Guidelines for the use and interpretation of assays for monitoring autophagy

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Daniel J. Klionsky,1,2,3,* Fabio C. Abdalla,4 Hagai Abeliovich,5 Robert T. Abraham,6 Abraham Acevedo-Arozena,7 Khosrow Adeli,8 Lotta Agholme,9 Maria Agnello,10 Patrizia Agostinis,11 Julio A. Aguirre-Ghiso,12 Hyung Jun Ahn,13 Ouardia Ait-Mohamed,14 Slimane Ait-Si-Ali,14 Takahiko Akematsu,15 Shizuo Akira,16 Hesham M. Al-Younes,17 Munir A. Al-Zeer,18 Matthew L. Albert,19 Roger L. Albin,20,21 Javier Alegre-Abarrategui,22 Maria Francesca Aleo,23 Mehrdad Alirezaei,24 Alexandru Almasan,25 Maylin Almonte-Becerril,26 Atsuo Amano,27 Ravi Amaravadi,28 Shoba Amarnath,29 Amal O. Amer,30,31 Nathalie Andrieu-Abadie,32,33 Vellareddy Anantharam,34 David K. Ann,35 Shailendra Anoopkumar-Dukie,36 Hiroshi Aoki,37 Nadezda Apostolova,38 Giuseppe Arancia,39 John P. Aris,40 Katsuhiko Asanuma,41 Nana Y.O. Asare,42 Hisashi Ashida,43 Valerie Askanas,44 David S. Askew,45 Patrick Auberger,46,47 Misuzu Baba,48 Steven K. Backues,1 Eric H. Baehrecke,49 Ben A. 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Xavier,988 Zhijun Xi,989 Pu Xia,990 Gengfu Xiao,991 Zhiping Xie,992 Zhonglin Xie,993 Da-zhi Xu,994 Jianzhen Xu,995 Liang Xu,996 Xiaolei Xu,997 Ai Yamamoto,252,364 Akitsugu Yamamoto,998 Shunhei Yamashina,999 Michiaki Yamashita,1000 Xianghua Yan,1001 Mitsuhiro Yanagida,1002 Dun-Sheng Yang,565 Elizabeth Yang,1003 Jin-Ming Yang,167,168 Shi Yu Yang,1004 Wannian Yang,1005 Wei Yuan Yang,151 Zhifen Yang,256 Meng-Chao Yao,1006 Tso-Pang Yao,780 Behzad Yeganeh,359 Wei-Lien Yen,1 Jia-jing Yin,589 Xiao-Ming Yin,1007 Ook-Joon Yoo,1008 Gyesoon Yoon,1009 Seung-Yong Yoon,502 Tomohiro Yorimitsu,1010 Yuko Yoshikawa,1011 Tamotsu Yoshimori,391 Kohki Yoshimoto,1012 Ho Jin You,1013 Richard J. Youle,702 Anas Younes,1014 Li Yu,160 Long Yu,1015 Seong-Woon Yu,503 Wai Haung Yu,251,252 Zhi-Min Yuan,1016 Zhenyu Yue,1017 Cheol-Heui Yun,1018 Michisuke Yuzaki,1019 Olga Zabirnyk,1020 Elaine Silva-Zacarin,4 David Zacks,1021 Eldad Zacksenhaus,1022 Nadia Zaffaroni,1023

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1Life Sciences Institute; University of Michigan; Ann Arbor, MI USA; 2Department of Molecular, Cellular and Developmental Biology; University of Michigan; Ann Arbor, MI USA; 3Department of Biological Chemistry; University of Michigan; Ann Arbor, MI USA; 4Laboratory of Structural and Functional Biology; Federal University of São Carlos (UFSCar); Campus Sorocaba; São Paulo State, Brazil; 5Department of Biochemistry and Food Science; Hebrew University; Rehovot, Israel; 6Oncology Research Unit; Pfizer Worldwide Research and Development; Pearl River, NY USA; 7MRC Mammalian Genetics Unit; Harwell, UK; 8Research Institute; The Hospital for Sick Children; University of Toronto; Ontario, Canada; 9Division of Geriatrics; Department of Clinical and Experimental Medicine; Faculty of Health Science; Linköping University; Linköping, Sweden; 10Department of STEMBIO (Cell Biology); University of Palermo; Palermo, Italy; 11Department of Molecular Cell Biology; Catholic University of Leuven; Leuven, Belgium; 12Department of Medicine and Department of Otolaryngology; Tisch Cancer Institute at Mount Sinai; Mount Sinai School of Medicine; New York University; New York, NY USA; 13Center for Theragnosis; Biomedical Research Institute; Korea Institute of Science and Technology; Seoul, South Korea; 14Laboratoire Epigénétique et Destin Cellulaire; UMR7216; Centre National de la Recherche Scientifique (CNRS); Université Paris Diderot Sorbonne Paris Cité; Paris, France; 15Department of Biology; York University; Toronto, Ontario Canada; 16Laboratory of Host Defense; WPI Immunology Frontier Research Center, and Department of Host Defense; Research Institute for Microbial Diseases; Osaka University; Osaka, Japan; 17Department of Biological Sciences; Faculty of Science; University of Jordan; Amman, Jordan; 18Department of Molecular Biology; Max Planck Institute for Infection Biology; Berlin, Germany; 19Laboratory of Dendritic Cell Biology; Department of Immunology; Institut Pasteur and INSERM U818; Paris, France; 20Department of Neurology; University of Michigan; Ann Arbor, MI USA; 21Geriatrics Research, Education and Clinical Center; VAAAHS; Ann Arbor, MI USA; 22Oxford Parkinson's Disease Centre; Department of Physiology, Anatomy and Genetics; University of Oxford; Oxford, UK; 23Department of Biomedical Sciences and Biotechnologies; School of Medicine; University of Brescia; Brescia, Italy; 24Department of Immunology and Microbial Science; Scripps Research Institute; La Jolla, CA USA; 25Department of Cancer Biology; Cleveland Clinic; Cleveland, OH USA; 26Infectomic and Molecular Pathogenesis Departament; Centro de investigación y de Estudios avanzados del Instituto Politécnico Nacional (CINVESTAV-IPN); México City, México; 27Department of Preventive Dentistry; Osaka University Graduate School of Dentistry; Osaka, Japan; 28Department of Medicine; Perelman School of Medicine; University of Pennsylvania; Philadelphia, PA USA; 29Experimental Transplantation and Immunology Branch; National Cancer Institute; National Institutes of Health; Bethesda, MD USA; 30The Department of Microbial Infection and Immunity; the Department of Internal Medicine; Center for Microbial Interface Biology; The Ohio State University; Columbus, OH USA; 31Davis Heart and Lung Research Institute; The Ohio State University; Columbus, OH USA; 32INSERM UMR1037; University Paul Sabatier; Toulouse, France; 33University Paul Sabatier; Toulouse, France; 34Department of Biomedical Science; Iowa Center for Advanced Neurotoxicology; Iowa State University; Ames, IA USA; 35Department of Molecular Pharmacology; Beckman Research Institute; City of Hope; Duarte, CA USA; 36Griffith Health Institute and School of Pharmacy; Griffith University; Gold Coast, Australia; 37Department of Neurosurgery; Brain Research Institute; Niigata University; Niigata, Japan; 38Departamento de Farmacologia; Facultad de Medicina; Universitat de Valencia; Valencia, Spain; 39Ultrastructural Methods for Innovative Cancer Therapies Unit; Technology and Health Department; Italian National Institute of Health; Rome, Italy; 40Department of Anatomy and Cell Biology; University of Florida College of Medicine; Gainesville, FL USA; 41Division of Nephrology; Department of Internal Medicine; Juntendo University Faculty of Medicine; Tokyo, Japan; 42Department of Chemical Toxicology; Norwegian Institute of Public Health; Oslo, Norway; 43Graduate School of Biostudies; Kyoto University; Kyoto, Japan; 44Department of Neurology; USC Neuromuscular Center; University of Southern California Keck School of Medicine; Los Angeles, CA USA; 45Department of Pathology & Laboratory Medicine; University of Cincinnati College of Medicine; Cincinnati, OH USA; 46INSERM U895; Centre Mediterraneen de Medecine Moleculaire (C3M); University of Nice Sophia Antipolis; Nice, France; 47University of Nice Sophia Antipolis; Nice, France; 48Collaborative Open Research Center; Research Institute for Science and Technology; Kogakuin University; Tokyo, Japan; 49Department of Cancer Biology; University of Massachusetts Medical School; Worcester, MA USA; 50Biotechnology Research and Training Center; William C. Friday Laboratory; University of North Carolina Pembroke; Pembroke, NC USA; 51Department of Nephrology; State Key Laboratory of Kidney Diseases; Chinese PLA General Hospital; Beijing, China; 52Cytologie et Cytopathologie Neuronales; INCI - CNRS UPR 3212; Université de Strasbourg; Strasbourg, France; 53Division of Hematology; The Ohio State University, Columbus; OH USA; 54Department of Biochemistry and Molecular Biology; University of Arkansas for Medical Sciences; Little Rock, AR USA; 55Department of Biomolecular Sciences; University of Urbino "Carlo Bo"; Urbino, Italy; 56Telethon Institute of Genetics and Medicine (TIGEM); Federico II University; Naples, Italy; 57Department of Pediatrics; Federico II University; Naples, Italy; and Jan and Dan Duncan Neurological Research Institute; Baylor College of Medicine; Houston, TX USA; 58Department of Biological Sciences; University of Toledo; Toledo, OH USA; 59MRC Toxicology Unit; University of Leicester; Lancaster Road; Leicester, UK; 60Semmelweis University; Department of Biochemistry, Molecular Biology and Pathobiochemistry; Budapest, Hungary; 61Department of Genetics, Development and Cell Biology, and Plant Sciences Institute; Iowa State University; Ames, IA USA; 62Department of Experimental Therapeutics; MD Anderson Cancer Center; Houston, TX USA; 63Catholic University of Louvain; Institute of Life Sciences; UCL/ ISV; Louvain-la-Neuve, Belgium; 64Department of Anatomy; Yong Loo Lin School of Medicine; National University of Singapore; Singapore; 6535 INSERM UMR-S984; and Université Paris Sud; Châtenay-Malabry, France; 66INRA UMR 1019; Unité de Nutrition Humaine; and CRNH; Université d'Auvergne; Clermont-Ferrand, France; 67College of Nanoscale Science and Engineering; University at Albany; SUNY; Albany, NY USA; 68Institute for Pathobiochemistry; University Medical Center of the Johannes Gutenberg-University; Mainz, Germany; 69Institute of Biochemistry II; Medical School Goethe University; Frankfurt, Germany; 70Laboratoire de Biochimie Médicale; Rouen University Hospital and NeoVasc EA 4309; University of Rouen; Rouen, France; 71Veterinary Microbiology and Preventive Medicine; Iowa State University; Ames, IA USA; 72Westmead Millennium Institute; University of Sydney; Westmead Institute for Cancer Research; Westmead, NSW, Australia; 73Vaccine Manufacturing Sciences and Commercialization; Merck Manufacturing Division; West Point, PA USA; 74Department of Health Sciences; Magna Graecia University; Catanzaro, Italy; 75INRA; UMR866 Dynamique Musculaire et Métabolisme; Université Montpellier 1; Montpellier, France; 76Department of Experimental Medicine and Biochemical Sciences; University of Rome "Tor Vergata"; Rome, Italy; 77DIMNP; UMR5235; CNRS; Université de Montpellier 2; Montpellier, France; 78Department of Biochemistry; Comenius University; Bratislava, Slovak Republic; 79Basic Medical Sciences; Western University of Health Sciences; Pomona, CA USA; 80Centre d'études d'agents Pathogènes et Biotechnologies pour la Santé; CNRS UM1; UM2; Montpellier, France; 81Department of Microbiology and Immunology; Indiana University School of Medicine; Indianapolis, IN USA; 82Department of Hematology and Medical Oncology; Winship Cancer Institute; Emory University School of Medicine; Atlanta, GA USA; 83Department of Biomedical Sciences; University of Padova; Padova, Italy; 84Department of Medicine; University of Chicago; Chicago, IL USA; 85Department of Oncology; University Children's Hospital; University of Zurich; Zurich, Switzerland; 86Department of Biological Sciences and Cellular and Molecular Therapy Center - Universidade Federal de São Paulo; São Paulo, Brazil; 87Department of Veterinary Medicine; University of Maryland; College Park, MD USA; 88Department of Cell Proliferation and Development; Centro de Investigaciones Biológicas; CIB-CSIC; Madrid, Spain; 89Université de Franche-Comté; Laboratoire de Biochimie; EA3922; "Estrogènes; Expression Génique et Pathologies du Système Nerveux Central"; IFR133; U.F.R. Sciences et Techniques; Besançon, France; 90Department of Plant Biology and Forest Genetics; Uppsala BioCentre; Swedish University of Agricultural Sciences; Uppsala, Sweden; 91Systems Biology of Cell Death Mechanisms; German Cancer Research Center (DKFZ); and Medical Faculty; University of Heidelberg; Heidelberg, Germany; 92Dipartment Scienze Mediche e Biologiche; Università degli Studi di Udine; Udine, Italy; 93Centre for Cancer Biomedicine; University of Oslo and Department of Biochemistry; Oslo University Hospital; Oslo, Norway; 94Department of Cell and Developmental Biology; UNC School of Medicine; Chapel Hill, NC USA; 95Research Unit for Tropical Diseases; de Duve Institute and Laboratory of Biochemistry; Université Catholique de Louvain; Brussels, Belgium; 96Department of Cell and Regenerative Biology; Paul Carbone Cancer Center; University of Wisconsin School of Medicine and Public Health; Madison, WI USA; 97University of Nice-Sophia Antipolis; Institute of Research on Cancer and Ageing of Nice (IRCAN); INSERM CNRS; Nice, France; CHU-Nice; Laboratoire de Pathologie Clinique et Expérimentale; IFR-50; Nice, France; ARC 9; Villejuif, France; 98Virginia Commonwealth University; Departments of Pharmacology and Toxicology; Massey Cancer Center; Goodwin Research Laboratories; Richmond, VA USA; 99Department of Anesthesiology; University of Rochester Medical Center; Rochester, NY USA; 100Department of Microbial Pathogenesis; Genentech, Inc.; South San Francisco, CA USA; 101Cell Biology Program; Hospital for Sick Children; Department of Molecular Genetics; University of Toronto; Ontario, Canada; 102Division of Pharmacology; Faculty of Health Sciences; Linköping University; Linköping, Sweden; 103Regenerative Medicine Program; Ottawa Hospital Research Institute; Ottawa, ON Canada; 104Department of Genetics; University of North Carolina; Chapel Hill, NC USA; 105Laboratory of Molecular and Cellular Signaling; Department of Molecular Cell Biology; Catholic University of Leuven; Leuven, Belgium; 106Department of Neurodegenerative Diseases; Hertie-Institute for Clinical Brain Research; University of Tuebingen; and DZNE; German Center for Neurodegenerative Diseases; Tuebingen, Germany; 107Department of Medicine I; Division of Oncology; Institute of Cancer Research; Medical University of Vienna; Vienna, Austria;

108Department of Internal Medicine; Division of Pulmonary, Allergy, Critical Care & Sleep Medicine; The Ohio State University; Columbus, OH USA; 109Department of Genetics and Evolution; University of Geneva; Geneva, Switzerland; 110Laboratory of Genetics and Molecular Hematology; University of São Paulo School of Medicine; São Paulo, Brazil; 111Skirball Institute of Biomolecular Medicine; Department of Microbiology; New York University School of Medicine; New York, NY USA; 112Department of Metabolism and Diabetes; Institute of Clinical and Experimental Medicine; Prague, Czech Republic; 113Department of Molecular Pharmacology; Albert Einstein College of Medicine; Bronx, NY USA; 114Vanderbilt Eye Institute; Vanderbilt University; Nashville, TN USA; 115Department of Cell Biology and Neuroscience; Rutgers University; Piscataway, NJ USA; 116Department of Cancer Biology; Kimmel Cancer Center; Thomas Jefferson University; Philadelphia, PA USA; 117Instituto de Investigaciones Biomédicas "Alberto Sols"; Consejo Superior de Investigaciones Científicas (CSIC) and Universidad Autónoma de Madrid (UAM); Madrid, Spain; 118Team "Mitochondries, Stress et Morts Cellulaires"; Institut de Biochimie et de Génétique Cellulaires (IBGC); UMR5095; CNRS & Université Bordeaux-Ségalen; Bordeaux, France; 119Royal Veterinary College; University of London; Consortium for Mitochondrial Research; University College London; London UK; 120EBRI-Rita Levi Montalcini Foundation; Rome, Italy; 121Institute of Parasitology and Biomedicine "Lopez-Neyra"-CSIC; Department of Cell Biology and Immunology; Granada, Spain; 122Department of Biochemistry; University of Rome "Tor Vergata"; Rome, Italy; 123Department of Pediatrics; Xiangya Hospital; Central South University; China; 124Department of Plant, Soil, and Entomological Sciences; University of Idaho; Moscow, ID USA; 125GI Tract Programme, Institute of Food Research; Norwich School of Medicine, The University of East Anglia; Faculty of Health; Norwich Research Park; Norwich, UK; 126Department of Mucosal Immunology; Norwich School of Medicine; The University of East Anglia–Faculty of Health; Norwich Research Park; Norwich, UK; 127Faculty of Medicine and Center for Neurosciences and Cell Biology; University of Coimbra; Coimbra, Portugal; 128Department of Medicine; Division of Hematology/Oncology; University of Texas Health Science Center at San Antonio; San Antonio, TX USA; 129Department of Molecular Biology and Microbiology; Case Western Reserve University; Cleveland, OH USA; 130Muscle Biology Unit; Departement of Experimental Medical Science; Lund University; Lund, Sweden; 131Instituto de Microbiologia; Universidade Federal do Rio de Janeiro; Rio de Janeiro, Brazil; 132Department of Medical Biosciences; University of Modena and Reggio Emilia; Italy; 133Department of Cell Biology; University Medical Center Groningen; Groningen, The Netherlands; 134Department of Human Pathology; University of Messina; Messina, Italy; 135Vita-Salute San Raffaele University & San Raffaele Scientific Institute; Milan, Italy; 136Institute of Neuroscience; Department of Cell Biology, Physiology and Immunology; Universitat Autònoma de Barcelona; Barcelona, Spain; 137Department of Health Sciences; Università del Piemonte Orientale "A. Avogadro"; Novara, Italy; 138Department of Cell Biology and Institute of Biomembranes; University Medical Centre Utrecht; Utrecht, The Netherlands; 139Department of Biochemistry and Cell Biology and Institute of Biomembranes; Utrecht University; Utrecht, The Netherlands; 140Dulbecco Telethon Institute–IRCCS Santa Lucia Foundation; University of Rome Tor Vergata; Rome, Italy; 141Department of Biology; University of Rome Tor Vergata; Rome, Italy; 142Laboratory of Intracellular Parasites; Rocky Mountain Laboratories; National Institute of Allergy and Infectious Diseases; National Institutes of Health; Hamilton, MT USA; 143Division of Radiation Biology & Molecular Environmental Research; Department of Radiation Oncology; University of Tuebingen; Tuebingen, Germany; 144Department of Pharmacology and Cardiovascular Research Institute; Medical School; Chonbuk University; Chonbuk, Korea; 145Department of Pathology; Kaohsiung Medical University Hospital; Kaohsiung, Taiwan; 146Division of Biology; California Institute of Technology; Pasadena, CA USA; 147Strathclyde Institute of Pharmacy and Biomedical Sciences; University of Strathclyde; Glasgow, Scotland UK; 148Laboratory of Neurodegenerative Diseases; Department of Anatomy; Li Ka Shing Faculty of Medicine; The University of Hong Kong; Hong Kong, China; 149Department of Chemistry; The University of Hong Kong; Hong Kong; 150Department of Pharmacology; College of Medicine; National Taiwan University; Taipei, Taiwan; 151Institute of Biological Chemistry; Academia Sinica; Taipei, Taiwan; 152Key Laboratory of Cell Differentiation and Apoptosis of Chinese Ministry of Education; Shanghai Jiao Tong University School of Medicine; Shanghai, China; 153Department of Pathology and Immunology; Baylor College of Medicine; Houston, TX USA; 154State Key Laboratory of Membrane Biology; Institute of Zoology; Chinese Academy of Sciences; 155College of Life Sciences; Nankai University; 156Institute of Biomedical Sciences; Academia Sinica; Taipei, Taiwan; 157MOE Key Laboratory of Laser Life Science & Institute of Laser Life Science; South China Normal University; Guangzhou, China; 158The Children's Hospital; Zhejiang University School of Medicine; Hangzhou, China; 159Department of Hematology; Xijing Hospital; Fourth Military Medical University; Xi'an, China; 160School of Life Sciences; Tsinghua University; Beijing, China; 161Department of Immunology; Peking University; Center for Human Disease Genomics; Beijing, China; 162Department of Internal Medicine; University of Michigan Medical School; Ann Arbor, MI USA; 163Department of Radiation Oncology; Mackay Memorial Hospital; Taipei, Taiwan; 164Department of Botany and Plant Pathology; Purdue University; West Lafayette, IN USA; 165Department of Biochemistry and Molecular Biology; University of Louisville; Louisville, KY USA; 166School of Biomedical Sciences; The Chinese University of Hong Kong; Shatin, Hong Kong; 167 Department of Pharmacology; The Pennsylvania State University College of Medicine; Penn State Hershey Cancer Institute; Hershey, PA USA; 168Penn State Hershey Cancer Institute; Hershey, PA USA; 169Department of Cancer Biology and Genetics; Memorial Sloan-Kettering Cancer Center; New York, NY USA; 170Department of Surgery; Yonsei University College of Medicine; Seoul, Korea; 171Department of Microbiology; University of Pennsylvania; Philadelphia, PA USA; 172Faculty of Health Sciences and Medicine; Bond University; Queensland, Australia; 173Department of Biochemistry; Li Ka Shing Faculty of Medicine; The University of Hong Kong; Hong Kong, China; 174INSERM U1053; and Université Bordeaux Segalen; Bordeaux, France; 175Department of Cellular and Molecular Physiology; Penn State University College of Medicine; Hershey, PA USA; 176Graduate School of Life and Environmental Sciences; University of Tsukuba; Ibaraki, Japan; 177Department of Pharmacology; Emory University School of Medicine; Atlanta, GA USA; 178Institute of Pharmacology; School of Medicine; National Yang-Ming University; Taipei, Taiwan; 179School of Biomedical Sciences; The Chinese University of Hong Kong; Hong Kong, China; 180Graduate School of East-West Medical Science; Kyung Hee University; Yongin, Korea; 181Division of Pulmonary and Critical Care Medicine; Brigham & Women's Hospital; Harvard Medical School; Boston, MA USA; 182Department of Obstetrics and Gynecology; Samsung Medical Center; Sungkyunkwan University School of Medicine; Seoul, Korea; 183Institute for Medical Sciences; Ajou University School of Medicine; Suwon, Korea; 184Renal Division; Department of Medicine; Brigham and Women's Hospital; Harvard Medical School; Boston, MA USA; 185INSERM U753 PR1; Institut Gustave Roussy; Villejuif, France; 186Department of Environmental Health; University of Cincinnati; Cincinnati, OH USA; 187Department of Pharmacology; Tartu University; Tartu, Estonia; 188Department of Pathology; University of Pittsburgh School of Medicine; Pittsburgh, PA USA; 189Center for Neuroscience; University of Pittsburgh School of Medicine; Pittsburgh, PA USA; 190Immunology Research Center; National Health Research Institutes; Miaoli County, Taiwan; 191Department of Biochemistry; National Defense Medical Center; Taipei, Taiwan; 192School of Life Sciences and Biotechnology; Korea University; Seoul, Korea; 193Department of Microbiology; Ajou University School of Medicine; Suwon, Korea; 194School of Biological Sciences; University of Hong Kong; Pokfulam, Hong Kong, China; 195Department of Structures, Function and Biological Technologies; University of Naples "Federico II"; Naples, Italy; 196IRCCS San Raffaele; Rome, Italy; 197Physiological Laboratory; Institute of Translational Medicine; University of Liverpool; Liverpool, UK; 198Safar Center for Resuscitation Research; Pittsburgh, PA USA; 199Département de Biologie Cellulaire et de Morphologie; Université de Lausanne; Lausanne, Switzerland; 200Department of Oncology; Lombardi Comprehensive Cancer Center; Georgetown University; Washington D.C. USA; 201INSERM U984; University of Paris-South; Paris, France; 202Department of Molecular Biology; Princeton University; Princeton, NJ USA; 203Laboratorio de Biología Celular y Molecular-Instituto de Histología y Embriología; Universidad Nacional de Cuyo-CONICET; Mendoza, Argentina; 204Department of Genetics and Microbiology; University of Pavia; Pavia, Italy; 205Pharmacology; Faculty of Pharmacy; Università del Piemonte Orientale "A. Avogadro"; Novara, Italy; 206Cell Biology and Gene Expression Section; Laboratory of Neurogenetics; National Institute on Aging; National Institutes of Health; Bethesda, MD USA; 207Department of Molecular Microbiology and Immunology; Johns Hopkins University; Baltimore, MD USA; 208Departamento Enfermedades Cardiovasculares; Facultad de Medicina; Pontificia Universidad Catolica de Chile; Santiago, Chile; 209Institut Pasteur; Unité des Interactions Bactéries-Cellules; Paris, France; 210INSERM; U604; INRA; USC2020; Paris, France; 211Department of Experimental Medicine and Oncology; University of Torino; Torino, Italy; 212Larry Hillblom Islet Research Center; David Geffen School of Medicine; University of California; Los Angeles, CA USA; 213Departamento de Morfología y Biología Celular; Universidad de Oviedo; Oviedo, Spain; 214Departamento de Biología y Cs Ambientales; Facultad de Ciencias; Universidad de Valparaíso; Valparaíso, Chile; 215Division of Applied Medicine; University of Aberdeen; Aberdeen, UK; 216Keck Graduate Institute of Applied Sciences; Claremont, CA USA; 217Instituto de Bioquimica Vegetal y Fotosintesis (CSIC-Universidad de Sevilla); Sevilla, Spain; 218Department of Biochemistry; University of Johannesburg; Johannesburg, South Africa; 219Department of Anatomy and Structural Biology; Albert Einstein College of Medicine; Bronx, NY USA; 220Department of Developmental and Molecular Biology; Albert Einstein College of Medicine; Bronx, NY USA; 221Marion Bessin Liver Research Center; Albert Einstein College of Medicine; Bronx, NY USA; 222Department of Surgery; University of Iowa; Iowa City, IA USA; 223Department of Medicine; Albert Einstein College of Medicine; Bronx, NY USA; 224Experimental Neurology Unit; European Centre for Brain Research; Santa Lucia Foundation; Rome, Italy; 225Pathogénie Bactérienne Intestinale USC-INRA 2018; Clermont-Ferrand, France; 226JE2526 Université Auvergne; Clermont-Ferrand, France; 227Cardiovascular Research Center; University of Connecticut School of Medicine; Farmington, CT USA; 228Department of Human Biology; University of Cape Town; Cape Town, South Africa; 229Division of Molecular Pathology; Cancer Therapeutics and Clinical Studies; Institute of Cancer Research and Royal Marsden Hosptial; Sutton, Surrey UK; 230Department of Public Health and Cell Biology; University of Rome Tor Vergata; Rome, Italy; 231Department of Neuro-Oncology; MD Anderson Cancer Center; University of Texas; Houston, TX USA; 232Virology Division; Department of Infectious Diseases and Immunology; Utrecht University; Utrecht, The Netherlands; 233Department of Pathology and Animal Health; University of Naples "Federico II"; Naples, Italy; 234Cancer Center Karolinska; Department of Oncology-Pathology; Karolinska Institute; Stockholm, Sweden; 235Department of Experimental Pathology; University of Pisa Medical School; Pisa, Italy; 236Department of Pathology; University of California; San Francisco, CA USA; 237Department of Biochemistry; Tufts University School of Medicine; Boston, MA USA; 238CNRS UMR 5293; Institut des Maladies Neurodégénératives; Université Bordeaux Segalen; Bordeaux, France; 239Department of Physiology; University of Melbourne; Parkville, Victoria Australia; 240Laboratorio Nazionale Consorzio Interuniversitario Biotecnologie; Trieste, Italy; 241Fungal Patho-Biology Group; Temasek Life Sciences Laboratory; National University of Singapore; Singapore; 242Freiburg Institute for Advanced Studies (FRIAS); School of Life Sciences (LifeNet); Freiburg, Germany; 243Department of

Neurosurgery; VCU Institute of Moleular Medicine; VCU Massey Cancer Center; Virginia Commonwealth University; School of Medicine; Richmond, VA USA; 244Centre for Cancer Biology; SA Pathology; University of Adelaide; SA, Australia; 245School of Molecular and Biomedical Science; University of Adelaide; SA, Australia; 246Department of Molecular Genetics and Microbiology; University of New Mexico Health Science Center; Albuquerque, NM USA; 247Department of Biochemistry and Molecular Biology; Louisiana State University–Health Sciences Center-School of Medicine; New Orleans, LA USA; 248Department of Biochemistry & Molecular Biology; Monash University; Clayton Campus; Melbourne, Victoria Australia; 249ARC Centre of Excellence in Structural and Functional Microbial Genomics; Monash University; Clayton Campus; Melbourne, Victoria Australia; 250Unit of Allergy and Immunotoxicology; Ageing Research Center (Ce.S.I.); Università G. d'Annunzio Foundation; Chieti, Italy; 251Taub Institute for Alzheimer's Disease Research; Columbia University Medical Center; New York, NY USA; 252Department of Pathology and Cell Biology; Columbia University Medical Center; New York, NY USA; 253Institute of Cell Biology and Neurobiology; EMMA-CNR; Campus "A. Buzzati Traverso"; Rome, Italy; 254Department of Pharmacological and Toxicological Chemistry; University of Chile; Santiago, Chile; 255Department of Biochemistry and Molecular Biology; School of Medicine; University of Alcalá; Madrid, Spain; 256Sanford-Burnham Medical Research Institute; La Jolla, CA USA; 257Departamento de Biología Molecular; Centro de Biologia Molecular Severo Ochoa (UAM-CSIC); Universidad Autonoma de Madrid; Madrid, Spain; 258Molecular Signaling; Frankfurt Institute for Molecular Life Sciences; Frankfurt am Main, Germany; 259Department of Plant Biology and The Genome Center; College of Biological Sciences; University of California; Davis, CA USA; 260Department of Pharmacology, Toxicology and Therapeutics; The University of Kansas Medical Center; Kansas City, KS USA; 261Department of Medicine; Case Western Reserve University; University Hospitals of Cleveland; Cleveland, OH USA; 262Department of Pharmacology; Case Western Reserve University; University Hospitals of Cleveland; Cleveland, OH USA; 263Department of Pathology; Case Western Reserve University; University Hospitals of Cleveland; Cleveland, OH USA; 264Comprehensive Cancer Center; Case Western Reserve University; University Hospitals of Cleveland; Cleveland, OH USA; 265University Hospitals of Cleveland; Cleveland, OH USA; 266Center for Cardiovascular Research; Washington University School of Medicine; Saint Louis, MO USA; 267Department of Internal Medicine; Washington University School of Medicine; Saint Louis, MO USA; 268INSERM U916 VINCO; Université Bordeaux; Bordeaux, France; 269CNRS UMR 8126; Université Paris-Sud 11; Institut Gustave Roussy; Villejuif, France; 270Department of Cellular Biology and Anatomy; Georgia Health Sciences University; Charlie Norwood VA Medical Center; Augusta, GA USA; 271Charlie Norwood VA Medical Center; Augusta, GA USA; 272Lilly Research Laboratories; Eli Lilly and Company; Indianapolis, IN USA; 273General and Molecular Pathophysiology Department; Bogomoletz Institute of Physiology; Kiev, Ukraine; 274Department of Pediatrics and Communicable Diseases; University of Michigan Medical School; Ann Arbor, MI USA; 275Department of Cell Biology; University of Massachusetts Medical School; Worcester, MA USA; 276Department of Human Virology; INSERM U758; Ecole Normale Supèrieure de Lyon; Lyon, France; 277Department of Biochemistry and Molecular Biology; Tongji Medical College; Huazhong University of Techology and Science; Wuhan, Hubei China; 278Division of Hematology and Central laboratory of Hematology; Lausanne University Hospital; Lausanne, Switzerland; 279Centre de Recherche des Cordeliers; INSERM; U872; Paris, France; 280Université Pierre et Marie Curie; Paris, France; 281Department of Biochemistry; University of Tuebingen; Tuebingen, Germany; 282Division of Renal Diseases and Hypertension; University of Colorado at Denver; Aurora, CO USA; 283Department of Developmental and Cell Biology; University of California; Irvine, CA USA; 284Department of Biología Celular, Inmunología y Neurociencias; Facultad de Medicina; Universidad de Barcelona–IDIBAPS; Barcelona, Spain; 285Institute of Biochemistry I; Medical Faculty; University of Cologne; Cologne, Germany; 286Department of Medicine; Baylor College of Medicine; Houston, TX USA; 287Department of Melanoma Medical Oncology; University of Texas; MD Anderson Cancer Center; Houston, TX USA; 288Laboratory of Translational Oncology and Experimental Cancer Therapeutics; Department of Medicine (Hematology/Oncology); Penn State College of Medicine; Penn State Hershey Medical Center; Hershey PA USA; 289Department of Biological Chemistry; The Weizmann Institute of Science; Rehovot, Israel; 290European Institute of Oncology, and Department of Biomolecular Sciences and Biotechnologies; University of Milan; Milan, Italy; 291Buck Institute for Research on Aging; Novato, CA USA; 292Department of Microbiology, Tumor and Cell Biology; Karolinska Institutet; Stockholm, Sweden; 293Department of Physiological Sciences; University of Stellenbosch; Stellenbosch, South Africa; 294Department of Pharmacology; The B. Rappaport Faculty of Medicine; Technion-Israel Institute of Technology; Haifa, Israel; 295Latvian Biomedical Research and Study Centre; Riga, Latvia; 296Department of Biosciences; University of Helsinki; Helsinki, Finland; 297Center for Applied Proteomics and Molecular Medicine; George Mason University; Manassas, VA USA; 298Department of Physiology and Biophysics; University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School; Piscataway, NJ USA; 299Liver Cancer Institute; Zhongshan Hospital; Fudan University; Shanghai, China; 300Departments of Neurology, Pediatrics, Neurological Surgery and Brain Tumor Research Center; Helen Diller Family Comprehensive Cancer Center, University of California; San Francisco, CA USA; 301Center for Biomedical Engineering and Technology (BioMET); University of Maryland, Baltimore; Baltimore, MD USA; 302Department of Physiology; University of Maryland, Baltimore; Baltimore, MD USA; 303The First Affiliated Hospital of Guangzhou University of Chinese Medicine; Guangzhou, China; 304MRC Centre for Developmental Neurobiology; King's College London, UK; 305Cell Death and Metabolism; Danish Cancer Society Research Center; Copenhagen, Denmark; 306Section of Molecular Biology; Division of Biological Sciences; University of California; San Diego; La Jolla, CA USA; 307INSERM U851; Université de Lyon, and the Université Lyon1; Lyon, France; 308Department of Cellular Biology; University of Georgia; Athens, GA USA; 309Immunobiology Section; Laboratory of Parasitic Diseases; National Institute of Allergy and Infectious Diseases; National Institutes of Health; Bethesda, MD USA; 310Department of Physiology and Biophysics; State University of New York at Buffalo; Buffalo, NY USA; 311Guangdong Provincial Key Lab of Biotechnology for Plant Development; School of Life Sciences; South China Normal University; Guangzhou, China; 312Department of Obstetrics & Gynecology; Shanghai First People's Hospital; Shanghai Jiao Tong University; Shanghai, China; 313Departments of Biochemistry and Molecular Biology, Apoptosis and Genomics Research Group of the Hungarian Academy of Sciences; Research Center for Molecular Medicine; University of Debrecen; Debrecen, Hungary; 314Cell & Molecular Biology Joint Doctoral Program; Department of Biology; San Diego State University; San Diego, CA USA; 315Department of Biological Sciences; Hunter College; City University of New York; New York, NY USA; 316National Institute for Infectious Diseases IRCCS L. Spallanzani; Rome, Italy; 317Department of Cell and Developmental Biology; University of Michigan Medical School; Ann Arbor, MI USA; 318Gladstone Institutes; Taube-Koret Center for Huntington's Disease and the Hellman Alzheimer's Disease Program; University of California; San Francisco, CA USA; 319Laboratory of Molecular Biology; National Heart, Lung, and Blood Institute; National Institutes of Health; Bethesda, MD USA; 320Bioscience Center; San Diego State University; San Diego, CA USA; 321Departments of Medicine and Cell Biology; Marc and Ruti Bell Program in Vascular Biology; New York University School of Medicine; New York, NY USA; 322Department of Human and Molecular Genetics; VCU Institute of Moleular Medicine; VCU Massey Cancer Center; Virginia Commonwealth University; School of Medicine; Richmond, VA USA; 323Laboratory of Molecular and Cellular Neuroscience; Rockefeller University; New York, NY USA; 324Institute for Behavioral Genetics; University of Colorado; Boulder, CO USA; 325McGill Parkinson Program; Department of Neurology & Neurosurgery; Montreal Neurological Institute; McGill University; Montreal, Quebec Canada; 326Department of Human Morphology; University of Pisa and IRCCS Neuromed; Pisa, Italy; 327University Hospital Heidelberg; Department of Surgery/Research Lab; Heidelberg, Germany; 328Department of Pharmacology and Experimental Neuroscience; University of Nebraska Medical Center; Omaha, NE USA; 329Redox Biology Center and School of Veterinary Medicine and Biomedical Sciences; University of Nebraska–Lincoln; Lincoln, NE USA; 330Biotech Research and Innovation Centre; University of Copenhagen; Copenhagen, Denmark; 331Department of Molecular and Cellular Medicine; University of Leuven; Leuven, Belgium; 332Centro de Investigación Biomédica en Red de Enfermedades Neurodegenerativas (CIBERNED); Departamento Bioquímica y Biología Molecular y Genética; E.U. Enfermería; Universidad de Extremadura; Cáceres, Spain; 333Department of Bacteriology; Graduate school of Medical Sciences; Kyushu University; Kagoshima, Japan; 334Laboratory of Emerging Infectious Diseases; Department of Frontier Veterinary Medicine; Kagoshima University; Kagoshima, Japan; 335Department of Pediatrics; Jichi Medical Universtiy; Tochigi, Japan; 336Department of Developmental Biology and Neurosciences; Graduate School of Life Sciences; Tohoku University; Miyagi, Japan; 337Institute of Physiological Chemistry; Hannover Medical School; Hannover, Germany; 338Laboratory of Cell Physiology; University of Louvain; Brussels, Belgium; 339Department of Physiological Sciences; Faculty of Veterinary Medicine; Warsaw University of Life Sciences; Warsaw, Poland; 340UMR CNRS 7622; Biologie du Développement; Université Pierre et Marie Curie; Paris, France; 341Department of Biological Sciences & Bioengineering; Indian Institute of Technology; Kanpur, India; 342Laboratory of Genetics; University of Wisconsin; Madison, WI USA; 343MRC Protein Phosphorylation Unit; College of Life Sciences; University of Dundee; Dundee, Scotland UK; 344Department of Neurology; University of Massachusetts Medical School; Worcester, MA USA; 345CAS Key Laboratory of Pathogenic Microbiology and Imuunology; Institute of Microbiology; Chinese Academy of Sciences (CAS); Beijing, China; 346Oncology and Pharmacology; UT Southwestern Medical Center; Dallas, TX USA; 347Centro Estudios Moleculares de la Celula; Facultad Ciencias Quimicas y Farmaceuticas; Universidad de Chile; Santiago, Chile; 348Department of Leukemia; University of Texas; MD Anderson Cancer Center; Houston, TX USA; 349Department of Biochemistry; School of Medicine; Boston University; Boston, MA USA; 350Laboratory of Dermatology; Department of Oncology; University of Leuven; Leuven, Belgium; 351Department of Medicine; University of Illinois at Chicago; Chicago, IL USA; 352Center of Immunology of Marseille Luminy; Aix-Marseille Univ; INSERM U631; and CNRS UMR6102; Marseille, France; 353Department of Molecular Cardiology; Randall Division for Cell and Molecular Biophysics and Cardiovascular Division; Kings College; London UK; 354Department of Biology; University of Kentucky; Lexington, KY USA; 355Department of Clinical Neurosciences; Institute of Neurology; University College London; London UK; 356Department of Cell Biology; Harvard Medical School; Boston, MA USA; 357Department of Cellular Molecular Medicine; University of Ottawa; Ottawa, Ontario Canada; 358Department of Pathology; University of Michigan; Ann Arbor, MI USA; 359Department of Physiology; Manitoba Institute of Child Health; University of Manitoba; Manitoba, Canada; 360Department of Medicine; University of California San Diego School of Medicine; La Jolla, CA USA; 361Department of Therapeutic Research and Medicine Evaluation; Istituto Superiore di Sanita; Rome, Italy; 362Department of Pathology; Democritus University of Thrace and University General Hospital of Alexandroupolis; Alexandroupolis, Greece; 363Biochemistry and Medical Genetics; Manitoba Institute of Cell Biology; University of Manitoba; Winnipeg, Manitoba Canada; 364Department of Neurology; Columbia University Medical Center; New York, NY USA; 365School of Health and Medicine; Lancaster University; Lancaster UK; 366Department of Medicine; Columbia University College of Physicians and Surgeons; New York, NY USA; 367Department of Immunology; Medical

University of Warsaw; Warsaw, Poland; 368Renal Research Institute; New York Medical College; Valhalla, NY USA; 369Department of Genetics; University of Texas MD Anderson Cancer Center; Houston, TX USA; 370Biology Department; Faculty of Science; Ege University; Bornova, Izmir Turkey; 371Institut de Recherche en Cancérologie de Montpellier; INSERM U896; Université Montpellier1; Montpellier, France; 372Department of Pathophysiology; School of Pharmacy and Biochemistry; University of Buenos Aires; Buenos Aires, Argentina; 373Instituto de Ciencias de la Vid y del Vino; CSIC-UR-CAR; Logroño, Spain; 374Department of Developmental Genetics and Gene Control; Centre for Genetics and Genomics; The University of Nottingham; Queen's Medical Centre; Nottingham UK; 375The Henry M. Jackson Foundation for the Advancement of Military Medicine; Uniformed Services University of the Health Sciences; Bethesda, MD USA; 376Genome Sciences Center; BC Cancer Agency; Vancouver, BC Canada; 377Molecular Cardiology Research Institute; Tufts Medical Center and Department of Medicine; Tufts University School of Medicine; Boston, MA USA; 378Biological Sciences and Bioengineering Program; Sabancı University; Orhanlı-Tuzla, Istanbul, Turkey; 379Department of Neurobiology and Behavior; University of California; Irvine, CA USA; 380Agricultural Institute of Slovenia; Ljubljana, Slovenia; 381University of Strasbourg; Immunologie et Chimie Thérapeutiques; CNRS; Strasbourg, France; 382Department of Pediatrics; Children's Hospital; University of Iowa; Iowa City, IA USA; 383Signaling Networks Program; Division of Oncology; Department of Medicine I; Comprehensive Cancer Center; Medical University Vienna & Ludwig Boltzmann Cluster Oncology; Vienna, Austria; 384Department of Pharmacology and Moores Cancer Center; University of California San Diego; La Jolla, CA USA; 385AstraZeneca; Cancer&Infection Research Area; Cheshire UK; 386Department of Medicine; the David Geffen School of Medicine; University of California; Los Angeles, CA USA; 387Department and Laboratory of Intensive Care Medicine; Katholieke Universiteit Leuven; Leuven, Belgium; 388Skaggs School of Pharmacy and Pharmaceutical Sciences; University of California San Diego; San Diego, CA USA; 389Department of Internal Medicine; Manitoba Institute of Child Health; University of Manitoba; Winnipeg, Manitoba Canada; 390Department of Microbiology; Montana State University; Bozeman, MT USA; 391Department of Genetics; Osaka University Graduate School of Medicine; Osaka, Japan; 392Institute of Pharmacology, Toxicology and Biochemical Pharmaceutics; Zhejiang University; Hangzhou, China; 393Discovery & ADMET-TOX Systems; Life Technologies; Madison, WI USA; 394Department of Oral and Craniofacial Molecular Biology; Massey Cancer Center; Virginia Commonwealth University; Richmond, VA USA; 395Third Department of Internal Medicine; School of Medicine; University of Occupational and Environmental Health; Kitakyushu, Japan; 396Department of Cardiology; Angiology and Pulmology; University of Heidelberg; Heidelberg, Germany; 397Molecular Oncology Laboratories; Oxford University Department of Oncology; Weatherall Institute of Molecular Medicine; John Radcliffe Hospital; Oxford UK; 398Immunology Research Centre; School of Biochemistry & Immunology; Trinity College Dublin; Dublin, Ireland; 399Center for Plant Science Innovation; University of Nebraska-Lincoln; Lincoln, NE USA; 400Division of Sensory and Motor Systems; Tokyo Metropolitan Institute of Medical Science; Tokyo, Japan; 401VA Boston Healthcare System; Boston, MA USA; 402Divison of Hypertension and Nephrology; National Cerebral and Cardiovascular Center; Osaka, Japan; and Department of Cell Signaling; Gifu University Graduate School of Medicine; Gifu, Japan; 403Molecular Oncology; Moffitt Cancer Center; Tampa, FL USA; 404Department of Internal Medicine; University of Texas Southwestern Medical Center; Dallas, TX USA; 405Department of Immunology; Duke University Medical Center; Durham, NC USA; 406Department of Nephrology; CRCHUM; Montréal, QB Canada; 407Department of Pharmacology; University of Colorado School of Medicine; Aurora, CO USA; 408College of Medical; Veterinary & Life Sciences; Institute of Cancer Sciences; University of Glasgow; Glasgow UK; 409Department of Cellular and Structural Biology; University of Texas Health Science Center at San Antonio; San Antonio, TX USA; 410Department of Molecular Genetics; The Ohio State University; Columbus, OH USA; 411Biomedical Neuroscience Institute; ICBM; Faculty of Medicine; University of Chile; Santiago, Chile; 412Department of Molecular Biology; Institute of Parasitology and Biomedicine "López-Neyra"; IPBLN-CSIC; Granada, Spain; 413Division of Cardiology; University of Texas Southwestern Medical Center; Dallas, TX USA; 414INSERM ERI 21/EA2319; and the Laboratoire de Pathologie Clinique et Expérimentale; IFR-50; Nice, France; 415Cellular Senescence Group A210; German Cancer Research Center; DKFZ-ZMBH Alliance; Heidelberg, Germany; 416Molecular Cell Biology; Institute for Cell Biology; University Bonn; Bonn, Germany; 417GCP center; Cancer Center of Sun Yat-sen University; Guangzhou, China; 418School of Kinesiology and Health Science; York University; Toronto; ON Canada; 419Harvard University; School of Public Health; Boston, MA USA; 420Department of Internal Medicine; University Medical Center Groningen; Groningen, The Netherlands; 421Department of Molecular Biomedicine; LEO Pharma A/S; Ballerup, Denmark; 422Neurology and Anesthesiology; Shock Trauma & Anesthesiology Research Center; University of Maryland School of Medicine; Baltimore, MD USA; 423Department of Biochemistry and Molecular Biology; University of New Mexico School of Medicine; Albuquerque, NM USA; 424Laboratory of Cancer Immunobiology; Earle A. Chiles Research Institute; Providence Portland Medical Center; Portland, OR USA; 425Department of Neurosurgery; University of Michigan; Ann Arbor, MI USA; 426The State Key Laboratory of Biotherapy; West China Hospital; Sichuan University; Chengdu, China; 427Department of Medicine; Mayo Clinic and Mayo Cancer Center; Rochester, MN USA; 428Department of Life Science and Institute of Zoology; National Taiwan University; Taipei, Taiwan; 429Renal Division; University Hospital Freiburg; and BIOSS Centre for Biological Signalling Studies; Albert-Ludwigs-University; Freiburg, Germany; 430School of Biological Sciences and Research Center for Functional Cellulomics; Seoul National University; Seoul, Korea; 431Department of Obstetrics and Gynecology; Chang Gung Memorial Hospital at Taipei; Taipei, Taiwan; 432Department of Experimental Cancer Research; University of Edinburgh; Institute of Genetics and Molecular Medicine; Cell Signalling Unit; Edinburgh UK; 433Department of Pharmacology; Infection Signaling Network; Research Center; College of Medicine; Chungnam National University; Daejeon, Korea; 434Department of Biochemistry; University of Washington; Seattle, WA USA; 435Critical Care Department; McGill University; Montreal, QB Canada; 436School of Biological and Biomedical Sciences; University of Durham; Durham UK; 437Institute for Innovative Cancer Research; University of Ulsan College of Medicine; and Asan Medical Center; Seoul, Korea; 438Department of Pathology and Immunology; Washington University School of Medicine; St. Louis, MO USA; 439Department of Medicine II; Endocrinology and Hypertension; Tokyo Women's Medical University; Tokyo, Japan; 440Department of Molecular and Integrative Physiology, and the Division of Nephrology; Department of Internal Medicine; University of Michigan Medical School; Ann Arbor, MI USA; 441Department of Oral Microbiology; Division of Oral Infections and Health Sciences; Asahi University School of Dentistry; Mizuho, Japan; 442INSERM U624 and University of Méditerranée; Marseille, France; 443Division of Life Science and State Key Laboratory of Molecular Neuroscience; The Hong Kong University of Science and Technology; Hong Kong, China; 444Department of Geriatric Medicine and Nephrology; Osaka University Graduate School of Medicine; Suita, Japan; 445Department of Applied Plant Science; Graduate School of Agricultural Sciences; Tohoku University; Miyagi, Japan; 446Department of Immunology; Nagoya University Graduate School of Medicine; Nagoya University; Nagoya, Japan; 447Department of Immunobiology; Yale University School of Medicine; New Haven, CT USA; 448Division of General Thoracic Surgery; School of Medicine; Keio University; Tokyo, Japan; 449Centre for Biotechnology; University of Turku; Turku, Finland; 450Department of Neurology; University of Texas Medical ranch; Galveston, TX USA; 451Department of Microbiology and Molecular Genetics; Medical College of Wisconsin; Milwaukee, WI USA; 452Laboratory of Experimental-Hemato-Oncology; Department of Oncology; Public Research Center for Health (CRP-Santé); Luxembourg City, Luxembourg; 453Department of Neurology; University Medical School; Goethe University; Frankfurt/Main, Germany; 454Department of Physiology; Seoul National University College of Medicine; Seoul, Korea; 455Laboratory of Veterinary Biochemistry and Molecular Biology; College of Veterinary Medicine; Chungbuk National University; Cheongju, Korea; 456Key Laboratory of Hepatosplenic Surgery; Department of General Surgery; the First Affiliated Hospital of Harbin Medical University; Harbin, China; 457Department of Biochemistry; University of Texas Health Science Center; San Antonio, TX USA; 458Department of Urologic Surgery; Vanderbilt University Medical Center; Nashville TN USA; 459Department of Nutrition Science; Purdue University; West Lafayette, IN USA; 460Department of Cell Biology; Memorial Sloan-Kettering Cancer Center; New York, NY USA; 461State Key Laboratory of Mycology; Institute of Microbiology; Chinese Academy of Sciences; Beijing, China; 462Departamento de Microbiología y Genética; Universidad de Salamanca; Salamanca, Spain; 463Department of Pharmacology; University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School; Piscataway, New Jersey USA; 464Department of Biological Sciences; Korea Advanced Institute of Science and Technology; Daejeon, South Korea; 465Biochemistry Department; Institute of Medical Biology; University of Tromsø; Tromsø, Norway; 466Department of Medicine; University of Pittsburgh; Pittsburgh, PA USA; 467Cell Biology Program; Sickkids; Departments of Paediatrics and Physiology; University of Toronto; Toronto, ON Canada; 468Department of Pathology and Cell Biology; Thomas Jefferson University; Philadelphia, PA USA; 469Department of Physiology; University of Pretoria; Pretoria, South Africa; 470Department of Anatomy, Cell and Developmental Biology; Eötvös Loránd University; Budapest, Hungary; 471University Institute of Pathology; CHUV–University of Lausanne; Lausanne, Switzerland; 472Functional Food Technology Research Group; Korea Food Research Institute; Korea; 473Department of Biological Science; Seoul National University; Seoul, Korea; 474Department of Ophthalmology; University of Eastern Finland and Kuopio University Hospital; Kuopio, Finland; 475Department of Degenerative Neurological Diseases; National Institute of Neuroscience; National Center of Neurology and Psychiatry (NCNP); Tokyo, Japan; 476Department of Applied Biological Chemistry; Niigata University; Niigata, Japan; 477Experimental Pathology; Department of Clinical and Experimental Medicine; Faculty of Health Science; Linköping University; Linköping, Sweden; 478Laboratory of Biological Diversity; National Institute for Basic Biology; Okazaki, Japan; 479Institute of Environmental Medicine; Karolinska Institutet; Stockholm, Sweden; 480Gifu University Graduate School of Medicine; Division of Cardiology; Gifu, Japan; 481Howard Hughes Medical Institute; Division of Genetics; Brigham and Women's Hospital; Department of Genetics; Harvard Medical School; Boston, MA USA; 482Division of Medical Sciences; Humphrey Oei Institute of Cancer Research; National Cancer Centre Singapore; Singapore; 483Department of Chemistry and Biology; Korea Science Academy of KAIST; Busan, Korea; 484The DAMP Laboratory; Department of Surgery; Hillman Cancer Center; University of Pittsburgh Cancer Institute; University of Pittsburgh; Pittsburgh, PA USA; 485Department of Clinical Chemistry and Laboratory Medicine; Kyushu University Graduate School of Medical Sciences; Fukuoka, Japan; 486Department of Immunology; St. Jude Children's Research Hospital; Memphis, TN USA; 487Department of Orthopaedic Surgery; Tohoku University School of Medicine; Miyagi, Japan; 488Division of Medical Oncology; Department of Internal Medicine; University of Medicine and Dentistry of New Jersey; Robert Wood Johnson Medical School; Piscataway; and The Cancer Institute of New Jersey; New Brunswick, NJ USA; 489Central Arkansas Veterans Healthcare System and Department of Medicine; University of Arkansas for Medical Sciences; Little Rock, AR USA; 490Division of Biochemistry; Institute for Chemical Research; Kyoto University; Kyoto, Japan; 491Department of Biochemistry and Molecular Biology; College of Medicine; Chang Gung University; Taoyuan, Taiwan; 492B cell Molecular Immunology Section; Laboratory of

Immunoregulation; NIAID; National Institutes of Health; Bethesda, MD USA; 493Department of Laboratory Medicine and Pathology; University of Minnesota Cancer Center; Minneapolis, MN USA; 494Fraunhofer Group “Extracorporeal Immune Modulation (EXIM)”; Division of Nephrology; Department of Internal Medicine; Rostock, Germany; 495Department of Pharmacology; Wayne State University School of Medicine; Detroit, MI USA; 496Department of Molecular Biology; Genetic Engineering and Biotechnology Research Institute; Menoufiya University; El-Sadat City, Egypt; 497Molecular Cell Biology; Groningen Biomolecular Sciences and Biotechnology Institute (GBB) & Kluyver Centre for Genomics of Industrial Fermentation; University of Groningen; Haren, The Netherlands; 498Section of Cell and Developmental Biology; University of California, San Diego; La Jolla, CA USA; 499Faculty of Pharmaceutical Sciences; Hokkaido University; Sapporo, Japan; 500Department of Biochemistry and Institute of Health Sciences; Gyeongsang National University School of Medicine; JinJu, Korea; 501Department of Biochemistry, Molecular Biology & Biophysics; University of Minnesota; Minneapolis, MN USA; 502Cell Dysfunction Research Center (CDRC); Department of Anatomy and Cell Biology; University of Ulsan College of Medicine; Seoul, Korea; 503Department of Brain Science; Daegu Gyeongbuk Institute of Science and Technology; Daegu, Korea; 504Department of Dental Pharmacology; College of Dentistry; Wonkwang University; Chonbuk, South Korea; 505Department of Surgery; University of Florida; Gainesville, FL USA; 506Fight against Angiogenesis-Related Blindness
(FARB) Laboratory; Clinical Research Institute; Seoul National University & Department of Ophthalmology; Seoul National University College of Medicine; Seoul, Korea; 507Department of Surgery; University of Ulsan College of Medicine and Asan Medical Center; Seoul, Korea; 508Department of Human Genetics; University of Michigan; Ann Arbor, MI USA; 509Department of Biochemistry; University of Toronto; Toronto ON Canada; 510Department of Biochemistry and Molecular Biology; University of Ulsan College of Medicine; Seoul, Korea; 511Ilsong Institute of Life Science; College of Medicine; Hallym University; Anyang, Korea; 512Department of Microbiology; College of Medicine; Hallym University; Anyang, Korea; 513Department of Chemical Biochemical and Environmental Engineering; University of Maryland, Baltimore County; Baltimore, MD USA; 514Department of Molecular Genetics; Weizmann Institute of Science; Rehovot, Israel; 515Division of Genomic Stability and DNA Repair; Department of Radiation Oncology; Dana-Farber Cancer Institute; Harvard Medical School; Boston, MA USA; 516Tumour Cell Death Laboratory; Beatson Institute for Cancer Research; Glasgow UK; 517Brown University; Department of Radiation Oncology; Warren Alpert Medical School and Rhode Island Hospital; Providence, RI USA; 518Merck Serono; Merck KGaA; Darmstadt, Germany; 519University of Manitoba; Department of Pharmacology & Therapeutics; Institute of Cardiovascular Sciences; St. Boniface General Hospital Research Centre; Winnipeg, Manitoba Canada; 520Department of Biotechnology; University of Tokyo; Tokyo, Japan; 521Department of Molecular Miocrobiology and Immunology; Graduate School of Biomedical Sciences; Nagasaki University; Nagasaki, Japan; 522Institute for Immunology; University of Munich; Munich, Germany; 523Department of Pharmacology and Toxicology; University of Arizona; Tucson, AZ USA; 524Division of Molecular Neurology; University Hospital; Erlangen, Germany; 525Department of Cell Biology; Centro de Investigación Príncipe Felipe; Valencia, Spain; 526Department of Anatomical and Cellular Pathology; The State Key Laboratory in Oncology in Southern China; The Chinese University of Hong Kong; Hong Kong, China; 527Department of Neurology; University Medicine Göttingen; Göttingen, Germany; 528Department of Neurology; University of Ulsan College of Medicine; Seoul, Korea; 529Department of Cell Biology and Neuroscience; Juntendo University School of Medicine; Tokyo, Japan; 530Protein Metabolism Project; Tokyo Metropolitan Institute of Medical Science; Tokyo, Japan; 531Department of Biochemistry; Juntendo University School of Medicine; Tokyo, Japan; 532Biotechnology Research Division; National Fisheries Research and Development Institute (NFRDI); Busan, Korea; 533Department of Otorhinolaryngology; Union Hospital of Tongji Medical College; Huazhong Science and Technology University; Wuhan, China; 534Institute for Ageing and Health; Newcastle University; Newcastle upon Tyne
UK; 535Graduate School of Biomedical Sciences; Hiroshima University; Hiroshima, Japan; 536Department of Radiotherapy–Oncology; Democritus University of Thrace; Alexandroupolis, Greece; 537Max F. Perutz Laboratories; University of Vienna; Vienna, Austria; 538Massachusetts General Hospital; Harvard Medical School; Department of Neurology; MassGeneral Institute for Neurodegenerative Disease; Charlestown, MA USA; 539Departments of Neuroscience and Cell Biology; University of Texas Southwesten Medical Center; Dallas, TX USA; 540Centre de Génétique et de Physiologie Moléculaire et Cellulaire; CNRS-UMR5534; Université Lyon I; Villeurbanne; Université de Lyon; Lyon, France; 541Department of Neurology; Brigham and Women’s Hospital; Harvard Medical School; Boston, MA USA; 542Faculty of Medicine of the University of Paris Descartes; Paris, France; 543Institute for Medical Microbiology, Immunology and Hygiene; University of Cologne; Cologne, Germany; 544Signaling Program; Babraham Institute; Cambridge UK; 545Division of Developmental Biology; Cincinnati Children’s Hospital Research Foundation; Cincinnati, OH USA; 546Institute of Biochemistry and Biophysics; Polish Academy of Sciences; Warsaw, Poland; 547Department of Anatomical Sciences and Neurobiology; University of Louisville School of Medicine; Louisville, KY USA; 548Department of Basic Sciences; The Commonwealth Medical College; Scranton, PA USA; 549Department of Pathology; St. Jude Children’s Research Hospital; Memphis, TN USA; 550Department of Biochemistry & Molecular Medicine; University of California, Davis Cancer Center; Sacramento, CA USA; 551Department of Biotechnology; Yonsei University; College of Life Science & Biotechnology; Seoul, Korea; 552Departments of Pediatrics, Cellular & Molecular Medicine, and Neurosciences; and the Institute for Genomic Medicine; University of California, San Diego; La Jolla, CA USA; 553Rady Children’s Hospital; San Diego, CA USA; 554Center for Infection and Immunity of Lille; Institut Pasteur de Lille; CNRS UMR8204; INSERM U1019; University Lille Nord de France; Lille, France; 555Laval Universtity Cancer Research Center; Québec, Canada; 556School of Biochemistry; University of Bristol; University Walk; Bristol UK; 557Nuclear Organisation and Oncogenesis Unit; INSERM U993; Institut Pasteur; Paris, France; 558Department of Translational Medecine; Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC); INSERMU964; CNRS UMR7104; Université de Strasbourg; Illkirch, France; 559School of Biomedical Sciences; The University of Nottingham; Queen’s Medical Centre; Nottingham UK; 560Instituto de Biología Molecular y Celular del Cáncer; Consejo Superior de Investigaciones Científicas (CSIC); Universidad de Salamanca; Salamanca, Spain; 561Institute of Health Sciences; Shanghai Jiao Tong University School of Medicine; Chinese Academy of Sciences; Shanghai, China; 562Shanghai Institutes for Biological Sciences; Chinese Academy of Sciences; Shanghai, China; 563Translational Cancer Therapeutics Laboratory; Cancer Research UK London Research Institute; London, England UK; 564Department of Internal Medicine; Yonsei University College of Medicine; Seoul, Korea; 565Department of Psychiatry; Nathan Kline Institute for Psychiatric Research; New York University School of Medicine; Orangeburg, NY USA; 566Division of Life Sciences; University of Incheon; Incheon, Korea; 567Department of Medicine; Samsung Medical Center; Seoul, Korea; 568Department of Pathology; College of Medicine; The Catholic University of Korea; Seoul, South Korea; 569 Department of Aging and Geriatrics; Division of Biology of Aging; University of Florida; Gainesville, FL USA; 570University of Rennes-1; IRSET; INSERM U1085; Team DRaTE; Rennes, France; 571Centre de Génétique Moléculaire; CNRS UPR3404 Université Paris Sud; Gif-sur-Yvette, France; 572Department of Biological Sciences; University of Arkansas; Fayetteville, AR USA; 573Department of Microbiology and Immunology; College of Medicine; National Cheng Kung University; Tainan, Taiwan; 574Biomedical Sciences Institute; and Departments of Biochemistry and Molecular Biology; Shanghai Medical College; Fudan University; Shanghai, China; 575Department of Microbiology and Immunology; Dartmouth Medical School; Lebanon, NH USA; 576Universidad de Santiago de Compostela; Laboratorio de Parasitología; Instituto de Investigación y Análisis Alimentarios; La Coruña, Spain; 577Departments of Pharmaceutical & Biomedical Sciences and Biochemistry & Molecular Biology; Medical University of South Carolina; Charleston, SC USA; 578Department of Biochemistry and Molecular Biology; APHP; University of Paris 11; Inserm U1004; Villejuif, France; 579Department of Surgery and Cancer Biology; The University of Chicago; Chicago, IL USA; 580Department of Cancer Biology; University of Texas; MD Anderson Cancer Center; Houston, TX USA; 581Department of Radiation Oncology; Rush University Medical Center; Chicago, IL USA; 582Department of Microbiology; Howard Hughes Medical Institute; University of Texas Southwestern Medical Center; Dallas, TX USA; 583 Department of Pharmacology; Nathan Kline Institute for Psychiatric Research; New York University School of Medicine; Orangeburg, NY USA; 584Department of Genetics; University of Wisconsin; Madison, WI USA; 585Department of General Surgery; The Central Hospital of Yongzhou City; Hunan, China; 586Key Laboratory of Insect Developmental and Evolutionary Biology; Institute of Plant Physiology and Ecology; Shanghai, China; 587Department of Chemistry; Hanshan Normal University; Chaozhou, China; 588Department of Pharmacology; School of Basic Medical Sciences; Peking University; Beijing, China; 589Department of Endocrinology; The First Affiliated Hospital of Harbin Medical University; Harbin, China; 590Department of Integrative Biology and Pharmacology; University of Texas Health Science Center at Houston; Houston, TX USA; 591Department of Molecular Microbiology & Immunology; University of Southern California; Keck School of Medicine; Los Angeles, CA USA; 592Cardiovascular Health Research Center; Sanford Research; University of South Dakota; and University of South Dakota Sanford School of Medicine; Sioux Falls, SD USA; 593Institute of Cellular and Organismic Biology; Academia Sinica; Taipei, Taiwan; 594 Department of Molecular Pathololgy and Neuropathology; Medical University Lodz; Lodz, Poland; 595Department of Biomedical Science & Technology; Konkuk University; Seoul, Korea; 596Department of Physiology; Yong Loo Lin School of Medicine; National University of Singapore; Singapore; 597Department of Biochemistry; Cancer Research Institute and Infection Signaling Network Research Center; College of Medicine; Chungnam National University; Daejeon, Korea; 598Institute of Clinical Medicine; College of Medicine; National Cheng Kung University; Tainan, Taiwan; 599Biotechnology Institute; Zhejiang University; Hangzhou, China; 600Beijing NMR Center; College of Chemistry and Molecular Engineering; Peking University; Beijing, China; 601Department of Cancer Signaling; Genentech; South San Francisco, CA USA; 602Department of Molecular and Cellular Biology; Baylor College of Medicine; Houston, TX USA; 603Institute of Biophysics; Federal University of Rio de Janeiro; Rio de Janeiro, Brazil; 604The Eunice Kennedy Shriver National Institute of Child Health and Development; National Institutes of Health; Bethesda, MD USA; 605Department of Stem Cell Biology; Kimmel Cancer Center; Thomas Jefferson University; Philadelphia, PA USA; and Breakthrough Breast Cancer Research Unit; University of Manchester; Manchester UK; 606Department of Ophthalmology; Duke University; Durham, NC USA; 607Department of Neurology; Second Affiliated Hospital; and Institute of Neuroscience; Soochow University; Suzhou, China; 608College of Life Science; Central China Normal University; Wuhan, China; 609Center for Cancer and Stem Cell Biology; Institute of Biosciences and Technology; Texas A&M Health Science Center; Houston, TX USA; 610Department of Biochemistry and Cell Biology; Stony Brook University; Stony Brook, NY USA; 611Metabolism & Cancer Susceptibility Section; Basic Research Laboratory; Center for Cancer Research; NCI–Frederick; Frederick, MD USA; 612Division of Natural Science; College of Liberal Education; Shu-Te University; Taiwan; 613Department of Biology; Queens College and Graduate Center of CUNY; Flushing, NY USA; 614Departamento de Bioquímica y Biología Molecular; Instituto Universitario de Oncología; Universidad

de Oviedo; Oviedo, Spain; 615University of Turin; Turin, Italy; 616Department of Immunology; University of Pittsburgh; Pittsburgh, PA USA; 617Department of Pathology; Stanford University School of Medicine; Stanford, CA USA; 618Department of Radiation Oncology; Thomas Jefferson University & Hospitals; Philadelphia, PA USA; 619Department of Pathology; Albert Einstein College of Medicine; Bronx, NY USA; 620Laboratory of Systems Biology; Van Andel Research Institute; Grand Rapids, MI USA; 621Ben May Department for Cancer Research; University of Chicago; Gordon Center for Integrative Sciences; Chicago, IL USA; 622Institute for Molecular Biosciences; University of Graz; Graz, Austria; 623European Institute for Research in Cystic Fibrosis; San Raffaele Scientific Institute; Milan, Italy; 624Department of Biology; University of Modena and Reggio Emilia; Modena, Italy; 625Medical Genetics Branch and UDP Translational Medicine; National Human Genome Research Institute; National Institutes of Health; Bethesda, MD USA; 626San Raffaele Sulmona Institute; L'Aquila, Italy; 627Hefei National Laboratory for Physical Sciences at Microscale; and School of Life Sciences; University of Science & Technology of China; Hefei, Anhui China; 628DZNE (German Center for Neurodegenerative Diseases); and CAESAR Research Center; Bonn, Germany; 629Pediatric Research and Electron Microscopy Unit; Ruth and Bruce Rappaport Faculty of Medicine; Technion-Israel Institute of Technology; Haifa, Israel; 630Nanjing Agricultural University; College of Veterinary Medicine; Nanjing, China; 631Department of Neurology; Emory University School of Medicine; Atlanta, GA USA; 632The Feinstein Institute for Medical Research; Manhasset, NY USA; 633University of Ottawa Heart Institute; Ottawa, ON Canada; 634Department of Medical and Molecular Genetics; Kings College London; London UK; 635Department of Endocrinology and Metabolism; University of Pisa; Pisa, Italy; 636School of Clinical Medicine; Cambridge Institute for Medical Research (CIMR); University of Cambridge; Cambridge, UK; 637Department of Genomics; Agricultural Biotechnology Research Institute of Iran; Karaj, Iran; 638INSERM; U848; Institut Gustave Roussy; Villejuif (Paris), France; 639 Institute of Molecular Biology and Biotechnology; Foundation for Research and Technology-Hellas & Medical School; University of Crete; Heraklion, Crete Greece; 640Department of Genetics; The Smurfit Institute; Trinity College; Dublin, Ireland; 641University Montpellier 2; Institut des Sciences de l'Evolution; Montpellier, France; 642Department of Pharmacology; University of Antwerp; Antwerp, Belgium; 643Neurodegenerative Diseases Research Group; Vall d'Hebron Research Institute-CIBERNED; Barcelona, Spain; 644Department of General Pathology; University of Pisa; Pisa, Italy; 645National Institute of Health; Department of Therapeutic Research and Medicine Evaluation; Section of Cell Aging and Degeneration; Rome, Italy; 646Laboratory of Toxicology; Osaka Prefecture University Graduate School of Life and Environmental Biosciences; Izumisano, Japan; 647Department de Biochimie; Université de Lausanne; Lausanne, Switzerland; 648Institute of Developmental Biology and Cancer Research; University of Nice; CNRS-UMR 6543; Centre Antoine Lacassagne; Nice, France; 649Departments of Urology and Cancer Biology; University of Texas MD Anderson Cancer Center; Houston, TX USA; 650Malaghan Institute of Medical Research; Wellington, New Zealand; 651Department of Biomedical Science; Bond University; Robina, Queensland Australia; 652Department of Pathobiology; Lerner Research Institute; Cleveland Clinic; Cleveland, OH USA; 653Cork Cancer Research Centre; University College, Cork; Cork, Ireland; 654Department of Neurology; Vanderbilt University; Nashville, TN USA; 655Departments of Pediatrics and Biochemistry & Molecular Biology; The Atlantic Research Centre; Dalhousie University; Halifax, NS Canada; 656Department of Medical Biochemistry; Academic Medical Center; Amsterdam, The Netherlands; 657The Graduate Center of the City University of New York; New York, NY USA; 658Department of Cell Biology; Yale University; New Haven, CT USA; 659IDI-IRCCS Laboratory; University Tor Vergata; Rome, Italy; 660Department of Neurobiology; Hospital Ramon y Cajal; Madrid, Spain; 661Catalan Institute of Oncology (ICO); Girona Biomedical Research Institute (IDIBGi); Catalonia, Spain; 662Laboratório de Biologia Celular; Instituto Oswaldo Cruz–FIOCRUZ; Rio de Janeiro, RJ Brasil; 663Department of Medical Genetics; Cambridge Institute for Medical Research; University of Cambridge; Cambridge UK; 664Department of Hematology/Oncology; University of Cincinnati; Cincinnati, OH USA; 665Department of Biochemistry and Molecular Biology; Thomas Jefferson University; Philadelphia, PA USA; 666Bernhard-Nocht-Institut für Tropenmedizin; Tropenmedizinische Grundlagenforschung; Hamburg, Germany; 667Department of Pharmacology; Second Military Medical University; Shanghai, China; 668Institute of Developmental Biology; School of Life Science; Shandong University; Jinan, China; 669URBC-NARILIS; University of Namur–FUNDP; Namur, Belgium; 670Department of Molecular Immunology and Gene Therapy; Max-Delbrück-Center for Molecular Medicine; Berlin, Germany; 671Department of Human Pathology and Oncology; Section of Pathological Anatomy; University of Siena; Siena, Italy; 672Lab of Integrin Signaling; Van Andel Research Institute; Grand Rapids, MI USA; 673First Department of Internal Medicine; Democritus University of Thrace; Alexandroupolis, Greece; 674Department of Biochemistry; Tokyo Medical University; Tokyo, Japan; 675Department of Physiology and Cell Biology; Tokyo Medical and Dental University; Tokyo, Japan; 676Institute of Research on Cancer and Ageing of Nice (IRCAN); INSERM; CNRS; Université de Nice-Sophia Antipolis; Faculté de Médecine; Nice, France; and Equipe Labellisée par l; Villejuif, France; 677Department of Clinical and Experimental Medicine; Division of Cell Biology; Linköping University; Linköping, Sweden; 678Grup de Recerca en Patologia Pancreatica; Institut de Recerca Hospital Universitari Vall d'Hebron; Barcelona, Spain; 679Ecole Normale Supérieure de Lyon; Laboratory of Molecular Biology of the Cell; CNRS UMR 5239; Lyon; France; 680Center for Medical Science; International University of Health and Welfare; Tochigi, Japan; 681Department of Internal Medicine; University of Iowa; Iowa City, IA USA; 682Department of Anatomy and Neurobiology; University of Maryland; Baltimore, MD USA; 683Plymouth Marine Laboratory; Plymouth UK; 684Systems Biology of Personalized Therapy; Virology Department; Faculty of Microbiology and Centro de Investigación en Estructuras Microscópicas CIEMIC; University of Costa Rica; Costa Rica; 685Department of Regulatory Biology; Graduate School of Science and Engineering; Saitama University; Saitama, Japan; 686Section of Microbiology; Centre for Molecular Microbiology and Infection; Imperial College London; London UK; 687Wellcome Trust Centre for Molecular Parasitology; Institute of Infection; Immunity and Inflammation; College of Medical, Veterinary and Life Sciences; University of Glasgow; Glasgow UK; 688Department of Neuroscience; Georgetown University Medical Center; Washington D.C. USA; 689Division of Infectious Diseases; Brigham and Women's Hospital and Department of Medicine Harvard Medical School; Boston, MA USA; 690Viral Immunobiology; Institute of Experimental Immunology; University of Zürich; Zürich, Switzerland; 691Novartis Institutes for Biomedical Research; Cambridge, MA USA; 692Molecular and Cellular Oncogenesis; The Wistar Institute; Philadelphia, PA USA; 693DAHFMO-Unit of Histology and Medical Embryology; Sapienza University of Rome; Rome, Italy; 694Department of Obstetrics and Gynecology; Washington University School of Medicine; St. Louis, MO USA; 695Department of Neurology; Tokai University School of Medicine; Kanagawa, Japan; 696Laboratory of Molecular and Cellular Biology; Faculty of Life Sciences; Kyoto Sangyo University; Kyoto, Japan; 697Central Laboratory of Hematology; University Hospital of Lausanne; Lausanne, Switzerland; 698Department of Neurobiology; Gifu University Graduate School of Medicine; Gifu, Japan; 699Department of Immunology; Juntendo University School of Medicine; Tokyo, Japan; 700Frontier Research Center; Tokyo Institute of Technology; Yokohama, Japan; 701Department of Cell Biology, Microbiology, and Molecular Biology; University of South Florida; Tampa, FL USA; 702Biochemistry Section; Surgical Neurology Branch; National Institute of Neurological Disorders and Stroke; National Institutes of Health; Bethesda, MD USA; 703Cancer Research UK; Cambridge Research Institute; Li Ka Shing Centre; Cambridge UK; 704Department of Medicine; Radboud University Nijmegen Medical Centre; Nijmegen, The Netherlands; 705Department of Genetics, Cell Biology & Development; University of Minnesota; Minneapolis, MN USA; 706Department of Cell & Molecular Biology; Lindsley F. Kimball Research Institute; New York Blood Center; New York, NY USA; 707Department of Medical Genetics; University of Tuebingen; Tuebingen, Germany; 708Department of Medical Microbiology, Immunology, and Cell Biology; Southern Illinois University School of Medicine; Simmons Cancer Institute; Springfield, IL USA; 709Department of Neuromuscular Research; National Institute of Neuroscience; National Center of Neurology and Psychiatry (NCNP); Kodaira, Tokyo Japan; 710Donnelly Centre; Banting and Best Department of Medical Research and the Department of Molecular Genetics; University of Toronto; Toronto; ON Canada; 711Department of Neuroscience; College of Medicine; McKnight Brain Institute of the University of Florida; Gainesville, FL USA; 712School of Medicine; University of Split; Split, Croatia; 713Department of Parasitology; National Institute of Infectious Diseases; Tokyo, Japan; and University of Tsukuba; Ibaraki, Japan; 714Laboratory for Structural Neuropathology; RIKEN Brain Science Institute; Wako, Saitama Japan; 715Center for Plant Molecular Biology; Eberhard-Karls-University; Tübingen, Germany; 716Departments of Pathology & Laboratory Medicine; University of Wisconsin; Madison; WI USA; 717Department of Physiology; South Texas Research Facility; University of Texas Health Science Center; San Antonio, TX USA; 718Department of Microbiology and Immunology; Institute of Medical Science; University of Tokyo; Tokyo, Japan; 719Department of Gene Therapy; Institute of DNA Medicine; The Jikei University School of Medicine; Tokyo, Japan; 720Department of Pediatrics; The Jikei University School of Medicine; Tokyo, Japan; 721Laboratory of Mitochondrial Dynamics; Graduate School of Frontier Biosciences; Osaka University; Osaka, Japan; 722Department of Radiation Oncology; Case Western Reserve University; Cleveland, OH USA; 723Department of Biochemistry; and Environmental Health Sciences; Case Western Reserve University; Cleveland, OH USA; 724University of Copenhagen; Department of Agriculture and Ecology; Frederiksberg C, Denmark; 725Ecole Polytechnique Fédérale de Lausanne; School of Life Sciences; Global Health Institute; Lausanne, Switzerland; 726Sanford-Burnham Medical Research Institute at Lake Nona; Orlando FL USA; 727Pacific Biosciences Research Center; University of Hawaii; Honolulu, HI USA; 728Cardiovascular Division; King's College London; London UK; 729AC Immune SA; Ecole Polytechnique Fédérale de Lausanne (EPFL); Lausanne, Switzerland; 730INSERM U775; Université Paris Descartes; Paris, France; 731Department of Microbiology Immunology and Parasitology; Louisiana State University Health Sciences Center; School of Medicine; New Orleans, LA USA; 732Department of Neurology; Baylor College of Medicine; Houston, TX USA; 733Department of Genetics; Louisiana State University Health Sciences Center; New Orleans, LA USA; 734Department of Cell Biology and Biophysics; University of Athens; Panepistimiopolis Zografou, Athens Greece; 735Molecular & Clinical Pharmacology; Faculty of Medicine; University of Chile; Santiago, Chile; and Santo Tomas University; Viña del Mar, Chile; 736Division of Biological Science and Technology; Yonsei University; Wonju, Korea; 737Departmenet of Internal Medicine; University of Goettingen; Göttingen, Niedersachsen Germany; 738UNC Mcallister Heart Institute; University of North Carolina at Chapel Hill; Chapel Hill, NC USA; 739Department of Dermatology and the Yale Cancer Center; Yale School of Medicine; New Haven, CT USA; 740Institute of Entomology; Central China Normal University; Wuhan, China; 741Department of Pediatrics; University of Pittsburgh School of Medicine and Children's Hospital of Pittsburgh; Pittsburgh, PA USA; 742Department of Ecology; University of Calabria; Rende (CS), Italy; 743Department of Biology; College of Sciences; University of Texas at San Antonio; San Antonio, TX USA; 744Cancer and Stem Cell Biology Program; Duke-NUS Graduate Medical School; Singapore; 745Institute of Biochemistry; ETH Zurich; Zurich, Switzerland; 746Department of Medical Oncology; VU University Medical Center; Amsterdam, The Netherlands; 747Department of Biology; University of Copenhagen; Copenhagen, Denmark; 748Departments of Ophthalmology and Biochemistry and Molecular Biology; University of

Debrecen; Medical and Health Science Center; Debrecen, Hungary; 749GéPITOs UMR 6235 CNRS; Faculté de Médecine; Université de Nice Sophia Antipolis; Nice, France; 750CNRS; UMR 8122; Institut Gustave Roussy; Villejuif, France; 751Department of Neurobiology; Tel-Aviv University; Ramat-Aviv, Tel-Aviv Israel; 752Università degli Studi di Torino; Neuroscience Institute Cavalieri-Ottolenghi (NICO); Orbassano (TO), Italy; 753Jules Stein Eye Institute; University of California Los Angeles; Los Angeles, CA USA; 754Robert H. Lurie Comprehensive Cancer Center and Division of Hematology/Oncology; Northwestern University Medical School; Chicago, IL USA; 755Institute of Microbiology and Genetics; Department Genetics of Eukaryotic Microorganisms; Georg-August-University; Goettingen, Germany; 756University Toulouse III; CRCT; Institut Claudius Regaud; Toulouse, France; 757Universita' degli Studi di Milano; Dipartimento di Endocrinologia; Fisiopatologia e Biologia Applicata; Centro di Eccellenza sulle Malattie Neurodegenerative; Universita' degli Studi di Milano; Milano, Italy; 758EA4530; Dynamique des microtubules en physiopathologie; Université Paris-Sud 11; Chatenay-Malabry, France; 759Centro Andaluz de Biología Molecular y Medicina Regenerativa; CSIC; Sevilla, Spain; 760Department of Molecular and Cellular Biochemistry; The Ohio State University; Columbus, OH USA; 761Division of Experimental Medicine; Beth Israel Deaconess Medical Center; Harvard Medical School; Boston, MA USA; 762Institute for Research in Ophthalmology (IRO); Sion, Switzerland; 763Center for Molecular Microbiology and Department of Oral Biology; University of Florida; Gainesville, FL USA; 764Department of Molecular Biology; University of Tuebingen; Tuebingen, Germany; 765Center for Motor Neuron Biology and Disease; Columbia University; New York, NY USA; 766Cardiovascular Research Institute and Departments of Physiology & Emergency Medicine; Wayne State University School of Medicine; Detroit, MI USA; 767Laboratory of Cell Biology; National Heart, Lung, and Blood Institute; National Institutes of Health; Bethesda, MD USA; 768Division of Nutritional Sciences; Cornell University; Ithaca, NY USA; 769Institute of Basic Medical Sciences & School of Basic Medicine; Chinese Academy of Medical Sciences and Peking Union Medical College; Departments of Physiology & Pathology; Beijing, China; 770Department of Pharmacology; Soochow University School of Pharmaceutical Science; Suzhou, China; 771The Samuel Lunenfeld Research Institute; University of Toronto; Toronto, ON Canada; 772Arthritis and Rheumatism Branch; National Institute of Arthritis and Musculoskeletal and Skin Diseases; National Institutes of Health; Bethesda, MD USA; 773Department of Medicine; University of British Columbia; Vancouver, BC Canada; 774Department of Environmental Medicine; Lung Biology and Disease Program; University of Rochester Medical Center; Rochester, NY USA; 775Institute of Cellular and Molecular Anatomy; Clinics of the JWG-University; Frankfurt, Germany; 776Monash Micro Imaging; Monash University; Clayton Campus; Melbourne, Victoria; Australia; 777Department of Microbiology; The University of Chicago; Chicago, IL USA; 778MRC Laboratory of Molecular Biology; Cambridge UK; 779Laboratory of Biochemistry; Center for Drug Evaluation and Research; Food and Drug Administration; Bethesda, MD USA; 780Department of Pharmacology and Cancer Biology; Duke University Medical Center; Durham, NC USA; 781Neurodegenerative Disease Research; EMD Serono Research Institute, Inc.; Billerica, MA USA; 782Department of Pathology, Microbiology, and Immunology; University of South Carolina School of Medicine; Columbia, SC USA; 783Department of Biology; University of Waterloo; Waterloo, ON Canada; 784Division of Tumor Virology; German Cancer Research Center; Heidelberg and Department of Biology; University of Mainz; Mainz, Germany; 785Mitochondrial Biology; Frankfurt Institute for Molecular Life Sciences; and Mitochondriale Biologie; Zentrum für Molekulare Medizin; Goethe Universität Frankfurt am Main; Frankfurt am Main, Germany; 786Institute of Environmental Health Sciences; Wayne State University; Detroit, MI USA; 787Pharmacology; University of Wyoming College of Health Sciences; Laramie, WY USA; 788Kovler Diabetes Center; University of Chicago; Chicago, IL USA; 789Departamento de Morfologia; Universidade Federal de Minas Gerais; Minas Gerais, Brasil; 790Department of Pediatrics; Cincinnati Children's Hospital; Cincinnati, OH USA; 791Department of Biochemistry and Molecular Biology; University of British Columbia; Vancouver, BC Canada; 792Department of Urology; Comprehensive Cancer Center; University of Michigan; Ann Arbor, MI USA; 793Service de Dermatologie; Hôpital Archet II; CHU Nice, France; 794Centro de Investigaciones Biológicas (CSIC) and Ciber de Enfermedades Raras; Madrid, Spain; 795Ophthalmology, and Neurosciences Division; Medical University of South Carolina; Charleston, SC USA; 796Department of Pharmaceutical and Biomedical Sciences; South Carolina College of Pharmacy; University of South Carolina; Columbia, SC USA; 797Department of Animal Histology and Embryology; University of Silesia; Katowice, Poland; 798Unité de Recherche 04; Vieillissement; Stress et Inflammation; Université Pierre et Marie Curie (UPMC); Paris, France; 799Department of Radiation Oncology (Maastro Lab); GROW School for Oncology & Developmental Biology; Maastricht University; Maastricht, The Netherlands; 800Departments of Molecular Genetics and Anatomic Pathology; Cleveland Clinic; Lerner Research Institute; Taussig Cancer Center; Cleveland, OH USA; 801Institute for Medical Microbiology, Virology and Hygiene; University Medical Center Eppendorf; Hamburg, Germany; 802Dearptment of Clinical Biochemisty and National Institute of Biotechnology Negev; Ben-Gurion University; Beer-Sheva, Israel; 803Department of Medical Biology and Genetics; Faculty of Medicine in Hradec Králové; Charles University; Hradec Králové, Czech Republic; 804Boston University; School of Medicine; Section of Molecular Medicine; Boston, MA USA; 805Department of Pharmacobiology; University of Calabria; Arcavacata di Rende; Italy; 806Department of Biology; Massachusetts Institute of Technology; Whitehead Institute; Cambridge, MA USA; 807Whitehead Institute; Cambridge, MA USA; 808Department of Cell Biology and Molecular Medicine; Cardiovascular Research Institute; University of Medicine and Dentistry of New Jersey–New Jersey Medical School; Newark, NJ USA; 809Department of Diagnostic and Therapeutic Sciences; Meikai University School of Dentistry; Sakado, Japan; 810Division of Applied Life Sciences; Graduate School of Agriculture; and Research Unit for Physiological Chemistry; the Center for the Promotion of Interdisciplinary Education and Research; Kyoto University; Kyoto, Japan; 811Department of Systems Biology; Agricultural Biotechnology Research Institute of Iran; Karaj, Iran; 812Samantha Dickson Brain Cancer Unit; UCL Cancer Institute; London UK; 813Division of Neuroscience; Beckman Research Institute of the City of Hope; Duarte, CA USA; 814Department of Hematology, Oncology and Molecular Medicine; Istituto Superiore di Sanità; Rome, Italy; 815Centro Andaluz de Biología del Desarrollo (CABD); Universidad Pablo de Olavide-Consejo Superior de Investigaciones Científicas-Junta de Andalucía; Sevilla, Spain; 816Laboratorio de Oncologia Molecular; CRIB-PcyTA; Universidad de Castilla la Mancha; Albacete, Spain; 817Dulbecco Telethon Institute; 818Venetian Institue of Molecular Medicine; Padova, Italy; 819Department of Pharmacology and Toxicology; James Graham Brown Cancer Center and Owensboro Cancer Research Program; University of Louisville; Louisville, KY USA; 820Department of Pediatrics; Stanford University; Stanford, CA USA; 821Department of Immunology and Microbiology; Albert Einstein College of Medicine; Bronx, NY USA; 822Jawaharlal Nehru University; School of Life Sciences; New Delhi, India; 823Departments of Immunology and Surgery; Stanford University; Stanford, CA USA; 824Department of Biochemistry and Biophysics; Vilnius University; Vilnius, Lithuania; 825Laboratory of Molecular Traffic; Institute for Molecular and Cellular Regulation; Gunma University; Maebashi, Gunma Japan; 826Division of Gastroenterology and Hepatology; University Hospital Zürich; Zürich, Switzerland; 827Departments of Veterinary Sciences and Molecular Biology; University of Wyoming; Laramie, WY USA; 828Departments of Genome Analysis and Neurology; Academic Medical Center; Amsterdam, The Netherlands; 829Sektion Experimentelle Anästhesiologie; Universitätsklinikum Ulm; Ulm, Germany; 830Experimental Tumor Pathololgy; Institute of Pathology; University of Erlangen-Nürnberg; Erlangen, Germany; 831Department of Cellular Biology and Anatomy; Medical College of Georgia; Augusta, GA USA; 832Ecole Polytechnique Fédérale de Lausanne (EPFL); Lausane, Switzerland; 833DAGZ; Department of Applied Genetics and Cell Biology; University of Natural Resources and Life Sciences Vienna (BOKU); Vienna, Austria; 834Memorial Hospital Research Laboratories; Memorial Sloan-Kettering Cancer Center; Medicine; New York, NY USA; 835Department of Cell Physiology and Medicine; University of Geneva; Geneva, Switzerland; 836Department of Molecular Physiology and Biophysics; and Department of Cancer Biology; Vanderbilt University School of Medicine; Nashville, TN USA; 837Department of Cell Biology; Institute for Cancer Research; Rikshospitalet-Radiumhospitalet HF; Montebello, Oslo Norway; 838Molecular & Clinical Pharmacology; Faculty of Medicine; University of Chile; Santiago, Chile; 839INRA; UR1067 Nutrition Métabolisme et Aquaculture; Saint-Pée-sur-Nivelle, France; 840Department of Surgery; St. Jude Children's Research Hospital; Memphis, TN USA; 841Department of Pathology; Drexel University College of Medicine; Philadelphia, PA, USA; 842Integrating Proteomics & Metabolomics Research Team; Korea Basic Science Institute; Seoul, Korea; 843Department of Pharmaceutical Sciences; Wayne State University College of Pharmacy; Detroit, MI USA; 844Department of Dermatology; University of Wisconsin School of Medicine & Public Health; Madison, WI USA; 845The Near-Future Locomoter Organ Medicine Creation Course (Kusunoki Kai); Graduate School of Medical and Dental Science; Kagoshima University; Kagoshima, Japan; 846Department of Pathology; University of Alabama at Birmingham; Birmingham, AL USA; 847Department of Orthopaedic Surgery; Jefferson Medical College; Thomas Jefferson University; Philadelphia, PA USA; 848Department of Biochemistry and Molecular Biology; IMRIC; The Hebrew University-Hadassah Medical School; Jerusalem, Israel; 849Howard Hughes Medical Institute; Molecular and Cell Biology Laboratory; The Salk Institute for Biological Studies; La Jolla, CA USA; 850Critical Care Medicine Department; Clinical Center; National Institutes of Health; Bethesda, MD USA; 851Department of Epidemiology and Public Health; Yong Loo Lin School of Medicine; National University of Singapore; Singapore; 852University of Southern California School of Pharmacy; Los Angeles, CA USA; 853Synaptic Function Section; National Institute of Neurological Disorders and Stroke; National Institutes of Health; Bethesda, MD USA; 854Division of Pediatric Surgery; Department of Surgery; Medical College of Wisconsin; Milwaukee, WI USA; 855National Institute of Floricultural Science; National Agriculture and Food Research Organization (NARO); Tsukuba, Japan; 856Laboratory of Molecular and Cellular Biology; Graduate School of Human Health Science; University of Nagasaki; Nagayo, Nagasaki Japan; 857Institute of Biomedical Sciences; College of Life Sciences; National Chung Hsing University; Taichung, Taiwan; 858Department of Biochemistry; School of Medicine; Taipei Medical University; Taipei, Taiwan; 859Department of Pathological Cell Biology; Tokyo Medical and Dental University; Tokyo, Japan; 860Laboratory of Bioindustrial Genomics; Graduate School of Agricultural Science; Tohoku University; Miyagi, Japan; 861Department of Pharmacology and Experimental Therapeutics; Tufts University School of Medicine; Boston, MA USA; 862Department of Biochemistry and Goodman Cancer Center; McGill University; Montreal, QB Canada; 863Institute of Cell Biology; National Academy of Sciences of Ukraine; Lviv, Ukraine; and University of Rzeszow; Rzeszow, Poland; 864Cancer Genetics; Kolling Institute of Medical Research; University of Sydney and Royal North Shore Hospital; New South Wales, Australia; 865MRC Human Immunology Unit; Weatherall Institute of Molecular Medicine; University of Oxford; Oxford UK; 866Nuffield Department of Medicine; Oxford, UK; 867Institute of Pharmacology; University of Bern; Bern, Switzerland; 868Laboratory of Signal-Dependent Transcription; DTP; Consorzio Mario Negri Sud; Santa Maria Imbaro (Ch); Italy; and IRCCS "Saverio de Bellis"; Castellana Grotte (Ba), Italy; 869Department of Biochemistry; Institute of Basic Medical Sciences; University of Oslo; Oslo, Norway; 870Department of Genetics; Harvard Medical School; Boston, MA USA; 871Department of Medicine and Diabetes Research Center; Albert Einstein College of Medicine; Bronx, NY USA; 872Wilmer Eye Institute; Johns Hopkins University School of Medicine; Baltimore, MD USA; 873Division of Oncology; Mayo Clinic and Mayo Cancer

Center; Rochester,MN USA; 874Department of Health Technology and Informatics; Hong Kong Polytechnic University; Hung Hom; Kowloon, Hong Kong China; 875Department of Biochemistry and Microbiology; Institute of Chemical Technology; Prague, Czech Republic; 876A.N. Belozersky Institute of Physico-Chemical Biology; Moscow State University; Moscow, Russia; 877Department of Pharmacology; Federal University of São Paulo; School of Medicine; São Paulo, Brazil; 878Institute of Molecular Biosciences and Center for Emerging and Neglected Infectious Diseases; Mahidol University; Thailand; 879Melanoma Laboratory; Spanish Cancer Research Centre; Madrid, Spain; 880Department of Biochemistry; University of Geneva; Geneva, Switzerland; 881Department of Neurology; The Second Hospital of Hebei Medical University; Institute of Cardiocerebrovascular Disease; and Neurological Laboratory of Hebei Province; Shijiazhuang, Hebei China; 882Departments of Gynecologic Oncology and Cancer Biology; Center for RNA Interference and Non-Coding RNA; MD Anderson Cancer Center; Houston, TX USA; 883Pediatric Infectious Disease; University of California, San Diego; La Jolla, CA USA; and Rady Children's Hospital; San Diego, CA USA; 884Department of Anaesthesiology and Intensive Care Medicine; Campus Virchow-Klinikum and Campus Charité Mitte; Charité-Universitaetsmedizin Berlin; Berlin, Germany; 885Department of Neuroscience; Mayo Clinic; Jacksonville, FL USA; 886Laboratory of Immune Cell Biology; National Cancer Institute; National Institutes of Health; Bethesda, MD USA; 887Laboratory of Neurodegenerative Diseases; Biomedical Research Foundation of the Academy of Athens; Athens, Greece; 888Department of Psychiatry and Human Behavior; University of California, Irvine; Irvine, CA USA; 889Department of Neurosurgery; Charité–University Medicine Berlin; Berlin, Germany; 890Division of Medicine; University College London; UK; 891Nanotechnology Characterization Laboratory; Advanced Technology Program; SAIC-Frederick, Inc.; NCI–Frederick; Frederick, MD USA; 892Laboratory of Translational Research; Clinical Research; National Cancer Institute of Brazil; Rio de Janeiro, Brazil; 893Institute of Molecular Medicine; Heinrich-Heine-University; Düsseldorf, Germany; 894Departments of Ophthalmology and Medicine; Case Western Reserve University School of Medicine; Institute of Pathology; Cleveland, OH USA; 895Department of Colorectal Surgery and Key Laboratory of Biotherapy of Zhejiang Province; Sir Run Run Shaw hospital; Zhejiang University; Hangzhou, Zhejiang China; 896Department of Psychiatry; Columbia University Medical Center; New York, NY USA; 897Department of Microbiol Immunology; University of Texas Medical Branch; Galveston, TX USA; 898The State Key Laboratory Breeding Base of Basic Science of Stomatology (Hubei-MOST); Key Laboratory of Oral Biomedicine Ministry of Education; School & Hospital of Stomatology; Wuhan University; Wuhan, China; 899Institute of Digestive Diseases; Li Ka Shing Institute of Health Science and Department of Medicine & Therapeutics; The Chinese University of Hong Kong; Hong Kong, China; 900Bioimaging Center; Graduate School of Frontier Sciences; University of Tokyo; Chiba, Japan; 901Department of Molecular Bacteriology and Immunology; Graduate School of Medicine; University of the Ryukyus; Okinawa, Japan; 902Department of Microbiology and Immunology; University of Michigan; Ann Arbor, MI USA; 903Department of Biology; University of York; Heslington, York UK; 904Department of Cell & Developmental Biology; Consortium for Mitochondrial Research; University College London; London UK; 905Division of Cardiology; Department of Medicine; The University of Texas Medical School at Houston; Houston, TX USA; 906Department of Experimental Medicine; University of Rome "Sapienza"; Rome, Italy; 907Department of Genetics; Eötvös Loránd University; Budapest, Hungary; 908Laboratory of Plant Pathology; Graduate School of Agriculture; Kyoto University; Kyoto, Japan; 909Department of Bioscience & Biotechnology; Kyushu University; Kyushu, Japan; 910Department of Molecular Biodefense Research; Yokohama City University Graduate School of Medicine; Yokohama, Japan; 911School of Biosciences; University of Exeter; Exeter UK; 912Department of Microbiology; Yong Loo Lin School of Medicine; National University of Singapore; Singapore; 913Department of Molecular Oncology; Institute of Development, Aging and Cancer; Tohoku University; Miyagi, Japan; 914State Key Laboratory of Plant Cell and Chromosome Engineering; Chinese Academy of Sciences; Beijing, China; 915Institute of Genetics and Developmental Biology; Chinese Academy of Sciences; Beijing, China; 916Laboratory of Biomembranes; Department of Biochemistry and Cell Biology; National Institute of Infectious Diseases; Tokyo, Japan; 917Laboratory of Experimental Therapeutics and Molecular Imaging; Neuroscience Center; Massachusetts General Hospital and Harvard Medical School; Charlestown, MA USA; 918CRUK Institute for Cancer Studies; University of Birmingham; Birmingham UK; 919Departments of Medicine; Molecular Genetics and Microbiology; Division of Geriatrics; and Center for the Study of Aging and Human Development; Duke University Medical Center; Durham, NC USA; and GRECC; VA Medical Center; Durham, NC USA; 920Developmental Neurobiology; St. Jude Children's Research Hospital; Memphis, TN USA; 921Department of Clinical Pathology and Cytology; Karolinska University Hospital in Huddinge; Stockholm, Sweden; 922Department of Biotechnology and Life Sciences; University of Insubria; Varese, Italy; 923Centre of Microbial and Plant Genetics (CMPG); Katholieke Universiteit Leuven; Leuven, Belgium; 924Department of Pharmacology; University of Colorado School of Medicine; Denver, CO USA; 925Department Biochemistry II; Georg-August-Universitaet; Goettingen, Germany; 926Department of Neurology; Okayama University Medical School; Okayama City, Japan; 927Department of Biochemistry; University of Cambridge; Cambridge UK; 928Secretory Pathways Laboratory; Cancer Research UK London Research Institute; London, England UK; 929Faculty of Life Sciences; University of Manchester; Manchester, Lancashire UK; 930Molecular and Computational Biology Program; University of Southern California; Los Angeles, CA USA; 931Institute of Microbiology and Immunology; School of Medicine; University of Belgrade; Belgrade, Serbia; 932Laboratory of Respiratory Phisiology; Carlos Chagas Filho Biophysics Institute; Federal University of Rio de Janeiro; Rio de Janeiro, Brasil; 933Department of Life Sciences and Institute of Genome Sciences; National Yang-Ming University; Taipei, Taiwan; 934Experimental Oncology/Hematology; Departments of Clinical Research and Medical Oncology; Inselspital; Bern University Hospital; Bern, Switzerland; 935Laboratory of Immunology; Graduate School of Biomedical Sciences; Tokyo Medical and Dental University; Tokyo, Japan; 936Division of Transplantation and Division of Surgical Oncology; University of Pittsburgh Medical Center; Pittsburgh, PA USA; 937Department of Biochemistry and Molecular and Structural Biology; J. Stefan Institute; Ljubljana, Slovenia; 938Department of Biology; Francis Marion University; Florence, SC USA; 939Department of Physiology and Biophysics School of Medicine; University of Louisville; Louisville, KY USA; 940Department of Neurology; Jungers Center for Neurosciences Research; Oregon Health & Science University; Portland, OR USA; 941IRCCS Casa Sollievo della Sofferenza; CSS–Mendel Laboratory; San Giovanni Rotondo; and University of Messina; Messina, Italy; 942Mann Laboratory; Department of Plant Sciences; University of California; Davis, CA USA; 943Microbiology and Immunology Departments; University of Colorado School of Medicine; Aurora, CO USA; 944Departments of Molecular and Cell Biololgy and Immunology/Surgery; VU University Medical Center; Amsterdam, The Netherlands; 945Liver Cell Biology Laboratory; Department of Cell Biology; Vrije Universiteit Brussel; Brussels, Belgium; 946Molecular Signalling and Cell Death Unit; Department for Molecular Biomedical Research; VIB; Ghent University; Ghent, Belgium; 947Department of Neurology; University Hospitals Leuven; Leuven, Belgium; 948Hospital Clínic; Division of Gastroenterology; CiberEHD; Barcelona; Catalonia Spain; 949Department of Biochemistry and Molecular Biology I; Complutense University; School of Biology; Madrid, Spain; 950Department of Biochemistry and Molecular Biology; Autonomous University of Barcelona (UAB); and Catalan Institution for Research and Advanced Studies (ICREA); Barcelona, Spain; 951INSERM U1048; Toulouse, France; 952Department of Pediatrics; Laboratory of Oncohematology; University of Padova; Padova, Italy; 953Experimental Neurorehabilitation Lab; IRCSS Santa Lucia Foundation; Rome, Italy; 954Laboratory of Plant Ecological Physiology; Komarov Botanical Institute; Russian Academy of Sciences; St. Petersburg, Russia; 955Visual Neuroscience & Molecular Biology Group; School of Optometry & Vision Sciences; Cardiff University; Cardiff UK; 956Texas A&M Institute for Biosciences and Technology; Houston, TX USA; 957Molecular Biology & Biochemistry; University of California, Irvine; Irvine, CA USA; 958Department of Neurology; University of Bonn; Bonn, Germany; 959Department of Medical Oncology; the 3rd Affiliated Hospital of Sun Yatsen University; Guangzhou, China; 960The Cleveland Clinic Foundation; Biomedical Engineering; Cleveland; OH USA; 961Department of Stem Cell Biology & Regenerative Medicine; Thomas Jefferson University; Philadelphia, PA USA; 962Department of Cell Biology; Duke University Medical Center; Durham, NC USA; 963Department of Emergency Medicine; North Shore University Hospital; Manhasset, NY USA; 964Institute of Biotechnology and Department of Life Science; National Tsing Hua University; HsinChu, Taiwan; 965Department of Dermatology; University of Texas Southwestern Medical Center; Dallas, TX USA; 966Protein Quality Control and Degradation Research Center; Division of Basic Biomedical Sciences; Sanford School of Medicine of the University of South Dakota; Vermillion, SD USA; 967Department of Environmental and Occupational Health; National Cheng Kung University; Medical College; Tainan, Taiwan; 968Institute of Medicinal Biotechnology; Chinese Academy of Medical Sciences & Peking Union Medical College; Beijing, China; 969Department of Cancer Biology; Dana-Farber Cancer Institute; Harvard Medical School; Boston, MA USA; 970Department of Cardiothoracic Surgery; Changzheng Hospital; The Second Military Medical University; Shanghai, China; 971Department of Cell Biology; Nijmegen Centre for Molecular Life Sciences Radboud University Nijmegen Medical Centre; Nijmegen, The Netherlands; 972Department of Pathology; University of Utah; School of Medicine; Salt Lake City, UT USA; 973Juntendo University Graduate School of Medicine; Department of Metabolism and Endocrinology; Tokyo, Japan; 974Randall Division for Cell and Molecular Biophysics; Kings College; London UK; 975House Research Institute; Los Angeles, CA USA; 976Tumor Immunology and Gene Therapy Center; Eastern Hepatobiliary Hospital; Second Military Medical University; Shanghai, China; 977Department of Neurology; Washington University School of Medicine; Saint Louis, MO USA; 978School of Life Science; University of Science & Technology of China; Hefei, Anhui China; 979MRC Centre for Developmental and Biomedical Genetics; Department of Biomedical Science; University of Sheffield; Sheffield UK; 980University of East Anglia; Faculty of Health; Biomedical Research Centre; Norwich, East Anglia UK; 981Edinburgh CRUK Research Centre; Institute of Genetics and Molecular Medicine; University of Edinburgh; Edinburgh UK; 982Institute of Physical Biology; Heinrich-Heine-Universität; Düsseldorf, Germany; and Institute of Complex Systems; ICS-6; Research Centre Jülich; Jülich, Germany; 983Laboratory of Clinical Infectious Diseases; National Institute of Allergy and Infectious Diseases; National Institutes of Health; Bethesda, MD USA; 984Hypoxia and Microenvironment Program; Ontario Cancer Institute/Princess Margaret Hospital; University Health Network; Departments of Radiation Oncology and Medical Biophysics; University of Toronto; ON Canada; 985The Second Affiliated Clinical College of Fujian University of Traditional Chinese Medicine; Fuzhou, China; 986Key Laboratory of Biomedical Information Engineering of Ministry of Education; School of Life Science and Technology; Xi'an Jiaotong University; Xi'an, China; 987School of Biological Sciences; Life Sciences Building; Highfield Campus; University of Southampton; Southampton UK; 988Center for Computational and Integrative Biology and Gastrointestinal Unit; Massachusetts General Hospital; Harvard Medical School; Boston, MA USA; 989Department of Urology; Peking University First Hospital; Beijing, China; 990Centenary Institute and Sydney Medical School University of Sydney; Sydney, Australia; 991State Key Laboratory of Virology; Wuhan Institute of Virology; Chinese Academy of Sciences; Wuhan, China; 992School of Medicine; Nankai University; Tianjin, China; 993Section of Molecular Medicine; Department of Medicine; University of Oklahoma Health Sciences Center; Oklahoma City, OK US; 994State Key Laboratory of

Oncology in South China; Department of Gastric and Pancreatic Surgery; Cancer Center; Sun Yat-sen University; Guangzhou, China; 995College of Bioengineering; Henan University of Technology; Zhengzhou, China; 996Departments of Molecular Biosciences, Urology and Radiation Oncology; University of Kansas; Lawrence, KS USA; 997Department of Biochemistry and Molecular Biology; Mayo Clinic College of Medicine; Rochester, MN USA; 998Department of Cell Biology; Nagahama Institute of Bio-Science and Technology; Nagahama, Japan; 999Department of Gastroenterology; Juntendo University School of Medicine; Tokyo, Japan; 1000National Research Institute of Fisheries Science; Yokohama, Japan; 1001College of Animal Sciences and Technology; Huazhong Agricultural University; Wuhan, China; 1002OIST Promotion Corporaton; Okinawa, Japan; 1003Department of Pediatrics; Vanderbilt Ingram Cancer Center; Vanderbilt University School of Medicine; Nashville, TN USA; 1004Division of Surgery & Interventional Science; University College London; London UK; 1005Weis Center for Research; Geisinger Clinic; Danville, PA USA; 1006Institute of Molecular Biology; Academia Sinica; Taipei, Taiwan; 1007Department of Pathology and Laboratory Medicine; Indiana University School of Medicine; Indianapolis, IN USA; 1008BioMedical Research Center; Graduate School of Medical Science and Engineering; Korea Advanced Institute of Science and Technology; Daejeon, Korea; 1009Department of Biochemistry; Ajou University School of Medicine; Suwon, Korea; 1010Department of Life Sciences; Graduate School of Arts and Sciences; University of Tokyo; Tokyo, Japan; 1011School of Food and Nutritional Sciences; University of Shizuoka; Shizuoka, Japan; 1012RIKEN; Plant Science Center; Tsurumi-ku, Yokohama Japan; 1013Department of Pharmacology; DNA Repair Research Center; Chosun University School of Medicine; Gwangju, Korea; 1014Department of Lymphoma/Myeloma; MD Anderson Cancer Center; Houston, TX USA; 1015State Key Laboratory of Genetics Engineering; School of Life Sciences; Fudan University; Shanghai, China; 1016Department of Radiation Oncology; University of Texas Health Science Center San Antonio; San Antonio, TX USA; 1017Department of Neurology & Neuroscience; Friedman Brain Institute; Mount Sinai School of Medicine; New York, NY USA; 1018Protein Engineering and Comparative Immunology; Graduate School of Agricultural Biotechnology; Seoul National University; Seoul, Korea; 1019Department of Neurophysiology; Keio University School of Medicine; Tokyo, Japan; 1020Metabolism and Cancer Susceptibility Section; Laboratory of Comparative Carcinogenesis; Center for Cancer Research; NCI–Frederick; National Institutes of Health; Frederick, MD USA; 1021Department of Ophthalmology and Visual Sciences; University of Michigan School of Medicine; Ann Arbor, MI USA; 1022Division of Cell & Molecular Biology; Toronto General Research Institute–University Health Network; Toronto, ON Canada; 1023Department of Experimental Oncology and Molecular Medicine; Fondazione IRCCS Istituto Nazionale Tumori; Milan, Italy; 1024Department of Surgery; University of Pittsburgh; Pittsburgh, PA USA; 1025Department of Neuroscience; University of Virginia; Charlottesville, VA USA; 1026National Institute of Biological Sciences; Beijing, China; 1027Laboratory of Cerebrovascular Pharmacology; College of Pharmaceutical Science; Soochow University; Suzhou, China; 1028Department of Pharmacology and Chemical Biology; University of Pittsburgh School of Medicine; Pittsburgh, PA USA; 1029Department of Molecular Cell Biology; Leiden University Medical Center; Leiden, The Netherlands; 1030Key Laboratory of Plant Resources Conservation and Sustainable Utilization; South China Botanical Garden; Chinese Academy of Sciences; Guangzhou, China; 1031School of Medicine and Public Health; University of Newcastle; New South Wales, Australia; 1032Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education); Peking University Health Science Center; Beijing, China; 1033Department of Biochemistry and Molecular Biology; Peking University Health Science Center; Beijing, China; 1034Department of Pathology; University of Oklahoma Health Sciences Center; Oklahoma City, OK USA; 1035Qiushi Academy for Advanced Studies; Zhejiang University; Hangzhou, China; 1036Department of Molecular and Cell Biology; University of California; Berkeley, CA USA; 1037Center for Brain Repair and Rehabilitation; Institute of Neuroscience and Physiology; University of Gothenburg; Gothenburg, Sweden; 1038State Key Laboratory of Oncology in South China; Department of Medical Oncology; Cancer Center; Sun Yat-sen University; Guangzhou, China; 1039Department of Molecular Genetics & Microbiology; Stony Brook University; Stony Brook, NY USA; 1040Institute for Research in Biomedicine; CIBERDEM; and Universitat de Barcelona; Barcelona, Spain; 1041Max Planck Institute of Psychiatry; Munich, Germany; 1042Department of General Surgery; University of Pittsburgh Medical Center and VA Pittsburgh Healthcare System; Pittsburgh, PA USA

Keywords: autolysosome, autophagosome, flux, LC3, lysosome, phagophore, stress, vacuole

Abbreviations: 3-MA, 3-methyladenine; AMPK, AMP-activated protein kinase; Ape1, aminopeptidase I; Atg, autophagy-related; AV, autophagic vacuole; CLEAR, coordinated lysosomal enhancement and regulation; CLEM, correlative light and electron microscopy; CMA, chaperone-mediated autophagy; Cvt, cytoplasm-to-vacuole targeting; DQ-BSA, dequenched bovine serum albumin; e-MI, endosomal microautophagy; EBSS, Earle's balanced salt solution; FACS, fluorescence-activated cell sorter; GFP, green fluorescent protein; IMP, intramembrane particle; LAMP2, lysosome-associated membrane protein 2; LAP, LC3-associated phagocytosis; LC3, microtubule-associated protein 1 light chain 3 (MAP1LC3); MDC, monodansylcadaverine; mRFP, monomeric red fluorescent protein; MTOR, mechanistic target of rapamycin; NVJ, nucleus-vacuole junction; RPS6KB, ribosomal protein S6 kinase, 70kDa, polypeptide 2; PAS, phagophore assembly site; PE, phosphatidylethanolamine; PMN, piecemeal microautophagy of the nucleus; PtdIns3K, phosphatidylinositol 3-kinase; PtdIns3KC3, PtdIns3K class III; Rluc, Renilla reniformis luciferase; SD, standard deviation; SOD, superoxide dismutase; TEM, transmission electron microscopy; tfLC3, tandem fluorescent LC3; TORC1, TOR complex I; TR-FRET, time-resolved fluorescence resonance energy transfer; UPR, unfolded protein response

In 2008 we published the first set of guidelines for standardizing research in autophagy. Since then, research on this topic has continued to accelerate, and many new scientists have entered the field. Our knowledge base and relevant new technologies have also been expanding. Accordingly, it is important to update these guidelines for monitoring autophagy in different organisms. Various reviews have described the range of assays that have been used for this purpose. Nevertheless, there continues to be confusion regarding acceptable methods to measure autophagy, especially in multicellular eukaryotes. A key point that needs to be emphasized is that there is a difference between measurements that monitor the numbers or volume of autophagic elements (e.g., autophagosomes or autolysosomes) at any stage of the autophagic process vs. those that measure flux through the autophagy pathway (i.e., the complete process); thus, a block in macroautophagy that results in autophagosome accumulation needs to be differentiated from stimuli that result in increased autophagic activity, defined as increased autophagy induction coupled with increased delivery to, and degradation within, lysosomes (in most higher eukaryotes and some protists such as Dictyostelium) or the vacuole (in plants and fungi). In other words, it is especially important that investigators new to the field understand that the appearance of more autophagosomes does not necessarily equate with more autophagy. In fact, in many cases, autophagosomes accumulate because of a block in trafficking to lysosomes without a concomitant change in autophagosome biogenesis, whereas an increase in autolysosomes may reflect a reduction in degradative activity. Here, we present a set of guidelines for the selection and interpretation of methods for use by investigators who aim to examine macroautophagy and related processes, as well as for reviewers who need to provide realistic and reasonable critiques of papers that are focused on these processes. These guidelines are not meant to be a formulaic set of rules, because the appropriate assays depend in part on the question being asked and the system being used. In addition, we emphasize that no individual assay is guaranteed to be the most appropriate one in every situation, and we strongly recommend

the use of multiple assays to monitor autophagy. In these guidelines, we consider these various methods of assessing autophagy and what information can, or cannot, be obtained from them. Finally, by discussing the merits and limits of particular autophagy assays, we hope to encourage technical innovation in the field.

*Correspondence to: Daniel J. Klionsky; Email: klionsky@umich.edu

Introduction

Many researchers, especially those new to the field, need to determine which criteria are essential for demonstrating autophagy, either for the purposes of their own research, or in the capacity of a manuscript or grant review.[1] This is an important issue, particularly considering that each of us may have his/her own opinion regarding the answer. Unfortunately, the answer is in part a "moving target" as the field evolves,2 and this can be extremely frustrating for researchers who may think they have met those criteria, only to find out that the reviewers of their paper have different ideas.

Conversely, as a reviewer, it is tiresome to raise the same objections repeatedly, wondering why researchers have not fulfilled some of the basic requirements for establishing the occurrence of an autophagic process. In addition, drugs that potentially modulate autophagy are increasingly being used in clinical trials, and screens are being performed for new drugs that can modulate autophagy for therapeutic purposes. Clearly it is important to determine whether these drugs are truly affecting autophagy based on a set of accepted criteria. Accordingly, we describe here a basic set of contemporary guidelines that can be used by researchers to plan and interpret their experiments, by clinicians to evaluate the literature with regard to autophagymodulating therapies, and by both authors and reviewers to justify or criticize an experimental approach.

Several fundamental points must be kept in mind as we establish guidelines for the selection of appropriate methods to monitor autophagy.[1] Importantly, there are no absolute criteria for determining autophagic status that are applicable in every biological or experimental context. This is because some assays are inappropriate, problematic or may not work at all in parti- cular cells, tissues or organisms.[3-6] In addition, these guidelines are likely to evolve as new methodologies are developed and current assays are superseded. Nonetheless, it is useful to estab- lish guidelines for acceptable assays that can reliably monitor autophagy in many experimental systems. It is important to note that in this set of guidelines the term "autophagy" generally refers to macroautophagy; other autophagy-related processes are specifically designated when appropriate.

For the purposes of this review, the autophagic compartments (Fig. 1) are referred to as the sequestering (pre-autophagosomal) phagophore (previously called the isolation or sequestration membrane7,8),9 the autophagosome,10 the amphisome (generated by fusion of autophagosomes or amphisomes with a lysosome), and the autophagic body (generated by fusion and release of the internal autophagosomal compartment into the vacuole in fungi and plants; autophagic bodies are not formed within lysosomes/ autolysosomes because these lytic organelles are typically smaller than autophagosomes[13]).[8,10] One critical point is that autophagy is a highly dynamic, multi-step process. Like other cellular pathways, it can be modulated at several steps, both positively and negatively. An accumulation of autophagosomes [measured by transmission electron microscopy (TEM) image analysis, as fluorescent GFP-MAP1LC3 (GFP-LC3) dots, or as LC3 lipida- tion on a western blot], could, for example, reflect induction of autophagy, reduction in autophagosome turnover,[14-16] or the inability of turnover to keep pace with increased autophagosome formation (Fig. 1).[17] For example, inefficient fusion with endo- somes and/or lysosomes, respectively, or perturbation of the transport machinery,[18] would inhibit autophagosome maturation to amphisomes or autolysosomes, whereas decreased flux could also be due to inefficient degradation of the cargo once fusion has occurred.[19]

Accordingly, the use of autophagy markers such as LC3-II needs to be complemented by assays to estimate overall auto- phagic flux, or flow, to permit a correct interpretation of the results. That is, autophagic activity includes not just the increased synthesis or lipidation of Atg8/LC3 (LC3 is a mammalian homolog of yeast Atg8), or an increase in the formation of autophagosomes, but, most importantly, flux through the intere system, including lysosomes or the vacuole, and the subsequent release of the breakdown products. Therefore, autophagic substrates need to be monitored dynamically over time to verify that they have reached the lysosome/vacuole, and, when appropri- ate, are degraded. By responding to perturbations in the extracellular environment, cells tune autophagic flux to meet intracellular metabolic demands. The impact of autophagic flux on cell death and human pathologies therefore demands accurate tools to measure not only the current flux of the system, but also its capacity,[20] and its response time, when exposed to a defined insult.[21]

One approach is to measure the rate of general protein breakdown by autophagy.[8,22] Alternatively, it is possible to arrest the autophagic flux at a given point, and then record the time-dependent accumulation of an organelle, an

organelle marker, a cargo marker or the entire cargo at the point of blockage; however, the latter assumes there is no feedback of the accumulating structure on its own rate of formation.[23] Along the same lines, one can follow the time-dependent decrease of an autophagy- degradable marker (with the caveat that the potential contribution of other proteolytic systems needs to be experimentally addressed). In theory, this can be achieved by blocking autophagic sequestration at specific steps of the pathway (e.g., blocking further induction or nucleation of new phagophores) and by measuring the decrease of markers distal to the block point.[14,16,24]

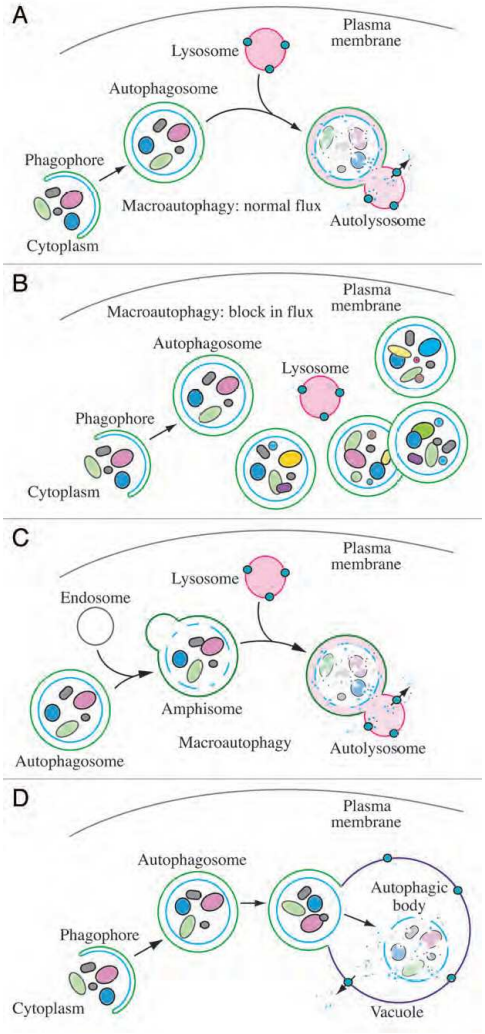

Figure 1. Schematic model demonstrating the induction of autophagosome formation when turnover is blocked vs. normal autophagic flux, and illustrating the morphological intermediates of macroautophagy. (A) The initiation of autophagy includes the formation of the phagophore, the initial sequestering compartment, which expands into an autophagosome. Completion of the autophagosome is followed by fusion with lysosomes and degradation of the contents, allowing complete flux, or flow, through the entire pathway. This is a different outcome than the situation shown in (B) where induction results in the initiation of autophagy, but a defect in autophagosome turnover due, for example, to a block in fusion with lysosomes or disruption of lysosomal functions will result in an increased number of autophagosomes. In this scenario, autophagy has been induced, but there is no or limited autophagic flux. (C) An autophagosome can fuse with an endosome to generate an amphisome, prior to fusion with the lysosome. (D) Schematic drawing showing the formation of an autophagic body in plants and fungi. The large size of the plant and fungal vacuole relative to autophagosomes allows the release of the single-membrane autophagic body within the vacuole lumen. In cells that lack vacuolar hydrolase activity, or in the presence of inhibitors that block hydrolase activity, intact autophagic bodies accumulate within the vacuole lumen and can be detected by light microscopy. The lysosome of most higher eukaryotes is too small to allow the release of an autophagic body.

The key issue is to differentiate between the often transient accumulation of autophagosomes due to increased induction, from accumula- tion due to inefficient completion of autophagy, by measuring both the levels of autophagosomes at static time points and by addressing changes in the rates of autophagic degradation of cellular components.[19] Both processes have been used to estimate "autophagy," but unless the experiments can relate changes in autophagosome numbers to a direct or indirect measurement for autophagic flux, they may be difficult to interpret.[25] A general caution regarding the use of the term "steady state" is warranted at this point. It should not be assumed that an autophagic system is at steady-state in the strict biochemical meaning of this term, as this implies that the level of autophagosomes does not change with time, and the flux through the system is constant. In these

guidelines, we use steady-state to refer to the baseline range of autophagic flux in a system that is not subjected to specific perturbations that increase or decrease that flux.

Autophagic flux refers to the entire process of autophagy including the delivery of cargo to lysosomes (via fusion of the latter with autophagosomes or amphisomes) and its subsequent breakdown and release of the resulting macromolecules back into the cytosol (this may be referred to as productive or complete autophagy). Thus, increases in the level of phosphatidylethanolamine (PE)-modified Atg8/LC3 (Atg8–PE/LC3-II), or even the appearance of autophagosomes are not measures of autophagic flux per se, but can reflect the induction of autophagic sequestration and/or inhibition of autophagosome or amphisome clearance. Also, it is important to realize that while formation of Atg8–PE/LC3-II appears to correlate with the induction of autophagy, we do not know, at present, the actual mechanistic relationship between Atg8–PE/LC3-II formation and the rest of the autophagic process; indeed, it may be possible to execute "self-eating" in the absence of LC3-II.26 As a final note, we also recommend that researchers refrain from the use of the expression "percent autophagy" when describing experimental results, as in "The cells displayed a 25% increase in autophagy." In contrast, it is appropriate to indicate that the average number of GFP-Atg8/LC3 puncta per cell is increased or a certain percentage of cells display punctate GFP-Atg8/LC3 that exceeds a particular threshold (and this threshold should be clearly defined in the methods), or that there is a particular increase or decrease in the rate of degradation of long-lived proteins, as these are the actual measurements being quantified.

In the previous version of these guidelines,1 the methods were separated into two main sections—steady-state and flux. In some instances, a lack of clear distinction between the actual methodologies and their potential uses made such a separation somewhat artificial. For example, fluorescence microscopy was initially listed as a steady-state method, although this approach can clearly be used to monitor flux as described in this article, especially when considering the increasing availability of new technologies such as microfluidics. Furthermore, the use of multiple time points and/or lysosomal fusion/degradation inhibitors can turn even a typically static method such as TEM into one that monitors flux.

Therefore, although we maintain the importance of monitoring autophagic flux and not just induction, this revised set of guidelines does not separate the methods based on this criterion. Readers should be aware that this article is not meant to present protocols, but rather guidelines, including information that is typically not presented in protocol papers. For detailed informa- tion on experimental procedures we refer readers to various protocols that have been published elsewhere.[27-42] Collectively, we propose the following guidelines for measuring various aspects of selective and nonselective autophagy in eukaryotes.

A. Methods for Monitoring Autophagy

1. Transmission electron microscopy. Autophagy was first detected by TEM in the 1950s (reviewed in ref. 8). The focal degradation of cytoplasmic areas sequestered by the phagophore, which matures into the autophagosome, is the morphological hallmark of autophagy. TEM can be used to monitor both selective and nonselective autophagy. In the case of selective autophagy, the cargo should correspond to the specific substrate being targeted for sequestration—bulk cytoplasm is essentially excluded. In contrast, during nonselective autophagy, the content of the autophagosome is morphologically identical to the cytoplasm, containing similar densities of ribosomes, and intact sequestered organelles are clearly identifiable. Therefore, the use of TEM is a valid and important method both for the qualitative and quantitative analysis of changes in various autophagic structuresthat sequentially form the phagophore, autophagosome, amphisome, autolysosome and autophagic body (Fig. 1).[43] The maturation from the phagophore through the autolysosome is a dynamic and continuous process[44] and, thus, the classification of compartments into discrete morphological subsets can be problematic; however, some basic guidelines can be offered.

Autophagosomes (also referred to as initial autophagic vacuoles, AVi) have a double membrane that is usually at least partly visible as two parallel membrane bilayers separated by an electron-lucent cleft (Fig. 2A).[45,46] Autophagosomes contain cytosol and/or organelles that look morphologically intact, i.e., similar to the cytosol and organelles elsewhere in the cell.[43,47] Amphisomes[48] can sometimes be identified by the presence of small internal vesicles inside the autophagosome/autophagic vacuole (AV).[49] These internal vesicles are delivered into the lumen by fusion with multivesicular endosomes. Late/degradative autophagic vacuoles and autolysosomes (AVd) usually have only one limiting membrane, and contain cytoplasmic material and/or organelles at various stages of degradation (Fig. 2A and B).[43,47] It should be emphasized that not all vesicles containing electron- dense amorphous material are autolysosomes. The cytoplasmic origin of the contents must still be morphologically identifiable, if morphology is the only criterion that is being used for the identification of autolysosomes. For many biological and patho- logical situations, examination of both early and late autophagic structures yields valuable data regarding the overall autophagy/ lysosomal status in the cells.[17] Along these lines, it is possible to use immunocytochemistry to follow particular cytosolic protein such as CuZn

superoxide dismutase (SOD) and carbonic anhy- drase to determine the stage of autophagy; the former is much more resistant to lysosomal degradation.[50] In some autophagy-inducing conditions it is possible to observe multi-lamellar membrane structures in addition to the conventional double-membrane autophagosomes. The nature of these structures is not fully understood. They may indeed be multiple double layers of phagophores[51] and positive for LC3,[52] or mere artifacts of fixation. TEM observations of platinum-carbon replicas obtained by the freeze fracture technique can also supply useful ultrastructural information on the autophagic process. In quickly frozen and fractured cells the fracture runs preferentially along the hydro- phobic plane of the membranes, allowing characterization of the limiting membranes of the different types of autophagic vacuoles and visualization of their limited protein intramembrane particles (IMPs, or integral membrane proteins). Several studies have been performed using this technique on yeast,[53] as well as on mammalian cells or tissue, first on mouse exocrine pancreas,[54] then on mouse and rat liver,[55,56] mouse seminal vesicle epithe- lium[24,51] or cancer cell lines (e.g., breast cancer MDA-MB-231)[57] to investigate the various phases of autophagosome maturation, and to reveal useful details about the origin and evolution of their limiting membranes.

The phagophore and the limiting membranes of autophago- somes contain few, or no detectable, IMPs (Fig. 3A and B), when compared with other cellular membranes and to the mem- branes of lysosomes. In subsequent stages of the autophagic process the fusion of the autophagosome with an endosome and a lysosome results in increased density of IMPs in the mem- brane of the formed autophagic compartments (amphisomes, autolysosomes; Fig. 3C).[8,24,53-56,61,62]

Autolysosomes are generally delimited by a single membrane because, in addition to the engulfed material, the inner membrane is degraded by the lytic enzymes. Similarly, the limiting membrane of autophagic bodies in yeast and plants is also quickly broken down under normal conditions. Autophagic bodies can be stabilized, however, by the addition of phenylmethylsulphonylfluoride (PMSF) or genetically by the deletion of the yeast PEP4 gene.

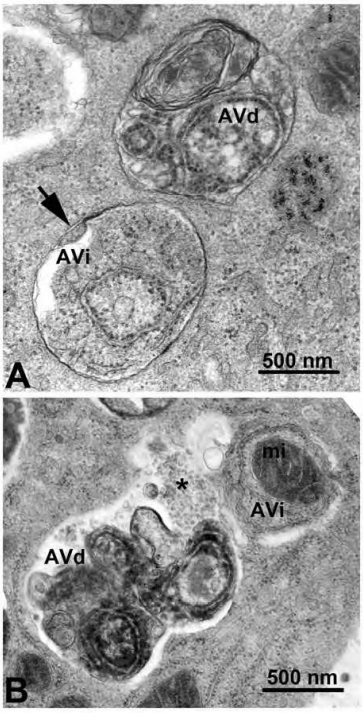

Figure 2. TEM images of autophagic vacuoles in isolated mouse hepatocytes. (A) One autophagosome or early autophagic vacuole (AVi) and one degradative autophagic vacuole (AVd) are shown. The AVi can be identified by its contents (morphologically intact cytoplasm, inlcuding ribosomes, and rough endoplasmic reticulum), and the limiting membrane that is partially visible as two bilayers separated by a narrow electron-lucent cleft, i. e., as a double membrane (arrow). The AVd can be identified by its contents, partially degraded, electron-dense rough endoplasmic reticulum. The vesicle next to the AVd is an endosomal/ lysosomal structure containing 5-nm gold particles that were added to the culture medium to trace the endocytic pathway. (B) One AVi, containing rough endoplasmic reticulum and a mitochondrion, and one AVd, containing partially degraded rough endoplasmic reticulum, are shown. Note that the limiting membrane of the AVi is not clearly visible, possibly because it is tangentially sectioned. However, the electron-lucent cleft between the two limiting membranes is visible and helps in the identification of the AVi. The AVd contains a region filled by small internal vesicles (asterisk), indicating that the AVd has fused with a multivesicular endosome. mi, mitochondrion. Image provided by E.-L. Eskelinen.

Thus, another method to consider for monitoring autophagy in plants and yeast is to count autophagic bodies by TEM using at least two time points. The advantage of this approach is that it can provide accurate information on flux even when the autophagosomes are abnor- mally small.[63,64] Thus, although a high frequency of “abnormal” structures presents a challenge, TEM is still very helpful in analyzing autophagy.

Cautionary notes: Although TEM is one of the most widely used methodologies to monitor autophagy, it is also one of the most problematic due to misinterpretations mostly deriving from methodological artifacts.[45,46,65,66] Care in the choice of sample to be analyzed is critical to the success of TEM studies for autophagy. Whereas fixation of in vitro samples is relatively straightforward, fixation of excised tissues requires care to avoid sampling a nonrepresentative or uninformative section of tissue. For instance, if 95% of a tumor is necrotic, TEM analysis of the necrotic core may not be informative, and if the sampling is from the viable rim, this needs to be specified when reported.

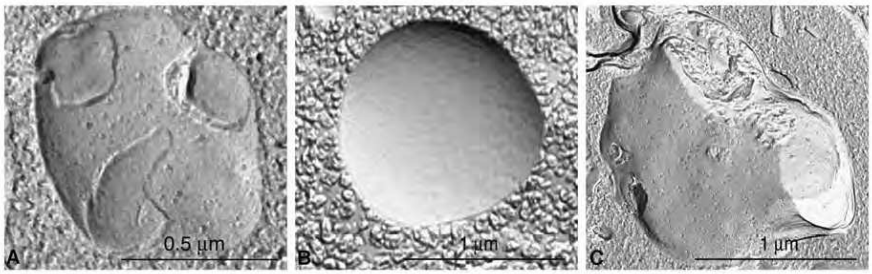

Figure 3. Different autophagic vesicles observed after freeze fracturing in cultured osteosarcoma cells after treatment with the autophagy inducer voacamine.59 (A) Early autophagosome delimited by a double membrane. (B) Inner monolayer of an autophagosome membrane deprived of protein particles. (C) Autolysosome delimited by a single membrane rich in protein particles. In the cross-fractured portion (on the right) the profile of the single membrane and the inner digested material are easily visible. Images provided by S. Meschini, M. Condello and A. Giuseppe.

Ex vivo tissue should be fixed immediately and systematically across samples to avoid changes in autophagy that may occur simply due to elapsed time ex vivo. It is recommended that for tissue samples, perfusion fixation should be used when possible. For yeast, rapid freezing techniques such as high pressure freezing followed by freeze substitution (i.e., dehydration at low temperature) may be particularly useful.

Due to the high potential for sampling artifacts, careful selection of appropriate nonbiased methods of quantification and orphometric/stereological analyses is essential.[67-69] Data obtained simply by scoring for the presence or absence of autophagic vacuoles (autophagosomes, autolysosomes) in the section of a cell leads to unreliable results due to variability in cell areas, and autophagic vacuole profiles in the sections. It is more reliable to quantify autophagosome (and/or autolysosome) profiles per total cytoplasmic or cellular area in sections, which still includes an unaccounted variability in the profile size of the autophagic element. The best approach is to estimate the volume occupied by autophagic structures (as percent of cytoplasmic or cellular volume, using volumetric morphometry/stereology) in at least 20 cell profiles per sample (the number needed should be dictated by some form of power analysis, which indicates that the data are significant).[20,65,68,70,71] During quantification it is important to make sure that each imaged cell profile is captured and scored at the same magnification, and that every cell profile in the thin section has an equal probability to be included in the counting. The accurate identification of the autophagosome is a pre- requisite for a valid analysis. An additional complication, however, is that maturation of metazoan autophagosomes involves a transition from a double-membrane compartment to single-membrane structures (i.e., amphisomes and autolysosomes).[72] In addition, not all double-membrane structures areautophagosomes. Thus, double membranes cannot be relied upon as the sole means for the ultrastructural identification of autophagy-related structures, and it is important to employ expert analysis to avoid misinterpretation of micrographs.[46,65,66] In some cases, it may be prudent to employ tomographic reconstructions of the TEM images to confirm that the autophagic compartments are spherical and are not being confused with endomembrane cisternae or damaged mitochondria with similar appearance in thin-sections (e.g., see ref.73), but this is obviously a timeconsuming approach requiring sophisticated equipment. In addition, interpretation of tomographic images can be problematic. For example, starvation-induced autophagosomes should contain cytoplasm (i.e., cytosol and possibly organelles), but autophagosome-related structures involved in specific types of autophagy should show the selective cytoplasmic target, but may be relatively devoid of cytoplasm. Such processes include selective peroxisome or mitochondria degradation (pexophagy or mitophagy, respectively),[74,75] targeted degradation of pathogenic microbes (xenophagy),[76-81] as well as the yeast biosynthetic cytoplasm-to-vacuole targeting (Cvt) pathway.[82] Furthermore, some pathogenic microbes express membrane-disrupting factors during infection (e.g., phospholipases) that disrupt the normal double-membrane architecture of autophagosomes.[83] It is not even clear if the sequestering compartments used for specific organelle degradation or xenophagy should be termed autophago- somes or if alternate terms such as pexophagosome,[84] mitophago some and xenophagosome should be used, even though the membrane and mechanisms involved in their formation may be identical to those for starvation-induced autophagosomes; for example, the double-membrane vesicle of the Cvt pathway is referred to as a Cvt vesicle.

It can also be difficult to determine whether material present within a phagosomal structure reflects self-eating, or is from a heterophagic (consumption of components from outside the cell) process. A prominent example is related to apoptosis.[85,86] Apoptotic bodies from neighboring cells are readily phagocytosed by surviv- ing cells of the same tissue. Immediately after phagocytic uptake of apoptotic bodies, phagosomes have double limiting membranes. The inner one is from the plasma membrane of the apoptotic body and the outer one is that of the phagocytizing cell. The early heterophagic vacuole formed in this way may appear similar to an autophagosome or, in a later stage, an early autolysosome in that it contains recognizable cytoplasmic material. A major difference, however, is that the surrounding membranes are the thicker (plasma membrane type), rather than the thinner sequestration membrane type (9–10 nm, vs. 7–8 nm, respectively).[66] A good feature to distinguish between autophago- somes and double plasma membrane-bound structures is the lack of the distended empty space (characteristic for the sequestr- ation membranes of autophagosomes) between the two mem- branes of the phagocytic vacuoles. In addition, engulfed apoptotic bodies usually have a larger average size than autophagosomes.[87] The problem of heterophagic elements interfering with the identification of autophagic ones is most prominent in cell types with particularly intense heterophagic activity (such as macro- phages, and amoeboid or ciliate protists). Special attention has to be paid to this problem in cell cultures or in vivo treatments (e.g., with toxic or chemotherapeutic agents) causing extensive apoptosis.

To decide about the lytic nature of a vacuolar compartment, demonstration of the presence of lysosomal enzymes by traditional (enzyme) cytochemistry or immunocytochemistry is also feasible for identifying post-fusion autophagic compartments. However, when heterophagy and autophagy are going on in parallel, the fusion of secondary lysosomes from both sources happens without distinction. The result will be a mixture of degradative products that may be derived both from heterophagy and autophagy, making it impossible to determine the proportion derived from a single process.

There are numerous structures in cells that resemble, or can be confused with, autophagic vesicles. The most common organelles are mitochondria, and ER, and also (depending on their struc- ture) plastids in plants. Due to the cisternal structure of the ER, double membrane-like structures surrounding mitochondria or other organelles are often observed after sectioning,[88] but these can also correspond to cisternae of the ER coming into and out of the section plane.[45] If there are ribosomes associated with these membranes they can help distinguish them from the ribosome- free double-membrane of the phagophore and autophagosome. Observation of a mixture of early and late autophagosomes that is modulated by time point of collection and/or brief pulses of bafilomycin A_1 (a V-ATPase inhibitor) to trap the cargo in a recognizable early state[41] increases the confidence that an autophagic process is being observed. Criteria for distinguishing specialized structures such as melanosomes containing electron dense pigment from autophagosomes should be specified prior to scoring and included in a methods section when reporting the results. Considering that swollen mitochondria can encom- pass most of the internal structure of small autophagosomes, the possibility exists for scoring errors in every study. To minimize the impact of such errors, exact specification of autophagic elements must be applied. Efforts should be made to clarify the nature of questionable structures by extensive preliminary com- parison in many test areas. Elements that still remain question- able should be categorized into special groups and measured separately. Should their later identification become possible, they can be added to the proper category or, if not, kept separate.

Uncertainties of identification and special features of the autophagic process may be clarified by immuno-TEM with gold-labeling,[89,90] using antibodies, for example, to cargo proteins of cytoplasmic origin and to LC3 to verify the autophagic nature of the compartment. Although labeling of LC3 can be difficult, good antibodies exist to visualize the GFP moiety of GFP-LC3 reporter constructs.[91] Antibodies against an abundant cytosolic protein will result in high background labeling; however, organelle markers work well. Because there are very few characterized proteins that remain associated with the completed structure, the choices for confirmation of its autophagic nature are limited. Furthermore, autophagosome-associated proteins may be cell type-specific. At any rate, the success of this methodology depends on the quality of the antibodies and also on the TEM preparation and fixation procedures utilized. With immuno- TEM, authors should provide controls showing that labeling is specific. This may require a quantification of staining over different cellular compartments.

In addition, statistical information should be provided due to the necessity of showing only a selective number of sections. Again, we note that for quantitative data it is preferable to use proper volumetric analysis rather than just counting numbers of sectioned objects. On the one hand, it must be kept in mind that even volumetric morphometry/stereology only shows either steady-state levels, or a snapshot in a changing dynamic process.

Such data by themselves are not informative regarding auto autophagic flux, unless performed over multiple time points. Alternatively, investigation in the presence and absence of flux inhibitors can reveal the dynamic changes in various stages of the autophagic process.[14,20,92,93] For example, if the turnover of autolysosomes is very rapid, a low number/volume will not necessarily be an accurate reflection of low autophagic activity.

On the other hand, quantitative analyses indicate that auto- phagosome volume in many cases does correlate with the

rates of protein degradation.[94-96] One potential compromise is to perform whole cell quantification of autophagosomes using fluorescence methods, with qualitative verification by TEM,[97] to show that the changes in fluorescent puncta reflect increases in autophagic structures.

One additional caveat with TEM, an to some extent with confocal fluorescence microscopy, is that the analysis of a single plane within a cell can be misleading and may make the identification of autophagic structures difficult. Confocal microscopy and fluorescence microscopy with deconvolution software (or with much more work, 3-dimensional TEM) can be used to generate multiple/serial sections of the same cell to reduce this concern; however, in some cases where there is sufficient structural resolution, analysis of a single plane with multiple cells can suffice given practical limitations. Newer EM technologies, including focused ion beam dual-beam EM, should make it much easier to apply three-dimensional analyses. An additional methodology to assess autophagosome accumulation is correlative light and electron microscopy, CLEM, which is helpful in confirming that fluorescent structures are autophagosomes.[98,99]

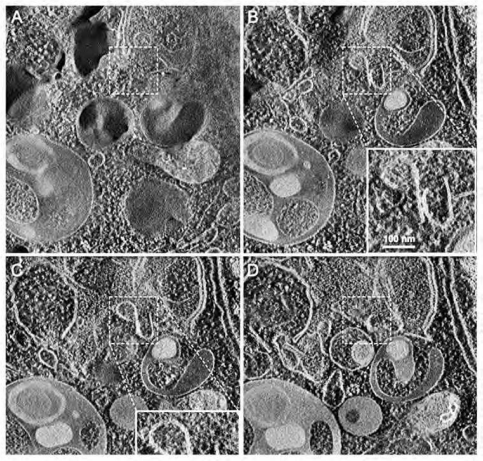

Figure 4. Cryoelectron microscopy can be used as a three-dimensional approach to monitor the autophagic process. Four computed sections of an electron tomogram of the autophagic vesicle-rich cytoplasm in a hemophagocyte of a semi-thin section after high-pressure freezing preparation. The dashed area is membrane-free (1A) but tomography reveals newly formed or degrading membranes with a parallel stretch (1B). In another computed section (1C) of the same tomogram, the parallel stretch of the two membranes shows only one membrane bilayer, and this membrane composition has disappeared in the computed section shown in (1D). Image published previously104 and provided by M. Schneider and P. Walter.

Along these lines, the new mini Singlet Oxygen Generator (miniSOG) fluorescent flavoprotein, which is less than half the size of GFP, provides an additional means to genetically tag proteins for CLEM analysis under conditions that are particularly suited to subsequent TEM analysis.100 Combinatorial assays using tandem mRFP-GFP-LC3 (see Tandem mRFP/ mCherry-GFP fluorescence microscopy) along with static TEM images should help in the analysis of flux and the visualization of cargo structures.[101] Another technique that has proven quite useful for analyzing the complex membrane structures that participate in autophagy is three-dimensional electron tomography,[102,103] and cryoelectron microscopy (Fig. 4). Finally, although an indirect measurement, a comparison of the ratio of autophagosomes to autolysosomes by TEM can support altera- tions in autophagy identified by other procedures.[105] In this case it is important to always compare samples to the control of the same cell type and in the same growth phase, as the ratio of autophagosome/autolysosome varies in a cell context-dependent fashion, depending on their clearance activity. It may also be necessary to distinguish autolysosomes from telolysosomes/late secondary lysosomes (the former are actively engaged in degradation, whereas the latter have reached an end point in the break-down of lumenal contents and are also referred to as residual bodies; see Tissue fractionation) because lysosome numbers generally increase when autophagy is induced.

With regard to immunohistochemistry using SOD1 as a marker to follow autophagy, it should be noted that a portion of the CuZn superoxide dismutase is also associated with various organelles, including the ER, mitochondria and peroxisomes. In addition, the wild-type SOD1 protein can be oxidized, and this form (SODox) is associated with sporadic cases of amyotrophic interfere with autophagy, and, if so, it may not be a good choice for a marker to monitor steady-state autophagic flux.

Conclusion: EM can be an extremely informative method for monitoring autophagy; however, it must be performed with extreme caution and rigor to avoid bias, and to ensure correct identification and quantification of autophagic compartments. With TEM, immunogold labeling is strongly recommended as it generally provides the most unequivocal results. Whenever possible, EM should not be the sole method used to monitor autophagy, but rather should be complemented by additional assays as described in this article.

2Atg8/LC3 detection and quantification. Atg8/LC3 is the most widely monitored autophagy-related protein. In this section we

describe multiple assays that utilize this protein, separating the descriptions into several subsections for ease of discussion.

a Western blotting and ubiquitin-like protein conjugation systems. The Atg8/LC3 protein is a ubiquitin-like protein that can be conjugated to PE (and possibly to phosphatidylserine[107]). In yeast and several other organisms, the conjugated form is referred to as Atg8–PE. The mammalian homologs of Atg8 constitute a family of proteins subdivided in two subfamilies: LC3 (microtubule-associated protein 1 light chain 3) and GABARAP ($GABA_A$ receptor-associated protein). The former is comprised of LC3A, B, B2 and C, whereas the latter family includes GABARAP, GABARAPL1/Atg8L/GEC1 ($GABA_A$ receptor-associated protein like 1/Glandular Epithelial Cell 1), GABARAPL2/GATE-16/GEF2 ($GABA_A$ receptor-associated protein like 2/Golgi-associated ATPase enhancer of 16 kDa/ganglioside expression factor 2) and GABARAPL3 ($GABA_A$ receptor-associated protein like 3).[108] The nonlipidated and lipidated forms are usually referred to as LC3-I and LC3-II, or GABARAP and GABARAP–PE, etc. The positions of both Atg8/LC3-I (approximately 18 kDa) and Atg8–PE/LC3-II (approximately 16 kDa) should be indicated on western blots whenever both are detectable.

The mammalian Atg8 homologs share from 29% to 94% sequence identity and have all, apart from GABARAPL3, been demonstrated to be involved in autophagosome biogenesis.[109] The LC3 proteins are involved in phagophore formation, with participation of GABARAP subfamily members in later stages of autophagosome formation, in particular phagophore elongation and closure.[110] Nevertheless, in most published studies, LC3 has been the primary Atg8 homolog examined in mammalian cells and the one that is typically characterized as an autophagosome marker per se, making this factor the most relevant for this discussion (note that although this protein is referred to as "Atg8" in many other systems, for simplicity we primarily refer to it here as LC3 to distinguish it from the yeast protein). LC3, like the other Atg8 homologs, is initially synthesized in an unprocessed form, proLC3, which is converted into a proteolytically processed form lacking amino acids from the C terminus, LC3-I, and is finally modified into the PE-conjugated form, LC3-II (Fig. 5). bafilomycin A_1) or by inhibiting lysosome-mediated proteolysis (e.g., with the cysteine protease inhibitor E-64d, the aspartic protease inhibitor pepstatin A, or chloroquine[117]). Atg8–PE/LC3-II is the only protein marker that is reliably associated with completed autophagosomes, but is also localized to phagophores. In yeast, Atg8 amounts increase at least 10-fold when autophagy is induced.[113] In mammalian cells, however, the total levels of LC3 do not necessarily change in a predictable manner, as there may be increases in the conversion of LC3-I to LC3-II, or a decrease in LC3-II relative to LC3-I if degradation of LC3-II via lysosomal turnover is particularly rapid. Both of these events can be seen sequentially in several cell types as a response to total nutrient and serum starvation. In cells of neuronal origin a high ratio of LC3-I to LC3-II is a common finding.[114] For instance, SH-SY5Y neuroblastoma cell lines display only a slight increase of LC3-II after nutrient deprivation, whereas LC3-I is clearly reduced. This is likely related to a high basal autophagic flux, as suggested by the higher increase in LC3-II when cells are treated with NH4Cl,[115,116] although cellspecific differences in transcriptional regulation of LC3 may also play a role. The pattern of LC3-I to LC3-II conversion seems not only to be cell specific, but also related to the kind of stress to which cells are subjected. For example, the same SH-SY5Y cells display a strong increase of LC3-II when treated with the mitochondrial uncoupler CCCP, a well-known inducer of mitophagy. Thus, neither assessment of LC3-I consumption nor the evaluation of LC3-II levels would necessarily reveal a slight induction of autophagy (e.g., by rapamycin). Also, there is not always a clear precursor/product relationship between LC3-I and LC3-II, because the conversion of the former to the latter is cell type-specific and dependent on the treatment used to induce autophagy. Accumulation of LC3-II can be obtained by interrupting the autophagosome-lysosome fusion step (e.g., by depolymerizing acetylated microtubules with vinblastine, or by raising the lysosomal pH with the lysosomal proton pump inhibitor bafilomycin A1) or by inhibiting lysosome-mediated proteolysis (e.g., with the cysteine protease inhibitor E-64d, the aspartic protease inhibitor pepstatin A, or chloroquine117). Western blotting can be used to monitor changes in LC3 amounts (Fig. 5);[25,118] however, even if the total amount of LC3 does increase, the magnitude of the response is generally less than that documented in yeast. It is worth noting that since the conjugated forms of the GABARAP subfamily members are usually undetectable without induction of autophagy in mammalian cells,[119] these proteins might be more suitable than LC3 to study and quantify subtle changes in the autophagic flux.

In most organisms, Atg8/LC3 is initially synthesized with a C-terminal extension that is removed by the Atg4 protease. Accordingly, it is possible to use this processing event to monitor Atg4 activity. For example, when GFP is fused at the C terminus of Atg8 (Atg8-GFP), the GFP moiety is removed in the cytosol to generate free Atg8 and GFP. This processing can be easily monitored by western blot.[120] It is also possible to use assays with an artificial fluorogenic substrate, or a fusion of LC3B to phospholipase A(2) that allows the release of the active phospholipase for a subsequent fluorescent assay. Another method to monitor ATG4 activity in vivo uses the release of Gaussia luciferase from the C terminus of LC3 that is tethered to actin.[123] Note that there are four Atg4 homologs in mammals, and they have different activities with regard to the Atg8 subfamilies of proteins.[124] ATG4A is able to leave the GABARAP subfamily, but has very limited activity toward the LC3 subfamily, whereas ATG4B is apparently active against most or all of these proteins. The ATG4C and ATG4D isoforms have minimal activity for any of the Atg8 homologs. In particular because a C-terminal fusion will be cleaved immediately by Atg4, researchers should be careful to correctly specify whether they are using

GFP-Atg8/LC3 (an N-terminal fusion, which can be used to monitor various steps of autophagy) or Atg8/LC3-GFP (a C-terminal fusion, which can only be used to monitor Atg4 activity).[125]

Cautionary notes: There are several important caveats to using Atg8/LC3-II or GABARAP-PE to visualize fluctuations in autophagy. First, changes in LC3-II amounts are tissue- and cell context-dependent.[112,126] Indeed, in some cases, autophagosome accumulation detected by TEM does not correlate well with the amount of LC3-II (Tallóczy Z, de Vries RLA, Sulzer D, unpublished results; Eskelinen E-L, unpublished results). This is particularly evident in those cells that show low levels of LC3-II (based on western blotting) because of an intense autophagy flux that consumes this protein,[127] or in cell lines having high levels of LC3-II that are tumor-derived, such as MDA-MB-231.[112] Conversely, the detectable formation of LC3-II is not sufficient evidence for autophagy, without careful quantification. For example, homozygous deletion of Becn1 does not prevent the formation of LC3-II in embryonic stem cells even though autophagy is substantially reduced, whereas deletion of Atg5 results in the complete absence of LC3-II (see Fig. 5A and Supplemental Data in ref. [128]). The same is true for the generation of Atg8–PE in yeast in the absence of ATG6 (see Fig. 7 in ref. [129]). Thus, it is important to remember that not all of the autophagy-related proteins are required for Atg8/LC3 processing, including lipidation.[129] Vagaries in the detection and amounts of LC3-I vs. LC3-II present technical problems. For example, LC3-I is very abundant in brain tissue, and the intensity of the LC3-I band may obscure detection of LC3-II, unless the polyacrylamide crosslinking density is optimized. Conversely, certain cell lines have much less visible LC3-I compared with LC3-II. In addition, tissues may have asynchronous and heterogeneous cell populations, and this may present challenges when analyzing LC3 by western blotting.

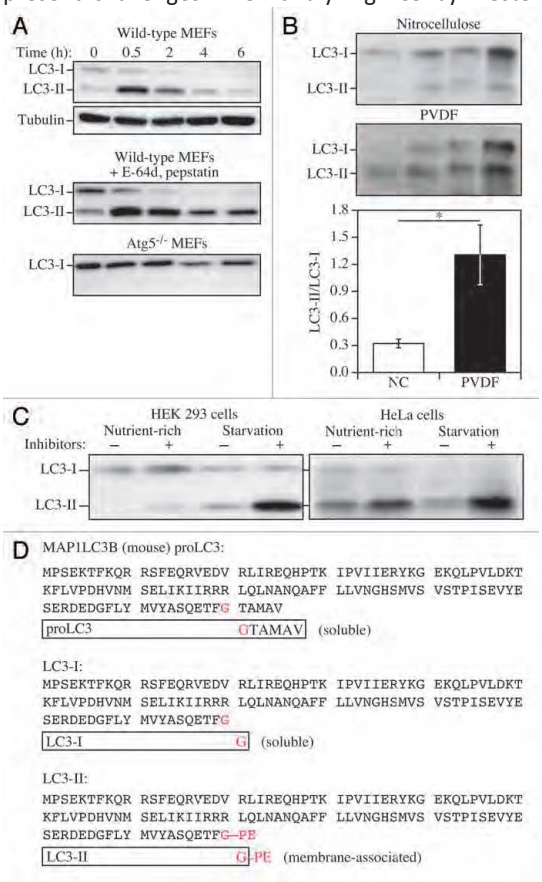

Figure 5. LC3-I conversion and LC3-II turnover. (A) Expression levels of LC3-I and LC3-II during starvation. Atg5+/+ (wild-type) and Atg52/2 MEFs were cultured in DMEM without amino acids and serum for the indicated times, and then subjected to immunoblot analysis using anti-LC3 antibody and anti-tubulin antibody. E-64d (10 mg/ml) and pepstatin A (10 mg/ml) were added to the medium where indicated. Positions of LC3-I and LC3-II are indicated. The inclusion of lysosomal protease inhibitors reveals that the apparent decrease in LC3-II is due to lysosomal degradation as easily seen by comparing samples with and without inhibitors at the same time points (the overall decrease seen in the presence of inhibitors may reflect decreasing effectiveness of the inhibitors over time). Monitoring autophagy by following steady-state amounts of LC3-II without including inhibitors in the analysis can result in an incorrect interpretation that autophagy is not taking place (due to the apparent absence of LC3-II). Conversely, if there are high levels of LC3-II but there is no change in the presence of inhibitors this may indicate that induction has occurred but that the final steps of autophagy are blocked, resulting in stabilization of this protein. This figure was modified from data previously published in reference 25 and is reproduced by permission of Landes Bioscience, copyright 2007. (B) Lysates of 4 human adipose tissue biopsies were resolved on two 12% polyacrylamide gels, as described previously.[111] Proteins were transferred in parallel to either a PVDF or a nitrocellulose membrane, and blotted with anti-LC3 antibody, and then identified by reacting the membranes with an HRPconjugated anti-rabbit IgG antibody, followed by ECL. The LC3-II/LC3-I ratio was calculated based on densitometry analysis of both bands. *p , 0.05. (C) HEK 293 and HeLa cells were cultured in nutrient-rich medium (DMEM containing10% FCS) or incubated for 4 h in starvation conditions (Krebs-Ringer medium) in the absence (-) or presence (+) of E-64d

and pepstatin at 10 mg/ml each (Inhibitors). Cells were then lysed and the proteins resolved by SDS-PAGE. Endogenous LC3 was detected by immunoblotting. Positions of LC3-I and LC3-II are indicated. In the absence of lysosomal protease inhibitors, starvation results in a modest increase (HEK 293 cells) or even a decrease (HeLa cells) in the amount of LC3-II. The use of inhibitors reveals that this apparent decrease is due to lysosome-dependent degradation. This figure was modified from data previously published in reference ,112 and is reproduced by permission of Landes Bioscience, copyright 2005. (D) Sequence and schematic representation of the different forms of LC3B. The sequence for the nascent (proLC3) from mouse is shown. The glycine at position 120 indicates the cleavage site for ATG4. After this cleavage, the truncated LC3 is referred to as LC3-I, which is still a soluble form of the protein. Conjugation to PE generates the membrane associated LC3-II (equivalent to Atg8–PE).

Second, LC3-II also associates with the membranes of non- autophagic structures. For example, some members of the c-protocadherin family undergo clustering to form intracellular tubules that emanate from lysosomes.[130] LC3-II is recruited to these tubules, and appears to promote or stabilize membrane expansion. Furthermore, LC3 can be recruited directly to bacteria-containing phagosome membranes under certain immune activating conditions, for example, TLR-mediated stimulation, in a process designated LC3-associated phagocytosis (LAP)[131,132] and also to apoptotic cell-containing phagosome membranes,[133,134] macropinosomes,[133] and to single-membrane entotic vacuoles.[133] TEM analysis of murine macrophage-like RAW 264.7 cells infected with Burkholderia pseudomallei reveals that intracellular bacteria colocalize with GFP-LC3 puncta. However, the TEM analysis further shows that bacteria are either free in the cytosol, or sequestered in single-membrane phagosomes rather than within canonical double-membrane autophagosomes.[135] Therefore, in studies of infection of mammalian cells by bacterial pathogens the identity of the LC3-II- labeled compartment as an autophagosome should be confirmed by a second method, such as TEM. It is also worth noting that autophagy induced in response to bacterial infection is not directed solely against the bacteria but can also be a response to remnants of the phagocytic membrane.[136]

Similar cautions apply with regard to viral infection, as coronaviruses induce the formation of double-membrane vesicles that are coated with LC3-I, and this nonlipidated form of LC3 plays an autophagyindependent role in viral replication.137 Along these lines, with herpes simplex virus infection, an LC3+ autophagosome-like organelle derived from nuclear membranes is observed that contains viral proteins.[138]

Third, caution must be exercised in general when evaluating LC3 by western blotting, and appropriate standardization controls are necessary. For example, LC3-I may be less sensitive to detection by certain anti-LC3 antibodies. Moreover, LC3-I is more labile than LC3-II, being more sensitive to freezingthawing and to degradation in SDS sample buffer, so fresh samples should be boiled and assessed as soon as possible and should not be subjected to repeated freeze-thaw cycles. A general point to consider when examining transfected cells concerns the efficiency of transfection. A western blot will detect LC3in the entire cell population, including those that are not transfected. Thus, if transfection efficiency is too low, it may be necessary to use methods, such as fluorescence microscopy, that allow autophagy to be monitored in single cells. The critical point is that the analysis of the gel shift of transfected LC3 or GFP-LC3 can be employed to follow LC3 lipidation only in readily transfected cells.[139] When dealing with animal tissues, western blotting of LC3 should be performed on frozen biopsy samples homogenized in the presence of general protease inhibitors (Isidoro C, personal communication; see also Human).[140] Caveats regarding detection of LC3 by western blotting have been covered in a review.25 For example, PVDF membranes may result in a stronger LC3-II retention than nitrocellulose membranes, possibly due to a higher affinity for hydrophobic proteins (Fig. 5B; Kovsan J, Rudich A, personal communication), and Triton X-100 may not efficiently solubilize LC3-II in some systems.[141] Heating in the presence of 1% SDS, or analysis of membrane fractions,[142] may assist in the detection of this protein.

Another important issue concerns the quantification of changes in LC3-II. The previous version of these guidelines specifically stated that the levels of LC3-II should be compared with actin (and here we would modify this to include other appropriate “housekeeping” proteins) and not to that of LC3-I. As a general rule, this still holds true, but there are some exceptions. For example, in some cases actin levels decrease when autophagy is induced, so that it may be more appropriate to determine the ratio of LC3-II to LC3-I. Either method has its potential advantages and disadvantages. For example, if the amount of LC3-I is high relative to LC3-II (as in brain tissues, where the LC3-I signal can be overwhelming), it can be difficult to quantify the change in LC3-II relative to LC3-I. Conversely, by ignoring the level of LC3-I in favor of LC3-II the researcher may miss part of the overall picture of the cellular autophagic response.

Fourth, LC3 is expressed as four isoforms in mammalian cells, LC3A, LC3B, LC3B2 and LC3C,[143,144] which exhibit different tissue distributions, and it may be necessary to use different antisera or antibodies that distinguish among these isoforms.

A point of caution along these lines is that the increase in LC3AII vs. LC3B-II levels may not display equivalent changes in all organisms under autophagy-inducing conditions, and it should not be assumed that LC3B is the optimal protein to monitor.[145] This supports the important notion that the LC3 isoforms display different functions, but these are yet to be fully elucidated. The commercialized anti-LC3B antibodies also recognize LC3A, but do not recognize LC3C, which shares less sequence homology. It is important to note that LC3C possesses in its primary amino acid sequence the DYKD motif that is recognized with a high affinity by anti-FLAG antibodies. Thus, the standard anti-FLAG M2 antibody can detect and immunoprecipitate overexpressed LC3C, and caution has to be taken in experiments using FLAG-

tagged proteins (Biard-Piechaczyk M, Espert L, personal communication). In addition, it is important to keep in mind the presence of the other subfamily of Atg8 proteins, the GABARAP subfamily (see above).109,146 Certain types of mitophagy induced by NIX/BNIP3L are highly dependent on GABARAP and less dependent on LC3 proteins.[147] Furthermore, commercial antibodies for GABARAPL1 also recognize GABARAP,108 which might lead to misinterpretation of experiments, in particular those using immunohistochemical techniques. The problem with cross-reactivity of the anti-GABARAPL1 antibody can be overcome when analyzing these proteins by western blot because they can be resolved during SDS-PAGE using high concentration (15%) gels, with GABARAP migrating faster than GABARAPL1 (Boyer-Guittaut M, personal communication). We therefore advise caution in choosing antibodies for western blotting and immunofluorescence experiments and in interpreting results based on stated affinities of antibodies unless these have been clearly determined. As with any western blot, proper methods of quantification must be used, which are, unfortunately, often not well disseminated, and the readers are referred to an excellent paper on this subject (see ref.148). Unlike the other members of the GABARAP family, almost no information is available on GABARAPL3, perhaps because it is not yet possible to differentiate between GABARAPL1 and GABARAPL3 proteins, which have 94% identity. As stated by the laboratory that described the cloning of the human GABARAPL1 and GABARAPL3 genes,[146] their expression patterns are apparently identical. It is worth
noting that GABARAPL3 is the only gene of the GABARAP subfamily that seems to lack an ortholog in mice.146 GABARAPL3 might therefore be considered as a pseudogene without an intron that is derived from GABARAPL1. Hence, until new data are published, GABARAPL3 should not be considered as the fourth member of the GABARAP family.

Fifth, in non-mammalian species, the discrimination of Atg8–PE from the nonlipidated form can be complicated by their nearly identical SDS-PAGE mobilities and the presence of multiple isoforms (e.g., there are 9 in Arabidopsis). In yeast, it is possible to resolve Atg8 (the nonlipidated form) from Atg8– PE by including 6 M urea in the SDS-PAGE separating gel,[149] or by using a 15% resolving gel without urea (Reggiori F, personal communication). Similarly, urea combined with prior treatment of the samples with (or without) phospholipase D (that will remove the PE moiety) can often resolve the ATG8 species in plants.[150,151] It is also possible to label cells with radioactive ethanolamine, followed by autoradiography to identify Atg8–PE, and a C-terminal peptide can be analyzed by mass spectrometry to identify the lipid modification at the terminal glycine residue. Furthermore, Atg8–PE aberrantly migrates faster than unconjugated Atg8 during SDS-PAGE, even though the former has a larger molecular mass. Special treatments are not needed for the separation of mammalian LC3-I from LC3-II.

Finally, we would like to point out that one general issue with regard to any assay is that it could introduce some type of stress—for example, mechanical stress due to lysis, temperature stress due to heating or cooling a sample, or oxidative stress on a microscope slide, which could lead to potential artifacts including the induction of autophagy.[152] This point is not intended to limit the use of any specific methodology, but rather to note that there are no perfect assays; special care should be taken with cells in suspension, however, as the stress resulting from centrifugation can induce autophagy. Therefore, it is important to verify that the positive (e.g., treatment with rapamycin, torin1 or other inducers) and negative (e.g., inhibitor treatment) controls behave as expected in any assays being utilized. Similarly, plasmid transfection or nucleofection can result in the potent induction of autophagy (based on increases in LC3-II or SQSTM1 degradation). In some cell types, the amount of autophagy induced by transfection of a control empty vector may be so high that it is virtually impossible to examine the effect of enforced gene expression on autophagy (Levine B, personal communication). This effect is generally not observed with siRNA transfection; however, it is an issue for plasmid shRNA transfection and for plasmid expression constructs. The use of endotoxin-free DNA reduces, but does not eliminate, this problem.

Finally, the precise composition of media components can have profound effects on basal autophagy levels and may need to be modified empirically depending on the cell lines being used.

Conclusion: Atg8/LC3 is often an excellent marker for auto- phagy; however, it must be kept in mind that there are multiple LC3 isoforms, there is a second family of mammalian Atg8-like proteins (GABARAPs), and antibody affinity (for LC3-I vs. LC3-II) and specificity (for example, for LC3A vs. LC3B) must be considered and/or determined.

b Turnover of LC3-II/Atg8–PE. Autophagic flux can be measured by inferring LC3-II/Atg8–PE turnover by western blot (Fig. 5C)[112] in the presence and absence of lysosomal, or vacuolar, degradation. The relevant parameter in this assay is the difference in the amount of LC3-II in the presence and absence of saturating levels of inhibitors, which can be used to examine the transit of LC3-II through the autophagic pathway; if flux is occurring, the amount of LC3-II will be higher in the presence of the inhibitor.[112] Lysosomal degradation can be prevented through the use of protease inhibitors (e.g., pepstatin A and E-64d), compounds such as bafilomycin A_1, chloroquine or NH_4Cl that neutralize the lysosomal pH,[18,114,153,154] or by treatment with agents that block fusion of autophagosomes with lysosomes (note that bafilomycin A will ultimately cause a fusion block as well as neutralize the pH).[155,156] Alternatively, knocking down or knocking out lysosome-associated membrane protein-2 (LAMP2) represents a genetic approach to block the fusion of autophagosomes and lysosomes [for example, inhibiting

LAMP2 in myeloid leukemic cells results in a marked increase of GFP-LC3 dot formation and endogenous LC3-II protein compared with control cells upon autophagy induction during myeloid differentiation (Tschan MP, unpublished data)].157 This approach, however, may be complicated by the compensatory upregulation of macroautophagy that occurs when chaperonemediated autophagy is blocked,[158] unless the LAMP2B isoform is specifically knocked down.[159]

Generally, an increase in the levels of LC3-II observed with a particular treatment condition in the presence of bafilomycin A_1, compared with the treatment alone, is indicative of some degree of flux through the system (i.e., compound/drug treat- ment plus bafilomycin A_1 should result in a higher amount of LC3-II than compound/drug treatment alone); however, a treat- ment condition increasing LC3-II on its own that has no difference in LC3-II in the presence of bafilomycin A_1 com- pared with treatment alone may suggest a block in autophagy at the terminal stages.[6] This procedure has been validated with several autophagy modulators.[160] With each of these techniques, it is essential to avoid assay saturation. The duration of the bafilomycin A_1 treatment needs to be relatively short (1–2 h) to allow comparisons of the amount of LC3 that is lysosomally degraded over a given time frame under one treatment condition to another treatment condition. Positive control experiments using treatment with known autophagy inducers, along with bafilomycin A_1 vs. vehicle, are important to demonstrate the utility of this approach in each experimental context. The same type of assay monitoring the turnover of Atg8–PE can be used to monitor flux in yeast, by comparing the amount of Atg8 present in a wild-type vs. a pep4D strain following autophagy induction.[161]

An additional methodology for monitoring autophagy relies on the observation that a subpopulation of LC3-II exists in a cytosolic form (LC3-IIs) in some cell types.[162] The amount of cytosolic LC3-IIs and the ratio between LC3-I and LC3-IIs appears to correlate with changes in autophagy and may provide a more accurate measure of autophagic flux than ratios based on the total level of LC3-II.[162] The validity of this method has been demonstrated by comparing autophagic proteolytic flux in rat hepatocytes and hepatoma cells. One advantage of this approach is that it does not require the presence of autophagic or lysosomal inhibitors to block the degradation of LC3-II.

Finally, autophagic flux can be monitored based on the turnover of LC3-II, by utilizing a luminescence-based assay. For example, a reporter assay based on the degradation of Renilla reniformis luciferase (Rluc)-LC3 fusion proteins is well suited for screening compounds affecting autophagic flux.[163] In this assay, Rluc is fused N-terminally to either wild-type LC3 (LC3wt) or a lipidation-deficient mutant of LC3 (G120A). Since Rluc-LC3wt, in contrast to Rluc-LC3-G120A, specifically associates with the autophagosomal membranes, Rluc-LC3wt is more sensitive to autophagic degradation. A change in autophagy-dependent LC3-turnover can thus be estimated by monitoring the change in the ratio of luciferase activities between the two cell populations expressing either Rluc-LC3wt or Rluc-LC3-G120A.

In its simplest form, the Rluc-LC3-assay can be used to estimate autophagic flux at a single time point by defining the luciferase activities in cell extracts. Moreover, the use of a live cell luciferase substrate makes it possible to monitor changes in autophagic activity in living cells in real time. This method has been successfully used to identify positive and negative regulators of autophagy from cells treated with microRNA, siRNA and small molecule libraries.[163-166]

Cautionary notes: The main caveat regarding the measurement of LC3-IIs/LC3-I is that this method has only been tested in isolated rat hepatocytes and H4-II-E cells. Thus, it is not yet known whether it is generally applicable to other cell types, and a soluble form of LC3-II (i.e., LC3-IIs) is not observed in many standard cell types including HeLa, HEK 293 and PC12. In addition, the same concerns apply regarding detection of LC3-I by western blotting. It should be noted that the LC3-IIs/ LC3-I ratio must be analyzed using the cytosolic fractions rather than the total homogenates. Furthermore, the same caveats mentioned above regarding the use of LC3 for qualitatively monitoring autophagy also apply to the use of this marker for evaluating flux.

The use of a radioactive pulse-chase analysis provides an alternative to lysosomal protease inhibitors,[113] although such inhibitors should still be used to verify that degradation is lysosome-dependent. In addition, drugs must be used at concentrations and for time spans that are effective in inhibiting fusion or degradation, but that do not provoke cell death. Thus, these techniques may not be practical in all cell types or in tissues from whole organisms where the use of protease inhibitors is problematic, and where pulse labeling requires artificial short-term that may induce autophagy. Another concern when monitoring flux via LC3-II turnover may be seen in the case of a partial autophagy block; in this situation, agents that disrupt autophagy (e.g., bafilomycin A1) will still result in an increase in LC3-II, which may be interpreted as the complete absence of a block resulting from the mutant or compound being tested. Thus, care is needed in interpretation. Furthermore, for characterizing new autophagy modulators, it is ideal to test autophagic flux at early (e.g., 4 h) and late (e.g., 24 h) timepoints, since in certain instances, such as with calcium phosphate precipitates, a compound may increase or decrease flux at these two time-points, respectively.[167] Finally, many of the chemicals used to inhibit autophagy, such as bafilomycin A1, NH4Cl (see Autophagy inhibitors and inducers below) or chloroquine, also directly inhibit the endocytosis/unocating of viruses (Smith DR, personal comunication) and other endocytic eventis requiring low pH, as well as exit from the Golgi (Tooze S, personal comuinication), and as such could be used only with extreme caution in studies investigating autophagy-viruses interactions.

One additional consideration is that it may not be absolutely necessary to follow LC2-II turnover if other substrates are being

monitored simultaneously. For example, an increase of LC3-II levels in combination with the lysosomal (or ideally autophagy-specific) removal of an autophagic substrate (such as an organelle[168,169]) that is not a good proteasomal substrate provides an independent assessment of autophagic flux. However, due to the fact that LC3 might be coupled to endosomal membranes and not just autophagosomes, and the levels of well-characterized autophagosome substrates such as p62 can also be affected by proteasome inhibitors,170 it is probably prudent to monitor both turnover of LC3-II and an autophagosome substrate in parallel.

Another issue relates to the use of protease inhibitors (see Autophagy inhibitors and inducers below). When using lysosomal protease inhibitors, it is of fundamental importance to assess proper conditions of inhibitor concentration and time of pre-incubation to ensure full inhibition of lysosomal cathepsins. In this respect, 1 h of pre-incubation with 10 mg/ml E-64d is sufficient in most cases, since this inhibitor is membrane permeable and rapidly accumulates within lysosomes. On the other hand, pepstatin A is membrane impermeable (ethanol or preferably DMSO must be employed as a vehicle) and requires a prolonged incubation (8 h) and a relatively high con- centration (50 mg/ml) to fully inhibit lysosomal cathepsin D (Fig. 6). An incubation of this duration, however, can be pro- blematic due to indirect effects (see GFP-Atg8/LC3 lysosomal delivery and proteolysis). Also, note that the relative amount of lysosomal cathepsins B and D is cell-specific and changes with culture conditions. In contrast to the protease inhibitors, chloroquine (10 mM) or bafilomycin A_1 (1–100 nM) can be added to cells immediately prior to autophagy induction.

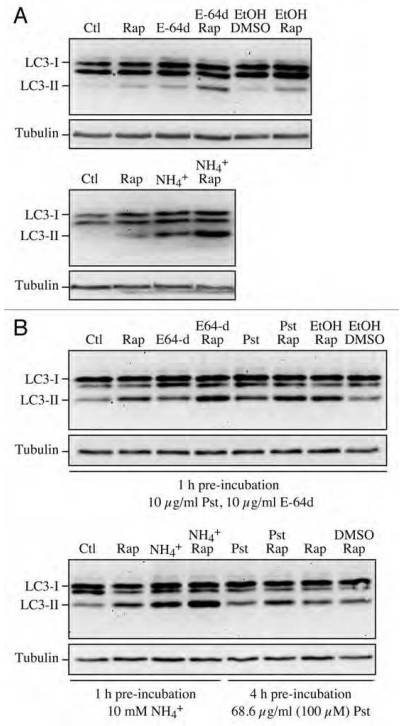

Figure 6. Effect of different inhibitors on LC3-II accumulation. SH-SY5Y human neuroblastoma cells were plated and allowed to adhere for a minimum of 24 h, then treated in fresh medium. Treatments were as follows: rapamycin (Rap), (A) 1 mM, 4 h or (B) 10 mM, 4 h; E-64d, final concentration 10 mg/ml from a 1 mg/ml stock in ethanol (ETOH); NH4Cl (NH4 +), final concentration 10 mM from a 1 M stock in water; pepstatin A (Pst), final concentration 10 mg/ml from a 1 mg/ml stock in ethanol, or 68.6 mg/ml from a 6.86 mg/ml stock in DMSO; ethanol or DMSO, final concentration 1%. Pre-incubations in (B) were for 1 or 4 h as indicated. Ten mM NH4Cl (or 30 mM chloroquine, not shown) were the most effective compounds for demonstrating the accumulation of LC3-II. E-64d was also effective in preventing the degradation of LC3-II, with or without a preincubation, but ammomium chloride (or chloroquine) may be more effective. Pepstatin A at 10 mg/ml with a 1 h pre-incubation was not effective at blocking degradation, whereas a 100 mM concentration with 4 h pre-incubation had a partial effect. Thus, alkalinizing compounds are more effective in blocking LC3-II degradation, and pepstatin A must be used at saturating conditions to have any noticeable effect. Images provided by C. Isidoro. Note that the band running just below LC3-I at approximately 17.5 kDa may be a processing intermediate of LC3-I; it is detectable in freshly prepared homogenates, but is less visible after the sample is subjected to a freeze-thaw cycle.

Conclusion: It is important to be aware of the difference between monitoring the steady-state level of Atg8/LC3 and autophagic flux; the latter can be determined by following Atg8/ LC3 in the absence and presence of autophagy inhibitors, and/or by examining the autophagy-dependent degradation of appropriate substrates. In particular, if there is any evidence of an increase in LC3-II (or autophagosomes), it is essential to determine whether this represents increased flux, or a block in fusion or degradation through the use of inhibitors such as chloroquine or bafilomycin A_1.

c GFP-Atg8/LC3 lysosomal delivery and proteolysis. GFP-LC3B (hereafter referred to as GFP-LC3) has also been used to follow flux. First, when GFP-Atg8 or GFP-LC3 is delivered to a lysosome/vacuole the Atg8/LC3 part of the chimera is sensitive to

degradation, whereas the GFP protein is relatively resistant to hydrolysis (note, however, that GFP fluorescence is quenched by low pH; see GFP-Atg8/LC3 fluorescence microscopy and Tandem mRFP/mCherry-GFP fluorescence microscopy). Therefore, the appearance of free GFP on western blots can be used to monitor lysis of the inner autophagosome membrane and breakdown of the cargo in metazoans (Fig. 7A),[161,171,173] or the delivery of autophagosomes to, and the breakdown of autophagic bodies within, the yeast and plant vacuole.[151,161,174] Reports on Dictyostelium and mammalian cells highlight the importance of lysosomal pH as a critical factor in the detection of free GFP that results from the degradation of fused proteins. In these cell types, free GFP fragments are only detectable in the presence of nonsaturating levels of lysosomotropic compounds (NH_4Cl or chloroquine) or under conditions that attenuate lysosomal acidity; otherwise, the autophagic/degradative machinery appears to be too efficient to allow the accumulation of the proteolytic fragment (Fig. 7B and C).[36,172] Hence, a reduction in the intensity of the free GFP band may indicate reduced flux, but it may also be due to efficient turnover. Using a range of concentrations and treatment times of compounds that inhibit autophagy can be useful in distinguishing between these possibilities.[175] Since the pH in the yeast vacuole is higher than that in mammalian or Dictyostelium lysosomes, the levels of free GFP fragments are detectable in yeast without the necessity of using lysosomotropic compounds.[29] Additionally, in yeast the diffuse fluorescent haze from the released GFP moiety within the vacuole lumen can be observed by fluorescence microscopy.

The movement of GFP-LC3 to lysosomes also can be monitored by fluorescence microscopy, although, as mentioned above, the GFP fluorescent signal is more sensitive to acidic pH than other fluorophores (see GFP-Atg8/LC3 fluorescence micro- scopy). A time-course evaluation of the cell population showing GFP-LC3 puncta can serve to monitor the autophagy flux, since a constant increase in the number of cells accumulating GFP-LC3 puncta is suggestive of defective fusion of autophagosomes with lysosomes, and conversely a decline implies that GFP-LC3 is consumed within newly formed autolysosomes. In either case, it can be problematic to use GFP fluorescence to follow flux, as new GFP-LC3 is continuously being synthesized. A potential solution to this problem for following fluorescence is to use a photoactivatable version of the fluorescent protein,[176] which allows this assay to be performed essentially as a pulse/chase analysis. Another alternative is to follow flux using GFP-LC3 fluorescence by adding lysosomal protease or fusion inhibitors to cells expressing GFP-LC3 and monitoring changes in the number of puncta. In this case, the presence of lysosomal inhibitors should increase the number of GFP-LC3-positive structures, and the absence of an effect on the total number of GFP-LC3 puncta or on the percentage of cells displaying numerous puncta is indicative of a defect(s) in autophagic flux.[177] The combination of protease inhibitors (to prevent the degradation of GFP) or compounds that modify lyso- somal pH such as NH_4Cl or chloroquine, or compounds such as bafilomycin A_1 or others that block fusion of autophagosomes with lysosomes (e.g., vinblastine) may be most effective in preventing lysosome-dependent decreases in GFP-LC3 puncta. However, because the stability of GFP is affected by lysosomal pH, we advise the use of protease inhibitors whether or not lysosomotropic compounds or fusion inhibitors are included (although lysosomotropic compounds should help stabilize GFP by neutralizing the pH in the lysosome, they do not have an immediate effect on lysosomal hydrolase activity).

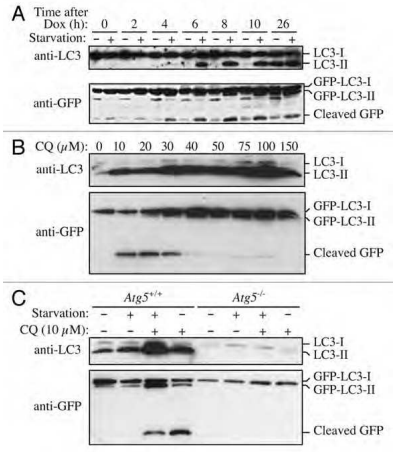

Figure 7. GFP-LC3 processing can be used to monitor delivery of autophagosomal membranes. (A) $Atg5^{2/2}$ MEFs engineered to express Atg5 under the control of the Tet-off promoter were grown in the presence of doxycyline (10 ng/ml) for one week to suppress autophagy. Cells were then cultured in the absence of drug for the indicated times, with or without a final 2 h starvation. Protein lysates were analyzed by western blot using anti-LC3 and anti-GFP antibodies. The positions of untagged and GFP-tagged LC3-I and LC3-II, and free GFP are indicated. This figure was modified from data previously published in reference 171, FEBS Letters, 580, Hosokawa N, Hara Y, Mizushima N, Generation of cell lines with tetracycline-regulated autophagy and a role for

autophagy in controlling cell size, pp. 2623–2629, copyright 2006, with permission from Elsevier. (B) Differential role of unsaturating and saturating concentrations of lysosomal inhibitors on GFP- LC3 cleavage. HeLa cells stably transfected with GFP-LC3 were treated with various concentrations of chloroquine (CQ) for 6 h. Total lysates were prepared and subjected to immunoblot analysis. (C) CQ-induced free GFP fragments require classical autophagy machinery. Wild-type and Atg5$^{2/2}$ MEFs were first infected with adenovirus GFP-LC3 (100 viral particles per cell) for 24 h. The cells were then either cultured in regular culture medium with or without CQ (10 mM), or subjected to starvation in EBSS buffer in the absence or presence of CQ for 6 h. Total lysates were prepared and subjected to immunoblot analysis. (B and C) are modified from the data previously published in reference 172.

Cautionary notes: The GFP-Atg8 processing assay is used routinely to monitor autophagy in yeast. One caveat, however, is that this assay is not always performed in a quantitative manner (for example, western blot exposures need to be in the linear range). Accordingly, an enzymatic assay such as the Pho8D60 assay may be preferred (see Autophagic protein degradation),[178,179] especially when the differences in autophagic activity need to be precisely determined; however, appropriate caution must be used as with any enzyme assay regarding, for example, substrate concentrations and linearity (note that an equivalent assay has not been developed for higher eukaryotic cells). The main limitation of the GFP-LC3 processing assay in mammalian cells is that it seems to depend on cell type and culture conditions (Hosokawa N, Mizushima N, unpublished data). Apparently, GFP is more sensitive to mammalian lysosomal hydrolases than to the degradative milieu of the yeast vacuole. Alternatively, the lower pH of lysosomes relative to that of the vacuole may contribute to differences in detecting free GFP. Under certain conditions [such as Earle's balanced salt solution (EBSS)-induced starvation] in some cell lines, when the lysosomal pH becomes particularly low, free GFP is undetectable because both the LC3-II and free GFP fragments are quickly degraded.[172] Therefore, if this method is used it should be accompanied by immunoblotting including controls to address the stability of nonlysosomal GFP such as GFP-LC3-I. It should also be noted that free GFP can be detected when cells are treated with nonsaturating doses of lysosomal inihibitors such as chloroquine, E-64d and bafilomycin A_1. The saturating concentrations of these lysosomal inhibitors vary in different cell lines, and it would be better to use a saturating concentration of lysosomal inhibitors when performing an autophagic flux assay.[172] Therefore, caution must be exercised in interpreting the data using this assay; it would be helpful to combine an analysis of GFP-LC3 processing with other assays such as the monitoring of endogenous LC3-II by western blot.

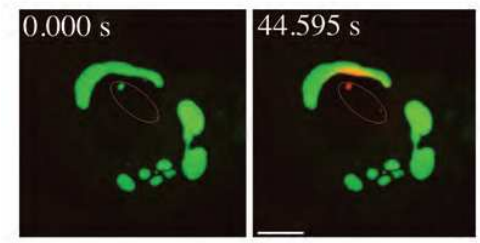

Figure 8. Movement of activated pDendra2-hp62 (orange) from the nucleus (middle) to the aggregate in ARPE-19 cells, revealed by confocal microscopy. Cells were exposed to 5 mM MG132 for 24 h to induce the formation of perinuclear aggregates.185 The cells were then exposed to a UV pulse (the UV-induced area is shown by red lines that are inside of the nucleus) that converts Dendra2 from green to red, and the time shown after the pulse is indicated. p62 is present in a small nuclear aggregrate, and is shuttled from the nucleus to a perinuclear large protein aggregate (detected as red). Scale bar, 5 mm. Image provided by K. Kaarniranta.

Along these lines, a caution concerning the use of the EGFP fluorescent protein for microscopy is that this fluorophore has a relatively neutral pH optimum for fluorescence,[180] so that its signal may diminish quickly during live cell imaging due to the acidic environment of the lysosome. It is possible to circumvent this latter problem by imaging paraformaldehyde-fixed cultures that are maintained in a neutral pH buffer, which retains EGFP fluorescence (Kleinman M, Reiners JJ, personal communication). Alternatively, it may be preferable to use a different fluorophore such as monomeric red fluorescent protein (mRFP) or mCherry, which retain fluorescence even at acidic pH.[181] On the one hand, a putative advantage of mCherry over mRFP is its enhanced photostability and intensity, which are an order of magnitude higher (and comparable to GFP), enabling acquisition of images at similar exposure settings as are used for GFP, thus minimizing potential bias in interpretation.[182] On the other hand, caution is required when evaluating the localization of mCherry fusion proteins during autophagy due to the persistence of the mCherry signal in acidic environments, all tagged proteins are prone to show enrichment in lysosomes during nonspecific autophagy of the cytoplasm, especially higher expression levels. In addition, red fluorescent proteins (even the monomeric forms) can be toxic due to aggregation.[183] Dendra2 is an improved version of the green-to-red photoswitchable fluorescent protein Dendra, which is derived from the octocoral Dendronephthya sp.[184] Dendra2 is capable of irreversible photoconversion from a green to a red fluorescent form, but can be used also as normal GFP or RFP vector. This modified version of the fluorophore has certain properties including a monomeric state, low phototoxic activation and efficient chromophore maturation, which make it suitable for real-time tracking of LC3 and p62 (Fig. 8; Kaarniranta K, personal

communication). Another alternative to mRFP or mCherry is to use the Venus variant of YFP, which is brighter than mRFP and less sensitive to pH than GFP.186 The pH optimum of EGFP is important to consider when using GFP-LC3 constructs, as the original GFP-LC3 marker187 uses the EGFP variant, which may result in a reduced signal upon the formation of amphisomes or autolysosomes. An additional caveat when using the photoactivatable construct PA-GFP180 is that the process of activation by photons may induce DNA damage, which could, in turn, elicit induction of autophagy. Also, GFP is relatively resistant to denaturation, and boiling for 5 min may be needed to prevent the folded protein from being trapped in the stacking gel during SDS-PAGE. As noted above (see Western blotting and ubiquitin-like protein conjugation systems), Atg4 cleaves the residue(s) that follow the C-terminal glycine of Atg8/LC3 that will be conjugated to PE. Accordingly, it is critical that any chimeras be constructed with the fluorescent tag at the N-terminus of Atg8/LC3. Finally, lysosomal inhibition needs to be carefully controlled. Prolongewd inhibition of lysosomal hydrolases (>6h) is likely to induce a secondary autophagic response triggered by the accumulated undigested autophagy cargo. This secondary autophagic response can complicate the analysis of the autophagy flux, making it appear more vigorous than it would in the absence of the lysosomal inhibitors.

Conclusion: The GFP-Atg8/LC3 processing assay (the genera- tion of free GFP within the vacuole/lysosome) is a convenient way to monitor autophagy, but it does not work in all cell types, and is not as easy to quantify as enzyme-based assays. d. GFP-Atg8/LC3 fluorescence microscopy. LC3B, or the protein tagged at its N terminus with a fluorescent protein such as GFP

(GFP-LC3), has been used to monitor autophagy through indirect immunofluorescence or direct fluorescence microscopy (Fig. 9), measured as an increase in punctate LC3 or GFP- LC3.[187,188] The detection of GFP-LC3/Atg8 is also useful for in vivo studies using transgenic organisms such as Caenorhabditis elegans,[189] Dictyostelium discoideum,[190] filamentous ascomy- cetes,[191-195] Ciona intestinalis,[196] Drosophila melanogaster,[197-199] Arabidopsis thaliana,[200] Leishmania major[202,203] and mice.[126] It is also possible to use anti-LC3/Atg8 antibodies for immuno-cytochemistry or immunohistochemistry,[140,204-209] procedures that have the advantages of detecting the endogenous protein, obviating the need for transfection and or the generation of a transgenic organism, as well as avoiding potential artifacts resulting from overexpression (for example, high levels of overexpressed GFP-LC3 can result in its nuclear localization, although the protein can still relocate to the cytosol upon starvation), but we note that it is not always possible to detect endogenous Atg8/LC3. The use of imaging cytometry allows rapid and quantitative measures of the number of LC3 puncta and their relative number in individual or mixed cell types, using computerized assessment, enumeration, and data display (e.g., see refs. 142 and 210). In this respect, the alternative use of an automated counting system may be helpful for obtaining an objective number of puncta per cell. For this purpose, the WatershedCounting3D plug-in for ImageJ may be useful.[211,212]

Monitoring the endogenous protein, however, obviously depends on the ability to detect it in the system of interest. If the endogenous amount is below the level of detection, the use of an exogenous construct is warranted. In this case, it is important to consider the use of stable transformants vs. transient trans- fections. On the one hand, stable transformants may have reduced background resulting from the lower gene expression, and there is also the advantage of eliminating artifacts resulting from recent exposure to transfection reagents (see below).

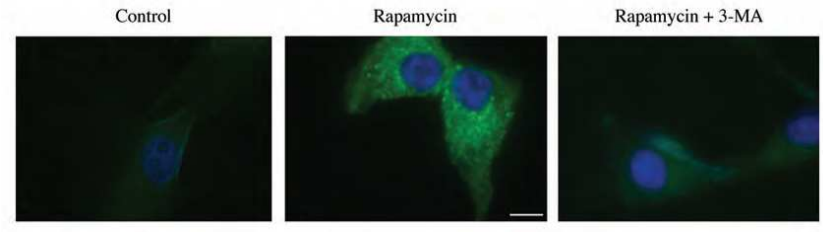

Figure 9. Changes in the detection and localization of GFP-LC3 upon the induction of autophagy. U87 cells stably expressing GFP-LC3 were treated with PBS, rapamycin (200 nM), or rapamycin in combination with 3-MA (2 mM) for 24 h. Representative fluorescence images of cells counterstained with DAPI (nuclei) are shown. Scale bar, 10 mm. This figure was modified from Figure 6 published in Badr et al. Lanatoside C sensitizes glioblastoma cells to tumor necrosis factor–related apoptosis-inducing ligand and induces an alternative cell death pathway. Neuro-Oncology, 13:1213–24, 2011, by permission of Oxford University Press.

Furthermore, with stable transformants more cells can be easily analyzed because nearly 100% of the population will express tagged LC3. On the other hand, one disadvantage of stable transfectants is that the the integration sites cannot always be predicted, and expression levels may not be optimal. Therefore, it is worth considering the use of stable episomal plasmids that avoid the problem of unsuitable integration.[181] An important advantage of transient transfection is that this approach is better for examining the immediate effects of the transfected protein on autophagy, although it restricts the length of time that the analysis can be performed, and consideration must be given to the induction of autophagy resulting from exposure to the transfection reagents (see below). In addition, a double transfection can be used (e.g., with GFP-LC3 and the protein of interest) to visually tag the cells that express

the protein being examined. In conclusion, there is no simple rule for the use of stable vs. transient transfections. When stable transfections are utilized, it is worthwhile screening for clones that give the best signal to noise ratio, and when transient transfections are used, it is worthwhile optimizing the GFP-LC3 DNA concentration to give the best signal to noise ratio. In clones, the uniformity of expression of GFP-LC3 also makes "thresholding" when scoring puncta-positive cells (see below) much easier. However, there is also a need to beware of the frequent unrepresentative behavior of mammalian cell lines when selecting a single cell clone from a parental pool; therefore, it may be better to use a pool of multiple selected clones to avoid possible artifacts that can arise from the selection and propagation of individual clones from a single transfected cell (although the use of a pool is also problematic as its composition will change over time). Optimization, together with including the appropriate controls, will help overcome the effects of the inherent variability in these analyses.

An additional use of GFP-LC3 is to monitor colocalization with a target during autophagy-related processes such as organelle degradation or the sequestration of pathogenic microbes.[213-215] Preincubation of cells stably expressing GFP-LC3 with leupeptin can help stabilize the GFP-LC3 signal during fluorescence micro- scopy, especially under conditions of induced autophagic flux. Leupeptin is an inhibitor of lysosomal cysteine and serine proteases and will therefore inhibit degradation of membrane-conjugated GFP-LC3 that is present within autolysosomes. Cautionary notes: Quantification of autophagy by measuring GFP-LC3 puncta (or LC3 by immunofluorescence) can be more tedious, depending on the method used (e.g., high-throughput image analysis can obviously be rapid and efficient), than monitoring LC3-II by western blot; however, the former may be more sensitive and quantitative. Ideally, it is preferable to include both assays and to compare the two sets of results. In addition, if GFP-LC3 is being quantified, it is better to deter- mine the number of puncta corresponding to GFP-LC3 on a per cell basis (or per cell area basis if the cell population is in culture or the cell composition of the tissue is not homogenous, as well as in the case of plant cells, which tend to grow) rather than simply the total number of cells displaying puncta. This latter point is critical because even in nutrient-rich conditions, cells display some basal level of GFP-LC3 puncta, unless they are lacking autophagy-related genes (and even in the latter case it is possible to get puncta of GFP-LC3 depending on the specific conditions). There are, however, practical issues with counting puncta manually and reliably, especially if there are large numbers per cell [although this may be more accurate than relying on a software program, in which case it is important to ensure that only appropriate dots are being counted; applicable programs include ImageJ, Imaris, which may be more accurate (Ktistakis NT, personal communication), and the open-source software CellProfiler[216]]. Moreover, when autophagosome-lysosome fusion is blocked, larger autophagosomes are detected, possibly due to autophagosome-autophagosome fusion. Although it is possible to detect changes in the size of GFP-Atg8/LC3 puncta by fluorescence microscopy, it is not possible to correlate size with autophagy activity without additional assay methods. Size deter- minations can be problematic by fluorescence microscopy unless careful standardization is performed,[217] and size estimation is not recommended as a method for monitoring autophagy; however, it is possible to quantify the fluorescence intensity of GFP-Atg8/ LC3 at specific puncta, which does provide a valid measure of protein recruitment.[218] In addition to autophagosome size, the number of puncta visible to the eye will also be influenced by both the level of expression of GFP-LC3 in a given cell and by the exposure time of the microscope, if using widefield microscopy. In many cell types it may be possible to establish a cut-off value for the number of puncta per cell in conditions of "low" and "high" autophagy.[219] This can be tested empirically by exposing cells to autophagy-inducing and blocking agents. Thus, cell populations showing significantly greater proportions of cells with autophagosome numbers higher than the cut-off in perturbation conditions compared with the control cells could provide quantitative evidence of altered autophagy. It is then possible to score the population as the percentage of cells displaying numerous autophagosomes. This approach will only be feasible if the background number of puncta is relatively low. For this method, it is particularly important to count a large number of cells and multiple representative sections of the sample (probably on the order of 50 or more, preferably in at least three different trials, depending on the particular system and experiment, but the critical point is that this determination should be based on statistical power analysis). Accordingly, high-content imaging analysis methods are extremely applicable to provide reliable values.

Such methods enable quantification of GFP-LC3 puncta (or overall fluorescence intensity) in thousands of cells per sample (e.g., see refs.164,175 and 220). When using automated analysis methods, care must be taken to manually evaluate parameters used to establish background cutoff values for different treatment conditions and cell types. Another note of caution is that treatments affecting cell morphology, leading to the "roundingup" of cells for example, can result in apparent changes in the number of GFP-LC3 puncta per cell. To avoid misinterpretation of results due to such potential artifacts, manual review of cell images is highly recommended. To allow comparisons by other researchers attempting to repeat these experiments, it is critical that the authors also specify the baseline number of puncta that are used to define "normal" or "low" autophagy. Furthermore, the cells should also be counted using unbiased procedures (e.g., using a random start point followed by inclusion of all cells at regular intervals), and statistical information should be provided for both baseline and altered conditions, as these assays can be highly

variable. One possible method to obtain unbiased counting of GFP-LC3 puncta in a large number of cells is to perform multispectral imaging flow cytometry (see Autophagic flux determination using flow and multispectral imaging cytometry).[221] This method can also be used for endogenous LC3, and, therefore, is useful for non-transfected primary cells.[222] Multispectral imaging flow cytometry allows characterization of single cells within a population by assessing a combination of morphology and immunofluorescence patterns, thereby providing statistically meaningful data.[223] For adherent cell cultures, one caution for flow cytometry is that the techniques necessary to produce single cell suspensions can cause significant injury to the cells, leading to secondary changes in autophagy. Therefore, staining for plasma membrane permeabilization (e.g., cell death) before vs. after isolation is an important control.

An important caveat in the use of GFP-LC3 is that this chimera can associate with aggregates, especially when expressed at high levels in the presence of aggregate-prone proteins, which can lead to a misinterpretation of the results.[224] Of note, GFP-LC3 can associate with ubiquitinated protein aggregates;[225] however, this does not occur if the GFP-LC3 is expressed at low levels (Rubinsztein DC, unpublished observations). These aggregates have been described in many systems, and are also referred to as Aggresome-Like Induced Structures or ALIS,[225-227] dendritic cell ALIS,[228] p62 bodies/sequestosomes[229] and inclusions. Inhibition of autophagy in vitro and in vivo leads to the accumulation of these aggregates, suggesting a role for autophagy in mediating their clearance.[225,226,230-232] One way to control for background levels of puncta is to determine fluorescence from untagged GFP. The adaptor protein SQSTM1/p62 is required for the formation of ubiquitinated protein aggregates in vitro (see SQSTM1/p62 and related LC3 binding protein turnover assays).[229] In this case, the interaction of SQSTM1 with both ubiquitinated proteins and LC3 is thought to mediate delivery of these aggregates to the autophagy system.[233,234]

Many cellular stresses can induce the formation of aggregates, including transfection reagents,[225] or the introduction of foreign DNA (especially if the DNA is not extracted endotoxin free). p62-positive aggregates are also formed by proteasome inhibition or puromycin treatment. Calcium phosphate transfection of COS7 cells or lipofectamine transfection of MEFs (Pinkas-Kramarski R, personal communica- tion), primary neurons (La Spada AR, personal communication) or neuronal cells (Chu CT, personal communication) transiently increases basal levels of GFP-LC3 puncta and/or the amount of LC3-II. One solution is to examine GFP-LC3 puncta in cells stably expressing GFP-LC3; however, as transfection-induced increases in GFP-LC3 puncta and LC3-II are often transient, another approach is to use cells transfected with GFP, and cells subjected to a mock time-matched transfection as background (negative) controls. A lipidation-defective LC3 mutant where glycine 120 is mutated to alanine is targeted to these aggregates independently of autophagy (likely via its interaction with SQSTM1, see above) and as a result this mutant can serve as another valuable control.[225] When carrying out transfections it may be necessary to alter the protocol depending on the level of background fluorescence. For example, changing the medium and waiting 24 to 48 h after the transfection can help to reduce the background level of GFP-LC3 puncta that is due to the transfection reagent (Colombo MI, personal communication). Similarly, when using an mCherry-GFP-SQSTM1 double tag (see Tandem mRFP/mCherry-GFP fluorescence microscopy) in transient transfections it is best to wait 48 h after transfection to reduce the level of aggregate formation and potential inhibition of autophagy (Johansen T, personal communication). Another consideration is that in addition to transfection, viral infection can activate stress pathways in some cells and possibly induce autophagy, again emphasizing the importance of appropriate controls, such as control viruses expressing GFP.[235]

Ubiquitinated protein aggregate formation and clearance appear to represent a cellular recycling process. Aggregate formation can occur when autophagy is either inhibited or when its capacity for degradation is exceeded by the formation of proteins delivered to the aggregates. In principle, formation of GFP-LC3-positive aggregates represents a component of the autophagy process. However, the formation of GFP-LC3-positive ubiquitinated protein aggregates does not directly reflect either the induction of autophagy (or autophagosome formation), or flux through the system. Indeed, formation of ubiquitinated protein aggregates that are GFP-LC3 positive can occur in autophagy-deficient cells.[225] Therefore, it should be remembered that GFP-LC3 puncta likely represent a mix of ubiquitinated protein aggregates in the cytosol, ubiquitinated protein aggregates within autophagosomes and more “conventional” phagophores and autophagosomes bearing other cytoplasmic cargo (this is one example where CLEM could help in resolving this question). In Dictyostelium, inhibition of autophagy leads to huge ubiquitinated protein aggregates containing p62 and GFP-Atg8, when the latter is coexpressed; the size of the aggregates and the typical presence of one structure per cell make them easily distinguishable from autophagosome. Saponin treatment has been used to reduce background fluorescence under conditions where no aggregation of GFP-LC3 is detected in hepatocytes, GFP-LC3 stably-transfected HEK 293235 and human osteosarcoma cells, and in nontransfected cells;236 however, treatment with saponin and other detergents can provoke artifactual GFPLC3 puncta formation,237 and controls need to be included in such experiments in light of these findings. In general, it is preferable to include additional assays that measure autophagy rather than relying solely on monitoring GFP-LC3. In addition, we recommend that researchers validate their assays by demonstrating the absence or reversal of GFP-LC3 puncta formation in cells treated with pharmacological or RNA interference-based

autophagy inhibitors (Table 1). For example, 3-MA is commonly used to inhibit starvation- or rapamycin-induced autophagy,[256] but some data indicate that this compound can also have stimulatory effects (see Autophagy inhibitors and inducers).[257] Another general limitation of the GFP-LC3 assay is that it requires a system amenable to the introduction of an exogenous gene. Accordingly, the use of GFP-LC3 in primary non-transgenic cells is more challenging. Here again, controls need to be included to verify that the transfection protocol itself does not artifactually induce GFP-LC3 puncta or cause LC3 aggregation. Furthermore, transfection should be performed with low levels of constructs, and the transfected cells should be followed to determine (1) when sufficient expression for detection is achieved, and (2) that during the time frame of the assay, basal GFP-LC3 puncta remain appropriately low. In addition, the demonstration of a reduction in the number of induced GFP-LC3 puncta under conditions of autophagy inhibition is helpful. For some primary cells, delivering GFP-LC3 to precursor cells by infection with recombinant lentivirus, retrovirus or adenovirus,258 and subsequent differentiation into the cell type of interest, is a powerful alternative to transfection of the already differentiated cell type.91

To implement the scoring of autophagy via fluorescence microscopy, one option is to measure pixel intensity. Since the expression of GFP-LC3 may not be the same in all cells—as discussed above—it is possible to use specific imaging software to calculate the standard deviation (SD) of pixel intensity within the fluorescence image and divide this by the mean intensity of the pixels within the area of analysis. This will provide a ratio useful for establishing differences in the degree of autophagy between cells. Cells with increased levels of autophagic activity, and hence a greater number of autophagosomes in their cytosol, are associated with a greater variability in pixel intensity (i.e., a high SD). Conversely, in cells where autophagy is not occurring, GFP-LC3 is uniformly distributed throughout the cytosol and a variation in pixel intensity is not observed (i.e., a low SD) (Campanella M, personal communication). Although LC3-II is primarily membrane-associated, it is not necessarily associated with autophagosomes as is often assumed; the protein is also found on phagophores, the precursors to autophagosomes, as well as on amphisomes and phagosomes, as well as on amphisomes and phagosomes (see Western blotting and ubiquitin-like protein conjugation systems)[132, 259, 260]. Along these lines, yeast Atg8 can associate with the vacuole membrane independent of lipidation, so that a punctate pattern does not necessarily correspond to autophagic compartments.[261] Thus, the use of additional markers is necessary to specify the identity of an LC3-positive structure; for example, ATG12–ATG5-ATG16L1 would be present on a phagophore, but not aa autophagosome. In addition, the site(s) of LC3 conjugation to PE is not definitively known and levels of Atg8–PE/LC3-II can increase even in autophagy mutants that cannot form autophagosomes.[262] One method that can be used to examine LC3-II membrane association is differential extraction in Triton X-114, which can be used with mammalian cells,258 or western blot analysis of total membrane fractions following solubilization with Triton X-100, which is helpful in plants.[150,151] Another approach is to examine colocalization of LC3 with ATG5 (or other ATG proteins); the ATG12–ATG5 conjugate does not remain associated with autophagosomes, meaning that colocalized structures would correspond to phagophores. Importantly, we stress again that numbers of GFP-LC3 puncta, similar to steady-state LC3-II levels, reflect only a snapshot of the numbers of autophagy-related structures (e.g., autophagosomes) in a cell, and not autophagic flux.

Finally, we offer a general note of caution with regard to using GFP. First, the GFP tag is large, in particular relative to the size of LC3; therefore, it is possible that a chimera may behave differently from the native protein in some respects. Second, GFP is not native to most systems, and as such it may be recognized as an aberrant protein and targeted for degradation, which has obvious implications when studying autophagy. Third, some forms of GFP tend to oligomerize, which may interfere with protein function and/or localization. Fourth, EGFP inhibits polyubiquitination[263] and may cause defects in other cellular processes. Fifth, not all LC3 puncta represent LC3-II and correspond to autophagosomes.[137,264,265] Accordingly it would be prudent to complement any assays that rely on GFP fusions (to Atg8/LC3 or any protein) with additional methods that avoid the use of this fluorophore.

Table 1. Genetic and pharmacological regulation of autophagy†

Method	Comments
1. 3-methyladenine	A PtdIns3K inhibitor that effectively blocks an early stage of autophagy by inhibiting the class III PtdIns3K, but is not a specific autophagy inhibitor. 3-MA also inhibits the class I PtdIns3K and can thus, at suboptimal concentrations in long-term experiments, promote autophagy in some systems, as well as affect cell survival through AKT and other kinases.
2. 10-NCP	10-(4′-N-diethylamino)butyl)-2-chlorophenoxazine; an AKT inhibitor that induces autophagy in neurons.238
3. 17-AAG	An inhibitor of the HSP90-CDC37 chaperone complex, induces autophagy in certain systems (e.g., neurons), but impairs starvation-induced autophagy and mitophagy in others by promoting the turnover of ULK1.239
4. ATG4C74A	An active site mutant of ATG4 that is defective for autophagy.240

5. Bafilomycin A1 A V-ATPase inhibitor that causes an increase in lysosomal/vacuolar pH, and ultimately blocks fusion of autophagosomes with the vacuole.

6. Calcium An autophagy activator that can be released from ER or lysosomal stores under stress conditions; however, calcium can also inhibit autophagy.[241]

7. Chloroquine, NH4Cl Lysosomotropic compounds that elevate/neutralize the lysosomal/vacuolar pH.

8. Deletion This method provides the most direct evidence for the role of an autophagic component; however, more than one gene involved in autophagy should be targeted to avoid indirect effects.

9. E-64d A membrane-permeable cysteine protease inhibitor that can block the activity of a subset of lysosomal hydrolases; should be used in combination with pepstatin A for inhibiting lysosomal protein degradation.

10. Knockdown This method provides relatively direct evidence for the role of an autophagic component. However, the efficiency of knockdown varies, as does the stability of the targeted protein. In addition, more than one gene involved in autophagy should be targeted to avoid indirect effects.

11. KU-0063794 An MTOR inhibitor that binds the catalytic site and activates autophagy.242,243

12. Leupeptin An inhibitor of cysteine, serine and threonine proteases that can be used in combination with pepstatin A and/or E-64d to block lysosomal protein degradation. Leupeptin is not membrane permeable, so its effect on cathepsins may depend on endocytic activity.

13. microRNA Can be used to reduce the levels of target mRNA(s) or block translation.

14. NAADP-AM Activates the lysosomal two pore channel and induces autophagy.244

15. NED-19 Inhibits the lysosomal two-pore channel and NAADP- induced autophagy.244

16. NVP-BEZ235 A dual inhibitor of p110 and the MTOR catalytic site that activates autophagy.245,246

17. Pathogen-derived ICP34.5. vBCL2, vFLIP, influenza M2, and HIV Nef autophagy inhibitor transfection.

18. Pepstatin A An aspartyl protease inhibitor that can be used to partially block lysosomal degradation; should be used in combination with other inhibitors such as E-64d. Pepstatin A is not membrane permeable.

19. Protease inhibitors These chemicals inhibit the degradation of autophagic substrates within the lysosome/vacuole lumen. A combination of inhibitors (e.g., leupeptin, pepstatin A and E-64d) is needed for complete blockage of degradation.

20. Rapamycin Inhibits MTOR by binding to RAPTOR, thus inducing autophagy, but only provides partial inhibition.

21. Resveratrol A natural polyphenol that induces autophagy via activation of AMPK.247,248

22. RNAi Can be used to inhibit gene expression.

23. RSVAs Synthetic small-molecule analogs of resveratrol that potently activate AMPK and induce autophagy.249

24. Thapsigargin An inhibitor of the sarcoplasmic/endoplasmic reticulum Ca2+ ATPase (SERCA) that inhibits autophagic sequestration through the depletion of intracellular Ca2+ stores;[250] however, thapsigargin may also block fusion of autophagosomes with endosomes by interfering with recruitment of RAB7, resulting in autophagosome accumulation.251 Long-term thapsigargin treatment may induce ER stress and a secondary stimulation of autophagy.

25. Torin1 A catalytic MTOR inhibitor that induces autophagy and provides more complete inhibition than rapamycin (it inhibits all forms of MTOR).[252]

26. Trehalose An inducer of autophagy that may be relevant for the treatment of different neurodegenerative diseases.[253,254]

27. Tunicamycin A glycosylation inhibitor that induces autophagy due to ER stress.[255]

28. Vinblastine A depolymerizer of both normal and acetylated microbtubles that interferes with autophagosome-lysosome fusion.[156]

29. Wortmannin An inhibitor of PtdIns 3-kinase that blocks autophagy, but is not a specific inhibitor (see 3-MA above).

†This table is not meant to be complete, as there are many compounds and genetic methods that regulate autophagy, and new ones are being discovered
routinely.

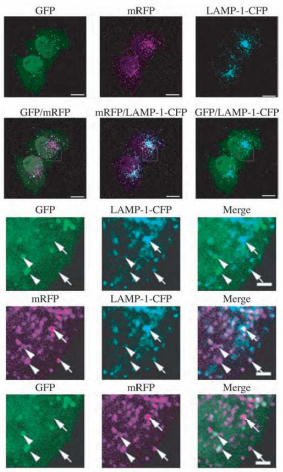

Figure 10. The GFP and mRFP signals of tandem fluorescent LC3 (tfLC3, mRFP-GFP-LC3) show different localization patterns. HeLa cells were cotransfected with plasmids expressing either tfLC3 or LAMP1-CFP. Twenty-four hours after the transfection, the cells were starved in Hanks' solution for 2 h, fixed and analyzed by microscopy. The lower panels are a higher magnification of the upper panels. Bar, 10 mm in the upper panels and 2 mm in the lower panels. Arrows in the lower panels point to (or mark the location of) typical examples of colocalized signals of mRFP and LAMP1. Arrowheads point to (or mark the location of) typical examples of colocalized particles of GFP and mRFP signals. This figure was previously published in reference ,181 and is reproduced by permission of Landes Bioscience, copyright 2007.

Similarly, with the emergence of "super-resolution" microscopy methods such as photoactivated localization microscopy (PALM), new tags are being used (e.g., the EosFP green to red photoconvertible fluorescent protein, or the Dronpa GFP-like protein) that will need to be tested and validated.[266]

Conclusion: GFP-LC3 provides a marker that is relatively easy to use for monitoring autophagy induction (based on the appearance of puncta), or colocalization; however, it is not a preferred method for determining flux. In addition, it is recommended to use additional assays along with GFP-LC3 fluorescence microscopy to monitor autophagy.

e. Tandem mRFP/mCherry-GFP fluorescence microscopy. A fluorescence assay that is particularly designed to monitor flux relies on the use of a tandem monomeric RFP-GFP-tagged LC3 (tfLC3; Fig. 10).181 The GFP signal is sensitive to the acidic nd/or proteolytic conditions of the lysosome lumen, whereas mRFP is more stable. Therefore, colocalization of both GFP and mRFP fluorescence indicates a compartment that has not fused with a lysosome, such as the phagophore or an autophagosome. In contrast, an mRFP signal without GFP corresponds to an amphisome or autolysosome. Other fluorophores such as mCherry are also suitable instead of mRFP,[229] and an imagerecognition algorithm has been developed to quantify flux of the reporter to acidified compartments.[242,267] One of the major advantages of the tandem mRFP/mCherry-GFP reporter method is that it enables simultaneous estimation of both the induction of autophagy and flux through autophagic compartments in essentially native conditions, without requiring any drug treatment. The use of more than one time point allows visualization of increased early autophagosomes followed by increases in late autophagosomes as an additional assurance that flux has been maintained.[268] In addition, this method can be used to monitor autophagy in high-throughput drug screening studies.[242] The quantification of "yellow only" and "red only" dots in a stable tandem-fluorescent LC3-reporter cell line can be automated by a Cellomics microscope that can be used to assess a huge population of cells (1,000 or more) over a large number of random fields of view. This can give rise to more accurate data than can be achieved by manual assessment of a few selected cells.[167, 269]

An alternative dual fluorescence assay involves the Rosella biosensor. This assay monitors the uptake of material to the lysosome/vacuole and complements the use of the tandem mRFP/mCherry-GFP reporter. The assay is based upon the genetically encoded dual color-emission biosensor Rosella, a fusion between a relatively pH-stable fast-maturing RFP variant, and a pHsensitive GFP variant. When targeted to specific cellular compartments or fused to an individual protein, the Rosella biosensor provides information about the identity of the cellular component being delivered to the vacuole/lysosome for degradation. Importantly, the pH-sensitive dual color fluorescence emission provides information about the environment of the biosensor during autophagy of various cellular components. In yeast, Rosella has been successfully used to monitor autophagy of cytosol, mitochondria (mitophagy) and the nucleus (nucleophagy).[270,271] Furthermore, the Rosella biosensor can be used as a reporter under various conditions including nitrogen depletion-dependent induction of autophagy.[270,271] The Rosella biosensor can also be expressed in mammalian cells to follow nonselective autophagy (cytoplasmic turnover), or mitophagy.[271]

Cautionary notes: The use of tandem mRFP/mCherry-GFPLC3/Atg8 reporters in live imaging experiments can be complicated by the motion of LC3/Atg8 puncta. As a consequence, conventional confocal microscopy may not allow visualization of colocalized mRFP/mCherry-GFP puncta. In this case, GFP or colocalized puncta represent newly formed autophagic structures whereas mRFP/mCherry-only puncta are ambiguous. Spinning disk confocal microscopy, or rapid acquisition times may be required for imaging tandem mRFP/mCherry-GFP proteins, although these techniques require a brighter fluorescent signal associated with what may be undesirably higher levels of transgene expression. One solution is to use the mTagRFP-mWasabi-LC3 chimera,[272a] as mTagRFP is brighter than mRFP1 and mCherry, and mWasabi is brighter than EGFP.[272b] Another possibility is to use fixed cells; however, this presents an additional concern: The use of tandem mRFP/mCherry-GFP relies on the quenching of the GFP signal in the acidic autolysosome; however, fixation solutions are often neutral or weak bases, which will increase the pH of the entire cell. Accordingly, the GFP signal may be restored after fixation (Fig. 11), which would cause an underestimation of the amount of signal that corresponds only to RFP (i.e., in the autolysosome). Thus, the tissue or cell samples must be properly processed to avoid losing the acidic environment of the autolysosomes. In addition, there may be weak fluorescence of EGFP even in an acidic environment (pH between 4 and 5).[180,258] Therefore, it may be desirable to choose a monomeric green fluorescent protein that is more acid sensitive than EGFP for assaying autophagic flux.

Another caution in the interpretation of the tandem fluorescent marker is that colocalization of GFP and mRFP/mCherry might also be seen in the case of impaired proteolityc degradation within autolysosomes, or altered lysosomal pH. Finally, expression of tandem mRFP-GFP-LC3 is toxic to some cancer cell lines relative to GFP-LC3 or RFP-LC3 (KS Choi, personal communication). The cytotoxicity of DsRed and its variants such as mRFP1 is associated with downregulation of BCL2L1 (Bcl-xL).[273] In contrast to mRFP-GFP-LC3, overexpression of mTagRFP-mWasabi-LC3 does not appear to be toxi to Hela cells (Lin J, personal communication). The Rosella assay has not been tested in a wide range of mammalian cell types. Accordingly, the sensitivity and the specificity of the assay must be verified independently until this method has been tested more extensively and used more widely. Finally, it is ideal to capture the dynamic behavior of autophagy in real time, in order to generate data revealing the rate of formation and clearance of autophagosomes over time, rather than single data points. For example, by acquiring signals from two fluorescent constructs in real time, the rate of change in colocalization signal as a measure of the fusion rate and recycling rate between autophagosomes and lysosomes can be assessed. Importantly, due to the integral dynamic relationship of autophagic flux with the onset of apoptosis and necrosis it is advantageous to monitor cell death and autophagic flux parameters concomitantly over time, which FRET-based reporter constructs make possible.[274]

Conclusion: The use of tandem fluorescent constructs, which display different emission signals depending on the environment (in particular, GFP fluorescence is particularly sensitive to an acidic pH), provides a convenient way to monitor autophagy flux in many cell types.

f. Autophagic flux determination using flow and multispectral imaging cytometry. Whereas fluorescence microscopy, in combination with novel autophagy probes, has permitted single cell analysis of autophagic flux, automation for allowing medium to high-throughput analysis has been challenging. A number of methods have been developed that allow the determination of autophagic flux using the fluorescence-activated cell sorter (FACS),[154,223,236,275-277] and commercial kits are now available for monitoring autophagy by flow cytometry. These approaches make it possible to capture high-content images of cells in flow (up to 1,000 cells/sec), and are particularly useful for cells that grow in suspension. Optimization of image analysis permits the study of cells with heterogeneous LC3 puncta, thus making it possible to quantify autophagic flux accurately in situations that might perturb normal processes (e.g., microbial infection).[277,278] Since EGFP-LC3 is a substrate for autophagic degradation, total fluorescence intensity of EGFP-LC3 can be used to indicate levels of autophagy in living mammalian cells.[275] When autophagy is induced, the decrease in total cellular fluorescence can be precisely quantified in large numbers of cells to obtain robust data. In another approach, soluble EGFP-LC3-I can be depleted from the cell by a brief saponin extraction so that the total fluorescence of EGFP-LC3 then represents that of EGFP-LC3-II alone (Fig. 12A).[235,236] Since EGFP-LC3 transfection typically results in high relative levels of EGFP-LC3-I, this treatment significantly reduces the background fluorescence due to non-autophagosomeassociated reporter protein.

By comparing these treatments in presence or absence of lysosomal degradation inhibitors, subtle changes in the flux rate of the GFP-LC3 reporter construct can be detected. If it is not desirable to treat cells with lysosomal inhibitors to determine rates of autophagic flux, a tandem mRFP/ mCherry-EGFP-LC3 (or similar) construct can also be used for autophagic flux measurements in FACS experiments (see Tandem mRFP/mCherry-GFP fluorescence microscopy).[276]

These methods, however, require the cells of interest to be transfected with reporter constructs. Since the saponin extraction method can also be combined with intracellular staining for endogenous LC3 protein, subtle changes in autophagic flux can be measured without the need for reporter transfections (Fig. 12B). This enables investigations of autophagic flux in a wide variety of cell types and tissues.

Cautionary notes: Care must be taken when applying flow cytometry measurements to adherent cells, particularly neurons and other cells with interdigitated processes, as the preparation of single cell suspensions entails significant levels of

plasma membrane disruption and injury that can secondarily induce autophagy.

Users of the saponin extraction method should carefully titrate saponin concentrations and times of treatment in order to ensure specific extraction of LC3-I in their systems. Also, it has been observed in some cell types that saponin treatment can lead to non-autophagic aggregation of LC3,[237] which should be con- trolled for in these assays (see GFP-Atg8/LC3 fluorescence microscopy).

Cell membrane permeabilization with digitonin and extraction of the nonmembrane-bound form of LC3 allows combined staining of membrane-associated LC3-II protein and any markers for detection of autophagy in relation to other cellular events/ processes. Based on this approach a method for monitoring autophagy in different stages of the cell cycle was developed.[279] Thus, the presence of basal or starvation-induced autophagy is detected in G_1, S and G_2/M phases of the cell cycle in MEFs with doxycycline-regulated ATG5 expression. In these experiments cells were gated based on their DNA content, and the relative intensity of GFP-LC3-II and LC3-II expression. This approach might also be used for the detection of autophagic flux in different stages of the cell cycle or subG_1 apoptotic cell population by measuring accumulation of LC3-II in the presence or absence of lysosomal inhibitors.

Although GFP-LC3 can be used as a reporter for flow cytometry, it is more stable (which is not necessarily ideal for flux measurements) than GFP-SQSTM1 or GFP-NBR1 (NBR1 is a selective autophagic substrate with structural similarity to p62280); GFP-p62 displays the largest magnitude change following the induction of autophagy by amino acid deprivation or rapamycin treatment, and may thus be a better marker for following autophagic flux by this method (confirmed in SH-SY5Y neuronal cell lines stably expressing GFP-p62; Valente EM, personal communication).

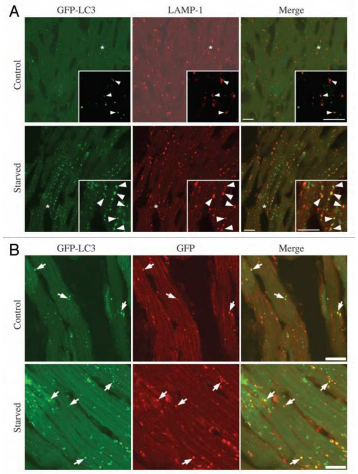

Figure 11. GFP fluorescence in the autolysosome can be recovered upon neutralization of the pH. (A) GFP-LC3 emits green fluorescence in the autolysosomes of post-mortem processed heart sections. Cryosections of 3.8% paraformaldehyde fixed ventricular myocardium from 3-week old GFP-LC3 transgenic mice at the baseline (control) or starved for 24 h (starved) were processed for immunostaining using a standard protocol (buffered at pH 7.4). Most of the GFP-LC3 puncta are positive for LAMP1, suggesting that the autolysosomes had recovered GFP fluorescence. (B) Colocalization between GFP-LC3 direct fluorescence (green) and indirect immunostaining for GFP (red). Sections processed as in (A) were immunostained for GFP using a red fluorescence-tagged secondary antibody, and the colocalization with GFP fluorescence was examined by confocal microscopy. Almost all of the red puncta emit green fluorescence. Images provided by X. Wang.

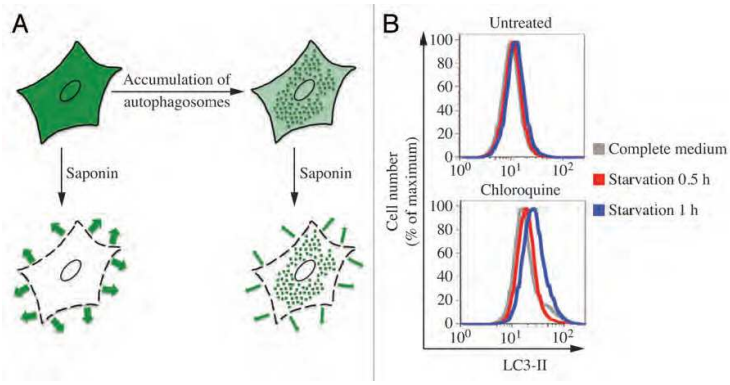

Figure 12. Saponin extraction allows quantification of LC3-II fluorescence by FACS. (A) Schematic diagram of the effects of the saponin wash. Due to the reorganization of the EGFP-LC3 reporter protein, induction of autophagosome formation does not change the total levels of fluorescence in EGFP-LC3-transfected cells. However, extraction of EGFP-LC3-I with saponin results in a higher level of fluorescence in cells with proportionally higher levels of EGFP-LC3-II-containing autophagosomes. This figure was previously published in reference.236 (B) Saponin extraction can also be used to measure flux of endogenous LC3 protein. Human osteosarcoma cells were starved of amino acids and serum by incubation in EBSS, for the indicated times in the presence or absence of a 1 h chloroquine (50 mM) treatment. Cells were then washed with PBS containing 0.05% saponin and processed for FACS analysis for endogenous LC3. These data are provided by K.E. Eng and G.M. McInerney.

However, p62 changes can be cell type and context specific. In some cell types, there is no change in overall p62 levels despite strong levels of autophagy induction, verified by the tandem mRFP/mCherry-GFP-LC3 reporter as well as Atg7- and lysosomedependent turnover of cargo proteins (Chu CT, personal observation). In other contexts, a robust loss of p62 does not correlate with increased autophagic flux as assessed by a luciferasebased measure of flux.166 Thus, appropriate positive and negative controls, and assessment of p62 mRNA levels, may be needed prior to the use of p62 as a flux indicator in a particular cellular context.

Conclusion: Medium-to high-throughput analysis of autophagy is possible using flow and multispectral imaging cytometry (Fig. 13). The advantage of this approach is that larger numbers of cells can be analyzed with regard to GFP-LC3 puncta, cell morphology and/or autophagic flux, and concomitant detection of surface markers can be included, potentially providing more robust data than is achieved with other methods.

g. Immunohistochemistry. Immunodetection of ATG proteins (particularly LC3 and BECN1) has been reported as a prognostic factor in various human carcinomas, including lymphoma,[140,282] breast carcinoma,283 endometrial adenocarcinoma,[284,285] head and neck squamous cell carcinoma,[286,287] hepatocellular carcinoma,288,289 gliomas,[290] non-small cell lung carcinomas,[291] pancreatic[292] and colon adenocarcinomas,[293-295] as well as in cutaneous and uveal melanomas.[296,297] Importantly, this kind of assay should be performed as recommended by the Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK). As we identify new drugs for modulating autophagy in clinical applications, this type of information may prove useful in the identification of subgroups of patients for targeted therapy.[299-301]

In mouse and rat tissues, endogenous LC3, ATG4B, and ATG9A have been detected by immnohistochemical analyses using both paraffin sections and cryosections.[208,302-304] When autophagosomes are absent, the localization pattern of LC3 in the cells of various tissues is diffuse and cytosolic. Moreover, intense fibrillary staining of LC3 is detectable along dendrites of intact neurons, whereas granular staining for LC3 appears mainly in the perikarya of neurons in cathepsin D- or cathepsins B- and L-deficient mouse brains.[208] In developing retinal tissue in chicken, BECN1 and AMBRA1 are detected by immunofluore- scence.[305,306] Finally, in non-mammalian vertebrates, BECN1 was detected during follicular atresia in the ovary of three fish species using paraffin sections; a punctate immunostaining for BECN1 was scattered throughout the cytoplasm of the follicular cells when they were in intense phagocytic activity for yolk removal (Rizzo E, unpublished results).

Cautionary notes: One problem with immunohistochemistry for LC3 is that in some tissues this protein can be localized in structures other than autophagosomes. For example, in murine hepatocytes and cardiomyocytes under starved conditions, endogenous LC3 is detected not only in autophagosomes but also on lipid droplets.[307] In neurons in ATG7-deficient mice, LC3 is accumulated in ubiquitin- and SQSTM1-positive aggregates.[308]

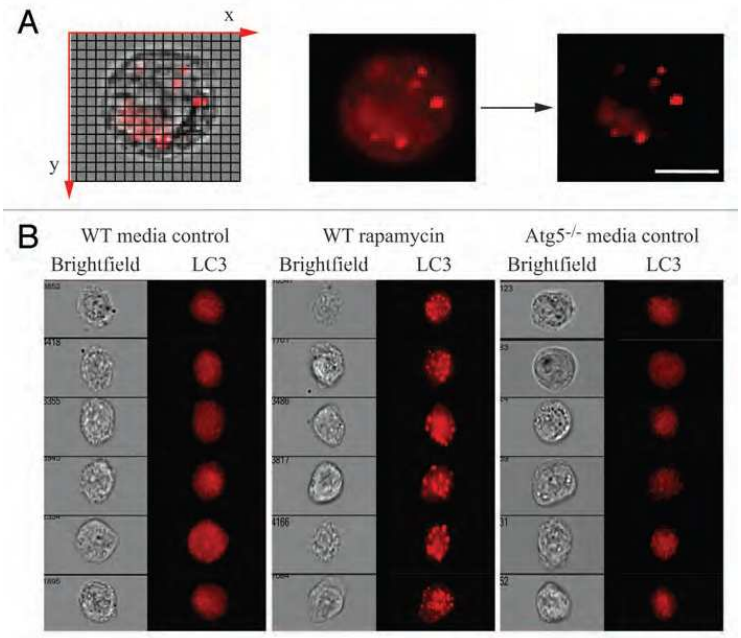

Figure 13. Assessing autophagy with multispectral imaging cytometry. (A) Bright Detail Intensity (BDI) measures the foreground intensity of bright puncta (that are three pixels or less) within the cell image. For each cell, the local background around the spots is removed before intensity calculation. Thus, autophagic cells with puncta have higher BDI values. (B) Media control (untreated wild type), rapamycin-treated wildtype and Atg52/2 MEFs were gated based on BDI. Representative images of cells with high or low BDI values. Scale bar, 10 mm. Images provided by M.L. Albert.

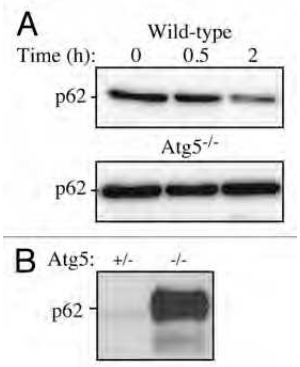

Figure 14. Regulation of the p62 protein during autophagy. (A) The level of p62 during starvation. Atg5+/+ and Atg52/2 MEFs were cultured in DMEM without amino acids and serum for the indicated times, and then subjected to immunoblot analysis using anti-p62 antibody (Progen Biotechnik). This figure was previously published in reference 25 and is reproduced by permission of Landes Bioscience, copyright 2007. (B) The level of p62 in the brain of neural-cell specific Atg5 knockout mice. This image was generously provided by Dr. Taichi Hara (Tokyo Medical and Dental University).

Thus, immunodetection of LC3 in cytoplasmic granules is not sufficient to monitor autophagy in vivo.

Conclusion: It has not been clearly demonstrated that immunohistochemistry of ATG proteins in tissues corresponds to autophagy activity, and this area of research needs to be further explored before we can make specific recommendations.

3. SQSTM1/p62 and related LC3 binding protein turnover assays. In addition to LC3, SQSTM1/p62, or other receptors such as NBR1, can also be used as a protein marker, at least in certain settings.[25,309] The SQSTM1 protein serves as a link between LC3 and ubiquitinated substrates.[98] SQSTM1 and SQSTM1-bound polyubiquitinated proteins become incorpo- rated into the completed autophagosome and are degraded in autolysosomes, thus serving as a readout of autophagic degrada- tion (Fig. 14). Inhibition of autophagy correlates with increased levels of SQSTM1 in mammals and Drosophila, suggesting that steady-state levels of this protein reflect the autophagic status.[304,310-315] Similarly, decreased SQSTM1 levels are associated with autophagy activation. The phosphorylation of SQSTM1 at Ser403 appears to regulate its role in the autophagic clearance of ubiquitinated proteins, and anti-phospho-SQSTM1/p62 anti- bodies can be used to detect the modified form of the protein.[234]

Cautionary notes: SQSTM1 contains an LC3 interacting motif as well as a ubiquitin binding domain, and appears to act by linking ubiquitinated substrates with the autophagic machinery. Nonetheless, it would be prudent to keep in mind that SQSTM1 contains domains that interact with several signaling molecules,[316] and SQSTM1 may be part of the mechanistic target of rapamycin (MTOR) complex 1 (TORC1).[317] Thus, it may have additional functions that need to be considered with regard to its role in autophagy. In the context of autophagy as a stress response, the complexity of using SQSTM1 as an autophagy marker is underscored by its capacity to modulate the NFE2L2/NRF2 anti-oxidant response pathway through a KEAP1 binding domain.[318] In fact, SQSTM1 may, itself, be transcriptionally induced by NFE2L2.[319] Furthermore, it is necessary to examine endogenous SQSTM1 because overexpression of this protein leads to the formation of protein inclusions. In fact, even endogenous SQSTM1 becomes Triton X-100-insoluble in the presence of protein aggregates and when autophagic degradation is inhibited; thus, results with this protein are often context-dependent. In addition, SQSTM1 participates in proteasomal degradation, and its level may also increase when the proteasome is inhibited.[320] Accordingly, the SQSTM1 degradation rate should be analyzed in the presence of an inhibitor such as epoxomicin or lactacystin to determine the contribution from the proteasome (see Autophagy inhibitors and inducers for potential problems with MG132).[321] SQSTM1 is also a substrate for CASP6/caspase 6 and CASP8/caspase 8 (as well as CAPN1/calpain 1), which may confound its use in examining cell death and autophagy.[322] Another issue is that some phosphatidylinositol 3-kinase (PtdIns3K) inhibitors such as LY294002, and to a lesser extent wortmannin (but apparently not 3-MA)[256] can inhibit protein synthesis;[323] this might in turn affect the turnover of SQSTM1 and LC3, which could influence conclusions that are drawn from the status of these proteins regarding autophagy flux or aggresome-like induced structures (ALIS) formation. Accordingly, it may be advisable to measure protein synthesis and proteasome activity along with autophagy under inhibitory or activating conditions. With regard to protein synthesis, it is worth noting that this can be monitored through a nonradioactive method.[324] Finally, SQSTM1 may be transcriptionally upregulated under certain conditions,[227,325] further complicating the interpretation of results. For example, SQSTM1 upregulation, and at least transient increases in the amount of SQSTM1, is seen in some situations where there is an increase in autophagic flux.[326-328] Of interest, SQSTM1 hyperexpression at both gene and protein levels can be observed in muscle atrophy induced by cancer, though not by glucocorticoids, suggesting that the

stimulus inducing autophagy may also be relevant to the differential regulation of autophagy-related prtoteins (Penna F, Costelli P, unpublished observations). One solution to problems relating relating to variations in p62 synthesis is to use a HaloTag1-p62 chimera.[329] The chimeric protein can be covalently labeled
with HaloTag1 ligands, and the loss of signal can then be monitored without interference by subsequent changes in protein synthesis. Similarly, a stable cell line expressing EGFP-tagged p62 under the control of in inducible promoter can be used to assess the rates of p62 degradation, taking into account the limitations outlined above (see Autophagic flux determination using flow and multispectral imaging cytometry).[281]

Western blot analysis using NP40 or Triton X-100 lysis in autophagic conditions typically shows a reduction in SQSTM1 levels. However, this does not necessarily indicate that SQSTM1 is degraded, because SQSTM1 aggregates are insoluble in these detergent lysis conditions.[227,331] Moreover, in some instances SQSTM1 levels do not change in the soluble fractions despite autophagic degradation, a finding that might be explained by simultaneous transcriptional induction of the gene encoding SQSTM1, since the soluble fraction accounts only for the diffuse or free form of SQSTM1. Accumulation of SQSTM1 in the Triton X-100-insoluble fraction can be observed when autophagy- mediated degradation is inhibited. Under conditions of higher autophagic flux, accumulation of SQSTM1 in Triton X-100- insoluble fractions may not be observed and SQSTM1 levels may be reduced or maintained. The simplest approach to circumvent many of these problems is by using lysis buffer that allows identification of the entire cellular pool of SQSTM1 (e.g., containing 1% SDS); however, additional assessment of both Triton X-100-soluble and -insoluble fractions will provide further information regarding the extent of SQSTM1 oligomerization.[308] To conclusively establish SQSTM1 degradation by autophagy, SQSTM1 levels in both Triton X-100-soluble and-insoluble fractions need to be determined upon treatment with autophagy inducers in combination with autophagy inhibitors, such as those that inhibit the autolysosomal degradation steps (e.g., protease inhibitors, chloroquin or bafilomycin A_1, or genetically by knocking out or knocking down LAMP2). Additionally, an alteration in the levels of SQSTM1 may not be immediately evident with changes observed in autophagic flux upon certain chemical perturbations (Sarkar S, personal communication). Whereas LC3 changes may be rapid, clearance of autophagy substrates may require a longer time. Therefore, if LC3 changes are assessed at 6 h or 24 h after a drug treatment, SQSTM1 levels can be tested not only at the same time points, but also at later time points (24 h or 48 h) for determining the maximal impact on substrate clearance. An alternative method is immunostaining for SQSTM1 with and without autophagy inhibitors, which will appear as either a diffuse or punctate pattern. Experiments with autophagy inducers and inhibitors, in combination with western blot and immunostaining analyses, best establish autophagic degradation based on SQSTM1 turnover. A final point, however, is that empirical evidence suggests that the species-specificity of antibodies for detecting p62 recognize both human and mouse SQSTM1, whereas others detect the human, but not the mouse protein (see the Autophagy Forum at https://www.landesbioscience.com/journals/autophagy/forum/ for information pertaining to anti-SQSTM1/p62 antibodies).[332] Another issue with detecting SQSTM1 in the context of human diseases is that it can be mutated (e.g., in Paget disease of bone).[333] Thus, care should be taken to ensure that potential mutations are not affecting the epitopes that are recognized by anti- SQSTM1 antibodies when using western blotting to detect this protein.

Conclusion: There is not always a clear correlation between increases in LC3-II and decreases in SQSTM1. Thus, although analysis of SQSTM1 can assist in assessing the impairment of autophagy or autophagy flux, we recommend using SQSTM1 only in combination with other methods such as LC3-II turnover to monitor flux.

4. TOR, AMPK and Atg1/ULK1. Atg1/ULK1 are central components in autophagy that likely act at more than one stage of the process. There are multiple ULK isoforms in mammalian cells including ULK1, ULK2, ULK3, ULK4 and STK36.[334] ULK3 is a positive regulator of the Hedgehog signaling pathway,[335] and its overexpression induces both autophagy and senescence.[336] Along these lines, ectopic ULK3 displays a punctate pattern upon starvation-induced autophagy induction.[336] ULK3, ULK4 and STK36, however, lack the domain present on ULK1 and ULK2 that bind ATG13 and RB1CC1/FIP200.[337] Thus, ULK3 may play a role that is restricted to senescence, and that is independent of the core autophagy machinery. ULK2 has a higher degree of identity with ULK1 than any of the other homologs, and they may have similar functions that are tissue specific; however, ULK1 may be the predominant isoform involved in autophagy, as knockdown of ULK2 does not affect movement of ATG9.[338] The stability and activation of ULK1, but not ULK2, is dependent on its interaction with the HSP90-CDC37 chaperone complex. Pharmacological or genetic inhibition of the chaperone complex increases proteasome-mediated turnover of ULK1, impairing its kinase activity and ability to promote both starvation-induced autophagy and mitophagy.[239]

AMP-activated protein kinase (AMPK) is a multimeric serine/threonine protein kinase comprising a- (catalytic), β- (scaffold), and c- (regulatory) subunits. The enzyme activity of AMPK is absolutely dependent on phosphorylation of the a-subunit on Thr172,[339,340] and can, therefore, be conveniently monitored by western blotting with a phosphospecific antibody against this site. In some cells, Thr172 is phosphorylated by the Ca2+-activated protein kinase CaMKKβ; in other cells by the constitutively active kinase LKB1, regulation of AMPK activity being performed

primarily by Thr172-dephosphorylating protein phosphatases such as PP1 and PP2A.341 Thr172 dephosphorylation is modulated by adenine nucleotides that bind competitively to regulatory sites in the c-subunit. AMP and ADP inhibit dephosphorylation and promote AMPK activity, whereas Mg2+-ATP has the opposite effect.340 AMPK thus acts as a fine-tuned sensor of the overall cellular energy charge that regulates cellular metabolism to maintain energy homeostasis. Activation of AMPK is also associated with the phosphorylation of downstream enzymes involved in ATP-consuming processes, such as fatty acid (acetyl-CoA carboxylase) and cholesterol (hydroxymethylglutaryl-CoA dehydrogenase) biosynthesis. The role of AMPK in autophagy is complex, and highly dependent on both cell type and metabolic conditions. In liver cells, AMPK suppresses autophagy at the level of cargo sequestration as indicated by the rapid sequestration-inhibitory effects of a variety of AMPK activators, whereas it appears to stimulate autophagy in many other cell types, including fibroblasts, colon carcinoma cells and skeletal muscle.[342-351] Autophagy-promoting effects of AMPK are most evident in cells cultured in a complete medium with serum and amino acids, where cargo sequestration is otherwise largely suppressed.348 Presumably, AMPK antagonizes the autophagy-inhibitory effect of amino acids (at the level of phagophore assembly) by phosphorylating proteins involved in TORC1 signaling, such as TSC2352 and RAPTOR[353] as well the TORC1 target ULK1 (see below).[354-356] Compound C is an effective and widely used inhibitor of activated (phosphorylated) AMPK.357 However, being a nonspecific inhibitor of oxidative phosphorylation,[358,359] this drug has been observed to inhibit autophagy under conditions where AMPK is already inactive or knocked out,360 and has even been shown to stimulate autophagy by an AMP-independent mechanism. 359,361 Compound C thus cannot be used as a stand-alone indicator of AMPK involvement. TORC1 is an autophagy-suppressive regulator that integrates growth factor, nutrient and energy signals. In most systems, inhibition of MTOR leads to induction of autophagy, and AMPK activity is generally antagonistic toward MTOR function. TORC1 mediates the autophagy-inhibitory effect of amino acids, which stimulate the MTOR protein kinase through a Rag GTPase dimer. Insulin and growth factors activate TORC1 through upstream kinases including AKT (protein kinase B), and extracellular signal regulated kinase (ERK) when the energy supply is sufficient, whereas energy depletion may induce AMPK-mediated TORC1 inhibition and autophagy stimulation, for example, during glucose starvation. Amino acid starvation, on the other hand, can strongly induce autophagy even in cells completely lacking AMPK catalytic activity.[362]

AMPK and TORC1 regulate autophagy through coordinated phosphorylation of ULK1; under glucose starvation, AMPK promotes autophagy by directly activating ULK1 through phosphorylation, although the exact AMPK-mediated ULK1 phosphorylation site(s) remains unclear.[351,354-356] Under condi- tions of nutrient sufficiency, high TORC1 activity prevents ULK1 activation by phosphorylating alternate ULK1 residues and disrupting the interaction between ULK1 and AMPK.

There are commercially available phospho-specific antibodies that recognize different forms of ULK1. For example, phosphorylation at Ser555, an AMPK site, is indicative of increased autophagy in response to nutrient stress, whereas Ser757 is targeted by MTOR to inhibit autophagy. Even the autophagy-suppressive effects of AMPK could, conceivably, be mediated through ULK1 phosphorylation, for example, at the inhibitory site Ser638.[363] AMPK inhibits MTOR by phosphorylating and activating TSC2.[364] Therefore, AMPK is involved in processes that synergize to activate autophagy, by directly activating ULK1, and indirectly impairing MTOR-dependent inhibition of ULK1. The identification of ULK1 as a direct target of TORC1 and AMPK represents a significant step toward the definition of new tools to monitor the induction of autophagy. However, further studies directed at identifying physiological substrates of ULK1 will be essential to understand how ULK1 activation results in initiation of the autophagy program.

Along these lines, ULK1 phosphoryl- ates AMBRA1,[365] and the MYLK/MLCK-like protein Sqa,[366] as well as ATG13 and RB1CC1/FIP200.[367-370] In addition, ULK1 binds to, and phosphorylates, RPTOR, leading to inhibition of TORC1.[371] Furthermore, ULK1 itself appears to be able to mediate inhibitory AMPK phosphorylation to generate a negative feedback loop.[372]

TORC1 activity can be monitored by following the phospho- rylation of its substrates, such as EIF4EBP1 (4E-BP1/PHAS-I) and RPS6KB1 (p70S6 kinase) or the latter's downstream target, the ribosomal protein S6 (RPS6), for which good commercial antibodies are available.[373-375] In mammalian cells, the analysis should focus on the phosphorylation of RPS6KB1 at Thr389, and EIF4EBP1 at Thr37 and Thr46, which are directly phosphory- lated by TORC1.[376] The TORC1-dependent phosphorylation of EIF4EBP1 can be detected as a molecular mass shift by western blot.[375] Examining the phosphorylation status of RPS6KB1 and EIF4EBP1 may be a better method for monitoring TORC1 activity than following the phosphorylation of proteins such as RPS6, because the latter is not a direct substrate of TORC1 (although RPS6 phosphorylation is a good readout for RPS6KB1/ 2 activities, which are directly dependent on MTOR), and it can also be phosphorylated by other kinases such as RPS6KA/RSK. Furthermore, the mechanisms that determine the selectivity as well as the sensitivity of TORC1 for its substrates seem to be dependent on the integrity and configuration of TORC1. For example, rapamycin strongly reduces RPS6KB1 phosphorylation, whereas its effect on EIF4EBP1 is more variable. In the case of rapamycin treatment, EIF4EBP1 can be phosphorylated by TORC1 until rapamycin disrupts TORC1 dimerization and its integrity, whereas RPS6KB1 phosphorylation is quickly reduced when rapamycin simply interacts

with MTOR in TORC1 (see Autophagy inhibitors and inducers for information on catalytic MTOR inhibitors such as torin1).[376] Since it is likely that other inhibitors, stress, and stimuli may also affect the integrity of TORC1, a decrease or increase in the phosphorylation status of one TORC1 substrate does not necessarily correlate with changes in others, including ULK1. Therefore, reliable antiphospho- ULK1 antibodies should be used to directly examine the phosphorylation state of ULK1, along with additional experi approaches to analyze the role of the MTOR complex in regulating autophagy. Activation/assembly of the Atg1 complex in yeast (composed of at least Atg1-Atg13-Atg17-Atg29-Atg31) or the ULK1 complex in mammals (ULK1-FIP200-ATG13-ATG101) is one of the first steps of autophagy induction. Therefore, activation of this complex can be assessed to monitor autophagy induction. In yeast, dephosphorylation of Atg13 is associated with activation/assembly of the core complex, which can be assessed by immunoprecipitation or western blotting.[377-380] In addition, the autophosphorylation of Atg1 at Thr226 is required for its kinase activity and for autophagy induction; this can be detected using phosphospecific antibodies, by immunoprecipitation or western blotting (Fig. 15).[381,382] In mammalian cells, the phosphorylation status of ULK1 at the activating sites (Ser317, 777, 467, 555, 637, or Thr574) or dephosphorylation at inactivating sites (Ser638, 757) can be determined using phospho-specific antibodies,355,356 or by western blotting.383 In general, the core complex is stable in mammalian cells, although, as noted above, upstream inhibitors (MTOR) or activators (AMPK) may interact dynamically with it, thereby determining the status of autophagy.

One additional topic that bears on ULK1 concerns the process of LC3-associated phagocytosis. LAP is a type of phagocytosis in macrophages that involves the conjugation of LC3 to single- membrane pathogen-containing phagosomes, a process that promotes phagosome acidification and fusion with lysosomes.[131] Most of the core autophagy components are required for LAP, but the two processes can be distinguished by the presence or absence, respectively, of a double-membrane sequestering vesicle. ULK1 is not needed for LAP, which provides a more convenient means for distinguishing between the two processes.[134]

Cautionary notes: A decrease in TORC1 activity is a good measure for autophagy induction; however, TORC1 activity does not necessarily preclude autophagy induction because there are TOR-independent mechanisms that induce autophagy.[384-387]

Along these lines, whereas in most systems inhibition of MTOR leads to the induction of autophagy, there are instances in commonly used cancer cell lines in which MTOR appears to be a positive effector.[388] Furthermore, in adult skeletal muscle, autophagy gene upregulation and autophagosome formation is independent of TORC1 but partially dependent on MTOR complex 2 (TORC2), as shown by the finding that autophagosome formation is increased by knockdown of RICTOR, a component of TORC2, but not TORC1.346 In addition, TORC1 is downstream of AKT; however, oxidative stress inhibits MTOR, thus allowing autophagy, despite the concomitant activation of AKT.[115] Also, persistent autophagy induction can cause negative feedback that results in the reactivation of MTOR under conditions of ongoing starvation.[389] Thus, it is necessary to be cautious in deciding how to monitor the MTOR pathway, and to verify that the pathway being analyzed displays MTORdependent inhibition.

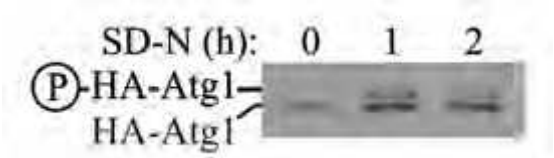

Figure 15. S. cerevisae cells transformed with a plasmid encoding HA-Atg1 were cultured to mid-log phase and shifted to SD-N (minimal medium lacking nitrogen that induces a starvation response). Immunoblotting was done with anti-HA antibody. The upper band corresponds to autophosphorylation of Atg1. This figure was modified from data previously published in reference ,381 and is reproduced by permission of the American Society for Cell Biology, copyright 2011.

One problem in monitoring assembly of the ULK1 complex is the low abundance of endogenous ULK1 in many systems, which makes it difficult to detect phospho-ULK1 by western blot analysis. In addition, Atg1/ULK1 is phosphorylated by multiple kinases, and the amount of phosphorylation at different sites can increase or decrease during autophagy induction. Thus, although there is an increase in phosphorylation at the activating sites upon induction, the overall phosphorylation states of ULK1 and ATG13 are decreased under conditions that lead to induction of autophagy; therefore, monitoring changes in phosphorylation by following molecular mass shifts upon SDS-PAGE may not be informative. In addition, such phospholtylation/dephosphorylation events are expected to occur relatively early (1-2h) in the signaling cascade of autophagy. Therefore, it is necessary to optimize treatment time conditions. Finally, in Arabidopsis and possibly other eukaryotes, the ATG1 and ATG13 proteins are targets of autophagy, which means that their levels may drop substantially under conditions that induce autophagic turnover.174At present, the use of Agt1/ULK1 kinase activity as a tool to monitor autophagy is limited because only a few physiological substrates have been identified, and the importance of the Atg1/ULK1-dependent phosphorylation has not been determined. Nonetheless, Atg1/ULK1 kinase activity appears to increase when autophagy is induced,

irrespective of the pathway leading to induction. As additional physiological substrates of Atg1/ULK1 are identified it will be possible to follow their phosphorylation in vivo as is done with analyses for MTOR. Nonetheless, it must be kept in mind that monitoring changes in the activity of Atg1/ULK1 is not a direct assay for autophagy, although such changes may correlate with autophagy activity. Furthermore, in some cells ULK1 has functions in addition to autophagy, such as in axonal transport and outgrowth, and its activity state may thus reflect its role in these processes.390-395 Accordingly, other methods as described throughout these guidelines should also be used to follow autophagy directly.

Finally, there is not a complete consensus on the specific residues of ULK1 that are targeted by AMPK or MTOR. Similarly, apparently contradictory data have been published regarding the association of AMPK and MTOR with the ULK1 kinase complex under different conditions. Therefore, caution should be used in monitoring ULK1 phosphorylation or the status of ULK1 association with AMPK until these issues are resolved.

Conclusion: Assays for Atg1/ULK1 can provide detailed insight into the induction of autophagy, but are not a direct measurement of the process. Similarly, analysis of MTOR substrates such as S6K1 and EIF4EBP1 are not recommended readouts for autophagy, and need to be combined with other assays that directly monitor autophagy activity.

5. Additional autophagy-related markers. Although Atg8/LC3 has been the most extensively used protein for monitoring autophagy, other proteins can also be used for this purpose. Here, we discuss some of the more commonly used or bettercharacterized possibilities.

a. Atg9. Atg9 is the only integral membrane Atg protein that is essential for autophagosome formation. ATG9 displays partial colocalization with GFP-LC3.[396] Perhaps the most unique feature of Atg9, however, is that it localizes to multiple discrete puncta, whereas most Atg proteins are detected primarily in a single punctum or diffusely within the cytosol. Yeast Atg9 may cycle between the phagophore assembly site (PAS, also referred to as the phagophore assembly site) and peripheral reservoirs;[397] the latter correspond to tubulovesicular clusters that are precursors to the phagophore.[398] Anterograde movement to the PAS is dependent on Atg11, Atg23, Atg27 and actin. Retrograde movement requires Atg1-Atg13, Atg2-Atg18 and the PtdIns3K complex I.[399] Mutants such as atg1D accumulate Atg9 exclusively at the PAS, and this phenotype forms the basis of the transport of Atg9 after knocking out ATG1 (TAKA) assay.[63] In brief, this is an epistasis analysis in which a double-mutant strain is constructed (one of the mutations being atg1D) that expresses Atg9-GFP. If the second mutated gene encodes a protein that is needed for Atg9 anterograde transport, the double mutant will display multiple tg9-GFP puncta. In contrast, if the protein acts along with or after Atg1, all of the Atg9-GFP will be confined to the PAS.

Monitoring the localization of ATG9 has not been used extensively in higher eukaryotes, but this protein displays the same type of dependence on Atg1/ULK1 for cycling as seen in yeast,[396,399] suggesting that it is possible to follow this protein as an indication of ULK1 and ATG13 function.[370 b]. Atg12–Atg5. ATG5, ATG12, and ATG16L1, associate with the phagophore and have been detected by fluorescence or immunofluorescence (Fig. 16).[400,401] Endogenous ATG5, ATG12 or ATG16L1 puncta formation can be followed to monitor autophagy upregulation. Under physiological conditions, the endogenous proteins are predominantly diffusely distributed throughout the cytoplasm. Upon induction of autophagy, for example during starvation, there is a marked increase in the proportion of cells with punctate ATG5, ATG12 and ATG16L1. Furthermore, inhibitors of autophagosome formation result in a block in this starvation-induced puncta formation, and this assay is very robust in mammalian cells. Atg12–Atg5 conjugation has been used in some studies to measure autophagy. In Arabidopsis and some mammalian cells it appears that essentially all of the ATG5 and ATG12 proteins exist in the conjugated form and the expression levels do not change, at least during short-term starvation.[150,400-402] Therefore, monitoring ATG12–ATG5 conjugation per se may not be a useful method for following the induction of autophagy. It is worth noting, however, that in some cell lines free ATG5 can be detected,[403] suggesting that the amount of free ATG5 may be cell line-dependent. One final parameter that may be considered is that the total amount of the ATG12–ATG5 conjugate may increase following prolonged starvation as has been observed in hepatocytes and both mouse and human fibroblasts (Cuervo AM, personal communication; Sarkar S, personal communication).

c. ATG14. Yeast Atg14 is the autophagy-specific subunit of the Vps34 complex I,[404] and a human homolog, named ATG14 (ATG14L/BARKOR), has been identified.[405-408] ATG14 localizes primarily to phagophores. The C-terminal fragment of the protein is named the BATS domain (BARKOR/ATG14(L) autophagosome targeting sequence), and is able to direct GFP and BECN1 to autophagosomes in the context of a chimeric protein.[409] Currently, a good antibody that can be used to detect the endogenous ATG14 does not exist. ATG14-GFP or BATS-GFP detected by fluorescence microscopy or TEM can be used as a phagophore marker; however, ATG14 is not localized exclusively

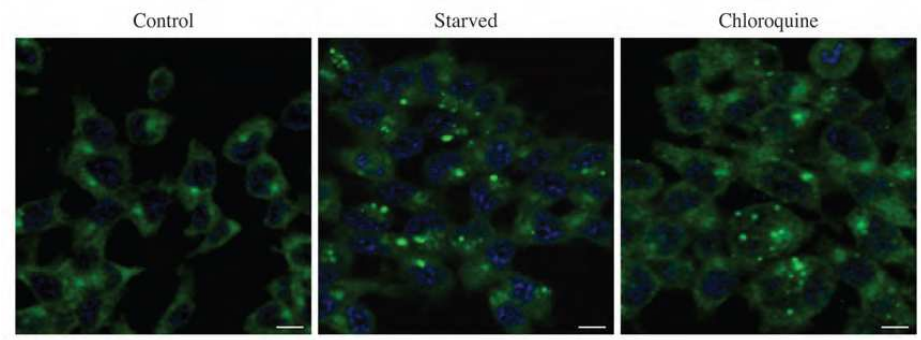

Figure 16. Confocal microscopy image of HCT116 cells immunostained with human-specific antibody to ATG12. Cells were starved for 8 h or treated with chloroquine (50 mM) for 3 h. Scale bar, 10 mm. Image provided by M. Llanos Valero, M.A de la Cruz and R. Sanchez-Prieto.

to phagophores, as it can also be detected on mature autophagosomes as well as the ER.[409,410] Accordingly, detection of ATG14 should be performed in combination with other phagophore and autophagosome markers.

d. ATG16L1. ATG16L1 has been used to monitor the movement of plasma membrane as a donor for autophagy, and thus an early step in the process; ATG16L1 is located on phagophores, but not on completed autophagosomes.[269,411] ATG16L1 can be detected by immuno-TEM, by immunostaining of Flag epitope-tagged ATG16L1, and/or by the use of GFP-tagged ATG16L1.

e. Atg18/WIPI family. Yeast Atg18412,[413] and Atg21262 (or the mammalian WIPI homologs[414]) are required for both macroautophagy (i.e., nonspecific sequestration of cytoplasm) and autophagy-related processes (e.g., the Cvt pathway,[415,416] specific organelle degradation,74 and autophagic elimination of invasive microbes[77,78,80,81,417).412] These proteins bind phosphatidylinositol 3-phosphate (PtdIns3P) that is present at the phagophore and autophagosome[418,419] and also PtdIns(3,5)P2. Human WIPI-1 and WIPI-2 function downstream of the phosphatidylinositol 3-kinase class III (PtdIns3KC3) complex I [PtdIns3KC3, BECN1, PIK3R4 (VPS15), ATG14] and upstream of both the ATG12 and LC3 ubiquitin-like conjugation systems.[418,420,421]

Upon the initiation of the autophagic pathway, WIPI1 and WIPI2 bind PtdIns3P and accumulate at limiting membranes, such as those of the endoplasmic reticulum, where they participate in the formation of omegasomes and/or autophagosomes. On the basis of quantitative fluorescence microscopy, this specific WIPI protein localization has been used as an assay to monitor autophagy in human cells.[419] Using either endogenous WIPI1 or WIPI2, detected by indirect fluorescence microscopy or EM, or transiently or stably expressed tagged fusions of GFP to WIPI1 or WIPI2, basal autophagy can be detected in cells that display WIPI puncta at autophagosomal membranes. In circumstances of increased autophagic activity, such as nutrient starvation or rapamycin administration, the induction of autophagy is reflected by the elevated number of cells that display WIPI puncta when compared with the control setting. Also, in circumstances of reduced autophagic activity such as wortmannin treatment, the reduced number of WIPI puncta-positive cells reflects the inhibition of autophagy. Basal, induced and inhibited formation of WIPI puncta closely correlate with both the protein level of LC3-II and the formation of GFP-LC3 puncta.419,421 Accordingly, WIPI puncta can be assessed as an alternative to LC3. Automated imaging and analysis of fluorescent WIPI-1 (Fig. 17) or WIPI2 puncta represents an efficient and reliable opportunity to combine the detection of WIPI proteins with other parameters. It should be noted that there are two isoforms of WIPI2 (2B and 2D),[421] and in C. elegans WIPI4 (EPG-6) has been identified as the WIPI homolog required for autophagy.[422] Thus, these proteins, along with the currently uncharacterized WIPI3, provide additional possibilities for monitoring phagophore and autophagosome formation.

Cautionary notes: With regard to detection of the WIPI proteins, endogenous WIPI1 puncta cannot be detected in many cell types,[418] and the level of transiently expressed GFP-WIPI1 puncta is cell context-dependent;[418,419] however, it has been used in human and mouse cell systems[348,419] and mCherry-Atg18 also works in transgenic Drosophila (Juhász G, personal communication), although one caution with regard to the latter is that GFPAtg18 expression can induce autophagy in the fat body of fed larvae (Kiger A, unpublished observation). GFP-WIPI1 and GFPWIPI2 have been detected on the completed (mature) autophagosome by freeze-fracture analysis,60 but endogenous WIPI2 has not been detected on mRFP-LC3- or LAMP2-positive autophagosomes or autolysosomes using immunolabeling.[418] Accordingly, it may be possible to follow the formation and subsequent disappearance of WIPI puncta to monitor autophagy induction and flux using specific techniques. As with GFP-LC3, overexpression of WIPI1 or WIPI2 can lead to the formation of aggregates, which are stable in the presence of PtdIns3K inhibitors.

f. BECN1/Atg6. BECN1 (Atg6) and VPS34 are essential partners in the autophagy interactome that signals the onset of autophagy,404,423,424 and many researchers use this protein as a way to monitor autophagy. BECN1 is inhibited by its binding to the anti-apoptotic protein BCL2.425 Autophagy is induced by the release of BECN1 from BCL2 by pro-apoptotic BH3 proteins, phosphorylation of BECN1 by DAPK (at Thr119, located in the BH3 domain),[426] or phosphorylation of BCL2 by JNK1 (at Thr69, Ser70 and Ser87).[427,428] The relationship between BECN1 and BCL2 is more

complex in developing cerebellar neurons as it appears that the cellular levels of BCL2 are, in turn, post-translationally regulated by an autophagic mechanism linked to a switch from immaturity to maturity.[429,430]
It is important to be aware, however, that certain forms of macroautophagy are induced in a BECN1-independent manner, and are not blocked by PtdIns3K inhibitors.[97,431] Interestingly, caspase-mediated cleavage of BECN1 inactivates BECN1-induced autophagy and enhances apoptosis in several cell types,432 emphasizing that the crosstalk between apoptosis and autophagy is complex.
Although a population of BECN1 may localize in proximity to the trans-Golgi network,[433] it is also present at the ER and mitochondria.425 In keeping with these observations, in cerebellar organotypic cultures BECN1 co-immunoprecipitates with BCL2 that is primarily localized at the mitochondria and ER, and in a mouse model of neurodegeneration autophagic vacuoles in Purkinje neurons contain partially digested organelles that are immunoreactive for BCL2.[430,434] In addition, BECN1 and VPS34 can be present in multiple complexes, so caution must be exercised when monitoring localization. On induction of autophagy by various stimuli the presence of BECN1- and VPS34-positive macroaggregates can be detected in the region of the Golgi complex by immunofluorescence.[115,435]
Thus, BECN1-GFP puncta detected by fluorescence microscopy or TEM may serve as an additional marker for autophagy induction;[436] however, as with any GFP chimeras there is a concern that the GFP moiety interferes with correct localization of BECN1. To
demonstrate that BECN1 or PtdIns3K macroaggregates are an indirect indication of ongoing autophagy, it is mandatory to show their specific association with the process by including

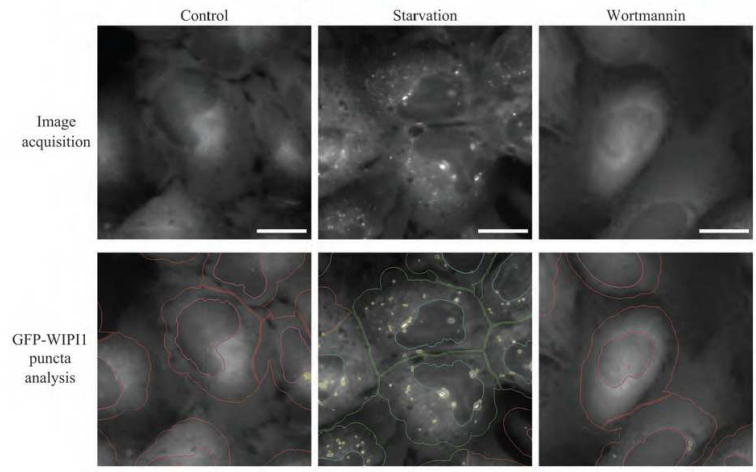

Figure 17. Automated WIPI1 puncta image acquisition and analysis monitors the induction and inhibition of autophagy. Stable U2OS clones expressing GFP-WIPI1 were selected using 0.6 mg/ml G418 and then cultured in 96-well plates. Cells were treated for 3 h with nutrient-rich medium (control), nutrient-free medium (EBSS), or with 233 nM wortmannin. Cells were fixed in 3.7% paraformaldehyde and stained with DAPI (5 mg/ml in PBS). An automated imaging and analysis platform was used to determine the number of both GFP-WIPI1 puncta-positive cells and the number of GFP-WIPI1 puncta per individual cell.348 Cells without GFP-WIPI1 puncta are highlighted in red (cell detection) and purple (nuclei detection), whereas GFP-WIPI1 puncta-positive cells are highlighted in yellow (GFP-WIPI1 puncta detection), green (cell detection) and blue (nuclei detection). Bars, 20 mm. These images were provided by S. Pfisterer and T. Proikas-Cezanne.

appropriate controls with inhibitors (e.g., 3-MA) or autophagy gene silencing. When a BECN1-independent autophagy pathway is induced, such aggregates are not formed regardless of the fact that the cell expresses BECN1 (e.g., as assessed by western blotting) (Isidoro C, personal communication).
g. DFCP1. DFCP1 (double FYVE-containing protein 1) binds PtdIns3P that localizes to the ER and Golgi. The ER population of DFCP1 is involved in formation of the omegasome.[437] Starvation induces the translocation of DFCP1 to punctuate structures on the ER. DFCP1 partially colocalizes with WIPI1 upon nutrient starvation,[421] and also with WIPI2.[418]
h. DRAM. DRAM (damage-regulated autophagic modulator) is a gene induced by activated p53 in response to different types of cellular stress, including DNA damage.[438,439] DRAM is a small hydrophobic protein with six transmembrane domains. It is detected as a subpopulation in the Golgi and cis-Golgi, colocalizing with giantin and GM130, and also in early and late endosomes and lysosomes, colocalizing with EEA1 and LAMP2.[439] The elimination of DRAM by siRNA blocks autophagy, [439,440] as effectively as elimination of BECN1, indicating it is an essential component for this process, although its mechanism of action is not known. The time course of autophagy as a consequence of DRAM activation can be monitored following the disappearance of the VRK1 protein, a direct target of this process, by immunoblot.[439] Detection of DRAM RNA is very easy by qRT-PCR during autophagy;[438,439] however, detection of the DRAM protein is very difficult because of its small size and hydrophobicity, which makes generation of specific antibodies a complicated process, and in general these have very low sensitivity.

Conclusion: Proteins other than Atg8/LC3 can be monitored to follow autophagy, and can be important tools to define specific steps of the process. For example, WIPI puncta formation can be used to monitor autophagy, but, similar to Atg8/LC3, should be examined in the presence and absence of inhibitors. Analysis of WIPI puncta should be combined with other assays because individual members of the WIPI family might also participate in additional, uncharacterized functions apart from their role in autophagy. At present, we caution against the use of changes in BECN1 localization as a marker of autophagy induction.

6. Transcriptional and translational regulation. The induction of autophagy in certain scenarios is accompanied by an increase in the mRNA levels of certain autophagy genes, such as ATG8/Lc3[441,442] and Atg12,[443] and an autophagy-dedicated microarray was developed as a high-throughput tool to simultaneously monitor the transcriptional regulation of all genes involved in, and related to, autophagy.[444] The gene that shows the greatest transcriptional regulation in the liver (in response to starvation and circadian signals) is Ulk1, but others also show more limited changes in mRNA levels including Gabarapl1, Bnip3 and, to a minor extent, Lc3B (Lin JD, personal communication). In several mouse and human cancer cell lines, ER stress and hypoxia increase the transcription of LC3, ATG5 and ATG12 by a mechanism involving the unfolded protein response (UPR). Similarly, a stimulus-dependent increase in LC3B expression is detected in neutrophils undergoing autophagy induction.[445]

Increased expression of Atg5 in vivo after optic nerve axotomy in mice[446] and increased expression of Atg7, Becn1 and Lc3A during neurogenesis at different embryonic stages in the mouse olfactory bulb are also seen.[447] LC3 and ATG5 are not required for the initiation of autophagy, but mediate phagophore expansion and autophagosome formation. In this regard, the transcriptional induction of LC3 may be necessary to replenish the LC3 protein that is turned over during extensive ER stress- and hypoxia-induced autophagy.[443,448] Thus, assessing the mRNA levels of LC3 and other autophagy-related genes by northern blot or qRT-PCR may provide correlative data relating to the induction of autophagy. It is not clear if these changes are sufficient to induce autophagy, however, and therefore these are not direct measurements.

Of note, large changes in Atg gene transcription just prior to Drospohila salivary gland cell death (that is accompanied by an increase in autophagy) are detected in Atg2, Atg4, Atg5 and Atg7, whereas there is no significant change in Atg8a or Atg8b.[449,450] Autophagy is critical for Drosophila midgut cell death, which is accompanied by transcriptional upregulation of all of the Atg genes tested, including Atg8a (Fig. 18).[199,451] Similarly, in the silkworm (B. mori) larval midgut the occurrence of autophagy is accompanied by an upregulation of Atg5, Atg6 and Atg8 mRNAin Atg2, Atg4, Atg5 and Atg7, whereas there is no significant change in Atg8a or Atg8b.[449,450] Autophagy is critical for Drosophila midgut cell death, which is accompanied by transcriptional upregulation of all of the Atg genes tested, including Atg8a (Fig. 18).[199,451] Similarly, in the silkworm (B. mori) larval midgut the occurrence of autophagy is accompanied by an upregulation of Atg5, Atg6 and Atg8 mRNA levels.[452] Transcriptional upregulation of Drosophila Atg8a and Atg8b is also observed in the fat body following induction of autophagy at the end of larval development,453 Atg5, Atg6, Atg8a and Atg18 are upregulated in the ovary of starved flies,[454] and an increase in Drosophila Atg8b is observed in cultured Drosophila l(2)mbn cells following starvation (Gorski S, personal communication).

An upregulation of plant ATG8 may be needed during the adaptation to reproductive growth; a T-DNA inserted mutation of rice ATG8b blocked the change from vegetative growth to reproductive growth in both homozygous and heterozygous plant lines (Zhang M-Y, unpublished results). Similarly, the upregulation of autophagy-related genes (Lc3, Gabarapl1, Bnip3, Atg4b, Atg12l) has been documented at the transcriptional and translational level in several other species (e.g., mouse, rat, trout, Arabidopsis and maize) under conditions of ER stress,[443] and diverse types of prolonged (several days) catabolic situations including cancer cachexia, diabetes mellitus, uremia and fasting.[151,346,455-457] Along these lines, ATG9 and ATG16L1 are transcriptionally upregulated upon influenza virus infection (Khalil H, personal communication), and in C. elegans, the FOXA transcription factor PHA-4 regulates the expression of several autophagy-related genes.[458] Such prolonged induction of the expression of ATG genes has been thought to allow the replenishment of critical proteins (e.g., LC3 and GABARAP) that are destroyed during autophagosome fusion with the lysosome. [459] The polyamine spermidine increases life span and induces autophagy in cultured yeast and mammalian cells, as well as in nematodes and flies; in aging yeast, spermidine treatment triggers epigenetic deacetylation of histone H3 through inhibition of histone acetyltransferases, leading to significant upregulation of various autophagy-related transcripts.[460] In addition to the ATG genes, transcriptional upregulation of VMP1 (a protein that is involved in autophagy regulation and that remains associated with the completed autophagosome) can be detected in mammalian cells subjected to rapamycin treatment or starvation, and in tissues undergoing disease-induced autophagy. [461] VMP1 is an essential autophagy gene that is conserved from Dictyostelium to mammals,[232,462] and the VMP1 protein regulates early steps of the autophagic pathway.420 VMP1 is poorly expressed in mammalian cells under nutrient-normal conditions, but is highly upregulated in cells undergoing autophagy, and the expression of VMP1 induces autophagosome formation.

A gene regulatory network, named CLEAR (coordinated lysosomal enhancement and regulation) that controls both lysosome

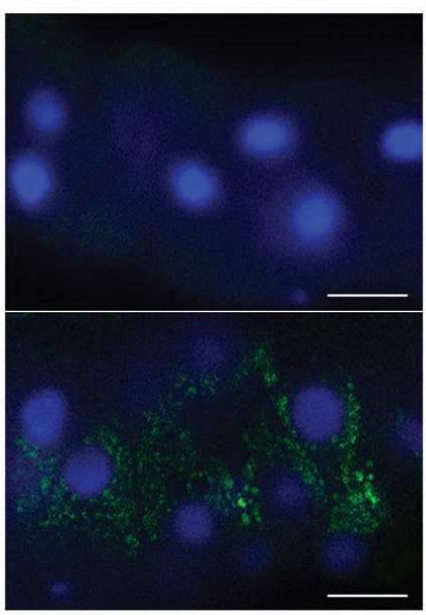

Figure 18. pGFP-Atg8a can be used to monitor autophagy in Drosophila melanogaster. The autophagosome marker pGFP-Atg8a, results in expression of Atg8a fused to GFP from the endogenous Atg8a promoter.199 Live imaging of gastric ceca from Drosophila melanogaster midgut pGFP-Atg8a puncta (green) and Hoechst 33342 (blue). Midgut from early third instar larvae prior to the onset of cell death (top) and from dying midgut at 2 h after puparium formation (bottom). Bar, 25 mm. Figure provided by D. Denton and S. Kumar.

and autophagosome biogenesis was identified using a systemsbiology approach.[463-465] The basic helix-loop-helix transcription factor EB (TFEB) acts as a master gene of the CLEAR network and positively regulates the expression of both lysosomal and autophagy genes, thus linking the biogenesis of two distinct types of cellular compartments that cooperate in the autophagic pathway. TFEB activity is regulated by starvation and is controlled by ERK2-mediated phosphorylation at a specific serine residue,[463] and can thus serve as a new tool for monitoring transcriptional regulation connected with autophagy. Along these lines, the erythroid transcription factor GATA1 and its coregulator Friend of GATA1 induce the transcription of multiple genes encoding autophagy components; this developmentally regulated transcriptional response is coupled to increases in autophagosome number as well as the percent of cells that contain autophagosomes.[466] Finally, C/EBPβ is a transcription factor that regulates autophagy in response to the circadian cycle.[467] Although less work has been done on post-transcriptional regulation, several studies implicate microRNAs in controlling the expression of proteins associated with autophagy.[164,468-470]

Cautionary notes: Most of the ATG genes do not show significant changes in mRNA levels when autophagy is induced. Even increases in LC3 mRNA can be quite modest and are cell type- and organism-dependent.[471] In addition, it is generally better to follow protein levels because that is the ultimate readout that is significant with regard to the initiation and completion of autophagy, although ATG protein amounts do not always change significantly and the extent of increase is again cell type- and tissue-dependent. In some cases (e.g., yeast ATG14), increased transcription is not accompanied by increased protein levels, apparently due to changes in translation efficiency under starvation conditions (Abeliovich H, unpublished data). Finally, changes in autophagy protein levels are not sufficient evidence of autophagy induction, and must be accompanied by additional assays as described herein. Thus, monitoring changes in mRNA levels for either ATG genes or autophagy regulators may provide some evidence for autophagy induction, but should be used along with other methods.

Another general caution pertains to the fact that in any cell culture system different types of cells (for example, those undergoing autophagy or not) exist simultaneously. Therefore, only an average level of protein or mRNA expression can be evaluated with most methods. This means that the results regarding specific changes in autophagic cells could be hidden due to the background of the average data. Along these lines, experiments using single-cell real-time PCR to examine gene expression in individual cardiomyocytes with and without signs of autophagy revealed that the transcription of MTOR markedly and significantly increased in autophagic cells in intact cultures (spontaneously undergoing autophagy) as well as in cultures treated with proteasome inhibitors to induce autophagy (Dosenko V, personal communication). Finally, researchers need to realize that mammalian cell lines may have mutations that alter autophagy signaling or execution; this problem can be avoided by using primary cells. Conclusion: Although there are changes in ATG gene expression that coincide with, and may be needed for, autophagy, this has not been carefully studied experimentally. Therefore, at the present time we do not recommend the monitoring of ATG gene transcription as a general readout for autophagy unless there is clear documentation that the change(s) correlates with autophagy activity.

7. Autophagic protein degradation. Protein degradation assays represent a well-established methodology for measuring autophagic flux, and they allow good quantification. The general strategy is first to label cellular proteins by incorporation of a radioactive amino acid (e.g., [14C]-leucine, [14C]-valine or [35S]-methionine; although valine may be preferred over leucine due to the strong inhibitory effects of the latter on autophagy), preferably for a long time to achieve sufficient labeling of the longlived proteins that best represent autophagic substrates, and then to follow this with a long cold-chase so that the assay starts well after labeled short-lived proteins are degraded (which occurs predominantly via the proteasome). Next, the time-dependent release of acid-soluble radioactivity from the labeled protein in intact cells or perfused organs is measured.[3,472,473] Note that the inclusion of the appropriate unlabeled amino acid (i.e., valine, leucine or methionine) in the starvation medium at a concentration equivalent to that of other aminoacids in the chase medium is necessary; otherwise the release ^{14}C –aminoacid is effectively re-incorporated into cellular proteins, which results in a significant underestimation of protein degradation. The turnover of specific proteins can also be measured in a pulse-chase regimen using the Tet-ON/OFF system and subsequent western blot analysis [474,475]

In this type of assay a considerable fraction of the measured degradation will be non-autophagic and thus, it is important to also measure, in parallel, cell samples treated with autophagysuppressive concentrations of 3-MA or amino acids, or obtained from mutants missing central ATG components (however, the latter assumes that non-autophagic proteolytic activity remains unchanged, which is unlikely); these values are then subtracted from the total. The complementary approach of using compounds that block other degradative pathways, such as proteasome inhibitors, may cause unexpected results and should generally be avoided due to crosstalk among the degradative systems. For example, blocking proteasome function may activate autophagy. [476-479] Thus, when using inhibitors it is critical to know whether the inhibitors being used alter autophagy in the particular cell type and context being examined. In addition, because 3-MA could have some autophagy-independent effects in particular settings it is advisable to verify that the 3-MA-sensitive degradation is also sensitive to general lysosomal inhibitors (such as NH4Cl or leupeptin). The use of stable isotopes, such as 13C and 15N, in quantitative mass spectrometry-based proteomics allows the recording of degradation rates of thousands of proteins simultaneously. These assays may be applied to autophagy-related questions enabling researchers to investigate differential effects in global protein or even organelle degradation studies.[480,481] SILAC (stable isotopelabeling with amino acids in cell culture) can also provide comparative information between different treatment conditions, or between a wild type and mutant.

Another assay that could be considered relies on the limited proteolysis of a betaine homocysteine methyltransferase (BHMT) fusion protein. The 44-kDa full-length BHMT protein is cleaved in hepatocyte amphisomes in the presence of leupeptin to generate 32-kDa and 10-kDa fragments.[482-485] Accumulation of these fragments is time-dependent and is blocked by treatment with autophagy inhibitors. A modified version of this marker, GST-BHMT, can be expressed in other cell lines where it behaves similar to the wild-type protein.[486] Other substrates may be considered for similar types of assays. For example, the neomycin phosphotransferase II-GFP (NeoR-GFP) fusion protein is a target of autophagy.[487] Transfection of lymphoblastoid cells with a plasmid encoding NeoR-GFP followed by incubation in the presence of 3-MA leads to an accumulation of the NeoR-GFP protein as measured by flow cytometry.[488] A similar western blot assay is based on the degradation of a cytosolic protein fused to GFP. This method has been used in yeast and Dictyostelium cells using GFP-PgkA and GFP-Tkt-1 (phosphoglycerate kinase and transketolase, respectively). In this case the relative amount of the free GFP and the complete fusion protein is the relevant parameter for quantification; although it may not be possible to detect clear changes in the amount of the full-length chimera, especially under conditions of limited flux.29,36 As described above for the marker GFP-Atg8/LC3, nonsaturating levels of lysosomal inhibitors are also needed in Dictyostelium cells to slow down the autophagic degradation, allowing the accumulation and detection of free GFP. It should be noted that this method monitors bulk autophagy since it relies on the passive transit of a cytoplasmic marker to the lysosome. Consequently, it is important to determine that the marker is distributed homogeneously in the cytoplasm. One of the most useful methods for monitoring autophagy in S. cerevisiae is the Pho8D60 assay. PHO8 encodes the vacuolar alkaline phosphatase, which is synthesized as a zymogen before finally being transported to and activated in the vacuole.[489] A molecular genetic modification that eliminates the first 60 amino acids prevents the mutant (Pho8D60) from entering the endoplasmic reticulum, leaving the zymogen in the cytosol. When autophagy is induced, the mutant zymogen is delivered to the vacuole nonselectively inside autophagosomes along with other cytoplasmic material. The resulting activation of the zymogen can be easily measured by assays for alkaline phosphatase.[178] To minimize background activity, it is preferable to have the gene encoding cytosolic alkaline phosphatase (PHO13) additionally deleted (although this is not necessary when assaying certain substrates).

Cautionary notes: Measuring the degradation of long-lived proteins requires prior radiolabeling of the cells, and subsequent separation of acid-soluble from acid-insoluble radioactivity. The labeling can be done with relative ease both in cultured cells, and in live animals.3 In cells, it is also possible to measure the release of an unlabeled amino acid by chromatographic methods, thereby obviating the need for prelabeling;[490] however, it is important to keep in mind that amino acid release is also regulated by protein synthesis, which in turn is modulated by many different

factors. In either case, one potential problem is that the released amino acid may be further metabolized. For example, branched chain amino acids are good indicators of proteolysis in hepatocytes, but not in muscle cells where they are further oxidized (Meijer AJ, personal communication). In addition, the amino acid can be reincorporated into protein; for this reason, such experiments can be performed in the presence of cycloheximide, but this raises additional concerns (see Turnover of autophagic compartments). In the case of labeled amino acids, a non labeled chase is added where the tracer amino acid is present in excess (being cautious to avoid using an amino acid that inhibits autophagy), or by use of single pass perfused organs or superfused cells.[491,492] The perfused organ system also allows for testing the reversibility of effects on proteolysis and the use of autophagy-specific inhibitors in the same experimental preparation, which are crucial controls for proper assessment. If the autophagic protein degradation is low (as it will be in cells in replete medium), it may be difficult to measure it reliably above the relatively high background of non-autophagic degradation. It should also be noted that the usual practice of incubating the cells under "degradation conditions," that is, in a saline buffer, indicates the potential autophagic capacity (maximal attainable activity) of the cells rather than the autophagic activity that prevails in vivo or under rich culture conditions. Finally, inhibition of a particular degradative pathway is typically accompanied by an increase in a separate pathway as the cell attempts to compensate for the loss of degradative capacity.[158,478,493] This compensation might interfere with control measurements under conditions that attempt to inhibit macroautophagy; however, as the latter is the major degradative pathway, the contributions of other types of degradation over the course of this type of experiment are most often negligible. The Pho8D60 assay requires standard positive and negative controls (such as an atg1D strain), and care must be taken to ensure the efficiency of cell lysis. Glass beads lysis works well in general, provided that the agitation speed of the instrument is adequate. Instruments designed for liquid mixing with lower speeds should be avoided. We also recommend against holding individual sample tubes on a vortex, as it is difficult to maintain reproducibility; devices or attachments are available to allow multiple tubes to be agitated simultaneously.

Conclusion: Measuring the turnover of long-lived proteins is a good method for determining autophagic flux.

8. Selective types of autophagy. Although autophagy can be nonspecific, in particular during starvation, there are many examples of selective types of autophagy.

a. The Cvt pathway, mitophagy, pexophagy and piecemeal microautophagy of the nucleus in yeast. The precursor form of aminopeptidase I (prApe1) is the major cargo of the Cvt pathway in yeast, a biosynthetic autophagy-related pathway.[82] The propeptide of prApe1 is proteolytically cleaved upon vacuolar delivery, and the resulting shift in molecular mass can be monitored by western blot. Under starvation conditions, prApe1 can enter the vacuole through nonspecific autophagy, and thushas been used as a marker for both the Cvt pathway and autophagy. The yeast Cvt pathway is unique in that it is a biosynthetic route that utilizes the autophagy-related protein machinery, whereas other types of selective autophagy are degradative. The latter include pexophagy, mitophagy, reticulophagy,ribophagy and xenophagy, and each process has its own marker proteins, although these are typically variations of other assays used to monitor the Cvt pathway or autophagy. One common type of assay involves the processing of a GFP chimera similar to the GFP-Atg8/LC3 processing assay (see GFP-Atg8/LC3 lysosomal delivery and proteolysis). For example, yeast pexophagy utilizes the processing of Pex14-GFP and Pot1 (thiolase)-GFP,[494,495] whereas mitophagy can be monitored by the generation of free GFP from Om45-GFP, Idh1-GFP, Idp1-GFP or mito-DHFR-GFP.[496-500] Localization of these mitochondriallytargeted proteins (or specific MitoTracker dyes) or similar organelle markers such as those for the peroxisome [e.g., GFPSKL with Ser-Lys-Leu at the C terminus that acts as a peroxisomal targeting signal, acyl-CoA oxidase 3 (Aox3-EYFP) that allows simultaneous observation of peroxisome-vacuole dynamics with the single FITC filter set, or GFP-catalase] can also be followed by fluorescence microscopy.[412,495,501-503] In addition, yeast mitophagy requires both the Slt2 and Hog1 signaling pathways; the activation and phosphorylation of Slt2 and Hog1 can be monitored with commercially available phospho-specific antibodies (Fig. 19).[381] It is also possible to monitor pexophagy in yeasts by the disappearance of activities of specific peroxisome markers such as catalase, alcohol oxidase or amine oxidase in cell-free extracts,[504] or permeabilized cell suspensions. Catalase activity, however, is a useful marker only when peroxisomal catalases are the only such enzymes present. In S. cereviseae there are two genes encoding catalase activity, and only one of these gene products is localized in peroxisomes. Plate assays for monitoring the activity of peroxisomal oxidases in yeast colonies are also available.[501,505] The decrease in the level of endogenous proteins such as alcohol oxidase or Pot1 can be followed by western blotting,[412,506-509] TEM,510 fluorescence microscopy[412,511,512] or laser confocal scanning microscopy of GFP-labeled peroxisomes.[513,514] In yeast, nonspecific autophagy can be induced by nitrogen starvation conditions, whereas degradative types of selective autophagy generally require a carbon source change or ER stress for efficient induction. For example, to induce a substantial level of mitophagy, cells need to be precultured in a nonfermentable carbon source such as lactate or glycerol to stimulate the proliferation of mitochondria. After sufficient mitochondria proliferation, shifting the cells back to a fermentable carbon source such as glucose will cause the autophagic degradation of superfluous mitochondria.[497] It should be noted that in addition to carbon source change, simultaneous nitrogen starvation is also required for efficient mitophagy induction. This is possibly because excessive mitochondria can be segregated into daughter cells by cell division if growth continues.[478] A similar carbon source change from oleic acid or methanol to

ethanol or glucose (with or without nitrogen starvation) can be used to assay for pexophagy.[515] In addition, mitophagy can also be induced by culturing the cells in a nonfermentable carbon source to postlog phase. In this case, mitophagy may be induced because the energy demand is lower at post-log phase and the mitochondrial mass exceeds the cell's needs.[75,516,517] It has been suggested by several workers in the field that this type of mitophagy, also known as "stationary phase mitophagy," reflects a quality-control function that culls defective mitochondria that accumulate in nondividing, respiring cells.[518] Similar, pexophagy can be induced by culturing the cells in a peroxisome proliferation medium to post-log phase (Farré J-C, unpublished results). Along these lines, it should also be realized that selective types of autophagy continuously occur at a low level under noninducing conditions. Thus, organelles such as peroxisomes have a finite life span and are turned over at a slow rate by autophagy-related pathways.[519] Piecemeal microautophagy of the nucleus (PMN, also micronucleophagy) is another selective autophagic subtype, which targets portions of the nucleus for degradation.[520-522] In S. cerevisiae, the nuclear outer membrane, which is continuous with the nuclear ER, forms contact sites with the vacuolar membrane. These nucleus-vacuole junctions (NVJs) are generated by interaction of the outer nuclear membrane protein Nvj1 with the vacuolar protein Vac8.[523] Nvj1 further recruits the ERmembrane protein Tsc13, which is involved in the synthesis of very-long-chain fatty acids (VLCFAs) and Osh1, a member of a family of oxysterol-binding proteins. Upon starvation the NVJs bulge into the vacuole and subsequently a PMN-vesicle pinches off into the vacuole. PMN vesicles thus contain nuclear material and are limited by three membranes with the outermost derived from the vacuole, and the two inner ones from the nuclear ER. It is not clear which nuclear components are removed by PMN, but since PMN is not a cell death mechanism per se, most likely superfluous material is recycled. During PMN the NVJs are selectively incorporated into the PMN vesicles and degraded. Accordingly, PMN can be monitored using the proteins that are associated with the NVJs as markers. To quantitatively follow PMN, an assay analogous to the above-described GFP-Atg8/LC3

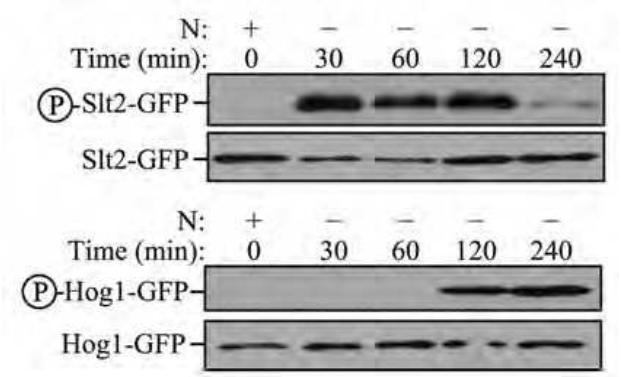

Figure 19. S. cerevisae cells were cultured to mid-log phase and shifted to SD-N for the indicated times. Samples were taken before (+) and at the indicated times after (–) nitrogen starvation. Immunoblotting was done with anti-phospho-Slt2 and anti-phospho-Hog1 antibody. This figure was modified from data previously published in reference,381 and is reproduced by permission of the American Society for Cell Biology, copyright 2011.

processing assay has been established using either GFP-Osh1 or Nvj1-GFP. These GFP chimeras are, together with the PMNvesicles, degraded in the vacuole. Thus, the formation of the relatively proteolysis-resistant GFP detected in western blots correlates with the PMN rate. In fluorescence microscopy, PMN can be visualized with the same constructs, and a chimera of mCherry fused to a nuclear localization signal (NLS-mCherry) can also be used. To assure that the measured PMN rate is indeed due to selective micronucleophagy, appropriate controls such as cells lacking Nvj1 or Vac8 should be included. Detailed protocols for the described assays are provided in ref.[524]

b. Reticulophagy and ribophagy. Activation of the UPR in the ER in yeast induces a type of selective macroautophagy of the ER.[525-527] This process is termed reticulophagy to be consistent with the terms pexophagy and mitophagy.[527] Reticulophagyassociated autophagosomes are lamellar-membraned structures that contain ER proteins. In theory, since reticulophagy is selective, it should be able to sequester parts of the ER that are damaged, and eliminate protein aggregates that cannot be removed in other ways. Reticulophagy may also serve to limit the UPR, by reducing the ER to a normal level after a particular stress condition has ended. Some of the mutated dysferlin protein, LGMB2B/Miyoshi type muscle dystrophy, which accumulates in the ER, is degraded by ER-stress mediated reticulophagy.[528] In addition to activation of the UPR, PtdIns3P and its binding proteins could be good markers for reticulophagy.[437] Autophagy is also used for the selective removal of ribosomes.[529] This process can be monitored by western blot, following the generation of free GFP from Rpl25-GFP or the disappearance of ribosomal subunits such as Rps3. Vacuolar localization of Rpl25-GFP can also be seen by fluorescence microscopy.

Cautionary notes: The Cvt pathway has been demonstrated to occur only in yeast. In addition, the sequestration of prApe1 is specific, even under starvation conditions, as it involves the recognition of the propeptide by a receptor, Atg19, which in turn interacts with the scaffold protein Atg11.[530,531] Thus, unless the propeptide is removed, prApe1 is recognized as a selective substrate. Overexpression of prApe1 saturates import by the Cvt pathway, and the precursor

form accumulates, but is rapidly matured upon autophagy induction.[218] In addition, mutants such as vac8D and tlg2D accumulate prApe1 under rich conditions, but not during autophagy.[380,532] Accordingly, it is possible to monitor the processing of prApe1 when overexpressed, or in certain mutant strains to follow autophagy induction. However, even the latter conditions may be misleading, as they do not indicate the size of the autophagosome. The Cvt complex (prApe1 bound to Atg19) is smaller than typical peroxisomes or mitochondrial fragments that are subject to autophagic degradation. Accordingly, particular mutants may display complete maturation of prApe1 under autophagy-inducing conditions, but may still have a defect in other types of selective autophagy, as well as being unable to induce a normal level of nonspecific autophagy.[63] For this reason, it is good practice to evaluate autophagosome size and number by TEM. Actually, it is much simpler to monitor autophagic bodies (rather than autophagosomes) in yeast. First, the vacuole is easily identified, making the identification of autophagic bodies much simpler. Second, autophagic bodies can be accumulated within the vacuole, allowing for an increased sample size. It is best to use a strain background that is pep4D vps4D to prevent the breakdown of the autophagic bodies, and to eliminate confounding vesicles from the multivesicular body pathway. One caveat to the detection of autophagic bodies, however, is that they may coalesce in the vacuole lumen, making it difficult to obtain an accurate quantification. In general, when working with yeast it is preferable to use strains that have the marker proteins integrated into the chromosome rather than relying on plasmid-based expression, because plasmid numbers can vary from cell to cell. The GFP-Atg8, or similar, processing assay is easy to perform and is suitable for analysis by microscopy as well as western blotting; however, particular care is needed to obtain quantitative data for GFPAtg8, Pex14-GFP or Om45-GFP, etc. processing assays (see cautionary notes for GFP-Atg8/LC3 lysosomal delivery and proteolysis). An alternative is an organelle targeted Pho8D60 assay. For example, mitoPho8D60 can be used to quantitatively measure mitophagy.498 In addition, for the GFP-Atg8 processing assay, 2 h of starvation is generally sufficient to detect a significant level of free (i.e., vacuolar) GFP by western blotting as a measure of nonselective autophagy. For selective types of autophagy, the length of induction needed for a clearly detectable free GFP band will vary depending on the rate of cargo delivery/degradation. Usually 6 h of mitophagy induction is needed to be able to detect free GFP (e.g., from Om45-GFP) by western blot under starvation conditions, whereas stationary phase mitophagy typically requires 3 d before a free GFP band is observed.

c. Vacuole import and degradation pathway. In yeast, gluconeogenic enzymes such as fructose-1,6-bisphosphatase (Fbp1, also called FBPase), malate dehydrogenase (Mdh2), isocitrate lyase (Icl1) and phosphoenolpyruvate carboxykinase (Pck1) constitute the cargo of the vacuole import and degradation (Vid) pathway.[533] These enzymes are induced when yeast cells are glucose starved (grown in a medium containing 0.5% glucose and potassium acetate). Upon replenishing these cells with fresh glucose (a medium containing 2% glucose), these enzymes are degraded in either the proteasome[534-536] or the vacuole[533,537] depending on the duration of starvation. Following glucose replenishment after 3 d glucose starvation, the gluconeogenic enzymes are delivered to the vacuole for degradation.[538] These enzymes are sequestered in specialized 30- to 50-nm vesicles called Vid vesicles.[539] Vid vesicles can be purified by fractionation and gradient centrifugation; western blotting analysis using antibodies against organelle markers and Fbp1, and the subsequent verification of fractions by EM facilitate their identification.[539] Furthermore, the amount of marker proteins in the cytosol compared with the Vid vesicles can be examined by differential centrifugation. In this case, yeast cells are lysed and subjected to differential centrifugation. The Vid vesicle-enriched pellet fraction and the cytosolic supernatant fraction are examined with antibodies against Vid24, Vid30, Sec28 and Fbp1.[540-542] Vid/endosomes containing their cargo aggregate around endocytic vesicles forming on the plasma membrane and are released into the cytoplasm. The Vid/endosomes can be purified byfractionation and density gradient centrifugation.[543] The fractions containing purified Vid/endosomes can be identified by western blot analysis using antibodies against Vid24, Fbp1 and the endosomal marker Pep12. The distribution of Vid vesicles containing cargo destined for endosomes, and finally for the vacuole, can be examined using FM 4–64, a lipophilic dye that stains endocytic compartments and the vacuole limiting membrane.[544] In these experiments, starved yeast cells are replenished with fresh glucose and FM 4–64, and cells are collected at appropriate time points for examination by fluorescence microscopy.[541] The site of degradation of the cargo in the vacuole can be determined by studying the distribution of Fbp1-GFP, or other Vid cargo markers in wild-type and pep4D cells.[545] Cells can also be examined for the distribution of Fbp1 at the ultrastructural level by immuno-TEM.[543] As actin patch polymerization is required for the delivery of cargo to the vacuole in the Vid pathway, distribution of Vid vesicles containing cargo and actin patches can be examined by actin staining (with phalloidin conjugated to rhodamine) using fluorescence microscopy.[543] The distribution of GFP tagged protein and actin is examined by fluorescent microscopy. GFPVid24, Vid30-GFP and Sec28-GFP colocalize with actin during prolonged glucose starvation and for up to 30 min following glucose replenishment in wild-type cells; however, colocalization is less obvious by the 60 min time point.[540,543]

d. Mammalian mitophagy and peroxisome degradation. There is no consensus at the present time with regard to the best method for monitoring mammalian mitophagy. As with any organelle-specific form of autophagy, it is necessary to demonstrate: i) increased levels of autophagosomes containing mitochondria, ii) maturation of these autophagosomes to culminate with mitochondrial degradation, which can be blocked by specific inhibitors of

autophagy or of lysosomal degradation, and iii) whether the changes are due to selective mitophagy or increased mitochondrial degradation during generalized autophagy. Techniques to address each of these points have been reviewed.[41]

Ultrastructural analysis at early time points can be used to establish selective mitophagy, although a maturation inhibitor may be needed to trap early autophagosomes with recognizable cargo (Fig. 20). Depending on the use of specific imaging techniques, dyes for living cells or antibodies for fixed cells have to be chosen. In any case, transfection of the autophagosomal marker GFP-LC3 and visualization of mitochondria (independent of their mitochondrial membrane potential) makes it possible to determine the association of these two cellular components. This may appear as fluorescence colocalization or as rings of GFP-LC3 surrounding mitochondria in higher resolution images. For live cell imaging microscopy, a method that marks mitochondria independently of mitochondrial membrane potential, such as MitoTracker1 Green FM or transfection with a matrixtargeted fluorescent protein, should be used to detect mitochondrial structures. Antibodies that specifically recognize mitochondrial proteins such as VDAC, TOM20 or complex IV subunit I may be used to visualize mitochondria in immunohistochemical experimental procedures.[546,547] Colocalization analyses of mitochondria and autophagosomes provide an indication of the degree of sequestration. TEM can be used to demonstrate the presence of mitochondria within autophagosomes (referred to as mitophagosomes during mitophagy), and this can be coupled with bafilomycin A1 treatment to prevent fusion with the lysosome.[41] The fusion process of mitophagosomes with hydrolasecontaining lysosomes represents the next step in the degradation process. To monitor the amount of fused organelles via live cell imaging microscopy, MitoTracker1 Green FM, as a mitochondrial marker without significant membrane potential dependence and LysoTracker1 Red DND-99 may be used to visualize the fusion process (Fig. 21). Independent of the cell-type specific concentration used for both dyes, we recommend exchanging MitoTracker1 Green FM with normal medium after incubation

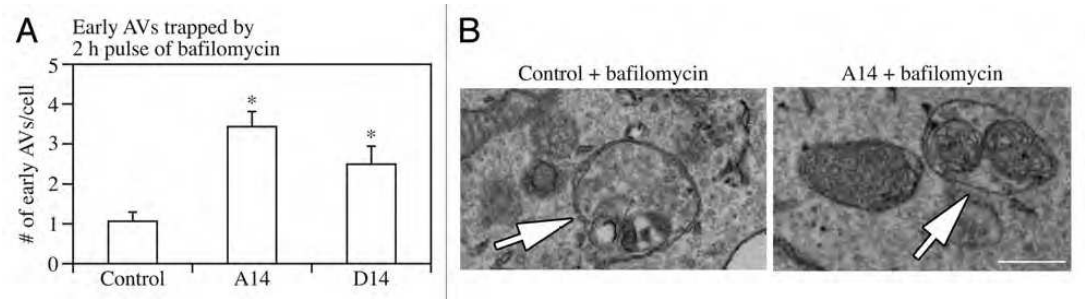

Figure 20. Autophagosomes with recognizable cargo are rare in cells. (A) To assess relative rates of autophagosome formation, the fusion inhibitor bafilomycin A1 (10 nM) was applied for 2 h prior to fixation with 2% glutaraldehyde in order to trap newly formed autophagosomes. Two different PINK1 shRNA lines exhibit increased AV formation over 2 h compared with the control shRNA line. *p . 0.05 vs. Control. (B) Autophagosomes in bafilomycin A1-treated control cells contain a variety of cytoplasmic structures (left, arrow), while mitochondria comprise a prominent component of autophagosomes in A14 bafilomycin A1-treated (PINK1 shRNA) cells (right, arrow). Scale bar, 500 nm. These data indicate induction of selective mitophagy in PINK1-deficient cells. This figure was modified from Figure 2 published in Chu CT. A pivotal role for PINK1 and autophagy in mitochondrial quality control: implications for Parkinson disease. Human Molecular Genetics 2010; 19:R28-R37.

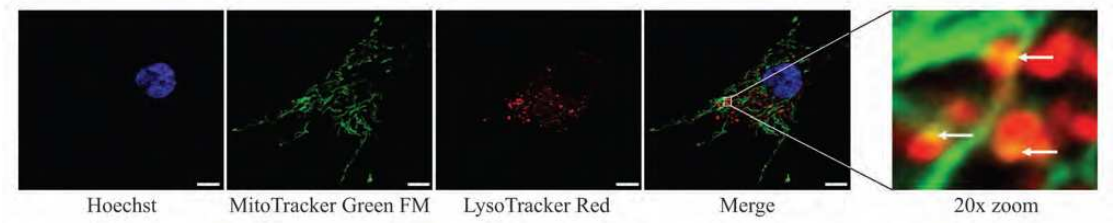

Figure 21. Human fibroblasts showing colocalization of mitochondria with lysosomes. The degree of colocalization of mitochondria with lysosomes in human fibroblasts was measured via live cell imaging microscopy at 37°C and 5% CO2 atmosphere using the ApoTome1 technique. LysoTracker1 Red DND-99 staining was applied to mark lysosomal structures (red), and MitoTracker1 Green FM to visualize mitochondria (green). Hoechst 33342 dye was used to stain nuclei (blue). A positive colocalization is indicated by yellow signals (merge) due to the overlap of LysoTracker1 Red and MitoTracker1 Green staining (white arrows). Scale bar, 10 mm. Statistical evaluation is performed by calculating the Pearson's coefficient for colocalizing pixels. Image provided by L. Burbulla and R. Krüger.

with the dye, whereas it is best to maintain the LysoTracker1 Red staining in the incubation medium during the acquisition of images. For immuncytochemical experiments, antibodies specific for mitochondrial proteins and an antibody against the lysosomalassociated membrane protein 1 (LAMP1) can be used. Overlapping signals appear as a merged color and can be used as indicators for successful fusion of autophagosomes that contain mitochondria with lysosomal structures.[548] To measure the correlation between two variables by imaging techniques, such as the colocalization of two different stainings, we recommend some form of correlation analysis to assess the value correlating with the strength of the association. This may use, for example, ImageJ software, or other colocalization

scores that can be derived from consideration not only of pixel colocalization, but also from a determination that the structures have the appropriate shape. During live-cell imaging, the two structures should move together in more than one frame. Mitophagy can also be quantitatively monitored using a mitochondria-targeted version of the pHdependent Keima protein.[549] The peak of the excitation spectrum of the protein shifts from 440 nm to 586 nm when mitochondria are delivered to acidic lysosomes, which allows easy quantification of mitophagy (Fig. 22). However, it should be noted that long exposure times of the specimen to intense laser light lead to a similar spectral change. The third and last step of the degradation process is the monitoring of the amount of remaining mitochondria by analyzing the mitochondrial mass. This final step provides the opportunity to determine the efficiency of degradation of dysfunctional, aged or impaired mitochondria. Mitochondrial mass can either be measured by a FACS technique using MitoTracker1 Green FM or, on a single cell basis, by either live cell imaging or immuncytochemistry with use of MitoTracker1 Green FM or MitoTracker1 Red FM for the former, and antibodies specifically raised against different mitochondrial proteins for the latter. Immunoblot analysis of the levels of mitochondrial proteins from different mitochondrial subcompartments is valuable for validating the data from FACS or microscopy studies. EM can also be used to verify loss of entire mitochondria, and PCR (or fluorescence microscopy) to quantify mitochondrial DNA. In addition to monitoring the steady-state levels of different steps of mitophagy, investigation of the mitophagic flux is needed to decipher which steps within this process potentially fail to

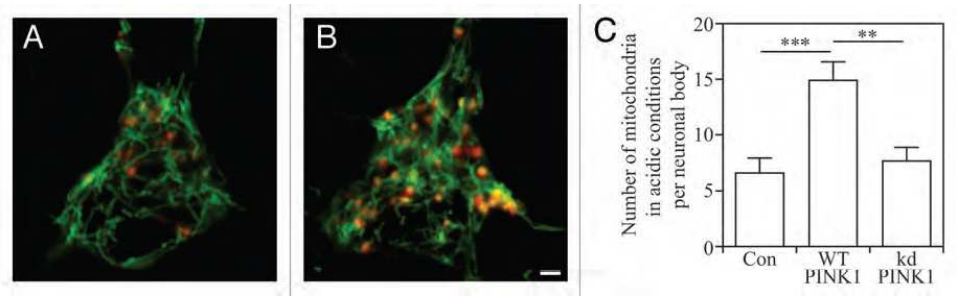

Figure 22. Detection of mitophagy in primary cortical neurons using mitochondria-targeted Keima. Neurons transfected with mito-Keima were visualized using 458 nm (green, mitochondria at neutral pH) and 561 nm (red, mitochondria in acidic pH) laser lines and 575 nm band pass filter. Compared with the control (A) wild-type PINK1 overexpression (B) increases the number of the mitochondria exposed to acidic conditions. Scale bar, 2 mm. (C) Quantification of red dots suggests increased mitophagy in wild-type PINK1 but not in the kinase dead PINK1K219M-overexpressing neurons. Figure provided by V. Choubey and A. Kaasik.

result in efficient mitochondrial degradation. Therefore, appropriate treatment may be applied to prevent mitochondrial degradation at distinct steps of the process. Certain cellular models require stress conditions to measure the mitochondrial degradation capacity, as basal levels are too low to reliably assess organelle clearance. Hence, it may be useful to pretreat the cells with uncoupling agents, such as CCCP that stimulate mitochondrial degradation and allow measurements of mitophagic activity; however, it should be kept in mind that, although helpful to stimulate mitochondrial degradation, this treatment is not physiological. Another method to induce mitophagy is by expressing and activating a mitochondriallylocalized fluorescent protein photosensitizer such as Killer Red.[550] The excitation of Killer Red results in an acute increase of superoxide that causes mitochondrial damage resulting in mitophagy (Kim PK, unpublished results). The advantage of using a genetically encoded photosensitizer is that it allows for both spatial and temporal control in inducing mitophagy. Relatively little work has been done in the area of mammalian peroxisome degradation by autophagy, and at present it is not known if this is a selective process. Typically, peroxisomes are induced by treatment with hypolipidemic drugs such as clofibrate or dioctyl phthalate, and degradation is induced by drug withdrawal, although starvation without prior proliferation can also be used. Loss of peroxisomes can be followed enzymatically or by immunoblot, monitoring enzymes such as fatty acyl-CoA oxidase (note that this enzyme is sometimes abbreviated “AOX,” but should not be confused with the enzyme alcohol oxidase that is frequently used in assays for yeast pexophagy) or catalase, and also by EM.[551,552] Finally, a HaloTag1-PTS1 marker that is targeted to peroxisomes has been used to fluorescently label the organelle.553 Cautionary notes: There are many assays that can be used to monitor specific types of autophagy, but caution must be used in choosing an appropriate marker(s). It is best to monitor more than one protein, and to include an inner membrane or matrix component in the analysis. In particular, it is not sufficient to follow a single mitochondrial outer membrane protein because these can be degraded independently of mitophagy. Although the localization of PARK2 (PARKIN) to mitochondria as monitored by fluorescence microscopy is associated with the early stages of protonophore uncoupler (CCCP)-driven mitochondria degradation,[169] this by itself cannot be used as a marker for mitophagy, as these events can be dissociated.[554] PARK2 translocates to damaged mitochondria and ubiquitinates a wide range of outer membrane proteins including VDAC1, MFN1/2 and TOMM20 (TOM20).[547,555-557] This results in the preferential degradation of mitochondrial outer membrane proteins by the proteasome, while inner membrane proteins and

mitochondrial DNA[558] remain intact. Monitoring loss of a single protein such as TOM20 by western blot or fluorescence microscopy to follow mitophagy may thus be misleading.[555] MitoTracker dyes are widely used to stain mitochondria and, when colocalized with GFP-LC3, they can function as a marker for mitophagy. However, staining with certain MitoTracker dyes depends on mitochondrial activity and membrane potential, so that damaged, or sequestered nonfunctional mitochondria may not be stained. Although it is widely assumed that macroautophagy is the major mechanism for degradation of entire organelles, there are multiple mechanisms that may account for the disappearance of mitochondrial markers. These include proteasomal degradation of outer membrane proteins and/or proteins that fail to correctly translocate into the mitochondria, degradation due to proteases within the mitochondria, and reduced biosynthesis or import of proteins. In addition to mitophagy, mitochondria can be eliminated by extrusion from the cell (mitoptosis).[559] Thus, it is advisable to use a variety of complementary methods to monitor mitochondria loss including TEM, single cell analysis of LC3 fluorescent puncta, and western blot, in conjunction with flux inhibitors and specific inhibitors of autophagy induction compared with inhibitors of the other major degradation systems (see cautions in Autophagy inhibitors and inducers). Likewise, although the mechanism(s) of peroxisome degradation in mammals awaits further elucidation, it can occur by both autophagic and proteasome-dependent mechanisms.560 Thus, controls are needed to determine the extent of degradation that is due to the proteasome. Moreover, two additional degradation mechanisms have been suggested: the action of the peroxisomespecific Lon protease and the membrane disruption effect of 15-lipoxygenase.[561]

e. Aggrephagy. Aggrephagy is the selective removal of aggregates by macroautophagy.[562] This process can be followed in vitro (in cell culture) and in vivo (in mice) by monitoring the levels of an aggregate-prone protein such as an expanded polyglutamine (polyQ)-containing protein or mutant SNCA (a-synuclein). Levels are quantified by immunofluorescence or traditional immunoblot. Similarly, fluorescently tagged aggregated proteins such as polyQ80-CFP can be monitored via immunoblot and immunofluorescence. A polyQ80-luciferase reporter, which forms aggregates, can also be used to follow aggrephagy.[563] A nonaggregating polyQ19-luciferase or untagged full-length luciferase serves as a control. The ratio of luciferase activity from these two constructs can be calculated to determine autophagic flux. Autophagic degradation of endogenous aggregates such as lipofuscin can in some cell types be monitored by fluorescence microscopy, utilizing the autofluorescence of lipofuscin particles. The amount of lipofuscin in primary human adipocytes can be reduced by activation of autophagy, and the amount of lipofuscin is dramatically reduced in adipocytes from patients with type 2 diabetes and chronically enhanced autophagy.[209] Cautionary notes: Caution must be used when performing immunoblots of aggregated proteins, as many protein aggregates fail to enter the resolving gel and are retained in the stacking gel. In addition, the polyQ80-luciferase in the aggregated state lacks luciferase activity whereas soluble polyQ80-luciferase retains activity. Therefore, caution must be used when interpreting results with these vectors, as treatments that increase aggrephagy or enhance protein aggregation can lead to a decrease in luciferase activity.564 Finally, soluble polyQ reporters can bedegraded by the proteasome; thus, changes in the ratio of polyQ19-luciferase:polyQ80-luciferase may also reflect proteasomal effects and not just changes in autophagic flux.

f. Xenophagy. The macroautophagy pathway has emerged as an important cellular factor in both innate and adaptive immunity. Many in vitro and in vivo studies have demonstrated that genes encoding macroautophagy components are required for host defense against infection by bacteria, parasites and viruses. Macroautophagy may be defective in human diseases such as inflammatory bowel disease, since genes encoding essential macroautophagy components have been linked to the disease. Xenophagy is often used as a term to describe autophagy of microbial pathogens, mediating their capture and delivery to lysosomes for degradation. Since xenophagy presents an immune defense, it is not surprising that microbial pathogens have evolved strategies to overcome it. The interactions of such pathogens with the autophagy system of host cells are complex and have been the subject of several excellent reviews.[76-81,565-570] Here we will make note of a few key considerations when studying interactions of microbial pathogens with the autophagy system.

LC3 is commonly used as a marker of macroautophagy. However, studies have established that LC3 can promote phagosome maturation independently of macroautophagy through LC3-associated phagocytosis (see cautionary notes in Atg8/LC3 detection and quantification). Other studies show that macroautophagy of Salmonella Typhimurium is dependent on Atg9, an essential macroautophagy gene, whereas LC3 recruitment to bacteria does not require Atg9.571 In contrast, macroautophagy of these bacteria requires ubiquitination of target proteins (not yet identified) and recruitment of three ubiquitin-binding adaptor proteins, p62/SQSTM1,[572] NDP52[573] and OPTN.[574] Therefore, the currently available criteria to differentiate LAP from macroautophagy include: i) LAP involves LC3 recruitment to bacteria in a manner that requires reactive oxygen species production by an NADPH oxidase. It should be noted that most cells express at least one member of the NADPH oxidase (also known as NOX) family. Targeting expression of the common p22phox subunit is an effective way to disrupt the NOX NADPH oxidases. Scavenging of reactive oxygen species by antioxidants such as resveratrol and a-tocopherol is also an effective way to inhibit LAP. In contrast, N-acetylcysteine, which raises cellular glutathione levels, does not inhibit LAP.[575] ii) Macroautophagy of bacteria requires Atg9, while LAP apparently does not.571 iii) LAP involves singlemembrane structures. For LAP, CLEM (with LC3 as a

marker) is expected to show single-membrane structures that are LC3+ with LAP.131 In contrast, macroautophagy is expected to generate double-membrane structures surrounding cargo (which may include single membrane phagosomes, giving rise to triplemembrane structures[571]). iv) Macroautophagy of bacteria requires protein ubiquitination and ubiquitin-binding adaptors (p62/SQSTM1, NDP52, OPTN, and possibly others), whereas LAP does not. It is anticipated that more specific markers of LAP will be identified as these phagosomes are further characterized. Nonmotile Listeria monocytogenes can be targeted to doublemembrane autophagosomes upon antibiotic treatment,[576] which indicates that macroautophagy serves as a cellular defense to microbes in the cytosol. However, subsequent studies have revealed that macroautophagy can also target pathogens within phagosomes, damaged phagosomes or the cytosol. Therefore, when studying microbial interactions by EM, many structures can be visualized, with any number of membranes encompassing microbes, all of which may be LC3+.[577] As discussed above, singlemembrane structures that are LC3+ may arise through LAP, and we cannot rule out the possibility that both LAP and macroautophagy may operate at the same time to target the same phagosome. Viruses can also be targeted by autophagy, and in turn can act to inhibit autophagy. For example, infection of a cell by influenza and dengue viruses[578] or enforced expression of the hepatitis B virus C protein[579] have profound consequences for autophagy, as viral proteins such as NS4A stimulate autophagy and protect the infected cell against apoptosis, thus extending the time in which the virus can replicate. Conversely, the herpes simplex virus ICP34.5 protein inhibits autophagy by targeting BECN1.[580] While the impact of ICP34.5's targeting of BECN1 on virus replication in cultured permissive cells is minimal, it has a significant impact upon pathogenesis in vivo, most likely through interfering with activation of CD4+ T cells.[581,582] Care must be taken in determining the role of autophagy in virus replication, as some viruses such as vaccinia use doublemembrane structures that form independently of the autophagy machinery.[583] Similarly, dengue virus replication, which appears to involve a double-membrane compartment, requires the ER rather than autophagosomes,[584] whereas coronaviruses use a nonlipidated version of LC3 (see Atg8/LC3 detection and quantification)[137] Yet another type of variation is seen with hepatitis C virus, which requires BECN1, ATG4B, ATG5 and ATG12 for initiating replication, but does not require these proteins once an infection is established.[585] Finally, it is important to realize that there may be other macroautophagy-like pathways that have yet to be characterized.

For example, in response to cytotoxic stress (treatment with etoposide), autophagosomes are formed in an ATG5- and ATG7-independent manner.[26] While this does not rule out involvement of other macroautophagy regulators/components in the formation of these autophagosomes, it does establish that the canonical macroautophagy pathway involving LC3 conjugation is not involved. In contrast, RAB9 is required for this alternative pathway, potentially providing a useful marker for analysis of these structures. Returning to xenophagy, Mycobacterium tuberculosis can be targeted to autophagosomes in an ATG5-independent manner.[586] Furthermore, up to 25% of intracellular Salmonella typhimurium are observed in multilamellar membrane structures resembling autophagosomes in Atg52/2 MEFs.[572] These findings indicate that an alternate macroautophagy pathway is relevant to host-pathogen interactions. Moreover, differences are observed that depend on the cell type being studied. Yersinia pseudotuberculosis is targeted to autophagosomes where they can replicate in bone marrow-derived macrophages,[587] whereas in RAW264.7 and J774 cells, bacteria are targeted both to autophagosomes, andLC3-negative, single-membrane vacuoles (Lafont F, personal communication).

g. Lipophagy. The specific macroautophagic degradation of lipid droplets represents another type of selective autophagy.588 Lipophagy requires the core autophagic machinery and can be monitored by following triglyceride content, or total lipid levels using BODIPY 493/503 or HCS LipidTOX neutral lipid stains with fluorescence microscopy, cell staining with Oil Red O, or ideally label-free techniques such as CARS or SRS microscopy. TEM can also be used to monitor lipid droplet size and number. Cautionary notes: With regard to changes in the cellular neutral lipid content, the presence and potential activation of cytoplasmic lipases that are unrelated to lysosomal degradation must be considered.

h. Zymophagy. Zymophagy is a protective mechanism induced during pancreatitis that results in the selective degradation of activated zymogen granules, which are deleterious for the pancreatic acinar cells.[589] This process can be monitored by TEM, identifying autophagosomes containing secretory granules, by following p62 degradation by western blot, and by examining the subcellular localization of VMP1-EGFP, which relocates to granular areas of the cell upon zymophagy induction. Colocalization of trypsinogen (which is packaged within zymogen granules) and LC3, or of GFP-ubiquitin (which is recruited to the activated granules) with RFP-LC3 can also be observed by indirect or direct immunofluorescence microscopy, respectively.

i. Allophagy. In metazoans, mitochondria, and hence mitochondrial DNA, from the sperm is eliminated by an autophagic process. This process of allogeneic (nonself) organelle autophagy is termed “allophagy.”[590,591] During allophagy in C. elegans, both paternal mitochondria and membranous organelles (a spermspecific membrane compartment) are eliminated by the 16-cell stage (100–120 min post-fertilization).[592,593] The degradation process can be monitored in living embryos with GFP: ubiquitin, which appears in the vicinity of the sperm chromatin (labeled for example with mCherry-histone H2B) on the membranous organelles within 3 min after fertilization. GFP fusions and antibodies specific for LGG-1 and LGG-2 (Atg8/LC3 homologs), which appear next to the sperm DNA, membranous

organelles and mitochondria (labeled with CMXRos or mitochondriatargeted GFP) within 15 to 30 min post-fertilization, can be used to verify the autophagic nature of the degradation. TEM can also be utilized to demonstrate the presence of mitochondria within autophagosomes in the early embryo.

Conclusion: There are many assays that can be used to monitor specific types of autophagy, but caution must be used in choosing an appropriate marker(s). The potential role of other degradative pathways for any individual organelle or cargo marker should be considered, and it is advisable to use more than one marker or technique.

9. Autophagic sequestration assays. Autophagic activity can also be monitored by the sequestration of autophagic cargo, using either an (electro)injected, inert cytosolic marker such as [3H]raffinose,[594] or an endogenous cytosolic protein such as lactate dehydrogenase (LDH),[595] in the latter case along with treatment with a protease inhibitor (e.g., leupeptin) to prevent intralysosomal degradation of the protein marker. The assay simply measures the transfer of cargo from the soluble (cytosol) to the insoluble (sedimentable) cell fraction (which includes autophagic compartments), with no need for a sophisticated subcellular fractionation (a filtration assay would presumably work just as well as centrifugation, although it would be necessary to verify that the filtration membrane does not destroy the integrity of the postnuclear supernatant compartments). The cargo marker can be quantified by an enzymatic assay, or by western blotting. In principle, any intracellular component can be used as a cargo marker, but cytosolic enzymes having low sedimentable backgrounds are preferable. Membrane-associated markers are less suitable, and proteins such as LC3, which are part of the sequestering system itself, will have a much more complex relationship to the autophagic flux than a pure cargo marker such as LDH. In yeast, sequestration assays are typically done by monitoring protease protection of an autophagosome marker or a cargo protein. For example, prApe1, and GFP-Atg8 have been used to follow completion of the autophagosome.596 The relative resistance or sensitivity to an exogenous protease in the absence of detergent is an indication of whether the autophagosome (or other sequestering vesicle) is complete or incomplete, respectively. Thus, this method also distinguishes between a block in autophagosome formation vs. fusion with the vacuole. The critical issues to keep in mind involve the use of appropriate control strains and/or proteins, and deciding on the correct reporter protein. In addition to protease protection assays, sequestration can be monitored by fluorescence microscopy during pexophagy of methanol-induced peroxisomes, using GFP-Atg8 as a pexophagosome marker, and BFP-SKL to label the peroxisomes. The vacuolar sequestration process during micropexophagy can also be monitored by formation of the vacuolar sequestration membrane (VSM) stained with FM 4–64.[501,507] Sequestration assays can be designed to measure flux through individual steps of the autophagy pathway. For example, intralysosomally degraded sequestration probes such as [14C]-lactate or LDH will mark prelysosomal compartments in the absence of degradation inhibitors. Hence, their accumulation in such compartments can be observed when fusion with lysosomes suppressed, for example, by a microtubule inhibitor such as vinblastine.[597] Furthermore, lactate hydrolysis can be used to monitor the overall autophagic pathway (autophagic lactolysis).[598] One caveat, however, is that inhibitors may affect sequestration indirectly, for example, by modifying the uptake and metabolism (including protein synthesis) of autophagy-suppressive amino acids (see Autophagy inhibitors and inducers). A variation of this approach applicable to mammalian cells includes live cell imaging. Autophagy induction is monitored as the movement of cargo, such as mitochondria, to GFP-LC3- colocalizing compartments, and then fusion/flux is measured by delivery of cargo to lysosomal compartments.[258,599] In addition, sequestration of fluorescently tagged cytosolic proteins into membranous compartments can be measured, as fluorescent puncta become resistant to the detergent digitonin.[600] Use of multiple time points and monitoring colocalization of a particularcargo with GFP-LC3 and lysosomes can also be used to assess sequestration of cargo with autophagosomes as well as delivery to lysosomes.[601]

Cautionary notes: The electro-injection of radiolabeled probes is technically demanding, but the use of an endogenous cytosolic protein probe is very simple and requires no pretreatment of the cells other than with a protease inhibitor. Another concern with electro-injection is that it can affect cellular physiology, so it is necessary to verify that the cells behave properly under control situations such as amino acid deprivation. An alternate approach for incorporating exogenous proteins into mammalian cell cytosol is to use “scrape-loading,” a method that works for cells that are adherent to tissue culture plates.[602] Finally, these assays work well with hepatocytes but may be problematic with other cell types, and it can be difficult to load the cell while retaining the integrity of the compartments in the post-nuclear supernatant (Tooze S, unpublished results). General points of caution to be addressed with regard to live cell imaging relate to photobleaching of the fluorophore, cell injury due to repetitive imaging, autofluorescence in tissues containing lipofuscin, and the pH sensitivity of the fluorophore. There are several issues to keep in mind when monitoring sequestration by the protease protection assay in yeast.[596] First, as discussed in Selective types of autophagy, prApe1 is not an accurate marker for nonspecific autophagy; import of prApe1 utilizes a receptor (Atg19) and a scaffold (Atg11) that make the process specific. In addition, vesicles that are substantially smaller than autophagosomes can effectively sequester the Cvt complex. Another problem is that prApe1 cannot be used as an autophagy reporter for mutants that are not defective in the Cvt pathway, although this can be bypassed by using a vac8D background.[603] At present, the prApe1 assay cannot be used in any system other than yeast. The GFP-Atg8 protease protection assay avoids these problems, but the signal-to-noise ratio is typically substantially higher. In

theory, it should be possible to use this assay in other cell types, but at present no publications report its use other than in yeast.

Conclusion: Sequestration assays present another valid method for monitoring autophagy, and in particular for discriminating between conditions where the autophagosome is complete (but not fused with the lysosome/vacuole) or open (i.e., a phagophore). These assays can also be modified to measure autophagic flux.

10. Turnover of autophagic compartments. Inhibitors of autophagic sequestration (e.g., amino acids, 3-MA or wortmannin) can be used to monitor the disappearance of autophagic elements (phagophores, autophagosomes, autolysosomes) to estimate their half-life by TEM morphometry/stereology. The turnover of the autophagosome or the autolysosome will be differentially affected if fusion or intralysosomal degradation is inhibited.[14,16,24,604]

The duration of such experiments is usually only a few hours; therefore, long-term side effects or declining effectiveness of the inhibitors can be avoided. It should be noted that fluorescence microscopy has also been used to monitor the half-life of autophagosomes, monitoring GFP-LC3 in the presence and absence of bafilomycin A1 or following GFP-LC3 after starvation and recovery in amino acid-rich medium (see Atg8/LC3 detection and quantification).[18,605]

Cautionary notes: The inhibitory effect must be strong and the efficiency of the inhibitor needs to be tested under the experimental conditions to be employed. Cycloheximide is sometimes used as an autophagy inhibitor, but this is problematic because of the many potential indirect effects. Cycloheximide inhibits translational elongation, and therefore protein synthesis. In addition, it decreases the efficiency of protein degradation in several cell types (Cuervo AM, personal communication) including hematopoietic cells (Edinger A, personal communication). Treatment with cycloheximide causes a potent increase in TORC1 activity, which can decrease autophagy in part as a result of the increase in the amino acid pool resulting from suppressed protein synthesis (Shen H-M, personal communication).[606,607] In addition, at high concentrations (in the millimolar range) cycloheximide inhibits complex I of the mitochondrial respiratory chain,[608,609] but this is not a problem, at least in hepatocytes, at low concentrations (10 -20 mM) that are sufficient to prevent protein synthesis (Meijer AJ, personal communication).

Conclusion: The turnover of autophagic compartments is a valid, but less preferred method, for monitoring flux, and cycloheximide must be used in these experiments with caution.

11. Autophagosome-lysosome colocalization and dequenching assay. Another method to demonstrate the convergence of the autophagic pathway with a functional degradative compartment is to incubate cells with the bovine serum albumin derivative dequenched (DQ)-BSA that has been labeled with the redfluorescent BODIPY TR-X dye; this conjugate will accumulate in lysosomes. The labeling of DQ-BSA is so extensive that the fluorophore is self-quenched. Proteolysis of this compound results in dequenching and the release of brightly fluorescent fragments. Thus, DQ-BSA is useful for detecting intracellular proteolytic activity as a measure of a functional lysosome.[610] Furthermore, DQ-BSA labeling can be combined with GFPLC3 to monitor colocalization, and thus visualize the convergence, of amphisomes with a functional degradative compartment (DQ-BSA is internalized by endocytosis). This method can also be used to visualize fusion events in real-time experiments by confocal microscopy (live cell imaging). Along similar lines, other approaches for monitoring convergence are to follow the colocalization of RFP-LC3 and LysoSensor Green (Bains M, Heidenreich KA, personal communication), mCherry-LC3 and LysoSensor Blue,[259] or tagged versions of LC3 and LAMP1 (Macleod K, personal communication) or CD63258 as a measure of the fusion of autophagosomes with lysosomes. It is also possible to trace autophagic events by visualizing the pH-dependent excitation changes of the coral protein Keima.[549] This quantitative technique is capable of monitoring the fusion of autophagosomes with lysosomes, that is, the formation of an autolysosome, and the assay does not depend on the analysis of LC3.

Cautionary notes: Some experiments require the use of inhibitors (e.g., 3-MA or wortmannin) or overexpression of proteins (e.g., RAB7 dominant negative mutants) that may also affect theendocytic pathway or the delivery of DQ-BSA to lysosomes (e.g., wortmannin causes the swelling of late endosomes611). In this case, the lysosomal compartment can be labeled with DQ-BSA overnight before treating the cells with the drugs, or prior to the transfection.

Conclusion: DQ-BSA provides a relatively convenient means for monitoring lysosomal protease function, and can also be used to follow the fusion of amphisomes with the lysosome. Colocalization of autophagosomes (fluorescently tagged LC3) with lysosomal proteins or dyes can also be monitored.

12. Tissue fractionation. The study of autophagy in the organs of larger animals, in large numbers of organisms with very similar characteristics, or in tissue culture cells provides an opportunity to use tissue fractionation techniques as has been possible with autophagy in rat liver.[34,48,612-617] Because of their sizes [smaller than nuclei but larger than membrane fragments (microsomes)], differential centrifugation can be used to obtain a subcellular fraction enriched in mitochondria and organelles of the autophagic-lysosomal system, which can then be subjected to density gradient centrifugation to enrich autophagosomes, amphisomes, autolysosomes and lysosomes.[34,48,617-621] Any part of such a fraction can be considered to be a representative sample of tissue constituents and used in quantitative biochemical, centrifugational and morphological studies of autophagic particle populations.

The simplest studies of the autophagic process take advantage of sequestered marker enzymes, changes in location of these enzymes, differences in particle/compartment size and differential sensitivity of particles of different sizes to mechanical and osmotic stress (for example, acid hydrolases are found primarily in membrane-bound compartments and their latent activities cannot be measured unless these membranes are lysed). Such a change in enzyme accessibility can be used to follow the time course of an exogenously induced, or naturally occurring, autophagic process.[612,614,616] Quantitative localization of enzymatic activity (or any other marker) to specific cytoplasmic particle populations and changes in the location of such markers during autophagy can be performed using rate sedimentation ultracentrifugation (see Autophagic sequestration assays).[618] Similar results can be obtained with isopycnic centrifugation where particles enter a density gradient (sometimes made with sucrose but iso-osmotic media such as iodixanol, metrizamide and Nycodenz may be preferred as discussed below under Cautionary notes) and are centrifuged until they reach locations in the gradient where their densities are equal to those of the gradient.[618] The fractionation of organelles can also be evaluated by protein-correlation-profiling (PCP), a quantitative mass spectrometry-based proteomics approach. Similar to the biochemical assays described above, gradient profiles of marker proteins can be recorded and compared with proteins of interest.[278] Compared with classical biochemical approaches, PCP allows the proteomewide recording of protein gradient profiles. Particle populations in subcellular fractions evaluated with quantitative biochemical and centrifugational approaches can also be studied with quantitative morphological methods. Detailed morphological study of the particle populations involved in the autophagic process usually requires the use of EM. The thin sections required for such studies pose major sampling problems in both intact cells[622] and subcellular fractions.[618] With the latter, 2,000,000 sections can be obtained from each 0.1 ml of pellet volume, so any practical sample size is an infinitesimally small subsample of the total sample.[618] However, through homogenization and resuspension, complex and heterogeneous components of subcellular fractions become randomly distributed throughout the fraction volume. Therefore, any aliquot of that volume can be considered a random sample of the whole volume. What is necessary is to conserve this property of subcellular fractions in the generation of a specimen that can be examined with the electron microscope. This can be done with the use of a pressure filtration procedure.[618,623] Because of the thinness of the sections, multiple sections of individual particles are possible so morphometric/stereological methods[622] must be used to determine the volume occupied by a given class of particles, as well as the size distribution and average size of the particle class. From this information the number of particles in a specific particle class can be calculated.[624] Examination of individual profiles gives information on the contents of different types of particles and their degree of degradation, as well as their enclosing membranes.[612,614]

Cautionary notes: When isolating organelles from tissues and cells in culture it is essential to use disruption methods that do not alter the membrane of lysosomes and autophagosomes, compartments that are particularly sensitive to some of those procedures. For example teflon/glass motor homogenization is suitable for tissues with abundant connective tissue, such as liver, but for circulating cells or cells in culture, disruption by nitrogen cavitation is a good method to preserve lysosomal membrane stability;[625] however, this method is not suitable for small samples and may not be readily available. Other methods, including “Balch” or “Dounce” homogenizers also work well.[626,627] During the isolation procedure it is essential to always use iso-osmotic solutions to avoid hypotonic or hypertonic disruption of the organelles. In that respect, because lysosomes are able to take up sucrose if it is present at high concentrations, the use of sucrose gradients for the isolation of intact lysosome-related organelles is strongly discouraged. As with the isolation of any other intracellular organelle, it is essential to assess the purity of each preparation, as there is often considerable variability from experiment to experiment due to the many steps involved in the process. Correction for purity can be done through calculation of recovery (percentage of the total activity present in the homogenate) and enrichment (dividing by the specific activity in the homogenate) of enzymes or protein markers for those compartments (e.g., β-hexosaminidase is routinely used to assess lysosomal purity, but enzymes such as cathepsin B may also be used and may provide more accurate readouts).625 Because of the time-consuming nature of quantitative morphological studies, such studies should not be performed until simpler biochemical procedures have established the circumstances most likely to give meaningful morphometric/stereological results. Finally, it is worthwhile noting that not all lysosomes are alike. For example, there are differences among primary lysosomes, autolysosomes and telolysosomes. Furthermore, what we refer to as “lysosomes” are actually a very heterogeneous pool of organelles that simply fulfill five classical criteria, having a pH , 5.6, mature cathepsins, the presence of LAMP proteins, a single membrane, and the absence of endosomal and recycling compartment markers (e.g., the mannose-6-phosphate receptor or RAB5). But even applying those criteria we can separate lysosomes with clear differences in their proteome and other properties, and these distinct populations of lysosomes are likely to participate in different functions in the cell (see Chaperonemediated autophagy).[628]

Conclusion: Considering the limited methods available for in vivo analysis of autophagy, tissue fractionation is a valid, although relatively laborious, method for monitoring autophagy. Care must be taken to ensure that sample analysis is representative.

13. Analyses in vivo. Monitoring autophagic flux in vivo or in organs is one of the least developed areas at present, and ideal methods relative to the techniques possible with cell culture may not exist. Importantly, the level of basal autophagy, time course of autophagic induction, and the bioavailabilty of autophagystimulating and inhibiting drugs is likely tissue specific. Moreover, basal autophagy or sensitivity to autophagic induction may vary with animal age, sex or strain background. Therefore methods may need to be optimized for the tissue of interest. One method for in vivo studies is the analysis of GFP-LC3/Atg8 (see GFP-Atg8/LC3 fluorescence microscopy above). Autophagy can be monitored in tissue (e.g., skeletal muscle, liver and retina) in vivo in transgenic mice systemically expressing GFP-LC3,[126,446] or in other models by transfection with GFP-LC3 plasmids.[346] In addition, tissue-specific GFP-LC3 mice have been generated for monitoring cardiac myocytes.629,630 In these settings, GFP fluorescent puncta are indicative of autophagic structures. In addition, cleavage of GFP-LC3 to generate free GFP can be evaluated. This has been successfully performed in mouse liver,[172,631] suggesting the GFP-LC3 cleavage assay may also be applied to in vivo studies. Alternatively, confocal laser scanning microscopy, which makes it possible to obtain numerous sections and substantial data about spatial localization features, can be a suitable system for studying autophagic structures (especially for whole mount embryo in vivo analysis).[632] In addition, this method can be used to obtain quantitative data through densitometric analysis of fluorescent signals.[633] Another possibility is immunohistochemical staining, an important procedure that may be applicable to human studies as well considering the role of autophagy in neurodegeneration, myopathies and cardiac disease where samples may be limited to biopsy/autopsy tissue. Immunodetection of LC3 as definite puncta is possible in paraffin-embedded tissue sections and fresh frozen tissue, by either immunohistochemistry or immunofluorescence; [140,634-637] however, this methodology has not received extensive evaluation, and does not lend itself well to dynamic assays. Other autophagic substrates can be evaluated via immunohistochemistry and include p62, NBR1, ubiquitinated inclusions and protein aggregates. Similarly, autophagy can be evaluated by measuring levels of these autophagic substrates via traditional immunoblot; however, their presence or absence needs to be cautiously interpreted as some of these substrates can accumulate with either an increase or a decrease in autophagic flux (see p62 and related LC3 binding protein turnover assays). Bone marrow transfer has been used to document in vivo the role of autophagy in the reverse cholesterol transport pathway from peripheral tissues or cells (e.g., macrophages) to the liver for secretion in bile and for excretion,[638] and a study shows that transglutaminase type 2 protein levels decrease in mouse liver in vivo upon starvation in an autophagy-dependent manner (and in human cell lines in vitro in response to various stimuli; Piacentini M, personal communication), presenting additional possible methods for following autophagy activity. It is also possible to analyze tissues ex vivo, and these studies can be particularly helpful in assessing autophagic flux as they avoid the risks of toxicity and bioavailabilty of compounds such as bafilomycin A1 or other autophagy inhibitors. Along these lines, autophagic flux can be determined by western blot in retinas placed in culture for 4 h with protease inhibitors. This method could be used in tissues that can remain “alive” for several hours in culture such as the retina (Boya P, unpublished observations) and brain slices (Desai S, unpublished observations). Several studies have demonstrated the feasibility of monitoring autophagic flux in vivo in skeletal muscle. Starvation is one of the easiest and most rapid methods for stimulating the autophagic machinery in skeletal muscles. Twelve h of fasting in mice may be sufficient to trigger autophagy in muscle,639,640 but the appropriate time should be determined empirically. Data about the autophagic flux can be obtained by treating mice with, for example, chloroquine,[640] leupeptin[641] or colchicine[153] and then monitoring the change in accumulation of LC3 (see cautionary notes). This type of analysis can also be done with liver, by comparing the LC3-II level in untreated liver (obtained by a partial hepatectomy) to that following subsequent exposure to chloroquine (Skop V, Papackova Z, Cahová M, personal communication). Additional reporter assays to monitor autophagy flux in vivo need to be developed, including tandem fluorescent-LC3 transgenic mice, or viral vectors to express this construct in vivo in localized areas. Some biochemical assays may be used to at least provide indirect correlative data relating to autophagy, in particular when examining the role of autophagy in cell death. For example, cellular viability is related to high cathepsin B activity and low cathepsin D activities.[642] Therefore, the appearance of the opposite levels of activities may be one indication of the initiation of autophagy (lysosome)-dependent cell death. The question of “high” vs. “low” activities can be determined by comparison to the same tissue under control conditions, or to a different tissue in the same organism, depending on the specific question. With regard to living mammals, a minimally invasive method that may be used even in humans is to measure the arterio-venous amino acid exchange rate in the peripheral tissues as a measure of postabsorptive protein catabolism. In humans, the insulin- and amino acid-sensitive postabsorptive (autophagic) net protein catabolism in the peripheral (mostly skeletal muscle) tissue canbe measured by determining the amino acid exchange rate across the lower extremities, as defined by the difference between the plasma amino acid concentrations in the femoral artery and femoral vein multiplied by the blood flow.[643-645] Amino acid exchange studies show that the peripheral tissues take up amino acids during the post-prandial (fed) state and release amino acids in the postabsorptive (fasted) state, i.e., in a state with relatively low plasma insulin and amino acid levels. This post-absorptive release of amino acids is strongly inhibited by infusion of insulin or by exogenous supply of amino acids, suggesting that it is mainly mediated by a

lysosomal/autophagic mechanism of protein catabolism.[643-650] However, the relative contribution of autophagy to the post-absorptive release of amino acids may be changed in disease states (see cautionary notes).

Cautionary notes: The major hurdle with in vivo analyses is the identification of autophagy-specific substrates and the ability to "block" autophagosome degradation with a compound such as bafilomycin A1. Regardless, it is still essential to adapt the same rigors for measuring autophagic flux in vitro to measurements made with in vivo systems. Moreover, as with cell culture, in order to substantiate a change in autophagic flux it is not adequate to rely solely on the analysis of static levels or changes in LC3-II protein levels on western blot using tissue samples. To truly measure in vivo autophagic flux using LC3-II as a biomarker, it is necessary to block lysosomal degradation of the protein. Several studies have successfully done this in select tissues in vivo. Certain general principles need to be kept in mind: a) Any autophagic blocker, whether leupeptin, bafilomycin A1, chloroquine or microtubule depolarizing agents such as colchicine or vinblastine must significantly increase basal LC3-II levels. The turnover of LC3-II or rate of basal autophagic flux is not known for tissues in vivo, and therefore short treatments (e.g., 4 h) may not be as effective as blocking for longer times (e.g., 12 to 24 h); b) the toxicity of the blocking agent needs to be considered (e.g., treating animals with bafilomycin A1 for 2 h can be quite toxic) and food intake must be monitored. If long-term treatment is needed to see a change in LC3-II levels, then confirmation that the animals have not lost weight may be needed. Mice may lose a substantial portion of their body weight when deprived of food for 24 h, and starvation is a potent stimulus for the activation of autophagy.

c) The bioavailability of the agent needs to be considered. For example, many inhibitors such as bafilomycin A1 or chloroquine have relatively poor bioavailability to the central nervous system. To overcome this problem, intracerebroventricular injection can be performed. When analyzing autophagic flux in vivo, one major limitation is the variability between animals. Different animals do not always activate autophagy at the same time. To improve the statistical relevance and avoid unclear results, these experiments should be repeated more than once and each experiment should include several animals. Induction of autophagy in a time-dependent manner by fasting mice for different times requires appropriate caution. Mice are nocturnal animals, so they preferentially move and eat during the night, while they mostly rest during daylight. Therefore, in such experiments it is better to start food deprivation early in the morning, in order to avoid the possibility that the animals have already been fasting for several hours. The use of chloroquine is technically easier, since it only needs one intraperitoneal injection per day, but the main concern is that chloroquine has some toxicity. Chloroquine suppresses the immunological response in a manner that is not due to its pHdependent lysosomotropic accumulation (chloroquine interferes with lipopolysaccharide-induced TNF-a gene expression by a nonlysosmotropic mechanism),[651] as well as through its pHdependent inhibition of antigen presentation.[652] Therefore, chloroquine treatment should be used for short times and at doses that do not induce severe collateral effects, which may invalidate the measurement of the autophagic flux, and care must be exercised in using chloroquine for studies on autophagy that involve immunological aspects. It is also important to have timematched controls for in vivo analyses. That is, having only a zero hour time point control is not sufficient because there may be substantial diurnal changes in basal autophagy.[467] For example, variations in basal flux in the liver associated with circadian rhythm may be several fold, which can equal or exceed the changes due to starvation. Along these lines, in order to allow comparisons of a single time-point it is important to specify what time of day the measurement is taken, and the lighting conditions under which the animals are housed. It is also important that the replicate experiments are conducted at the same time of day. Controlling for circadian effects can greatly reduce the mouse-to mouse variability in autophagy markers and flux (Haspel JA, Choi AMK, personal communication).

The amino acid exchange rate, which has been suggested as a minimal invasive marker for measuring autophagic flux in vivo should be used with special caution. Postprandial suppression of amino acid release by peripheral tissues is strongly mediated by insulin and amino acids and is therefore thought to be mediated by autophagy (see above). However, the ubiquitin-proteasome system likely confounds this investigation, as this pathway can also be inhibited by insulin and amino acids.[653] In addition, multiple disease states have been associated with an altered activity of the ubiquitin-proteasome system, further complicating the use of the amino acid exchange rate as a marker of autophagy.[654] When analyzing basal autophagic level in vivo using GFPLC3 transgenic mice,[126] one pitfall is that GFP-LC3 expression is driven by the cytomegalovirus enhancer and β-actin (CAG) promoter, so that the intensity of the GFP signal may not always represent the actual autophagic activity, but rather the CAG promoter activity in individual cells. For example, GFP-LC3 transgenic mice exhibit prominent fluorescence in podocytes, but rarely in tubular epithelial cells in the kidney,126 but a similar GFP pattern is observed in transgenic mice carrying CAG promoter-driven non-tagged GFP.[655] Furthermore, proximal tubule-specific ATG5-deficient mice656 display a degeneration phenotype earlier than podocyte-specific ATG5-deficient mice,[657] suggesting that autophagy, and hence LC3 levels, might actually be more prominent in the former. One caution in using approaches that monitor ubiquitinated aggregates is that the accumulation of ubiquitin may indicate a block in autophagy, inhibition of proteasomal degradation, or may correspond to structural changes in the substrate proteins www.landesbioscience. that hinder their degradation. In addition, only cytosolic and not nuclear ubiquitin is subject to autophagic degeneration. It is helpful to analyze aggregate degradation in an autophagy-deficient control strain, such as an autophagy mutant mouse, whenever

possible to determine whether an aggregate is being degraded by an autophagic mechanism. This type of control will be impractical for some tissues such as those of the central nervous system because the absence of autophagy leads to rapid degeneration. Accordingly, the use of Atg16l1 hypomorphs or Becn1/becn1 heterozygotes may help circumvent this problem.

Conclusion: Although the techniques for analyzing autophagy in vivo are not as advanced as those for cell culture, it is still possible to follow this process (including flux) by monitoring, for example, GFP-LC3 by fluorescence microscopy, and p62 and NBR1 by immunohistochemistry and/or western blotting.

14. Cell death. In a limited number of cases, autophagy has been established as the cause of cell death[97,199,658-664] although opposite results have been reported using analogous experimental settings.[665] Furthermore, many of the papers claiming a causative role of autophagy in cell death fail to provide adequate evidence.[666] Other papers suffer from ambiguous use of the term "autophagic cell death," which was coined in the 1970s[667] in a purely morphological context to refer to cell death with autophagic features (especially the presence of numerous secondary lysosomes); this was sometimes taken to suggest a role of autophagy in the cell death mechanism, but death-mediation was not part of the definition.[668] Unfortunately, the term "autophagic cell death" is now used in at least three different ways: a) autophagyassociated cell death (the original meaning); b) autophagymediated cell death (which could involve a standard mechanism of cell death such as apoptosis, but triggered by autophagy); c) a distinct mechanism of cell death, independent of apoptosis or necrosis. Clearly claim (b) is stronger than claim (a), and needs to be justified by proof that inhibiting autophagy, through either genetic or chemical means, prevents cell death.[669] Claim (c) is still stronger, because, even if the cell death is blocked by autophagy inhibition, proof needs to be provided that the cell death mechanism is not apoptosis or necrosis.[670] In view of the current confusion it may be preferable to replace the term "autophagic cell death" by other terms such as "autophagy-associated cell death" or "autophagy-mediated cell death," unless the criteria in claim (c) above have been satisfied. Along these lines, it is preferable to use the term "autophagy-dependent cell death" instead of "autophagymediated cell death" when it is proven that autophagy is a prerequisite for the occurrence of cell death, but it is not proven that autophagy mechanistically mediates the switch to cell death. A special caution should also be taken when describing developmental programmed cell death in plants, which is in most cases executed by the growing lytic vacuoles and therefore is referred to as "vacuolar cell death."[671] Although the morphology of vacuolar cell death resembles a combination of macro- and microautophagy, there is no genetic evidence yet that this death requires the core autophagic machinery. Finally, the relationship between autophagy and cell death may significantly differ as a function of the model system being studied. For example, upon induction by starvation of multicellular development in the protest Dictyostelium, autophagy (or at least Atg1) is required to protect against starvation-induced cell death, allowing vacuolar developmental cell death to take place instead.[672,673] Autophagy may be involved not only in allowing this death to occur, but also in the vacuolization process itself.[674]

Cautionary notes: In brief, rigorous criteria must be met in order to establish a death-mediating role of autophagy, as this process typically promotes cell survival. These include a clear demonstration of autophagic flux as described in this article, as well as verification that inhibition of autophagy prevents cell death [claim (b) above; if using a knockdown approach, at least two ATG genes should be targeted], and that other mechanisms of cell death are not responsible [claim (c) above]. As part of this analysis, it is necessary to examine the effect of the specific treatment, conditions or mutation on cell viability using several methods.[675] In the case of postmitotic cells such as neurons or retinal cells, cell death—and cell rescue by autophagy inhibition—can usually be established in vivo by morphological analysis,[676] and in culture by cell counts and/or measurement of the release of an enzyme such as LDH into the medium at early and late time points; however, a substantial amount of neuronal cell death occurs during neurogenesis, making it problematic to carry out a correct analysis in vivo or ex vivo.[677,678] In populations of rapidly dividing cells, the problems may be greater. A commonly used method is the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, or a related assay using a similar, or a water-soluble, tetrazolium salt. The main concern with the MTT assay is that it measures mitochondrial activity, but does not allow a precise determination of cellular viability or cell death, whereas methods that show cell death directly (e.g., trypan blue exclusion) fail to establish the viability of the remaining cell population.679 Accordingly, a preferred alternative is to accurately quantify cell death by appropriate cytofluorometric or microscopy assays.[675] Moreover, long-term clonogenic assays should be employed when possible to measure the effective functional survival of cells.

Conclusion: In most systems, ascribing death to autophagy based solely on morphological criteria is insufficient; autophagic cell death can only be demonstrated as death that is suppressed by the inhibition of autophagy, through either genetic or chemical means.[669] In addition, more than one assay should be used to measure cell death.

15. Chaperone-mediated autophagy. The primary characteristic that makes chaperone-mediated autophagy (CMA) different from the other autophagic variants described in these guidelines is that it does not require formation of intermediate vesicular compartments (autophagosomes or microvesicles) for the import of cargo into lysosomes.[680,681] Instead, the CMA substrates are translocated across the lysosomal membrane through the action of HSPA8 (HSC70) located in the cytosol and lysosome lumen, and the lysosome membrane protein LAMP2A. To date, CMA has only

been identified in mammalian cells, and accordingly this section refers only to studies in mammals. The following methods are commonly utilized to determine if a protein is a CMA substrate (see ref.682 for experimental details): (a) Analysis of the amino acid sequence of the protein toidentify the presence of a KFERQ-related motif that is an absolute requirement for all CMA substrates.[683] (b) Colocalization studies with lysosomal markers (typically LAMP2A and/or LysoTracker) to identify a fraction of the protein associated with lysosomes. The increase in association of the putative substrate under conditions that upregulate CMA (such as prolonged starvation) or upon blockage of lysosomal proteases (to prevent the degradation of the protein) helps support the hypothesis that the protein of interest is a CMA substrate. However, association with lysosomes is necessary but not sufficient to consider a protein an authentic CMA substrate, because proteins delivered by other pathways to lysosomes will also behave in a similar manner. A higher degree of confidence can be attained if the association is preferentially with the subset of lysosomes active for CMA (i.e., those containing HSPA8 in their lumen), which can be separated from other lysosomes following published procedures.[628] (c) Co-immunoprecipitation of the protein of interest with cytosolic HSPA8. Due to the large number of proteins that interact with this chaperone, it is usually better to perform affinity isolation with the protein of interest and then analyze the isolated proteins for the presence of HSPA8 rather than vice versa. (d) Co-immunoprecipitation of the protein of interest with LAMP2A.[684] Due to the fact that the only antibodies specific for the LAMP2A variant (the only one of the three LAMP2 variants involved in CMA[105,685]) are generated against the cytosolic tail of LAMP2A, where the substrate also binds, it is necessary to affinity isolate the protein of interest and then analyze for the presence of LAMP2A. Immunoblot for LAMP2A in the precipitate can only be done with the antibodies specific for LAMP2A and not just those that recognize the lumenal portion of the protein that is identical in the other LAMP2 variants. If the protein of interest is abundant inside cells, co-immunoprecipitations with LAMP2A can be done in total cellular lysates, but for low abundance cellular proteins, preparation of a membrane fraction (enriched in lysosomes) by differential centrifugation may facilitate the detection of the population of the protein bound to LAMP2A. (e) Selective upregulation and blockage of CMA to demonstrate that degradation of the protein of interest changes with these manipulations. Selective chemical inhibitors for CMA are not currently available. Note that general inhibitors of lysosomal proteases (e.g., bafilomycin A1, NH4Cl, leupeptin) also block the degradation of proteins delivered to lysosomes by other autophagic and endosomal pathways. The most selective way to block CMA is by knockdown of LAMP2A, which causes this protein to become a limiting factor.105 The other components involved in CMA, including HSPA8, HSP90, GFAP, and eF1a are all multifunctional cellular proteins, making it difficult to interpret the effects of knockdowns. Overexpression of LAMP2A[684] is also a better approach to upregulate CMA than the use of chemical modulators. The two compounds demonstrated to affect degradation of long-lived proteins in lysosomes,[686] 6-aminonicotinamide and geldanamycin, lack selectivity, as they affect many other cellular processes. In addition, in the case of geldanamycin, the effect on CMA can be the opposite (inhibition rather than stimulation) depending on the cell type (this is due to the fact that the observed stimulation of CMA is actually a compensatory response to the blockage of HSP90 in lysosomes, and different cells activate different compensatory responses).[687] (f) The most conclusive way to prove that a protein is a CMA substrate is by reconstituting its direct translocation into lysosomes using a cellfree system.[682] This method is only possible when the protein of interest can be purified, and it requires the isolation of the population of lysosomes active for CMA. Internalization of the protein of interest inside lysosomes upon incubation with the isolated organelle can be monitored using protease protection assays (in which addition of an exogenous protease removes the protein bound to the cytosolic side of lysosomes, whereas it is inaccessible to the protein that has reached the lysosomal lumen; note that pre-incubation of lysosomes with lysosomal protease inhibitors before adding the substrate is required to prevent the degradation of the translocated substrate inside lysosomes).[688] The use of exogenous protease requires numerous controls (see ref.682) to guarantee that the amount of protease is sufficient to remove all the substrate outside lysosomes, but will not penetrate inside the lysosomal lumen upon breaking the lysosomal membrane. The difficulties in the adjustment of the amount of protease, has led to the development of a second method that is more suitable for laboratories that have no previous experience with these procedures. In this case, the substrate is incubated with lysosomes untreated or previously incubated with inhibitors of lysosomal proteases, and uptake is determined as the difference of protein associated with lysosomes not incubated with inhibitors (in which the only remaining protein will be the one associated with the cytosolic side of the lysosomal membrane) and those incubated with the protease inhibitors (which contain both the protein bound to the membrane and that translocated into the lumen).[689] Confidence that the lysosomal internalization is by CMA increases if the uptake of the substrate can be competed with proteins previously identified as substrates for CMA (e.g., glyceraldehyde-3-phosphate dehydrogenase or ribonuclease A, both commercially available as purified proteins), but is not affected by the presence of similar amounts of nonsubstrate proteins (such as ovalbumin or cyclophilin A). Blockage of uptake by pre-incubation of the lysosomes with antibodies against the cytosolic tail of LAMP2A also reinforces the hypothesis that the protein is a CMA substrate. In other instances, rather than determining if a particular protein is a CMA substrate, the interest may be to analyze possible changes in CMA activity under different conditions or in response to different modifications. We enumerate here the methods, from lower to higher complexity that can be utilized to measure CMA in cultured cells and in tissues (see ref.682 for

detailed experimental procedures). (a) Measurement of changes in the intracellular rates of degradation of long-lived proteins, when combined with inhibitors of other autophagic pathways, can provide a first demonstration in support of changes that are due to CMA. For example, CMA is defined as lysosomal degradation upregulated in response to serum removal but insensitive to PtdIns3K inhibitors. (b) Measurement of levels of CMA components is insufficient to conclude changes in CMA because this does not provide functional information, and changes in CMA components can also occur under other conditions. However, analysis of the levels of LAMP2A can be used to support changes in CMA detected by other procedures. Cytosolic levels of HSPA8 remain constant and are not limiting for CMA, thus providing no information about this pathway. Likewise, changes in total cellular levels of LAMP2A do not have an impact on this pathway unless they also affect their lysosomal levels (i.e., conditions in which LAMP2A is massively overexpressed lead to its targeting to the plasma membrane where it cannot function in CMA). It is advisable that changes in the levels of these two CMA components are confirmed to occur in lysosomes, either by colocalization with lysosomal markers when using image-based procedures or by performing immunoblot of a lysosomal enriched raction (purification of this fraction does not require the large amounts of cells/tissue necessary for the isolation of the subset of lysosomes active for CMA). (c) Tracking changes in the subset of lysosomes active for CMA. This group of lysosomes is defined as those containing HSPA8 in their lumen (note that LAMP2A is present in both lysosomes that are active and inactive for CMA, and it is the presence of HSPA8 that confers CMA capability). Immunogold or immunofluorescence against these two proteins (LAMP2A and HSPA8) makes it possible to quantify changes in the levels of these lysosomes present at a given time, which correlates well with CMA activity.628 (d) Analysis of lysosomal association of fluorescent artificial CMA substrates. Two different fluorescent probes have been generated to track changes in CMA activity in cultured cells using immunofluorescence or FACS analysis.[628] These probes contain the KFERQ and context sequences in frame with photoswitchable or photoactivated fluorescent proteins. Activation of CMA results in the mobilization of a fraction of the cytosolic probe to lysosomes and the subsequent change from a diffuse to a punctate pattern. CMA activity can be quantified as the number of fluorescent puncta per cell or as the decay in fluorescence activity over time because of degradation of the artificial substrate. Because the assay does not allow measuring accumulation of the substrate (which must unfold for translocation), it is advisable to perform a timecourse analysis to determine gradual changes in CMA activity. Antibodies against the fluorescent protein in combination with inhibitors of lysosomal proteases can be used to monitor accumulation of the probe in lysosomes over a period of time, but both the photoswitchable and the unmodified probe will be detected by this procedure.690 As for any other fluorescence probe based on analysis of intracellular “puncta” it is essential to include controls to confirm that the puncta are indeed lysosomes (colocalization with LysoTracker or LAMPs and lack of colocalization with markers of cytosolic aggregation such as ubiquitin), and do not reach the lysosomes through other autophagic pathways (insensitivity to PtdIns3K inhibitors and sensitivity to LAMP2A knockdown are good controls in this respect). (e) Direct measurement of CMA using in vitro cell free assays. Although the introduction of the fluorescent probes should facilitate measurement of CMA in many instances, they are not applicable for tissue samples. In addition, because the probes measure binding of substrate to lysosomal membranes it is important to confirm that enhanced binding does not result from defective translocation. Lastly, the in vitro uptake assays are also the most efficient way to determine primary changes in CMA independently of changes in other proteolytic systems in the cells. These in vitro assays are the same ones described in the previous section on the identification of proteins as substrates of CMA, but are performed in this case with purified proteins previously characterized to be substrates for CMA. In this case the substrate protein is always the same and what changes is the source of lysosomes (from the different tissues or cells that are to be compared). As described in the previous section, binding and uptake can be analyzed separately using lysosomes previously treated or not with protease inhibitors. The analysis of the purity of the lysosomal fractions prior to performing functional analysis is essential to conclude that changes in the efficiency to take up the substrates results from changes in CMA rather than from different levels of lysosomes in the isolated fractions. Control of the integrity of the lysosomal membrane and sufficiency of the proteases are also essential to discard the possibility that degradation is occurring outside lysosomes because of leakage, or that accumulation of substrates inside lysosomes is due to enhanced uptake rather than to decreased degradation.

Cautionary notes: The discovery of a new selective form of protein degradation in mammals named endosomal-microautophagy (e-MI)691 has made it necessary to reconsider some of the criteria that applied in the past for the definition of a protein as a CMA substrate. The KFERQ-like motif, previously considered to be exclusive for CMA, is also used to mediate selective targeting of cytosolic proteins to the surface of late endosomes. Once there, substrates can be internalized in microvesicules that form from the surface of these organelles in an ESCRT-dependent manner. HSPA8 has been identified as the chaperone that binds this subset of substrates and directly interacts with lipids in the late endosomal membrane, acting thus as a receptor for cytosolic substrates in this compartment. At a practical level, to determine if a KFERQ-containing protein is being degraded by CMA or e-MI the following criteria can be applied: (a) Inhibition of lysosomal proteolysis (for example with NH4Cl and leupeptin) blocks degradation by both pathways. (b) Knockdown of LAMP2A inhibits CMA but not e-MI. (c) Knockdown of components of ESCRTI and II (e.g., VPS4 and TG101) inhibits e-MI but not CMA. (d) Interfering with the capability to unfold the substrate protein blocks its

degradation by CMA, but does not affect e-MI of the protein. In this respect soluble proteins, oligomers and protein aggregates can undergo e-MI, but only soluble proteins can be CMA substrates. (e) In vitro uptake of e-MI substrates can be reconstituted using isolated late endosomes whereas in vitro uptake of CMA substrates can only be reconstituted using lysosomes. Another pathway that needs to be considered relative to CMA is chaperone-assisted selective autophagy (CASA).[692] CASA is dependent on HSPA8 and LAMP2 (although it is not yet known if it is dependent solely on the LAMP2A isoform). Thus, a requirement for these two proteins is not sufficient to conclude that a protein is degraded by CMA.

Conclusion: One of the key issues with the analysis of CMA is verifying that the protein of interest is an authentic susbstrate. Methods for monitoring CMA that utilize fluorescent probes areavailable that eliminate the need for the isolation of CMAcompetent lysosomes, one of the most difficult aspects of assaying this process.

B. Comments on Additional Methods

1. Acidotropic dyes. Among the older methods for following autophagy is staining with acidotropic dyes such as monodansylcadaverine (MDC),[693] acridine orange,694 Neutral Red,[632] LysoSensor Blue[695] and LysoTracker Red.[198,696] Cautionary notes: Although MDC was first described as a specific marker of autophagic vacuoles[697] subsequent studies have suggested that this, and other acidotropic dyes, are not specific markers for early autophagosomes,[258] but rather label later stages in the degradation process. For example, autophagosomes are not acidic, and MDC staining can be seen in autophagy-defective mutants[401] and in the absence of autophagy activation.[698] MDC may also show confounding levels of background labeling unless narrow bandpass filters are used. However, in the presence of vinblastine, which blocks fusion with lysosomes, MDC labeling increases, suggesting that under these conditions MDC can label latestage autophagosomes.[699] Along these lines, cells that overexpress a dominant negative version of RAB7 (the T22N mutant) show colocalization of this protein with MDC; in this case fusion with lysosomes is also blocked700 indicating that MDC does not just label lysosomes. Finally, MDC labeling could be considered to be an indicator of autophagy when the increased labeling of cellular compartments by this dye is prevented by treatment with autophagy inhibitors such as wortmannin or 3-MA. Overall, staining with MDC or its derivative monodansylamylamine (MDH)693 is not, by itself, a sufficient method for monitoring autophagy. Similarly, LysoTracker Red, Neutral Red and acridine orange are not ideal markers for autophagy because they primarily detect lysosomes. These markers are, however, useful for monitoring selective autophagy when used in conjunction with protein markers or other dyes. For example, increased colocalization of mitochondria with both GFP-LC3 and LysoTracker can be used as evidence of autophagic cargo delivery to lysosomes. Moreover, LysoTracker Red has been used to provide correlative data on autophagy in Drosophila melanogaster fat body cells (Fig. 23).197,198 However, additional assays, such as GFP-Atg8/LC3 fluorescence or EM, should be used to substantiate results obtained with acidotropic dyes whenever possible. One important caution when co-imaging with LysoTracker Red and a green-fluorescing marker (e.g., GFP-LC3 or MitoTracker Green) is that it is necessary to control for rapid red-to-green photoconversion of the LysoTracker, which can otherwise result in an incorrect interpretation of colocalization.701 Some of the confusion regarding the interpretation of results with these dyes stems in part from the nomenclature in this field. Indeed, the discussion of acidotropic dyes points out why it is advisable to differentiate between the terms “autophagosome” and “autophagic vacuole,” although they are occasionally, and incorrectly, used interchangeably. The autophagosome is the sequestering compartment generated by the phagophore. The fusion of an autophagosome with an endosome or a lysosome generates an amphisome or an autolysosome, respectively. The early autophagosome is not an acidic compartment, whereas amphisomes and autolysosomes are acidic. Earlier names for these compartments are “initial autophagic vacuole (AVi),” “intermediate autophagic vacuole (AVi/d)” and “degradative autophagic vacuole (AVd),” respectively. Thus, acidotropic dyes can stain late autophagic vacuoles (in particular autolysosomes), but not the initial autophagic vacuole, the early autophagosome. With the above caveats in mind, the combined use of early and late markers of autophagy is highly encouraged, and when quantifying mammalian lysosomes, it is important to keep in mind that increases in both lysosome size and number are frequently observed. Finally, in order to avoid confusion with the plant and fungal vacuole, the equivalent organelle to the lysosome, we recommend the use of the term “autophagosome” instead of “autophagic vacuole,” and the use of “autophagic compartment” when the specific nature of the structure is not known. Conclusion: Given the development of better techniques that are indicators of autophagy, the use of acidotropic dyes to study this process is discouraged, and relying entirely on such dyes is not acceptable.

2. Autophagy inhibitors and inducers. In many situations it is important to demonstrate an effect resulting from inhibition or stimulation of autophagy (see ref.702 for a partial listing of regulatory compounds), and a few words of caution are worthwhile in this regard. Most chemical inhibitors of autophagy are not entirely specific, and it is important to consider possible doseand time-dependent effects. Accordingly, it is generally preferable to analyze specific loss-of-function Atg mutants. However, it must

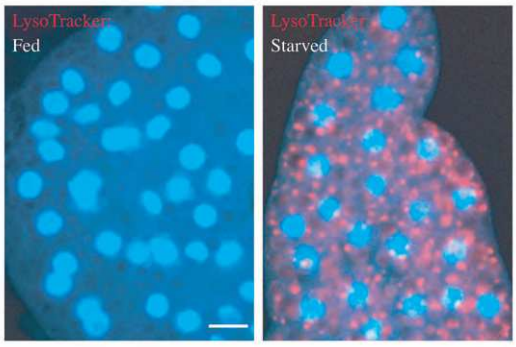

Figure 23. LysoTracker Red stains lysosomes and can be used to monitor autophagy in Drosophila. Live fat body tissues from Drosophila were stained with LysoTracker Red (red) and Hoechst 33342 (blue) to stain the nucleus. Tissues were isolated from fed (left) or 3 h starved (right) animals. Bar, 25 mm. This figure was modified from data presented in reference ,198 Developmental Cell, 7, Scott RC, Schuldiner O, Neufeld TP, Role and regulation of starvationinduced autophagy in the Drosophila fat body, pp. 167–78, copyright 2004, with permission from Elsevier

be kept in mind that some apparently specific Atg gene products may have autophagy-independent roles (e.g., ATG5 in cell death, and the VPS34-containing complexes—including BECN1—in apoptosis, endosomal function and protein trafficking). [403,432,703-706] Therefore, the experimental conditions of inhibitor application and their side effects must be carefully considered. In addition, it must be emphasized once again that autophagy, as a multistep process, can be inhibited at different stages. Sequestration inhibitors, including 3-MA, LY294002 and wortmannin, inhibit class I as well as class III PtdIns3Ks.[134,257,707] The class I enzymes generate products [PtdIns(3,4,5)P3] that inhibit autophagic sequestration, whereas the class III product (PtdIns3P) generally stimulates autophagic sequestration. The overall effect of these inhibitors is typically to block autophagy because the class III enzymes that are required to activate autophagy act downstream of the negative regulatory class I enzymes, although cell death may ensue in cell types that are dependent upon high levels of AKT for survival. The effect of 3-MA (but not that of wortmannin) is further complicated by the fact that it has different temporal patterns of inhibition, causing a long-term suppression of the class I PtdIns3K, but only a transient inhibition of the class III enzyme. In cells incubated in a complete medium for extended periods of time, 3-MA may, therefore (particularly at suboptimal concentrations), promote autophagy by inhibition of the class I enzyme.[257] Thus, wortmannin may be considered as an alternative to 3-MA for autophagy inhibition.[257] However, wortmannin can induce the formation of vacuoles that may have the appearance of autophagosomes, although they are swollen late endocytic compartments.[611] Furthermore, studies have demonstrated that inhibition of autophagy with 3-MA or wortmannin can have effects on cytokine transcription, processing and secretion, particularly IL-1 family members,[708-710] but 3-MA also inhibits the secretion of some cytokines (e.g., TNF, IL-6) in an autophagy-independent manner (Harris J, unpublished observations). Thus, in studies where the effect of autophagy inhibition on specific cellular processes is being investigated, is is important to confirm results using other methods, such as RNA silencing. Due to these issues, it is of great interest that inhibitors with specificity for the class III PtdIns3Ks, and their consequent effects on autophagy, have been described.165 Cycloheximide, a commonly used protein synthesis inhibitor in mammals, is also an inhibitor of sequestration in vivo,[14-16,92,604,711-715] and in various cell types in vitro,[344,716] and it has been utilized to investigate the dynamic nature of the regression of various autophagic elements.[14-16,24,92,712,713] The mechanism of action of cycloheximide in short-term experiments is not clear, but it has no direct relation to the inhibition of protein synthesis.[344] This latter activity, however, may complicate certain types of analysis when using this drug. A significant challenge for a more detailed analysis of the dynamic role of autophagy in physiological and pathophysiological processes, for instance with regard to cancer and cancer therapy, is to find more specific inhibitors of autophagy signaling which do not affect other signaling cascades. For example, in the context of cellular radiation responses it is well known that PtdIns3Ks (e.g., ATM, DNA-PKcs), in addition to signaling through the PtdIns3K-AKT pathway, have a major role in the regulation of DNA-damage repair.[717] However, 3-MA, which is a nonspecific inhibitor of class 3 PtdIns3Ks, can alter the function of other classes of this enzyme, which are involved in the DNAdamage repair response. This is of particular importance for investigations into the role of radiation-induced autophagy in cellular radiation sensitivity or resistance.[718,719] Most other inhibitory drugs act at post-sequestration steps. These types of agents have been used in many experiments to both inhibit endogenous protein degradation and to increase the number of autophagic compartments. They cause the accumulation of sequestered material in either autophagosomes or autolysosomes, or both, because they allow autophagic sequestration to proceed. The main categories of these types of inhibitors include the vinca alkaloids (e.g., vinblastine) and other microtubule poisons that inhibit fusion, inhibitors of lysosomal enzymes (e.g., leupeptin, pepstatin A and E-64d), and compounds that elevate lysosomal pH (e.g., inhibitors of vacuolar-type ATPases such as bafilomycin A1 and concanamycin A (another V-ATPase inhibitor), and weak base amines including methyl- or propylamine, chloroquine, and Neutral Red, some of which slow down fusion). Ammonia is a very useful agent for the elevation of lysosomal pH in short-term experiments, but has been reported to cause a stimulation of autophagy

during long-term incubation of cells in a full medium,[720] under which conditions a good alternative might be methylamine or propylamine.[721] Along these lines, it should be noted that the half-life of glutamine in cell culture media is approximately two weeks due to chemical decomposition, which results in media with lowered glutamine and elevated ammonia concentrations that can affect the autophagic flux (either inhibiting or stimulating autophagy, depending on the concentration722). Thus, the use of freshly prepared cell culture media with glutamine is advised, to help reduce experimental variation. A special note of caution is also warranted in regard to chloroquine. Although this chemical is commonly used as an autophagy inhibitor, chloroquine may initially stimulate autophagy (Dorsey FC, personal communication; Franco R, personal communication).

Some data suggest that some nanomaterials may also be novel inhibitors of autophagy, by as yet unidentified mechanisms.[723] It is worth noting that lysosomal proteases fall into three general groups, cysteine, aspartic acid and serine proteases. Therefore, the fact that leupeptin, a serine and cysteine protease inhibitor, has little or no effect does not necessarily indicate that lysosomal degradation is not taking place; a combination of leupeptin, pepstatin A and E-64d may be a more effective treatment. However, it should also be pointed out that these protease inhibitors can exert inhibitory effects not only on lysosomal proteases, but also on cytosolic proteases, that is, degradation of proteins might be blocked through inhibition of cytosolic instead of lysosomal proteases. Conversely, it should be noted that MG132 (Z-leu-leu-leu-al) and its related peptide aldehydes are commonly used as proteasomal inhibitors, but they can also inhibit certain lysosomal hydrolases such as cathepsins and calpains.[724] Thus, any positive results using MG132 do not ruleout the possibility of involvement of the autophagy-lysosome system. Therefore, even if MG132 is effective, it is important to confirm the result using more specific proteasomal inhibitors such as lactacystin or epoxomicin. Finally, there are significant differences in cell permeability among protease inhibitors. For example, E-64d is membrane permeable, whereas leupeptin and pepstatin A are not (although there are derivatives that display greater permeability such as pepstatin A methyl ester).[725] Thus, when analyzing whether a protein is an autophagy substrate, caution should be taken in utilizing these protease inhibitors to block autophagy. As with the PtdIns3K inhibitors, many autophagy-suppressive compounds are not specific. For example, okadaic acid[726] is a powerful general inhibitor of both type 1 (PP1) and type 2A (PP2A) protein phosphatases.[727] Bafilomycin A1 and other compounds that raise the lysosomal pH may have indirect effects on any acidified compartments. Moreover, treatment with bafilomycin A1 for extended periods (18 h) can cause significant disruption of the mitochondrial network in cultured cells (Gegg ME, personal communication), and either bafilomycin A1 or concanamycin A cause swelling of the Golgi in plants,[728] and increase cell death by apoptosis in cancer cells (Rao VA, personal communication). Bafilomycin A1 is often used at a final concentration of 100 nM, but much lower concentrations such as 1 nM may be sufficient to inhibit autophagic-lysosomal degradation and are less likely to cause indirect effects;[154,729,730] however, appropriate inhibitory concentrations should be empirically determined for each cell type.6 One final caution with regard to bafilomycin A1 is that activation of lysosomally localized TORC1 depends on an active V-ATPase.[364] This means that treatment with bafilomycin A1 could elevate the autophagic flux through MTOR inhibition, while simultaneously interfering with flux by inhibiting fusion. Accordingly, other compounds may be preferred for flux measurements. Thus, although these various agents can inhibit different steps of the autophagic pathway, their potential side effects must be considered in interpretation of the secondary consequences of autophagy inhibition, especially in long-term studies. For example, lysosomotropic compounds can increase the rate of autophagosome formation by inhibiting TORC1.[731] Along these lines, chloroquine treatment may cause an apparent increase in the formation of autophagosomes possibly by blocking fusion with the lysosome (F.C. Dorsey and J.L. Cleveland, personal communication). This conclusion is supported by the finding that chloroquine reduces the colocalization of LC3 and LysoTracker despite the presence of autophagosomes and lysosomes (Simon AK, personal communication). Concanamycin A blocks sorting of vacuolar proteins in plant cells in addition to inhibiting vacuolar acidification.[732] Furthermore, in addition to causing the accumulation of autophagic compartments, many of these drugs seem to be stimulators of sequestration in many cell types, especially in vivo.[93,235,604,712,716,733-737] Although it is clear why these drugs cause the accumulation of autophagic compartments, it is not known why they stimulate sequestration. One possibility, at least for hepatocytes, is that the inhibition of protein degradation reduces the intracellular amino acid pool, which in turn upregulates sequestration. A time-course study of the changes in both the intra- and extracellular fractions may provide accurate information regarding amino acid metabolism. For these various reasons, it is important to include appropriate controls; along these lines, MTOR inhibitors such as rapamycin or amino acid deprivation can be utilized as positive controls for inducing autophagy. In many cell types, however, the induction of autophagy by rapamycin is relatively slow, or transient, allowing more time for indirect effects; thus, rapamycin may fail to activate autophagy in cultured primary neurons, despite its potent stimulation of autophagy in some cancer cell lines,[238,738,739] and, similarly, it does not induce autophagy in human neuroblastoma SH-SY5Y cells, which can differentiate into neuron-like cells (Diaz-Nido J, personal communication). Along these lines, glucose depletion may be much more efficient at inducing autophagy than rapamycin or amino acid starvation in neurons in culture (Germain M, Slack R, personal communication); although a number of compounds can also be quite efficient autophagy inducers in neurons including the calpain inhibitor calpeptin. [238,740,741] Several small molecule inhibitors,

including torin1, PP242, KU-0063794, PtdIns-103 and NVP-BEZ235, have been developed that target the catalytic domain of MTOR in an ATPcompetitive manner.[154,252,742-745] In comparison to rapamycin, these catalytic MTOR inhibitors are more potent, and hence are stronger autophagy agonists in most cell lines.[242,252,746] The use of these second-generation MTOR inhibitors may reveal that some reports of mTOR-independent autophagy may actually reflect the use of the relatively weak inhibitor rapamycin. Furthermore, the use of these compounds has revealed a role for TORC1 and TORC2 as independent regulators of autophagy.[747] Finally, a specialized class of compounds with a,β-unsaturated ketone structure tends to induce autophagic cell death, accompanied by changes in mitochondrial morphology; since the cytotoxic action of these compounds is efficiently blocked by N-acetyl-L-cysteine, the β-position in the structure may interact with an SH group of the targeted molecules.[748] Due to the potential pleiotropic effects of various drug treatments, it is incumbent upon the researcher to demonstrate that autophagy is indeed inhibited, by using the methodologies described herein. Accordingly, it is critical to verify the effect of a particular biochemical treatment with regard to its effects on autophagy induction or inhibition when using a cell line that was previously uncharacterized for the chemical being used. Similarly, cytotoxicity of the relevant chemical should be assessed. The use of gene deletions/inactivations (e.g., in primary or immortalized atg2/2 MEFs,[401] plant T-DNA or transposon insertion mutants,[200,749] or in vivo using transgenic knockout models750,751 including Cre-lox based “conditional” knockouts230,231) or functional knockdowns (e.g., with RNAi against ATG genes) is the preferred approach when possible because these methods allow a more direct assessment of the resulting phenotype; however, different floxed genes are deleted with varying efficiency, and the proportion deleted must be carefully quantified (Hwang S, Virgin IV HW, personal communication).

Studies also suggest that microRNAs may be used for blocking gene expression.[164,468,469,752] In certain contexts, it is advisablewhen using a knockout or knockdown approach to examine multiple autophagy-related genes to exclude the possibility that the phenotype observed is due to effects on a non-autophagic function(s) of the corresponding protein, especially when examining the possibility of autophagic cell death (in contrast, if examining whether perturbation induces clearance of a substrate via autophagy, a single Atg gene knockout is probably sufficient). This is particularly the case in evaluating BECN1, which interacts with anti-apoptotic BCL2 family proteins,[425] or when low levels of a target protein are sufficient for maintaining autophagy as is the case with ATG5.[171] With regard to ATG5, a better approach may be to use a dominant negative (K130R) version.[706,753,754] Along these lines, and as stated above for the use of inhibitors, when employing a knockout or especially a knockdown approach, it is again incumbent upon the researcher to demonstrate that autophagy is actually inhibited, by using the methodologies described herein. Finally, we note that the long-term secondary consequences of gene knockouts or knockdowns are likely much more complex than the immediate effects of the actual autophagy inhibition. To overcome this concern, inducible knockout systems might be useful.[171,315] One additional caveat to knockdown experiments is that pathogen-associated molecular pattern (PAMP) recognition pathways can be triggered by doublestranded RNAs (dsRNA), like siRNA probes, or the viral vector systems that deliver shRNA.[755] Some of these, like TLR-mediated RNA recognition,[756] can influence autophagy by either masking any inhibitory effect or compromising autophagy independent of the knockdown probe. Therefore, nontargeting (scrambled) siRNA or shRNA controls should be used with the respective transfection or transduction methods in the experiments that employ ATG knockdown. Another strategy to specifically interfere with autophagy is to use dominant negative inhibitors. Delivery of these agents by transient transfection, adenovirus, or TAT-mediated protein transduction offers the possibility of their use in cell culture or in vivo.[753] However, since autophagy is an essential metabolic process for many cell types and tissues, loss of viability due to autophagy inhibition always has to be a concern when analyzing cell death-unrelated questions. In this respect it is noteworthy that some cell-types of the immune system such as dendritic cells260 seem to tolerate loss of autophagy fairly well, whereas others such as T and B cells are compromised in their development and function after autophagy inhibition.[757,758] In addition to pharmacological inhibition, RNA silencing, gene knockout and dominant negative RAB and ATG protein expression, pathogen-derived autophagy inhibitors can also be considered to manipulate autophagy. Along these lines ICP34.5, viral BCL2 homologs and viral FLIP of herpesviruses block autophagosome formation,[425,580,759] whereas M2 of influenza virus and HIV Nef block autophagosome degradation.[278,760] However, as with other tools discussed in this section, transfection or transduction of viral autophagy inhibitors should be used in parallel with other means of autophagy manipulation, because these proteins are used for the regulation of usually more than one cellular pathway by the respective pathogens. There are fewer compounds that act as inducers of autophagy, but the initial characterization of this process was due in large part to the inducing effects of glucagon, which appears to act through indirect inhibition of MTOR via the activation of LKB1-AMPK.[615,616,761] Currently, the most commonly used inducer of autophagy is rapamycin, an allosteric inhibitor of TORC1, although one caution is that MTOR is a major regulatory protein that is part of the insulin signaling pathway, and it controls processes other than autophagy, so that rapamycin will ultimately affect many metabolic pathways.[379,762-764] In particular, the strong effects of MTOR on protein synthesis may be a confounding factor when analyzing the effects of rapamycin. MTORindependent regulation can be achieved through lithium, sodium valproate and carbamazepine, compounds that lower the myoinositol-1,4,5-triphosphate levels.[765] In vivo treatment of embryos with cadmium results in an

increase in autophagy, probably to counter the stress, allowing cell survival through the elimination/recycling of damaged structures.[632] Autophagy may also be regulated by the release of calcium from the endoplasmic reticulum under stress conditions;[212,726,766] however, additional calcium signals from other stores such as the mitochondria and lysosomes could also play an important role in autophagy induction. The activation of the lysosomal two pore channel (TPC), by nicotinic acid adenine dinucleotide phosphate (NAADP) induces autophagy, which can selectively be inhibited by the TPC blocker NED-19, or by pre-incubation with BAPTA, showing that lysosomal calcium also modulates autophagy.[244] Another way to induce autophagy, both in cultured cells and in vivo, is through transcriptional control. For example, this can be achieved either through overexpression or post-translational activation of the gene encoding TFEB (see Transcriptional and translational regulation), a transcriptional regulator of the biogenesis of both lysosomes and autophagosomes.[463,464] Similarly, adenoviral-mediated expression of the transcription factor C/EBPβ induces autophagy in hepatocytes.467 Relatively little is known about direct regulation via the ATG proteins, but there is some indication that tamoxifen acts to induce autophagy by increasing the expression of BECN1 in MCF7 cells.[767] However, in U87MG cells treated with tamoxifen BECN1 does not appear to be upregulated, whereas the levels of LC3-II and p62 are increased, while LAMP2B is downregulated and cathepsin D and L activities are almost completely blocked (Choi KS, personal communication). Thus, the effect of tamoxifen may differ depending on the cell type. Other data suggest that tamoxifen acts by blocking cholesterol biosynthesis, and that the sterol balance may determine whether autophagy acts in a protective vs. cytotoxic manner.[768,769] Finally, screens have identified small molecules that induce autophagy independently of rapamycin, and allow the removal of misfolded or aggregateprone proteins,[770,771] suggesting that they may prove useful in therapeutic applications. However, caution should be taken because of the crosstalk between autophagy and the proteasomal system. For example, trehalose, an mTOR-independent autophagy inducer,253 can compromise proteasomal activity in cultured primary neurons.[739]

Conclusion: Rapamycin is much less effective at inhibiting MTOR and inducing autophagy than catalytic inhibitors, and the latter should therefore be considered for use instead of rapamycin; however, it must be kept in mind that catalytic inhibitors also affect mTORC2. The main concern with pharmacological manipulations is pleiotropic effects of the compound being used. Accordingly, genetic confirmation is preferred whenever possible.

3. Basal autophagy. Basal levels of LC3-II or GFP-LC3 puncta may change according to the time after addition of fresh medium to cells, and this can lead to misinterpretations of what basal autophagy means. This is particularly important when comparing the levels of basal autophagy between different cell populations (such as knockout vs. wild-type clones). If cells are very sensitive to nutrient supply and display a high variability of basal autophagy, the best experimental condition is to monitor the levels of basal autophagy at different times after the addition of fresh medium. One example is the chicken lymphoma DT40 cells (see Chicken B-lymphoid DT40 cells below) and their knockout variant for all three inositol 1,4,5-trisphosphate receptor isoforms. [241,772,773] In these cells, no differences in basal levels of LC3-II can be observed up to 4 h after addition of fresh medium, but differences can be observed after longer times (Vicencio JM, Szabadkai G, personal communication). This concept should also be applied to experiments in which the effect of a drug upon autophagy is the subject of study. If the drugs are added after a time in which basal autophagy is already high, then the effects of the drug can be masked by the cell's basal autophagy, and wrong conclusions may be drawn. To avoid this, fresh medium should be added first in order to reduce and equilibrate basal autophagy in cells under all conditions, and then the drugs can be added. The basal autophagy levels of the cell under study must be identified beforehand in order to know the time needed to reduce basal autophagy. A similar caution must be exercised with regard to cell culture density and hypoxia. When cells are grown in normoxic conditions at high cell density, HIF-1a is stabilized at levels similar to that obtained with low-density cultures under hypoxic conditions.[774] This results in the induction of BNIP3 and BNIP3L/NIX and "hypoxia"-induced autophagy, even though the conditions are theoretically normoxic.[775] Therefore, researchers need to be careful about cell density in order to avoid accidental induction of autophagy. It should be realized that also in yeast species, medium changes can trigger a higher "basal" level of autophagy in the cells. In the methylotrophic yeast species Pichia pastoris and Hansenula polymorpha a shift of cells grown in batch from glucose to methanol results in stimulation of autophagy.[776,777] A shift to a new medium can be considered a stress situation. Thus, it appears to be essential to cultivate the yeast cells for a number of hours to stabilize the level of basal autophagy, before performing experiments intended to study levels of (selective) autophagy (e.g., pexophagy). Finally, plant root tips cultured in nutrient-sufficient medium display constitutive autophagic flux (i.e., a basal level), which is enhanced in nutrient-deprived medium.[696,778,779]

Conclusion: The levels of basal autophagy can vary substantially and can mask the effects of the experimental parameters being tested. Changes in media and growth conditions need to be examined empirically to determine affects on basal autophagy and the appropriate times for subsequent manipulations.

4. Experimental systems. Throughout these guidelines we have noted that it is not possible to state explicit rules that can be applied to all experimental systems. For example, some techniques may not work in particular cell types or organisms. In each case, efficacy of autophagy promotors, inhibitors and measurement technique must be empirically determined, which is why it is important to include appropriate controls. Differences may also be seen between in vivo or perfused organ studies and cell culture analyses. For example, insulin has no effect on proteolysis in suspended

rat hepatocytes, in contrast to the result with perfused rat liver. The insulin effect reappears, however, when isolated hepatocytes are incubated in stationary dishes780,781 or are allowed to settle down on the matrix (Häussinger D, personal communication).

The reason for this might be that autophagy regulation by insulin and some amino acids requires volume sensing via integrin-matrix interactions and also intact microtubules. [782-784] Along these lines, the use of whole embryos makes it possible to investigate autophagy in multipotent cells, which interact among themselves in their natural environment, bypassing the disadvantages of isolated cells that are deprived of their normal network of interactions.[632] In general, it is important to keep in mind that results from one particular system may not be generally applicable to others.

Conclusion: Although autophagy is conserved from yeast to human, there may be tremendous differences in the specific details among systems. Thus, results based on one system should not be assumed to be applicable to another.

5. Nomenclature. In order to minimize confusion regarding nomenclature, we make the following notes: In general, we follow the conventions established by the nomenclature committees for each model organism whenever appropriate guidelines are available, and briefly summarize the information here using "ATG1" as an example for yeast and mammals. The standard nomenclature of autophagy-related genes, mutants and proteins for yeast is ATG1, atg1 (or atg1D in the case of deletions) and Atg1, respectively, according to the guidelines adopted by the Saccharomyces Genome Database (http://www.yeastgenome.org/gene_guidelines.shtml). For mammals we follow the recommendations of the International Committee on Standardized Genetic Nomenclature for Mice (www.informatics.jax.org/mgihome/nomen/), which dictates the designations Atg1, atg1 and ATG1 (for all rodents), respectively, and the guidelines for human genes established by the HUGO Nomenclature Committee (http:// www.genenames.org/guidelines.html), which states that human gene symbols are in the form ATG1, and recommends that proteins use the same designation without italics, as with ATG1.

C. Methods and Challenges of Specialized Model Systems There are now a large number of model systems being used to study autophagy. These guidelines cannot cover every detail, and this article is not meant to provide detailed protocols. Nonetheless, we think it is useful to briefly discuss what techniques can be used in these systems, and to highlight some of the specific concerns and/or challenges. We also refer readers to the threevolumes of Methods in Enzymology that provide additional information for "nonstandard" model systems.[38-40]

1. C. elegans. C. elegans has a single ortholog of most yeast Atg proteins; however, two nematode homologs exist for Atg4 and Atg8.[785] Multiple studies have established C. elegans as a useful multicellular genetic model to delineate the autophagy pathway and associated functions (see for example refs.189, 462, 592, 593 and 786). The LGG-1/Atg8/LC3 reporter is the most commonly used tool to detect autophagy in C. elegans. Similar to Atg8, which is incorporated into the double membrane of autophagic vesicles during autophagy,[113,187,442] the C. elegans LGG-1 localizes into cytoplasmic puncta under conditions known to induce autophagy. Fluorescent reporter fusions of LGG-1/Atg8 with GFP, DsRED or mCherry have been used to monitor autophagosome formation in vivo, in the nematode. These reporters can be expressed either in specific cells and tissues or throughout the animal.[189,593,787,788] LGG-2 is the second LC3 homolog and is also a convenient marker for autophagy either fused to GFP,789 especially when expressed from an integrated transgene to prevent its germline silencing,[592] or using specific antibodies.[592] The exact function of LGG-1 vs. LGG-2 remains to be addressed. For observing autophagy by GFP-LC3 fluorescence in C. elegans, it is best to use integrated versions of GFPLC3592,593,790 (GFP::LGG-1 and GFP::LGG-2; Fig. 24) rather than extrachromosomal transgenic strains189,789 because the latter show variable expression among different animals or mosaic expression (Kang C, personal communication; Galy V, personal communication). It is also possible to carry out indirect immunofluorescence microscopy using antibodies against endogenous LGG-1,[462,593] or LGG-2.[592] In addition, with the integrated version, or with antibodies directed against endogenous LGG-1, it is possible to perform a western blot analysis for lipidation, at least in embryos,[790] and in the whole animal,[593] respectively. Finally, we point out the increasing availability of instruments that are capable of "super-resolution" fluorescence microscopy, which will further enhance the value and possibilities afforded by this technology.[791,792]

2. Chicken B-lymphoid DT40 cells and retina. The chicken B-lymphoid DT40 cell line represents a suitable tool for the analysis of autophagic processes in a nonmammalian vertebrate system. In DT40 cells, foreign DNA integrates with a very high frequency by homologous recombination compared with random integration. This makes the cell line a very valuable tool for the generation of cellular gene knockouts. Generally, the complete knockout of autophagy-regulatory proteins is preferable compared with RNAi-mediated knockdown, since in some cases these proteins function normally when expressed at reduced levels.[171] Different Atg-deficient DT40 cell lines already exist, including atg132/2, ulk12/2, ulk22/2, ulk1/22/2,793 becn12/2, and fip2002/2 (Stork B, personal communication). Many additional nonautophagy-related gene knockout DT40 cell lines have been generated and are commercially available.[794] DT40 cells are highly proliferative (the generation time is approximately 10 h) and knockout cells can be easily reconstituted with cDNAs by retroviral gene transfer for the mutational analysis of signaling pathways. DT40 cells mount an autophagic response upon starvation in EBSS,[793] and autophagy can be analyzed by a variety of assays in this cell line. Steady-state methods that can be used include TEM, LC3 western blotting and fluorescence microscopy; flux measurements include monitoring LC3-II turnover and tandem mRFP/mCherry-GFPLC3 fluorescence microscopy.

Using atg132/2 and ulk1/22/2 DT40 cells, it was shown that ATG13 and its binding capacity for FIP200 are mandatory for both basal and starvation-induced autophagy in this cell line, whereas ULK1/2 and in vitro-mapped ULK1-dependent phosphorylation sites of ATG13 appear to be dispensable for these processes.793 Another useful system is chick retina, which can be used for monitoring autophagy at different stages of development. For example, lipidation of LC3 is observed during starvation, and can be blocked with a short-term incubation with 3-MA.305,306 LEP-100 antibody is commercially available for the detection of this lysosomal protein.

Cautionary notes: Since the DT40 cell line derives from a chicken bursal lymphoma, not all ATG proteins and autophagyregulatory proteins are detected by the commercially available antibodies produced against their mammalian orthologs. The chicken genome is almost completely assembled, which facilitates the design of targeting constructs. However, in the May 2006 chicken (Gallus gallus) v2.1 assembly 5% of the sequence has not been anchored to specific chromosomes, and this might also include autophagy regulatory genes. It is possible that there is some divergence within the signaling pathways between mammalian and nonmammalian model systems. One example might be the role of ULK1/2 in starvation-induced autophagy described above. Additionally, neither rapamycin nor torin1 seem to be potent inducers of autophagy in DT40 cells, although MTOR activity is completely repressed as detected by phosphorylated RPS6KB2 western blotting.[793] Finally, DT40 cells represent a transformed cell line, being derived from an avian leukosis virus (ALV)-induced bursal lymphoma. Thus, DT40 cells release ALV nto the medium, and the 3'-long-terminal repeat has integrated upstream of the c-myc gene, leading to an increased c-myc expression.795 Both circumstances might influence basal and starvation-induced autophagy.

3. Chlamydomonas. It is possible to detect Atg8 modification as well as an increase in the amount of the protein by western blotting in response to autophagy activation.[207] Detection of Atg8 by immunofluorescence microscopy assays is also a reliable method to study autophagy, although it is recommended that this be combined with western blot analysis.

4. Drosophila. Drosophila provides an excellent system for in vivo analysis of autophagy. In this case, the problem of animalto-animal variability can be circumvented by the use of clonal mutant cell analysis, a major advantage of this model system. In this scenario, somatic clones of cells are induced that either overexpress the gene of interest, or silence the gene through expression of a transgenic RNA interference construct, or homozygous mutant cells are generated. These gain- or loss-offunction clones are surrounded by wild-type cells, which serve as an internal control for autophagy induction. In such an analysis,

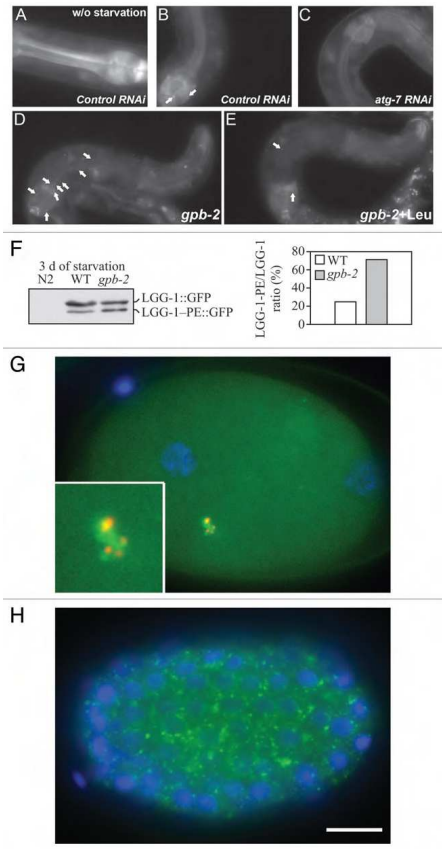

Figure 24. GFP::LGG-1 and GFP::LGG-2 are autophagy markers in C. elegans. (A–F) Animals were generated that carry an integrated transgene expressing a GFP-tagged version of lgg-1, the C. elegans ortholog of mammalian MAP1LC3. Representative green fluorescence images in the pharyngeal muscles of (A) control RNAi animals without starvation, (B) control RNAi animals after 9 d of starvation, (C) atg-7 RNAi animals after 9 d of starvation, (D) starvation-hypersensitive gpb-2 mutants without leucine after 3 d of starvation, and (E) gpb-2 mutants with leucine after 3 d of

starvation. The arrows show representative GFP::LGG-1-positive punctate areas that label pre-autophagosomal and autophagosomal structures. (F) The relative levels of PE-conjugated and unconjugated GFP::LGG-1 were determined by western blotting. These figures were modified from data previously published in Kang, C., Y.J. You, and L. Avery. 2007. Dual roles of autophagy in the survival of Caenorhabditis elegans during starvation. Genes & Development. 21:2161–71, Copyright G 2007, Genes & Development by Cold Spring Harbor Laboratory Press and Kang, C., and L. Avery. 2009. Systemic regulation of starvation response in Caenorhabditis elegans. Genes & Development. 23:12–7, Copyright G 2011, Genes and Development by Cold Spring Harbor Laboratory Press, ww.genesdev.org. (G and H) GFP:LGG-2 serves as a marker for autophagosomes in early C. elegans embryos. (G) GFP::LGG-2 expressed in the germline from an integrated transgene reveal the formation of autophagosomes (green) around sperm inherited membranous organelles (red). DNA of the two pronuclei is stained (blue). (H) Later during development, GFP::LGG-2-positive structures are present in all cells of the embryo. Scale bar, 10 mm. Images provided by V. Galy.

autophagy in these genetically distinct cells is always compared with neighboring cells of the same tissue, thus eliminating most of the variability and also ruling out potential non-cell-autonomous effects that may arise in mutant animals. Along these lines, clonal analysis should be an integral part of in vivo Drosophila studies when possible. LC3-II western blotting using antibodies against mammalian proteins does not work in Drosophila (Baehrecke E, Denton D, Kumar S, Neufeld T, unpublished results). Western blotting has been used successfully in Drosophila by monitoring flies expressing human GFPLC3101,[197] or using an antibody directed against the endogenous Atg8 protein.[454,796] In addition, cultured Drosophila (S2) cells can be stably transfected with GFP fused to Drosophila Atg8a, which generates easily resolvable GFP-Atg8a and GFP-Atg8a–PE forms that respond to autophagic stimuli (Wilkinson S, personal communication). Similarly, cultured Drosophila cells (l(2)mbn or S2) stably transfected with EGFP-humanLC3B respond to autophagy stimuli (nutrient deprivation) and inhibitors (3-MA, bafilomycin A1) as expected, and can be used to quantify GFP-LC3 puncta, which works best using fixed cells with the aid of an anti-GFP antibody.[797] However, in the Drosophila eye, overexpression ofGFP-Atg8 results in a significant increase in Atg8–PE by western blot, and this occurs even in control flies in which punctuate GFP-Atg8 is not detected by immunofluorescence (Fanto M, unpublished results), and in transfected Drosophila Kc167 cells, uninducible but persistent GFP-Atg8 puncta are detected (Kiger A, unpublished results). In contrast, expression of GFP-LC3 under the control of the rh1 promoter in wild-type flies did not result in the formation of LC3-II detectable by western blot, nor the formation of punctate staining; however, increased GFP-LC3 puncta by immunofluorescence or LC3-II by western blot were observed upon activation of autophagy (Mollereau B, unpublished results). Autophagy can also be monitored with mCherry-Atg18, which is displayed in punctate patterns that are very similar to mCherry-Atg8a (Juhász G, personal communication). Tandem fluorescence reporters have been established in Drosophila in vivo, where GFP-mCherry-Atg8a is expressed in the nurse cells of the developing egg chamber.[662] A Drosophila transgenic line (Ref(2)P-GFP) and a specific antibody against Ref(2)P, the Drosophila p62 homolog, are available to follow p62 expression and localization.[312,368] 5. Filamentous fungi. As in yeast, autophagy is involved in nutrient recycling during starvation.[193,194,798-801] In addition, macroautophagy seems to be involved in many normal developmental processes such as sexual and asexual reproduction, where there is a need for reallocation of nutrients from one part of the mycelium to another to supply the developing spores and spore-bearing structures.[194,798,799,801,802] Similarly, autophagy also affects conidial germination under nitrogen-limiting conditions.[194] In Podospora anserina, autophagy has been studied in relation to incompatibility reactions between mating strains where it seems to play a prosurvival role.[192,802] Of special interest to many researchers of autophagy in filamentous fungi has been the possible involvement of autophagy in plant pathogen infection and growth inside the host.[193,798,799,803-805] Autophagy also appears to be necessary for the development of aerial hyphae,[194,798,802,804] and for appresorium function in Magnaporthe oryzae and Colletotrichum orbiculare.[193,803,804] Some of these effects could be caused by the absence of autophagic processing of storage lipids (lipophagy) to generate glycerol for increasing turgor.[798,804,805] Methods for functional analysis of autophagy have been covered in a review article.[806] Most studies on autophagy in filamentous fungi have involved deleting some of the key genes necessary for autophagy, followed by an investigation of what effects this has on the biology of the fungus. Most commonly, ATG1 and/or ATG8 has been deleted.[193,798,799,801,802,804] To confirm that the deletion(s) affects autophagy, the formation of autophagic bodies in the wild type and the mutant can be compared. In filamentous fungi the presence of autophagic bodies can be detected using MDC staining,[193,798] TEM193,[799] or fluorescence microscopy to monitor Atg8 tagged with a fluorescent protein.[194,801,802] This type of analysis is most effective after increasing the number of autophagic bodies by starvation, in combination with decreasing the degradation of the autophagic bodies through the use of the protease inhibitor PMSF,[193,799,801,802] or alternatively by adding the autophagyinducing drug rapamycin.[194,798] In filamentous fungi it might also be possible to detect the accumulation of autophagic bodies in the vacuoles using differential interference contrast (DIC) microscopy.[801,802] Additional information regarding the timing of autophagy induction can be gained by monitoring transcript accumulation of ATG1 and/or ATG8 using qRT-PCR.[799]
6. Honeybee. The reproductive system of bees, or insects with meroistic polytrophic ovaries, with regard to the ovary developmental cycle can be a very useful tool to analyze and monitor physiological autophagy. Both queen and worker ovaries of Africanized A. mellifera display time-regulated features of cell death that are, however, linked to external stimuli.807 Features of apoptosis and autophagy are frequently associated with the degeneration process in bee organs, but only more recently has the role of autophagy been highlighted in degenerating bee tissues. TEM is the primary method currently being used to monitor autophagy by following the formation of autophagosomes and

autolysosomes. This can be combined with cytochemical and immunohistochemical detection of acid phosphatase as a marker for autolysosomes.[808,809] Acidotropic dyes can also be used to follow autophagy in bee organs, as long as the cautions noted in this article are followed. The honeybee genome has been sequenced, and differential gene expression has been used to monitor Atg18 in bees parasitized by Varroa destructor.[810]

7. Human. Considering that much of the research conducted today is directed at understanding the functioning of the human body, in both normal and disease states, it is pertinent to include humans and primary human tissues and cells as important models for the investigation of autophagy. Although clinical studies are not readily amenable to these types of analyses, it should be kept in mind that the TORC1 inhibitor rapamycin is available as a clinically approved drug (e.g., sirolimus). Furthermore, fresh biopsies of some human tissues are possible to obtain. Blood, in particular, as well as samples of adipose and muscle tissues, can be obtained from needle biopsies or from elective surgery. For example, in a large study, adipocytes were isolated from pieces of adipose tissue (obtained during surgery) and examined for insulin signaling and autophagy. It was demonstrated that autophagy was strongly upregulated (based on LC3 flux, EM, and lipofuscin degradation) in adipocytes obtained from obese patients with type 2 diabetes compared with nondiabetic subjects.209 The major caveat of the work concerning autophagy on human tissue is the problem of postmortem times and fixation. Postmortem times are typically longer in autopsy material than when surgical biopsies are obtained. For tumors, careful sampling to avoid necrosis, hemorrhagic areas and non-neoplastic tissue is required. The problem of fixation is that it can diminish the antibody binding capability; in addition, especially in autopsies, material is not obtained immediately after death.[811,812] The possibilities of postmortem autolysis and fixation artifacts must always be taken into consideration when interpreting changes attributed to autophagy.813 Analyses of these types of samples require not only special antigen retrieval techniques, but also histopathological experience to interpret autophagy studies by immunohistochemistry, immunofluorescence or TEM. The situation is even worse with TEM, where postmortemdelays can cause vacuolization. Researchers experienced in the

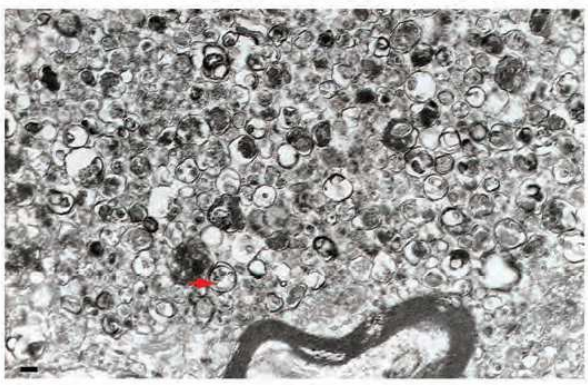

Figure 25. A large dystrophic neurite from a brain biopsy of a patient with Gerstmann-Sträussler-Scheinker (GSS) disease not unlike those reported for Alzheimer disease.814 This structure is filled with innumerable autophagic vacuoles, some of which are covered by a double membrane. Electron dense lysosomal-like structures are also visible. The red arrow points to a doublemembrane autophagic vacuole. Scale bar, 200 nm. Image provided by P. Liberski.

analysis of TEM images corresponding to autophagy should be able to identify these potential artifacts because autophagic vacuoles should contain cytoplasm. While brain biopsies may be usable for high quality TEM (Figs. 25 and 26), this depends upon proper handling at the intraoperative consultation stage, and such biopsies are performed infrequently except for brain tumor diagnostic studies. An analysis that examined liver and skeletal muscle from critically ill patients utilized tissue biopsies that were taken within 30 ± 20 min after death and were flash-frozen in liquid nitrogen followed by storage at -80°C.[815] Samples could subsequently be used for EM and western blot analysis. A major limitation of studying patient biopsies is that only static measurements can be performed. This limitation does not apply, however, for dynamic experiments on tissue biopsies or cells derived from biopsies, as described above.209 Multiple measurements over time, especially when deep (vital) organs are involved, are impossible and ethically not justifiable. Hence, quantitative flux measurements are virtually impossible in patients. To overcome these problems to the extent possible and to gain a more robust picture of the autophagic status, observational studies need to include two different aspects. First, a static marker for phagophore or autophagosome formation needs to be measured. This can be done by assessing ultrastructural changes with TEM and/or on the molecular level by measuring LC3-II protein levels. Second, accumulation of autophagy substrates, such as p62 and (poly)ubiquitinated proteins can provide information on the overall efficacy of the pathway and can be a surrogate marker of the consequences of altered autophagic flux, especially when autophagy is insufficient, although these changes can also be affected by the ubiquitin-proteasome system as mentioned above. In addition, and even more so when problems with specific pathways are suspected (e.g., mitophagy), specific substrates of these pathways should be determined. Again, none of

these measurements on its own provides enough information on (the efficacy of) autophagy, because other processes may confound

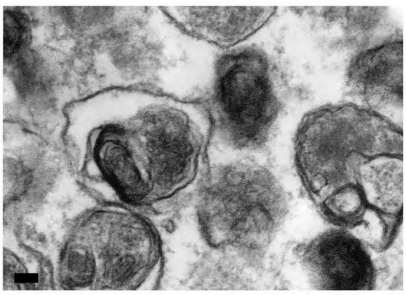

Figure 26. A high-power electron micrograph from a brain biopsy showing autophagic vacuoles in a case of ganglioglioma. Scale bar, 200 nm. Image provided by P. Liberski.

every single parameter. However, the combination of multiple analyses should be informative. Although still in its infancy with regard to autophagy, it is worth pointing out that mathematical modeling has the power to bridge whole body in vivo data with in vitro data from tissues and cells. The usefulness of so-called hierarchical or multilevel modeling has thus been demonstrated when examining the relevance of insulin signaling to glucose uptake in primary human adipocytes compared with whole-body glucose homeostasis.[816] The amino acid exchange rate, which has been suggested as a minimal invasive marker for measuring autophagic flux in vivo should be used with special caution, as its utility is debatable in disease models (see Analyses in vivo). A stepwise process can be proposed for linking changes in the autophagic pathway to changes in disease outcome. First, in an observational study, the changes in the autophagic pathway (see above) should be quantified and linked to changes in disease outcome. To prove causality, a subsequent autophagy-modifying intervention should be tested in a randomized study. Before an intervention study is performed in human patients, the phenotype of (in)active autophagy contributing to poor outcome should be established in a validated animal model of the disease. For the validation of the hypothesis in an animal model, a similar two-step process is suggested, with the assessment of the phenotype in a first stage, followed by a proof-of-concept intervention study (see Large animals).

8. Hydra. Hydra is a freshwater cnidarian animal that provides a unique model system to test autophagy either in the context of nutrient deprivation, as these animals easily survive several weeks of starvation,[817,818] or in the context of regeneration, because in the absence of protease inhibitors, bisection of the animals leads to an uncontrolled wave of autophagy; in the latter case, an excess of autophagy in the regenerating tip immediately after amputation is deleterious.[819-821] Most components of the autophagy and MTOR pathways are evolutionarily conserved in Hydra.818 For steady-state measurements, autophagy can be monitored by western blot for ATG8/LC3, by immunofluorescence (using antibodies to ATG8/LC3, LBPA or RSK), or with dyes such as MitoFluor Red[589] and LysoTracker Red. Flux measurements can be made by following ATG8/LC3 turnover using lysosomal protease inhibitors (leupeptin and pepstatin A), or in vivo labeling using LysoTracker Red. It is also possible to monitor MTOR activity with phosphospecific antibodies to RPS6KB2 kinase and EIF4EBP1, or to examine gene expression by semiquantitative RT-PCR, using primers that are designed for Hydra. Autophagy can be induced by RNAi-mediated knockdown of Kazal1,[819,820] or with rapamycin treatment, and can be inhibited with wortmannin or bafilomycin A1.[817,818]

9. Large animals. Assessment of autophagy (and, in particular, autophagic flux) in clinically relevant large animal models is critical in establishing its (patho)physiological role in multiple disease states. For example, evidence obtained in swine suggests that upregulation of autophagy may protect the heart against damage caused by acute myocardial infarction or “heart attack.”[822] Autophagy also plays an important role in the development and remodeling of the bovine mammary gland. In vitro studies with the use of a three-dimensional culture model of bovine mammary epithelial cells (MECs) have shown that this process is involved in the formation of fully developed alveoli-like structures.[823] Earlier studies show that intensified autophagy is observed in bovine MECs at the end of lactation and during the dry period, when there is a decrease in the levels of lactogenic hormones, increased expression of auto/paracrine apoptogenic peptides, increased influence of sex steroids and enhanced competition between the intensively developing fetus and the mother organism for nutritional and bioactive compounds.[824,825] These studies were based on some of the methods described elsewhere in these guidelines, including GFP-Atg8/LC3 fluorescence microscopy, TEM, and western blotting of LC3 and BECN1. Creation of a specific GFPLC3 construct by insertion of cDNA encoding bovine LC3 into the pEGFP-C1 vector makes it possible to observe induction of autophagy in bovine MECs in a more specific manner than can be achieved by immunofluoresce techniques, in which the antibodies do not show specific reactivity to bovine cells and tissues.823,825 However, it is important to remember that definitive confirmation of cause-and-effect is challenging for studies on large animals, given the lack or poor availability of specific antibodies and other molecular tools, the frequent inability to utilize genetic approaches, and the often

prohibitive costs of administering pharmacological inhibitors in these translational preparations. In contrast with cell culture experiments, precise monitoring of autophagic flux is practically impossible in large animals. Theoretically, repetitive analyses of small tissue biopsies should be performed to study ultrastructural and molecular alterations over time in the presence or absence of an autophagy inhibitor (e.g., chloroquine). However, several practical problems impede applicability of this approach. First, repetitive sampling of small needle biopsies in the same animal (a major challenge by itself) could be assumed to induce artifacts following repetitive tissue destruction, especially when deep (vital) organs are involved. In addition, chemical inhibitors of autophagy have considerable side effects and toxicity, hampering their usage. Also, the general physical condition of an animal may confound results obtained with administration of a certain compound, for instance altered uptake of the compound when perfusion is worse. Therefore, in contrast to cells, where it is more practical to accurately document autophagic flux, we suggest the use of a stepwise approach in animal models to provide a proof-of-concept with an initial evaluation of sequellae of (in)active autophagy and the relation to the outcome of interest. First, prior to an intervention, the static ultrastructural and molecular changes in the autophagic pathway should be documented and linked to the outcome of interest (organ function, muscle mass or strength, survival, etc.). These changes can be evaluated by light microscopy, EM and/or by molecular markers such as LC3-II. In addition, the cellular content of specific substrates normally cleared by autophagy should be quantified, as such measurement, despite the static nature, could provide a clue about the results of altered autophagic flux in vivo. These autophagic substrates can include p62 and (poly) ubiquitinated substrates or aggregates, but also specific substrates such as damaged mitochondria. As noted above, measurement ofthese autophagic substrates is mainly informative when autophagic flux is prohibited/insufficient, and, individually, all have specific limitations for interpretation. As mentioned several times in these guidelines, no single measurement provides enough information on its own to reliably assess autophagy, and all measurements should be interpreted in view of the whole picture. In every case, both static measurements reflecting the number of autophagosomes (ultrastructural and/or molecular) and measurements of autophagic substrates as surrogate markers of autophagic flux need to be combined. Depending on the study hypothesis, essential molecular markers can further be studied to pinpoint at which stage of the process autophagy may be disrupted. After having identified a potential role of autophagy in mediating an outcome in a clinically relevant animal model, an autophagy-modifying intervention should be tested. For this purpose, an adequately designed, randomized controlled study of sufficient size on the effect of a certain intervention on the phenotype and outcome can be performed in a large animal model. Alternatively, the effect of a genetic intervention can be studied in a small animal model with clinical relevance to the studied disease. As mentioned above, exact assessment of autophagic flux requires multiple time points, which cannot be done in the same animal. Alternatively, different animals can be studied for different periods of time. Due to the high variability between animals, however, it is important to include a sufficiently high number of animals per time point. This thus limits feasibility and the number of time points that can be investigated. The right approach to studying autophagy in large animals likely differs depending on the question that is being addressed. Several shortcomings regarding the methodology, inherent to working with large animals, can be overcome by an adequate study design. As for every study question, the use of an appropriate control group with a sufficient number of animals is crucial in this regard.

10. Lepidoptera. Some of the earliest work in the autophagy field was performed in the area of insect metamorphosis.[667] Microscopy and biochemical research revealed autophagy during the metamorphosis of American silkmoths and the tobacco hornworm, Manduca sexta, and included studies of the intersegmental muscles, but they did not include molecular analysis of autophagy. Overall, these tissues cannot be easily maintained in culture, and antibodies against mammalian proteins do not often work. Accordingly, these studies were confined to biochemical measurements and electron micrographs. During metamorphosis, the bulk of the larval tissue is removed by autophagy and other forms of proteolysis.[826] Bombyx mori is now used as a representative model among Lepidoptera, for studying not only the regulation of autophagy in a developmental setting, but also the relations between autophagy and apoptosis. The advantages of this model are the large amount of information gathered on its developmental biology, physiology and endocrinology, the availability of numerous genetic and molecular biology tools, and a completely sequenced genome.[827] The basic studies of B. mori autophagy have been performed in four main larval systems: the silk gland, the fat body, the midgut and the ovary. The methods used for these studies are comparatively similar, starting from EM, which is the most widely used method to follow the changes of various autophagic structures and other features of the cytosol and organelles that are degraded during autophagy.[452,828-831] Immuno-TEM also can be used, when specific antibodies for autophagic markers are available. Acidotropic dyes such as MDC and LysoTracker Red staining have been used as markers for autophagy in silk moth egg chambers combined always with additional assays.[828,829] Acidic phosphatase also can be used as a marker for autolysosomal participation in these tissues.[452,830,832] Systematic cloning and analysis revealed that homologs of most of the Atg genes identified in other insect species such as Drosophila are present in B. mori, and 11 Atg genes have now been identified in the silkworm genome, as well as other genes involved in the MTOR signal transduction pathway.[833,834] Variations in the expression of several of these genes have been monitored not only in silkworm larval organs, where autophagy is associated with development,[452,833-835] but also in the fat body of larvae undergoing starvation.[833] In the IPLB-LdFB cell

line, derived from the fat body of the caterpillar of the gypsy moth Lymantria dispar, indirect immunofluorescence experiments have demonstrated an increased number of Atg8-positive dots in cells with increased autophagic activity; however, western blotting did not reveal the conversion of Atg8 into Atg8–PE. Instead, a single band with an approximate molecular mass of 42 kDa was observed that was independent of the percentage of cells displaying punctate Atg8 (Malagoli D, unpublished results). In contrast, with B. mori midgut, the use of an antibody specific for BmAtg8 makes it possible to monitor BmAtg8 processing to BmAtg8–PE by western blotting.[452] Thus, the utility of monitoring Atg8 in insects may depend on the particular organism and antibody.

11. Neotropical teleosts. In tropical environments, fish have developed different reproductive strategies, and many species have the potential for use as biological model in cell and molecular biology, especially for studying the mechanisms that regulate gametogenesis and embryo development. In these fish, the ovary is a suitable experimental model system for studying autophagy and its interplay with cell death programs due to the presence of postovulatory follicles (POFs) and atretic follicles (AFs), which follow different routes during ovarian remodeling after spawning. 836 In the fish reproductive biology, POFs are excellent morphological indicators of spawning, whereas AFs are relevant biomarkers of environmental stress. In addition, many freshwater teleosts of commercial value do not spawn spontaneously in captivity, providing a suitable model for studying the mechanisms of follicular atresia under controlled conditions.[837] When these species are subjected to induced spawning, the final oocyte maturation (resumption of meiosis) occurs, and POFs are formed and quickly reabsorbed in ovaries after spawning.838 Assessment of autophagy in fish has been primarily made using TEM at different times of ovarian regression.839 Due to the difficulty of obtaining antibodies specific for each fish species, immunodetection of ATG-proteins (mainly LC3 and BECN1) by immunohistochemistry associated with analyses by western blotting can be performed using antibodies that are commercially available for other vertebrates. Such studies suggest dual roles for autophagy infollicular cells;[836] however, evaluation of the autophagic flux in different conditions is critical for establishing its physiological role during follicular regression and ovarian remodeling after spawning. Given the ease of obtaining samples and monitoring them during development, embryos of these fish are also suitable models for studying autophagy that is activated in response to different environmental stressors, particularly in studies in vivo.

12. Odontoblasts. Odontoblasts are long-lived dentin-forming postmitotic cells, which evolved from neural crest cells early during vertebrate evolution. These cells are aligned at the periphery of the dental pulp and are maintained during the entire healthy life of a tooth. As opposed to other permanent postmitotic cells such as cardiac myocytes or central nervous system neurons, odontoblasts are significantly less protected from environmental insult, such as dental caries and trauma. Mature odontoblasts develop a well-characterized autophagic-lysosomal system, including a conspicuous autophagic vacuole that ensures turnover and degradation of cell components. Immunocytochemical and TEM studies make it possible to monitor age-related changes in autophagic activity in human odontoblasts.[840]

13. Planarians. Planarians are one of the favorite model systems in which to study regeneration and stem cell biology, and represent a unique model where it is possible to investigate autophagy in the context of regeneration, stem cells and growth. Currently the method used to detect autophagy is TEM. A detailed protocol adapted to planarians has been described.[841,842] However, complementary methods to detect autophagy are also needed, since TEM cannot easily distinguish between activation and blockage of autophagy, which would both be observed as an accumulation of autophagosomes. Detection of autophagy by other methods are being developed (González-Estévez C, personal communication), including immunohistochemistry and western blotting approaches for the planarian homolog of LC3. Several commercial antibodies against human LC3 have been tried for cross-reactivity without success (see the Autophagy Forum for details) and three planarian-specific antibodies have been generated. Some preliminary results show that LysoTracker Red can be a useful technique on whole-mount planarians. Interestingly, most of the components of the autophagy and MTOR signaling machinery are evolutionarily conserved in planarians, and an RNA interference screen is being performed. Whether autophagy genes vary at the mRNA level during starvation and after depletion of MTOR signaling components is also currently being investigated (González-Estévez C, personal communication).

14. Plants. The fluorophore of the red fluorescent protein shows a relatively high stability under acidic pH conditions. Thus, chimeric RFP fusion proteins that are sequestered within autophagosomes and delivered to the plant vacuole can be easily detected by fluorescence microscopy. Furthermore, fusion proteins with some versions of RFP tend to form intracellular aggregates, allowing the development of a visible autophagic assay for plant cells.[843] For example, fusion of cytochrome b5 and the original (tetrameric) RFP generate an aggregated cargo protein that displays cytosolic puncta of red fluorescence and, following vacuolar delivery, diffuse staining throughout the vacuolar lumen. This system allows autophagy to be monitored through fluorescence microscopy with minimum damage to intact plant cells. In addition, the size difference between the intact and processed cargo protein allows the quantification of autophagic degradation through the detection of RFP after separation of total protein by gel electrophoresis, similar to the GFP-Atg8/LC3 processing assay described for yeast and mammals. Arabidopsis cells can be stably transfected with GFP fused to plant ATG8, and the lipidated and nonlipidated forms can be separated by SDS-PAGE.[150] Furthermore, the GFP-ATG8 processing assay is particularly robust in Arabidopsis and can be observed not only by

western blotting but also by a diffuse fluorescent haze that appears in vacuoles together with punctuate GFP fluorescence, which is presumed to represent free soluble GFP and GFP-ATG8–PE still bound to autophagic bodies, respectively. [151,174] Thus, as with other systems, autophagosome formation in plants can be monitored through the use of fluorescent protein fusions to ATG8, and by TEM (Fig. 27). A tandem fluorescence reporter system is also available in Arabidopsis.[844]

Furthermore, it is possible to use plant homologs of p62 and NBR1 (JOKA2 in tobacco[845] and NBR1 in Arabidopsis[844]) as markers for autophagy when constructed as fluorescent chimeras. It is worth noting that in some plant models including Arabidopsis[200,402] and somatic embryos of Picea abies (Norway spruce) (Minina A, Bozhkov PV, personal communication), the high turnover rate of Atg8-decorated autophagosomes precludes their detection in untreated cells using fluorescent markers (GFP/RFP-Atg8, MDC or LysoTracker) and necessitates pretreatment with concanamycin A. However, the latter treatment will increase vacuolar pH, which may subsequently interfere with its detection by MDC and LysoTracker. In some systems, including fungi and plants, the size of the vacuole is sufficiently large such that fusion of the autophagosome

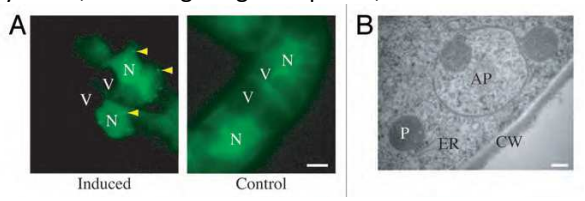

Figure 27. Detection of macroautophagy in tobacco BY-2 cells. (A) Induction of autophagosomes in tobacco BY-2 cells expressing YFP-NtAtg8 (shown in green for ease of visualization) under conditions of nitrogen limitation (Induced). Arrowheads indicate autophagosomes that can be seen as a bright green dot. No such structure was found in cells grown in normal culture medium (Control). Bar, 10 mm. N, nucleus; V, vacuole. (B) Ultrastructure of an autophagosome in a tobacco BY-2 cell cultured for 24 h without a nitrogen source. Bar, 200 mm. AP, autophagosome; P, plastid; CW, cell wall. This image was provided by Dr. Kiminori Toyooka, (RIKEN Plant Science Center).

results in the release of the inner vesicle into the organelle lumen; the resulting single-membrane vesicle is termed an autophagic body (Fig. 1).[200,846] The accumulation of autophagic bodies can be detected by light microscopy in cells that lack vacuolar hydrolase activity (e.g., the pep4D yeast mutant) or in the presence of inhibitors that interfere with hydrolase activity (e.g., PMSF or concanamycin). Using Nomarski optics (differential interference contrast) it is easy to distinguish and quantify yeast vacuoles that lack autophagic bodies from those that have accumulated them, and the same is true for plants. Other methods described throughout these guidelines can also be used in plants. For example, in tobacco cells cultured in sucrose starvation medium, the net degradation of cellular proteins can be measured by a standard protein assay; this degradation is inhibited by 3-MA and E-64c (an analog of E-64d), and is thus presumed to be due to autophagy.[847] In addition, the cytoplasm in tobacco cells that is present in transvacuolar strands that cross the large central vacuole can be seen by TEM to disappear during starvation-induced autophagy.[847] Cautionary notes: Although the detection of vacuolar RFP can be applied to both plant cell lines and to intact plants, it is not practical to measure RFP fluorescence in intact plant leaves, due to the very high red fluorescence of chloroplasts. Furthermore, different autophagic induction conditions cause differences in protein synthesis rates; thus, special care should be taken to monitor the efficiency of autophagy by quantifying the intact and processed cargo proteins. With regard to autophagic body accumulation, it is difficult to quantify their number and/or volume, although their presence or absence can be examined by light microscopy or TEM. In addition, the accumulation of autophagic bodies requires the inhibition of vacuolar hydrolase activity. Therefore, to demonstrate turnover, the assay must be performed either in the absence and presence of appropriate inhibitors, or in both a wild-type strain and a strain with a mutation/deletion in a gene encoding a vacuolar hydrolase(s) (see Selective types of autophagy). Otherwise, accumulation of autophagic bodies could instead indicate a defect in the lysis/ degradation step of autophagy. Finally, this method is not well suited for systems other than plants or fungi because lysosomes are too small for detection by standard (i.e., nonfluorescence) light microscopy, and fusion with autophagosomes does not generate autophagic bodies (Fig. 1).

15. Protists. An essential role of autophagy during the differentiation of parasitic protists (formerly called protozoa) is clearly emerging. Only a few of the known ATG genes are present in these organisms, which raises the question about the minimal system that is necessary for the normal functioning of autophagy. The reduced complexity of the autophagic machinery in many protists provides a simplified model to investigate the core mechanisms of autophagosome formation necessary for selective proteolysis, and will open a completely new area in autophagy research. Some of the standard techniques used in other systems can be applied to protists including indirect immunofluorescence using antibodies generated against ATG8, the generation of stable lines expressing mCherry- or GFP-fused ATG8 for live microscopy and immuno-TEM analyses. Extrachromosomal constructs of GFP-ATG8 also work well with lower eukaryotes, [201-203] as do other fluorescently-tagged ATG proteins including ATG5 and ATG12. The unicellular amoeba Dictyostelium discoideum provides another useful system for monitoring autophagy.848 The primary advantage of Dictyostelium is that it has a unique life cycle that involves a transition from a unicellular to a multicellular form. Upon starvation, up to 100,000 single cells aggregate by chemotaxis and form a multicellular

structure that undergoes morphogenesis and cell-type differentiation. Development proceeds via the mound stage, the tipped aggregate and a motile slug and culminates with the formation of a fruiting body that is composed of a ball of spores supported by a thin, long stalk made of vacuolized dead cells. Development is dependent on autophagy and, at present, all of the generated mutants in Dictyostelium autophagy genes display developmental phenotypes of varying severity.[848,849] D. discoideum is also a versatile model to study infection with human pathogens and the role of autophagy in the infection process. The susceptibility of D. discoideum to microbial infection and its strategies to counteract pathogens are similar to those in higher eukaryotes.850 Along these lines, Dictyostelium utilizes some of the proteins involved in autophagy that are not present in S. cerevisiae including Atg101, Uvrag and Vmp1, in addition to the core Atg proteins. Autophagy can be monitored in Dictyostelium by fluorescence microscopy of GFP-Atg8, by TEM and by western blot to detect free GFP or the loss of p62.[848] One cautionary note with regard to the use of GFP-ATG8 in protists is that these organisms display some "nonclassical" variations in their ATG proteins. For example, Leishmania contains many apparent ATG8-like proteins (the number varying per species; e.g., up to 25 in L. major) grouped in four families, but only one labels true autophagosomes even though the others form puncta,202 and ATG12 requires truncation to provide the C-terminal glycine before it functions in the canonical way. Unusual variants in protein structures also exist in other protists, including the malaria parasite Plasmodium falciparum, which expresses ATG8 with a terminal glycine not requiring cleavage to become functional in autophagy.[851] Thus, in each case care needs to be applied and the use of the protein to monitor autophagy validated. In addition, due to possible divergence in the upstream signaling kinases, classical inhibitors such as 3-MA, or inducers like rapamycin, which are not as potent for trypanosomes[852] or apicomplexan parasites as in mammalian cells or yeast, must be used with caution (Coppens I, personal communication);[201] however, RNAi knockdown of TORC1 (e.g., TOR1 or RAPTOR) is effective in inducing autophagy. The scuticociliate Philasterides dicentrarchi has proven to be a good experimental organism for identifying autophagy-inducing drugs or for autophagy initiation by starvation-like conditions, since this process can be easily induced and visualized in this ciliate.[853] In scuticociliates, the presence of autophagic vacuoles can be detected by TEM, fluorescence microscopy or confocal laser scanning microscopy by using dyes such as MitoTracker Deep Red FMG and MDC.

Finally, a novel autophagy event has been found in Tetrahymena thermophila, which is a free-living ciliated protist. Aremarkable, virtually unique feature of the ciliates is that they maintain spatially differentiated germline and somatic nuclear genomes within a single cell. The germline genome is housed in the micronucleus, while the somatic genome is housed in the macronucleus. These nuclei are produced during sexual reproduction (conjugation), which involves not only meiosis and mitosis of the micronucleus and its products, but also degradation of some of these nuclei as well as the parental old macronucleus. Hence, there should be a mechanism governing the degradation of these nuclei. The inhibition of PtdIns3Ks with wortmannin or LY294002 results in the accumulation of additional nuclei during conjugation.[854] During degradation of the parental old macronucleus, the envelope of the nucleus becomes MDC- and LysoTracker Red-stainable without sequestration of the nucleus by a double membrane and with the exposure of certain sugars and phosphatidylserine on the envelope.[855] Subsequently, lysosomes fuse only to the old parental macronucleus,but other co-existing nuclei such as developing new macro- and micronuclei are unaffected.[855] This evidence suggests that selective autophagy may be involved in the regulation of the degradation of nuclei during conjugation, but its mechanism apparently differs from the classical pathway. Indeed, no ATG genes homologous to those in yeast are characterized in this organism to date.

16. Rainbow trout. Salmonids (e.g., salmon, rainbow trout) experience long periods of fasting often associated with seasonal reductions in water temperature and prey availability or spawning migrations. As such, they represent an interesting model system for studying and monitoring the long-term induction of autophagy. Moreover, the rainbow trout (Oncorhynchus mykiss) displays unusual metabolic features that may allow us to gain a better understanding of the nutritional regulation of this degradative system (i.e., a high dietary protein requirement, an important use of amino acids as energy sources, and an apparent inability to metabolize dietary carbohydrates). It is also probably one of the most deeply studied fish species with a long history of research performed in physiology, nutrition, ecology, genetics, pathology, carcinogenesis and toxicology.[856] Its relatively large size compared with model fish such as zebrafish or medaka, makes rainbow trout a particularly well-suited alternative model to carry out biochemical and molecular studies on specific tissues or cells that are impossible to decipher in small fish models. The genomic resources in rainbow trout are now being extensively developed; a high-throughput DNA sequencing program of EST has been initiated associated with numerous transcriptomics studies,[857-860] and the full genome sequence is now available. Most components of the autophagy and associated signaling pathways (AKT, TOR, AMPK, FOXO) are evolutionarily conserved in rainbow trout;[457,861-863] however, not all ATG proteins and autophagy-regulatory proteins are detected by the commercially available antibodies produced against their mammalian orthologs. Nonetheless, the expressed sequence transcript databases facilitate the design of targeting constructs. For steady-state measurement, autophagy can be monitored by western blot or by immunofluorescence using antibodies to ATG8/LC3.[863] Flux measurements can be made in a trout cell culture model (e.g., in primary culture of trout myocytes) by following ATG8/LC3 turnover in the absence and presence of bafilomycin A1. It is also possible to monitor the mRNA levels of

ATG genes by real-time PCR using primer sequences chosen from trout sequences available in the above-mentioned expressed sequence transcript database. A major challenge in the near future will be to develop for this model the use of RNAi-mediated gene silencing in order to analyze the role of some signaling proteins in the control of autophagy, and also the function of autophagy-related genes in this species.

17. Sea urchin. Sea urchin embryo is an appropriate model system for studying and monitoring autophagy and other defense mechanisms activated during physiological development and in response to stress.[632] This experimental model offers the possibility of detecting LC3 through both protein gel blot and immunofluorescence in situ analysis. Furthermore, in vivo staining of autolysosomes with acidotropic dyes can also be performed. Studies on whole embryos make it possible to obtain qualitative and quantitative data for autophagy and also to get information about spatial localization aspects in cells that interact among themselves in their natural environment.

Cautionary notes include the standard recommendation that it is always preferable to combine molecular and morphological parameters to support the validity of the data.

18. Ticks. In the hard tick Haemaphysalis longicornis, endogenous autophagy-related proteins (Atg6 and Atg12) can be detected by western blotting and/or by immunohistochemical analysis of midgut sections.[864,865] It is also possible to detect endogenous Atg3 and Atg8 by western blotting using antibodies produced against the H. longicornis proteins (Umemiya-Shirafuji R, unpublished results). Commercial antibodies against mammalian ATG orthologs (ATG3, ATG5, and BECN1) can be also used for western blotting; however, when the tick samples include blood of a host animal, the animal species immunized with autophagy-related proteins should be checked before use to avoid nonspecific background cross-reactivity. In addition to these methods, TEM is recommended to detect autophagosomes and autolysosomes. Although acidotropic dyes can be useful as a marker for autolysosomes in some animals, careful attention should be taken when using the dyes in ticks. Since the midgut epithelial cells contain acidic organelles (e.g., lysosomes) that are related to blood digestion during blood feeding, this method may cause confusion. It is difficult to distinguish between autophagy (autolysosomes) and blood digestion (lysosomes) with acidotropic dyes. Another available monitoring method is to assess the mRNA levels of tick ATG genes by real-time PCR.866 However, this method should be used along with other approaches such as western blotting, immunostaining, and TEM as described in this article. Unlike model insects, such as Drosophila, powerful genetic tools to assess autophagy are still not established in ticks. However, RNAi-mediated gene silencing is currently being developed to analyze the function of autophagy-related genes in ticks during nonfeeding periods (Umemiya-Shirafuji R, unpublished results).

19. Food biotechnology. Required for yeast cell survival under a variety of stress conditions, autophagy has the potential to contribute to the outcome of many food fermentation processes. For example, autophagy induction is observed during the primary fermentation of synthetic grape must,[867] and during sparkling wine production (secondary fermentation).[868] A number of genome-wide studies have identified vacuolar functions and autophagy as relevant processes during primary wine fermentation or for ethanol tolerance, based on gene expression data or cell viability of knockout yeast strains;[867,869-873] however, understanding the actual relevance of autophagy in yeast-driven food fermentation processes would require addressing this issue experimentally using some of the methods available for S. cerevisiae as described in these guidelines. Autophagy is a target for some widespread food preservatives used to prevent yeast-dependent spoilage. For example, the effect of benzoic acid is exacerbated when concurrent with nitrogen starvation.[874] This opened the way to devise strategies to improve the usefulness of sorbic and benzoic acid, taking advantage of their combination with stress conditions that would require functional autophagy for yeast cell survival.[875] Practical application of these findings would also require extending this research to other relevant food spoilage yeast species, which would be of obvious practical interest. In the food/health interface, the effect of some food bioactive compounds on autophagy in different human cell types has already attracted some attention.[876,877] Interpreting the results of this type of research, however, warrants two cautionary notes.[878] First, the relationship between health status and autophagic activity is obviously far from being direct. Second, experimental design in this field must take into account the actual levels of these molecules in the target organs after ingestion, as well as exposure time and their transformations in the human body. In addition, attention must be paid to the fact that several mechanisms might contribute to the observed biological effects. Thus, relevant conclusions about the actual involvement of autophagy on the health-related effect of food bioactive compounds would only be possible by assaying the correct molecules in the correct concentrations.

20. Zebrafish. Zebrafish have many characteristics that make them a valuable vertebrate model organism for the analysis of autophagy. For example, taking advantage of the transparency f embryos, autophagosome formation can be visualized in vivo during development using transgenic GFP-Lc3 and GFP-Gabarap fish. The addition of 1-phenyl-2-thiourea (PTU) to media inhibits melanogenesis allowing visualization of later-stage embryos. Lysosomes can also be readily detected in vivo by the addition of LysoTracker Red to fish media prior to visualization. Additionally, protocols have been developed to monitor Lc3 protein levels and conjugation to PE by western blot analysis using commercially available Lc3 antibodies.[35,879] Because of their translucent character and external fertilization nd development, this vertebrate has proved an exceptional choice for developmental research. In situ hydridization of whole embryos can be performed to determine expression patterns. Knockdown of gene function is performed by treatment with

morpholinos; the core autophagy machinery protein Gabarap,880 and regulatory proteins such as the phosphinositide phosphatase Mtmr14,[881] Raptor and Mtor,[882] have all been successfully knocked down by morpholino treatment. However, research is currently somewhat hampered by the deficiency of efficient methods for targeted gene mutations and deletions. Zebrafish are ideal organisms for in vivo drug discovery and/or verification because of their relatively small size and aqueous habitat, and several chemicals have been identified that modulate zebrafish autophagy activity.[879] Many chemicals can be added to the media and are absorbed directly through the skin. Because of simple drug delivery and the onset of neurodegenerative disease phenotypes at the larval stage, zebrafish are a promising organism for the study of autophagy's role in neurodegenative disease.
Along these lines, a zebrafish model of Huntington disease has been developed.[740]
Conclusions and Future Perspectives There is no question that research on the topic of autophagy has expanded dramatically since the publication of the first set of guidelines.1 To help keep track of the field we have published a glossary of autophagy-related molecules and processes,[883,884] and there are now databases that are specifically dedicated to autophagy including the Human Autophagy-dedicated Database (HADb; www.autophagy.lu) and the Autophagy Database (http://tp-apg.genes.nig.ac.jp/autophagy/). With this continued influx of new researchers we think it is critical to try to define standards for the field. Accordingly, we have highlighted the uses and caveats of an expanding set of recommended methods for monitoring macroautophagy in awide range of systems (Table 2). Importantly, investigators need to determine whether they are evaluating levels of early or late autophagic compartments, or autophagic flux. If the question being asked is whether a particular condition changes autophagic flux (i.e., the rate of delivery of autophagy substrates to lysosomes or the vacuole, followed by degradation), then assessment of steady-state levels of autophagosomes (e.g., by counting GFP-LC3 puncta, monitoring the amount of LC3-II without examining turnover, or by single time point electron micrographs) is not sufficient as an isolated approach. In this case it is also necessary to directly measure the flux of autophagosomes and/or autophagy cargo (e.g., in wild-type cells compared with autophagydeficient cells, the latter generated by treatment with an autophagy inhibitor or resulting from ATG gene knockdowns). Collectively, we strongly recommend the use of multiple assays whenever possible, rather than relying on the results from a single method.
As a final reminder, we stated at the beginning of this article that this set of guidelines is not meant to be a formulaic compilation of rules, because the appropriate assays depend in part on the question being asked and the system being used. Rather, these guidelines are presented primarily to emphasize key issues that need to be addressed such as the difference between measuring autophagy components, and flux or substrate clearance; they are not meant to constrain imaginative approaches to monitoring autophagy. Indeed, it is hoped that new methods for monitoring autophagy will continue to be developed, and new findings may alter our view of the current assays. Similar to the process of autophagy, this is a dynamic field, and we need to remain flexible in the standards we apply.

Table 2. Recommended methods for monitoring autophagy

Method	Description
1. Electron microscopy	Quantitative electron microscopy, immuno-TEM; monitor autophagosome number, volume
2. Atg8/LC3 western blotting	The analysis is performed in the absence and presence of lysosomal protease or fusion inhibitors to monitor flux; an increase in the LC3-II amount in the presence of the inhibitor is usually indicative of flux
3. GFP-Atg8/LC3 lysosomal delivery and western blot	± lysosomal fusion or proteolysis degradation inhibitors; the generation of free GFP indicates lysosomal/vacuolar delivery
4. GFP-Atg8/LC3 fluorescence microscopy	Fluorescence microscopy, FACS to monitor vacuolar/lysosomal localization. Also, increase in punctate GFPAtg8/LC3 or Atg18/WIPI
5. Tandem mRFP/mCherry-GFP fluorescence	Flux can be monitored as a decrease in microscopy, Rosella green/red (yellow) fluorescence (phagophores, autophagosomes) and an increase in red fluorescence (autolysosomes)
6. p62 and related LC3 binding protein turnover	The amount of p62 increases when autophagy is inhibited and decreases when autophagy is induced
7. MTOR, AMPK and Atg1/ULK1 kinase activity	western blot, immunoprecipitation or kinase assays
8. WIPI fluorescence microscopy	Quantitative fluorescence analysis using endogenous WIPI proteins, or GFP- or Myc-tagged versions. Suitable for highthroughput imaging procedures.
9. Transcriptional and translational regulation	northern blot, or qRT-PCR, autophagy-dedicated microarray
10. Autophagic protein degradation	Turnover of long-lived proteins to monitor flux
11. Pex14-GFP, GFP-Atg8, Om45-GFP,	A range of assays can be used to monitor mitoPho8D60 selective types of autophagy. These typically involve proteolytic maturation of a resident enzyme or degradation of a chimera, which can be followed enzymatically or by western blot
12. Autophagic sequestration assays	Lysosomal accumulation by biochemical or multilabel fluorescence techniques, and TEM with a maturation inhibitor
13. Turnover of autophagic compartments	Electron microscopy with morphometry/stereology
14. Autophagosome-lysosome colocalization	Fluorescence microscopy and dequenching assay

15. Sequestration and processing assays Chimeric RFP fluorescence and processing, in plants and light and electron microscopy
16. Tissue fractionation Centrifugation, western blot and electron microscopy
17. Degradation of endogenous lipofuscin Fluorescence microscopy

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References

1. Klionsky DJ, Abeliovich H, Agostinis P, Agrawal DK, Aliev G, Askew DS, et al. Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes. Autophagy 2008; 4:151-75; PMID:18188003
2. Klionsky DJ. Autophagy: from phenomenology to molecular understanding in less than a decade. Nat Rev Mol Cell Biol 2007; 8:931-7; PMID:17712358;http://dx.doi.org/10.1038/nrm2245
3. Klionsky DJ, Cuervo AM, Seglen PO. Methods for monitoring autophagy from yeast to human.Autophagy 2007; 3:181-206; PMID:17224625
4. Mizushima N. Methods for monitoring autophagy. Int J Biochem Cell Biol 2004; 36:2491-502; PMID: 15325587; http://dx.doi.org/10.1016/j.biocel.2004.02.005
5. Mizushima N, Yoshimori T, Levine B. Methods in mammalian autophagy research. Cell 2010; 140:313-26; PMID:20144757; http://dx.doi.org/10.1016/j.cell.2010.01.028
6. Rubinsztein DC, Cuervo AM, Ravikumar B, Sarkar S, Korolchuk V, Kaushik S, et al. In search of an "autophagomometer". Autophagy 2009; 5:585-9; PMID:19411822; http://dx.doi.org/10.4161/auto.5.5.8823
7. Klionsky DJ. The autophagosome is overrated! Autophagy 2011; 7:353-4; PMID:21258205; http://dx.doi.org/10.4161/auto.7.4.14730
8. Eskelinen E-L, Reggiori F, Baba M, Kovács AL, Seglen PO. Seeing is believing: the impact of electron microscopy on autophagy research. Autophagy 2011; 7:935-56; PMID:21566462; http://dx.doi.org/10.4161/auto.7.9.15760
9. Seglen PO. Regulation of autophagic protein degradation in isolated liver cells. In: Glaumann H and Ballard FJ, eds. Lysosomes: Their Role in Protein Breakdown.London: Academic Press, 1987:369-414.
10. De Duve C, Wattiaux R. Functions of lysosomes. Annu Rev Physiol 1966; 28:435-92; PMID:5322983; http://dx.doi.org/10.1146/annurev.ph.28.030166.002251
11. Dunn WA, Jr.. Autophagy and related mechanisms of lysosome-mediated protein degradation. Trends Cell Biol 1994; 4:139-43; PMID:14731737; http://dx.doi.org/10.1016/0962-8924(94)90069-8
12. Gordon PB, Seglen PO. Prelysosomal convergence of autophagic and endocytic pathways. Biochem Biophys Res Commun 1988; 151:40-7; PMID:3126737;http://dx.doi.org/10.1016/0006-291X(88)90556-6
13. Dice JF. Artophagy: the art of autophagy–macroautophagy. Interview by Daniel J. Klionsky. Autophagy 2010; 6:320-1; PMID:20118653; http://dx.doi.org/10.4161/auto.6.3.11263
14. Kovács J, Fellinger E, Kárpáti AP, Kovács AL, László L, Réz G. Morphometric evaluation of the turnover of autophagic vacuoles after treatment with Triton X-100 and vinblastine in murine pancreatic acinar and seminal vesicle epithelial cells. Virchows Arch B Cell Pathol Incl Mol Pathol 1987; 53:183-90; PMID: 2888237; http://dx.doi.org/10.1007/BF02890242
15. Kovács J, Fellinger E, Kárpáti PA, Kovács AL, László L. The turnover of autophagic vacuoles: evaluation by quantitative electron microscopy. Biomed BiochimActa 1986; 45:1543-7; PMID:3579875
16. Kovács J, László L, Kovács AL. Regression of autophagic vacuoles in pancreatic acinar, seminal vesicle epithelial, and liver parenchymal cells: a comparative morphometric study of the effect of vinblastine and leupeptin followed by cycloheximide treatment. Exp Cell Res 1988; 174: 244-51; PMID:3335225; http://dx.doi.org/10.1016/0014-4827(88)90158-9
17. Chu CT. Autophagic stress in neuronal injury and disease. J Neuropathol Exp Neurol 2006; 65:423-32; PMID:16772866; http://dx.doi.org/10.1097/01.jnen.0000229233.75253.be
18. Fass E, Shvets E, Degani I, Hirschberg K, Elazar Z. Microtubules support production of starvation induced autophagosomes but not their targeting and fusion with lysosomes. J Biol Chem 2006; 281:36303-16; PMID:16963441; http://dx.doi.org/10.1074/jbc.M607031200
19. Kovács AL, Reith A, Seglen PO. Accumulation of autophagosomes after inhibition of hepatocytic protein degradation by vinblastine, leupeptin or a lysosomotropic amine. Exp Cell Res 1982; 137:191-201; PMID:7056284; http://dx.doi.org/10.1016/0014-4827(82)90020-9
20. Tóth S, Nagy K, Pálfia Z, Réz G. Cellular autophagic capacity changes during azaserine-induced tumour progression in the rat pancreas. Up-regulation in all premalignant stages and down-regulation with loss of cycloheximide sensitivity of segregation along with malignant transformation. Cell Tissue Res2002; 309:409-16; PMID:12195297
21. Loos B, Engelbrecht AM. Cell death: a dynamic response concept. Autophagy 2009; 5:590-603; PMID:19363298; http://dx.doi.org/10.4161/auto.5.5.8479
22. Seglen PO, Gordon PB, Grinde B, Solheim A, Kovács AL, Poli A. Inhibitors and pathways of hepatocytic protein degradation. Acta Biol Med Ger 1981;40:1587-98; PMID:7342604
23. Ktistakis NT, Andrews S, Long J. What is the advantage of a transient precursor in autophagosome biogenesis? Autophagy 2011; 7:118-22; PMID:20935487; http://dx.doi.org/10.4161/auto.7.1.13697
24. Kovács AL, Réz G, Pálfia Z, Kovács J. Autophagy in the epithelial cells of murine seminal vesicle in vitro. Formation of large sheets of nascent isolation membranes, sequestration of the nucleus and inhibition by wortmannin and 3-ethyladenine. Cell Tissue Res 2000; 302:253-61; PMID:11131136
25. Mizushima N, Yoshimori T. How to interpret LC3 immunoblotting. Autophagy 2007; 3:542-5; PMID:17611390
26. Nishida Y, Arakawa S, Fujitani K, Yamaguchi H, Mizuta T, Kanaseki T, et al. Discovery of Atg5/Atg7-independent alternative macroautophagy. Nature 2009; 461:654-8; PMID:19794493; http://dx.doi.org/10.1038/nature08455
27. Kanki T, Kang D, Klionsky DJ. Monitoring mitophagy in yeast: the Om45-GFP processing assay. Autophagy 2009; 5:1186-9; PMID:19806021; http://dx.doi.org/10.4161/auto.5.8.9854

28. Grandér D, Kharaziha P, Laane E, Pokrovskaja K, Panaretakis T. Autophagy as the main means of cytotoxicity by glucocorticoids in hematological malignancies. Autophagy 2009; 5:1198-200; PMID:19855186; http://dx.doi.org/10.4161/auto.5.8.10122
29. Welter E, Thumm M, Krick R. Quantification of nonselective bulk autophagy in S. cerevisiae using Pgk1-GFP. Autophagy 2010; 6:794-7; PMID: 20523132; http://dx.doi.org/10.4161/auto.6.6.12348 30. Raju D, Jones NL. Methods to monitor autophagy in H. pylori vacuolating cytotoxin A (VacA)-treated cells. Autophagy 2010; 6:138-43; PMID:19875940; http://dx.doi.org/10.4161/auto.6.1.10222
31. Geng J, Klionsky DJ. Determining Atg protein stoichiometry at the phagophore assembly site by fluorescence microscopy. Autophagy 2010; 6:144-7; PMID:20131413; http://dx.doi.org/10.4161/auto.6.1.10249
32. Swanlund JM, Kregel KC, Oberley TD. Investigating autophagy: quantitative morphometric analysis using electron microscopy. Autophagy 2010; 6:270-7; PMID:19923921; http://dx.doi.org/10.4161/auto.6.2.10439
33. Zhang J, Ney PA. Reticulocyte mitophagy: monitoring mitochondrial clearance in a mammalian model. Autophagy 2010; 6:405-8; PMID:20200480; http://dx.doi.org/10.4161/auto.6.3.11245
34. Seglen PO, Brinchmann MF. Purification of autophagosomes from rat hepatocytes. Autophagy 2010; 6:542-7; PMID:20505360; http://dx.doi.org/10.4161/auto.6.4.11272
35. He C, Klionsky DJ. Analyzing autophagy in zebrafish. Autophagy 2010; 6:642-4; PMID:20495344; http://dx.doi.org/10.4161/auto.6.5.12092
36. Calvo-Garrido J, Carilla-Latorre S, Mesquita A, Escalante R. A proteolytic cleavage assay to monitor autophagy in Dictyostelium discoideum. Autophagy 2011; 7:1063-8; PMID:21876387; http://dx.doi.org/10.4161/auto.7.9.16629
37. Xu F, Liu XH, Zhuang FL, Zhu J, Lin FC. Analyzing autophagy in Magnaporthe oryzae. Autophagy 2011; 7:525-30; PMID:21317549; http://dx.doi.org/10.4161/auto.7.5.15020
38. Klionsky DJ. Autophagy: Lower Eukaryotes and Non-Mammalian Systems, Part A. Amsterdam: Academic Press/Elsevier, 2008.
39. Klionsky DJ. Autophagy in Disease and Clinical Applications, Part C. Amsterdam: Academic Press/Elsevier, 2008.
40. Klionsky DJ. Autophagy in Mammalian Systems, Part B.Amsterdam: Academic Press/Elsevier, 2008.
41. Zhu J, Dagda RK, Chu CT. Monitoring mitophagy in neuronal cell cultures. Methods Mol Biol 2011; 793:325-39; PMID:21913110; http://dx.doi.org/10.1007/978-1-61779-328-8_21
42. http://www.landesbioscience.com/journals/autophagy/protocols/
43. Ylä-Anttila P, Vihinen H, Jokitalo E, Eskelinen E-L. Monitoring autophagy by electron microscopy in Mammalian cells. Methods Enzymol 2009; 452:143-64; PMID:19200881; http://dx.doi.org/10.1016/S0076-6879(08)03610-0
44. Eskelinen E-L. Maturation of autophagic vacuoles in Mammalian cells. Autophagy 2005; 1:1-10; PMID:16874026; http://dx.doi.org/10.4161/auto.1.1.1270
45. Eskelinen E-L. To be or not to be? Examples of incorrect identification of autophagic compartments in conventional transmission electron microscopy of mammalian cells. Autophagy 2008; 4:257-60; PMID:17986849
46. Eskelinen E-L, Kovács AL. Double membranes vs. lipid bilayers, and their significance for correct identification of macroautophagic structures. Autophagy 2011; 7:931-2; PMID:21642767; http://dx.doi.org/10.4161/auto.7.9.16679
47. Eskelinen E-L. Fine structure of the autophagosome.
In: Deretic V, ed. Autophagosome and Phagosome. Totowa, NJ: Humana Press, 2008:11-28.
48. Berg TO, Fengsrud M, Strømhaug PE, Berg T, Seglen PO. Isolation and characterization of rat liver amphisomes. Evidence for fusion of autophagosomes with both early and late endosomes. J Biol Chem 1998; 273:21883-92; PMID:9705327; http://dx.doi.org/10.1074/jbc.273.34.21883
49. Eskelinen E-L. Macroautophagy in mammalian cells. In: Saftig P, ed. Lysosomes. Georgetown, TX:LandesBioscience/Eurekah.com, 2005.
50. Rabouille C, Strous GJ, Crapo JD, Geuze HJ, Slot JW. The differential degradation of two cytosolic proteins as a tool to monitor autophagy in hepatocytes by immunocytochemistry. J Cell Biol 1993; 120:897-908; PMID:8432730; http://dx.doi.org/10.1083/jcb.120.4.897
51. Kovács AL, Pálfia Z, Réz G, Vellai T, Kovács J. Sequestration revisited: integrating traditional electron microscopy, de novo assembly and new results. Autophagy 2007; 3:655-62; PMID:17603297
52. Gao W, Kang JH, Liao Y, Ding WX, Gambotto AA, Watkins SC, et al. Biochemical isolation and characterization of the tubulovesicular LC3-positive autophagosomal compartment. J Biol Chem 2010; 285:1371-83; PMID:19910472; http://dx.doi.org/10. 1074/jbc.M109.054197
53. Baba M, Osumi M, Ohsumi Y. Analysis of the membrane structures involved in autophagy in yeast by freeze-replica method. Cell Struct Funct 1995; 20:465-71; PMID:8825067; http://dx.doi.org/10.1247/csf.20.465
54. Réz G, Meldolesi J. Freeze-fracture of drug-induced autophagocytosis in the mouse exocrine pancreas. Lab Invest 1980; 43:269-77; PMID:7401637
55. Punnonen E-L, Pihakaski K, Mattila K, Lounatmaa K, Hirsimäki P. Intramembrane particles and filipin labelling on the membranes of autophagic vacuoles and lysosomes in mouse liver. Cell Tissue Res 1989; 258:269-76; PMID:2582478; http://dx.doi.org/10.1007/BF00239447
56. Fengsrud M, Erichsen ES, Berg TO, Raiborg C, Seglen PO. Ultrastructural characterization of the delimiting membranes of isolated autophagosomes and amphisomes by freeze-fracture electron microscopy. Eur J Cell Biol 2000; 79:871-82; PMID:11152279; http://dx.doi.org/10.1078/0171-9335-00125
57. Rao VA, Klein SR, Bonar SJ, Zielonka J, Mizuno N, Dickey JS, et al. The antioxidant transcription factor Nrf2 negatively regulates autophagy and growth arrest induced by the anticancer redox agent mitoquinone. J Biol Chem 2010; 285:34447-59; PMID:20805228; http://dx.doi.org/10.1074/jbc.M110.133579
58. Krick R, Mühe Y, Prick T, Bredschneider M, Bremer S, Wenzel D, et al. Piecemeal microautophagy of the nucleus: genetic and morphological traits. Autophagy 2009; 5:270-2; PMID:19182523; http://dx.doi.org/10.4161/auto.5.2.7639
59. Meschini S, Condello M, Calcabrini A, Marra M, Formisano G, Lista P, et al. The plant alkaloid voacamine induces apoptosis-independent autophagic cell death on both sensitive and multidrug resistant human osteosarcoma cells. Autophagy 2008; 4:1020-33; PMID:18838862
60. Proikas-Cezanne T, Robenek H. Freeze-fracture replica immunolabelling reveals human WIPI-1 and WIPI-2 as membrane proteins of autophagosomes. J Cell Mol Med 2011; 15:2007-10; PMID:21564513;http://dx.doi.org/10.1111/j.1582-4934.2011.01339.x
61. Kovács J, Réz G, Kovács AL, Csák J, Zboray G. Autophagocytosis: freeze-fracture morphology, effects of vinblastine and influence of transcriptional and translational inhibitors. Acta Biol Med Ger 1982;41:131-5; PMID:7113544
62. Hirsimäki Y, Hirsimäki P, Lounatmaa K. Vinblastineinduced autophagic vacuoles in mouse liver and Ehrlich ascites tumor cells as assessed by freeze-fracture electron microscopy. Eur J Cell Biol 1982; 27:298-301; PMID:7117273
63. Cheong H, Yorimitsu T, Reggiori F, Legakis JE, Wang C-W, Klionsky DJ. Atg17 regulates the magnitude of the autophagic response. Mol Biol Cell 2005; 16: 3438-53; PMID:15901835; http://dx.doi.org/10.1091/mbc.E04-10-0894
64. Xie Z, Nair U, Klionsky DJ. Atg8 controls phagophore expansion during autophagosome formation. Mol Biol Cell 2008; 19:3290-8; PMID:18508918; http://dx.doi.org/10.1091/mbc.E07-12-1292
65. Sigmond T, Fehér J, Baksa A, Pásti G, Pálfia Z, Takács-Vellai K, et al. Qualitative and quantitative characterization of autophagy in Caenorhabditis elegans by electron microscopy. Methods Enzymol 2008; 451:467-91; PMID:19185736; http://dx.doi.org/10. 1016/S0076-6879(08)03228-X

66. Kovács AL, Vellai T, Müller F. Autophagy in Caenorhabditis elegans. In: Klionsky DJ, ed. Autophagy. Georgetown, Texas: Landes Bioscience, 2004:217-23.
67. Weibel ER. Practical Methods for Biological Morphometry. Academic Press, New York, 1979.
68. Williams MA. Quantitative methods in biology: Practical methods in electron microscopy. Amsterdam, New York, Oxford: North-Holland Publishing Company, 1977.
69. Howard V, Reed MG. Unbiased stereology; three dimensional measurement in microscopy. U BiosScientific Publishers, 1998.
70. Punnonen EL, Reunanen H. Effects of vinblastine, leucine, and histidine, and 3-methyladenine on autophagy in Ehrlich ascites cells. Exp Mol Pathol 1990; 52:87-97; PMID:2307216; http://dx.doi.org/10.1016/0014-4800(90)90061-H
71. Kovács AL, László L, Fellinger E, Jakab A, Orosz A, Réz G, et al. Combined effects of fasting and vinblastine treatment on serum insulin level, the size of autophagic-lysosomal compartment, protein content and lysosomal enzyme activities of liver and exocrine pancreatic cells of the mouse. Comp Biochem Physiol B 1989; 94:505-10; PMID:2695284; http://dx.doi.org/10.1016/0305-0491(89)90189-2
72. Shelburne JD, Arstila AU, Trump BF. Studies on cellular autophagocytosis. The relationship of autophagocytosis to protein synthesis and to energy metabolism in rat liver and flounder kidney tubules in vitro. Am J Pathol 1973; 73:641-70; PMID:4767257
73. Reyes FC, Chung T, Holding D, Jung R, Vierstra R, Otegui MS. Delivery of prolamins to the protein storage vacuole in maize aleurone cells. Plant Cell 2011; 23:769-84; PMID:21343414; http://dx.doi.org/10.1105/tpc.110.082156
74. Dunn WA, Jr., Cregg JM, Kiel JAKW, van der Klei IJ, Oku M, Sakai Y, et al. Pexophagy: the selective autophagy of peroxisomes. Autophagy 2005; 1:75-83; PMID:16874024; http://dx.doi.org/10.4161/auto.1.2.1737
75. Wang K, Klionsky DJ. Mitochondria removal by autophagy. Autophagy 2011; 7:297-300; PMID: 21252623; http://dx.doi.org/10.4161/auto.7.3.14502
76. Bélanger M, Rodrigues PH, Dunn WA, Jr., Progulske-Fox A. Autophagy: a highway for Porphyromonas gingivalis in endothelial cells. Autophagy 2006; 2:165-70; PMID:16874051
77. Birmingham CL, Brumell JH. Autophagy recognizes intracellular Salmonella enterica serovar Typhimurium in damaged vacuoles. Autophagy 2006; 2:156-8;PMID:16874057
78. Colombo MI, Gutierrez MG, Romano PS. The two faces of autophagy: Coxiella and Mycobacterium. Autophagy 2006; 2:162-4; PMID:16874070
79. Ogawa M, Sasakawa C. Shigella and autophagy.Autophagy 2006; 2:171-4; PMID:16874102
80. Vergne I, Singh S, Roberts E, Kyei G,Master S, Harris J, et al. Autophagy in immune defense against Mycobacterium tuberculosis. Autophagy 2006; 2:175-8; PMID:16874111
81. Yoshimori T. Autophagy vs. Group A Streptococcus.Autophagy 2006; 2:154-5; PMID:16874113
82. Lynch-Day MA, Klionsky DJ. The Cvt pathway as a model for selective autophagy. FEBS Lett 2010; 584:1359-66; PMID:20146925; http://dx.doi.org/10.1016/j.febslet.2010.02.013
83. Birmingham CL, Canadien V, Gouin E, Troy EB, Yoshimori T, Cossart P, et al. Listeria monocytogenes evades killing by autophagy during colonization of host cells. Autophagy 2007; 3:442-51; PMID:17568179 84. Klionsky DJ. Protein transport from the cytoplasm into the vacuole. J Membr Biol 1997; 157:105-15; PMID:9151652; http://dx.doi.org/10.1007/s002329900220
85. Dini L, Pagliara P, Carlà EC. Phagocytosis of apoptotic cells by liver: a morphological study. Microsc Res Tech 2002; 57:530-40; PMID:12112436; http://dx.doi.org/10.1002/jemt.10107
86. Kroemer G, El-Deiry WS, Golstein P, Peter ME, Vaux D, Vandenabeele P, et al & Nomenclature Committee on Cell Death. Classification of cell death: recommendations of the Nomenclature Committee on Cell Death. Cell Death Differ 2005; 12(Suppl 2):1463-7; PMID:16247491; http://dx.doi.org/10.1038/sj.cdd.4401724
87. Réz G, Pálfia Z, Fellinger E. Occurrence and inhibition by cycloheximide of apoptosis in vinblastine-treated murine pancreas. A role for autophagy?Acta Biol Hung 1991; 42:133-40; PMID:1844306
88. Giammarioli AM, Gambardella L, Barbati C, Pietraforte D, Tinari A, Alberton M, et al. Differential effects of the glycolysis inhibitor 2-deoxy-D-glucose on the activity of pro-apoptotic agents in metastatic melanoma cells, and induction of a cytoprotective autophagic response. Int J Cancer 2012; In press; PMID:21913183
89. Mayhew TM. Quantitative immunoelectron microscopy: alternative ways of assessing subcellular patterns of gold labeling. Methods Mol Biol 2007; 369:309-29; PMID:17656757; http://dx.doi.org/10.1007/978-1-59745-294-6_15
90. Mayhew TM, Lucocq JM, Griffiths G. Relative labelling index: a novel stereological approach to test for non-random immunogold labelling of organelles and membranes on transmission electron microscopy thin sections. J Microsc 2002; 205:153-64; PMID: 11879430; http://dx.doi.org/10.1046/j.0022-2720.2001.00977.x
91. Schmid D, Pypaert M, Münz C. Antigen-loading compartments for major histocompatibility complex class II molecules continuously receive input from autophagosomes. Immunity 2007; 26:79-92; PMID: 17182262; http://dx.doi.org/10.1016/j.immuni.2006.10.018
92. Kovács J. Regression of autophagic vacuoles in seminal vesicle cells following cycloheximide treatment. Exp Cell Res 1983; 144:231-4; PMID:6840208; http://dx.doi.org/10.1016/0014-4827(83)90460-3
93. Réz G, Csák J, Fellinger E, László L, Kovács AL, Oliva O, et al. Time course of vinblastine-induced autophagocytosis and changes in the endoplasmic reticulum in murine pancreatic acinar cells: a morphometric and biochemical study. Eur J Cell Biol 1996; 71:341-50;PMID:8980904
94. Kovács AL, Grinde B, Seglen PO. Inhibition of autophagic vacuole formation and protein degradation by amino acids in isolated hepatocytes. Exp Cell Res 1981; 133:431-6; PMID:7238609; http://dx.doi.org/10.1016/0014-4827(81)90336-0
95. Mortimore GE, Hutson NJ, Surmacz CA. Quantitative correlation between proteolysis and macro- and microautophagy in mouse hepatocytes during starvation and refeeding. Proc Natl Acad Sci U S A 1983; 80:2179-83; PMID:6340116; http://dx.doi.org/10.1073/pnas.80.8. 2179
96. Mortimore GE, Lardeux BR, Adams CE. Regulation of microautophagy and basal protein turnover in rat liver. Effects of short-term starvation. J Biol Chem 1988; 263:2506-12; PMID:3257493
97. Zhu JH, Horbinski C, Guo F, Watkins S, Uchiyama Y, Chu CT. Regulation of autophagy by extracellular signal-regulated protein kinases during 1-methyl-4-phenylpyridinium-induced cell death. Am J Pathol 2007; 170:75-86; PMID:17200184; http://dx.doi.org/10.2353/ajpath.2007.060524
98. Bjørkøy G, Lamark T, Brech A, Outzen H, Perander M, Øvervatn A, et al. p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death. J Cell Biol 2005; 171:603-14; PMID:16286508; http://dx.doi.org/10.1083/jcb.200507002
99. Razi M, Tooze SA. Correlative light and electron microscopy. Methods Enzymol 2009; 452:261-75; PMID:19200888; http://dx.doi.org/10.1016/S0076-6879(08)03617-3
100. Shu X, Lev-Ram V, Deerinck TJ, Qi Y, Ramko EB, Davidson MW, et al. A genetically encoded tag for correlated light and electron microscopy of intact cells, tissues, and organisms. PLoS Biol 2011; 9:e1001041; PMID:21483721; http://dx.doi.org/10.1371/journal.pbio.1001041
101. Castillo K, Rojas-Rivera D, Lisbona F, Caballero B, Nassif M, Court FA, et al. BAX inhibitor-1 regulates autophagy by controlling the IRE1a branch of the unfolded protein response. EMBO J 2011; 30:4465-78; PMID:21926971; http://dx.doi.org/10.1038/emboj.2011.318
102. Ylä-Anttila P, Vihinen H, Jokitalo E, Eskelinen E-L. 3D tomography reveals connections between the phagophore and endoplasmic reticulum. Autophagy 2009; 5:1180-5; PMID:19855179; http://dx.doi.org/10.4161/auto.5.8.10274

103. Hayashi-Nishino M, Fujita N, Noda T, Yamaguchi A, Yoshimori T, Yamamoto A. Electron tomography reveals the endoplasmic reticulum as a membrane source for autophagosome formation. Autophagy 2010; 6:301-3; PMID:20104025; http://dx.doi.org/10.4161/auto.6.2.11134
104. Schneider EM, Lorezn M, Walther P. Autophagy as a hallmark of hemophagocytic diseases In: Gorbunov N, ed. Autophagy: Principles, Regulation and Roles in Disease: Nova Science Publishers, 2012.
105. Massey AC, Kaushik S, Sovak G, Kiffin R, Cuervo AM. Consequences of the selective blockage of chaperone-mediated autophagy. Proc Natl Acad Sci U S A 2006; 103:5805-10; PMID:16585521; http://dx.doi.org/10.1073/pnas.0507436103
106. Bosco DA, Morfini G, Karabacak NM, Song Y, Gros-Louis F, Pasinelli P, et al. Wild-type and mutant SOD1 share an aberrant conformation and a common pathogenic pathway in ALS. Nat Neurosci 2010; 13:1396-403; PMID:20953194; http://dx.doi.org/10.1038/nn.2660
107. Sou YS, Tanida I, Komatsu M, Ueno T, Kominami E. Phosphatidylserine in addition to phosphatidylethanolamine is an in vitro target of the mammalian Atg8 modifiers, LC3, GABARAP, and GATE-16. J Biol Chem 2006; 281:3017-24; PMID:16303767; http://dx.doi.org/10.1074/jbc.M505888200
108. Le Grand JN, Chakrama FZ, Seguin-Py S, Fraichard A, Delage-Mourroux R, Jouvenot M, et al. GABARAPL1 (GEC1): original or copycat? Autophagy 2011; 7:1098-107; PMID:21597319; http://dx.doi.org/10.4161/auto.7.10.15904
109. Kabeya Y, Mizushima N, Yamamoto A, Oshitani-Okamoto S, Ohsumi Y, Yoshimori T. LC3, GABARAP and GATE16 localize to autophagosomal membrane depending on form-II formation. J Cell Sci 2004; 117:2805-12; PMID:15169837; http://dx.doi.org/10.1242/jcs.01131
110. Weidberg H, Shvets E, Shpilka T, Shimron F, Shinder V, Elazar Z. LC3 and GATE-16/GABARAP subfamilies are both essential yet act differently in autophagosome biogenesis. EMBO J 2010; 29:1792-802; PMID: 20418806; http://dx.doi.org/10.1038/emboj.2010.74
111. Kovsan J, Blüher M, Tarnovscki T, Klöting N, Kirshtein B, Madar L, et al. Altered autophagy in human adipose tissues in obesity. J Clin Endocrinol Metab 2011; 96:E268-77; PMID:21047928; http://dx.doi.org/10.1210/jc.2010-1681
112. Tanida I, Minematsu-Ikeguchi N, Ueno T, Kominam E. Lysosomal turnover, but not a cellular level, of endogenous LC3 is a marker for autophagy. Autophagy 2005; 1:84-91; PMID:16874052; http://dx.doi.org/10.4161/auto.1.2.1697
113. Huang W-P, Scott SV, Kim J, Klionsky DJ. The itinerary of a vesicle component, Aut7p/Cvt5p, terminates in the yeast vacuole via the autophagy/Cvt pathways. J Biol Chem 2000; 275:5845-51; PMID: 10681575; http://dx.doi.org/10.1074/jbc.275.8.5845
114. Cai Q, Lu L, Tian J-H, Zhu Y-B, Qiao H, Sheng Z-H. Snapin-regulated late endosomal transport is critical for efficient autophagy-lysosomal function in neurons. Neuron 2010; 68:73-86; PMID:20920792; http://dx.doi.org/10.1016/j.neuron.2010.09.022
115. Castino R, Fiorentino I, Cagnin M, Giovia A, Isidoro C. Chelation of lysosomal iron protects dopaminergic SH-SY5Y neuroblastoma cells from hydrogen peroxide toxicity by precluding autophagy and Akt dephosphorylation. Toxicol Sci 2011; 123:523-41; PMID: 21742779; http://dx.doi.org/10.1093/toxsci/kfr179
116. Michiorri S, Gelmetti V, Giarda E, Lombardi F, Romano F, Marongiu R, et al. The Parkinsonassociated protein PINK1 interacts with Beclin1 and promotes autophagy. Cell Death Differ 2010; 17:962-74; PMID:20057503
117. Ahlberg J, Berkenstam A, Henell F, Glaumann H. Degradation of short and long lived proteins in isolated rat liver lysosomes. Effects of pH, temperature, and proteolytic inhibitors. J Biol Chem 1985; 260:5847-54; PMID:3988775
118. McLeland CB, Rodriguez J, Stern ST. Autophagy monitoring assay: qualitative analysis of MAP LC3-I to II conversion by immunoblot. Methods Mol Biol 2011; 697:199-206; PMID:21116969; http://dx.doi.org/10.1007/978-1-60327-198-1_21
119. Chakrama FZ, Seguin-Py S, Le Grand JN, Fraichard A, Delage-Mourroux R, Despouy G, et al. GABARAPL1 (GEC1) associates with autophagic vesicles. Autophagy 2010; 6:495-505; PMID: 20404487; http://dx.doi.org/10.4161/auto.6.4.11819
120. Kim J, Huang W-P, Klionsky DJ. Membrane recruitment of Aut7p in the autophagy and cytoplasm to vacuole targeting pathways requires Aut1p, Aut2p, and the autophagy conjugation complex. J Cell Biol 2001; 152:51-64; PMID:11149920; http://dx.doi.org/10.1083/jcb.152.1.51
121. Shu CW, Drag M, Bekes M, Zhai D, Salvesen GS, Reed JC. Synthetic substrates for measuring activity of autophagy proteases: autophagins (Atg4). Autophagy 2010; 6:936-47; PMID:20818167; http://dx.doi.org/10.4161/auto.6.7.13075
122. Li M, Chen X, Ye Q-Z, Vogt A, Yin X-M. A highthroughput FRET-based assay for determination of Atg4 activity. Autophagy 2012; 8; In press; PMID:22302004
123. Ketteler R, Seed B. Quantitation of autophagy by luciferase release assay. Autophagy 2008; 4:801-6;PMID:18641457
124. Li M, Hou Y, Wang J, Chen X, Shao ZM, Yin X-M. Kinetics comparisons of mammalian Atg4 homologues indicate selective preferences toward diverse Atg8 substrates. J Biol Chem 2011; 286:7327-38; PMID: 21177865; http://dx.doi.org/10.1074/jbc.M110. 199059
125. Klionsky DJ. For the last time, it is GFP-Atg8, not Atg8-GFP (and the same goes for LC3). Autophagy 2011; 7:1093-4; PMID:21993240; http://dx.doi.org/10.4161/auto.7.10.15492
126. Mizushima N, Yamamoto A, Matsui M, Yoshimori T, Ohsumi Y. In vivo analysis of autophagy in response to nutrient starvation using transgenic mice expressing a fluorescent autophagosome marker. Mol Biol Cell 2004; 15:1101-11; PMID:14699058; http://dx.doi. org/10.1091/mbc.E03-09-0704
127. Castino R, Lazzeri G, Lenzi P, Bellio N, Follo C, Ferrucci M, et al. Suppression of autophagy precipitates neuronal cell death following low doses of methamphetamine. J Neurochem 2008; 106:1426-39; PMID:18489716; http://dx.doi.org/10.1111/j.1471-4159.2008.05488.x
128. Matsui Y, Takagi H, Qu X, Abdellatif M, Sakoda H, Asano T, et al. Distinct roles of autophagy in the heart during ischemia and reperfusion: roles of AMP-activated protein kinase and Beclin 1 in mediating autophagy. Circ Res 2007; 100:914-22; PMID:17332429; http://dx.doi.org/10.1161/01.RES.0000261924.76669.36
129. Suzuki K, Kirisako T, Kamada Y, Mizushima N, Noda T, Ohsumi Y. The pre-autophagosomal structure organized by concerted functions of APG genes is essential for autophagosome formation. EMBO J 2001; 20:5971-81; PMID:11689437; http://dx.doi. org/10.1093/emboj/20.21.5971
130. Hanson HH, Kang S, Fernández-Monreal M, Oung T, Yildirim M, Lee R, et al. LC3-dependent intracellular membrane tubules induced by gamma-protocadherins A3 and B2: a role for intraluminal interactions. J Biol Chem 2010; 285:20982-92; PMID:20439459; http://dx.doi.org/10.1074/jbc.M109.092031
131. Sanjuan MA, Dillon CP, Tait SW, Moshiach S, Dorsey F, Connell S, et al. Toll-like receptor signaling in macrophages links the autophagy pathway to phagocytosis. Nature 2007; 450:1253-7; PMID: 18097414; http://dx.doi.org/10.1038/nature06421
132. Sanjuan MA, Milasta S, Green DR. Toll-like receptor signaling in the lysosomal pathways. Immunol Rev 2009; 227:203-20; PMID:19120486; http://dx.doi. org/10.1111/j.1600-065X.2008.00732.x
133. Florey O, Kim SE, Sandoval CP, Haynes CM, Overholtzer M. Autophagy machinery mediates macroendocytic processing and entotic cell death by targeting single membranes. Nat Cell Biol 2011; 13:1335-43; PMID:22002674; http://dx.doi.org/10.1038/ncb2363
134. Martinez J, Almendinger J, Oberst A,Ness R, Dillon CP, Fitzgerald P, et al. Microtubule-associated protein 1 light chain 3 alpha (LC3)-associated phagocytosis is required for the efficient clearance of dead cells. Proc Natl Acad Sci U S A 2011; 108:17396-401; PMID:21969579; http://dx.doi.org/10.1073/pnas.1113421108
135. Gong L, Cullinane M, Treerat P, Ramm G, Prescott M, Adler B, et al. The Burkholderia pseudomallei type III secretion system and BopA are required for evasion of LC3-associated phagocytosis. PLoS One 2011; 6: e17852; PMID:21412437; http://dx.doi.org/10.1371/journal.pone.0017852

136. Dupont N, Lacas-Gervais S, Bertout J, Paz I, Freche B, Van Nhieu GT, et al. Shigella phagocytic vacuolar membrane remnants participate in the cellular response to pathogen invasion and are regulated by autophagy. Cell Host Microbe 2009; 6:137-49; PMID:19683680;http://dx.doi.org/10.1016/j.chom.2009.07.005
137. Reggiori F, Monastyrska I, Verheije MH, Calì T, Ulasli M, Bianchi S, et al. Coronaviruses Hijack the LC3-I-positive EDEMosomes, ER-derived vesicles exporting short-lived ERAD regulators, for replication. Cell Host Microbe 2010; 7:500-8; PMID:20542253;http://dx.doi.org/10.1016/j.chom.2010.05.013
138. English L, Chemali M, Duron J, Rondeau C, Laplante A, Gingras D, et al. Autophagy enhances the presentation of endogenous viral antigens on MHC class I molecules during HSV-1 infection. Nat Immunol 2009; 10:480-7; PMID:19305394; http://dx.doi.org/10.1038/ni.1720
139. Plowey ED, Cherra SJ, 3rd, Liu YJ, Chu CT. Role of autophagy in G2019S-LRRK2-associated neurite shortening in differentiated SH-SY5Y cells. J Neurochem 2008; 105:1048-56; PMID:18182054;http://dx.doi.org/10.1111/j.1471-4159.2008.05217.x
140. Nicotra G, Mercalli F, Peracchio C, Castino R, Follo C, Valente G, et al. Autophagy-active beclin-1 correlates with favourable clinical outcome in non-Hodgkin lymphomas. Mod Pathol 2010; 23:937-50; PMID: 20473282; http://dx.doi.org/10.1038/modpathol.2010. 80
141. Tanida I, Ueno T, Kominami E. LC3 and Autophagy. Methods Mol Biol 2008; 445:77-88; PMID:18425443; http://dx.doi.org/10.1007/978-1-59745-157-4_4
142. Chu CT, Plowey ED, Dagda RK, Hickey RW, Cherra SJ, 3rd, Clark RS. Autophagy in neurite injury and neurodegeneration: in vitro and in vivo models. Methods Enzymol 2009; 453:217-49; PMID:19216909; http://dx.doi.org/10.1016/S0076-6879(08)04011-1
143. He H, Dang Y, Dai F, Guo Z, Wu J, She X, et al. Post-translational modifications of three members of the human MAP1LC3 family and detection of a novel type of modification for MAP1LC3B. J Biol Chem 2003; 278:29278-87; PMID:12740394; http://dx.doi.org/10.1074/jbc.M303800200
144. Shpilka T, Weidberg H, Pietrokovski S, Elazar Z. Atg8: an autophagy-related ubiquitin-like protein family. Genome Biol 2011; 12:226; PMID:21867568; http://dx.doi.org/10.1186/gb-2011-12-7-226
145. Zois CE, Koukourakis MI. Radiation-induced autophagy in normal and cancer cells: towards novel cytoprotection and radio-sensitization policies? Autophagy 2009; 5:442-50; PMID:19164950; http://dx.doi.org/10.4161/auto.5.4.7667
146. Xin Y, Yu L, Chen Z, Zheng L, Fu Q, Jiang J, et al. Cloning, expression patterns, and chromosome localization of three human and two mouse homologues of GABA(A) receptor-associated protein. Genomics 2001; 74:408-13; PMID:11414770; http://dx.doi.org/10.1006/geno.2001.6555
147. Novak I, Kirkin V, McEwan DG, Zhang J, Wild P, Rozenknop A, et al. Nix is a selective autophagy receptor for mitochondrial clearance. EMBO Rep 2010; 11:45-51; PMID:20010802; http://dx.doi.org/10.1038/embor.2009.256
148. Gassmann M, Grenacher B, Rohde B, Vogel J. Quantifying Western blots: pitfalls of densitometry. Electrophoresis 2009; 30:1845-55; PMID:19517440;http://dx.doi.org/10.1002/elps.200800720
149. Kirisako T, Ichimura Y, Okada H, Kabeya Y, Mizushima N, Yoshimori T, et al. The reversible modification regulates the membrane-binding state of Apg8/Aut7 essential for autophagy and the cytoplasm to vacuole targeting pathway. J Cell Biol 2000; 151:263-76; PMID:11038174; http://dx.doi.org/10.1083/jcb.151.2.263
150. Chung T, Phillips AR, Vierstra RD. ATG8 lipidation and ATG8-mediated autophagy in Arabidopsis require ATG12 expressed from the differentially controlled ATG12A AND ATG12B loci. Plant J 2010; 62:483- 93; PMID:20136727; http://dx.doi.org/10.1111/j.1365-313X.2010.04166.x
151. Chung T, Suttangkakul A, Vierstra RD. The ATG autophagic conjugation system in maize: ATG transcripts and abundance of the ATG8-lipid adduct are regulated by development and nutrient availability. Plant Physiol 2009; 149:220-34; PMID:18790996; http://dx.doi.org/10.1104/pp.108.126714
152. King JS, Veltman DM, Insall RH. The induction of autophagy by mechanical stress. Autophagy 2011; 7: 1490-9; PMID:22024750; http://dx.doi.org/10.4161/auto.7.12.17924
153. Ju JS, Varadhachary AS, Miller SE, Weihl CC. Quantitation of “autophagic flux” in mature skeletal muscle. Autophagy 2010; 6:929-35; PMID: 20657169; http://dx.doi.org/10.4161/auto.6.7.12785
154. Degtyarev M, De Mazière A, Orr C, Lin J, Lee BB, Tien JY, et al. Akt inhibition promotes autophagy and sensitizes PTEN-null tumors to lysosomotropic agents. J Cell Biol 2008; 183:101-16; PMID:18838554;http://dx.doi.org/10.1083/jcb.200801099
155. Klionsky DJ, Elazar Z, Seglen PO, Rubinsztein DC. Does bafilomycin A1 block the fusion of autophagosomes with lysosomes? Autophagy 2008; 4:849-50;PMID:18758232
156. Xie R, Nguyen S, McKeehan WL, Liu L. Acetylated microtubules are required for fusion of autophagosomes with lysosomes. BMC Cell Biol 2010; 11:89; PMID:21092184; http://dx.doi.org/10.1186/1471-2121-11-89
157. González-Polo RA, Boya P, Pauleau AL, Jalil A, Larochette N, Souquère S, et al. The apoptosis/autophagy paradox: autophagic vacuolization before apoptotic death. J Cell Sci 2005; 118:3091-102;PMID:15985464; http://dx.doi.org/10.1242/jcs. 02447
158. Cuervo AM, Stefanis L, Fredenburg R, Lansbury PT, Sulzer D. Impaired degradation of mutant alphasynuclein by chaperone-mediated autophagy. Science 2004; 305:1292-5; PMID:15333840; http://dx.doi.org/10.1126/science.1101738
159. Trincheri NF, Follo C, Nicotra G, Peracchio C, Castino R, Isidoro C. Resveratrol-induced apoptosis depends on the lipid kinase activity of Vps34 and on the formation of autophagolysosomes. Carcinogenesi 2008; 29:381-9; PMID:18048384; http://dx.doi.org/10.1093/carcin/bgm271
160. Sarkar S, Ravikumar B, Rubinsztein DC. Autophagic clearance of aggregate-prone proteins associated with neurodegeneration. Methods Enzymol 2009; 453:83-110; PMID:19216903; http://dx.doi.org/10.1016/S0076-6879(08)04005-6
161. Shintani T, Klionsky DJ. Cargo proteins facilitate the formation of transport vesicles in the cytoplasm to vacuole targeting pathway. J Biol Chem 2004; 279:29889-94; PMID:15138258; http://dx.doi.org/10.1074/jbc.M404399200
162. Karim MR, Kanazawa T, Daigaku Y, Fujimura S, Miotto G, Kadowaki M. Cytosolic LC3 ratio as a sensitive index of macroautophagy in isolated rat hepatocytes and H4-II-E cells. Autophagy 2007;3:553-60; PMID:17617739
163. Farkas T, Høyer-Hansen M, Jäättelä M. Identification of novel autophagy regulators by a luciferase-based assay for the kinetics of autophagic flux. Autophagy 2009; 5:1018-25; PMID:19652534; http://dx.doi.org/10.4161/auto.5.7.9443
164. Frankel LB, Wen J, Lees M, Høyer-Hansen M, Farkas T, Krogh A, et al. microRNA-101 is a potent inhibitor of autophagy. EMBO J 2011; 30:4628-41; PMID: 21915098; http://dx.doi.org/10.1038/emboj.2011. 331
165. Farkas T, Daugaard M, Jäättelä M. Identification of small molecule inhibitors of phosphatidylinositol 3-kinase and autophagy. J Biol Chem 2011; 286:38904-12; PMID:21930714
166. Szyniarowski P, Corcelle-Termeau E, Farkas T, Høyer-Hansen M, Nylandsted J, Kallunki T, et al. A comprehensive siRNA screen for kinases that suppress macroautophagy in optimal growth conditions. Autophagy 2011; 7:892-903; PMID:21508686;http://dx.doi.org/10.4161/auto.7.8.15770
167. Sarkar S, Korolchuk V, Renna M, Winslow A, Rubinsztein DC. Methodological considerations for assessing autophagy modulators: a study with calcium phosphate precipitates. Autophagy 2009; 5:307-13; PMID:19182529; http://dx.doi.org/10.4161/auto.5.3.7664
168. Iwata J, Ezaki J, Komatsu M, Yokota S, Ueno T, Tanida I, et al. Excess peroxisomes are degraded by autophagic machinery in mammals. J Biol Chem 2006; 281:4035-41; PMID:16332691; http://dx.doi.org/10.1074/jbc.M512283200
169. Narendra D, Tanaka A, Suen DF, Youle RJ. Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. J Cell Biol 2008; 183:795-803; PMID:19029340; http://dx.doi.org/10.1083/jcb.200809125

170. Nogalska A, Terracciano C, D'Agostino C, King Engel W, Askanas V. p62/SQSTM1 is overexpressed and prominently accumulated in inclusions of sporadic inclusion-body myositis muscle fibers, and can help differentiating it from polymyositis and dermatomyositis. Acta Neuropathol 2009; 118:407-13; PMID: 19557423; http://dx.doi.org/10.1007/s00401-009-0564-6
171. Hosokawa N, Hara Y, Mizushima N. Generation of cell lines with tetracycline-regulated autophagy and a role for autophagy in controlling cell size. FEBS Lett 2006; 580:2623-9; PMID:16647067; http://dx.doi. org/10.1016/j.febslet.2006.04.008
172. Ni HM, Bockus A, Wozniak AL, Jones K, Weinman S, Yin XM, et al. Dissecting the dynamic turnover of GFP-LC3 in the autolysosome. Autophagy 2011; 7:188-204; PMID:21107021; http://dx.doi.org/10.4161/auto.7.2.14181
173. Gutierrez MG, Saka HA, Chinen I, Zoppino FC, Yoshimori T, Bocco JL, et al. Protective role of autophagy against Vibrio cholerae cytolysin, a poreforming toxin from V. cholerae. ProcNatlAcad SciUSA 2007; 104:1829-34; PMID:17267617; http://dx.doi.org/10.1073/pnas.0601437104
174. Suttangkakul A, Li F, Chung T, Vierstra RD. The ATG1/ATG13 protein kinase complex is both a regulator and a target of autophagic recycling in Arabidopsis. Plant Cell 2011; 23:3761-79; PMID:21984698; http://dx.doi.org/10.1105/tpc.111.090993
175. Balgi AD, Fonseca BD, Donohue E, Tsang TC, Lajoie P, Proud CG, et al. Screen for chemical modulators of autophagy reveals novel therapeutic inhibitors of mTORC1 signaling. PLoS One 2009; 4:e7124; PMID:19771169; http://dx.doi.org/10.1371/journal.pone.0007124
176. Patterson GH, Lippincott-Schwartz J. Selective photolabeling of proteins using photoactivatable GFP. Methods 2004; 32:445-50; PMID:15003607; http://dx.doi.org/10.1016/j.ymeth.2003.10.006
177. Hamacher-Brady A, Brady NR, Gottlieb RA. Enhancing macroautophagy protects against ischemia/reperfusion injury in cardiac myocytes. J Biol Chem 2006; 281:29776-87; PMID:16882669; http://dx.doi.org/10.1074/jbc.M603783200
178. Noda T, Klionsky DJ. The quantitative Pho8Delta60 assay of nonspecific autophagy. Methods Enzymol 2008; 451:33-42; PMID:19185711; http://dx.doi.org/10.1016/S0076-6879(08)03203-5
179. Klionsky DJ. Monitoring autophagy in yeast: the Pho8Delta60 assay. Methods Mol Biol 2007; 390: 363-71; PMID:17951700; http://dx.doi.org/10.1007/978-1-59745-466-7_24
180. Patterson GH, Knobel SM, Sharif WD, Kain SR, Piston DW. Use of the green fluorescent protein and its mutants in quantitative fluorescence microscopy. Biophys J 1997; 73:2782-90; PMID:9370472; http://dx.doi.org/10.1016/S0006-3495(97)78307-3
181. Kimura S, Noda T, Yoshimori T. Dissection of the autophagosome maturation process by a novel reporter protein, tandem fluorescent-tagged LC3. Autophagy 2007; 3:452-60; PMID:17534139
182. Shaner NC, Campbell RE, Steinbach PA, Giepmans BN, Palmer AE, Tsien RY. Improved monomeric red, orange and yellow fluorescent proteins derived from Discosoma sp. red fluorescent protein. Nat Biotechnol 2004; 22:1567-72; PMID:15558047; http://dx.doi.org/10.1038/nbt1037
183. Strack RL, Keenan RJ, Glick BS. Noncytotoxic DsRed derivatives for whole-cell labeling. Methods Mol Biol 2011; 699:355-70; PMID:21116992; http://dx.doi.org/10.1007/978-1-61737-950-5_17
184. Gurskaya NG, Verkhusha VV, Shcheglov AS, Staroverov DB, Chepurnykh TV, Fradkov AF, et al. Engineering of a monomeric green-to-red photoactivatable fluorescent protein induced by blue light. Nat Biotechnol 2006; 24:461-5; PMID:16550175; http://dx.doi.org/10.1038/nbt1191
185. Ryhänen T, Hyttinen JM, Kopitz J, Rilla K, Kuusisto E, Mannermaa E, et al. Crosstalk between Hsp70 molecular chaperone, lysosomes and proteasomes in autophagy-mediated proteolysis in human retinal pigment epithelial cells. J Cell Mol Med 2009; 13 (9B):3616-31; PMID:19017362; http://dx.doi.org/10.1111/j.1582-4934.2008.00577.x
186. Rekas A, Alattia JR, Nagai T, Miyawaki A, Ikura M. Crystal structure of venus, a yellow fluorescent protein with improved maturation and reduced environmental sensitivity. J Biol Chem 2002; 277:50573-8; PMID: 12370172; http://dx.doi.org/10.1074/jbc.M209524200
187. Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T, et al. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. EMBO J 2000; 19:5720-8; PMID:11060023; http://dx.doi.org/10.1093/emboj/19.21.5720
188. Badr CE, Wurdinger T, Nilsson J, Niers JM, Whalen M, Degterev A, et al. Lanatoside C sensitizes glioblastoma cells to tumor necrosis factor-related apoptosisinducing ligand and induces an alternative cell death pathway. Neuro Oncol 2011; 13:1213-24; PMID: 21757445; http://dx.doi.org/10.1093/neuonc/nor067
189. Meléndez A, Tallóczy Z, Seaman M, Eskelinen E-L, Hall DH, Levine B. Autophagy genes are essential for dauer development and life-span extension in C. elegans. Science 2003; 301:1387-91; PMID:12958363; http://dx.doi.org/10.1126/science.1087782
190. Otto GP, Wu MY, Kazgan N, Anderson OR, Kessin RH. Macroautophagy is required for multicellular development of the social amoeba Dictyostelium discoideum. J Biol Chem 2003; 278:17636-45; PMID: 12626495; http://dx.doi.org/10.1074/jbc.M212467200
191. Liu XH, Liu TB, Lin FC. Monitoring autophagy in Magnaporthe oryzae. Methods Enzymol 2008; 451: 271-94; PMID:19185727; http://dx.doi.org/10.1016/S0076-6879(08)03219-9
192. Pinan-Lucarré B, Paoletti M, Dementhon K, Coulary-Salin B, Clavé C. Autophagy is induced during cell death by incompatibility and is essential for differentiation in the filamentous fungus Podospora anserina. Mol Microbiol 2003; 47:321-33; PMID:12519185; http://dx.doi.org/10.1046/j.1365-2958.2003.03208.x
193. Veneault-Fourrey C, Barooah M, Egan M, Wakley G, Talbot NJ. Autophagic fungal cell death is necessary for infection by the rice blast fungus. Science 2006; 312:580-3; PMID:16645096; http://dx.doi.org/10.1126/science.1124550
194. Kikuma T, Ohneda M, Arioka M, Kitamoto K. Functional analysis of the ATG8 homologue Aoatg8 and role of autophagy in differentiation and germination in Aspergillus oryzae. Eukaryot Cell 2006; 5:1328-36; PMID:16896216; http://dx.doi.org/10.1128/EC. 00024-06
195. Nolting N, Bernhards Y, Pöggeler S. SmATG7 is required for viability in the homothallic ascomycete Sordaria macrospora. Fungal Genet Biol 2009; 46:531-42; PMID:19351563; http://dx.doi.org/10.1016/j.fgb.2009.03.008
196. Baghdiguian S, Martinand-Mari C, Mangeat P. Using Ciona to study developmental programmed cell death. Semin Cancer Biol 2007; 17:147-53; PMID:17197195; http://dx.doi.org/10.1016/j.semcancer.2006.11.005
197. Rusten TE, Lindmo K, Juhász G, Sass M, Seglen PO, Brech A, et al. Programmed autophagy in the Drosophila fat body is induced by ecdysone through regulation of the PI3K pathway. Dev Cell 2004; 7:179-92; PMID: 15296715; http://dx.doi.org/10.1016/j.devcel.2004.07.005
198. Scott RC, Schuldiner O, Neufeld TP. Role and regulation of starvation-induced autophagy in the Drosophila fat body. Dev Cell 2004; 7:167-78; PMID:15296714; http://dx.doi.org/10.1016/j.devcel. 2004.07.009
199. Denton D, Shravage B, Simin R, Mills K, Berry DL, Baehrecke EH, et al. Autophagy, not apoptosis, is essential for midgut cell death in Drosophila. Curr Biol 2009; 19:1741-6; PMID:19818615; http://dx.doi.org/10.1016/j.cub.2009.08.042
200. Yoshimoto K, Hanaoka H, Sato S, Kato T, Tabata S, Noda T, et al. Processing of ATG8s, ubiquitin-like proteins, and their deconjugation by ATG4s are essential for plant autophagy. Plant Cell 2004; 16:2967-83; PMID:15494556; http://dx.doi.org/10. 1105/tpc.104.025395
201. Besteiro S, Brooks CF, Striepen B, Dubremetz J-F. Autophagy protein Atg3 is essential for maintaining mitochondrial integrity and for normal intracellular development of Toxoplasma gondii tachyzoites. PLoS Pathog 2011; 7:e1002416; PMID:22144900; http://dx.doi.org/10.1371/journal.ppat.1002416
202. Williams RA, Tetley L, Mottram JC, Coombs GH. Cysteine peptidases CPA and CPB are vital for autophagy and differentiation in Leishmania mexicana. Mol Microbiol 2006; 61:655-74; PMID:16803590; http://dx.doi.org/10.1111/j.1365-2958.2006.05274.x
203. Williams RA, Woods KL, Juliano L, Mottram JC, Coombs GH. Characterization of unusual families of ATG8-like proteins and ATG12 in the protozoan parasite Leishmania major. Autophagy 2009; 5:159-72; PMID:19066473; http://dx.doi.org/10.4161/auto.5.2. 7328

204. Elsässer A, Vogt AM, Nef H, Kostin S, Möllmann H, Skwara W, et al. Human hibernating myocardium is jeopardized by apoptotic and autophagic cell death. J Am Coll Cardiol 2004; 43:2191-9; PMID:15193679; http://dx.doi.org/10.1016/j.jacc.2004.02.053
205. Knaapen MW, Davies MJ, De Bie M, Haven AJ, Martinet W, Kockx MM. Apoptotic versus autophagic cell death in heart failure. Cardiovasc Res 2001; 51:304-12; PMID:11470470; http://dx.doi.org/10. 1016/S0008-6363(01)00290-5
206. Kostin S, Pool L, Elsässer A, Hein S, Drexler HC, Arnon E, et al. Myocytes die by multiple mechanisms in failing human hearts. Circ Res 2003; 92:715-24; PMID:12649263; http://dx.doi.org/10.1161/01.RES.0000067471.95890.5C
207. Pérez-Pérez ME, Florencio FJ, Crespo JL. Inhibition of target of rapamycin signaling and stress activate autophagy in Chlamydomonas reinhardtii. Plant Physiol 2010; 152:1874-88; PMID:20107021; http://dx.doi.org/10.1104/pp.109.152520
208. Koike M, Shibata M, Waguri S, Yoshimura K, Tanidal, Kominami E, et al. Participation of autophagy in storage of lysosomes in neurons from mouse models of neuronal ceroid-lipofuscinoses (Batten disease). Am J Pathol 2005; 167:1713-28; PMID:16314482; http://dx.doi.org/10.1016/S0002-9440(10)61253-9
209. Ost A, Svensson K, Ruishalme I, Brännmark C, Franck N, Krook H, et al. Attenuated mTOR signaling and enhanced autophagy in adipocytes from obese patients with type 2 diabetes. Mol Med 2010;16:235-46; PMID:20386866
210. Tang D, Kang R, Livesey KM, Cheh CW, Farkas A, Loughran P, et al. Endogenous HMGB1 regulates autophagy. J Cell Biol 2010; 190:881-92; PMID:20819940; http://dx.doi.org/10.1083/jcb.200911078
211. Gniadek TJ, Warren G. WatershedCounting3D: a new method for segmenting and counting punctuate structures from confocal image data. Traffic 2007; 8:339-46; PMID:17319897; http://dx.doi.org/10. 1111/j.1600-0854.2007.00538.x
212. Decuypere J-P, Welkenhuyzen K, Luyten T, Ponsaerts R, Dewaele M, Molgó J, et al. IP 3 receptor-mediated Ca (2+) signaling and autophagy induction are interrelated. Autophagy 2011; 7:1472-89; PMID:22082873; http://dx.doi.org/10.4161/auto.7.12.17909
213. Amer AO, Swanson MS. Autophagy is an immediate macrophage response to Legionella pneumophila. Cell Microbiol 2005; 7:765-78; PMID:15888080; http://dx.doi.org/10.1111/j.1462-5822.2005.00509.x
214. Gutierrez MG, Master SS, Singh SB, Taylor GA, Colombo MI, Deretic V. Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages. Cell 2004; 119:753-66; PMID:15607973; http://dx.doi.org/10.1016/j.cell.2004.11.038
215. Ogawa M, Sasakawa C. Intracellular survival of Shigella. Cell Microbiol 2006; 8:177-84; PMID: 16441429; http://dx.doi.org/10.1111/j.1462-5822.2005.00652.x
216. Kamentsky L, Jones TR, Fraser A, Bray MA, Logan DJ, Madden KL, et al. Improved structure, function and compatibility for CellProfiler: modular highthroughput image analysis software. Bioinformatics 2011; 27:1179-80; PMID:21349861; http://dx.doi.org/10.1093/bioinformatics/btr095
217. Wu JQ, Pollard TD. Counting cytokinesis proteins globally and locally in fission yeast. Science 2005; 310:310-4; PMID:16224022; http://dx.doi.org/10.1126/science.1113230
218. Geng J, Baba M, Nair U, Klionsky DJ. Quantitative analysis of autophagy-related protein stoichiometry by fluorescence microscopy. J Cell Biol 2008; 182:129-40; PMID:18625846; http://dx.doi.org/10.1083/jcb.200711112
219. Brady NR, Hamacher-Brady A, Yuan H, Gottlieb RA. The autophagic response to nutrient deprivation in the hl-1 cardiac myocyte is modulated by Bcl-2 and sarco/endoplasmic reticulum calcium stores. FEBS J 2007; 274:3184-97; PMID:17540004; http://dx.doi.org/10. 1111/j.1742-4658.2007.05849.x
220. Qadir MA, Kwok B, Dragowska WH, To KH, Le D, Bally MB, et al. Macroautophagy inhibition sensitizes tamoxifen-resistant breast cancer cells and enhances mitochondrial depolarization. Breast Cancer Res Treat 2008; 112:389-403; PMID:18172760; http://dx.doi.org/10.1007/s10549-007-9873-4
221. Dolloff NG, Ma X, Dicker DT, Humphreys RC, Li LZ, El-Deiry WS. Spectral imaging-based methods for quantifying autophagy and apoptosis. Cancer Biol Ther 2011; 12:349-56; PMID:21757995; http://dx.doi.org/10.4161/cbt.12.4.17175
222. Phadwal K, Alegre-Abarrategui J, Watson AS, Pike L, Anbalagan S, Hammond EM, et al. A novel method for autophagy detection in primary cells: Impaired levels of macroautophagy in immunosenescent T cells. Autophagy 2012; 8; In press; PMID:22302009
223. Lee HK, Lund JM, Ramanathan B, Mizushima N, Iwasaki A. Autophagy-dependent viral recognition by plasmacytoid dendritic cells. Science 2007; 315: 1398-401; PMID:17272685; http://dx.doi.org/10.1126/science.1136880
224. Kuma A, Matsui M, Mizushima N. LC3, an autophagosome marker, can be incorporated into protein aggregates independent of autophagy: caution in the interpretation of LC3 localization. Autophagy 2007; 3:323-8; PMID:17387262
225. Szeto J, Kaniuk NA, Canadien V, Nisman R, Mizushima N, Yoshimori T, et al. ALIS are stressinduced protein storage compartments for substrates of the proteasome and autophagy. Autophagy 2006; 2:189-99; PMID:16874109
226. Kaniuk NA, Kiraly M, Bates H, Vranic M, Volchuk A, Brumell JH. Ubiquitinated-protein aggregates form in pancreatic beta-cells during diabetes-induced oxidative stress and are regulated by autophagy. Diabetes 2007; 56:930-9; PMID:17395740; http://dx.doi.org/10.2337/db06-1160
227. Fujita K, Maeda D, Xiao Q, Srinivasula SM. Nrf2- mediated induction of p62 controls Toll-like receptor-4-driven aggresome-like induced structure formation and autophagic degradation. Proc Natl Acad Sci U S A 2011; 108:1427-32; PMID:21220332; http://dx.doi.org/10.1073/pnas.1014156108
228. Pierre P. Dendritic cells, DRiPs, and DALIS in the control of antigen processing. Immunol Rev 2005; 207:184-90; PMID:16181336; http://dx.doi.org/10.1111/j.0105-2896.2005.00300.x
229. Pankiv S, Clausen TH, Lamark T, Brech A, Bruun JA, Outzen H, et al. p62/SQSTM1 binds directly to Atg8/ LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. J Biol Chem 2007; 282: 24131-45; PMID:17580304; http://dx.doi.org/10.1074/jbc.M702824200
230. Hara T, Nakamura K, Matsui M, Yamamoto A, Nakahara Y, Suzuki-Migishima R, et al. Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. Nature 2006; 441:885-9; PMID:16625204; http://dx.doi.org/10.1038/nature04724
231. Komatsu M, Waguri S, Chiba T, Murata S, Iwata J, Tanida I, et al. Loss of autophagy in the central nervous system causes neurodegeneration in mice. Nature 2006; 441:880-4; PMID:16625205; http://dx.doi.org/10.1038/nature04723
232. Calvo-Garrido J, Escalante R. Autophagy dysfunction and ubiquitin-positive protein aggregates in Dictyostelium cells lacking Vmp1. Autophagy 2010; 6:100-9; PMID:20009561; http://dx.doi.org/10.4161/auto.6.1.10697
233. Bjørkøy G, Lamark T, Johansen T. p62/SQSTM1: a missing link between protein aggregates and the autophagy machinery. Autophagy 2006; 2:138-9;PMID:16874037
234. Matsumoto G, Wada K, Okuno M, Kurosawa M, Nukina N. Serine 403 phosphorylation of p62/SQSTM1 regulates selective autophagic clearance of ubiquitinated proteins. Mol Cell 2011; 44:279-89;PMID:22017874; http://dx.doi.org/10.1016/j.molcel.
2011.07.039235. Köchl R, Hu XW, Chan EYW, Tooze SA. Microtubules facilitate autophagosome formation and fusion of autophagosomes with endosomes. Traffic 2006; 7: 129-45; PMID:16420522; http://dx.doi.org/10.1111/ j.1600-0854.2005.00368.x
236. Eng KE, Panas MD, Hedestam GB, McInerney GM. A novel quantitative flow cytometry-based assay for autophagy. Autophagy 2010; 6:634-41; PMID: 20458170; http://dx.doi.org/10.4161/auto.6.5.12112
237. Ciechomska IA, Tolkovsky AM. Non-autophagic GFPLC3 puncta induced by saponin and other detergents. Autophagy 2007; 3:586-90; PMID:17786021

238. Tsvetkov AS, Miller J, Arrasate M, Wong JS, Pleiss MA, Finkbeiner S. A small-molecule scaffold induces autophagy in primary neurons and protects against toxicity in a Huntington disease model. Proc Natl Acad Sci U S A 2010; 107:16982-7; PMID:20833817; http://dx.doi.org/10.1073/pnas.1004498107
239. Joo JH, Dorsey FC, Joshi A, Hennessy-Walters KM, Rose KL, McCastlain K, et al. Hsp90-Cdc37 chaperone complex regulates Ulk1- and Atg13-mediated mitophagy. Mol Cell 2011; 43:572-85; PMID: 21855797; http://dx.doi.org/10.1016/j.molcel.2011.06.018
240. Fujita N, Hayashi-Nishino M, Fukumoto H, Omori H, Yamamoto A, Noda T, et al. An Atg4B mutant hampers the lipidation of LC3 paralogues and causes defects in autophagosome closure. Mol Biol Cell 2008; 19:4651-9; PMID:18768752; http://dx.doi.org/10.1091/mbc.E08-03-0312
241. Decuypere JP, Bultynck G, Parys JB. A dual role for Ca(2+) in autophagy regulation. Cell Calcium 2011; 50:242-50; PMID:21571367; http://dx.doi.org/10.1016/j.ceca.2011.04.001
242. Nyfeler B, Bergman P, Triantafellow E, Wilson CJ, Zhu Y, Radetich B, et al. Relieving autophagy and 4EBP1 from rapamycin resistance. Mol Cell Biol 2011; 31:2867-76; PMID:21576371; http://dx.doi.org/10.1128/MCB.05430-11
243. García-Martínez JM, Moran J, Clarke RG, Gray A, Cosulich SC, Chresta CM, et al. Ku-0063794 is a specific inhibitor of the mammalian target of rapamycin (mTOR). Biochem J 2009; 421:29-42; PMID:19402821; http://dx.doi.org/10.1042/BJ20090489
244. Pereira GJ, Hirata H, Fimia GM, do Carmo LG, Bincoletto C, Han SW, et al. Nicotinic acid adenine dinucleotide phosphate (NAADP) regulates autophagy in cultured astrocytes. J Biol Chem 2011; 286:27875-81; PMID:21610076; http://dx.doi.org/10.1074/jbc. C110.216580
245. Serra V, Markman B, Scaltriti M, Eichhorn PJ, Valero V, Guzman M, et al. NVP-BEZ235, a dual PI3K/ mTOR inhibitor, prevents PI3K signaling and inhibits the growth of cancer cells with activating PI3K mutations. Cancer Res 2008; 68:8022-30; PMID: 18829560; http://dx.doi.org/10.1158/0008-5472.CAN-08-1385
246. Liu TJ, Koul D, LaFortune T, Tiao N, Shen RJ, Maira SM, et al. NVP-BEZ235, a novel dual phosphatidylinositol 3-kinase/mammalian target of rapamycin inhibitor, elicits multifaceted antitumor activities in human gliomas. Mol Cancer Ther 2009; 8:2204-10; PMID:19671762; http://dx.doi.org/10.1158/1535-7163.MCT-09-0160
247. Vingtdeux V, Giliberto L, Zhao H, Chandakkar P, Wu Q, Simon JE, et al. AMP-activated protein kinase signaling activation by resveratrol modulates amyloidbeta peptide metabolism. J Biol Chem 2010; 285: 9100-13; PMID:20080969; http://dx.doi.org/10.1074/jbc.M109.060061
248. Puissant A, Auberger P. AMPK- and p62/SQSTM1-dependent autophagy mediate Resveratrol-induced cell death in chronic myelogenous leukemia. Autophagy 2010; 6:655-7; PMID:20458181; http://dx.doi.org/10.4161/auto.6.5.12126
249. Vingtdeux V, Chandakkar P, Zhao H, d'Abramo C, Davies P, Marambaud P. Novel synthetic smallmolecule activators of AMPK as enhancers of autophagy and amyloid-β peptide degradation. FASEB J 2011; 25:219-31; PMID:20852062; http://dx.doi.org/10.1096/fj.10-167361
250. Gordon PB, Holen I, Fosse M, Røtnes JS, Seglen PO. Dependence of hepatocytic autophagy on intracellularly sequestered calcium. J Biol Chem 1993; 268:26107-12; PMID:8253727
251. Ganley IG, Wong PM, Gammoh N, Jiang X. Distinct autophagosomal-lysosomal fusion mechanism revealed by thapsigargin-induced autophagy arrest. Mol Cell 2011; 42:731-43; PMID:21700220; http://dx.doi.org/10.1016/j.molcel.2011.04.024
252. Thoreen CC, Kang SA, Chang JW, Liu Q, Zhang J, Gao Y, et al. An ATP-competitive mammalian target of rapamycin inhibitor reveals rapamycin-resistant functions of mTORC1. J Biol Chem 2009; 284: 8023-32; PMID:19150980; http://dx.doi.org/10. 1074/jbc.M900301200
253. Sarkar S, Davies JE, Huang Z, Tunnacliffe A, Rubinsztein DC. Trehalose, a novel mTOR-independent autophagy enhancer, accelerates the clearance of mutant huntingtin and alpha-synuclein. J Biol Chem 2007; 282:5641-52; PMID:17182613; http://dx.doi.org/10.1074/jbc.M609532200
254. Casarejos MJ, Solano RM, Gómez A, Perucho J, de Yébenes JG, Mena MA. The accumulation of neurotoxic proteins, induced by proteasome inhibition, is reverted by trehalose, an enhancer of autophagy, in human neuroblastoma cells. Neurochem Int 2011; 58:512-20; PMID:21232572; http://dx.doi.org/10.1016/j.neuint.2011.01.008
255. Carpenter JE, Jackson W, Benetti L, Grose C. Autophagosome formation during varicella-zoster virus infection following endoplasmic reticulum stress and the unfolded protein response. J Virol 2011; 85:9414-24; PMID:21752906; http://dx.doi.org/10.1128/JVI.00281-11
256. Seglen PO, Gordon PB. 3-Methyladenine: specific inhibitor of autophagic/lysosomal protein degradation in isolated rat hepatocytes. Proc Natl Acad Sci U S A 1982; 79:1889-92; PMID:6952238; http://dx.doi.org/10.1073/pnas.79.6.1889
257. Wu YT, Tan HL, Shui G, Bauvy C, Huang Q, Wenk MR, et al. Dual role of 3-methyladenine in modulation of autophagy via different temporal patterns of inhibition on class I and III phosphoinositide 3-kinase. J Biol Chem 2010; 285:10850-61; PMID:20123989;http://dx.doi.org/10.1074/jbc.M109.080796
258. Bampton ET, Goemans CG, Niranjan D, Mizushima N, Tolkovsky AM. The dynamics of autophagy visualized in live cells: from autophagosome formation to fusion with endo/lysosomes. Autophagy 2005; 1:23-36; PMID:16874023; http://dx.doi.org/10.4161/auto.1.1.1495
259. Tormo D, Checińska A, Alonso-Curbelo D, Pérez-Guijarro E, Cañón E, Riveiro-Falkenbach E, et al. Targeted activation of innate immunity for therapeutic induction of autophagy and apoptosis in melanoma cells. Cancer Cell 2009; 16:103-14; PMID:19647221;http://dx.doi.org/10.1016/j.ccr.2009.07.004
260. Lee HK, Mattei LM, Steinberg BE, Alberts P, Lee YH, Chervonsky A, et al. In vivo requirement for Atg5 in antigen presentation by dendritic cells. Immunity 2010; 32:227-39; PMID:20171125; http://dx.doi. org/10.1016/j.immuni.2009.12.006
261. Tamura N, Oku M, Sakai Y. Atg8 regulates vacuolar membrane dynamics in a lipidation-independent manner in Pichia pastoris. J Cell Sci 2010; 123:4107-16; PMID:21045113; http://dx.doi.org/10.1242/jcs.070045
262. Strømhaug PE, Reggiori F, Guan J, Wang C-W, Klionsky DJ. Atg21 is a phosphoinositide binding protein required for efficient lipidation and localization of Atg8 during uptake of aminopeptidase I by selective autophagy. Mol Biol Cell 2004; 15:3553-66; PMID: 15155809; http://dx.doi.org/10.1091/mbc.E04-02-0147
263. Baens M, Noels H, Broeckx V, Hagens S, Fevery S, Billiau AD, et al. The dark side of EGFP: defective polyubiquitination. PLoS One 2006; 1:e54; PMID: 17183684; http://dx.doi.org/10.1371/journal.pone. 0000054
264. Calì T, Galli C, Olivari S, Molinari M. Segregation and rapid turnover of EDEM1 by an autophagy-like mechanism modulates standard ERAD and folding activities. Biochem Biophys Res Commun 2008; 371:405-10; PMID:18452703; http://dx.doi.org/10.1016/j.bbrc.2008.04.098
265. Al-Younes HM, Al-Zeer MA, Khalil H, Gussmann J, Karlas A, Machuy N, et al. Autophagy-independent function of MAP-LC3 during intracellular propagation of Chlamydia trachomatis. Autophagy 2011; 7:814-28; PMID:21464618; http://dx.doi.org/10.4161/auto. 7.8.15597
266. Shroff H, Galbraith CG, Galbraith JA, White H, Gillette J, Olenych S, et al. Dual-color superresolution imaging of genetically expressed probes within individual adhesion complexes. Proc Natl Acad Sci U S A 2007; 104:20308-13; PMID:18077327; http://dx.doi. org/10.1073/pnas.0710517105
267. Nicklin P, Bergman P, Zhang B, Triantafellow E, Wang H, Nyfeler B, et al. Bidirectional transport of amino acids regulates mTOR and autophagy. Cell 2009; 136:521-34; PMID:19203585; http://dx.doi. org/10.1016/j.cell.2008.11.044
268. Cherra SJ, 3rd, Kulich SM, Uechi G, Balasubramani M, Mountzouris J, Day BW, et al. Regulation of the autophagy protein LC3 by phosphorylation. J Cell Biol 2010; 190:533-9; PMID:20713600; http://dx.doi.org/10.1083/jcb.201002108
269. Sarkar S, Korolchuk VI, Renna M, Imarisio S, Fleming A, Williams A, et al. Complex inhibitory effects of nitric oxide on autophagy. Mol Cell 2011; 43:19-32; PMID:21726807; http://dx.doi.org/10.1016/j.molcel. 2011.04.029
270. Rosado CJ, Mijaljica D, Hatzinisiriou I, Prescott M, Devenish RJ. Rosella: a fluorescent pH-biosensor for reporting vacuolar turnover of cytosol and organelles in yeast. Autophagy 2008; 4:205-13; PMID:18094608

271. Mijaljica D, Rosado CJ, Devenish RJ, Prescott M. Biosensors for monitoring autophagy In: Serra PA, ed. Biosensors-Emerging Materials and Applications Croatia: InTech, 2011:383-400.
272. Chudakov DM, Matz MV, Lukyanov S, Lukyanov KA. Fluorescent proteins and their applications in imaging living cells and tissues. Physiol Rev 2010; 90:1103-63; PMID:20664080; http://dx.doi.org/10.1152/physrev.00038.2009
273. Zhou J, Lin J, Zhou C, Deng X, Xia B. Cytotoxicity of red fluorescent protein DsRed is associated with the suppression of Bcl-xL translation. FEBS Lett 2011; 585:821-7; PMID:21320495; http://dx.doi.org/10.1016/j.febslet.2011.02.013
274. Loos B, Genade S, Ellis B, Lochner A, Engelbrecht AM. At the core of survival: autophagy delays the onset of both apoptotic and necrotic cell death in a model of ischemic cell injury. Exp Cell Res 2011; 317:1437-53; PMID:21420401; http://dx.doi.org/10.1016/j.yexcr. 2011.03.011
275. Shvets E, Fass E, Elazar Z. Utilizing flow cytometry to monitor autophagy in living mammalian cells. Autophagy 2008; 4:621-8; PMID:18376137
276. Hundeshagen P, Hamacher-Brady A, Eils R, Brady NR. Concurrent detection of autolysosome formation and lysosomal degradation by flow cytometry in a high-content screen for inducers of autophagy. BMC Biol 2011; 9:38; PMID:21635740; http://dx.doi.org/10.1186/1741-7007-9-38
277. de la Calle C, Joubert PE, Law HK, Hasan M, Albert ML. Simultaneous assessment of autophagy and apoptosis using multispectral imaging cytometry. Autophagy 2011; 7:1045-51; PMID:21606680; http://dx.doi.org/10.4161/auto.7.9.16252
278. Gannagé M, Dormann D, Albrecht R, Dengjel J, Torossi T, Rämer PC, et al. Matrix protein 2 of influenza A virus blocks autophagosome fusion with lysosomes. Cell Host Microbe 2009; 6:367-80; PMID: 19837376; http://dx.doi.org/10.1016/j.chom.2009. 09.005
279. Kaminskyy V, Abdi A, Zhivotovsky B. A quantitative assay for the monitoring of autophagosome accumulation in different phases of the cell cycle. Autophagy 2011; 7:83-90; PMID:20980814; http://dx.doi.org/ 10.4161/auto.7.1.13893
280. Kirkin V, Lamark T, Sou YS, Bjørkøy G, Nunn JL, Bruun JA, et al. A role for NBR1 in autophagosomal degradation of ubiquitinated substrates. Mol Cell 2009; 33:505-16; PMID:19250911; http://dx.doi.org/10.1016/j.molcel.2009.01.020
281. Larsen KB, Lamark T, Øvervatn A, Harneshaug I, Johansen T, Bjørkøy G. A reporter cell system to monitor autophagy based on p62/SQSTM1. Autophagy 2010; 6:784-93; PMID:20574168; http://dx.doi.org/10.4161/auto.6.6.12510
282. Huang JJ, Li HR, Huang Y, Jiang WQ, Xu RH, Huang HQ, et al. Beclin 1 expression: a predictor of prognosis in patients with extranodal natural killer T-cell lymphoma, nasal type. Autophagy 2010; 6:777-83; PMID:20639699; http://dx.doi.org/10.4161/auto. 6.6.12784
283. Sivridis E, Koukourakis MI, Zois CE, Ledaki I, Ferguson DJ, Harris AL, et al. LC3A-positive light microscopy detected patterns of autophagy and prognosis in operable breast carcinomas. Am J Pathol 2010; 176:2477-89; PMID:20382705; http://dx.doi. org/10.2353/ajpath.2010.090049
284. Sivridis E, Giatromanolaki A, Liberis V, Koukourakis MI. Autophagy in endometrial carcinomas and prognostic relevance of 'stone-like' structures (SLS): what is destined for the atypical endometrial hyperplasia? Autophagy 2011; 7:74-82; PMID:21099253; http://dx.doi.org/10.4161/auto.7.1.13947
285. Giatromanolaki A, Koukourakis MI, Koutsopoulos A, Chloropoulou P, Liberis V, Sivridis E. High Beclin 1 expression defines a poor prognosis in endometrial adenocarcinomas. Gynecol Oncol 2011; 123:147-51; PMID:21741077; http://dx.doi.org/10.1016/j.ygyno. 2011.06.023
286. Chen Y, Lu Y, Lu C, Zhang L. Beclin-1 expression is a predictor of clinical outcome in patients with esophageal squamous cell carcinoma and correlated to hypoxiainducible factor (HIF)-1alpha expression. Pathol Oncol Res 2009; 15:487-93; PMID:19130303; http://dx.doi. org/10.1007/s12253-008-9143-8
287. Wan XB, Fan XJ, ChenMY, Xiang J, Huang PY, Guo L, et al. Elevated Beclin 1 expression is correlated with HIF-1alpha in predicting poor prognosis of nasopharyngeal carcinoma. Autophagy 2010; 6:395-404; PMID: 20150769; http://dx.doi.org/10.4161/auto.6.3.11303
288. Shi YH, Ding ZB, Zhou J, Qiu SJ, Fan J. Prognostic significance of Beclin 1-dependent apoptotic activity in hepatocellular carcinoma. Autophagy 2009; 5:380-2; PMID:19145109; http://dx.doi.org/10.4161/auto.5.3.7658
289. Ding ZB, Shi YH, Zhou J, Qiu SJ, Xu Y, Dai Z, et al. Association of autophagy defect with a malignant phenotype and poor prognosis of hepatocellular carcinoma. Cancer Res 2008; 68:9167-75; PMID:19010888; http://dx.doi.org/10.1158/0008-5472.CAN-08-1573
290. Pirtoli L, Cevenini G, Tini P, Vannini M, Oliveri G, Marsili S, et al. The prognostic role of Beclin 1 protein expression in high-grade gliomas. Autophagy 2009; 5:930-6; PMID:19556884; http://dx.doi.org/10.4161/ auto.5.7.9227
291. Karpathiou G, Sivridis E, Koukourakis MI, Mikroulis D, Bouros D, Froudarakis ME, et al. Light-chain 3A autophagic activity and prognostic significance in nonsmall cell lung carcinomas. Chest 2011; 140:127-34; PMID:21148243; http://dx.doi.org/10.1378/chest. 10-1831
292. Fujii S, Mitsunaga S, Yamazaki M, Hasebe T, Ishii G, Kojima M, et al. Autophagy is activated in pancreatic cancer cells and correlates with poor patient outcome. Cancer Sci 2008; 99:1813-9; PMID:18616529
293. Li BX, Li CY, Peng RQ, Wu XJ, Wang HY, Wan DS, et al. The expression of beclin 1 is associated with favorable prognosis in stage IIIB colon cancers. Autophagy 2009; 5:303-6; PMID:19066461; http://dx.doi. org/10.4161/auto.5.3.7491
294. Koukourakis MI, Giatromanolaki A, Sivridis E, Pitiakoudis M, Gatter KC, Harris AL. Beclin 1 overand underexpression in colorectal cancer: distinct patterns relate to prognosis and tumour hypoxia. Br J Cancer 2010; 103:1209-14; PMID:20842118; http://dx.doi.org/10.1038/sj.bjc.6605904
295. Giatromanolaki A, Koukourakis MI, Harris AL, Polychronidis A, Gatter KC, Sivridis E. Prognostic relevance of light chain 3 (LC3A) autophagy patterns in colorectal adenocarcinomas. J Clin Pathol 2010; 63:867-72; PMID:20876316; http://dx.doi.org/10.1136/jcp.2010.079525
296. Sivridis E, Koukourakis MI, Mendrinos SE, Karpouzis A, Fiska A, Kouskoukis C, et al. Beclin-1 and LC3A expression in cutaneous malignant melanomas: a biphasic survival pattern for beclin-1. Melanoma Res 2011; 21:188-95; PMID:21537144; http://dx.doi.org/10.1097/CMR.0b013e328346612c
297. Giatromanolaki AN, Charitoudis GS, Bechrakis NE, Kozobolis VP, Koukourakis MI, Foerster MH, et al. Autophagy patterns and prognosis in uveal melanomas Mod Pathol 2011; 24:1036-45; PMID:21499230; http://dx.doi.org/10.1038/modpathol.2011.63
298. McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM & Statistics Subcommittee of the NCI-EORTC Working Group on Cancer Diagnostics. Reporting recommendations for tumor marker prognostic studies (REMARK). J Natl Cancer Inst 2005; 97:1180-4; PMID:16106022; http://dx.doi.org/10.1093/jnci/dji237
299. Kuwahara Y, Oikawa T, Ochiai Y, Roudkenar MH, Fukumoto M, Shimura T, et al. Enhancement of autophagy is a potential modality for tumors refractory to radiotherapy. Cell Death Dis 2011; 2:e177; PMID: 21716292; http://dx.doi.org/10.1038/cddis.2011.56
300. Hou YJ, Dong LW, Tan YX, Yang GZ, Pan YF, Li Z, et al. Inhibition of active autophagy induces apoptosis and increases chemosensitivity in cholangiocarcinoma.
Lab Invest 2011; 91:1146-57; PMID:21647092; http://dx.doi.org/10.1038/labinvest.2011.97
301. O'Donovan TR, O'Sullivan GC, McKenna SL. Induction of autophagy by drug-resistant esophageal cancer cells promotes their survival and recovery following treatment with chemotherapeutics. Autophagy 2011; 7:509-24; PMID:21325880; http://dx.doi.org/10. 4161/auto.7.5.15066
302. Yoshimura K, Shibata M, Koike M, Gotoh K, Fukaya M, Watanabe M, et al. Effects of RNA interference of Atg4B on the limited proteolysis of LC3 in PC12 cells and expression of Atg4B in various rat tissues. Autophagy 2006; 2:200-8; PMID:16874114
303. Tamura H, Shibata M, Koike M, Sasaki M, Uchiyama Y. Atg9A protein, an autophagy-related membrane protein, is localized in the neurons of mouse brains. J Histochem Cytochem 2010; 58:443-53; PMID: 20124090; http://dx.doi.org/10.1369/jhc.2010.955690
304. Cui J, Bai XY, Shi S, Cui S, Hong Q, Cai G, et al. Agerelated changes in the function of autophagy in rat kidneys. Age (Dordr) 2011; In press; PMID:21455601;http://dx.doi.org/10.1007/s11357-011-9237-1

305. Mellén MA, de la Rosa EJ, Boya P. The autophagic machinery is necessary for removal of cell corpses from the developing retinal neuroepithelium. Cell Death Differ 2008; 15:1279-90; PMID:18369370; http://dx. doi.org/10.1038/cdd.2008.40
306. Mellén MA, de la Rosa EJ, Boya P. Autophagy is not universally required for phosphatidyl-serine exposure and apoptotic cell engulfment during neural development. Autophagy 2009; 5:964-72; PMID:19587526;http://dx.doi.org/10.4161/auto.5.7.9292
307. Shibata M, Yoshimura K, Furuya N, Koike M, Ueno T, Komatsu M, et al. The MAP1-LC3 conjugation system is involved in lipid droplet formation. Biochem Biophys Res Commun 2009; 382:419-23; PMID:19285958; http://dx.doi.org/10.1016/j.bbrc.2009.03.039
308. Komatsu M, Waguri S, Koike M, Sou YS, Ueno T, Hara T, et al. Homeostatic levels of p62 control cytoplasmic inclusion body formation in autophagy-deficient mice. Cell 2007; 131:1149-63; PMID:18083104; http://dx. doi.org/10.1016/j.cell.2007.10.035
309. Germain M, Nguyen AP, Le Grand JN, Arbour N, Vanderluit JL, Park DS, et al. MCL-1 is a stress sensor that regulates autophagy in a developmentally regulated manner. EMBO J 2011; 30:395-407; PMID: 21139567; http://dx.doi.org/10.1038/emboj.2010.327
310. Komatsu M, Wang QJ, Holstein GR, Friedrich VL, Jr., Iwata J, Kominami E, et al. Essential role for autophagy protein Atg7 in the maintenance of axonal homeostasis and the prevention of axonal degeneration. Proc Natl Acad Sci U S A 2007; 104:14489-94; PMID:17726112; http://dx.doi.org/10.1073/pnas. 0701311104
311. Wang QJ, Ding Y, Kohtz DS, Mizushima N, Cristea IM, Rout MP, et al. Induction of autophagy in axonal dystrophy and degeneration. J Neurosci 2006; 26: 8057-68; PMID:16885219; http://dx.doi.org/10. 1523/JNEUROSCI.2261-06.2006
312. Nezis IP, Simonsen A, Sagona AP, Finley K, Gaumer S, Contamine D, et al. Ref(2)P, the Drosophila melanogaster homologue of mammalian p62, is required for the formation of protein aggregates in adult brain. J Cell Biol 2008; 180:1065-71; PMID:
18347073; http://dx.doi.org/10.1083/jcb.200711108
313. Bartlett BJ, Isakson P, Lewerenz J, Sanchez H, Kotzebue RW, Cumming RC, et al. p62, Ref(2)P and ubiquitinated proteins are conserved markers of neuronal aging, aggregate formation and progressive autophagic defects. Autophagy 2011; 7:572-83; PMID:21325881; http://dx.doi.org/10.4161/auto.7.6.14943
314. Lee JH, Yu WH, Kumar A, Lee S, Mohan PS, Peterhoff CM, et al. Lysosomal proteolysis and autophagy require presenilin 1 and are disrupted by Alzheimer-related PS1 mutations. Cell 2010; 141: 1146-58; PMID:20541250; http://dx.doi.org/10.1016/j.cell.2010.05.008
315. Masiero E, Agatea L, Mammucari C, Blaauw B, Loro E, Komatsu M, et al. Autophagy is required to maintain muscle mass. Cell Metab 2009; 10:507-15; PMID:19945408; http://dx.doi.org/10.1016/j.cmet. 2009.10.008
316. Moscat J, Diaz-Meco MT. p62 at the crossroads of autophagy, apoptosis, and cancer. Cell 2009; 137: 1001-4; PMID:19524504; http://dx.doi.org/10.1016/j.cell.2009.05.023
317. Duran A, Amanchy R, Linares JF, Joshi J, Abu-Baker S, Porollo A, et al. p62 is a key regulator of nutrient sensing in the mTORC1 pathway. Mol Cell 2011; 44:134-46; PMID:21981924; http://dx.doi.org/10. 1016/j.molcel.2011.06.038
318. Komatsu M, Kurokawa H, Waguri S, Taguchi K, Kobayashi A, Ichimura Y, et al. The selective autophagy substrate p62 activates the stress responsive transcription factor Nrf2 through inactivation of Keap1. Nat Cell Biol 2010; 12:213-23; PMID:20173742
319. Jain A, Lamark T, Sjøttem E, Larsen KB, Awuh JA, Øvervatn A, et al. p62/SQSTM1 is a target gene for transcription factor NRF2 and creates a positive feedback loop by inducing antioxidant response elementdriven gene transcription. J Biol Chem 2010; 285: 22576-91; PMID:20452972; http://dx.doi.org/10.1074/jbc.M110.118976
320. Bardag-Gorce F, Francis T, Nan L, Li J, He Lue Y, French BA, et al. Modifications in P62 occur due to proteasome inhibition in alcoholic liver disease. Life Sci 2005; 77:2594-602; PMID:15964033; http://dx. doi.org/10.1016/j.lfs.2005.04.020
321. Myeku N, Figueiredo-Pereira ME. Dynamics of the degradation of ubiquitinated proteins by proteasomes and autophagy: association with sequestosome 1/p62. J Biol Chem 2011; 286:22426-40; PMID:21536669; http://dx.doi.org/10.1074/jbc.M110.149252
322. Norman JM, Cohen GM, Bampton ET. The in vitro cleavage of the hAtg proteins by cell death proteases. Autophagy 2010; 6:1042-56; PMID:21121091; http://dx.doi.org/10.4161/auto.6.8.13337
323. Lelouard H, Schmidt EK, Camosseto V, Clavarino G, Ceppi M, Hsu HT, et al. Regulation of translation is required for dendritic cell function and survival during activation. J Cell Biol 2007; 179:1427-39; PMID: 18166652; http://dx.doi.org/10.1083/jcb.200707166 324. Schmidt EK, Clavarino G, Ceppi M, Pierre P. SUnSET, a nonradioactive method to monitor protein synthesis. Nat Methods 2009; 6:275-7; PMID: 19305406; http://dx.doi.org/10.1038/nmeth.1314
325. Nakaso K, Yoshimoto Y, Nakano T, Takeshima T, Fukuhara Y, Yasui K, et al. Transcriptional activation of p62/A170/ZIP during the formation of the aggregates: possible mechanisms and the role in Lewy body formation in Parkinson's disease. Brain Res 2004; 1012:42-51; PMID:15158159; http://dx.doi.org/10. 1016/j.brainres.2004.03.029
326. Zheng Q, Su H, Ranek MJ, Wang X. Autophagy and p62 in cardiac proteinopathy. Circ Res 2011; 109:296-308; PMID:21659648; http://dx.doi.org/10.1161/CIRCRESAHA.111.244707
327. Colosetti P, Puissant A, Robert G, Luciano F, Jacquel A, Gounon P, et al. Autophagy is an important event for megakaryocytic differentiation of the chronic myelogenous leukemia K562 cell line. Autophagy 2009; 5:1092-8; PMID:19786835; http://dx.doi.org/10.4161/auto.5.8.9889
328. Toepfer N, Childress C, Parikh A, Rukstalis D, Yang W. Atorvastatin induces autophagy in prostate cancer PC3 cells through activation of LC3 transcription. Cancer Biol Ther 2011; 12:691-9; PMID:21768780;http://dx.doi.org/10.4161/cbt.12.8.15978
329. BenYounès A, Tajeddine N, Tailler M, Malik SA, Shen S, Métivier D, et al. A fluorescence-microscopic and cytofluorometric system for monitoring the turnover of the autophagic substrate p62/SQSTM1. Autophagy 2011; 7:883-91; PMID:21460612; http://dx.doi.org/10.4161/auto.7.8.15538
330. Bjørkøy G, Lamark T, Pankiv S, Øvervatn A, Brech A, Johansen T. Monitoring autophagic degradation of p62/SQSTM1. Methods Enzymol 2009; 452:181-97; PMID:19200883; http://dx.doi.org/10.1016/S0076-6879(08)03612-4
331. Lim J, Kim HW, Youdim MB, Rhyu IJ, Choe KM, Oh YJ. Binding preference of p62 towards LC3-ll during dopaminergic neurotoxin-induced impairment of autophagic flux. Autophagy 2011; 7:51-60; PMID: 21045561; http://dx.doi.org/10.4161/auto.7.1.13909
332. Waguri S, Komatsu M. Biochemical and morphological detection of inclusion bodies in autophagydeficient mice. Methods Enzymol 2009; 453:181-96; PMID:19216907; http://dx.doi.org/10.1016/S0076-6879(08)04009-3
333. Hocking LJ, Lucas GJ, Daroszewska A, Mangion J, Olavesen M, Cundy T, et al. Domain-specific mutations in sequestosome 1 (SQSTM1) cause familial and sporadic Paget's disease. Hum Mol Genet 2002; 11:2735-9; PMID:12374763; http://dx.doi.org/10.1093/hmg/11.22.2735
334. Mizushima N, Levine B. Autophagy in mammalian development and differentiation. Nat Cell Biol 2010; 12:823-30; PMID:20811354; http://dx.doi.org/10.1038/ncb0910-823
335. Maloverjan A, Piirsoo M, Michelson P, Kogerman P, Osterlund T. Identification of a novel serine/threonine kinase ULK3 as a positive regulator of Hedgehog pathway. Exp Cell Res 2010; 316:627-37; PMID: 19878745; http://dx.doi.org/10.1016/j.yexcr.2009.10.018
336. Young ARJ, Narita M, Ferreira M, Kirschner K, Sadaie M, Darot JF, et al. Autophagy mediates the mitotic senescence transition. Genes Dev 2009; 23:798-803; PMID:19279323; http://dx.doi.org/10. 1101/gad.519709
337. Chan EY, Tooze SA. Evolution of Atg1 function and regulation. Autophagy 2009; 5:758-65; PMID:19411825
338. Chan EY, Kir S, Tooze SA. siRNA screening of the kinome identifies ULK1 as a multidomain modulator of autophagy. J Biol Chem 2007; 282:25464-74; PMID:17595159; http://dx.doi.org/10.1074/jbc.M703663200

339. Hardie DG. AMP-activated protein kinase: an energy sensor that regulates all aspects of cell function. Genes Dev 2011; 25:1895-908; PMID:21937710; http://dx.doi.org/10.1101/gad.17420111
340. Carling D, Mayer FV, Sanders MJ, Gamblin SJ. AMPactivated protein kinase: nature's energy sensor. Nat Chem Biol 2011; 7:512-8; PMID:21769098; http://dx.doi.org/10.1038/nchembio.610
341. Samari HR, Møller MT, Holden L, Asmyhr T, Seglen PO. Stimulation of hepatocytic AMP-activated protein kinase by okadaic acid and other autophagysuppressive toxins. Biochem J 2005; 386:237-44; PMID: 15461583; http://dx.doi.org/10.1042/BJ20040609
342. Behrends C, Sowa ME, Gygi SP, Harper JW. Network organization of the human autophagy system. Nature 2010; 466:68-76; PMID:20562859; http://dx.doi.org/10.1038/nature09204
343. Chiacchiera F, Matrone A, Ferrari E, Ingravallo G, Lo Sasso G, Murzilli S, et al. p38alpha blockade inhibits colorectal cancer growth in vivo by inducing a switch from HIF1alpha- to FoxO-dependent transcription. Cell Death Differ 2009; 16:1203-14; PMID: 19343039; http://dx.doi.org/10.1038/cdd.2009.36
344. Kovács AL, Seglen PO. Inhibition of hepatocytic protein degradation by methylaminopurines and inhibitors of protein synthesis. Biochim Biophys Acta 1981; 676:213-20; PMID:7260116; http://dx.doi.org/ 10.1016/0304-4165(81)90189-6
345. Liu HY, Han J, Cao SY, Hong T, Zhuo D, Shi J, et al. Hepatic autophagy is suppressed in the presence of insulin resistance and hyperinsulinemia: inhibition of FoxO1-dependent expression of key autophagy genes by insulin. J Biol Chem 2009; 284:31484-92; PMID: 19758991; http://dx.doi.org/10.1074/jbc.M109.033936
346. Mammucari C, Milan G, Romanello V, Masiero E, Rudolf R, Del Piccolo P, et al. FoxO3 controls autophagy in skeletal muscle in vivo. Cell Metab 2007; 6:458-71; PMID:18054315; http://dx.doi.org/10. 1016/j.cmet.2007.11.001
347. Mihaylova MM, Vasquez DS, Ravnskjaer K, Denechaud PD, Yu RT, Alvarez JG, et al. Class IIa histone deacetylases are hormone-activated regulators of FOXO and mammalian glucose homeostasis. Cell 2011; 145:607-21; PMID:21565617; http://dx.doi.org/10.1016/j.cell.2011.03.043
348. Pfisterer SG, Mauthe M, Codogno P, Proikas-Cezanne T. Ca2+/calmodulin-dependent kinase (CaMK) signaling via CaMKI and AMP-activated protein kinase contributes to the regulation of WIPI-1 at the onset of autophagy. Mol Pharmacol 2011; 80:1066-75; PMID:21896713; http://dx.doi.org/10.1124/mol.111.071761
349. Rodgers JT, Lerin C, Gerhart-Hines Z, Puigserver P. Metabolic adaptations through the PGC-1 alpha and SIRT1 pathways. FEBS Lett 2008; 582:46-53; PMID: 18036349; http://dx.doi.org/10.1016/j.febslet.2007.11.034
350. Samari HR, Seglen PO. Inhibition of hepatocytic autophagy by adenosine, aminoimidazole-4-carboxamide riboside, and N6-mercaptopurine riboside. Evidence for involvement of amp-activated protein kinase. J Biol Chem 1998; 273:23758-63; PMID:9726984; http://dx.doi.org/10.1074/jbc.273.37.23758
351. Sanchez AM, Csibi A, Raibon A, Cornille K, Gay S, Bernardi H, et al. AMPK promotes skeletal muscle autophagy through activation of Forkhead FoxO3a and interaction with Ulk1. J Cell Biochem 2012; PMID:22006269; http://dx.doi.org/10.1002/jcb.23399
352. Inoki K, Zhu T, Guan KL. TSC2 mediates cellular energy response to control cell growth and survival. Cell 2003; 115:577-90; PMID:14651849; http://dx.doi.org/10.1016/S0092-8674(03)00929-2
353. Gwinn DM, Shackelford DB, Egan DF, Mihaylova MM, Mery A, Vasquez DS, et al. AMPK phosphorylation of raptor mediates a metabolic checkpoint. Mol Cell 2008; 30:214-26; PMID:18439900; http://dx.doi.org/10.1016/j.molcel.2008.03.003
354. Egan D, Kim J, Shaw RJ, Guan K-L. The autophagy initiating kinase ULK1 is regulated via opposing phosphorylation by AMPK and mTOR. Autophagy 2011; 7:643-4; PMID:21460621; http://dx.doi.org/10.4161/auto.7.6.15123
355. Egan DF, Shackelford DB, Mihaylova MM, Gelino S, Kohnz RA, Mair W, et al. Phosphorylation of ULK1 (hATG1) by AMP-activated protein kinase connects energy sensing to mitophagy. Science 2011; 331: 456-61; PMID:21205641; http://dx.doi.org/10.1126/science.1196371
356. Kim J, Kundu M, Viollet B, Guan K-L. AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. Nat Cell Biol 2011; 13:132-41; PMID:21258367; http://dx.doi.org/10.1038/ncb2152
357. Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, et al. Role of AMP-activated protein kinase in mechanism of metformin action. J Clin Invest 2001;108:1167-74; PMID:11602624
358. Emerling BM, Viollet B, Tormos KV, Chandel NS. Compound C inhibits hypoxic activation of HIF-1 independent of AMPK. FEBS Lett 2007; 581:5727-31; PMID:18036344; http://dx.doi.org/10.1016/j.febslet.2007.11.038
359. Vucicevic L, Misirkic M, Janjetovic K, Vilimanovich U, Sudar E, Isenovic E, et al. Compound C induces protective autophagy in cancer cells through AMPK inhibition-independent blockade of Akt/mTOR pathway. Autophagy 2011; 7:40-50; PMID:20980833;http://dx.doi.org/10.4161/auto.7.1.13883
360. Meley D, Bauvy C, Houben-Weerts JH, Dubbelhuis PF, Helmond MT, Codogno P, et al. AMP-activated protein kinase and the regulation of autophagic proteolysis. J Biol Chem 2006; 281:34870-9; PMID: 16990266; http://dx.doi.org/10.1074/jbc.M605488200
361. Grotemeier A, Alers S, Pfisterer SG, Paasch F, Daubrawa M, Dieterle A, et al. AMPK-independent induction of autophagy by cytosolic Ca2+ increase. Cell Signal 2010; 22:914-25; PMID:20114074; http://dx.doi.org/10.1016/j.cellsig.2010.01.015
362. Williams T, Forsberg LJ, Viollet B, Brenman JE. Basal autophagy induction without AMP-activated protein kinase under low glucose conditions. Autophagy 2009; 5:1155-65; PMID:19844161; http://dx.doi.org/10.4161/auto.5.8.10090
363. Shang L, Chen S, Du F, Li S, Zhao L, Wang X. Nutrient starvation elicits an acute autophagic response mediated by Ulk1 dephosphorylation and its subsequent dissociation from AMPK. Proc Natl Acad Sci U S A 2011; 108:4788-93; PMID:21383122; http://dx.doi.org/10.1073/pnas.1100844108
364. Zoncu R, Bar-Peled L, Efeyan A, Wang S, Sancak Y, Sabatini DM. mTORC1 senses lysosomal amino acids through an inside-out mechanism that requires the vacuolar H(+)-ATPase. Science 2011; 334:678-83; PMID:22053050; http://dx.doi.org/10.1126/science.1207056
365. Di Bartolomeo S, Corazzari M, Nazio F, Oliverio S, Lisi G, Antonioli M, et al. The dynamic interaction of AMBRA1 with the dynein motor complex regulates mammalian autophagy. J Cell Biol 2010; 191: 155-68; PMID:20921139; http://dx.doi.org/10.1083/jcb.201002100
366. Tang HW, Wang YB, Wang SL, Wu MH, Lin SY, Chen GC. Atg1-mediated myosin II activation regulates autophagosome formation during starvation-induced
autophagy. EMBO J 2011; 30:636-51; PMID:21169990; http://dx.doi.org/10.1038/emboj.2010.338
367. Jung CH, Jun CB, Ro S-H, Kim Y-M, Otto NM, Cao J, et al. ULK-Atg13-FIP200 complexes mediate mTOR signaling to the autophagy machinery. Mol Biol Cell 2009; 20:1992-2003; PMID:19225151; http://dx.doi.org/10.1091/mbc.E08-12-1249
368. Chang Y-Y, Neufeld TP. An Atg1/Atg13 complex with multiple roles in TOR-mediated autophagy regulation. Mol Biol Cell 2009; 20:2004-14; PMID:19225150;http://dx.doi.org/10.1091/mbc.E08-12-1250
369. Hosokawa N, Hara T, Kaizuka T, Kishi C, Takamura A, Miura Y, et al. Nutrient-dependent mTORC1 association with the ULK1-Atg13-FIP200 complex required for autophagy. Mol Biol Cell 2009; 20:1981-91; PMID:19211835; http://dx.doi.org/10.1091/mbc.E08-12-1248
370. Chan EYW, Longatti A, McKnight NC, Tooze SA. Kinase-inactivated ULK proteins inhibit autophagy via their conserved C-terminal domains using an Atg13-independent mechanism. Mol Cell Biol 2009; 29:157-71; PMID:18936157; http://dx.doi.org/10.1128/MCB.01082-08
371. Jung CH, Seo M, Otto NM, Kim DH. ULK1 inhibits the kinase activity of mTORC1 and cell proliferation. Autophagy 2011; 7:1212-21; PMID:21795849;http://dx.doi.org/10.4161/auto.7.10.16660

372. Löffler AS, Alers S, Dieterle AM, Keppeler H, Franz- Wachtel M, Kundu M, et al. Ulk1-mediated phosphorylation of AMPK constitutes a negative regulatory feedback loop. Autophagy 2011; 7:696-706; PMID:21460634; http://dx.doi.org/10.4161/auto.7.7.15451
373. Erlich S, Alexandrovich A, Shohami E, Pinkas-Kramarski R. Rapamycin is a neuroprotective treatment for traumatic brain injury. Neurobiol Dis 2007; 26:86-93; PMID:17270455; http://dx.doi.org/10.1016/j.nbd.2006.12.003
374. Lavieu G, Scarlatti F, Sala G, Carpentier S, Levade T, Ghidoni R, et al. Regulation of autophagy by sphingosine kinase 1 and its role in cell survival during nutrient starvation. J Biol Chem 2006; 281:8518- 27; PMID:16415355; http://dx.doi.org/10.1074/jbc.M506182200
375. Brunn GJ, Hudson CC, Sekulić A, Williams JM, Hosoi H, Houghton PJ, et al. Phosphorylation of the translational repressor PHAS-I by the mammalian target of rapamycin. Science 1997; 277:99-101; PMID:9204908; http://dx.doi.org/10.1126/science.277.5322.99
376. Yip CK, Murata K, Walz T, Sabatini DM, Kang SA. Structure of the human mTOR complex I and its implications for rapamycin inhibition. Mol Cell 2010; 38:768-74; PMID:20542007; http://dx.doi.org/10.1016/j.molcel.2010.05.017
377. Cheong H, Nair U, Geng J, Klionsky DJ. The Atg1 kinase complex is involved in the regulation of protein recruitment to initiate sequestering vesicle formation for nonspecific autophagy in Saccharomyces cerevisiae. Mol Biol Cell 2008; 19:668-81; PMID:18077553; http://dx.doi.org/10.1091/mbc.E07-08-0826
378. Kabeya Y, Kamada Y, Baba M, Takikawa H, Sasaki M, Ohsumi Y. Atg17 functions in cooperation with Atg1 and Atg13 in yeast autophagy. Mol Biol Cell 2005; 16:2544-53; PMID:15743910; http://dx.doi.org/10.1091/mbc.E04-08-0669
379. Kamada Y, Funakoshi T, Shintani T, Nagano K, Ohsumi M, Ohsumi Y. Tor-mediated induction of autophagy via an Apg1 protein kinase complex. J Cell Biol 2000; 150:1507-13; PMID:10995454; http://dx. doi.org/10.1083/jcb.150.6.1507
380. Scott SV, Nice DC, 3rd, Nau JJ, Weisman LS, Kamada Y, Keizer-Gunnink I, et al. Apg13p and Vac8p are part of a complex of phosphoproteins that are required for cytoplasm to vacuole targeting. J Biol Chem 2000; 275:25840-9; PMID:10837477; http://dx.doi.org/10.1074/jbc.M002813200
381. Mao K, Wang K, Zhao M, Xu T, Klionsky DJ. Two MAPK-signaling pathways are required for mitophagy in Saccharomyces cerevisiae. J Cell Biol 2011; 193:755-67; PMID:21576396; http://dx.doi.org/10.1083/jcb.201102092
382. Yeh YY, Wrasman K, Herman PK. Autophosphorylation within the Atg1 activation loop is required for both kinase activity and the induction of autophagy in Saccharomyces cerevisiae. Genetics 2010; 185:871-82; PMID:20439775; http://dx.doi.org/10.1534/genetics.110.116566
383. Singh K, Matsuyama S, Drazba JA, Almasan A. Autophagy-dependent senescence in response to DNA damage and chronic apoptotic stress. Autophagy 2012; 8; In press; PMID:22240589
384. Djavaheri-Mergny M, Amelotti M, Mathieu J, Besançon F, Bauvy C, Souquère S, et al. NF-kappaB activation represses tumor necrosis factor-alpha induced autophagy. J Biol Chem 2006; 281:30373-82; PMID:16857678; http://dx.doi.org/10.1074/jbc.M602097200
385. Liu Z, Lenardo MJ. Reactive oxygen species regulate autophagy through redox-sensitive proteases. Dev Cell 2007; 12:484-5; PMID:17419989; http://dx.doi.org/10.1016/j.devcel.2007.03.016
386. Scarlatti F, Bauvy C, Ventruti A, Sala G, Cluzeaud F, Vandewalle A, et al. Ceramide-mediated macroautophagy involves inhibition of protein kinase B and up-regulation of beclin 1. J Biol Chem 2004; 279: 18384-91; PMID:14970205; http://dx.doi.org/10.1074/jbc.M313561200
387. Scherz-Shouval R, Shvets E, Fass E, Shorer H, Gil L, Elazar Z. Reactive oxygen species are essential for autophagy and specifically regulate the activity of Atg4. EMBO J 2007; 26:1749-60; PMID:17347651; http://dx.doi.org/10.1038/sj.emboj.7601623
388. Zeng X, Kinsella TJ. Mammalian target of rapamycin and S6 kinase 1 positively regulate 6-thioguanineinduced autophagy. Cancer Res 2008; 68:2384-90; PMID:18381446; http://dx.doi.org/10.1158/0008-5472.CAN-07-6163
389. Yu L, McPhee CK, Zheng L, Mardones GA, Rong Y, Peng J, et al. Termination of autophagy and reformation of lysosomes regulated by mTOR. Nature 2010; 465:942-6; PMID:20526321; http://dx.doi.org/10.1038/nature09076
390. Mochizuki H, Toda H, Ando M, Kurusu M, Tomoda T, Furukubo-Tokunaga K. Unc-51/ATG1 controls axonal and dendritic development via kinesin-mediated vesicle transport in the Drosophila brain. PLoS One 2011; 6:e19632; PMID:21589871; http://dx.doi.org/10.1371/journal.pone.0019632
391. Wairkar YP, Toda H, Mochizuki H, Furukubo-Tokunaga K, Tomoda T, Diantonio A. Unc-51 controls active zone density and protein composition by downregulating ERK signaling. J Neurosci 2009; 29:517-28; PMID:19144852; http://dx.doi.org/10.1523/JNEUROSCI.3848-08.2009
392. Loh SH, Francescut L, Lingor P, Bähr M, Nicotera P. Identification of new kinase clusters required for neurite outgrowth and retraction by a loss-of-function RNA interference screen. Cell Death Differ 2008; 15:283-98; PMID:18007665; http://dx.doi.org/10.1038/sj.cdd.4402258
393. Zhou X, Babu JR, da Silva S, Shu Q, Graef IA, Oliver T, et al. Unc-51-like kinase 1/2-mediated endocytic processes regulate filopodia extension and branching of sensory axons. Proc Natl Acad Sci U S A 2007; 104:5842-7; PMID:17389358; http://dx.doi.org/10.1073/pnas.0701402104
394. Tomoda T, Kim JH, Zhan C, Hatten ME. Role of Unc51.1 and its binding partners in CNS axon outgrowth. Genes Dev 2004; 18:541-58; PMID: 15014045; http://dx.doi.org/10.1101/gad.1151204
395. Okazaki N, Yan J, Yuasa S, Ueno T, Kominami E, Masuho Y, et al. Interaction of the Unc-51-like kinase and microtubule-associated protein light chain 3 related proteins in the brain: possible role of vesicular transport in axonal elongation. Brain Res Mol Brain Res 2000; 85:1-12; PMID:11146101; http://dx.doi. org/10.1016/S0169-328X(00)00218-7
396. Young ARJ, Chan EYW, Hu XW, Köchl R, Crawshaw SG, High S, et al. Starvation and ULK1-dependent cycling of mammalian Atg9 between the TGN and endosomes. J Cell Sci 2006; 119:3888-900; PMID: 16940348; http://dx.doi.org/10.1242/jcs.03172
397. Reggiori F, Shintani T, Nair U, Klionsky DJ. Atg9 cycles between mitochondria and the pre-autophagosomal structure in yeasts. Autophagy 2005; 1:101-9; PMID:16874040; http://dx.doi.org/10.4161/auto.1.2.1840
398. Mari M, Griffith J, Rieter E, Krishnappa L, Klionsky DJ, Reggiori F. An Atg9-containing compartment that functions in the early steps of autophagosome biogenesis. J Cell Biol 2010; 190:1005-22; PMID: 20855505; http://dx.doi.org/10.1083/jcb.200912089
399. Reggiori F, Tucker KA, Stromhaug PE, Klionsky DJ. The Atg1-Atg13 complex regulates Atg9 and Atg23 retrieval transport from the pre-autophagosomal structure. Dev Cell 2004; 6:79-90; PMID:14723849; http://dx.doi.org/10.1016/S1534-5807(03)00402-7
400. Mizushima N, Kuma A, Kobayashi Y, Yamamoto A, Matsubae M, Takao T, et al. Mouse Apg16L, a novel WD-repeat protein, targets to the autophagic isolation membrane with the Apg12-Apg5 conjugate. J Cell Sci 2003; 116:1679-88; PMID:12665549; http://dx.doi.org/10.1242/jcs.00381
401. Mizushima N, Yamamoto A, Hatano M, Kobayashi Y, Kabeya Y, Suzuki K, et al. Dissection of autophagosome formation using Apg5-deficient mouse embryonic stem cells. J Cell Biol 2001; 152:657-68; PMID: 11266458; http://dx.doi.org/10.1083/jcb.152.4.657
402. Thompson AR, Doelling JH, Suttangkakul A, Vierstra RD. Autophagic nutrient recycling in Arabidopsis directed by the ATG8 and ATG12 conjugation pathways. Plant Physiol 2005; 138:2097-110; PMID:16040659; http://dx.doi.org/10.1104/pp.105.060673
403. Yousefi S, Perozzo R, Schmid I, Ziemiecki A, Schaffner T, Scapozza L, et al. Calpain-mediated cleavage of Atg5 switches autophagy to apoptosis. Nat Cell Biol 2006; 8:1124-32; PMID:16998475; http://dx.doi.org/10.1038/ncb1482
404. Kihara A, Noda T, Ishihara N, Ohsumi Y. Two distinct Vps34 phosphatidylinositol 3-kinase complexes function in autophagy and carboxypeptidase Y sorting in Saccharomyces cerevisiae. J Cell Biol 2001; 152:519-30; PMID:11157979; http://dx.doi.org/10. 1083/jcb.152.3.519
405. Matsunaga K, Saitoh T, Tabata K, Omori H, Satoh T, Kurotori N, et al. Two Beclin 1-binding proteins, Atg14L and Rubicon, reciprocally regulate autophagy at different stages. Nat Cell Biol 2009; 11:385-96; PMID:19270696; http://dx.doi.org/10.1038/ncb1846
406. Zhong Y, Wang QJ, Li X, Yan Y, Backer JM, Chait BT, et al. Distinct regulation of autophagic activity by Atg14L and Rubicon associated with Beclin 1-phosphatidylinositol-3-kinase complex. Nat Cell Biol 2009; 11:468-76; PMID:19270693; http://dx.doi.org/10.1038/ncb1854

407. Sun Q, Fan W, Chen K, Ding X, Chen S, Zhong Q. Identification of Barkor as a mammalian autophagyspecific factor for Beclin 1 and class III phosphatidylinositol 3-kinase. Proc Natl Acad Sci U S A 2008; 105:19211-6; PMID:19050071; http://dx.doi.org/10.1073/pnas.0810452105
408. Itakura E, Kishi C, Inoue K, Mizushima N. Beclin 1 forms two distinct phosphatidylinositol 3-kinase complexes with mammalian Atg14 and UVRAG. Mol Biol Cell 2008; 19:5360-72; PMID:18843052; http://dx.doi.org/10.1091/mbc.E08-01-0080
409. Fan W, Nassiri A, Zhong Q. Autophagosome targeting and membrane curvature sensing by Barkor/Atg14(L). Proc Natl Acad Sci U S A 2011; 108:7769-74; PMID: 21518905; http://dx.doi.org/10.1073/pnas.1016472108
410. Matsunaga K, Morita E, Saitoh T, Akira S, Ktistakis NT, Izumi T, et al. Autophagy requires endoplasmic reticulum targeting of the PI3-kinase complex via Atg14L. J Cell Biol 2010; 190:511-21; PMID: 20713597; http://dx.doi.org/10.1083/jcb.200911141
411. Ravikumar B, Moreau K, Jahreiss L, Puri C, Rubinsztein DC. Plasma membrane contributes to the formation of pre-autophagosomal structures. Nat Cell Biol 2010; 12:747-57; PMID:20639872; http://dx.doi.org/10.1038/ncb2078
412. Guan J, Stromhaug PE, George MD, Habibzadegah-Tari P, Bevan A, Dunn WA, Jr., et al. Cvt18/Gsa12 is required for cytoplasm-to-vacuole transport, pexophagy, and autophagy in Saccharomyces cerevisiae and Pichia pastoris. Mol Biol Cell 2001; 12:3821-38;PMID:11739783
413. Barth H, Meiling-Wesse K, Epple UD, Thumm M. Autophagy and the cytoplasm to vacuole targeting pathway both require Aut10p. FEBS Lett 2001; 508:23-8; PMID:11707261; http://dx.doi.org/10. 1016/S0014-5793(01)03016-2
414. Proikas-Cezanne T, Waddell S, Gaugel A, Frickey T, Lupas A, Nordheim A. WIPI-1alpha (WIPI49), a member of the novel 7-bladed WIPI protein family, is aberrantly expressed in human cancer and is linked to starvation-induced autophagy. Oncogene 2004; 23:9314-25; PMID:15602573; http://dx.doi.org/10.1038/sj.onc.1208331
415. Monastyrska I, Klionsky DJ. Autophagy in organelle homeostasis: peroxisome turnover. Mol Aspects Med 2006; 27:483-94; PMID:16973210; http://dx.doi.org/10.1016/j.mam.2006.08.004
416. Nair U, Klionsky DJ. Molecular mechanisms and regulation of specific and nonspecific autophagy pathways in yeast. J Biol Chem 2005; 280:41785-8; PMID: 16230342; http://dx.doi.org/10.1074/jbc.R500016200
417. Tallóczy Z, Virgin HW, 4th, Levine B. PKR-dependent autophagic degradation of herpes simplex virus type 1.Autophagy 2006; 2:24-9; PMID:16874088
418. Polson HE, de Lartigue J, Rigden DJ, Reedijk M, Urbé S, Clague MJ, et al. Mammalian Atg18 (WIPI2) localizes to omegasome-anchored phagophores and positively regulates LC3 lipidation. Autophagy 2010; 6:506-22; PMID:20505359; http://dx.doi.org/10.4161/auto.6.4.11863
419. Proikas-Cezanne T, Ruckerbauer S, Stierhof YD, Berg C, Nordheim A. Human WIPI-1 puncta-formation: a novel assay to assess mammalian autophagy. FEBS Lett 2007; 581:3396-404; PMID:17618624; http://dx.doi.org/10.1016/j.febslet.2007.06.040
420. Itakura E, Mizushima N. Characterization of autophagosome formation site by a hierarchical analysis of mammalian Atg proteins. Autophagy 2010; 6:764-76; PMID:20639694; http://dx.doi.org/10.4161/auto.6.6. 12709
421. Mauthe M, Jacob A, Freiberger S, Hentschel K, Stierhof YD, Codogno P, et al. Resveratrol-mediated autophagy requires WIPI-1 regulated LC3 lipidation in the absence of induced phagophore formation. Autophagy 2011; 7:1448-61; PMID:22082875; http://dx.doi.org/10.4161/auto.7.12.17802
422. Lu Q, Yang P, Huang X, Hu W, Guo B, Wu F, et al. The WD40 repeat PtdIns(3)P-binding protein EPG-6 regulates progression of omegasomes to autophagosomes. Dev Cell 2011; 21:343-57; PMID:21802374; http://dx.doi.org/10.1016/j.devcel.2011.06.024
423. Yang Z, Klionsky DJ. Mammalian autophagy: core molecular machinery and signaling regulation. Curr Opin Cell Biol 2010; 22:124-31; PMID:20034776; http://dx.doi.org/10.1016/j.ceb.2009.11.014
424. Cao Y, Klionsky DJ. Physiological functions of Atg6/Beclin 1: a unique autophagy-related protein. Cell Res 2007; 17:839-49; PMID:17893711; http://dx.doi.org/10.1038/cr.2007.78
425. Pattingre S, Tassa A, Qu X, Garuti R, Liang XH, Mizushima N, et al. Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. Cell 2005; 122:927-39; PMID:16179260; http://dx.doi.org/10.1016/j.cell.2005.07.002
426. Zalckvar E, Berissi H, Mizrachy L, Idelchuk Y, Koren I, Eisenstein M, et al. DAP-kinase-mediated phosphorylation on the BH3 domain of beclin 1 promotes dissociation of beclin 1 from Bcl-XL and induction of autophagy. EMBO Rep 2009; 10:285-92; PMID:19180116; http://dx.doi.org/10.1038/embor.2008.246
427. Wei Y, Sinha S, Levine B. Dual role of JNK1-mediated phosphorylation of Bcl-2 in autophagy and apoptosis regulation. Autophagy 2008; 4:949-51; PMID:18769111
428. Wei Y, Pattingre S, Sinha S, Bassik M, Levine B. JNK1-mediated phosphorylation of Bcl-2 regulates starvation-induced autophagy. Mol Cell 2008; 30:678-88; PMID:18570871; http://dx.doi.org/10.1016/j.molcel.2008.06.001
429. Lossi L, Gambino G, Ferrini F, Alasia S, Merighi A. Posttranslational regulation of BCL2 levels in cerebellar granule cells: A mechanism of neuronal survival. Dev Neurobiol 2009; 69:855-70; PMID:19672954; http://dx.doi.org/10.1002/dneu.20744
430. Lossi L, Gambino G, Salio C, Merighi A. Autophagy regulates the post-translational cleavage of BCL-2 and promotes neuronal survival. ScientificWorldJournal 2010; 10:924-9; PMID:20495771; http://dx.doi.org/ 10.1100/tsw.2010.82
431. Scarlatti F, Maffei R, Beau I, Codogno P, Ghidoni R. Role of non-canonical Beclin 1-independent autophagy in cell death induced by resveratrol in human breast cancer cells. Cell Death Differ 2008; 15:1318-29; PMID:18421301; http://dx.doi.org/10.1038/cdd.2008.51
432. Kang R, Zeh HJ, Lotze MT, Tang D. The Beclin 1 network regulates autophagy and apoptosis. Cell Death Differ 2011; 18:571-80; PMID:21311563; http://dx. doi.org/10.1038/cdd.2010.191
433. Kihara A, Kabeya Y, Ohsumi Y, Yoshimori T. Beclinphosphatidylinositol 3-kinase complex functions at the trans-Golgi network. EMBO Rep 2001; 2:330-5; PMID:11306555; http://dx.doi.org/10.1093/emboreports/kve061
434. Amritraj A, Peake K, Kodam A, Salio C, Merighi A, Vance JE, et al. Increased activity and altered subcellular distribution of lysosomal enzymes determine neuronal vulnerability in Niemann-Pick type C1-deficient mice. Am J Pathol 2009; 175:2540-56; PMID:19893049;http://dx.doi.org/10.2353/ajpath.2009.081096
435. Castino R, Bellio N, Follo C, Murphy D, Isidoro C. Inhibition of PI3k class III-dependent autophagy prevents apoptosis and necrosis by oxidative stress in dopaminergic neuroblastoma cells. Toxicol Sci 2010; 117:152-62; PMID:20525898; http://dx.doi.org/10.1093/toxsci/kfq170
436. Yue Z, Horton A, Bravin M, DeJager PL, Selimi F, Heintz N. A novel protein complex linking the delta 2 glutamate receptor and autophagy: implications for neurodegeneration in lurcher mice. Neuron 2002; 35:921-33; PMID:12372286; http://dx.doi.org/10.1016/S0896-6273(02)00861-9
437. Axe EL, Walker SA, Manifava M, Chandra P, Roderick HL, Habermann A, et al. Autophagosome formation from membrane compartments enriched in phosphatidylinositol 3-phosphate and dynamically connected to the endoplasmic reticulum. J Cell Biol 2008; 182:685-701; PMID:18725538; http://dx.doi. org/10.1083/jcb.200803137
438. Crighton D, Wilkinson S, O'Prey J, Syed N, Smith P, Harrison PR, et al. DRAM, a p53-induced modulator of autophagy, is critical for apoptosis. Cell 2006; 126:121-34; PMID:16839881; http://dx.doi.org/10.1016/j.cell.2006.05.034
439. Valbuena A, Castro-Obregón S, Lazo PA. Downregulation of VRK1 by p53 in response to DNA damage is mediated by the autophagic pathway. PLoS One 2011; 6:e17320; PMID:21386980; http://dx.doi.org/10.1371/journal.pone.0017320
440. Lorin S, Pierron G, Ryan KM, Codogno P, Djavaheri-Mergny M. Evidence for the interplay between JNK and p53-DRAM signalling pathways in the regulation of autophagy. Autophagy 2010; 6:153-4; PMID: 19949306; http://dx.doi.org/10.4161/auto.6.1.10537

441. Nara A, Mizushima N, Yamamoto A, Kabeya Y, Ohsumi Y, Yoshimori T. SKD1 AAA ATPasedependent endosomal transport is involved in autolysosome formation. Cell Struct Funct 2002; 27: 29-37; PMID:11937716; http://dx.doi.org/10.1247/csf.27.29
442. Kirisako T, Baba M, Ishihara N, Miyazawa K, Ohsumi M, Yoshimori T, et al. Formation process of autophagosome is traced with Apg8/Aut7p in yeast. J Cell Biol 1999; 147:435-46; PMID:10525546; http://dx. doi.org/10.1083/jcb.147.2.435
443. Kouroku Y, Fujita E, Tanida I, Ueno T, Isoai A, Kumagai H, et al. ER stress (PERK/eIF2alpha phosphorylation) mediates the polyglutamine-induced LC3 conversion, an essential step for autophagy formation. Cell Death Differ 2007; 14:230-9; PMID:16794605; http://dx.doi.org/10.1038/sj.cdd.4401984
444. Moussay E, Kaoma T, Baginska J, Muller A, Van Moer K, Nicot N, et al. The acquisition of resistance to TNFa in breast cancer cells is associated with constitutive activation of autophagy as revealed by a transcriptome analysis using a custom microarray. Autophagy 2011; 7:760-70; PMID:21490427; http:// dx.doi.org/10.4161/auto.7.7.15454
445. Mitroulis I, Kourtzelis I, Kambas K, Rafail S, Chrysanthopoulou A, Speletas M, et al. Regulation of the autophagic machinery in human neutrophils. Eur J Immunol 2010; 40:1461-72; PMID:20162553;http://dx.doi.org/10.1002/eji.200940025
446. Rodríguez-Muela N, Germain F, Mariño G, Fitze PS, Boya P. Autophagy promotes survival of retinal ganglion cells after optic nerve axotomy in mice. Cell Death Differ 2012; 19:162-9; PMID:21701497;http://dx.doi.org/10.1038/cdd.2011.88
447. Vázquez P, Arroba AI, Cecconi F, de la Rosa EJ, Boya P, De Pablo F. Atg5 and Ambra1 differentially modulate neurogenesis in neural stem cells. Autophagy 2012; 8; In press; PMID:22240590
448. Rouschop KM, van den Beucken T, Dubois L, Niessen H, Bussink J, Savelkouls K, et al. The unfolded protein response protects human tumor cells during hypoxia through regulation of the autophagy genes MAP1LC3B and ATG5. J Clin Invest 2010; 120:127-41; PMID:20038797; http://dx.doi.org/10. 1172/JCI40027
449. Gorski SM, Chittaranjan S, Pleasance ED, Freeman JD, Anderson CL, Varhol RJ, et al. A SAGE approach to discovery of genes involved in autophagic cell death. Curr Biol 2003; 13:358-63; PMID:12593804; http://dx.doi.org/10.1016/S0960-9822(03)00082-4
450. Lee C-Y, Clough EA, Yellon P, Teslovich TM, Stephan DA, Baehrecke EH. Genome-wide analyses of steroid- and radiation-triggered programmed cell death in Drosophila. Curr Biol 2003; 13:350-7; PMID: 12593803; http://dx.doi.org/10.1016/S0960-9822 (03)00085-X
451. Denton D, Shravage B, Simin R, Baehrecke EH,
Kumar S. Larval midgut destruction in Drosophila: dependent on caspases but suppressed by the loss of autophagy. Autophagy 2010; 6:163-5; PMID:20009534; http://dx.doi.org/10.4161/auto.6.1.10601
452. Franzetti E, Huang ZJ, Shi YX, Xie K, Deng XJ, Li JP, et al. Erratum to: Autophagy precedes apoptosis during the remodeling of silkworm larval midgut. Apoptosis 2012; In press; PMID:22223360; http://dx.doi.org/10.1007/s10495-011-0693-y
453. Juhász G, Puskás LG, Komonyi O, Erdi B, Maróy P, Neufeld TP, et al. Gene expression profiling identifies FKBP39 as an inhibitor of autophagy in larval Drosophila fat body. Cell Death Differ 2007; 14:1181-90; PMID:17363962; http://dx.doi.org/10.1038/sj.cdd. 4402123
454. Barth JM, Szabad J, Hafen E, Köhler K. Autophagy in Drosophila ovaries is induced by starvation and is required for oogenesis. Cell Death Differ 2011; 18:915-24; PMID:21151027; http://dx.doi.org/10. 1038/cdd.2010.157
455. Lecker SH, Jagoe RT, Gilbert A, Gomes M, Baracos V, Bailey J, et al. Multiple types of skeletal muscle atrophy involve a common program of changes in gene expression. FASEB J 2004; 18:39-51; PMID: 14718385; http://dx.doi.org/10.1096/fj.03-0610com
456. Phillips AR, Suttangkakul A, Vierstra RD. The ATG12-conjugating enzyme ATG10 Is essential for autophagic vesicle formation in Arabidopsis thaliana. Genetics 2008; 178:1339-53; PMID:18245858; http://dx.doi.org/10.1534/genetics.107.086199
457. Seiliez I, Gutierrez J, Salmerón C, Skiba-Cassy S, Chauvin C, Dias K, et al. An in vivo and in vitro assessment of autophagy-related gene expression in muscle of rainbow trout (Oncorhynchus mykiss). Comp Biochem Physiol B Biochem Mol Biol 2010; 157:258-66; PMID:20601058; http://dx.doi.org/10.1016/j.cbpb.2010.06.011
458. Lapierre LR, Gelino S, Meléndez A, Hansen M. Autophagy and lipid metabolism coordinately modulate life span in germline-less C. elegans. Curr Biol 2011; 21:1507-14; PMID:21906946; http://dx.doi.org/10.1016/j.cub.2011.07.042
459. Sandri M. Autophagy in health and disease. 3. Involvement of autophagy in muscle atrophy. Am J Physiol Cell Physiol 2010; 298:C1291-7; PMID: 20089936; http://dx.doi.org/10.1152/ajpcell.00531.2009
460. Eisenberg T, Knauer H, Schauer A, Büttner S, Ruckenstuhl C, Carmona-Gutierrez D, et al. Induction of autophagy by spermidine promotes longevity. Nat Cell Biol 2009; 11:1305-14; PMID:19801973; http://dx.doi.org/10.1038/ncb1975
461. Ropolo A, Grasso D, Pardo R, Sacchetti ML, Archange C, Lo Re A, et al. The pancreatitis-induced vacuole membrane protein 1 triggers autophagy in mammalian cells. J Biol Chem 2007; 282:37124-33; PMID: 17940279; http://dx.doi.org/10.1074/jbc.M706956200
462. Tian Y, Li Z, Hu W, Ren H, Tian E, Zhao Y, et al. C. elegans screen identifies autophagy genes specific to multicellular organisms. Cell 2010; 141:1042-55; PMID:20550938; http://dx.doi.org/10.1016/j.cell. 2010.04.034
463. Settembre C, Di Malta C, Polito VA, Garcia Arencibia M, Vetrini F, Erdin S, et al. TFEB links autophagy to lysosomal biogenesis. Science 2011; 332:1429-33; PMID:21617040; http://dx.doi.org/10.1126/science. 1204592
464. Sardiello M, Palmieri M, di Ronza A, Medina DL, Valenza M, Gennarino VA, et al. A gene network regulating lysosomal biogenesis and function. Science2009; 325:473-7; PMID:19556463
465. Palmieri M, Impey S, Kang H, di Ronza A, Pelz C, Sardiello M, et al. Characterization of the CLEAR network reveals an integrated control of cellular clearance pathways. Hum Mol Genet 2011; 20:3852-66; PMID: 21752829; http://dx.doi.org/10.1093/hmg/ddr306
466. Kang YA, Sanalkumar R, O'Geen H, Linnemann AK, Chang CJ, Bouhassira EE, et al. Autophagy driven by a master regulator of hematopoiesis. Mol Cell Biol 2012; 32:226-39; PMID:22025678; http://dx.doi.org/10.1128/MCB.06166-11
467. Ma D, Panda S, Lin JD. Temporal orchestration of circadian autophagy rhythm by C/EBPβ. EMBO J 2011; 30:4642-51; PMID:21897364; http://dx.doi.org/10.1038/emboj.2011.322
468. Brest P, Lapaquette P, Souidi M, Lebrigand K, Cesaro A, Vouret-Craviari V, et al. A synonymous variant in IRGM alters a binding site for miR-196 and causes deregulation of IRGM-dependent xenophagy in Crohn's disease. Nat Genet 2011; 43:242-5; PMID:21278745; http://dx.doi.org/10.1038/ng.762
469. Meenhuis A, van Veelen PA, de Looper H, van Boxtel N, van den Berge IJ, Sun SM, et al. MiR-17/20/93/ 106 promote hematopoietic cell expansion by targeting sequestosome 1-regulated pathways in mice. Blood 2011; 118:916-25; PMID:21628417; http://dx.doi.org/10.1182/blood-2011-02-336487
470. Roccaro AM, Sacco A, Jia X, Azab AK, Maiso P, Ngo HT, et al. microRNA-dependent modulation of histone acetylation in Waldenstrom macroglobulinemia. Blood 2010; 116:1506-14; PMID:20519629; http://dx.doi.org/10.1182/blood-2010-01-265686
471. Martinet W, De Meyer GR, Andries L, Herman AG, Kockx MM. In situ detection of starvation-induced autophagy. J Histochem Cytochem 2006; 54:85-96; PMID:16148314; http://dx.doi.org/10.1369/jhc.5A6743.2005
472. Pattingre S, Petiot A, Codogno P. Analyses of Galphainteracting protein and activator of G-protein-signaling-3 functions in macroautophagy. Methods Enzymol 2004; 390:17-31; PMID:15488168; http://dx.doi.org/10.1016/S0076-6879(04)90002-X
473. Bauvy C, Meijer AJ, Codogno P. Assaying of autophagic protein degradation. Methods Enzymol 2009; 452:47-61; PMID:19200875; http://dx.doi.org/10.1016/S0076-6879(08)03604-5

474. Ichimura Y, Kumanomidou T, Sou YS, Mizushima T, Ezaki J, Ueno T, et al. Structural basis for sorting mechanism of p62 in selective autophagy. J Biol Chem 2008; 283:22847-57; PMID:18524774; http://dx.doi.org/10.1074/jbc.M802182200
475. Kabuta T, Furuta A, Aoki S, Furuta K, Wada K. Aberrant interaction between Parkinson disease-associated mutant UCH-L1 and the lysosomal receptor for chaperone-mediated autophagy. J Biol Chem 2008; 283:23731-8; PMID:18550537; http://dx.doi.org/10.1074/jbc.M801918200
476. Ding WX, Ni HM, Gao W, Yoshimori T, Stolz DB, Ron D, et al. Linking of autophagy to ubiquitinproteasome system is important for the regulation of endoplasmic reticulum stress and cell viability. Am J Pathol 2007; 171:513-24; PMID:17620365; http://dx.doi.org/10.2353/ajpath.2007.070188
477. Iwata A, Riley BE, Johnston JA, Kopito RR. HDAC6 and microtubules are required for autophagic degradation of aggregated huntingtin. J Biol Chem 2005; 280:40282-92; PMID:16192271; http://dx.doi.org/10.1074/jbc.M508786200
478. Pandey UB, Nie Z, Batlevi Y, McCray BA, Ritson GP, Nedelsky NB, et al. HDAC6 rescues neurodegeneration and provides an essential link between autophagy and the UPS. Nature 2007; 447:859-63; PMID:17568747; http://dx.doi.org/10.1038/nature05853
479. Tomek K, Wagner R, Varga F, Singer CF, Karlic H, Grunt TW. Blockade of fatty acid synthase induces ubiquitination and degradation of phosphoinositide-3-kinase signaling proteins in ovarian cancer. Mol Cancer Res 2011; 9:1767-79; PMID:21970855; http://dx.doi.org/10.1158/1541-7786.MCR-10-0467
480. Zimmermann AC, Zarei M, Eiselein S, Dengjel J. Quantitative proteomics for the analysis of spatiotemporal protein dynamics during autophagy. Autophagy 2010; 6:1009-16; PMID:20603599; http://dx.doi.org/10.4161/auto.6.8.12786
481. Kristensen AR, Schandorff S, Høyer-Hansen M, Nielsen MO, Jäättelä M, Dengjel J, et al. Ordered organelle degradation during starvation-induced autophagy. Mol Cell Proteomics 2008; 7:2419-28; PMID: 18687634; http://dx.doi.org/10.1074/mcp.M800184-MCP200
482. Furuya N, Kanazawa T, Fujimura S, Ueno T, Kominami E, Kadowaki M. Leupeptin-induced appearance of partial fragment of betaine homocysteine methyltransferase during autophagic maturation in rat hepatocytes. J Biochem 2001; 129:313-20; PMID: 11173534; http://dx.doi.org/10.1093/oxfordjournals.jbchem.a002859
483. Ueno T, Ishidoh K, Mineki R, Tanida I, Murayama K, Kadowaki M, et al. Autolysosomal membrane-associated betaine homocysteine methyltransferase. Limited degradation fragment of a sequestered cytosolic enzyme monitoring autophagy. J Biol Chem 1999; 274:15222-9; PMID:10329731; http://dx.doi. org/10.1074/jbc.274.21.15222
484. Øverbye A, Sætre F, Hagen LK, Johansen HT, Seglen PO. Autophagic activity measured in whole rat hepatocytes as the accumulation of a novel BHMT fragment (p10), generated in amphisomes by the asparaginyl proteinase, legumain. Autophagy 2011; 7:1011-27; PMID:21610319; http://dx.doi.org/10.4161/auto.7.9.16436
485. Seglen PO, Øverbye A, Saetre F. Sequestration assays for mammalian autophagy. Methods Enzymol 2009; 452:63-83; PMID:19200876; http://dx.doi.org/10.1016/S0076-6879(08)03605-7
486. Mercer CA, Kaliappan A, Dennis PB. Macroautophagy-dependent, intralysosomal cleavage of a betaine homocysteine methyltransferase fusion protein requires stable multimerization. Autophagy 2008; 4:185-94; PMID:18059170
487. Nimmerjahn F, Milosevic S, Behrends U, Jaffee EM, Pardoll DM, Bornkamm GW, et al. Major histocompatibility complex class II-restricted presentation of a cytosolic antigen by autophagy. Eur J Immunol 2003; 33:1250-9; PMID:12731050; http://dx.doi.org/10. 1002/eji.200323730
488. Taylor GS, Long HM, Haigh TA, Larsen M, Brooks J, Rickinson AB. A role for intercellular antigen transfer in the recognition of EBV-transformed B cell lines by EBV nuclear antigen-specific CD4+ T cells. J Immunol 2006; 177:3746-56; PMID:16951335
489. Klionsky DJ, Emr SD. Membrane protein sorting: biosynthesis, transport and processing of yeast vacuolar alkaline phosphatase. EMBO J 1989; 8:2241-50;PMID:2676517
490. Venerando R, Miotto G, Kadowaki M, Siliprandi N, Mortimore GE. Multiphasic control of proteolysis by leucine and alanine in the isolated rat hepatocyte. Am J Physiol 1994; 266:C455-61; PMID:8141260
491. Häussinger D, Hallbrucker C, vom Dahl S, Lang F, Gerok W. Cell swelling inhibits proteolysis in perfused rat liver. Biochem J 1990; 272:239-42; PMID:2264828 492. vom Dahl S, Häussinger D. Cell hydration and proteolysis control in liver. Biochem J 1995; 312:988-9;PMID:8554549
493. Cuervo AM, Stefanis L, Fredenburg R, Lansbury PT, Sulzer D. Impaired degradation of mutant alphasynuclein by chaperone-mediated autophagy. Science 2004; 305:1292-5; PMID:15333840; http://dx.doi.org/10.1126/science.1101738
494. Reggiori F, Monastyrska I, Shintani T, Klionsky DJ. The actin cytoskeleton is required for selective types of autophagy, but not nonspecific autophagy, in the yeast Saccharomyces cerevisiae. Mol Biol Cell 2005; 16:5843-56; PMID:16221887; http://dx.doi.org/10.1091/mbc.E05-07-0629
495. Manjithaya R, Jain S, Farré JC, Subramani S. A yeast MAPK cascade regulates pexophagy but not other autophagy pathways. J Cell Biol 2010; 189:303-10; PMID:20385774; http://dx.doi.org/10.1083/jcb.200909154
496. Journo D, Mor A, Abeliovich H. Aup1-mediated regulation of Rtg3 during mitophagy. J Biol Chem 2009; 284:35885-95; PMID:19840933; http://dx.doi.org/10.1074/jbc.M109.048140
497. Kanki T, Klionsky DJ. Mitophagy in yeast occurs through a selective mechanism. J Biol Chem 2008; 283:32386-93; PMID:18818209; http://dx.doi.org/10.1074/jbc.M802403200
498. Kanki T, Wang K, Baba M, Bartholomew CR, Lynch-Day MA, Du Z, et al. A genomic screen for yeast mutants defective in selective mitochondria autophagy. Mol Biol Cell 2009; 20:4730-8; PMID:19793921;http://dx.doi.org/10.1091/mbc.E09-03-0225
499. Kanki T, Wang K, Cao Y, Baba M, Klionsky DJ. Atg32 is a mitochondrial protein that confers selectivity during mitophagy. Dev Cell 2009; 17:98-109; PMID:19619495; http://dx.doi.org/10.1016/j.devcel.2009.06.014
500. Okamoto K, Kondo-Okamoto N, Ohsumi Y. Mitochondria-anchored receptor Atg32 mediates degradation of mitochondria via selective autophagy. Dev Cell 2009; 17:87-97; PMID:19619494; http://dx.doi.org/10.1016/j.devcel.2009.06.013
501. Sakai Y, Koller A, Rangell LK, Keller GA, Subramani S. Peroxisome degradation by microautophagy in Pichia pastoris: identification of specific steps and morphological intermediates. J Cell Biol 1998; 141:625-36; PMID:9566964; http://dx.doi.org/10.1083/jcb.141.3.625
502. Nazarko TY, Nicaud JM, Sibirny AA. Observation of the Yarrowia lipolytica peroxisome-vacuole dynamics by fluorescence microscopy with a single filter set. Cell Biol Int 2005; 29:65-70; PMID:15763501; http://dx. doi.org/10.1016/j.cellbi.2004.11.014
503. Roetzer A, Gratz N, Kovarik P, Schüller C. Autophagy supports Candida glabrata survival during phagocytosis. Cell Microbiol 2010; 12:199-216; PMID:19811500; http://dx.doi.org/10.1111/j.1462-5822.2009.01391.x
504. Bormann C, Sahm H. Degradation of microbodies in relation to activities of alcohol oxidase and catalase in Candida boidinii. Arch Microbiol 1978; 117:67-72; PMID:678013; http://dx.doi.org/10.1007/BF00689353
505. Stasyk OV, Nazarko TY, Sibirny AA. Methods of plate pexophagy monitoring and positive selection for ATG gene cloning in yeasts. Methods Enzymol 2008; 451:229-39; PMID:19185724; http://dx.doi.org/10. 1016/S0076-6879(08)03216-3
506. Hutchins MU, Veenhuis M, Klionsky DJ. Peroxisome degradation in Saccharomyces cerevisiae is dependent on machinery of macroautophagy and the Cvt pathway. J Cell Sci 1999; 112:4079-87; PMID:10547367
507. Mukaiyama H, Oku M, Baba M, Samizo T, Hammond AT, Glick BS, et al. Paz2 and 13 other PAZ gene products regulate vacuolar engulfment of peroxisomes during micropexophagy. Genes Cells 2002; 7:75-90; PMID:11856375; http://dx.doi.org/ 10.1046/j.1356-9597.2001.00499.x
508. Tuttle DL, Dunn WA, Jr.. Divergent modes of autophagy in the methylotrophic yeast Pichia pastoris. J Cell Sci 1995; 108:25-35; PMID:7738102

509. Nazarko TY, Huang J, Nicaud JM, Klionsky DJ, Sibirny AA. Trs85 is required for macroautophagy, pexophagy and cytoplasm to vacuole targeting in Yarrowia lipolytica and Saccharomyces cerevisiae. Autophagy 2005; 1:37-45; PMID:16874038; http://dx.doi.org/10.4161/auto.1.1.1512
510. Veenhuis M, Douma A, Harder W, Osumi M. Degradation and turnover of peroxisomes in the yeast Hansenula polymorpha induced by selective inactivation of peroxisomal enzymes. Arch Microbiol 1983; 134: 193-203; PMID:6351780; http://dx.doi.org/10.1007/BF00407757
511. Monosov EZ, Wenzel TJ, Lüers GH, Heyman JA, Subramani S. Labeling of peroxisomes with green fluorescent protein in living P. pastoris cells. J Histochem Cytochem 1996; 44:581-9; PMID:8666743; http://dx.doi.org/10.1177/44.6.8666743
512. Wiemer EA, Wenzel T, Deerinck TJ, Ellisman MH, Subramani S. Visualization of the peroxisomal compartment in living mammalian cells: dynamic behavior and association with microtubules. J Cell Biol 1997; 136:71-80; PMID:9008704; http://dx.doi.org/10. 1083/jcb.136.1.71
513. Monastyrska I, van der Heide M, Krikken AM, Kiel JAKW, van der Klei IJ, Veenhuis M. Atg8 is essential for macropexophagy in Hansenula polymorpha. Traffic 2005; 6:66-74; PMID:15569246; http://dx.doi.org/10.1111/j.1600-0854.2004.00252.x
514. Devenish RJ, Prescott M, Turcic K, Mijaljica D. Monitoring organelle turnover in yeast using fluorescent protein tags. Methods Enzymol 2008; 451:109-31; PMID:19185717; http://dx.doi.org/10.1016/S0076-6879(08)03209-6
515. Farré JC, Manjithaya R, Mathewson RD, Subramani S. PpAtg30 tags peroxisomes for turnover by selective autophagy. Dev Cell 2008; 14:365-76; PMID:18331717; http://dx.doi.org/10.1016/j.devcel.2007.12.011
516. Kanki T, Klionsky DJ. The molecular mechanism of mitochondria autophagy in yeast. Mol Microbiol 2010; 75:795-800; PMID:20487284; http://dx.doi.org/10.1111/j.1365-2958.2009.07035.x
517. Tal R, Winter G, Ecker N, Klionsky DJ, Abeliovich H. Aup1p, a yeast mitochondrial protein phosphatase homolog, is required for efficient stationary phase mitophagy and cell survival. J Biol Chem 2007; 282:5617-24; PMID:17166847; http://dx.doi.org/10.1074/jbc.M605940200
518. Abeliovich H. Stationary-phase mitophagy in respiring Saccharomyces cerevisiae. Antioxid Redox Signal 2011; 14:2003-11; PMID:21194383; http://dx.doi.org/10. 1089/ars.2010.3807
519. Aksam EB, Koek A, Kiel JAKW, Jourdan S, Veenhuis M, van der Klei IJ. A peroxisomal lon protease and peroxisome degradation by autophagy play key roles in vitality of Hansenula polymorpha cells. Autophagy 2007; 3:96-105; PMID:17172804
520. Roberts P, Moshitch-Moshkovitz S, Kvam E, O'Toole E, Winey M, Goldfarb DS. Piecemeal microautophagy of nucleus in Saccharomyces cerevisiae. Mol Biol Cell 2003; 14:129-41; PMID:12529432; http://dx.doi.org/10.1091/mbc.E02-08-0483
521. Krick R, Muehe Y, Prick T, Bremer S, Schlotterhose P, Eskelinen E-L, et al. Piecemeal microautophagy of the nucleus requires the core macroautophagy genes.
Mol Biol Cell 2008; 19:4492-505; PMID:18701704;http://dx.doi.org/10.1091/mbc.E08-04-0363
522. Farré JC, Krick R, Subramani S, Thumm M. Turnover of organelles by autophagy in yeast. Curr Opin Cell Biol 2009; 21:522-30; PMID:19515549; http://dx.doi.org/10.1016/j.ceb.2009.04.015
523. Kvam E, Goldfarb DS. Structure and function of nucleus-vacuole junctions: outer-nuclear-membrane targeting of Nvj1p and a role in tryptophan uptake. J Cell Sci 2006; 119:3622-33; PMID:16912077; http://dx.doi.org/10.1242/jcs.03093
524. Millen JI, Krick R, Prick T, Thumm M, Goldfarb DS. Measuring piecemeal microautophagy of the nucleus in Saccharomyces cerevisiae. Autophagy 2009; 5:75-81; PMID:18989095; http://dx.doi.org/10.4161/auto.5.1.7181
525. Bernales S, McDonald KL, Walter P. Autophagy counterbalances endoplasmic reticulum expansion during the unfolded protein response. PLoS Biol 2006; 4:e423; PMID:17132049; http://dx.doi.org/10.1371/journal.pbio.0040423
526. Yorimitsu T, Nair U, Yang Z, Klionsky DJ. Endoplasmic reticulum stress triggers autophagy. J Biol Chem 2006; 281:30299-304; PMID:16901900; http://dx.doi.org/10.1074/jbc.M607007200
527. Klionsky DJ, Cuervo AM, Dunn WA, Jr., Levine B, van der Klei I, Seglen PO. How shall I eat thee?Autophagy 2007; 3:413-6; PMID:17568180
528. Fujita E, Kouroku Y, Isoai A, Kumagai H, Misutani A, Matsuda C, et al. Two endoplasmic reticulumassociated degradation (ERAD) systems for the novel variant of the mutant dysferlin: ubiquitin/proteasome ERAD(I) and autophagy/lysosome ERAD(II). Hum Mol Genet 2007; 16:618-29; PMID:17331981;http://dx.doi.org/10.1093/hmg/ddm002
529. Kraft C, Deplazes A, Sohrmann M, Peter M. Mature ribosomes are selectively degraded upon starvation by an autophagy pathway requiring the Ubp3p/Bre5p ubiquitin protease. Nat Cell Biol 2008; 10:602-10; PMID:18391941; http://dx.doi.org/10.1038/ncb1723
530. Yorimitsu T, Klionsky DJ. Atg11 links cargo to the vesicle-forming machinery in the cytoplasm to vacuole targeting pathway. Mol Biol Cell 2005; 16:1593-605;PMID:15659643; http://dx.doi.org/10.1091/mbc.
531. Shintani T, Huang W-P, Stromhaug PE, Klionsky DJ. Mechanism of cargo selection in the cytoplasm to vacuole targeting pathway. Dev Cell 2002; 3:825-37; PMID:12479808; http://dx.doi.org/10.1016/S1534-5807(02)00373-8
532. Abeliovich H, Darsow T, Emr SD. Cytoplasm to vacuole trafficking of aminopeptidase I requires a t-SNARE-Sec1p complex composed of Tlg2p and Vps45p. EMBO J 1999; 18:6005-16; PMID: 10545112; http://dx.doi.org/10.1093/emboj/18.21.6005
533. Brown CR, Chiang H-L. A selective autophagy pathway that degrades gluconeogenic enzymes during catabolite inactivation. Commun Integr Biol 2009; 2:177-83; PMID:19513275
534. Schüle T, Rose M, Entian KD, Thumm M, Wolf DH. Ubc8p functions in catabolite degradation of fructose-1, 6-bisphosphatase in yeast. EMBO J 2000; 19:2161-7; PMID:10811607; http://dx.doi.org/10.1093/emboj/19.10.2161
535. Schork SM, Thumm M, Wolf DH. Catabolite inactivation of fructose-1,6-bisphosphatase of Saccharomyces cerevisiae. Degradation occurs via the ubiquitin pathway.J Biol Chem 1995; 270:26446-50; PMID:7592860
536. Regelmann J, Schüle T, Josupeit FS, Horak J, Rose M, Entian KD, et al. Catabolite degradation of fructose-1,6-bisphosphatase in the yeast Saccharomyces cerevisiae: a genome-wide screen identifies eight novel GID genes and indicates the existence of two degradation pathways. Mol Biol Cell 2003; 14:1652-63; PMID: 12686616; http://dx.doi.org/10.1091/mbc.E02-08-0456
537. Hung GC, Brown CR, Wolfe AB, Liu J, Chiang HL. Degradation of the gluconeogenic enzymes fructose-1,6- bisphosphatase and malate dehydrogenase is mediated by distinct proteolytic pathways and signaling events. J Biol Chem 2004; 279:49138-50; PMID:15358789; http://dx.doi.org/10.1074/jbc.M404544200
538. Chiang H-L, Schekman R, Hamamoto S. Selective uptake of cytosolic, peroxisomal, and plasma membrane proteins into the yeast lysosome for degradation J Biol Chem 1996; 271:9934-41; PMID:8626630; http://dx.doi.org/10.1074/jbc.271.17.9934
539. Huang PH, Chiang H-L. Identification of novel vesicles in the cytosol to vacuole protein degradation pathway. J Cell Biol 1997; 136:803-10; PMID:9049246; http://dx.doi.org/10.1083/jcb.136.4.803
540. Alibhoy AA, Giardina BJ, Dunton DD, Chiang H-L. Vid30 is required for the association of Vid vesicles and actin patches in the vacuole import and degradation pathway. Autophagy 2012; 8; In press; PMID:22082961; http://dx.doi.org/10.4161/auto.8.1.18104
541. Brown CR, Wolfe AB, Cui D, Chiang H-L. The vacuolar import and degradation pathway merges with the endocytic pathway to deliver fructose-1,6-bisphosphatase to the vacuole for degradation. J Biol Chem 2008; 283:26116-27; PMID:18660504; http://dx.doi.org/10.1074/jbc.M709922200
542. Chiang MC, Chiang H-L. Vid24p, a novel protein localized to the fructose-1, 6-bisphosphatase-containing vesicles, regulates targeting of fructose-1,6-bisphosphatase from the vesicles to the vacuole for degradation. J Cell Biol 1998; 140:1347-56; PMID:9508768; http://dx.doi.org/10.1083/jcb.140.6.1347

543. Brown CR, Dunton D, Chiang H-L. The vacuole import and degradation pathway utilizes early steps of endocytosis and actin polymerization to deliver cargo proteins to the vacuole for degradation. J Biol Chem 2010; 285:1516-28; PMID:19892709; http://dx.doi.org/10.1074/jbc.M109.028241
544. Vida TA, Emr SD. A new vital stain for visualizing vacuolar membrane dynamics and endocytosis in yeast. J Cell Biol 1995; 128:779-92; PMID:7533169; http://dx.doi.org/10.1083/jcb.128.5.779
545. Brown CR, Hung GC, Dunton D, Chiang H-L. The TOR complex 1 is distributed in endosomes and in retrograde vesicles that form from the vacuole membrane and plays an important role in the vacuole import and degradation pathway. J Biol Chem 2010; 285:23359-70; PMID:20457600; http://dx.doi.org/10.1074/jbc.M109.075143
546. Geisler S, Holmström KM, Treis A, Skujat D, Weber SS, Fiesel FC, et al. The PINK1/Parkin-mediated mitophagy is compromised by PD-associated mutations. Autophagy 2010; 6:871-8; PMID:20798600;http://dx.doi.org/10.4161/auto.6.7.13286
547. Geisler S, Holmström KM, Skujat D, Fiesel FC, Rothfuss OC, Kahle PJ, et al. PINK1/Parkin-mediated mitophagy is dependent on VDAC1 and p62/SQSTM1. Nat Cell Biol 2010; 12:119-31; PMID:20098416; http://dx.doi.org/10.1038/ncb2012
548. Boya P, González-Polo RA, Casares N, Perfettini JL, Dessen P, Larochette N, et al. Inhibition of macroautophagy triggers apoptosis. Mol Cell Biol 2005; 25:1025-40; PMID:15657430; http://dx.doi.org/10.1128/MCB.25.3.1025-1040.2005
549. Katayama H, Kogure T, Mizushima N, Yoshimori T, Miyawaki A. A sensitive and quantitative technique for detecting autophagic events based on lysosomal delivery. Chem Biol 2011; 18:1042-52; PMID:21867919; http://dx.doi.org/10.1016/j.chembiol.2011.05.013
550. Yang JY, Yang WY. Spatiotemporally controlled initiation of Parkin-mediated mitophagy within single cells. Autophagy 2011; 7:1230-8; PMID:22011618;http://dx.doi.org/10.4161/auto.7.10.16626
551. Luiken JJ, van den Berg M, Heikoop JC, Meijer AJ. Autophagic degradation of peroxisomes in isolated rat hepatocytes. FEBS Lett 1992; 304:93-7; PMID: 1618306; http://dx.doi.org/10.1016/0014-5793(92) 80596-9
552. Yokota S. Formation of autophagosomes during degradation of excess peroxisomes induced by administration of dioctyl phthalate. Eur J Cell Biol 1993;61:67-80; PMID:8223709
553. Huybrechts SJ, Van Veldhoven PP, Brees C, Mannaerts GP, Los GV, Fransen M. Peroxisome dynamics in cultured mammalian cells. Traffic 2009; 10:1722-33; PMID:19719477; http://dx.doi.org/10. 1111/j.1600-0854.2009.00970.x
554. Lee JY, Nagano Y, Taylor JP, Lim KL, Yao TP. Diseasecausing mutations in parkin impair mitochondrial ubiquitination, aggregation, and HDAC6-dependent mitophagy. J Cell Biol 2010; 189:671-9; PMID:20457763; http://dx.doi.org/10.1083/jcb.201001039
555. Chan NC, Salazar AM, Pham AH, Sweredoski MJ, Kolawa NJ, Graham RL, et al. Broad activation of the ubiquitin-proteasome system by Parkin is critical for mitophagy. Hum Mol Genet 2011; 20:1726-37; PMID:21296869; http://dx.doi.org/10.1093/hmg/ddr048
556. Tanaka A, Cleland MM, Xu S, Narendra DP, Suen DF, Karbowski M, et al. Proteasome and p97 mediate mitophagy and degradation of mitofusins induced by
Parkin. J Cell Biol 2010; 191:1367-80; PMID: 21173115; http://dx.doi.org/10.1083/jcb.201007013
557. Yoshii SR, Kishi C, Ishihara N, Mizushima N. Parkin mediates proteasome-dependent protein degradation and rupture of the outer mitochondrial membrane. J
Biol Chem 2011; 286:19630-40; PMID:21454557; http://dx.doi.org/10.1074/jbc.M110.209338
558. Okatsu K, Saisho K, Shimanuki M, Nakada K, Shitara H, Sou YS, et al. p62/SQSTM1 cooperates with Parkin for perinuclear clustering of depolarized mitochondria. Genes Cells 2010; 15:887-900; PMID:20604804
559. Lyamzaev KG, Nepryakhina OK, Saprunova VB, Bakeeva LE, Pletjushkina OY, Chernyak BV, et al. Novel mechanism of elimination of malfunctioning mitochondria (mitoptosis): formation of mitoptotic bodies and extrusion of mitochondrial material from the cell. Biochim Biophys Acta 2008; 1777:817-25;PMID:18433711
560. Hara-Kuge S, Fujiki Y. The peroxin Pex14p is involved in LC3-dependent degradation of mammalian peroxisomes. Exp Cell Res 2008; 314:3531-41; PMID: 18848543; http://dx.doi.org/10.1016/j.yexcr.2008.09.015
561. Ezaki J, Kominami E, Ueno T. Peroxisome degradation in mammals. IUBMB Life 2011; 63:1001-8; PMID:21990012; http://dx.doi.org/10.1002/iub.537
562. Øverbye A, Fengsrud M, Seglen PO. Proteomic analysis of membrane-associated proteins from rat liver autophagosomes. Autophagy 2007; 3:300-22; PMID:17377489
563. Ju JS, Miller SE, Jackson E, Cadwell K, Piwnica-Worms D, Weihl CC. Quantitation of selective autophagic protein aggregate degradation in vitro and in vivo using luciferase reporters. Autophagy 2009; 5:511-9; PMID:19305149; http://dx.doi.org/10.4161/auto.5.4.7761
564. Fuentealba RA, Marasa J, Diamond MI, Piwnica-Worms D, Weihl CC. An aggregation sensing reporter identifies leflunomide and teriflunomide as polyglutamine aggregate inhibitors. Hum Mol Genet 2012; 21:664-80; PMID:22052286; http://dx.doi.org/10.1093/hmg/ddr500
565. Webster P. Cytoplasmic bacteria and the autophagic pathway. Autophagy 2006; 2:159-61; PMID:16874112 566. Dubuisson JF, Swanson MS. Mouse infection by Legionella, a model to analyze autophagy. Autophagy 2006; 2:179-82; PMID:16874080
567. Jordan TX, Randall G. Manipulation or capitulation: virus interactions with autophagy. Microbes Infect 2012; PMID:22051604; http://dx.doi.org/10.1016/j.micinf.2011.09.007
568. Knodler LA, Celli J. Eating the strangers within: host control of intracellular bacteria via xenophagy. Cell Microbiol 2011; 13:1319-27; PMID:21740500; http://dx.doi.org/10.1111/j.1462-5822.2011.01632.x 569. Levine B, Mizushima N, Virgin HW. Autophagy in immunity and inflammation. Nature 2011; 469: 323-35; PMID:21248839; http://dx.doi.org/10.1038/nature09782
570. Deretic V. Autophagy in immunity and cell-autonomous defense against intracellular microbes. Immunol Rev 2011; 240:92-104; PMID:21349088; http://dx.doi.org/10.1111/j.1600-065X.2010.00995.x
571. Kageyama S, Omori H, Saitoh T, Sone T, Guan JL, Akira S, et al. The LC3 recruitment mechanism is separate from Atg9L1-dependent membrane formation in the autophagic response against Salmonella. Mol Biol Cell 2011; 22:2290-300; PMID:21525242; http://dx.doi.org/10.1091/mbc.E10-11-0893
572. Zheng YT, Shahnazari S, Brech A, Lamark T, JohansenT, Brumell JH. The adaptor protein p62/SQSTM1 targets invading bacteria to the autophagy pathway. J Immunol 2009; 183:5909-16; PMID:19812211;http://dx.doi.org/10.4049/jimmunol.0900441
573. Thurston TL, Ryzhakov G, Bloor S, von Muhlinen N, Randow F. The TBK1 adaptor and autophagy receptor NDP52 restricts the proliferation of ubiquitin-coated bacteria. Nat Immunol 2009; 10:1215-21; PMID:19820708; http://dx.doi.org/10.1038/ni.1800
574. Wild P, Farhan H, McEwan DG, Wagner S, Rogov VV, Brady NR, et al. Phosphorylation of the autophagy receptor optineurin restricts Salmonella growth. Science 2011; 333:228-33; PMID:21617041; http://dx.doi.org/10.1126/science.1205405
575. Huang J, Canadien V, Lam GY, Steinberg BE, Dinauer MC, Magalhaes MA, et al. Activation of antibacterial autophagy by NADPH oxidases. Proc Natl Acad Sci U S A 2009; 106:6226-31; PMID:19339495; http://dx.doi.org/10.1073/pnas.0811045106
576. Rich KA, Burkett C, Webster P. Cytoplasmic bacteria can be targets for autophagy. Cell Microbiol 2003; 5:455-68; PMID:12814436; http://dx.doi.org/10. 1046/j.1462-5822.2003.00292.x
577. Shahnazari S, Brumell JH. Mechanisms and consequences of bacterial targeting by the autophagy pathway. Curr Opin Microbiol 2011; 14:68-75; PMID:21112809; http://dx.doi.org/10.1016/j.mib.2010.11.001

578. McLean JE, Wudzinska A, Datan E, Quaglino D, Zakeri Z. Flavivirus NS4A-induced autophagy protects cells against death and enhances virus replication. J Biol Chem 2011; 286:22147-59; PMID:21511946;http://dx.doi.org/10.1074/jbc.M110.192500
579. Mao Y, Da L, Tang H, Yang J, Lei Y, Tiollais P, et al. Hepatitis B virus X protein reduces starvation-induced cell death through activation of autophagy and inhibition of mitochondrial apoptotic pathway. Biochem Biophys Res Commun 2011; 415:68-74; PMID:22020078;http://dx.doi.org/10.1016/j.bbrc.2011.10.013
580. Orvedahl A, Alexander D, Tallóczy Z, Sun Q, Wei Y, Zhang W, et al. HSV-1 ICP34.5 confers neurovirulence by targeting the Beclin 1 autophagy protein. Cell Host Microbe 2007; 1:23-35; PMID:18005679;http://dx.doi.org/10.1016/j.chom.2006.12.001
581. Alexander DE, Ward SL, Mizushima N, Levine B, Leib DA. Analysis of the role of autophagy in replication of herpes simplex virus in cell culture. J Virol 2007; 81:12128-34; PMID:17855538; http://dx.doi.org/10.1128/JVI.01356-07
582. Leib DA, Alexander DE, Cox D, Yin J, Ferguson TA. Interaction of ICP34.5 with Beclin 1 modulates herpes simplex virus type 1 pathogenesis through control of CD4+ T-cell responses. J Virol 2009; 83:12164-71; PMID:19759141; http://dx.doi.org/10.1128/JVI.01676-09
583. Zhang H, Monken CE, Zhang Y, Lenard J, Mizushima N, Lattime EC, et al. Cellular autophagy machinery is not required for vaccinia virus replication and maturation. Autophagy 2006; 2:91-5; PMID:16874104
584. Heaton NS, Randall G. Dengue virus and autophagy. Viruses 2011; 3:1332-41; PMID:21994782; http://dx.doi.org/10.3390/v3081332
585. Dreux M, Gastaminza P, Wieland SF, Chisari FV. The autophagy machinery is required to initiate hepatitis C virus replication. Proc Natl Acad Sci U S A 2009; 106:14046-51; PMID:19666601; http://dx.doi.org/10.1073/pnas.0907344106
586. Collins CA, De Mazière A, van Dijk S, Carlsson F, Klumperman J, Brown EJ. Atg5-independent sequestration of ubiquitinated mycobacteria. PLoS Pathog 2009; 5:e1000430; PMID:19436699; http://dx.doi.org/10.1371/journal.ppat.1000430
587. Moreau K, Lacas-Gervais S, Fujita N, Sebbane F, Yoshimori T, Simonet M, et al. Autophagosomes can support Yersinia pseudotuberculosis replication in macrophages. Cell Microbiol 2010; 12:1108-23; PMID:20180800; http://dx.doi.org/10.1111/j.1462- 5822.2010.01456.x
588. Singh R, Kaushik S, Wang Y, Xiang Y, Novak I, Komatsu M, et al. Autophagy regulates lipid metabolism. Nature 2009; 458:1131-5; PMID:19339967;http://dx.doi.org/10.1038/nature07976
589. Grasso D, Ropolo A, Lo Ré A, Boggio V, Molejón MI, Iovanna JL, et al. Zymophagy, a novel selective autophagy pathway mediated by VMP1-USP9x-p62, prevents pancreatic cell death. J Biol Chem 2011; 286:8308-24; PMID:21173155; http://dx.doi.org/10.1074/jbc.M110.197301
590. Al Rawi S, Louvet-Vallée S, Djeddi A, Sachse M, Culetto E, Hajjar C, et al. Allophagy: A macroautophagic process degrading spermatozoid-inherited organelles. Autophagy 2012; In press.
591. Sato M, Sato K. Maternal inheritance of mitochondrial DNA: Degradation of paternal mitochondria by allogeneic organelle autophagy, allophagy. Autophagy 2012; 8; In press; PMID:22302002
592. Al Rawi S, Louvet-Vallée S, Djeddi A, Sachse M, Culetto E, Hajjar C, et al. Postfertilization autophagy of sperm organelles prevents paternal mitochondrial DNA transmission. Science 2011; 334:1144-7; PMID:22033522; http://dx.doi.org/10.1126/science.1211878
593. Sato M, Sato K. Degradation of paternal mitochondria by fertilization-triggered autophagy in C. elegans embryos. Science 2011; 334:1141-4; PMID:21998252; http://dx.doi.org/10.1126/science.1210333
594. Seglen PO, Gordon PB, Tolleshaug H, Høyvik H. Use of [3H]raffinose as a specific probe of autophagic sequestration. Exp Cell Res 1986; 162:273-7; PMID: 3940229; http://dx.doi.org/10.1016/0014-4827(86) 90446-5
595. Kopitz J, Kisen GO, Gordon PB, Bohley P, Seglen PO. Nonselective autophagy of cytosolic enzymes by isolated rat hepatocytes. J Cell Biol 1990; 111:941-53; PMID:2391370; http://dx.doi.org/10.1083/jcb.111.3.941
596. Nair U, Thumm M, Klionsky DJ, Krick R. GFP-Atg8 protease protection as a tool to monitor autophagosome biogenesis. Autophagy 2011; 7:1546-50; PMID: 22108003; http://dx.doi.org/10.4161/auto.7.12.18424
597. Plomp PJ, Gordon PB, Meijer AJ, Høyvik H, Seglen PO. Energy dependence of different steps in the autophagic-lysosomal pathway. J Biol Chem 1989; 264:6699-704; PMID:2708336
598. Høyvik H, Gordon PB, Berg TO, Strømhaug PE, Seglen PO. Inhibition of autophagic-lysosomal delivery and autophagic lactolysis by asparagine. J Cell Biol 1991; 113:1305-12; PMID:1904444; http://dx.doi.org/10.1083/jcb.113.6.1305
599. Rodriguez-Enriquez S, Kim I, Currin RT, Lemasters JJ. Tracker dyes to probe mitochondrial autophagy (mitophagy) in rat hepatocytes. Autophagy 2006;2:39-46; PMID:16874071
600. Lorenz H, Hailey DW, Lippincott-Schwartz J. Fluorescence protease protection of GFP chimeras to reveal protein topology and subcellular localization. Nat Methods 2006; 3:205-10; PMID:16489338; http://dx.doi.org/10.1038/nmeth857
601. Dagda RK, Zhu J, Kulich SM, Chu CT. Mitochondrially localized ERK2 regulates mitophagy and autophagic cell stress: implications for Parkinson's disease. Autophagy 2008; 4:770-82; PMID:18594198
602. McNeil PL, Murphy RF, Lanni F, Taylor DL. A method for incorporating macromolecules into adherent cells. J Cell Biol 1984; 98:1556-64; PMID:6201494;http://dx.doi.org/10.1083/jcb.98.4.1556
603. Kim J, Huang WP, Stromhaug PE, Klionsky DJ. Convergence of multiple autophagy and cytoplasm to vacuole targeting components to a perivacuolar membrane compartment prior to de novo vesicle formation. J Biol Chem 2002; 277:763-73; PMID:11675395;http://dx.doi.org/10.1074/jbc.M109134200
604. Kovács AL, László L, Kovács J. Effect of amino acids and cycloheximide on changes caused by vinblastine, leupeptin and methylamine in the autophagic/lysosomal system of mouse hepatocytes in vivo. Exp Cell Res 1985; 157:83-94; PMID:3972014; http://dx.doi.org/10.1016/0014-4827(85)90154-5
605. Swanson MS, Byrne BG, Dubuisson JF. Kinetic analysis of autophagosome formation and turnover in primary mouse macrophages. Methods Enzymol 2009; 452:383-402; PMID:19200894; http://dx.doi.org/10. 1016/S0076-6879(08)03623-9
606. Beugnet A, Tee AR, Taylor PM, Proud CG. Regulation of targets of mTOR (mammalian target of rapamycin) signalling by intracellular amino acid availability. Biochem J 2003; 372:555-66; PMID:12611592; http://dx.doi.org/10.1042/BJ20021266
607. Urban J, Soulard A, Huber A, Lippman S, Mukhopadhyay D, Deloche O, et al. Sch9 is a major target of TORC1 in Saccharomyces cerevisiae. Mol Cell 2007; 26:663-74; PMID:17560372; http://dx.doi.org/10.1016/j.molcel.2007.04.020
608. Jomain-Baum M, Garber AJ, Farber E, Hanson RW. The effect of cycloheximide on the interaction between mitochondrial respiration and gluconeogenesis in guinea pig and rat liver. J Biol Chem 1973; 248:1536-43; PMID:4348543
609. Garber AJ, Jomain-Baum M, Salganicoff L, Farber E, Hanson RW. The effects of cycloheximide on energy transfer in rat and guinea pig liver mitochondria. J BiolChem 1973; 248:1530-5; PMID:4144389
610. Mora R, Dokic I, Kees T, Hüber CM, Keitel D, Geibig R, et al. Sphingolipid rheostat alterations related to transformation can be exploited for specific induction of lysosomal cell death in murine and human glioma. Glia 2010; 58:1364-83; PMID:20607862
611. Bright NA, Lindsay MR, Stewart A, Luzio JP. The relationship between lumenal and limiting membranes in swollen late endocytic compartments formed after wortmannin treatment or sucrose accumulation. Traffic 2001; 2:631-42; PMID:11555417; http://dx.doi.org/10.1034/j.1600-0854.2001.20906.x
612. Deter RL. Quantitative characterization of dense body, autophagic vacuole, and acid phosphatase-bearing particle populations during the early phases of glucagon-induced autophagy in rat liver. J Cell Biol 1971; 48:473-89; PMID:4322760; http://dx.doi.org/10.1083/jcb.48.3.473

613. Deter RL. Analog modeling of glucagon-induced autophagy in rat liver. I. Conceptual and mathematical model of telolysosome-autophagosome-autolysosome interaction. Exp Cell Res 1975; 94:122-6; PMID: 1193121; http://dx.doi.org/10.1016/0014-4827(75) 90538-8
614. Deter RL. Analog modeling of glucagon-induced autophagy in rat liver. II. Evaluation of iron labeling as a means for identifying telolysosome, autophagosome and autolysosome populations. Exp Cell Res 1975; 94:127-39; PMID:172336; http://dx.doi.org/10.1016/0014-4827(75)90539-X
615. Deter RL, Baudhuin P, De Duve C. Participation of lysosomes in cellular autophagy induced in rat liver by glucagon. J Cell Biol 1967; 35:C11-6; PMID:6055998; http://dx.doi.org/10.1083/jcb.35.2.C11
616. Deter RL, De Duve C. Influence of glucagon, an inducer of cellular autophagy, on some physical properties of rat liver lysosomes. J Cell Biol 1967; 33:437-49; PMID:4292315; http://dx.doi.org/10. 1083/jcb.33.2.437
617. Strømhaug PE, Berg TO, Fengsrud M, Seglen PO. Purification and characterization of autophagosomes from rat hepatocytes. Biochem J 1998; 335:217-24; PMID:9761717
618. Deter RL. Electron microscopic evaluation of subcellular fractions obtained by ultracentrifugation. In: Hayat MA, ed. Principles and Techniques of Electron Microscopy. New York: Van Nostrand Reinhold Co., 1973:199-235.
619. Marzella L, Ahlberg J, Glaumann H. Isolation of autophagic vacuoles from rat liver: morphological and biochemical characterization. J Cell Biol 1982; 93:144-54; PMID:7068752; http://dx.doi.org/10.1083/jcb.93.1.144
620. Wattiaux R, Wattiaux-De Coninck S, Ronveaux-dupal M-F, Dubois F. Isolation of rat liver lysosomes by isopycnic centrifugation in a metrizamide gradient. J Cell Biol 1978; 78:349-68; PMID:211139; http://dx.doi.org/10.1083/jcb.78.2.349
621. Rodríguez-Navarro JA, Rodríguez L, Casarejos MJ, Solano RM, Gómez A, Perucho J, et al. Trehalose ameliorates dopaminergic and tau pathology in parkin deleted/tau overexpressing mice through autophagy activation. Neurobiol Dis 2010; 39:423-38; PMID: 20546895; http://dx.doi.org/10.1016/j.nbd.2010.05. 014
622. Weibel ER, Bolender RP. Stereological techniques for electron microscopic morphometry. In: Hayat MA, ed. Principles and Techniques of Electron Microscopy. New York: Van Nostrand Reinhold Co., 1973:237-96.
623. Baudhuin P, Evrard P, Berthet J. Electron microscopic examination of subcellular fractions. I. The preparation of representative samples from suspensions of particles. J Cell Biol 1967; 32:181-91; PMID:10976209; http://dx.doi.org/10.1083/jcb.32.1.181
624. Baudhuin P, Berthet J. Electron microscopic examination of subcellular fractions. II. Quantitative analysis of the mitochondrial population isolated from rat liver. J Cell Biol 1967; 35:631-48; PMID:4294244; http://dx.doi.org/10.1083/jcb.35.3.631
625. Storrie B, Madden EA. Isolation of subcellular organelles. Methods Enzymol 1990; 182:203-25; PMID:2156127; http://dx.doi.org/10.1016/0076-6879(90)82018-W
626. Balch WE, Rothman JE. Characterization of protein transport between successive compartments of the Golgi apparatus: asymmetric properties of donor and acceptor activities in a cell-free system. Arch Biochem Biophys 1985; 240:413-25; PMID:2990347; http://dx.doi.org/10.1016/0003-9861(85)90046-3
627. Graham JM. Isolation of lysosomes from tissues and cells by differential and density gradient centrifugation. In: Bonifacino JS, Dasso M, Harfod JB, Lippincott-Schwartz J and Yamada KM, eds. Current Protocols in Cell Biology: John Wiley & Sons, Inc., 2000:Unit 3.6.
628. Cuervo AM, Dice JF, Knecht E. A population of rat liver lysosomes responsible for the selective uptake and degradation of cytosolic proteins. J Biol Chem 1997; 272:5606-15; PMID:9038169; http://dx.doi.org/10.1074/jbc.272.9.5606
629. Iwai-Kanai E, Yuan H, Huang C, Sayen MR, Perry-Garza CN, Kim L, et al. A method to measure cardiac autophagic flux in vivo. Autophagy 2008; 4:322-9; PMID:18216495
630. Zhu H, Tannous P, Johnstone JL, Kong Y, Shelton JM, Richardson JA, et al. Cardiac autophagy is a maladaptive response to hemodynamic stress. J Clin Invest 2007; 117:1782-93; PMID:17607355; http://dx.doi.org/10.1172/JCI27523
631. Ding WX, Li M, Chen X, Ni HM, Lin CW, Gao W, et al. Autophagy reduces acute ethanol-induced hepatotoxicity and steatosis in mice. Gastroenterology 2010; 139:1740-52; PMID:20659474; http://dx.doi.org/10.1053/j.gastro.2010.07.041
632. Chiarelli R, Agnello M, Roccheri MC. Sea urchin embryos as a model system for studying autophagy induced by cadmium stress. Autophagy 2011; 7:1028-34; PMID:21628995; http://dx.doi.org/10.4161/auto. 7.9.16450
633. Morici G, Agnello M, Spagnolo F, Roccheri MC, Di Liegro CM, Rinaldi AM. Confocal microscopy study of the distribution, content and activity of mitochondria during Paracentrotus lividus development. J Microsc 2007; 228:165-73; PMID:17970916; http://dx.doi.org/10.1111/j.1365-2818.2007.01860.x
634. Martinet W, De Meyer GR, Andries L, Herman AG, Kockx MM. Detection of autophagy in tissue by standard immunohistochemistry: possibilities and limitations. Autophagy 2006; 2:55-7; PMID:16874065
635. Holt SV, Wyspianska B, Randall KJ, James D, Foster JR, Wilkinson RW. The development of an immunohistochemical method to detect the autophagyassociated protein LC3-II in human tumor xenografts. Toxicol Pathol 2011; 39:516-23; PMID:21441228; http://dx.doi.org/10.1177/0192623310396903
636. Kimura S, Fujita N, Noda T, Yoshimori T. Monitoring autophagy in mammalian cultured cells through the dynamics of LC3. Methods Enzymol 2009; 452:1-12; PMID:19200872; http://dx.doi.org/10.1016/S0076-6879(08)03601-X
637. Dehay B, Bové J, Rodríguez-Muela N, Perier C, Recasens A, Boya P, et al. Pathogenic lysosomal depletion in Parkinson's disease. J Neurosci 2010; 30:12535-44; PMID:20844148; http://dx.doi.org/10.1523/JNEUROSCI.1920-10.2010
638. Ouimet M, Franklin V, Mak E, Liao X, Tabas I, Marcel YL. Autophagy regulates cholesterol efflux from macrophage foam cells via lysosomal acid lipase. Cell Metab 2011; 13:655-67; PMID:21641547; http://dx.doi.org/10.1016/j.cmet.2011.03.023
639. Gomes LC, Di Benedetto G, Scorrano L. During autophagy mitochondria elongate, are spared from degradation and sustain cell viability. Nat Cell Biol 2011; 13:589-98; PMID:21478857; http://dx.doi.org/10.1038/ncb2220
640. Grumati P, Coletto L, Sabatelli P, Cescon M, Angelin A, Bertaggia E, et al. Autophagy is defective in collagen VI muscular dystrophies, and its reactivation rescues myofiber degeneration. Nat Med 2010; 16:1313-20; PMID:21037586; http://dx.doi.org/10.1038/nm.2247
641. Haspel J, Shaik RS, Ifedigbo E, Nakahira K, Dolinay T, Englert JA, et al. Characterization of macroautophagic flux in vivo using a leupeptin-based assay. Autophagy 2011; 7:629-42; PMID:21460622; http://dx.doi.org/10.4161/auto.7.6.15100
642. Uchiyama Y. Autophagic cell death and its execution by lysosomal cathepsins. Arch Histol Cytol 2001; 64:233-46; PMID:11575420; http://dx.doi.org/10.1679/aohc.64.233
643. Biolo G, Fleming RY, Maggi SP, Wolfe RR. Transmembrane transport and intracellular kinetics of amino acids in human skeletal muscle. Am J Physiol 1995; 268:E75-84; PMID:7840186
644. Svanberg E, Möller-Loswick AC, Matthews DE, Körner U, Andersson M, Lundholm K. The role of glucose, long-chain triglycerides and amino acids for promotion of amino acid balance across peripheral tissues in man. Clin Physiol 1999; 19:311-20; PMID: 10451792; http://dx.doi.org/10.1046/j.1365-2281. 1999.00183.x
645. Volpi E, Ferrando AA, Yeckel CW, Tipton KD, Wolfe RR. Exogenous amino acids stimulate net muscle protein synthesis in the elderly. J Clin Invest 1998; 101:2000-7; PMID:9576765; http://dx.doi.org/10. 1172/JCI939
646. Denne SC, Liechty EA, Liu YM, Brechtel G, Baron AD. Proteolysis in skeletal muscle and whole body in response to euglycemic hyperinsulinemia in normal adults. Am J Physiol 1991; 261:E809-14; PMID:1767841

647. Fukagawa NK, Minaker KL, Young VR, Matthews DE, Bier DM, Rowe JW. Leucine metabolism in aging humans: effect of insulin and substrate availability. Am J Physiol 1989; 256:E288-94; PMID:2645788
648. Gelfand RA, Barrett EJ. Effect of physiologic hyperinsulinemia on skeletal muscle protein synthesis and breakdown in man. J Clin Invest 1987; 80:1-6; PMID:3298320; http://dx.doi.org/10.1172/JCI113033
649. Mortimore GE, Pösö AR. Intracellular protein catabolism and its control during nutrient deprivation and supply. Annu Rev Nutr 1987; 7:539-64; PMID: 3300746; http://dx.doi.org/10.1146/annurev.nu.07. 070187.002543
650. Pozefsky T, Felig P, Tobin JD, Soeldner JS, Cahill GF, Jr. Amino acid balance across tissues of the forearm in postabsorptive man. Effects of insulin at two dose levels. J Clin Invest 1969; 48:2273-82; PMID:5355340; http://dx.doi.org/10.1172/JCI106193
651. Weber SM, Levitz SM. Chloroquine interferes with lipopolysaccharide-induced TNF-alpha gene expression by a nonlysosomotropic mechanism. J Immunol 2000; 165:1534-40; PMID:10903761
652. Paludan C, Schmid D, Landthaler M, Vockerodt M, Kube D, Tuschl T, et al. Endogenous MHC class II processing of a viral nuclear antigen after autophagy. Science 2005; 307:593-6; PMID:15591165; http://dx.doi.org/10.1126/science.1104904
653. Chotechuang N, Azzout-Marniche D, Bos C, Chaumontet C, Gaudichon C, Tomé D. Downregulation of the ubiquitin-proteasome proteolysis system by amino acids and insulin involves the adenosine monophosphate-activated protein kinase and mammalian target of rapamycin pathways in rat hepatocytes. Amino Acids 2011; 41:457-68; PMID:20957397;http://dx.doi.org/10.1007/s00726-010-0765-2
654. Bedford L, Lowe J, Dick LR, Mayer RJ, Brownell JE. Ubiquitin-like protein conjugation and the ubiquitinproteasome system as drug targets. Nat Rev Drug Discov 2011; 10:29-46; PMID:21151032; http://dx.doi.org/10.1038/nrd3321
655. Akagi Y, Isaka Y, Akagi A, Ikawa M, Takenaka M, Moriyama T, et al. Transcriptional activation of a hybrid promoter composed of cytomegalovirus enhancer and beta-actin/beta-globin gene in glomerular epithelial cells in vivo. Kidney Int 1997; 51:1265-9; PMID:9083295;http://dx.doi.org/10.1038/ki.1997.172
656. Kimura T, Takabatake Y, Takahashi A, Kaimori JY, Matsui I, Namba T, et al. Autophagy protects the proximal tubule from degeneration and acute ischemic injury. J Am Soc Nephrol 2011; 22:902-13; PMID: 21493778; http://dx.doi.org/10.1681/ASN.2010070705
657. Hartleben B, Gödel M, Meyer-Schwesinger C, Liu S, Ulrich T, Köbler S, et al. Autophagy influences glomerular disease susceptibility and maintains podocyte homeostasis in aging mice. J Clin Invest 2010; 120:1084-96; PMID:20200449; http://dx.doi.org/10.1172/JCI39492
658. Berry DL, Baehrecke EH. Growth arrest and autophagy are required for salivary gland cell degradation in Drosophila. Cell 2007; 131:1137-48; PMID:18083103; http://dx.doi.org/10.1016/j.cell.2007.10.048
659. Aits S, Gustafsson L, Hallgren O, Brest P, Gustafsson
M, Trulsson M, et al. HAMLET (human alphalactalbumin made lethal to tumor cells) triggers autophagic tumor cell death. Int J Cancer 2009; 124: 1008-19; PMID:19048621; http://dx.doi.org/10. 1002/ijc.24076
660. Koike M, Shibata M, Tadakoshi M, Gotoh K, Komatsu M, Waguri S, et al. Inhibition of autophagy prevents hippocampal pyramidal neuron death after hypoxic-ischemic injury. Am J Pathol 2008; 172: 454-69; PMID:18187572; http://dx.doi.org/10.2353/ajpath.2008.070876
661. Hou YC, Hannigan AM, Gorski SM. An executioner caspase regulates autophagy. Autophagy 2009; 5:530-3; PMID:19242106; http://dx.doi.org/10.4161/auto.5.4.8061
662. Nezis IP, Shravage BV, Sagona AP, Lamark T, Bjørkøy G, Johansen T, et al. Autophagic degradation of dBruce controls DNA fragmentation in nurse cells during late Drosophila melanogaster oogenesis. J Cell Biol 2010; 190:523-31; PMID:20713604; http://dx.doi.org/10.1083/jcb.201002035
663. Piras A, Gianetto D, Conte D, Bosone A, Vercelli A. Activation of autophagy in a rat model of retinal ischemia following high intraocular pressure. PLoS One 2011; 6:e22514; PMID:21799881; http://dx.doi.org/10.1371/journal.pone.0022514
664. Schwarze PE, Seglen PO. Reduced autophagic activity, improved protein balance and enhanced in vitro survival of hepatocytes isolated from carcinogen-treated rats. Exp Cell Res 1985; 157:15-28; PMID:2857648; http://dx.doi.org/10.1016/0014-4827(85)90148-X
665. Russo R, Berliocchi L, Adornetto A, Varano GP, Cavaliere F, Nucci C, et al. Calpain-mediated cleavage of Beclin-1 and autophagy deregulation following retinal ischemic injury in vivo. Cell Death Dis 2011; 2:e144; PMID:21490676; http://dx.doi.org/10.1038/cddis.2011.29
666. Denton D, Nicolson S, Kumar S. Cell death by autophagy: facts and apparent artefacts. Cell Death Differ 2012; PMID:22052193; http://dx.doi.org/10. 1038/cdd.2011.146
667. Beaulaton J, Lockshin RA. Ultrastructural study of the normal degeneration of the intersegmental muscles of Anthereae polyphemus and Manduca sexta (Insecta, Lepidoptera) with particular reference of cellular autophagy. J Morphol 1977; 154:39-57; PMID:915948; http://dx.doi.org/10.1002/jmor.1051540104
668. Clarke PG. Developmental cell death: morphological diversity and multiple mechanisms. Anat Embryol (Berl) 1990; 181:195-213; PMID:2186664; http://dx.doi.org/10.1007/BF00174615
669. Galluzzi L, Vitale I, Abrams JM, Alnemri ES, Baehrecke EH, Blagosklonny MV, et al. Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death 2012. Cell Death Differ 2012; 19:107-20; PMID:21760595; http://dx.doi.org/10.1038/cdd.2011.96
670. Kroemer G, Levine B. Autophagic cell death: the story of a misnomer. Nat Rev Mol Cell Biol 2008; 9:1004-10; PMID:18971948; http://dx.doi.org/10.1038/nrm2529
671. van Doorn WG, Beers EP, Dangl JL, Franklin-Tong VE, Gallois P, Hara-Nishimura I, et al. Morphological classification of plant cell deaths. Cell Death Differ 2011; 18:1241-6; PMID:21494263; http://dx.doi.org/10.1038/cdd.2011.36
672. Giusti C, Tresse E, Luciani MF, Golstein P. Autophagic cell death: analysis in Dictyostelium. Biochim Biophys Acta 2009; 1793:1422-31; PMID:19133302
673. Luciani MF, Giusti C, Harms B, Oshima Y, Kikuchi H, Kubohara Y, et al. Atg1 allows second-signaled autophagic cell death in Dictyostelium. Autophagy 2011; 7:501-8; PMID:21301205; http://dx.doi.org/10.4161/auto.7.5.14957
674. Uchikawa T, Yamamoto A, Inouye K. Origin and function of the stalk-cell vacuole in Dictyostelium. Dev Biol 2011; 352:48-57; PMID:21256841; http://dx.doi.org/10.1016/j.ydbio.2011.01.014
675. Galluzzi L, Aaronson SA, Abrams J, Alnemri ES, Andrews DW, Baehrecke EH, et al. Guidelines for the use and interpretation of assays for monitoring cell death in higher eukaryotes. Cell Death Differ 2009; 16:1093-107; PMID:19373242; http://dx.doi.org/10.1038/cdd.2009.44
676. Guimarães CA, Benchimol M, Amarante-Mendes GP, Linden R. Alternative programs of cell death in developing retinal tissue. J Biol Chem 2003; 278:41938-46; PMID:12917395; http://dx.doi.org/10.1074/jbc.M306547200
677. Lossi L, Gambino G, Mioletti S, Merighi A. In vivo analysis reveals different apoptotic pathways in pre and postmigratory cerebellar granule cells of rabbit. J Neurobiol 2004; 60:437-52; PMID:15307148; http:// dx.doi.org/10.1002/neu.20032
678. Lossi L, Alasia S, Salio C, Merighi A. Cell death and proliferation in acute slices and organotypic cultures of mammalian CNS. Prog Neurobiol 2009; 88:221-45; PMID:19552996; http://dx.doi.org/10.1016/j.pneurobio.2009.01.002
679. Thorburn A. I think autophagy controls the death of my cells: what do I do to get my paper published? Autophagy 2011; 7:455-6; PMID:21270514; http://dx.doi.org/10.4161/auto.7.5.14797
680. Kaushik S, Bandyopadhyay U, Sridhar S, Kiffin R, Martinez-Vicente M, Kon M, et al. Chaperonemediated autophagy at a glance. J Cell Sci 2011; 124:495-9; PMID:21282471; http://dx.doi.org/10. 1242/jcs.073874

681. Arias E, Cuervo AM. Chaperone-mediated autophagy in protein quality control. Curr Opin Cell Biol 2011; 23:184-9; PMID:21094035; http://dx.doi.org/10. 1016/j.ceb.2010.10.009
682. Kaushik S, Cuervo AM. Methods to monitor chaperone-mediated autophagy. Methods Enzymo 2009; 452:297-324; PMID:19200890; http://dx.doi.org/10.1016/S0076-6879(08)03619-7
683. Dice JF. Peptide sequences that target cytosolic proteins for lysosomal proteolysis. Trends Biochem Sci 1990; 15:305-9; PMID:2204156; http://dx.doi.org/10.1016/0968-0004(90)90019-8
684. Cuervo AM, Dice JF. A receptor for the selective uptake and degradation of proteins by lysosomes. Science 1996; 273:501-3; PMID:8662539; http://dx.doi.org/10.1126/science.273.5274.501
685. Cuervo AM, Dice JF. Unique properties of lamp2a compared to other lamp2 isoforms. J Cell Sci 2000;113:4441-50; PMID:11082038
686. Finn PF, Mesires NT, Vine M, Dice JF. Effects of small molecules on chaperone-mediated autophagy. Autophagy 2005; 1:141-5; PMID:16874031; http://dx.doi.org/10.4161/auto.1.3.2000
687. Bandyopadhyay U, Kaushik S, Varticovski L, Cuervo AM. The chaperone-mediated autophagy receptor organizes in dynamic protein complexes at the lysosomal membrane. Mol Cell Biol 2008; 28:5747-63; PMID:18644871; http://dx.doi.org/10.1128/MCB.02070-07
688. Aniento F, Emans N, Griffiths G, Gruenberg J. Cytoplasmic dynein-dependent vesicular transport from early to late endosomes. J Cell Biol 1993; 123:1373-87; PMID:8253838; http://dx.doi.org/10.1083/jcb.123.6.1373
689. Salvador N, Aguado C, Horst M, Knecht E. Import of a cytosolic protein into lysosomes by chaperonemediated autophagy depends on its folding state. J Biol Chem 2000; 275:27447-56; PMID:10862611
690. Koga H, Martinez-Vicente M, Macian F, Verkhusha VV, Cuervo AM. A photoconvertible fluorescent reporter to track chaperone-mediated autophagy. Nat Commun 2011; 2:386; PMID:21750540; http://dx.doi.org/10.1038/ncomms1393
691. Sahu R, Kaushik S, Clement CC, Cannizzo ES, Scharf B, Follenzi A, et al. Microautophagy of cytosolic proteins by late endosomes. Dev Cell 2011; 20:131-9; PMID:21238931; http://dx.doi.org/10.1016/j.devcel.2010.12.003
692. Arndt V, Dick N, Tawo R, Dreiseidler M, Wenzel D, Hesse M, et al. Chaperone-assisted selective autophagy is essential for muscle maintenance. Curr Biol 2010; 20:143-8; PMID:20060297; http://dx.doi.org/10. 1016/j.cub.2009.11.022
693. Niemann A, Baltes J, Elsässer HP. Fluorescence properties and staining behavior of monodansylpentane, a structural homologue of the lysosomotropic agent monodansylcadaverine. J Histochem Cytochem 2001; 49:177-85; PMID:11156686; http://dx.doi.org/10.1177/002215540104900205
694. Paglin S, Hollister T, Delohery T, Hackett N, McMahill M, Sphicas E, et al. A novel response of cancer cells to radiation involves autophagy and formation of acidic vesicles. Cancer Res 2001; 61:439-44; PMID:11212227
695. Florez-McClure ML, Linseman DA, Chu CT, Barker PA, Bouchard RJ, Le SS, et al. The p75 neurotrophin receptor can induce autophagy and death of cerebellar Purkinje neurons. J Neurosci 2004; 24:4498-509; PMID:15140920; http://dx.doi.org/10.1523/JNEUROSCI.5744-03.2004
696. Moriyasu Y, Hattori M, Jauh G-Y, Rogers JC. Alpha tonoplast intrinsic protein is specifically associated with vacuole membrane involved in an autophagic process. Plant Cell Physiol 2003; 44:795-802; PMID:12941871; http://dx.doi.org/10.1093/pcp/pcg100
697. Biederbick A, Kern HF, Elsässer HP. Monodansylcadaverine (MDC) is a specific in vivo marker for autophagic vacuoles. Eur J Cell Biol 1995; 66:3-14; PMID:7750517
698. Høyer-Hansen M, Bastholm L, Mathiasen IS, Elling F, Jäättelä M. Vitamin D analog EB1089 triggers dramatic lysosomal changes and Beclin 1-mediated autophagic cell death. Cell Death Differ 2005; 12:1297-309; PMID:15905882; http://dx.doi.org/10.1038/sj.cdd.4401651
699. Munafó DB, Colombo MI. A novel assay to study autophagy: regulation of autophagosome vacuole size by amino acid deprivation. J Cell Sci 2001; 114:3619-29; PMID:11707514
700. Gutierrez MG, Munafó DB, Berón W, Colombo MI. Rab7 is required for the normal progression of the autophagic pathway in mammalian cells. J Cell Sci 2004; 117:2687-97; PMID:15138286; http://dx.doi.org/10.1242/jcs.01114
701. Freundt EC, Czapiga M, Lenardo MJ. Photoconversion of Lysotracker Red to a green fluorescent molecule. Cell Res 2007; 17:956-8; PMID:17893709;http://dx.doi.org/10.1038/cr.2007.80
702. Rubinsztein DC, Gestwicki JE, Murphy LO, Klionsky DJ. Potential therapeutic applications of autophagy.
Nat Rev Drug Discov 2007; 6:304-12; PMID:17396135; http://dx.doi.org/10.1038/nrd2272
703. Funderburk SF, Wang QJ, Yue Z. The Beclin 1-VPS34 complex–at the crossroads of autophagy and beyond. Trends Cell Biol 2010; 20:355-62; PMID:20356743; http://dx.doi.org/10.1016/j.tcb.2010.03.002
704. Levine B, Sinha S, Kroemer G. Bcl-2 family members: dual regulators of apoptosis and autophagy. Autophagy 2008; 4:600-6; PMID:18497563705. Simonsen A, Tooze SA. Coordination of membrane events during autophagy by multiple class III PI3-kinase complexes. J Cell Biol 2009; 186:773-82; PMID: 19797076; http://dx.doi.org/10.1083/jcb.200907014
706. Pyo JO, Jang MH, Kwon YK, Lee HJ, Jun JI, Woo HN, et al. Essential roles of Atg5 and FADD in autophagic cell death: dissection of autophagic cell death into vacuole formation and cell death. J Biol Chem 2005; 280:20722-9; PMID:15778222; http://dx.doi.org/10.1074/jbc.M413934200
707. Petiot A, Ogier-Denis E, Blommaart EF, Meijer AJ, Codogno P. Distinct classes of phosphatidylinositol 3'-kinases are involved in signaling pathways that control macroautophagy in HT-29 cells. J Biol Chem 2000; 275:992-8; PMID:10625637; http://dx.doi.org/10.1074/jbc.275.2.992
708. Harris J, Hartman M, Roche C, Zeng SG, O'Shea A, Sharp FA, et al. Autophagy controls IL-1beta secretion by targeting pro-IL-1beta for degradation. J Biol Chem 2011; 286:9587-97; PMID:21228274; http://dx.doi.org/10.1074/jbc.M110.202911
709. Crişan TO, Plantinga TS, van de Veerdonk FL, Farcaş MF, Stoffels M, Kullberg BJ, et al. Inflammasomeindependent modulation of cytokine response by autophagy in human cells. PLoS One 2011; 6:e18666; PMID:21490934; http://dx.doi.org/10.1371/journal. pone.0018666
710. Kleinnijenhuis J, Oosting M, Plantinga TS, van der Meer JW, Joosten LA, Crevel RV, et al. Autophagy modulates the Mycobacterium tuberculosis-induced cytokine response. Immunology 2011; 134:341-8; PMID:21978003; http://dx.doi.org/10.1111/j.1365-2567.2011.03494.x
711. Cantino D, Mosso R, Baccino FM. Changes induced by fasting and cycloheximide in the vacuolar apparatus of rat hepatocytes. A morphometric investigation. Boll Soc Ital Biol Sper 1979; 55:1884-9; PMID:553561
712. Kovács J. Morphometric study of the effect of leupeptin, vinblastine, estron acetate and cycloheximide on the autophagic vacuole-lysosomal compartments in mouse seminal vesicle cells. Virchows Arch B Cell Pathol Incl Mol Pathol 1983; 42:83-93; PMID:6132491
713. Papadopoulos T, Pfeifer U. Regression of rat liver autophagic vacuoles by locally applied cycloheximide. Lab Invest 1986; 54:100-7; PMID:3941538
714. Rumpelt HJ, Albring M, Thoenes W. Prevention of D-galactosamine-induced hepatocellular autophagocytosis by cycloheximide. Virchows Arch B Cell Pathol 1974; 16:195-203; PMID:4216140; http://dx.doi.org/10.1007/BF02894074
715. Rumpelt HJ, Weisbach T. Effect of cycloheximide on glucagon-induced autophagy. Quantitative examinations on hepatocytes in the rat. Am J Pathol 1978; 91:49-56; PMID:645821
716. Kovács AL, Kovács J. Autophagocytosis in mouse seminal vesicle cells in vitro. Temperature dependence and effects of vinblastine and inhibitors of protein synthesis. Virchows Arch B Cell Pathol Incl Mol Pathol 1980; 32:97-104; PMID:6102826
717. Rodemann HP, Dittmann K, Toulany M. Radiationinduced EGFR-signaling and control of DNAdamage repair. Int J Radiat Biol 2007; 83:781-91; PMID:18058366; http://dx.doi.org/10.1080/09553000701769970

718. Chaachouay H, Ohneseit P, Toulany M, Kehlbach R, Multhoff G, Rodemann HP. Autophagy contributes to resistance of tumor cells to ionizing radiation. Radiother Oncol 2011; 99:287-92; PMID:21722986; http://dx.doi.org/10.1016/j.radonc.2011.06.002
719. Apel A, Herr I, Schwarz H, Rodemann HP, Mayer A. Blocked autophagy sensitizes resistant carcinoma cells to radiation therapy. Cancer Res 2008; 68:1485-94; PMID:18316613; http://dx.doi.org/10.1158/0008-5472.CAN-07-0562
720. Eng CH, Yu K, Lucas J, White E, Abraham RT. Ammonia derived from glutaminolysis is a diffusible regulator of autophagy. Sci Signal 2010; 3:ra31; PMID: 20424262; http://dx.doi.org/10.1126/scisignal.2000911
721. Seglen PO, Gordon PB. Effects of lysosomotropic monoamines, diamines, amino alcohols, and other amino compounds on protein degradation and protein synthesis in isolated rat hepatocytes. Mol Pharmacol 1980; 18:468-75; PMID:7464813
722. Cheong H, Lindsten T, Wu J, Lu C, Thompson CB. Ammonia-induced autophagy is independent of ULK1/ULK2 kinases. Proc Natl Acad Sci U S A 2011; 108:11121-6; PMID:21690395; http://dx.doi.org/10.1073/pnas.1107969108
723. Wei P, Zhang L, Lu Y, Man N, Wen L. C60(Nd) nanoparticles enhance chemotherapeutic susceptibility of cancer cells by modulation of autophagy. Nanotechnology 2010; 21:495101; PMID:21071824;
http://dx.doi.org/10.1088/0957-4484/21/49/495101
724. Lee DH, Goldberg AL. Proteasome inhibitors: valuable new tools for cell biologists. Trends Cell Biol 1998; 8:397-403; PMID:9789328; http://dx.doi.org/10.1016/S0962-8924(98)01346-4
725. Mehdi S. Cell-penetrating inhibitors of calpain. Trends Biochem Sci 1991; 16:150-3; PMID:1877091; http://dx.doi.org/10.1016/0968-0004(91)90058-4
726. Holen I, Gordon PB, Seglen PO. Inhibition of hepatocytic autophagy by okadaic acid and other protein phosphatase inhibitors. Eur J Biochem 1993; 215:113-22; PMID:8393787; http://dx.doi.org/10.1111/j.1432-1033.1993.tb18013.x
727. Sasaki K, Murata M, Yasumoto T, Mieskes G, Takai A. Affinity of okadaic acid to type-1 and type-2A protein phosphatases is markedly reduced by oxidation of its 27-hydroxyl group. Biochem J 1994; 298:259-62; PMID:8135728
728. Robinson DG, Albrecht S, Moriysu Y. The V-ATPase inhibitors concanamycin A and bafilomycin A lead to Golgi swelling in tobacco BY-2 cells. Protoplasma 2004; 224:255-60; PMID:15614486; http://dx.doi.org/10.1007/s00709-004-0070-6
729. Wu YC, Wu WK, Li Y, Yu L, Li ZJ, Wong CC, et al. Inhibition of macroautophagy by bafilomycin A1 lowers proliferation and induces apoptosis in colon cancer cells. Biochem Biophys Res Commun 2009; 382:451-6; PMID:19289106; http://dx.doi.org/10.1016/j.bbrc.2009.03.051
730. Yamamoto A, Tagawa Y, Yoshimori T, Moriyama Y, Masaki R, Tashiro Y. Bafilomycin A1 prevents maturation of autophagic vacuoles by inhibiting fusion between autophagosomes and lysosomes in rat hepatoma cell line, H-4-II-E cells. Cell Struct Funct 1998; 23:33-42; PMID:9639028; http://dx.doi.org/10.1247/csf.23.33
731. Ostenfeld MS, Høyer-Hansen M, Bastholm L, Fehrenbacher N, Olsen OD, Groth-Pedersen L, et al. Anti-cancer agent siramesine is a lysosomotropic detergent that induces cytoprotective autophagosome accumulation. Autophagy 2008; 4:487-99; PMID:18305408
732. Matsuoka K, Higuchi T, Maeshima M, Nakamura K. A vacuolar-type H+-ATPase in a nonvacuolar organelle is required for the sorting of soluble vacuolar protein precursors in tobacco cells. Plant Cell 1997; 9:533-46; PMID:12237363; http://dx.doi.org/10.1105/tpc.9.4.533
733. Arstila AU, Nuuja IJ, Trump BF. Studies on cellular autophagocytosis. Vinblastine-induced autophagy in the rat liver. Exp Cell Res 1974; 87:249-52; PMID: 4415937; http://dx.doi.org/10.1016/0014-4827(74) 90477-7
734. Hirsimäki Y, Arstila AU, Trump BF. Autophagocytosis: in vitro induction by microtuble poisons. Exp Cell Res 1975; 92:11-4; PMID:1169154; http://dx. doi.org/10.1016/0014-4827(75)90630-8
735. Kominami E, Hashida S, Khairallah EA, Katunuma N. Sequestration of cytoplasmic enzymes in an autophagic vacuole-lysosomal system induced by injection of leupeptin. J Biol Chem 1983; 258:6093-100; PMID:6133857
736. Réz G, Fellinger E, Réti M, Biczó I, Kovács AL. Time course of quantitative morphological changes of the autophagic-lysosomal compartment of murine seminal vesicle epithelial cells under the influence of vinblastine. J Submicrosc Cytol Pathol 1990; 22:529-34; PMID:2282639
737. Oliva O, Réz G, Pálfia Z, Fellinger E. Dynamics of vinblastine-induced autophagocytosis in murine pancreatic acinar cells: influence of cycloheximide posttreatments. Exp Mol Pathol 1992; 56:76-86; PMID: 1547871; http://dx.doi.org/10.1016/0014-4800(92) 90025-7
738. von Haefen C, Sifringer M, Menk M, Spies CD. Ethanol enhances susceptibility to apoptotic cell death via down-regulation of autophagy-related proteins. Alcohol Clin Exp Res 2011; 35:1381-91; PMID:21410490
739. Krüger U, Wang Y, Kumar S, Mandelkow EM. Autophagic degradation of tau in primary neurons and its enhancement by trehalose. Neurobiol Aging 2011; In press; PMID:22169203; http://dx.doi.org/10.1016/j.neurobiolaging.2011.11.009
740. Williams A, Sarkar S, Cuddon P, Ttofi EK, Saiki S, Siddiqi FH, et al. Novel targets for Huntington's disease in an mTOR-independent autophagy pathway. Nat Chem Biol 2008; 4:295-305; PMID:18391949; http://dx.doi.org/10.1038/nchembio.79
741. Palomo GM, Cerrato T, Gargini R, Diaz-Nido J. Silencing of frataxin gene expression triggers p53-dependent apoptosis in human neuron-like cells. Hum Mol Genet 2011; 20:2807-22; PMID:21531789; http://dx.doi.org/10.1093/hmg/ddr187
742. Feldman ME, Apsel B, Uotila A, Loewith R, Knight ZA, Ruggero D, et al. Active-site inhibitors of mTOR target rapamycin-resistant outputs of mTORC1 and mTORC2. PLoS Biol 2009; 7:e38; PMID:19209957; http://dx.doi.org/10.1371/journal.pbio.1000038
743. Fleming A, Noda T, Yoshimori T, Rubinsztein DC. Chemical modulators of autophagy as biological probes and potential therapeutics. Nat Chem Biol 2011; 7:9-17; PMID:21164513; http://dx.doi.org/10.1038/nchembio.500
744. Yu K, Toral-Barza L, Shi C, Zhang WG, Lucas J, Shor B, et al. Biochemical, cellular, and in vivo activity of novel ATP-competitive and selective inhibitors of the mammalian target of rapamycin. Cancer Res 2009; 69:6232-40; PMID:19584280; http://dx.doi.org/10.1158/0008-5472.CAN-09-0299
745. Chresta CM, Davies BR, Hickson I, Harding T, Cosulich S, Critchlow SE, et al. AZD8055 is a potent, selective, and orally bioavailable ATP-competitive mammalian target of rapamycin kinase inhibitor with in vitro and in vivo antitumor activity. Cancer Res 2010; 70:288-98; PMID:20028854; http://dx.doi.org/10.1158/0008-5472.CAN-09-1751
746. Roscic A, Baldo B, Crochemore C, Marcellin D, Paganetti P. Induction of autophagy with catalytic mTOR inhibitors reduces huntingtin aggregates in a neuronal cell model. J Neurochem 2011; 119:398-407; PMID:21854390; http://dx.doi.org/10.1111/j.1471-4159.2011.07435.x
747. Fan QW, Cheng C, Hackett C, Feldman M, Houseman BT, Nicolaides T, et al. Akt and autophagy cooperate to promote survival of drug-resistant glioma. Sci Signal 2010; 3:ra81; PMID:21062993; http://dx.doi.org/10.1126/scisignal.2001017
748. Sakagami H, Kawase M, Wakabayashi H, Kurihara T. Factors that affect the type of cell death induced by chemicals. Autophagy 2007; 3:493-5; PMID:17611389749. Doelling JH, Walker JM, Friedman EM, Thompson AR, Vierstra RD. The APG8/12-activating enzyme APG7 is required for proper nutrient recycling and senescence in Arabidopsis thaliana. J Biol Chem 2002;
277:33105-14; PMID:12070171; http://dx.doi.org/10.1074/jbc.M204630200
750. Fimia GM, Stoykova A, Romagnoli A, Giunta L, Di Bartolomeo S, Nardacci R, et al. Ambra1 regulates autophagy and development of the nervous system. Nature 2007; 447:1121-5; PMID:17589504
751. Kuma A, Hatano M, Matsui M, Yamamoto A, Nakaya H, Yoshimori T, et al. The role of autophagy during the early neonatal starvation period. Nature 2004; 432:1032-6; PMID:15525940; http://dx.doi.org/10. 1038/nature03029

752. Zhu H, Wu H, Liu X, Li B, Chen Y, Ren X, et al. Regulation of autophagy by a beclin 1-targeted microRNA, miR-30a, in cancer cells. Autophagy 2009; 5:816-23; PMID:19535919
753. Hamacher-Brady A, Brady NR, Logue SE, Sayen MR, Jinno M, Kirshenbaum LA, et al. Response to myocardial ischemia/reperfusion injury involves Bnip3 and autophagy. Cell Death Differ 2007; 14:146-57; PMID:16645637; http://dx.doi.org/10.1038/sj.cdd.4401936
754. Yang L, Li P, Fu S, Calay ES, Hotamisligil GS. Defective hepatic autophagy in obesity promotes ER stress and causes insulin resistance. Cell Metab 2010; 11:467-78; PMID:20519119; http://dx.doi.org/10.1016/j.cmet.2010.04.005
755. Poeck H, Besch R, Maihoefer C, Renn M, Tormo D, Morskaya SS, et al. 5'-Triphosphate-siRNA: turning gene silencing and Rig-I activation against melanoma. Nat Med 2008; 14:1256-63; PMID:18978796; http://dx.doi.org/10.1038/nm.1887
756. Delgado MA, Elmaoued RA, Davis AS, Kyei G, Deretic V. Toll-like receptors control autophagy. EMBO J 2008; 27:1110-21; PMID:18337753; http://dx.doi.org/10.1038/emboj.2008.31
757. Pua HH, Dzhagalov I, Chuck M, Mizushima N, He YW. A critical role for the autophagy gene Atg5 in T cell survival and proliferation. J Exp Med 2007; 204:25-31; PMID:17190837; http://dx.doi.org/10. 1084/jem.20061303
758. Miller BC, Zhao Z, Stephenson LM, Cadwell K, Pua HH, Lee HK, et al. The autophagy gene ATG5 plays an essential role in B lymphocyte development. Autophagy 2008; 4:309-14; PMID:18188005
759. Lee JS, Li Q, Lee JY, Lee SH, Jeong JH, Lee HR, et al. FLIP-mediated autophagy regulation in cell death control. Nat Cell Biol 2009; 11:1355-62; PMID:19838173; http://dx.doi.org/10.1038/ncb1980
760. Kyei GB, Dinkins C, Davis AS, Roberts E, Singh SB, Dong C, et al. Autophagy pathway intersects with HIV-1 biosynthesis and regulates viral yields in macrophages. J Cell Biol 2009; 186:255-68; PMID:19635843; http://dx.doi.org/10.1083/jcb.200903070
761. Kimball SR, Siegfried BA, Jefferson LS. Glucagon represses signaling through the mammalian target of rapamycin in rat liver by activating AMP-activated protein kinase. J Biol Chem 2004; 279:54103-9; PMID:15494402; http://dx.doi.org/10.1074/jbc.M410755200
762. Blommaart EF, Luiken JJ, Blommaart PJ, van Woerkom GM, Meijer AJ. Phosphorylation of ribosomal protein S6 is inhibitory for autophagy in isolated rat hepatocytes. J Biol Chem 1995; 270:2320-6; PMID:7836465; http://dx.doi.org/10.1074/jbc.270.5.2320
763. Klionsky DJ, Meijer AJ, Codogno P, Neufeld TP, Scott RC. Autophagy and p70S6 kinase. Autophagy 2005; 1:59-60, discussion 60-1; PMID:16874035; http://dx.doi.org/10.4161/auto.1.1.1536
764. Noda T, Ohsumi Y. Tor, a phosphatidylinositol kinase homologue, controls autophagy in yeast. J Biol Chem 1998; 273:3963-6; PMID:9461583; http://dx.doi.org/10.1074/jbc.273.7.3963
765. Sarkar S, Floto RA, Berger Z, Imarisio S, Cordenier A, Pasco M, et al. Lithium induces autophagy by inhibiting inositol monophosphatase. J Cell Biol 2005; 170:1101-11; PMID:16186256; http://dx.doi.org/10.1083/jcb.200504035
766. Høyer-Hansen M, Bastholm L, Szyniarowski P, Campanella M, Szabadkai G, Farkas T, et al. Control of macroautophagy by calcium, calmodulindependent
kinase kinase-beta, and Bcl-2. Mol Cell 2007; 25:193-205; PMID:17244528; http://dx.doi.org/10.1016/j.molcel.2006.12.009
767. Furuya N, Liang XH, Levine B. Autophagy and cancer. In: Klionsky DJ, ed. Autophagy. Georgetown, TX: Landes Bioscience, 2004:241-55.
768. de Medina P, Paillasse MR, S'egala G, Khallouki F, Brillouet S, Dalenc F, et al. Importance of cholesterol and oxysterols metabolism in the pharmacology of tamoxifen and other AEBS ligands. Chem Phys Lipids 2011; PMID:21641337; http://dx.doi.org/10.1016/j.chemphyslip.2011.05.005
769. de Medina P, Payré B, Boubekeur N, Bertrand-Michel J, Tercé F, Silvente-Poirot S, et al. Ligands of the antiestrogen-binding site induce active cell death and autophagy in human breast cancer cells through the modulation of cholesterol metabolism. Cell Death Differ 2009; 16:1372-84; PMID:19521424; http://dx.doi.org/10.1038/cdd.2009.62
770. Sarkar S, Perlstein EO, Imarisio S, Pineau S, Cordenier A, Maglathlin RL, et al. Small molecules enhance autophagy and reduce toxicity in Huntington's disease models. Nat Chem Biol 2007; 3:331-8; PMID:17486044; http://dx.doi.org/10.1038/nchembio883
771. Zhang L, Yu J, Pan H, Hu P, Hao Y, Cai W, et al. Small molecule regulators of autophagy identified by an image based high-throughput screen. Proc Natl Acad Sci U S A 2007; 104:19023-8; PMID:18024584; http://dx.doi.org/10.1073/pnas.0709695104
772. Cárdenas C, Miller RA, Smith I, Bui T, Molgó J, Müller M, et al. Essential regulation of cell bioenergetics by constitutive InsP3 receptor Ca2+ transfer to mitochondria. Cell 2010; 142:270-83; PMID:20655468; http://dx.doi.org/10.1016/j.cell.2010.06.007
773. Vicencio JM, Ortiz C, Criollo A, Jones AW, Kepp O, Galluzzi L, et al. The inositol 1,4,5-trisphosphate receptor regulates autophagy through its interaction with Beclin 1. Cell Death Differ 2009; 16:1006-17; PMID:19325567; http://dx.doi.org/10.1038/cdd.2009.34
774. Dayan F, Bilton RL, Laferrière J, Trottier E, Roux D, Pouyssegur J, et al. Activation of HIF-1alpha in exponentially growing cells via hypoxic stimulation is independent of the Akt/mTOR pathway. J Cell Physiol 2009; 218:167-74; PMID:18781596; http://dx.doi.org/10.1002/jcp.21584
775. Bellot G, Garcia-Medina R, Gounon P, Chiche J, Roux D, Pouysségur J, et al. Hypoxia-induced autophagy is mediated through hypoxia-inducible factor induction of BNIP3 and BNIP3L via their BH3 domains. Mol Cell Biol 2009; 29:2570-81; PMID:19273585; http://dx.doi.org/10.1128/MCB.00166-09
776. Yamashita S, Yurimoto H, Murakami D, Yoshikawa M, Oku M, Sakai Y. Lag-phase autophagy in the methylotrophic yeast Pichia pastoris. Genes Cells 2009; 14:861-70; PMID:19549169; http://dx.doi.org/10. 1111/j.1365-2443.2009.01316.x
777. van Zutphen T, Baerends RJ, Susanna KA, de Jong A, Kuipers OP, Veenhuis M, et al. Adaptation of Hansenula polymorpha to methanol: a transcriptome analysis. BMC Genomics 2010; 11:1; PMID:20044946; http://dx.doi. org/10.1186/1471-2164-11-1
778. Inoue Y, Suzuki T, Hattori M, Yoshimoto K, Ohsumi Y, Moriyasu Y. AtATG genes, homologs of yeast autophagy genes, are involved in constitutive autophagy in Arabidopsis root tip cells. Plant Cell Physiol 2006; 47:1641-52; PMID:17085765; http://dx.doi. org/10.1093/pcp/pcl031
779. Yano K, Suzuki T, Moriyasu Y. Constitutive autophagy in plant root cells. Autophagy 2007; 3:360-2; PMID:17426438
780. Gordon PB, Kisen GO, Kovács AL, Seglen PO. Experimental characterization of the autophagic-lysosomal pathway in isolated rat hepatocytes. Biochem
Soc Symp 1989; 55:129-43; PMID:2619764 781. Poli A, Gordon PB, Schwarze PE, Grinde B, Seglen PO. Effects of insulin and anchorage on hepatocytic protein metabolism and amino acid transport. J Cell Sci 1981; 48:1-18; PMID:7024288
782. Schliess F, Reissmann R, Reinehr R, vom Dahl S, Häussinger D. Involvement of integrins and Src in insulin signaling toward autophagic proteolysis in rat liver. J Biol Chem 2004; 279:21294-301; PMID:14985360; http://dx.doi.org/10.1074/jbc.M313901200
783. vom Dahl S, Dombrowski F, Schmitt M, Schliess F, Pfeifer U, Häussinger D. Cell hydration controls autophagosome formation in rat liver in a microtubuledependent way downstream from p38MAPK activation. Biochem J 2001; 354:31-6; PMID:11171076; http://dx.doi.org/10.1042/0264-6021:3540031
784. vom Dahl S, Stoll B, Gerok W, Häussinger D. Inhibition of proteolysis by cell swelling in the liver requires intact microtubular structures. Biochem J 1995; 308:529-36; PMID:7772037
785. Kovacs AL, Zhang H. Role of autophagy in Caenorhabditis elegans. FEBS Lett 2010; 584:1335-41; PMID:20138173; http://dx.doi.org/10.1016/j.febslet.2010.02.002
786. Zhang Y, Yan L, Zhou Z, Yang P, Tian E, Zhang K, et al. SEPA-1 mediates the specific recognition and degradation of P granule components by autophagy in C. elegans. Cell 2009; 136:308-21; PMID:19167332; http://dx.doi.org/10.1016/j.cell.2008.12.022

787. Morselli E, Maiuri MC, Markaki M, Megalou E,Pasparaki A, Palikaras K, et al. Caloric restriction and esveratrol promote longevity through the Sirtuin-1-dependent induction of autophagy. Cell Death Dis 2010; 1:e10; PMID:21364612; http://dx.doi.org/10. 1038/cddis.2009.8
788. Samara C, Syntichaki P, Tavernarakis N. Autophagy is required for necrotic cell death in Caenorhabditis elegans. Cell Death Differ 2008; 15:105-12; PMID:17901876; http://dx.doi.org/10.1038/sj.cdd.4402231
789. Alberti A, Michelet X, Djeddi A, Legouis R. The autophagosomal protein LGG-2 acts synergistically with LGG-1 in dauer formation and longevity in C. elegans. Autophagy 2010; 6:622-33; PMID: 20523114; http://dx.doi.org/10.4161/auto.6.5.12252
790. Kang C, You YJ, Avery L. Dual roles of autophagy in the survival of Caenorhabditis elegans during starvation. Genes Dev 2007; 21:2161-71; PMID:17785524;http://dx.doi.org/10.1101/gad.1573107
791. Willig KI, Kellner RR, Medda R, Hein B, Jakobs S, Hell SW. Nanoscale resolution in GFP-based microscopy. Nat Methods 2006; 3:721-3; PMID:16896340;http://dx.doi.org/10.1038/nmeth922
792. Huang B, Bates M, Zhuang X. Super-resolution fluorescence microscopy. Annu Rev Biochem 2009; 78:993-1016; PMID:19489737; http://dx.doi.org/10. 1146/annurev.biochem.77.061906.092014
793. Alers S, Löffler AS, Paasch F, Dieterle AM, Keppeler H, Lauber K, et al. Atg13 and FIP200 act independently of Ulk1 and Ulk2 in autophagy induction. Autophagy 2011; 7:1424-33; PMID:22024743;http://dx.doi.org/10.4161/auto.7.12.18027
794. Brown WR, Hubbard SJ, Tickle C, Wilson SA. The chicken as a model for large-scale analysis of vertebrate gene function. Nat Rev Genet 2003; 4:87-98; PMID: 12560806; http://dx.doi.org/10.1038/nrg998
795. Baba TW, Giroir BP, Humphries EH. Cell lines derived from avian lymphomas exhibit two distinct phenotypes. Virology 1985; 144:139-51; PMID: 2998040; http://dx.doi.org/10.1016/0042-6822(85) 90312-5
796. Shelly S, Lukinova N, Bambina S, Berman A, Cherry S. Autophagy is an essential component of Drosophila immunity against vesicular stomatitis virus. Immunity 2009; 30:588-98; PMID:19362021; http://dx.doi.org/10.1016/j.immuni.2009.02.009
797. Hou YC, Chittaranjan S, Barbosa SG, McCall K, Gorski SM. Effector caspase Dcp-1 and IAP protein Bruce regulate starvation-induced autophagy during Drosophila melanogaster oogenesis. J Cell Biol 2008; 182:1127-39; PMID:18794330; http://dx.doi.org/10. 1083/jcb.200712091
798. Josefsen L, Droce A, Sondergaard TE, Sørensen JL, Bormann J, Schäfer W, et al. Autophagy provides nutrients for nonassimilating fungal structures and is necessary for plant colonization but not for infection in the necrotrophic plant pathogen Fusarium graminearum. Autophagy 2012; 8; In press; PMID:22240663
799. Nadal M, Gold SE. The autophagy genes ATG8 and ATG1 affect morphogenesis and pathogenicity in Ustilago maydis. Mol Plant Pathol 2010; 11:463-78; PMID:20618705; http://dx.doi.org/10.1111/j.1364-3703.2010.00620.x
800. Pollack JK, Harris SD, Marten MR. Autophagy in filamentous fungi. Fungal Genet Biol 2009; 46:1-8; PMID:19010432; http://dx.doi.org/10.1016/j.fgb.2008.10.010
801. Richie DL, Fuller KK, Fortwendel J, Miley MD, McCarthy JW, Feldmesser M, et al. Unexpected link between metal ion deficiency and autophagy inAspergillus fumigatus. Eukaryot Cell 2007; 6:2437-47; PMID:17921348; http://dx.doi.org/10.1128/EC.00224-07
802. Pinan-Lucarré B, Balguerie A, Clavé C. Accelerated cell death in Podospora autophagy mutants. Eukaryot Cell 2005; 4:1765-74; PMID:16278443; http://dx.doi.org/10.1128/EC.4.11.1765-1774.2005
803. Asakura M, Ninomiya S, Sugimoto M, Oku M, Yamashita S, Okuno T, et al. Atg26-mediated pexophagy is required for host invasion by the plant pathogenic fungus Colletotrichum orbiculare. Plant Cell 2009; 21:1291-304; PMID:19363139; http://dx.doi.org/10.1105/tpc.108.060996
804. Liu X-H, Lu J-P, Zhang L, Dong B, Min H, Lin F-C. Involvement of a Magnaporthe grisea serine/threonine kinase gene, MgATG1, in appressorium turgor and pathogenesis. Eukaryot Cell 2007; 6:997-1005; PMID:17416896; http://dx.doi.org/10.1128/EC.00011-07
805. Nguyen LN, Bormann J, Le GT, Stärkel C, Olsson S, Nosanchuk JD, et al. Autophagy-related lipase FgATG15 of Fusarium graminearum is important for lipid turnover and plant infection. Fungal Genet Biol 2011; 48:217-24; PMID:21094265; http://dx.doi.org/10.1016/j.fgb.2010.11.004
806. Deng YZ, Ramos-Pamplona M, Naqvi NI. Methods for functional analysis of macroautophagy in filamentous fungi. Methods Enzymol 2008; 451:295-310; PMID: 19185728; http://dx.doi.org/10.1016/S0076-6879(08) 03220-5
807. Berger B, Abdalla FC, Cruz-Landim C. Effect of narcosis with CO2 on the ovarian development in queens of Apis mellifera (Hymenoptera, Apini). Sociobiology 2005; 45:261-70.
808. Silva-Zacarin ECM, Tomaino GA, Brocheto-Braga MR, Taboga SR, De Moraes RL. Programmed cell death in the larval salivary glands of Apis mellifera (Hymenoptera, Apidae). J Biosci 2007; 32:309-28; PMID:17435323; http://dx.doi.org/10.1007/s12038-007-0031-2
809. Gregorc A, Bowen ID. Programmed cell death in the honey-bee (Apis mellifera L.) larvae midgut. Cell Biol Int 1997; 21:151-8; PMID:9151991; http://dx.doi.org/10.1006/cbir.1997.0127
810. Navajas M, Migeon A, Alaux C, Martin-Magniette M, Robinson G, Evans J, et al. Differential gene expression of the honey bee Apis mellifera associated with Varroa destructor infection. BMC Genomics 2008; 9:301; PMID:18578863; http://dx.doi.org/10 1186/1471-2164-9-301
811. Spruessel A, Steimann G, Jung M, Lee SA, Carr T, Fentz AK, et al. Tissue ischemia time affects gene and protein expression patterns within minutes following surgical tumor excision. Biotechniques 2004; 36:1030-7; PMID:15211754
812. Espina V, Edmiston KH, Heiby M, Pierobon M, Sciro M, Merritt B, et al. A portrait of tissue phosphoprotein stability in the clinical tissue procurement process. Mol Cell Proteomics 2008; 7:1998-2018; PMID:18667411; http://dx.doi.org/10.1074/mcp.M700596-MCP200
813. Barth S, Glick D, Macleod KF. Autophagy: assays and artifacts. J Pathol 2010; 221:117-24; PMID: 20225337; http://dx.doi.org/10.1002/path.2694
814. Nixon RA, Wegiel J, Kumar A, Yu WH, Peterhoff C, Cataldo A, et al. Extensive involvement of autophagy in Alzheimer disease: an immuno-electron microscopy study. J Neuropathol Exp Neurol 2005; 64:113-22; PMID:15751225
815. Vanhorebeek I, Gunst J, Derde S, Derese I, Boussemaere M, Güiza F, et al. Insufficient activation of autophagy allows cellular damage to accumulate in critically ill patients. J Clin Endocrinol Metab 2011; 96:E633-45; PMID:21270330; http://dx.doi.org/10.1210/jc.2010-2563
816. Nyman E, Brännmark C, Palmér R, Brugård J, Nyström FH, Strålfors P, et al. A hierarchical wholebody modeling approach elucidates the link between in Vitro insulin signaling and in Vivo glucose homeostasis J Biol Chem 2011; 286:26028-41; PMID:21572040; http://dx.doi.org/10.1074/jbc.M110.188987
817. Buzgariu W, Chera S, Galliot B. Methods to investigate autophagy during starvation and regeneration in hydra. Methods Enzymol 2008; 451:409-37; PMID:19185734; http://dx.doi.org/10.1016/S0076-6879(08)03226-6
818. Chera S, Buzgariu W, Ghila L, Galliot B. Autophagy in Hydra: a response to starvation and stress in early animal evolution. Biochim Biophys Acta 2009; 1793:1432-43; PMID:19362111
819. Chera S, de Rosa R, Miljkovic-Licina M, Dobretz K, Ghila L, Kaloulis K, et al. Silencing of the hydra serine protease inhibitor Kazal1 gene mimics the human SPINK1 pancreatic phenotype. J Cell Sci 2006; 119:846-57; PMID:16478786; http://dx.doi.org/10. 1242/jcs.02807
820. Galliot B. Autophagy and self-preservation: a step ahead from cell plasticity? Autophagy 2006; 2:231-3;PMID:16874084
821. Galliot B, Miljkovic-Licina M, de Rosa R, Chera S. Hydra, a niche for cell and developmental plasticity. Semin Cell Dev Biol 2006; 17:492-502; PMID: 16807002; http://dx.doi.org/10.1016/j.semcdb.2006.05.005

822. Sala-Mercado JA, Wider J, Undyala VV, Jahania S, Yoo W, Mentzer RM, Jr., et al. Profound cardioprotection with chloramphenicol succinate in the swine model of myocardial ischemia-reperfusion injury. Circulation 2010; 122(Suppl):S179-84; PMID:20837911; http://dx.doi.org/10.1161/CIRCULATIONAHA.109.928242
823. Sobolewska A, Motyl T, Gajewska M. Role and regulation of autophagy in the development of acinar structures formed by bovine BME-UV1 mammary epithelial cells. Eur J Cell Biol 2011; 90:854-64; PMID:21868124; http://dx.doi.org/10.1016/j.ejcb.2011.06.007
824. Motyl T, Gajewska M, Zarzynska J, Sobolewska A, Gajkowska B. Regulation of autophagy in bovine mammary epithelial cells. Autophagy 2007; 3:484-6; PMID:17592247
825. Sobolewska A, Gajewska M, Zarzyńska J, Gajkowska B, Motyl T. IGF-I, EGF, and sex steroids regulate autophagy in bovine mammary epithelial cells via the mTOR pathway. Eur J Cell Biol 2009; 88:117-30; PMID:19013662; http://dx.doi.org/10.1016/j.ejcb.2008.09.004
826. Facey CO, Lockshin RA. The execution phase of autophagy associated PCD during insect metamorphosis. Apoptosis 2010; 15:639-52; PMID:20405221; http://dx.doi.org/10.1007/s10495-010-0499-3
827. Malagoli D, Abdalla FC, Cao Y, Feng Q, Fujisaki K, Gregorc A, et al. Autophagy and its physiological relevance in arthropods: Current knowledge and perspectives. Autophagy 2010; 6:575-88; PMID:20458176; http://dx.doi.org/10.4161/auto.6.5.11962
828. Mpakou VE, Nezis IP, Stravopodis DJ, Margaritis LH, Papassideri IS. Programmed cell death of the ovarian nurse cells during oogenesis of the silkmoth Bombyx mori. Dev Growth Differ 2006; 48:419-28; PMID: 16961589; http://dx.doi.org/10.1111/j.1440-169X. 2006.00878.x
829. Mpakou VE, Nezis IP, Stravopodis DJ, Margaritis LH, Papassideri IS. Different modes of programmed cell death during oogenesis of the silkmoth Bombyx mori. Autophagy 2008; 4:97-100; PMID:17986869
830. Sumithra P, Britto CP, Krishnan M. Modes of cell death in the pupal perivisceral fat body tissue of the silkworm Bombyx mori L. Cell Tissue Res 2010; 339:349-58; PMID:19949813; http://dx.doi.org/10.1007/s00441-009-0898-3
831. Tettamanti G, Grimaldi A, Casartelli M, Ambrosetti E, Ponti B, Congiu T, et al. Programmed cell death and stem cell differentiation are responsible for midgut replacement in Heliothis virescens during prepupal instar. Cell Tissue Res 2007; 330:345-59; PMID:17661086; http://dx.doi.org/10.1007/s00441-007-0449-8
832. Goncu E, Parlak O. Some autophagic and apoptotic features of programmed cell death in the anterior silk glands of the silkworm, Bombyx mori. Autophagy 2008; 4:1069-72; PMID:18838861
833. Zhou S, Zhou Q, Liu Y, Wang S,Wen D, He Q, et al. Two Tor genes in the silkworm Bombyx mori. Insect Mol Biol 2010; 19:727-35; PMID:20609020; http://dx.doi.org/10.1111/j.1365-2583.2010.01026.x
834. Zhang X, Hu ZY, Li WF, Li QR, Deng XJ, Yang WY, et al. Systematic cloning and analysis of autophagyrelated genes from the silkworm Bombyx mori. BMC Mol Biol 2009; 10:50; PMID:19470186; http://dx. doi.org/10.1186/1471-2199-10-50
835. Li Q, Deng X, Huang Z, Zheng S, Tettamanti G, Cao Y, et al. Expression of autophagy-related genes in the anterior silk gland of the silkworm (Bombyx mori) during metamorphosis. Can J Zool 2011; 89:1019-26; http://dx.doi.org/10.1139/z11-075
836. Thomé RG, Santos HB, Arantes FP, Domingos FF, Bazzoli N, Rizzo E. Dual roles for autophagy during follicular atresia in fish ovary. Autophagy 2009; 5:117-9; PMID:19011378; http://dx.doi.org/10.4161/auto. 5.1.7302
837. Santos HB, Thomé RG, Arantes FP, Sato Y, Bazzoli N, Rizzo E. Ovarian follicular atresia is mediated by heterophagy, autophagy, and apoptosis in Prochilodus argenteus and Leporinus taeniatus (Teleostei: Characiformes). Theriogenology 2008; 70:1449-60; PMID:18701155; http://dx.doi.org/10.1016/j.theriogenology.2008.06.091
838. Santos HB, Sato Y, Moro L, Bazzoli N, Rizzo E. Relationship among follicular apoptosis, integrin beta1 and collagen type IV during early ovarian regression in the teleost Prochilodus argenteus after induced spawning. Cell Tissue Res 2008; 332:159-70; PMID:18193286; http://dx.doi.org/10.1007/s00441-007-0540-1
839. Santos HB, Rizzo E, Bazzoli N, Sato Y, Moro L. Ovarian regression and apoptosis in the South American teleost Leporinus taeniatus Lutken (Characiformes, Anostomidae) from the São Francisco Basin. J Fish Biol 2005; 67:1446-59; http://dx.doi.org/10.1111/j.1095-8649.2005.00854.x
840. Couve E, Schmachtenberg O. Autophagic activity and aging in human odontoblasts. J Dent Res 2011; 90:523-8; PMID:21212314; http://dx.doi.org/10. 1177/0022034510393347
841. González-Estévez C. Autophagy in freshwater planarians. Methods Enzymol 2008; 451:439-65; PMID: 19185735; http://dx.doi.org/10.1016/S0076-6879 (08)03227-8
842. González-Estévez C, Felix DA, Aboobaker AA, Saló E. Gtdap-1 promotes autophagy and is required for planarian remodeling during regeneration and starvation. Proc Natl Acad Sci U S A 2007; 104:13373-8; PMID:17686979; http://dx.doi.org/10.1073/pnas. 0703588104
843. Toyooka K, Moriyasu Y, Goto Y, Takeuchi M, Fukuda H, Matsuoka K. Protein aggregates are transported to vacuoles by a macroautophagic mechanism in nutrient-starved plant cells. Autophagy 2006; 2:96-106; PMID:16874101
844. Svenning S, Lamark T, Krause K, Johansen T. Plant NBR1 is a selective autophagy substrate and a functional hybrid of the mammalian autophagic adapters NBR1 and p62/SQSTM1. Autophagy 2011; 7:993-1010; PMID:21606687; http://dx.doi.org/10.4161/auto.7.9.16389
845. Zientara-Rytter K, Lukomska J, Moniuszko G, Gwozdecki R, Surowiecki P, Lewandowska M, et al. Identification and functional analysis of Joka2, a tobacco member of the family of selective autophagy cargo receptors. Autophagy 2011; 7:1145-58; PMID:21670587; http://dx.doi.org/10.4161/auto.7.10.16617
846. Takeshige K, Baba M, Tsuboi S, Noda T, Ohsumi Y. Autophagy in yeast demonstrated with proteinasedeficient mutants and conditions for its induction. J Cell Biol 1992; 119:301-11; PMID:1400575; http://dx.doi.org/10.1083/jcb.119.2.301
847. Moriyasu Y, Ohsumi Y. Autophagy in tobacco suspension-cultured cells in response to sucrose starvation. Plant Physiol 1996; 111:1233-41; PMID:12226358
848. Calvo-Garrido J, Carilla-Latorre S, Kubohara Y, Santos-Rodrigo N, Mesquita A, Soldati T, et al. Autophagy in Dictyostelium: genes and pathways, cell death and infection. Autophagy 2010; 6:686-701; PMID:20603609; http://dx.doi.org/10.4161/auto.6.6. 12513
849. Tung SM, Unal C, Ley A, Peña C, Tunggal B, Noegel AA, et al. Loss of Dictyostelium ATG9 results in a pleiotropic phenotype affecting growth, development, phagocytosis and clearance and replication of Legionella pneumophila. Cell Microbiol 2010; 12: 765-80; PMID:20070309; http://dx.doi.org/10.1111/j.1462-5822.2010.01432.x
850. Bozzaro S, Eichinger L. The professional phagocyte Dictyostelium discoideum as a model host for bacterial pathogens. Curr Drug Targets 2011; 12:942-54; PMID:21366522; http://dx.doi.org/10.2174/138945011795677782
851. Duszenko M, Ginger ML, Brennand A, Gualdrón-López M, Colombo MI, Coombs GH, et al. Autophagy in protists. Autophagy 2011; 7:127-58; PMID:20962583; http://dx.doi.org/10.4161/auto.7.2.13310
852. Barquilla A, Crespo JL, Navarro M. Rapamycin inhibits trypanosome cell growth by preventing TOR complex 2 formation. Proc Natl Acad Sci U S A 2008; 105:14579-84; PMID:18796613; http://dx.doi.org/10.1073/pnas.0802668105
853. Morais P, Lamas J, Sanmartín ML, Orallo F, Leiro J. Resveratrol induces mitochondrial alterations, autophagy and a cryptobiosis-like state in scuticociliates. Protist 2009; 160:552-64; PMID:19640787; http://dx.doi.org/10.1016/j.protis.2009.04.004
854. Yakisich JS, Kapler GM. The effect of phosphoinositide 3-kinase inhibitors on programmed nuclear degradation in Tetrahymena and fate of surviving nuclei. Cell Death Differ 2004; 11:1146-9; PMID:15257301; http://dx.doi.org/10.1038/sj.cdd.4401473

855. Akematsu T, Pearlman RE, Endoh H. Gigantic macroautophagy in programmed nuclear death of Tetrahymena thermophila. Autophagy 2010; 6:901-11; PMID:20798592; http://dx.doi.org/10.4161/auto. 6.7.13287
856. Thorgaard GH, Bailey GS, Williams D, Buhler DR, Kaattari SL, Ristow SS, et al. Status and opportunities for genomics research with rainbow trout. Comp Biochem Physiol B Biochem Mol Biol 2002; 133:609-46; PMID:12470823; http://dx.doi.org/10.1016/S1096-4959(02)00167-7
857. Govoroun M, Le Gac F, Guiguen Y. Generation of a large scale repertoire of Expressed Sequence Tags (ESTs) from normalised rainbow trout cDNA libraries. BMC Genomics 2006; 7:196; PMID:16887034;http://dx.doi.org/10.1186/1471-2164-7-196
858. Rexroad CE, 3rd, Lee Y, Keele JW, Karamycheva S, Brown G, Koop B, et al. Sequence analysis of a rainbow trout cDNA library and creation of a gene index. Cytogenet Genome Res 2003; 102:347-54; PMID: 14970727; http://dx.doi.org/10.1159/000075773
859. Rise ML, von Schalburg KR, Brown GD, Mawer MA, Devlin RH, Kuipers N, et al. Development and application of a salmonid EST database and cDNA microarray: data mining and interspecific hybridization characteristics. Genome Res 2004; 14:478-90; PMID:14962987; http://dx.doi.org/10.1101/gr.1687304
860. Salem M, Rexroad CE, 3rd, Wang J, Thorgaard GH, Yao J. Characterization of the rainbow trout transcriptome using Sanger and 454-pyrosequencing approaches. BMC Genomics 2010; 11:564; PMID: 20942956; http://dx.doi.org/10.1186/1471-2164-11-564
861. Polakof S, Panserat S, Craig PM, Martyres DJ, Plagnes-Juan E, Savari S, et al. The metabolic consequences of hepatic AMP-kinase phosphorylation in rainbow trout. PLoS One 2011; 6:e20228; PMID: 21625448; http://dx.doi.org/10.1371/journal.pone.0020228
862. Seiliez I, Gabillard JC, Skiba-Cassy S, Garcia-Serrana D, Gutiérrez J, Kaushik S, et al. An in vivo and in vitro assessment of TOR signaling cascade in rainbow trout (Oncorhynchus mykiss). Am J Physiol Regul Integr Comp Physiol 2008; 295:R329-35; PMID:18434442; http://dx.doi.org/10.1152/ajpregu.00146.2008
863. Seiliez I, Gabillard J-C, Riflade M, Sadoul B, Dias K, Avérous J, et al. Amino acids downregulate the expression of several autophagy-related genes in rainbow trout myoblasts. Autophagy 2012; 8; In press;PMID:22252009
864. Umemiya R, Matsuo T, Hatta T, Sakakibara S, Boldbaatar D, Fujisaki K. Cloning and characterization of an autophagy-related gene, ATG12, from the threehost tick Haemaphysalis longicornis. Insect Biochem Mol Biol 2007; 37:975-84; PMID:17681237; http://dx.doi.org/10.1016/j.ibmb.2007.05.006
865. Kawano S, Umemiya-Shirafuji R, Boldbaatar D, Matsuoka K, Tanaka T, Fujisaki K. Cloning and characterization of the autophagy-related gene 6 from the hard tick, Haemaphysalis longicornis. Parasitol Res 2011; 109:1341-9; PMID:21537978; http://dx.doi.org/10.1007/s00436-011-2429-x
866. Umemiya-Shirafuji R, Matsuo T, Liao M, Boldbaatar D, Battur B, Suzuki HI, et al. Increased expression of ATG genes during nonfeeding periods in the tick Haemaphysalis longicornis. Autophagy 2010; 6:473-81; PMID:20404490; http://dx.doi.org/10.4161/auto.6.4.11668
867. Piggott N, Cook MA, Tyers M, Measday V. Genomewide fitness profiles reveal a requirement for autophagy during yeast fermentation. Genes Genomes Genetics 2011; 1:353-67.
868. Cebollero E, Gonzalez R. Induction of autophagy by second-fermentation yeasts during elaboration of sparkling wines. Appl Environ Microbiol 2006; 72:4121-7; PMID:16751523; http://dx.doi.org/10.1128/AEM.02920-05
869. Marks VD, Ho Sui SJ, Erasmus D, van der Merwe GK, Brumm J, Wasserman WW, et al. Dynamics of the yeast transcriptome during wine fermentation reveals a novel fermentation stress response. FEMS Yeast Res 2008; 8:35-52; PMID:18215224; http://dx.doi.org/10.1111/j.1567-1364.2007.00338.x
870. Mendes-Ferreira A, Sampaio-Marques B, Barbosa C, Rodrigues F, Costa V, Mendes-Faia A, et al. Accumulation of non-superoxide anion reactive oxygen species mediates nitrogen-limited alcoholic fermentation by Saccharomyces cerevisiae. Appl Environ Microbiol 2010; 76:7918-24; PMID:20952643; http://dx.doi.org/10.1128/AEM.01535-10
871. Rossignol T, Dulau L, Julien A, Blondin B. Genomewide monitoring of wine yeast gene expression during alcoholic fermentation. Yeast 2003; 20:1369-85; PMID:14663829; http://dx.doi.org/10.1002/yea.1046
872. Teixeira MC, Raposo LR, Mira NP, Lourenço AB, Sá-Correia I. Genome-wide identification of Saccharomyces cerevisiae genes required for maximal tolerance to ethanol. Appl Environ Microbiol 2009; 75:5761-72; PMID:19633105; http://dx.doi.org/10.1128/ AEM.00845-09
873. Yoshikawa K, Tanaka T, Furusawa C, Nagahisa K, Hirasawa T, Shimizu H. Comprehensive phenotypic analysis for identification of genes affecting growth under ethanol stress in Saccharomyces cerevisiae. FEMS Yeast Res 2009; 9:32-44; PMID:19054128; http://dx.doi.org/10.1111/j.1567-1364.2008.00456.x
874. Hazan R, Levine A, Abeliovich H. Benzoic acid, a weak organic acid food preservative, exerts specific effects on intracellular membrane trafficking pathways in Saccharomyces cerevisiae. Appl Environ Microbiol 2004; 70:4449-57; PMID:15294772; http://dx.doi.org/10.1128/AEM.70.8.4449-4457.2004
875. Winter G, Hazan R, Bakalinsky AT, Abeliovich H. Caffeine induces macroautophagy and confers a cytocidal effect on food spoilage yeast in combination with benzoic acid. Autophagy 2008; 4:28-36; PMID: 17952024
876. Singletary K, Milner J. Diet, autophagy, and cancer: a review. Cancer Epidemiol Biomarkers Prev 2008; 17:1596-610; PMID:18628411; http://dx.doi.org/10. 1158/1055-9965.EPI-07-2917
877. Su CL, Chen FN, Won SJ. Involvement of apoptosis and autophagy in reducing mouse hepatoma ML-1 cell growth in inbred BALB/c mice by bacterial fermented soybean products. Food Chem Toxicol 2011; 49:17-24; PMID:20732379; http://dx.doi.org/10.1016/j.fct. 2010.08.017
878. Abeliovich H, Gonzalez R. Autophagy in food biotechnology. Autophagy 2009; 5:925-9; PMID: 19556866; http://dx.doi.org/10.4161/auto.5.7.9213879. He C, Bartholomew CR, Zhou W, Klionsky DJ. Assaying autophagic activity in transgenic GFP-Lc3 and GFP-Gabarap zebrafish embryos. Autophagy 2009; 5:520-6; PMID:19221467; http://dx.doi.org/ 10.4161/auto.5.4.7768
879. He C, Bartholomew CR, Zhou W, Klionsky DJ. Assaying autophagic activity in transgenic GFP-Lc3 and GFP-Gabarap zebrafish embryos. Autophagy 2009; 5:520-6; PMID:19221467; http://dx.doi.org/ 10.4161/auto.5.4.7768
880. Komoike Y, Shimojima K, Liang JS, Fujii H, Maegaki Y, Osawa M, et al. A functional analysis of GABARA on 17p13.1 by knockdown zebrafish. J Hum Genet 2010; 55:155-62; PMID:20111057; http://dx.doi.org/10.1038/jhg.2010.1
881. Dowling JJ, Low SE, Busta AS, Feldman EL. Zebrafish MTMR14 is required for excitation-contraction coupling, developmental motor function and the regulation of autophagy. Hum Mol Genet 2010; 19:2668-81; PMID: 20400459; http://dx.doi.org/10.1093/hmg/ddq153
882. Makky K, Tekiela J, Mayer AN. Target of rapamycin (TOR) signaling controls epithelial morphogenesis in the vertebrate intestine. Dev Biol 2007; 303:501-13; PMID:17222402; http://dx.doi.org/10.1016/j.ydbio. 2006.11.030
883. Klionsky DJ, Baehrecke EH, Brumell JH, Chu CT, Codogno P, Cuervo AM, et al. A comprehensive glossary of autophagy-related molecules and processes (2nd edition). Autophagy 2011; 7:1273-94; PMID: 21997368; http://dx.doi.org/10.4161/auto.7.11.17661
884. Klionsky DJ, Codogno P, Cuervo AM, Deretic V, Elazar Z, Fueyo-Margareto J, et al. A comprehensive glossary of autophagy-related molecules and processes. Autophagy 2010; 6:438-48; http://dx.doi.org/10. 4161/auto.6.4.12244

Glossary

For a more complete listing of autophagy-related terms and definitions we refer readers to Klionsky DJ, Baehrecke EH, Brumell JH, Chu CT, Codogno P, Cuervo AM, etal. A comprehensive glossary of autophagy-related molecules and processes (2nd edition). Autophagy 2011; 7:1273–94.

Amphisome: Intermediate compartment formed by the fusion of an autophagosome with an endosome; this compartment has not yet fused with a lysosome (also referred to as an acidic late autophagosome).
Autolysosome: A degradative compartment formed by the fusion of an autophagosome or amphisome with a primary lysosome or telolysosome. Upon completion of degradation, or when degradation has reached an end point, this compartment (again) becomes a telolysosome (also referred to as a residual body).
Autophagosome: A cytosolic membrane-bound compartment typically denoted by a limiting double membrane. The early autophagosome in particular contains cytoplasmic components and organelles that are morphologically unchanged because the compartment has not fused with a lysosome and lacks proteolytic enzymes.
Autophagy: A collection of processes typically involving degradative delivery of a portion of the cytoplasm to lysosomes or the plant or fungal vacuole that does not involve direct transport through the endocytic or vacuolar protein sorting, Vps, pathways.
Chaperone-mediated autophagy (CMA): Import and degradation
of soluble cytosolic proteins by chaperone-dependent, direct translocation across the lysosomal membrane.
Cytoplasm-to-vacuole targeting (Cvt): A biosynthetic pathway in fungi that transports resident hydrolases to the vacuole through a selective autophagy-related process.
Lysosome: A degradative organelle in higher eukaryotes that compartmentalizes a range of hydrolytic enzymes and maintains a highly acidic pH. A primary lysosome is a relatively small compartment that has not yet participated in a degradation process, whereas secondary lysosomes are sites of present or past digestive activity. The secondary lysosomes include autolysosomes and telolysosomes. Autolysosomes/early secondary lysosomes are larger compartments actively engaged in digestion, whereas telolysosomes/late secondary lysosomes do not have significant digestive activity and contain residues of previous digestions. Both may contain material of either autophagic or heterophagic origin.
Macroautophagy: The largely nonspecific autophagic sequestration of cytoplasm into a double- or multiple-membranedelimited compartment (an autophagosome) of nonlysosomal/vacuolar origin and its subsequent degradation by the lysosomal system. Note that certain proteins and organelles may be selectively degraded via a macroautophagy-related process, and conversely, some cytosolic components such as cytoskeletal elements are selectively excluded.
Microautophagy: Uptake and degradation of cytoplasm by protrusion, invagination or septation of the lysosome or vacuole membrane.
Mitophagy: The selective autophagic sequestration and degradation of mitochondria.
Nucleophagy: The selective autophagic degradation of the nucleus.
Pexophagy: A selective type of autophagy involving the sequestration and degradation of peroxisomes; can occur by a micro- or macroautophagic process.
Phagophore: Membrane cisterna that has been implicated in an initial event during formation of the autophagosome. Previousl y referred to as the “isolation membrane.”
Phagophore assembly site (PAS): A perivacuolar compartment or location that is involved in the formation of Cvt vesicles and autophagosomes in yeast. The PAS may supply membranes during the formation process or may be an organizing center where most of the autophagic machinery resides, at least transiently.
Phosphatidylinositol 3-kinase (PtdIns3K): A family of enzymes that add a phosphate group to the 3' hydroxyls on the inositol ring of phosphoinositides. The class III phosphatidylinositol 3-kinases are stimulatory for autophagy, whereas class I enzymes are inhibitory.
Reticulophagy: A type of selective macroautophagy of the ER. Autophagy counterbalances endoplasmic reticulum expansion during the unfolded protein response. Activation of the UPR in yeast induces reticulophagy.
Ribophagy: The selective autophagic sequestration and degradation of ribosomes. Vacuole: The fungal and plant counterpart of the lysosome; this organelle also carries out storage and osmoregulatory functions. The plants’ bona fide equivalent of the lysosome is the lytic vacuole. Xenophagy: The selective degradation of microbes (e.g., bacteria, fungi, parasites and/or viruses) through an autophagyrelated mechanism.

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