Reactive Oxygen Species: Friends and Foes of Signal Transduction

Journal of Signal Transduction

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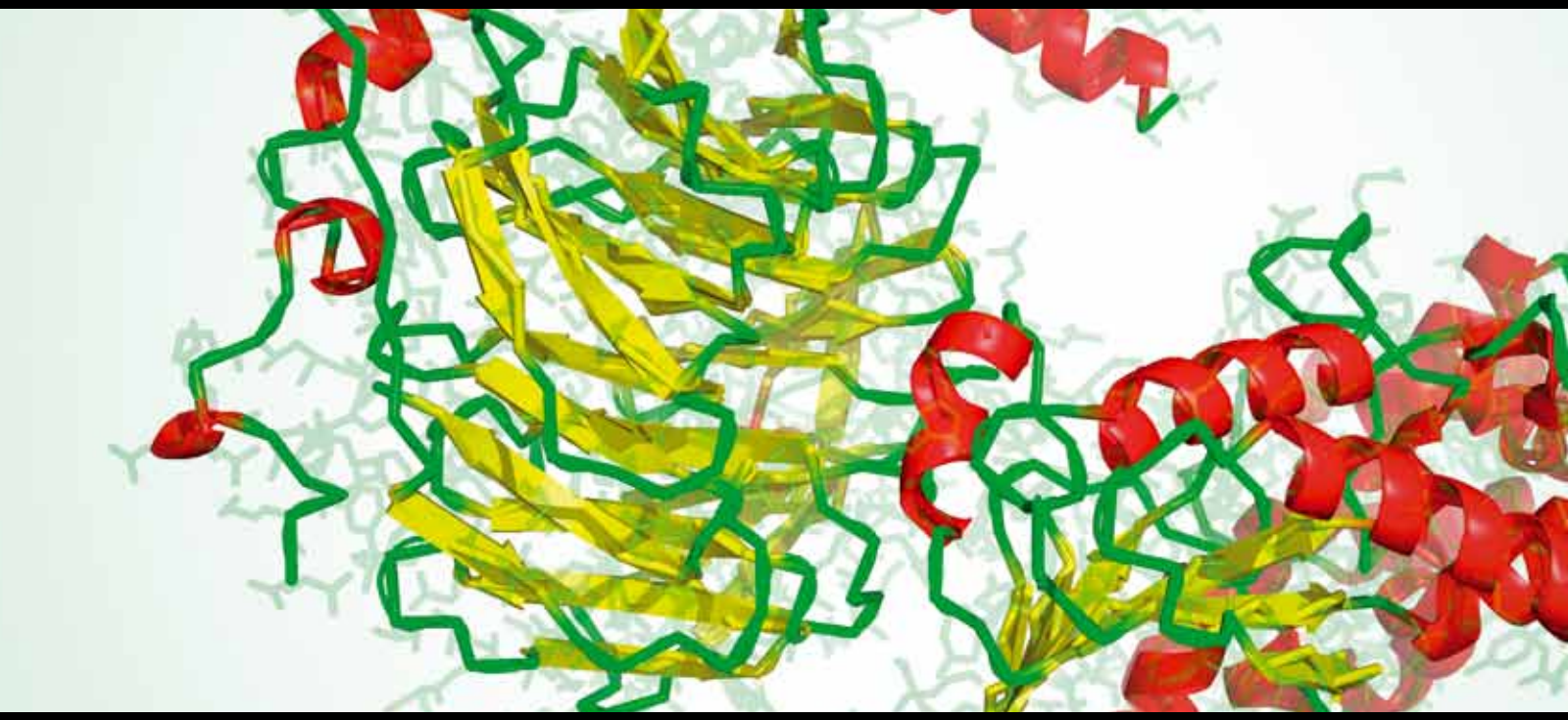
Guest Editors Saverio Francesco Retta, Paola Chiarugi, Lorenza Trabalzini, Paolo Pinton, and Alexey M. Belkin



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Editorial Board

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Editorial

Reactive Oxygen Species: Friends and Foes of Signal Transduction

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The maintenance of highly regulated mechanisms to control intracellular levels of reactive oxygen species (ROS) is essential for normal cellular homeostasis. Indeed, most ROS, including free radicals and peroxides, are produced at low level by normal aerobic metabolism and play an important role in the redox-dependent regulation of many signaling processes. In contrast, excessive accumulation of ROS, resulting from an imbalance between ROS production and scavenging, leads to a condition of oxidative stress that can cause extensive oxidative damage to most cellular components, including proteins, lipids, and DNA, and may have pathophysiological consequences. Remarkably, oxidative stress has been clearly implicated in aging and the pathogenesis of several human diseases, including cardiovascular, metabolic, inflammatory, and neurodegenerative diseases and cancer. Thus, ROS may function as friends or foes of signal transduction depending on specific threshold levels and cell context.

To highlight the important topics in this evolving field the Journal of Signal Transduction presents a special issue on the involvement of ROS in physiological and pathological signal transduction processes from prokaryotes to low and high eukaryotes.

In particular, the topics covered in this special issue include ROS-mediated signaling in bacteria (in the first paper), the mechanisms by which ROS affect *Neurospora crassa* light signal transduction (in the second paper), the interplay between ROS and mitochondria in the control of cell death and aging (in the third and fourth papers) and cancer progression (in the fifth and sixth papers), the role of ROS in nuclear transport (in the seventh paper), the interplay between ROS and Ras GTPases (in the eighth paper), the role of ROS in the crosstalk between integrins and cadherins (in the ninth paper), integrin signaling (in the tenth paper), and skeletal muscle signaling (in the eleventh paper). These articles describe our current understanding of this field. Furthermore, this special issue highlights the importance of gaining a greater understanding of the physiological and pathological role of ROS in the perspective of defining new therapeutic strategies based on redox regulation of signal transduction processes.

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Research Article

Mitochondrial Oxidative Stress due to Complex I Dysfunction Promotes Fibroblast Activation and Melanoma Cell Invasiveness

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Increased ROS (cellular reactive oxygen species) are characteristic of both fibrosis and tumour development. ROS induce the transdifferentiation to myofibroblasts, the activated form of fibroblasts able to promote cancer progression. Here, we report the role of ROS produced in response to dysfunctions of mitochondrial complex I, in fibroblast activation and in tumour progression. We studied human fibroblasts with mitochondrial dysfunctions of complex I, leading to hyperproduction of ROS. We demonstrated that ROS level produced by the mutated fibroblasts correlates with their activation. The increase of ROS in these cells provides a greater ability to remodel the extracellular matrix leading to an increased motility and invasiveness. Furthermore, we evidentiated that in hypoxic conditions these fibroblasts cause HIF-1α stabilization and promote a proinvasive phenotype of human melanoma cells through secretion of cytokines. These data suggest a possible role of deregulated mitochondrial ROS production in fibrosis evolution as well as in cancer progression and invasion.

1. Introduction

Mitochondrial-produced ROS have been recently involved in metastatic dissemination of cancer cells, as shown by Ishikawa et al. These authors described how replacing the endogenous mitochondrial DNA in a weakly metastatic tumour cell line with mitochondrial DNA from a highly metastatic cell line enhanced tumour progression through increased production of ROS and HIF-1α stabilization [1].

Recent studies demonstrate that tumour growth does not depend only on malignant cancer cells themselves but also on the surrounding tumour stroma. Indeed, tumour progression, growth, and spread is strictly dependent on angiogenesis and on cytokines and growth factors secreted by microenvironmental cells [2]. In this context, evidence is increasing that CAFs (cancer-associated fibroblasts) are key determinants in the malignant progression of cancer [3]. These fibroblasts, also commonly referred to as myofibroblasts, are the differentiated form of fibroblast that have acquired contractile and secretory characteristics [4]. They have been initially identified during wound healing [5], but are also present in the reactive tumour stroma, promoting tumour growth and progression [6]. Their role is linked to extracellular matrix deposition and secretion of MMPs (matrix metalloproteinases). Furthermore, activated fibroblasts influence cancer cells through the secretion of growth factors and are able to mediate EMT (epithelial mesenchymal transition) and stemness of tumor cells themselves, supporting their progression and the metastatic process. Transdifferentiation to myofibroblast is dependent on both exposure to MMPs and increased level of cellular ROS [7, 8]. Increased cellular ROS are characteristic of both fibrosis and malignancy. We have recently demonstrated that CAFs induce EMT of prostate cancer cells through a proinflammatory pathway involving COX-2 (cycloxygenase-2), NF-κB (nuclear factor-κB), and HIF-1α [9]. The secretion

of MMPs by CAFs induces a release of ROS in prostate carcinoma cells, which is mandatory for EMT, stemness, and dissemination of metastatic cells.

The aim of the present work is to assess the role of ROS produced in response to mitochondrial dysfunctions in fibroblast activation and in tumour progression.

Analysis of human fibroblasts with genetic dysfunctions of mitochondrial complex I show that ROS level produced by these fibroblasts correlate with their activation, leading to enhanced motility and invasiveness. Furthermore, in hypoxic conditions, we evidentiated that ROS generated by mitochondrial mutations promote a proinvasive phenotype of melanoma cells though HIF-1α stabilization and growth factor secretion.

2. Results

2.1. ROS Produced by Fibroblasts Carrying Mitochondrial Disfunctions Induce Transdifferentiation to Myofibroblasts. Our interest is to assess the role of mitochondrial oxidative stress for stromal fibroblast activation during tumour progression. To this end we used human fibroblasts carrying mitochondrial dysfunctions of complex I. In particular, fibroblasts mutated in the nuclear *NDUFS1* gene encoding for the 75 kDa-FeS protein (NDUFS1 Q522K and NDUFS1 R557X/T595A) of mitochondrial complex I, fibroblasts mutated in the nuclear *NDUFS4* gene encoding for the 18 kDa subunit (NDUFS4 W15X) of mitochondrial complex I, and fibroblasts mutated in the nuclear *PINK1* gene encoding for the PTEN induced Ser/Thr putative kinase1 localized in mitochondria (PINK W437X), in the same patient this mutation coexists with two homoplasmic mtDNA missense mutations in the *ND5* and *ND6* genes coding for two subunits of complex I [10, 11]. As control we used neonatal human dermal fibroblasts (HFY). Previously, it has been shown that mutation in *NDUSF4* gene results in complete suppression of the NADH-ubiquinone oxidoreductase activity of complex I, without any ROS accumulation [12]. The Q522K mutation in the *NDUFS1* gene results in a marked, but not complete, suppression of complex I activity with large accumulation of H_2O_2 and intramitochondrial superoxide ion [12]. Furthermore, it has been shown that the coexistence of the ND5 and ND6 mutations with the PINK1 mutation, contributes to enhanced ROS production by complex I and to a decrease in the Km for NADH [11, 13].

We first detected the superoxide ion production by flow cytometer analysis and confocal microscopy analysis using Mitosox as a redox-sensitive probe. As shown in Figures 1(a) and 1(b), mutations in *NDUFS1* genes and in *PINK* gene are associated with superoxide ion accumulation while *NDUFS4* gene mutation affects only marginally ROS production in agreement with previous data [12]. Recently, it has been demonstrated that the oxidative stress in the tumour stroma promotes the conversion of fibroblasts into myofibroblasts, a contractile and secretory form of fibroblasts [7]. To this purpose we analysed whether also ROS produced by mitochondrial disfunctions could affect the differentiation process of fibroblasts. We analysed the expression level of α-SMA (α-smooth muscle actin), a typical marker of myofibroblast differentiation, by western blot and confocal microscopy analyses (Figures 1(c) and 1(d)). The level of α-SMA in control and mutated fibroblasts correlates with ROS production, in particular, in fibroblasts carrying mutations in the *NDUFS1* gene (Q522K and R557X/T595A) and in the nuclear *PINK* gene (W437X) α-SMA is organized in stress fibers, thereby conferring to the differentiated myofibroblasts a strong contractile activity. Altogether, these data show that ROS produced by fibroblasts carrying mitochondrial genetic disfunctions correlates with their activation.

2.2. Mitochondrial ROS Production Induces an Increase in the Migration and Invasion Abilities of Myofibroblasts. During the conversion of fibroblasts into myofibroblasts, these cells acquire clear contractile and motile properties. To investigate whether fibroblasts carrying mitochondrial disfunctions and showing increased ROS level have modified their behaviour, we evaluated the migration and invasion abilities of these cells by Boyden assays. As shown in Figures 2(a) and 2(b), mutations in *NDUFS1* gene (Q522K and R557X/T595A) and in the nuclear *PINK* gene (W437X) increase both migration and invasion of fibroblasts while mutations in *NDUFS4* gene do not affect the contractile properties of cells, in agreement with their ROS production and α-SMA expression. Hence, we proposed that high mitochondrial generation of ROS converts fibroblasts into myofibroblasts, their activated form, causing an increase in the invasive and migratory abilities of these cells.

2.3. Hypoxic ROS Production in Mutated Fibroblasts Is Associated with HIF-1α Stabilization and Growth Factor Expression. Hypoxic conditions are able to induce a deregulation in mitochondrial ROS production, which control a variety of hypoxic responses, including the activation of HIF-1α transcription factor [14–16]. To this end, we analysed ROS production after culturing fibroblasts under hypoxic conditions (1% O_2). As shown in Figure 3(a), genetic mitochondrial disfunctions result in increased ROS production in hypoxic conditions. This effect is mainly evident for fibroblasts carrying mutations in nuclear *PINK1* gene. Noticeable, also fibroblasts mutated in *NDUFS4* gene show high level of ROS in hypoxic conditions. In agreement, in hypoxic conditions, all mutated fibroblasts show an increase of HIF-1α level (Figure 3(b)). Previous results from other laboratories indicated that the activated stroma secretes large amounts of VEGF-A (vascular endothelial growth factor-A), SDF1 (stromal cell-derived factor-1) and HGF (hepatocyte growth factor) leading to a significant increase in the invasive capacity of surrounding tumor cells [17–19]. In order to verify whether the increased ROS production in mutated fibroblasts correlates with a raise of these soluble growth factors and cytokines, we performed a real-time PCR analysis to quantify VEGF-A, SDF1, and HGF transcripts. As shown in Figures 4(a) and 4(c) mutated fibroblasts cultured in hypoxic conditions have higher level of transcripts for VEGF-A, SDF1, and HGF, acknowledged factors for the modulation of the response of tumour cells to activated fibroblasts.

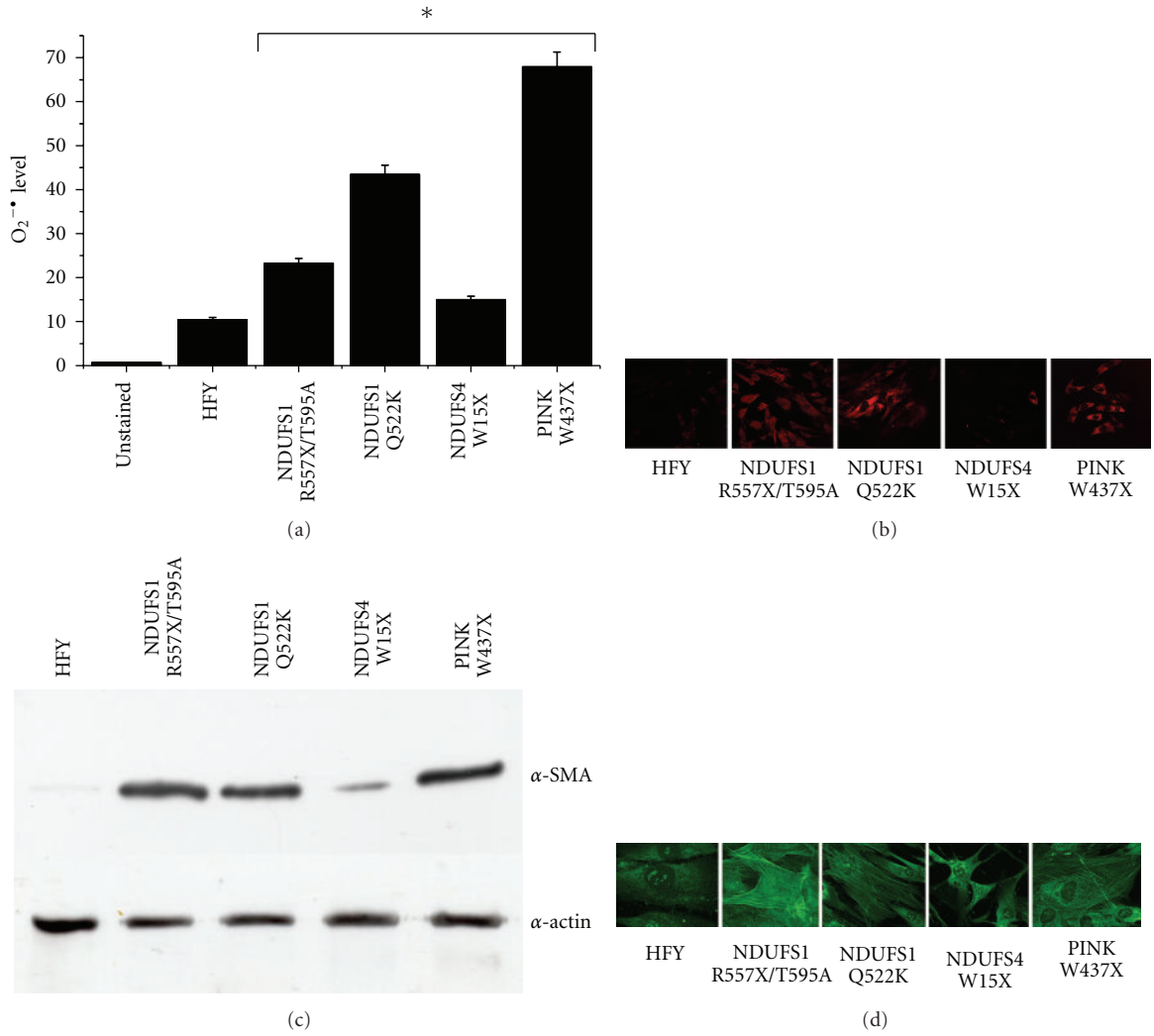
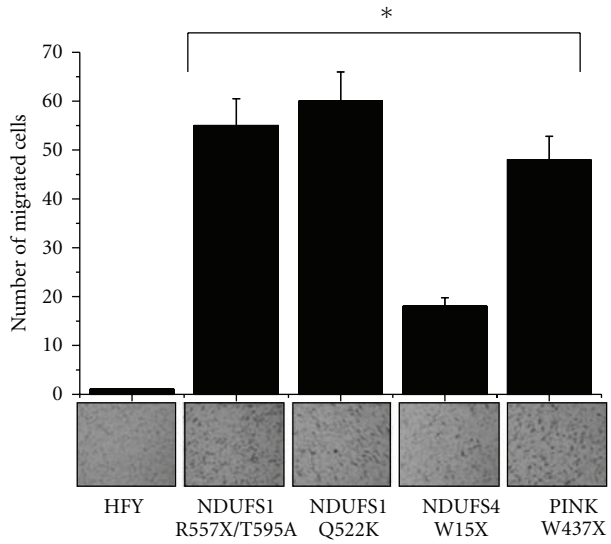


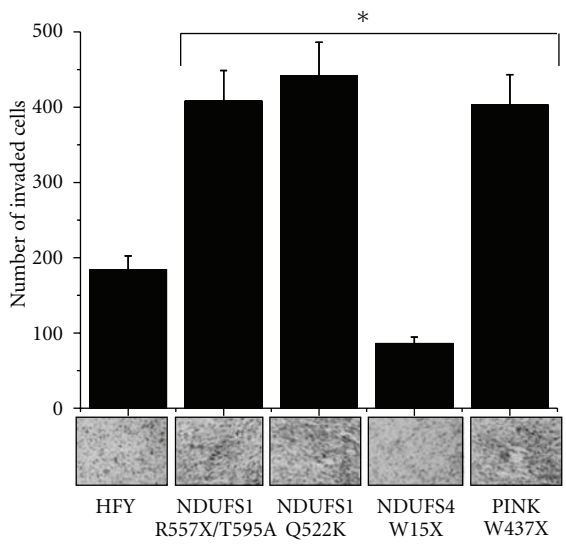
Figure 1: Oxygen superoxide level and α-SMA in fibroblasts carrying mitochondrial dysfunctions of complex I. (a) Flowcytometer analysis of $O_2^{\bullet -}$ level. Control fibroblasts (HFY) and fibroblasts carrying mutations in *NDUFS1* gene (Q522K and R557X/T595A), in *NDUFS4* gene (W15X), and in the nuclear *PINK* gene (W437X) were cultured for 48 hours in low glucose medium and then incubated with 5 mM Mitosox for 10 minutes at 37°C for detection of oxygen superoxide. A flowcytometer analysis is then performed. The results are representative of five experiments with similar results. $^*P < 0.005$ mutated fibroblasts versus control fibroblasts. (b) Fibroblasts seeded on glass coverslips are treated as in (a) and a confocal microscopy analysis is performed. (c) Analysis of α-SMA expression in control fibroblasts (HFY) and fibroblasts carrying mutations. Lysates of cells were subjected to α-SMA immunoblot analysis. An antiactin immunoblot was performed for normalization. (d) Analysis of α-SMA expression in control fibroblasts (HFY) and fibroblasts carrying mutations seeded on glass coverslips by confocal microscopy analysis.

2.4. The Conditioned Media of Mutated Fibroblasts Promotes Melanoma Cells Invasiveness. Recently, it has been demonstrated that high levels of mitochondrial ROS produced by cancer cells are linked to enhanced metastatic potential [1]. To this end we decided to investigate whether mitochondrial ROS derived from stromal components could, as well, influence the behavior of tumor cells. Thus, we analysed the ability of media from mutated fibroblasts cultured under hypoxic conditions to promote metastatic potential of cancer cells, using A375 cells, derived from human primary melanoma. As shown in Figure 5(a), left, while there are no differences in A375 human melanoma cells invasiveness when cultured with media from fibroblasts grown in normoxic condition, media from mutated fibroblasts cultured in hypoxic conditions cause an increase in A375 human melanoma cells invasiveness as examined by Boyden cell invasion assay (Figure 5(a), right).

To verify that the effect exerted by media from mutated fibroblasts on the invasiveness of melanoma cells are effectively due to their ROS production and HIF-1α stabilization, we analyzed HIF-1α level and melanoma cell invasion in the presence of NAC (N-acetyl cysteine), a ROS scavenger. As shown in Figures 5(b) and 5(c), NAC treatment blocks HIF-1α accumulation and reverts the increase of invasiveness of A375 treated with media from mutated fibroblasts cultured in hypoxic conditions. Hence, this evidence underlines the

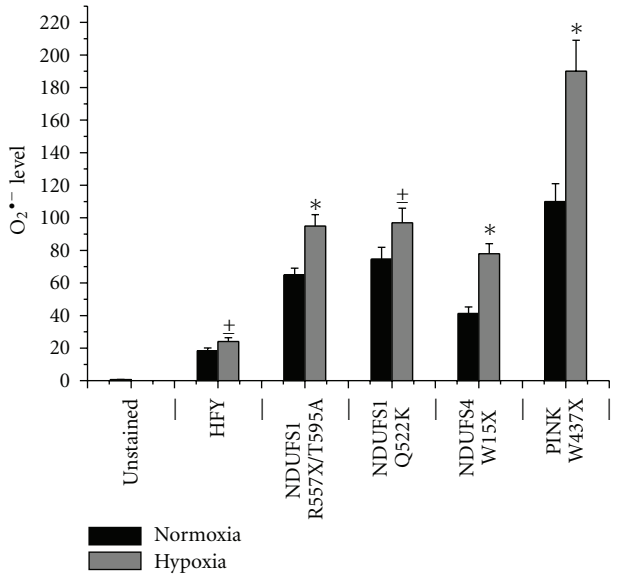


(a) Boyden cell migration assays of fibroblasts carrying mitochondrial dysfunctions of complex I. 15×10^3 fibroblasts, after 24 hours of serum starvation, were seeded into the upper compartment of Boyden chamber. Cells were allowed to migrate through the filter toward the lower compartment filled with complete medium. Cell invasion was evaluated after Diff-Quick staining by counting cell in six randomly chosen fields. The results are representative of four experiments with similar results

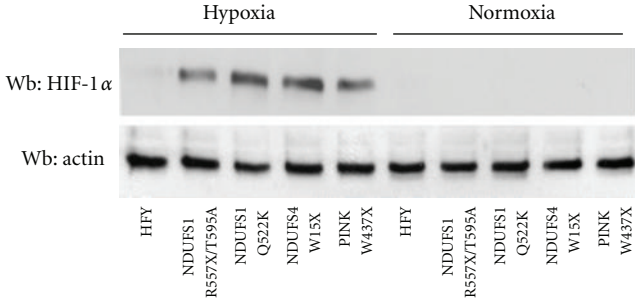


(b) Boyden cell invasion assays of fibroblasts carrying mitochondrial dysfunctions of complex I. Cells were treated as in (a). Cells were allowed to migrate through the filter coated with Matrigel toward the lower compartment filled with complete medium. Cell invasion was evaluated after Diff-Quick staining by counting cell in six randomly chosen fields. The results are representative of four experiments with similar results. $^*P < 0.005$ mutated fibroblasts versus control fibroblasts

Figure 2: Mutations in *NDUFS1* genes (Q522K and R557X/T595A) and in the nuclear *PINK* gene (W437X) increase fibroblasts migration and invasion.



(a) Fibroblasts were cultured in low glucose serum-free medium for 24 hours in normoxic or hypoxic condition (1% O_2) and then incubated with 5 μM Mitosox for 10 minutes at 37°C for detection of oxygen superoxide. A flowcytometer analysis was then performed. The results are representative of three experiments with similar results. $^*P < 0.005$ hypoxic fibroblasts versus normoxic fibroblasts



(b) Lysates of cells treated as in (a) were subjected to HIF-1α immunoblot analysis

Figure 3: Mutated fibroblasts induce HIF-1α stabilization in hypoxic conditions.

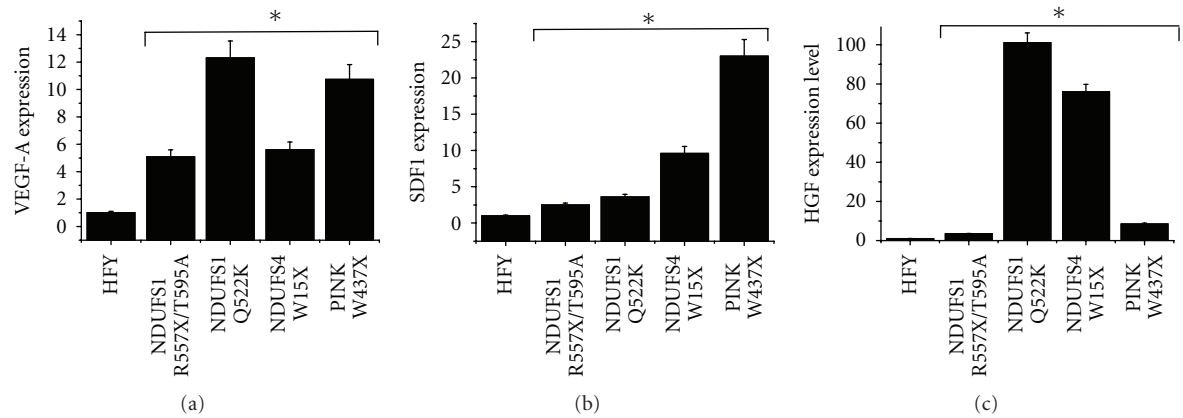


Figure 4: mRNA levels of VEGF-A, SDF-1, and HGF secreted by fibroblasts cultured in hypoxic condition. (a–c) Quantitative real-time reverse transcription PCR of RNA extracted from fibroblasts cultured for 24 hours in low glucose serum-free medium in hypoxic conditions using primers for human *VEGF-A* (a), *SDF-1* (b), and *HGF* (c) and *GAPDH* gene. Results were normalized first to GADPH expression levels and then displayed relative to level in HFY cells. Data are representative of three independent experiments. $^*P < 0.005$ mutated fibroblasts versus control fibroblasts.

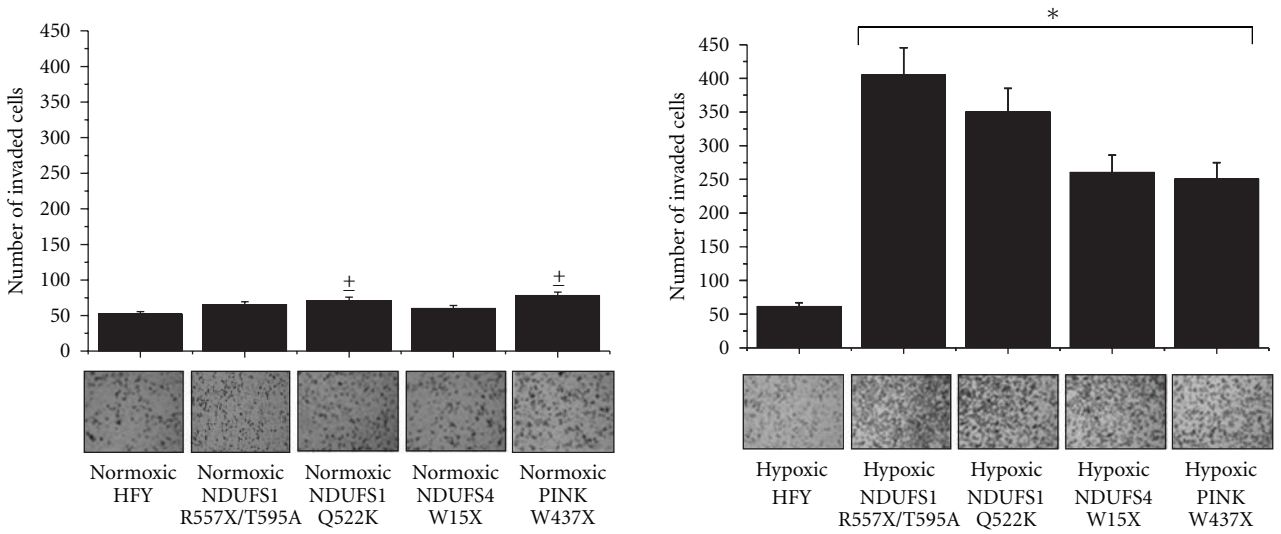
involvement of mitochondrial ROS production in regulating the aggressiveness of melanoma cells, likely modulating the delivery of key cytokines able to affect cancer cell invasiveness.

3. Discussion

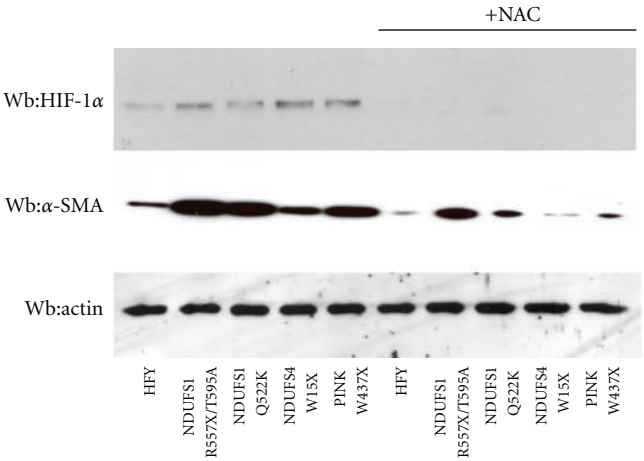
Data reported in this study clearly underscore the central role of mitochondrial ROS in the transdifferentiation of fibroblasts and in the stimulation of a pro-invasive phenotype of melanoma tumour cells.

Recent data unlighted that tumour microenvironment has a key role for the development of a variety of cancers promoting both tumour growth and metastatic dissemination. CAFs are the most common type of cells found in the reactive stroma of several human carcinomas. These activated fibroblasts express α-SMA, leading to the term "myofibroblasts," influence ECM turnover synthesizing both components of ECM itself and ECM-degrading enzymes, release a large amount of cytokines affecting cancer cells progress towards an aggressive phenotype. The origin of myofibroblasts are not fully understood. In culture the transdifferentiation of fibroblasts to myofibroblasts can be achieved by treatment with TGFβ (transforming growth factor β) [20], suggesting that similar pathways might be responsible for generation of myofibroblasts in tumours. Besides, emerging evidence indicates that also EMT, involving normal epithelial cells adjacent to the tumour, is a source of myofibroblasts in both fibrosis and cancer [21]. Furthermore, CAFs may arise directly from carcinoma cells through EMT [3, 7], allowing cancer cells to adopt a mesenchymal cell phenotype, with enhanced migratory capacity and invasiveness [22]. Indeed, mainly in breast cancers, have been reported CAF somatic mutations in *TP53* and *PTEN*, as well as gene copy number alteration at other loci in tumour stroma, [23, 24]. Actually, more recently, Radisky et al. [25], demonstrated that treatment of mouse mammary epithelial cells with MMP3 cause EMT through a pathways involving elevated ROS production and increased levels of Rac1b. The ROS increase in cells treated with MMP-3 was caused by mitochondrial activation and was found to be required for the induction of the mesenchymal vimentin as well as other myofibroblast genes. Thus, a new role for ROS in tumour progression is emerging, in addition to their well-known action in the oxidative damage to DNA. We have recently demonstrated that CAFs secrete MMPs which in turn stimulate ROS production in prostate carcinoma cells via a Rac1b/Cox2/HIF-1α pathway, finally leading to tumour growth and metastatic spreading [9]. Furthermore, Toullec et al., using a model of *junD*-deficient fibroblasts, demonstrated that the constitutive oxidative stress generated by inactivation of the *junD*-gene promotes the conversion of fibroblasts into myofibroblasts in a HIF1α and CXCL12-dependent pathway [19].

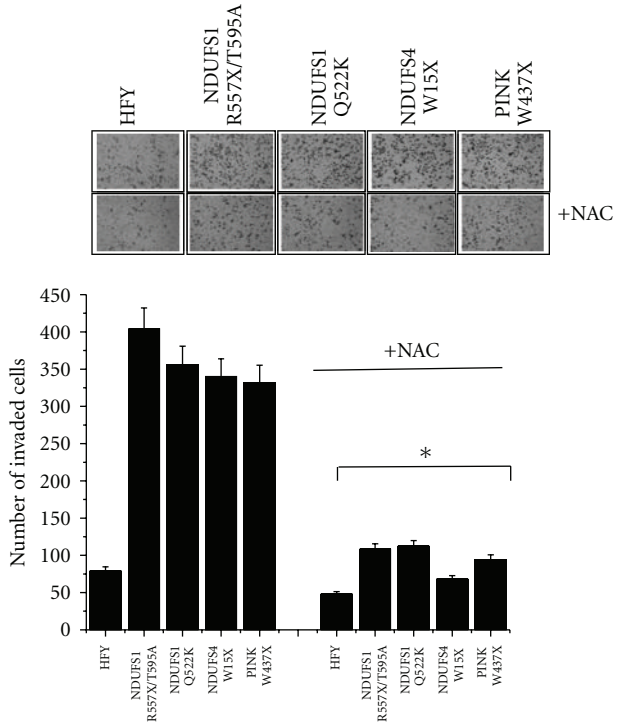
Mitochondria are the major site of ROS generation, which occurs mainly at complexes I and III of the respiratory chain. It is well known that mitochondrial disfunctions and increased ROS levels are present in many cancer cells. Pelicano et al. evidentiated a more invasive behavior of breast cancer cells in which oxidative stress is induced by inhibition of the electron transport complex I with rotenone [26]. A correlation between mutations in mitochondrial genes of complex I and cancer has been observed in various laboratories [1, 27, 28]. Recently, the group of G. Romeo reported, in the thyroid oncocytic cell line XTC.UC1, a dramatically decreased activity of complex I and III associated with ROS increase. Indeed, these defects are due to two mtDNA mutations: a frameshift mutation in the gene encoding ND1 subunit of complex I and a missense substitution in the cytochrome *b* gene, which affects the catalytic site involved in the electron transfer of complex III [29, 30]. Furthermore, mitochondria dysfunctions in a tumour cell line contribute to tumour progression by enhancing the metastatic potential of tumour cells [1]. Indeed, Ishikawa et al. demonstrated, by replacing the



(a) Boyden cell invasion assay. Control and mutated fibroblasts were incubated in low glucose serum-free media in normoxic or hypoxic conditions (1% O_2) for 24 hours. Media are then collected, and monolayers of A375 human melanoma cells were incubated in these conditioned media for 24 hours. 15×10^3A375 melanoma cells were seeded into the upper compartment of Boyden chambers. Cells were allowed to migrate through the filter toward the lower compartment filled with complete medium for 24 hours. Cell invasion was evaluated after Diff-Quick staining by counting cell in six randomly chosen fields. The results are representative of three experiments with similar results $^{\pm}P < 0.05$ media from mutated fibroblasts versus media from control fibroblasts, $^{*}P < 0.005$ media from mutated fibroblasts versus media from control fibroblasts



(b) Lysates of fibroblasts cultured in low glucose serum-free medium for 24 hours in hypoxic condition (1% O_2) with or without 20 mM NAC were subjected to HIF-1α and α-SMA immunoblot analysis



(c) Fibroblasts were cultured as in (b) A375 melanoma cells were then incubated for 24 hours with media collected from fibroblasts. 15×10^3A375 melanoma cells were seeded into the upper compartment of Boyden chambers coated with Matrigel. Cells were allowed to migrate through the filter toward the lower compartment filled with complete medium for 24 hours. Cell invasion was evaluated after Diff-Quick staining by counting cell in six randomly chosen fields. The results are representative of four experiments with similar results. $^{*}P < 0.005$ media from mutated fibroblasts versus media from control fibroblasts

Figure 5: Conditioned media from mutated fibroblasts promotes melanoma cells invasiveness; NAC treatment blocks HIF-1α stabilization and reverts the increase of melanoma cells invasiveness.

endogenous mtDNA in a mouse tumour cell line that was poorly metastatic with mtDNA from a cell line that was highly metastatic and viceversa, that the recipient tumour cells acquired the metastatic potential of the transferred mtDNA. Really, mtDNA containing mutations in the *ND6* (NADH dehydrogenase subunit 6) gene and hence a deficiency in respiratory complex I activity triggers an increase of ROS and higher expression levels of two genes associated with neoangiogenesis, namely, *HIF-1α* and *VEGF*. As already mentioned, mitochondrial alterations in cancer cells have been intensively studied to understand their role in tumour development and progression [31, 32], but, at least to our knowledge, this manuscript, for the first time, evidenced that mitochondrial dysfunctions in stromal cells affect the invasiveness of cancer cells. Indeed, our data show that ROS produced by mitochondrial dysfunction affect not only the migratory and invasive abilities of fibroblasts themselves but also the aggressiveness of melanoma cancer cells. In order to investigate the involvement of mitochondrial ROS derived from stromal cells in modulating the invasiveness of tumour cells, we used fibroblasts producing elevated mitochondrial ROS due to defects in NADH: ubiquinone oxidoreductase, or complex I. Dysfunctions of this complex cover more than 30% of hereditary mitochondrial encephalopathies [33–35]; furthermore, complex I defects have been also found in familiar Parkinson disease [11, 13], hereditary spastic paraplegia [36], Friedreich ataxia [37], and aging [38, 39]. We used fibroblasts from a baby diagnosed for Leigh syndrome (NDUFS4 W15X), from a baby diagnosed for leukodystrophy (NDUFS1 Q522K), from a child with complex I deficit (NDUFS1 R557X/T595A), and from a patient with familiar Parkinsons disease (PINK W437X) in which mutations in the *ND5* and *ND6* mitochondrial genes of complex I coexist with mutation in the nuclear phosphatase and tensin homolog- (PTEN-) induced serine/threonine putative kinase-1 (*PINK-1*) gene. We showed that in normoxic conditions all mutations present in these fibroblasts, with the exception of the mutation in the gene *NDUFS4*, produce an increase in ROS level, which correlates with the levels of α-SMA and hence with the degree of differentiation towards the myofibroblast-activated form. This differentiation represents also a key event during wound healing and tissue repair [4]. The deregulation of normal healing and continued exposure to chronic injury results in tissue fibrosis, massive deposition of ECM, scar formation, and organ failure. Indeed, oxidative stress, caused by increase in ROS is closely associated with fibrosis [40] and inhibitors of ROS have shown promise in clinical trials targeting this disease [41–43]. Herein, we demonstrate that mutated fibroblasts have a great ability to increase their motility and invasiveness. Overall, these findings indicate that increased mitochondrial ROS induce the transdifferentiation of fibroblasts to their activated form suggesting a possible involvement of these mutated fibroblast also in fibrogenic events.

The inappropriate induction of myofibroblasts that leads to organ fibrosis greatly enhances the risk of subsequent cancer development, by creating a stimulating microenvironment for epithelial tumor cells. Really, we show that exposure of melanoma cells to media of fibroblasts with mitochondrial dysfunctions cultured in hypoxic conditions promotes invasiveness of tumour cells. It is well known that in hypoxic condition, the low oxygen tension increases the generation of mitochondrial ROS that prevent hydroxylation of HIF1α protein, thus resulting in stabilization and activation of its transcriptional activity [14, 15, 44]. Recently, for instance, Klimova et al. demonstrated that ROS generated by mitochondrial complex III are required for the hypoxic activation of HIF1α [16]. Furthermore, Brunelle et al. showed that fibroblasts from a patient with Leigh's syndrome, which display residual levels of electron transport activity, stabilize HIF1α during hypoxia [45]. Really, we evidentiated in hypoxic conditions an extra ROS production in fibroblasts carrying mutations in mitochondrial complex I; this increase in ROS production is also evident in fibroblasts mutated in the *NDUFS4* gene that, conversely, have a low level of ROS in normoxic condition. The extra ROS production in hypoxic condition is associated with HIF1α stabilization. This stabilization is really dependent on mitochondrial mutations since it is absent in control fibroblasts and finally leads to the transcriptional activation of genes that allow cells to adapt to and survive in the hypoxic environment. We believe that this extra ROS production acts synergistically with the hypoxic condition in the promotion of a pro-invasive behavior of melanoma cancer cells. Neutralizing this extra ROS production with antioxidants allows the degradation of HIF1α and abolishes the aggressive behavior of melanoma cancer cells. Altogether we evidentiated that mitochondrial ROS produced by complex I defects of stromal components, namely, activated fibroblasts, are key molecules able to modulate the behavior of surrounding cancer cells, increasing their aggressiveness.

These data underline once again the close loop between tumor cells and stromal counterparts. It is well known that activated fibroblasts influence, through the secretion of soluble factors, the adhesive and migratory properties of cancer cells, which then in turn release cytokines influencing the behavior of stromal cells. Thus, to deeply explore the effect of mutated fibroblasts on surrounding tumour cells we investigated the transcriptional levels of some HIF1α target genes in fibroblasts cultured in hypoxic conditions. We observed an increase in VEGF-A, HGF, and SDF1 transcripts. These data are in keeping with those obtained by Orimo et al. [46], demonstrating that the coinjection of tumour cells with CAFs into nude mice generates larger xenografts with respect to those generated with normal fibroblasts. This event correlates with an increase in both cancer-cell proliferation and angiogenesis through the SDF-1 secretion. In agreement, Toullec et al. demonstrated that the same cytokine causes the conversion of fibroblasts into highly migrating myofibroblasts and subsequently promotes migration and dissemination of neoplastic cells [19]. Furthermore, Cat et al. showed that myofibroblasts secrete large amounts of HGF and VEGF resulting in a significant increase in the invasive capacity of surrounding tumor cells [17]. Besides, clinical studies show that the abundance of stromal myofibroblast is associated with disease recurrence, as shown for human colorectal cancers [47]. Really, our data, showing an increase in fibroblast secreted VEGF-A, HGF, and SDF1, are in

keeping with others emphasizing that tumour dissemination could be facilitated by the myofibroblastic component of the stroma through the secretion of invasion associated-secreted factors.

Altogether our findings suggest a possible role of ROS production, due to mitochondrial complex I dysfunctions of stroma, in fibroblast activation as well as in cancer progression and invasion.

4. Materials and Methods

4.1. Materials. Unless specified, all reagents were obtained from Sigma. Antibodies anti-HIF-1α were from BD Transduction Laboratories; antibodies anti-α-SMA were from Sigma; antibodies anti-actin were from Santa Cruz Biotechnology; MitoSOX Red mitochondrial superoxide indicator was from Molecular Probes.

4.2. Cell Culture. Control fibroblasts (neonatal human dermal fibroblasts) were from Cambrex. Fibroblasts carrying mitochondrial mutations in the nuclear *NDUFS4* gene-W15X and in the *NDUFS1* gene-Q522K (from M. Zeviani, C. Besta Neurological Institute Foundation, Milan) were cultivated and characterized by S. Scacco. Fibroblasts carrying mutation in the nuclear *PINK1* gene-W437X (from G. De Michele, Department of Neurological Sciences, Federico II University, Naples) were cultivated and characterized by A. M. Sardanelli. Fibroblasts carrying mitochondrial mutations in the nuclear *NDUFS1* gene (NDUFS1 R557X/T595A) were a generous gift from Fondazione Giuseppe Tomasello O.N.L.U.S. A375 human melanoma cells were from ATCC. Cells were cultured in DMEM supplemented with 10% fetal bovine serum and maintained in 5% CO_2 humidified atmosphere.

4.3. Mitochondrial Superoxide Detection. 25×10^3 cells were cultured for 48 hours in low glucose DMEM medium (5 mM glucose). Cells were then incubated for 10 minutes at 37°C with 5 μM MitoSox in PBS. Cells are trypsinized, centrifuged, washed with PBS, and resuspended in 300 μL PBS. A flowcytometer analysis was then performed (MitoSox excitation/emission: 510/580 nm). For the confocal microscope analysis of $O_2^{\bullet -}$ level, cells were seeded onto coverslips, incubated for 10 minutes at 37°C with 5 μM MitoSox in PBS, washed with PBS, and analysed with a laser scanning confocal microscope (model LEICA TCS SP2 with Acusto-Optic Beam Splitter) equipped with a five-lines Ar laser and two He/Ne lasers (lines 543 and 633 nm).

4.4. Western Blot Analysis. 1×10^6 cells were lysed for 20 minutes on ice in 500 μL of complete radioimmunoprecipitation assay (RIPA) lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP40, 2 mM EGTA, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin). Lysates were clarified by centrifuging, separated by SDS-PAGE, and transferred onto nitrocellulose. The immunoblots were incubated in 3% bovine serum albumin, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 0.1% Tween 20 for 1 hour at room temperature and were probed first with specific antibodies and then with secondary antibodies.

4.5. Immunohistochemistry. Fibroblasts were seeded onto coverslips, washed with PBS, and fixed in 3% paraformaldehyde for 20 minutes at 4°C. Fixed cells were permeabilized with three washes with TBST (50 mM Tris/HCl pH 7.4, 150 mM NaCl, 0.1% Triton X-100) and then blocked with 5.5% horse serum in TBST for 1 hour at room temperature. Cells were incubated with primary antibody, 1 : 100 dilution in TBS (50 mM Tris/HCl pH 7.4, 150 mM NaCl) containing 3% BSA overnight at 4°C. After extensive washes in TBST, cells were incubated with secondary antibodies for 1 hour at room temperature, washed, and mounted with glycerol plastine. Finally, cells were observed under a laser scanning confocal microscope (model LEICA TCS SP2 with Acusto-Optic Beam Splitter) equipped with a five-lines Ar laser and two He/Ne lasers (lines 543 and 633 nm).

4.6. Preparation of Conditioned Media. Conditioned media were obtained from fibroblasts as follow: fibroblasts were incubated in low glucose (5 mM glucose) serum-free media in normoxic or hypoxic conditions (1% O_2) for 24 hours. Media are then collected and monolayers of A375 human melanoma cells were incubated in these conditioned media for 24 hours.

4.7. In Vitro Boyden Migration and Invasion Assay. Fibroblasts were serum starved for 24 hours and then 15×10^3 cells were seeded onto Boyden chamber (8 mm pore size, 6.5 mm diameter) for the migration assay. For the invasion assay Boyden chambers are precoated with Matrigel (12.5 μg Matrigel/filter). In the lower chamber, complete medium was added as chemoattractant. Following 24 hours of incubation, the inserts were removed and the noninvading cells on the upper surface were removed with a cotton swab. The filters were then stained using the Diff-Quik kit (BD Biosciences) and photographs of randomly chosen fields are taken.

4.8. Real-Time PCR. Total RNA from fibroblasts was extracted using RNeasy (Qiagen) according to the manufacturer instructions. Strands of cDNA were synthesized using a high-capacity cDNA reverse transcription kit (Applied Biosystem) using 1 μg of total RNA. For quantification of VEGF-A, SDF-1, and HGF mRNA, real-time PCR, using Power SYBR green dye (Applied Biosystem) was done on a 7500 fast real-time PCR system (Applied Biosystem). The primers for VEGF-A were 5′TTCTGCTGTCTTGGG TGCAT-3′ (forward) and 5′TGTCCACCAGGGTCTCGATT-3′ (reverse). The primers for SDF-1 were 5′GTGTCACTG GCGACACGTAG-3′ (forward) and 5′TCCCATCCCACA GAGAGAAG-3′ (reverse). The primers for HGF were 5′CAT CAAATGTCAGCCCTGGAGTT-3′ (forward) and 5′CCT GTAGGTCTTTACCCCGATAGC-3′ (reverse). Data are normalised to those obtained with glyceraldehyde-3-phosphate dehydrogenase primers. Results (mean ± SD) are the mean of three different experiments.

Abbreviations

α-SMA: α-smooth muscle actin
CAF: Cancer associated fibroblast
ECM: Extracellular matrix
EMT: Ephitelial mesenchymal transition
HGF: Hepatocyte growth factor
HIF-1α: Hypoxia inducible factor-1α
MMP: Matrix metalloproteinase
NAC: N-acetyl cysteine
ROS: Reactive oxygen species
SDF1: Stromal cell-derived factor-1
VEGF: Vascular endothelial growth factor.

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Research Article

Redox Regulation of Nonmuscle Myosin Heavy Chain during Integrin Engagement

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On the basis of our findings reporting that cell adhesion induces the generation of reactive oxygen species (ROS) after integrin engagement, we were interested in identifying redox-regulated proteins during this process. Mass spectrometry analysis led us to identify nonmuscle myosin heavy chain (nmMHC) as a target of ROS. Our results show that, while nmMHC is reduced in detached/rounded cells, it turns towards an oxidized state in adherent/spread cells due to the integrin-engaged ROS machinery. The functional role of nmMHC redox regulation is suggested by the redox sensitivity of its association with actin, suggesting a role of nmMHC oxidation in cytoskeleton movement. Analysis of muscle MHC (mMHC) redox state during muscle differentiation, a process linked to a great and stable decrease of ROS content, shows that the protein does not undergo a redox control. Hence, we propose that the redox regulation of MHC in nonprofessional muscle cells is mandatory for actin binding during dynamic cytoskeleton rearrangement, but it is dispensable for static and highly organized cytoskeletal contractile architecture in differentiating myotubes.

1. Introduction

Studies over the past years have shown that reactive oxygen species (ROS) are involved in a diverse array of biological processes, including normal cell growth, induction and maintenance of cell transformation, programmed cell death, and cellular senescence. ROS are able to trigger such divergent responses probably through differences in the level and duration of the oxidant burst or in the cellular context accompanying oxidative stress. ROS include a variety of partially reduced oxygen metabolites (e.g., superoxide anions, hydrogen peroxide, and hydroxyl radicals) with a higher reactivity with respect to molecular oxygen. Oxidants can either be produced within cells by dysfunction of mitochondrial respiratory chain complexes or by cytosolic or membrane recruited enzymes such as NADPH oxidase, cyclooxygenases, lipoxygenases, and the NO synthase [1].

Oxidants have been proposed as intracellular messengers of a variety of physiological stimuli acting on cytosolic oxidases. Considerable progress has been made in identifying intracellular targets of ROS. Several findings support the idea that exogenous ROS or oxidants produced by activation of growth factors receptors or integrins can reversibly oxidize and hence inactivate redox-sensitive proteins. Proteins with low-pK_a cysteine residues, which are vulnerable to oxidation by hydrogen peroxide, include several transcription factors, such as the nuclear factor κ-B [2], activator protein 1 [3], hypoxia-inducible factor [4], p53 [5], the p21Ras family of proto-oncogenes [6], phosphotyrosine phosphatases (PTPs) [7], and src kinase family [8]. Our findings provide evidence that intracellular ROS are generated following integrin engagement and that these oxidant intermediates are necessary for integrin signalling during fibroblasts adhesion and spreading [9]. ROS production in response to integrin engagement represents signalling integration point between extracellular matrix (ECM) and growth factor signalling, and they are produced in Rac1 and 5- lipoxygenase- (5-LOX-) dependent manner [9]. A key role in the cytoskeleton redox regulation is played by a low molecular weight-phosphotyrosine phosphatase (LMW-PTP), which is oxidised/inhibited in response to ECM contact. Its inactivation prevents the dephosphorylation of two key regulators of cytoskeleton dynamics: the focal adhesion kinase (FAK) [9] and a GTPase activating protein for the GTPase RhoA (p190RhoGAP) [10]. Accordingly, the redox-dependent activation of FAK and p190RhoGAP leads

to focal adhesion formation, membrane ruffles development, and cell spreading [9, 11, 12]. Hence, both the small GTPases Rac1 and RhoA are critical regulators of redox-mediated actin cytoskeleton remodelling during cell spreading and migration.

Significantly, the key role of ROS in integrin signalling suggests that they may contribute to malignant growth and invasiveness through a deregulation of cell/matrix interaction and cell motility. We are now interested in identifying, among cytoskeleton proteins, the molecular targets of ROS in anchorage-dependent growth. To date, specific targets of integrin-generated ROS are LMW-PTP and SH2-PTP [9, 13], the tyrosine kinase Src [8, 9] and actin [9, 14, 15]. Oxidation of these proteins produces differential effects: (i) LMW-PTP and SH-PTP2 are inactivated through formation of an intramolecular disulfide [9, 13]; (ii) Src family kinases are conversely activated through a disulfide which blocks the protein in active state [8]; (iii) β-actin is oxidized through glutathionylation in a single sensitive cysteine, thus leading to increased polymerization and stress fiber formation [15].

We report herein that nonmuscle myosin heavy chain (nmMHC) is oxidized in the early stage of integrin-mediated adhesion in human fibroblasts. This redox control retains a functional role during cytoskeleton dynamic rearrangements in response to ECM contact, strongly affecting nmMHC binding of β-actin. Conversely, during stable and static cytoskeletal organization in contractile myotubes, the association of muscle MHC (mMHC) with actin is redox independent, suggesting a selective role of redox regulation of these proteins only during dynamic rearrangements of cytoskeleton.

2. Materials and Methods

2.1. Materials. Unless specified, all reagents were obtained from Sigma-Aldrich. Human fibroblasts were obtained as already described [16]. Anti-pan-actin and antimuscle Myosin Heavy Chain antibodies were from Santa Cruz Biotechnology, Nonmuscle myosin heavy chain antibodies were from Biomedical Technologies Inc. Anti-GSH antibodies were obtained from Virogen. N-(biotinoyl)-N′-(iodoacetyl)ethylene diamine (BIAM) was obtained from Molecular Probes. The streptavidin-horseradish (Strp-HRP) conjugate was from Biorad. Immunopure immobilized streptavidin was from Pierce Biotechnology. Blotting grade blocker nonfat dry milk was from Biorad. Secondary antibodies were from Amersham Bioscience. Sequencing grade modified trypsin was from Promega.

2.2. Cell Culture. Human fibroblasts were obtained as previously described [17]. Human fibroblasts and murine myoblasts C2C12 were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum at 37°C in a 5% CO_2 humidified atmosphere. To induce myogenic differentiation, C2C12 cells were grown until subconfluence and then cultured for six days in differentiating medium containing DMEM supplemented with 2% horse serum.

2.3. Cell Adhesion Assay. Human fibroblasts were serum deprived for 24 h and then detached with 0.25% trypsin for 1′. Trypsin digestion was then blocked by the use of 0.5 mg/mL soybean trypsin inhibitor. Cells were then centrifuged and diluted in fresh culture media and incubated for 30 minutes in gentle agitation at 37°C. The adherent/spread sample was obtained plated the cells on fibronectin-coated dishes for 45 minutes while the detached/cell rounded sample was plated for the same time on polylysine-coated plates. Nordihydroguaiaretic acid (NDGA) was added to the cells at the beginning of the suspension phase at a final concentration of 10 μM.

2.4. Intracellular H_2O_2 Assay. For the measure of ROS generation during adhesion, cells were serum deprived for 24 h, detached and incubated in gentle agitation for 30 minutes. Cells were then plated on fibronectin-coated dishes in serum-free medium, with or without NDGA 10 μM, and ROS assay was performed at different times of adhesion. Three minutes before the end, 5 μM DCF-DA was added. Cells were lysed in 1 mL of RIPA buffer containing 1% Triton X-100 and fluorescence was analysed immediately using a Perkin Elmer Fluorescence Spectrophotometer (excitation wavelength 488 nm, emission wavelength 510 nm). The values of fluorescence were normalized on the proteins content. The assay of ROS production in myoblasts and in six days differentiating myotubes were performed using the same protocol.

2.5. In Vivo BIAM Labelling of Proteins. Cells from adherent/spread and detached/rounded conditions are lysates in RIPA buffer (50 mM Tris-HCl, pH 7,5, 150 mM NaCl, 1% Triton, 2 mM EGTA) supplemented with BIAM (100 μM final concentration) and protease inhibitors cocktail (1 mM AEBSF, 8 μM aprotinin, 20 μM leupeptin, 40 μM bestatin, 15 μM pepstatin A, and 14 μM E-64). Lysates were then maintained on ice for 15 minutes and then centrifuge at 13000 rpm for 15′. For the binding of BIAM-labelled proteins with immobilized streptavidin, 30 μL of resin were added to the clarified samples and maintained overnight at 4°C in gentle agitation [12]. The resin was firstly washed four times with RIPA buffer and then resuspended in Laemmli sample buffer. The pattern of BIAM-labelled proteins were visualized by a Western blot analysis using horseradish peroxidase-streptavidin conjugate, washed and developed with the enhanced chemiluminescence kit.

2.6. Matrix Assisted Laser Desorption Ionization-Time of Light (MALDI-TOF) Sample Preparation. BIAM-labelled lysates from spread and rounded cells were run on SDS-PAGE. The gel was then stained by Coomassie blue solution, subjected to destaining solution for 24 h, and finally washed in water until completely equilibrated. The bands of interest were excised, transferred to an Eppendorf tube, and then washed twice with 50 mM NH_4HCO_3/acetonitrile (1 : 1), and they were shrunk with acetonitrile. After drying, samples were subjected to a reduction reaction in a buffer containing 10 mM dithiothreitol, 25 mM NH_4HCO_3 for 45 minutes at

56°C followed by an alkylation step in a buffer containing 55 mM Iodoacetic acid, 25 mM NH_4HCO_3 for 30 minutes at room temperature in the dark. After a final washing step, samples were dried up and trypsin digested for 24 h at 37°C. The peptides were then extracted from gel bands by sonification and by supplementing 50% acetonitrile and 1% trifluoroacetic acid (1 : 1 proportion with sample), and the supernatants were recovered and then dried.

2.7. MALDI-TOF Analysis. Spectrometric analysis were conducted on an Ultraflex MALDI-TOF (Bruker Daltonics) using a Scout ion source and operating in positive reflector mode. Samples were mixed with a-Cyano-4-hydroxycinnamic acid (1 : 1). 0.8 pmol/uL of sample were deposed with the dry droplet technique on an AnchorChip target. Peptides were identified within an error of 120 part per million. Mascot search algorithm parameters was set as following: carboxylation of cysteine and oxidation of methionine.

2.8. Immunoprecipitation and Western Blot Analysis. Immunoprecipitation was performed overnight using 2 μg/mL of specific antibodies. Immunocomplexes were collected on protein A-Sepharose, separated by SDS-poly-acrylamide gel electrophoresis, and transferred onto PVDF membrane. Immunoblots were probed firstly with specific antibodies in 2% nonfat dry milk, 0,05% Tween 20 in phosphate buffered saline buffer, and then with secondary antibodies conjugated with horseradish peroxidase, washed, and developed with the enhanced chemiluminescence kit.

2.9. Statistical Analysis. Data are presented as means ± S.D from at least three experiments. Analysis of densitometry was performed using Quantity One Software (Bio-Rad). Statistical analysis of the data was performed by Student's t-test. P values ≤ 0.05 were considered statistically significant.

3. Results and Discussion

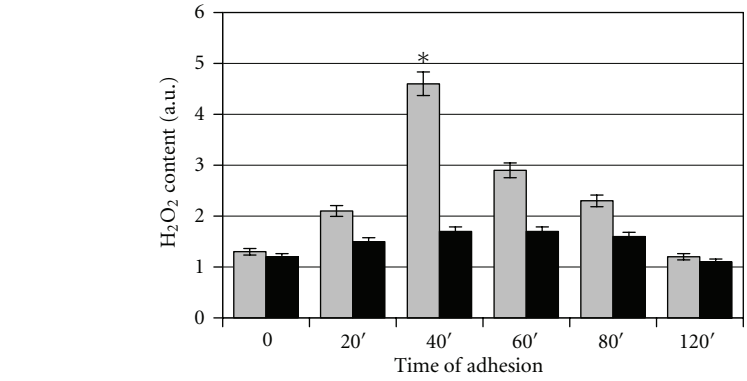
3.1. Redox Regulation of Nonmuscle MHC during Cell Adhesion. Firstly, we found that the engagement of integrin during cell adhesion induces in human fibroblasts a transient burst of ROS production (with a peak 40 minutes after fibronectin attachment) in keeping with what observed during spreading and adhesion of NIH-3T3 murine fibroblasts [9]. ROS burst was significantly inhibited by the use of NDGA which affects the activity of 5-LOX, thus confirming 5-LOX as the source of ROS production (Figure 1(a)) [9]. To study redox-regulated proteins during integrin-mediated cell adhesion, we used the BIAM-labelling technique. BIAM is a sulfhydryl-modifying reagent that selectively probes the thiolate form of cysteine residues, as already reported by Kim et al. [18]. Human fibroblasts were serum deprived for 24 h, detached, and maintained in suspension for 30 minutes to eliminate integrin signalling. For adherent/spread conditions, cells were left to adhere for 45 minutes on fibronectin-coated plates, while detached/rounded cells were seeded on polylysine-coated dishes. The analysis of redox-regulated proteins during integrin engagement shows the presence of a major band of about 200 KDa, differently labelled with BIAM in rounded and spread cells, suggesting a redox regulation of this protein during cell adhesion (Figure 1(b)). We repeated the experiment described above, performing a Coomassie staining of the SDS-PAGE gel, and the BIAM labelled band was excised and used for MALDI-TOF analysis. After trypsin digestion, the peptide fragments were probed on a MALDI-TOF mass spectrometer, and the fingerprint data were then submitted to the Mascot search algorithm. Figure 1(c) shows the analysis of the spectrum of the digested peptides that identify the protein as non-muscle myosin heavy chain (nmMHC) with a score of 107 (Figure 1(d)).

3.2. The Redox State of nmMHC Affects β-Actin Association. Firstly, we confirmed the data obtained by MALDI-TOF analysis by nmMHC immunoprecipitation. Results indicated that nmMHC is reduced in rounded cells and turns towards the oxidized form in spread cells. Furthermore, the treatment of the cells with NDGA, which abrogates ROS production by 5-LOX, rescues the reduced form of nmMHC (Figure 2(a)). In the same experimental setting, we found a redox regulation of β-actin, which became oxidised in spread cells through the binding with glutathione (Figure 2(b)).

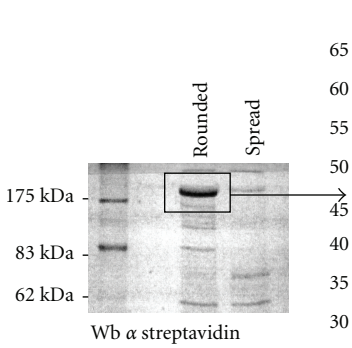
Myosin is the main motor protein of the cell and carries on this function through its binding with β-actin. The role of this association is the generation of the force responsible for cellular dynamic functions such as locomotion, cell division, and cytoplasmic contraction [19]. Therefore, we investigated whether the nmMHC oxidation, upon complete cell spreading, influences its association with β-actin. Results clearly show a redox sensitivity of the binding of nmMHC to β-actin. Indeed, treatment of spread fibroblasts with the 5-LOX inhibitor NDGA rescues the lack of binding between β-actin and nmMHC observed upon completion of the spreading process (Figure 2(c)).

In addition to other redox-regulated proteins of cytoskeleton, such as actin and profilin [15, 20, 21], our data add nmMHC as a new cytoskeletal protein undergoing redox regulation during spreading of human fibroblasts.

3.3. Redox State and mMHC/Actin Association in Differentiating Myotubes. In agreement with previous results [22], we observed that differentiation of murine myoblasts C2C12 is associated with a decrease of ROS content (Figure 3(a)). On the basis of these results, we speculated that muscle MHC (mMHC) might not be redox regulated in resting conditions, that is, when cytoskeleton does not undergo rearrangements due to the movement or spreading onto ECM. Therefore, we compared MHC redox state in spread or rounded fibroblasts and in differentiating myotubes. The results clearly showed that MHC in differentiating myotubes is mainly in its reduced form, while in spread fibroblasts MHC undergoes redox control (Figure 3(b)).



(a)



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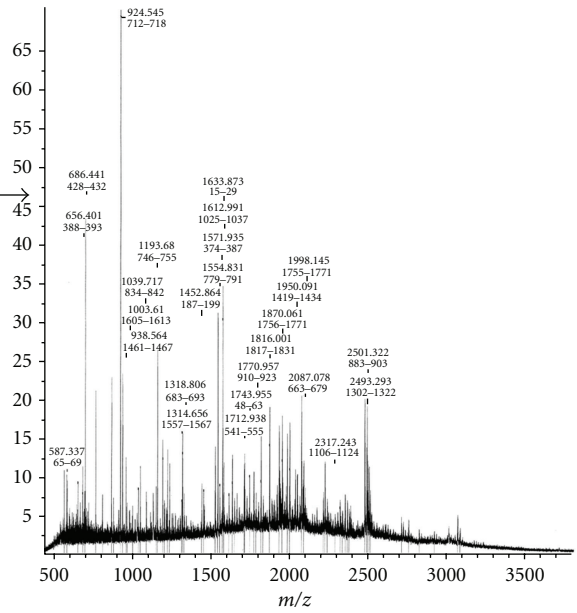

(c)

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111 YTYSGLFCVV INPYKNLPIY SEEIVEMYKG KKRHEMPPHI YAITDTAYRS MMQDREDQSI LCTGESGAGK TENTKKVIQY LAHVASSHKS KKDQGELERQ LLQANPILEA
221 FGNAKTVKND NSSRFGKFIR INFDVNGYIV GANIETYLLE KSRAIRQAKE ERTFHIFYYL LSGAGEHLKT DLLLEPYNKY RFLSNGHVTI PGQQDKDMFQ ETMEAMRIMG
331 IPEDEQMGLL RVISGVLQLG NIAFKKERNT DQASMPDNTA AQKVSHLLGI NVTDFTRGIL TPRIKVGRDY VQKAQTKEQA DFAIEALAKA TYERMFRWLV LRINKALDKT
441 KRQGASFIGI LDIAGFEIFD LNSFEQLCIN YTNEKLQQLF NHTMFILEQE EYQREGIEWN FIDFGLDLQP CIDLIEKPAG PPGILALLDE ECWFPKATDK SFVEKVVQEQ
551 GTHPKFQKPK QLKDKADFCI IHYAGKVDYK ADEWLMKNMD PLNDNIATLL HQSSDKFVSE LWKDVDRIIG LDQVAGMSET ALPGAFKTRK GMFRTVGQLY KEQLAKLMAT
661 LRNTNPNFVR CIIPNHEKKA GKLDPHLVLD QLRCNGVLEG IRICRQGFPN RVVFQEFRQR YEILTPNSIP KGFMDGKQAC VLMIKALELD SNLYRIGQSK VFFRAGVLAH
771 LEEERDLKIT DVIIGFQACC RGYLARKAFA KRQQQLTAMK VLQRNCAAYL RLRNWQWWRL FTKVKPLLNS IRHEDELLAK EAELTKVREK HLAAENRLTE METMQSQLMA
881 EKLQLQEQLQ AETELCAEAE ELRARLTAKK QELEEICHDL EARVEEEEER CQYLQAEKKK MQQNIQELEE QLEEEESARQ KLQLEKVTTE AKLKKLEEDQ IIMEDQNCKL
991 AKEKKLLEDR VAEFTTNLME EEEKSKSLAK LKNKHEAMIT DLEERLRREE KQRQELEKTR RKLEGDSTDL SDQIAELQAQ IAELKMQLAK KEEELQAALA RVEEEAAQKN
1101 MALKKIRELE TQISELQEDL ESERASRNKA EKQKRDLGEE LEALKTELED TLDSTAAQQE LRSKREQEVS ILKKTLEDEA KTHEAQIQEM RQKHSQAVEE LADQLEQTKR
1211 VKATLEKAKQ TLENERGELA NEVKALLQGK GDSEHKRKKV EAQLQELQVK FSEGERVRTE LADKVTKLQV ELDSVTGLLS QSDSKSSKLT KDFSALESQL QDTQELLQEE
1321 NRQKLSLSTK LKQMEDEKNS FREQLEEEEE AKRNLEKQIA TLHAQVTDMK KKMEDGVGCL ETAEEAKRRL QKDLEGLSQR LEEKVAAYDK LEKTKTRLQQ ELDDLLVDLD
1431 HQRQSVSNLE KKQKKFDQLL AEEKTISAKY AEERDRAEAE ARETKETKALS LARALEEAME QKAELERLNK QFRTEMEDLM SSKDDVGKSV HELEKSKRAL EQQVEEMKTQ
1541 LEELEDELQA TEDAKLRLEV NLQAMKAQFE RDLQGRDEQS EEKKKQLVRQ VREMEAELED ERKQRSMAMA ARKKLEMDLK DLEAHIDTAN KNREEAIKQL RKLQAQMKDC
1651 MRELDDTRAS REEILAQAKE NEKKLKSMEA EMIQLQEELA AAERAKRQAQ QERDELADEI ANSSGKGALA LEEKRRLEAR IALLEEELEE EQGNTELIND RLKKANLQID
1761 QINTDLNLER SHAQKNENAR QQLERQNKEL KAKLQEMESA VKSKYKASIA ALEAKIAQLE EQLDNETKER QAASKQVRRT EKKLKDVLLQ VEDERRNAEQ FKDQADKAST
1871 RLKQLKRQLE EAEEEAQRAN ASRRKLQREL EDATETADAM NREVSSLKNK LRRGDLPFVV TRRIVRKGTG DCSDEEVDGK ADGADAKAAE
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(d)

Figure 1: Identification of nmMHC as a redox-sensitive protein during cell adhesion. (a) Content of H_2O_2 in human fibroblasts during adhesion on fibronectin. Hydrogen peroxide production was assayed used DCF-DA. $^*P < 0.001$ versus time 0. (b) Cell lysates from rounded and spread human fibroblasts were labelled with BIAM and then added to immobilized streptavidin. The pattern of proteins were visualized by a treatment with HRP conjugate streptavidin. (c) Nonredundant (nrNCBI) database was scanned using MASCOT search algorithm. The nonmuscle myosin heavy chain IX of mus musculus (gi/20137006) was identified with a significant score of 115 at 120 ppm mass tolerance. The spectrum shows the matched peaks. (d) The primary structure of nmMHC is shown.

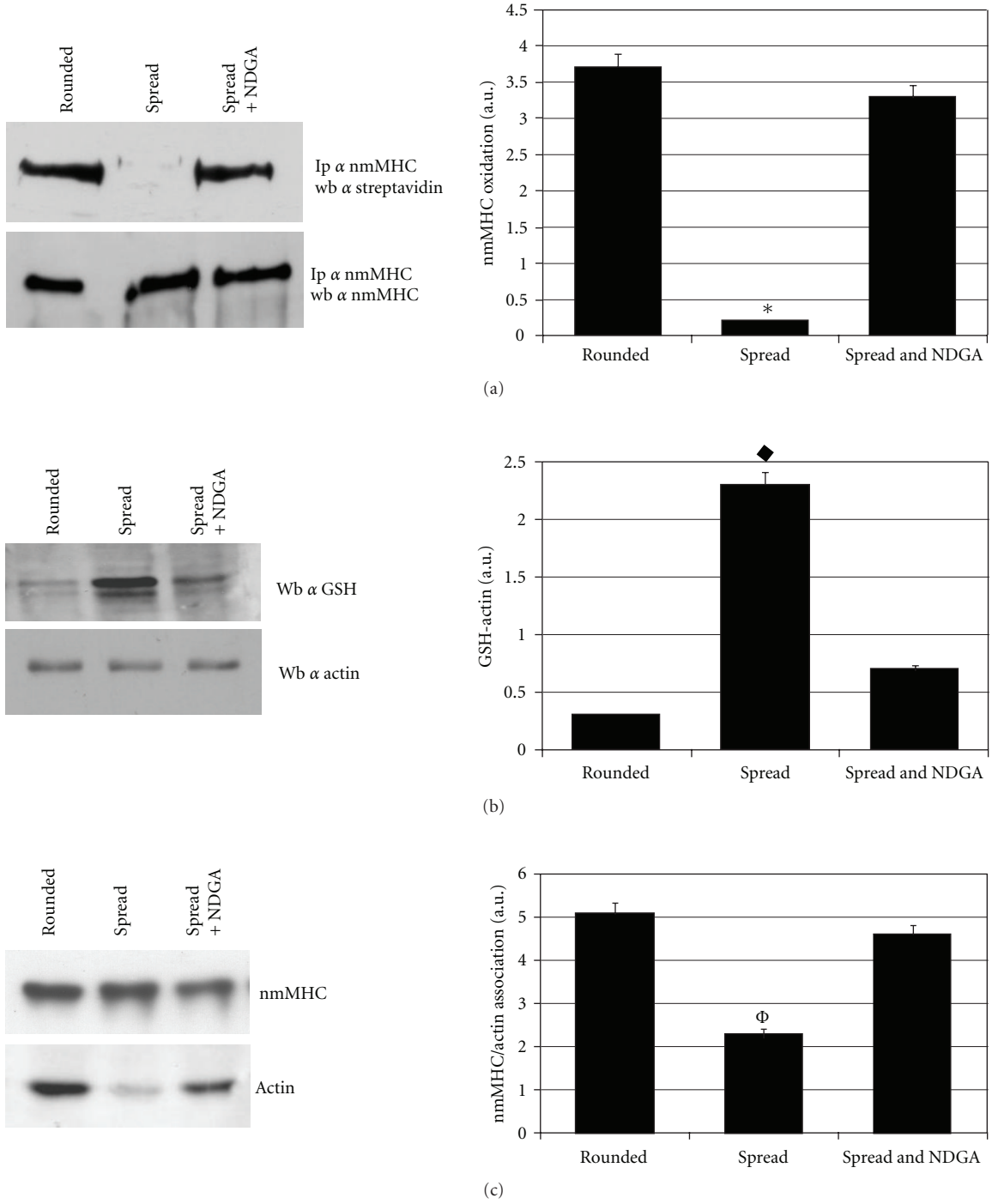
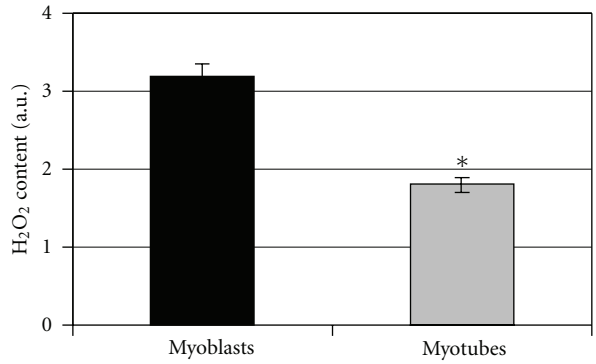
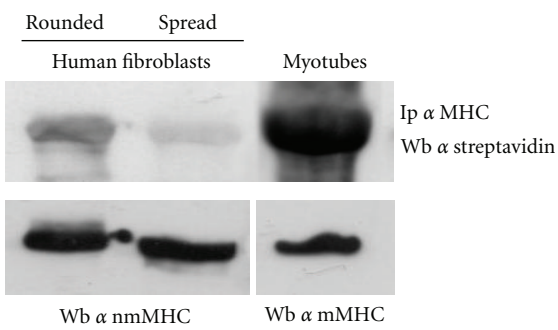
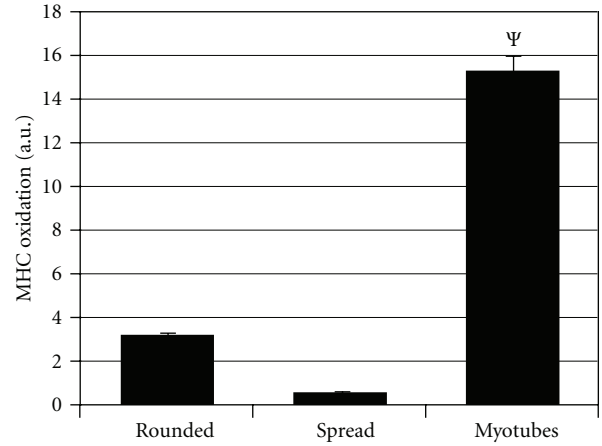


Figure 2: Analysis of nmMHC redox state and β-actin association in human fibroblasts. Cells lysates from detached and spread cells, treated with or without NDGA, were labelled with BIAM and nmMHC was immunoprecipitated with specific antibodies. (a) BIAM labelled pattern of nmMHC was revealed by western blot using HRP-streptavidin conjugate; amount of immunoprecipitated nmMHC was obtained probing the membrane with anti-nmMHC antibodies; the histogram shows the ratio between two corresponding samples after densitometric evaluation. $^{*}P < 0.001$ versus rounded. (b) Representative immunoblot showing actin glutathionylation in rounded and spread human fibroblasts. The histogram corresponds to the ratio between GSH-actin and total actin. $^{\blacklozenge}P < 0,001$ versus rounded. (c) Analysis of nmMHC-actin association. An anti-nmMHC immunoprecipitation was performed from rounded and spread cells treated with or without NDGA. The detection of actin associated with nmMHC was obtained with antiactin immunoblot, while anti-nmMHC immunoblot was used for normalization. The histogram corresponds to the ratio between the values obtained by densitometric analysis of two corresponding samples of the blots. $^{\Phi}P < 0.005$ versus rounded. Similar results were obtained in four independent experiments. a.u.: arbitrary units.



(a)





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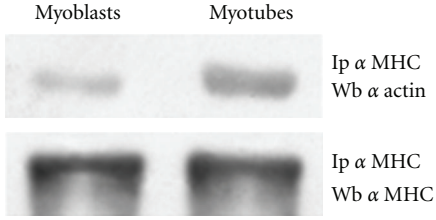

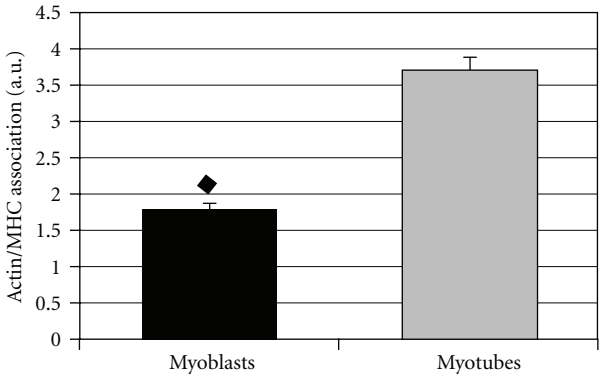

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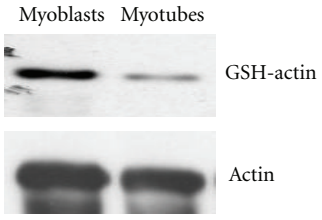
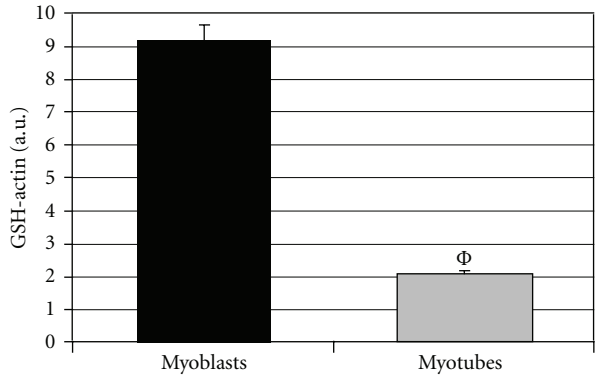




(d)

Figure 3: Continued.

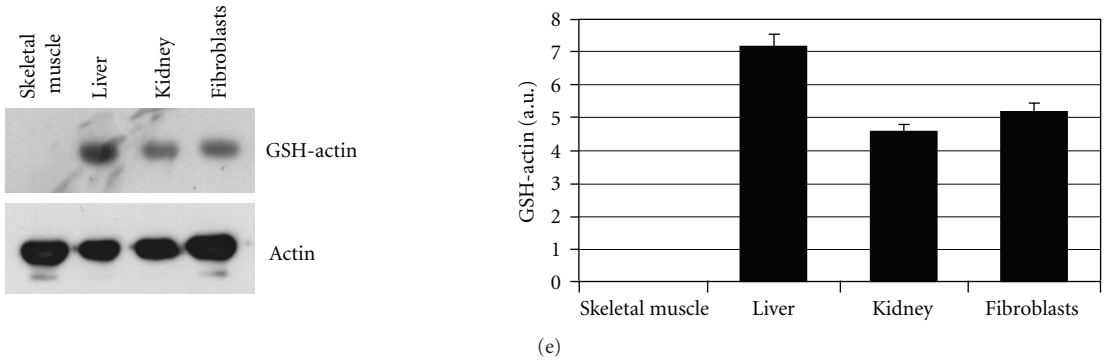


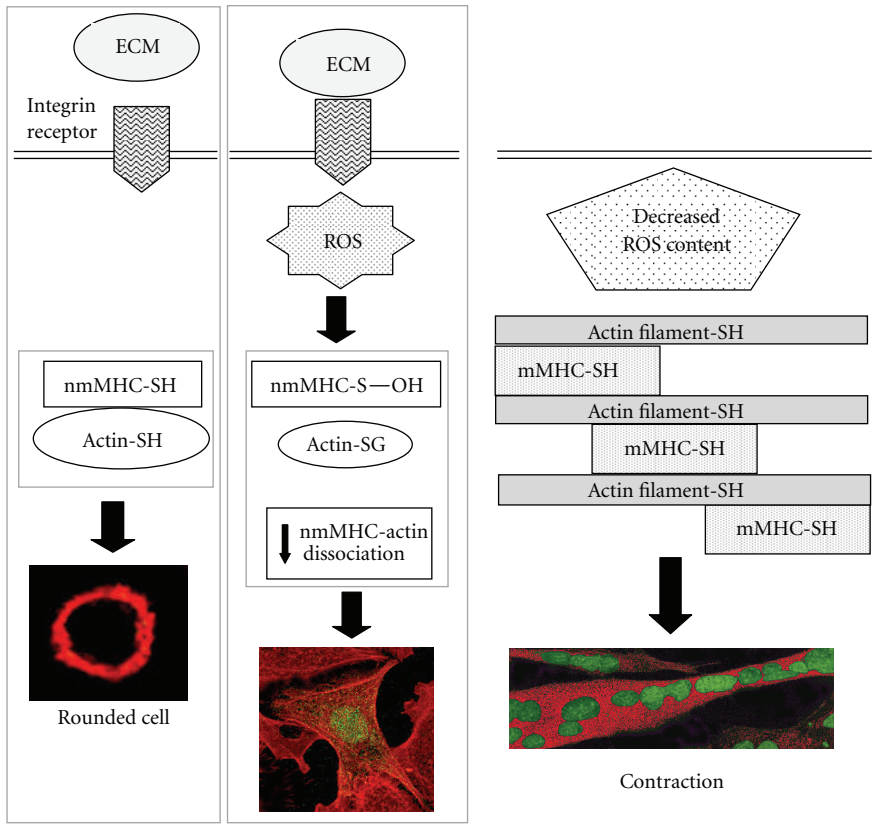
FIGURE 3: Analysis of the redox state of muscle MHC and actin in differentiating myotubes. (a) Analysis of H_2O_2 content in myoblasts and in differentiating myotubes after six days of differentiation. Hydrogen peroxide assay was performed with DCF-DA. $^{*}P < 0.005$ versus myoblasts. (b) Analysis of the redox state of MHC on rounded or spread human fibroblasts and in differentiating myotubes. BIAM labelled pattern of MHC was revealed by a Western blot using HRP-streptavidin conjugate after immunoprecipitation of nmMHC in human fibroblasts and mMHC in differentiating myotubes; MHC normalization was performed probing the membrane with specific anti-MHC antibodies; the histogram corresponds to the ratio between two corresponding values obtained by densitometric analysis. $^{\Psi}P < 0.001$. (c) Analysis of MHC-actin association in myoblasts and in differentiating myotubes. An anti-nmMHC immunoprecipitation was performed from myoblasts and from differentiating myotubes. The amount of actin associated with MHC was obtained with antiactin immunoblot, while the normalization was performed using anti-MHC antibodies. The histogram reports the ratio between the values obtained by densitometric analysis of the two corresponding values of the blots. $^{\blacklozenge}P < 0.0015$ versus myotubes. (d) and (e) Analysis of actin glutathionylation. Actin glutathionylation was assayed on growing C2C12 myoblasts, differentiating myotubes, human fibroblasts, and in murine skeletal muscle, liver, and kidney using anti-GSH antibodies. $^{\Phi}P < 0.001$ versus myotubes. a.u.: arbitrary units.

It is well known that muscle differentiation is accompanied with a dramatic and stable rearrangement of cytoskeleton architecture accompanied with the formation of contractile fibers. It is likely that binding of myosin to actin behaves as a cyclic event, and it is not under redox control. As expected, the results show that MHC/actin association is greatly improved in differentiating myotubes with respect to myoblasts (Figure 3(c)). Again, when ROS are high (as in spread fibroblasts or in undifferentiated myoblasts), β-actin is not bound to MHC. Conversely, when ROS are low (as in rounded/detached fibroblasts or in differentiating myotubes), actin strictly binds MHC, thereby underscoring the key importance of the redox control of the two proteins for their regulated interaction.

We have previously demonstrated that the ROS burst produced by ECM-integrin binding induces oxidation of β-actin, through glutathionylation of cysteine 374, which is an essential step for actomyosin disassembly [15]. Whether glutathionylation is important for its binding to myosin, we expected that in differentiating myotubes, where the association between these two proteins is essential for contraction, actin should be less glutathionylated. As expected, we found that actin glutathionylation is greater in myoblasts with respect to differentiating myotubes, in agreement with their ROS content in differentiated cells (Figure 3(d)). Furthermore, analysis of several murine tissues reveals that skeletal muscle fibres contains reduced actin, while in liver, kidney, and skin fibroblasts β-actin is oxidised/glutathionylated (Figure 3(e)). This suggests that both actin and MHC in skeletal muscle must be reduced to allow their continuous association for muscle contraction.

Although acute production of ROS have positive effects in skeletal muscle (such as for glucose uptake), ROS accumulation can provoke serious consequences for skeletal muscle physiology [23]. Beyond serious skeletal muscle pathologies (such as Duchenne muscular dystrophy and mitochondrial myopathies), in which an increased/uncontrolled production of ROS has been reported [24], oxidative molecules alter the physiology also in healthy muscle. Indeed, high amount of ROS induce an acute decrease in force production during repeated contractions, accompanied with a lower Ca^{2+} sensitivity of myofibrils [25]. In addition, several studies reported slower fatigue development in the presence of ROS scavengers [26]. In agreement with these observations and with our results, a decreased muscle contraction in oxidative conditions has been reported, suggesting that molecules involved in these process should not meet oxidation [27, 28].

The different behaviour of MHC in growing fibroblasts and in differentiating myotubes could be explained considering the function and architecture of cytoskeleton in these two situations. In undifferentiated growing cells, cytoskeleton is a dynamic structure which is subjected to continuous remodelling in response to extracellular signals to allow cell-shape changes associated with directed movement, secretion, or cell division (Figure 4(a)). Conversely, muscle differentiation is associated to a great cytoskeletal rearrangements that culminate with the formation of a static structure. In this view, it is possible that the actin/MHC binding does not need any further (i.e., transient) regulatory mechanism, as they are already associated to form stable structures (Figure 4(b)). On the contrary, in nonmuscle cells this association may be finely regulated, since both β-actin and MHC can be rapidly assembled and disassembled in response to extracellular signals. This control may be obtained through ROS generated by integrins during cell



(a) Moving cell: dynamic cytoskeletal architecture (b) Myotube: static cytoskeletal architecture

Figure 4: Proposed model for the MHC oxidation during fibroblasts adhesion and muscle differentiation. In a rounded cell, where level of ROS are low, both nmMHC and actin are reduced and associated ((a), left). In the early stage of fibroblast adhesion, the engagement of integrin receptors generates a burst of ROS leading to oxidation of nmMHC and decreased its binding with actin ((a), right). It is likely that the redox-dependent nmMHC/actin association is functional to the cytoskeleton dynamics during cell motility. Myotubes, where a stable and static cytoskeletal structure has been formed, are characterized by a low ROS content. In this environment, nmMHC is not redox-regulated and the increased nmMHC/actin association is a redox-independent mechanism that guarantees contraction (b).

adhesion, where oxidants might act as second messengers for actin/MHC cytoskeleton remodelling (Figure 4).

Taken together, these findings demonstrate that also proteins forming the cytoskeletal architecture could be ROS-regulated and that this control might be important for the cytoskeleton rearrangement during some cellular functions. Furthermore, these results open new perspectives for future investigations, such as the evaluation of ROS sensitivity of cytoskeletal proteins in cancer cells, a phenomenon linked both to a great ROS increase and a strong cytoskeleton rearrangements.

Abbreviations

BIAM: N-(biotinoyl)-N′-(iodoacetyl)ethylene diamine
FAK: Focal adhesion kinase
HRP: Horse radish peroxidase
GSH: Glutathione
LMW-PTP: Low molecular weight-phosphotyrosine phosphatase
5-LOX: 5-lipoxygenase
MHC: Myosin heavy chain
mMHC: muscle myosin heavy chain
nmMHC: Nonmuscle MHC
NDGA: Nordihydroguaiareticacid
ROS: Reactive oxygen species.

Acknowledgments

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Review Article

Molecular Crosstalk between Integrins and Cadherins: Do Reactive Oxygen Species Set the Talk?

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The coordinate modulation of the cellular functions of cadherins and integrins plays an essential role in fundamental physiological and pathological processes, including morphogenesis, tissue differentiation and renewal, wound healing, immune surveillance, inflammatory response, tumor progression, and metastasis. However, the molecular mechanisms underlying the fine-tuned functional communication between cadherins and integrins are still elusive. This paper focuses on recent findings towards the involvement of reactive oxygen species (ROS) in the regulation of cell adhesion and signal transduction functions of integrins and cadherins, pointing to ROS as emerging strong candidates for modulating the molecular crosstalk between cell-matrix and cell-cell adhesion receptors.

1. Introduction

The communication between signaling pathways, the so-called molecular crosstalk, plays a central role in cell biology, enabling the cell to couple the molecular functions of either near neighbors or distant cell components, with resulting synergistic or antagonistic effects and eventually appropriate biological outcomes.

Among the most important cellular crosstalk events is the signaling network that couples the molecular functions of adhesion receptors of the integrin and cadherin families. Indeed, acting in concert with growth factor receptor signaling pathways, this regulatory network is fundamentally important during the entire life of all metazoans, whereas its dysfunction almost invariably leads to developmental defects and/or diseases, including genetic diseases and cancer [1].

Integrins and cadherins are the major cell-extracellular matrix (ECM) and cell-cell adhesion receptors, respectively, and represent critical determinants of tissue architecture and function both in developing and adult organisms [2, 3].

Integrins are heterodimeric transmembrane glycoproteins composed of noncovalently linked α and β subunits, which are endowed with both structural and regulatory functions. They link the ECM to several distinct cytoplasmic proteins and the actin cytoskeleton at focal adhesions, thus serving as organizing centers for the assembly of structural and regulatory protein complexes at discrete cell-matrix adhesion sites and providing a mechanically sensitive system for mechanotransduction [4]. Furthermore, often acting in concert with growth factor receptors, they provide both outside-in and inside-out transmission of signals across the plasma membrane that control a number of critical cellular processes, including adhesion, cytoskeleton remodeling, migration, proliferation, differentiation, apoptosis, and gene expression [2, 5]. Specifically, integrin-mediated outside-in signaling stimulates tyrosine phosphorylation and activation of several proteins, including major components of focal adhesions, such as Src and FAK nonreceptor tyrosine kinases (PTK), and paxillin, as well as receptor tyrosine kinases (RPTK). In turn, these proteins are antagonized by nonreceptor (PTP) and receptor tyrosine phosphatases (RPTP), and entwined in a dynamic interplay with small GTPases and components of specialized plasma membrane and endosome microdomains, including caveolin-1, to form compartmentalized signaling platforms that allow for temporal and spatial coordination of specific downstream signaling events [6, 7].

Cadherins are single-pass transmembrane glycoproteins that support calcium-dependent, homophilic cell-cell adhesion. Together with their cytoplasmic domain interactors, such as β-catenin and p120^{ctn}, they constitute the core components of adherens junctions (AJs). These specialized adhesive structures link the cadherin homophilic adhesion to the actin cytoskeleton and are required for formation and maintenance of stable cell-cell adhesion and differentiated phenotype in all solid tissues [3, 8, 9]. Cadherin endocytosis and endosome-mediated trafficking has emerged as a major mechanism for controlling AJ remodeling [10–17]. Moreover, growing evidence demonstrates that cadherins can modulate the signaling activity of several proteins, including β-catenin, Ras and Rho family GTPases, PTK, RPTK, PTP, and RPTP, as well as mechanotransduction pathways that affect membrane and actin cytoskeleton dynamics [3, 18–21].

Although there is a large body of evidence supporting the existence of a fine-tuned crosstalk between members of these two adhesive receptor families, which influences their expression, turnover, positioning, and/or functions, and may enhance or suppress adhesion depending on the cellular and environmental context [1, 10, 17, 22–41], the molecules and molecular mechanisms involved in such important phenomenon are not completely defined. To clarify how this crosstalk is regulated remains therefore a fundamental challenge for basic and translational research, including research on tumor and vascular disease progression.

Multiple molecules and regulatory mechanisms have been placed at the heart of the molecular crosstalk between integrins and cadherins, including small GTPases of the Ras and the Rho families [10, 17, 42–45], nonreceptor kinases such as Src, FAK, Fer, and PI3K [27, 34, 46, 47], cell surface receptor-mediated pathways [48–50], and adhesion-dependent actomyosin traction forces [26, 34, 51].

Previously, we reported the pivotal role of the small GTPase Rap1 in regulating the crosstalk between cadherins and integrins, suggesting a model where Rap1 acts as a turnabout for endosome signaling and membrane trafficking pathways to orchestrate the delivery of cadherins and integrins to specific cell-cell and cell-matrix landmarks at the plasma membrane, respectively [10, 17].

Intriguingly, recent growing evidence suggests that reactive oxygen species (ROS) play an important role in both integrins, small GTPases, and cadherins functions, raising the possibility that ROS may contribute to the modulation of the molecular crosstalk between integrins and cadherins.

In this paper, we discuss the most recent advances on the role of ROS in outside-in and inside-out signal transduction events implicating integrins and cadherins, providing building bloks for the hypothesis that ROS constitute important players in the molecular crosstalk between these cell adhesion receptors.

2. ROS Metabolism and Signaling

ROS are a highly reactive group of oxygen-containing molecules, including free radicals and peroxides, such as superoxide anion ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2), which are generated constitutively, as common by-products of oxidative metabolism, or in response to the activation of several oxidative enzyme complexes [52–55].

The superoxide anion ($O_2^{\bullet -}$) is the key determinant of the overall effects of ROS. Indeed, even though it has a short half-life, $O_2^{\bullet -}$ is the precursor of all other major reactive oxygen species found in biological systems, including the powerful oxidants hydroxyl radical ($^{\bullet}OH$), hydrogen peroxide (H_2O_2), and peroxynitrite ($OONO^-$) [52–55]. It is generated by a number of sources located throughout the cell via the incomplete, one-electron reduction of molecular oxygen (O_2). Specifically, under physiological conditions the redox complexes I (NADH/ubiquinone oxidoreductase) and III (ubiquinol/cytochrome c oxidoreductase) of the mitochondrial electron transport chain are the major constitutive source, converting up to 5% of molecular O_2 to $O_2^{\bullet -}$ [56]. In addition, $O_2^{\bullet -}$ is produced by the activity of NAD(P)H oxidases, xanthine oxidases, cytochrome P450 monooxygenases, uncoupled NO synthase (NOS), myeloperoxidases, lipoxygenases, and cyclooxygenases [52–55], which can be induced by a variety of chemical and physical stimuli, including integrin ligands, growth factors, G-protein coupled receptor agonists, cytokines, neurotransmitters, metabolic factors, shear stress, ischemia/reperfusion, chemotherapeutics, and ionizing radiations, as well as aging [52–54, 57, 58]. Conversely, $O_2^{\bullet -}$ is rapidly removed by distinct superoxide dismutase (SOD) isoenzymes, located in the mitochondria (SOD2), cytoplasm (SOD1), and extracellular (SOD3) compartments, which catalyze the dismutation of $O_2^{\bullet -}$ into H_2O_2 and O_2. In turn, H_2O_2 is reduced to H_2O by the catalase and glutathione peroxidase enzymes. In addition, $O_2^{\bullet -}$ can be converted to hydroxyl radical ($^{\bullet}OH$) by the Fenton or Haber-Weiss reactions, or to peroxynitrite ($OONO^-$) by reacting with nitric oxide (NO) [59] (Figure 1).

It is now well established that physiologic concentrations of ROS are endowed with essential signaling properties, which are mainly due to the reversible oxidation of redox-sensitive molecular targets, thereby functioning as signaling molecules. Accordingly, it has been clearly demonstrated that ROS are involved in the redox-dependent regulation of multiple signal transduction pathways to fulfill a wide range of essential biological processes, including cell adhesion, migration, proliferation, differentiation, and survival [52–55]. However, at high levels, ROS are known to exert very damaging effects through oxidative stress. This is caused by an imbalance between the production of ROS and the ability of cellular antioxidant mechanisms to readily detoxify the reactive intermediates. Importantly, because $O_2^{\bullet -}$ can spontaneously react with NO to form $OONO^-$ at a rate 3 times faster than $O_2^{\bullet -}$ dismutation by SOD, modest increases of $O_2^{\bullet -}$ can result in a great reduction of NO bioavailability and increased formation of $OONO^-$, a very strong oxidant with the potential to produce multiple cytotoxic effects [60, 61]. In addition, $OONO^-$ can also trigger feedforward mechanisms that further amplify $O_2^{\bullet -}$ generation and oxidative stress, including the uncoupling of NO synthase (NOS) which produces $O_2^{\bullet -}$ instead of NO, thus amplifying the risk of cellular dysfunction and

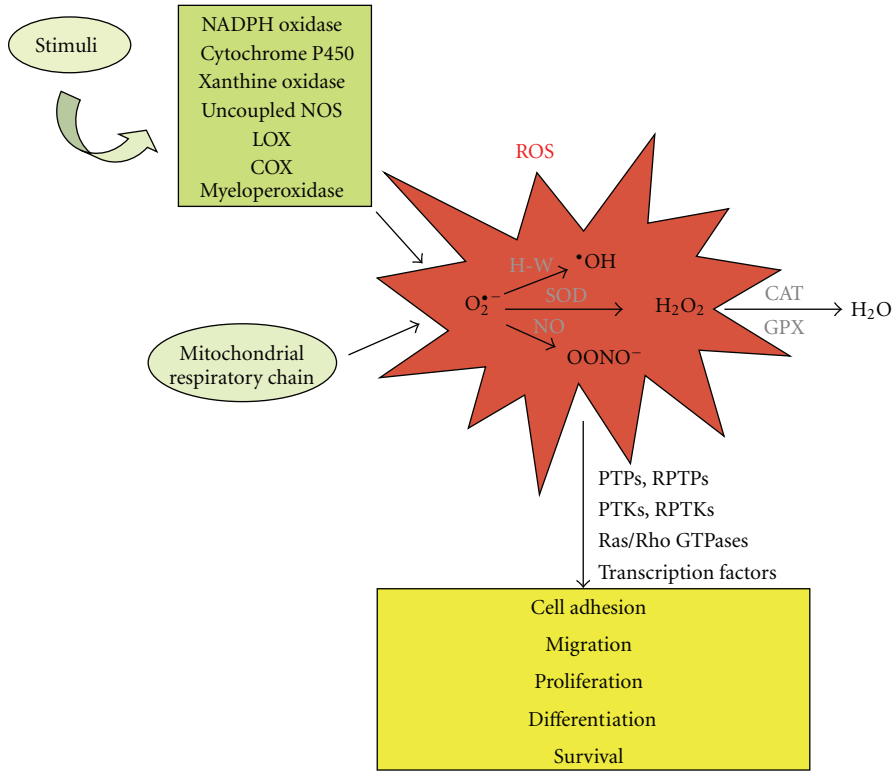

Figure 1: Schematic representation of ROS metabolism and signaling. The superoxide anion ($O_2^{\bullet -}$) is a key determinant of oxidative effects as well as the precursor of all other major reactive oxygen species, including hydroxyl radical ($^{\bullet}OH$), hydrogen peroxide (H_2O_2), and peroxynitrite ($OONO^-$). It is generated constitutively as by-product of oxidative metabolism, as well as upon stimuli triggering the activation of oxidative enzymes, including NADPH oxidases, xanthine oxidases, cytochrome P450 monooxygenases, uncoupled NO synthase (NOS), myeloperoxidases, lipoxygenases (LOX), and cyclooxygenases (COX). Conversely, $O_2^{\bullet -}$ is removed by superoxide dismutase (SOD) enzymes, which catalyze the dismutation of $O_2^{\bullet -}$ into H_2O_2 and O_2. In turn, H_2O_2 is reduced to H_2O by the catalase (CAT) and glutathione peroxidase (GPX) enzymes. At physiologic concentrations, ROS are endowed with essential signaling properties, being involved in the redox-dependent regulation of multiple signal transduction pathways to fulfill a wide range of essential biological processes, including cell adhesion, migration, proliferation, differentiation, and survival. However, at high levels, ROS exert very damaging effects through oxidative stress. H-W: Haber-Weiss reaction; NO: nitric oxide.

oxidative injury [52]. The maintenance of highly regulated mechanisms to control ROS levels and functional specificity is therefore essential for normal cellular homeostasis and proper response to environmental stimuli.

Among the major source of ROS, NADPH oxidases have been demonstrated to play a fundamental role in the compartmentalization of ROS production and redox signaling [7].

The NADPH oxidase (NOX) complex was originally identified in phagocytic leukocytes as an enzymatic defense system against infections required for the oxidative burst-dependent microbial killing [62, 63]. It is composed of membrane-associated and cytosolic components, which assembly to form the active NOX enzymatic complex in response to appropriate stimuli. Specifically, this complex consists of membrane-associated cytochrome b558, comprising the catalytic $gp91^{phox}$ (also known as NOX2) and regulatory $p22^{phox}$ subunits, and four cytosolic regulatory components, including $p40^{phox}$, $p47^{phox}$, $p67^{phox}$, and the small GTPase Rac1 [63]. Subsequently, NADPH oxidase complexes were also found in nonphagocytic cells, where several isoforms of the catalytic NOX2 protein were identified, including NOX1, NOX3, NOX4, and NOX5, and shown to localize in proximity of specific redox-sensitive molecular targets within discrete subcellular compartments, thereby facilitating the compartmentalization of redox signaling [7]. Indeed, NADPH oxidases can be targeted and activated within caveolae/lipid rafts, focal adhesions, cell-cell contacts, lamellipodial leading edges and membrane ruffles, endosomes, and the nucleus, allowing spatiotemporally confined ROS production and activation of specific redox signaling events [7].

Besides NADPH oxidase, an important role in the spatiotemporal regulation of ROS production is also played by enzymes involved in arachidonic acid (AA) metabolism, such as phospholipase A_2(PLA_2), lipooxygenases (LOX), and cyclooxygenases (COX), suggesting that a complex regulatory network may take place for proper modulation of redox signaling [64].

Accumulating evidence points to PTPs as the major redox-sensitive molecular targets of ROS [65]. This protein family is indeed characterized by the presence in the active

site of a highly conserved sequence motif containing a Cys residue that is essential for catalysis and very susceptible to reversible inactivating oxidation by ROS. In turn, oxidative inactivation of PTPs promotes phosphorylation-dependent downstream signaling events. In addition to PTPs, other important signaling proteins have been shown to act as endogenous redox sensors for mediating ROS signaling, including RPTKs, cytoplasmic kinases, small GTPases of the Ras and Rho families, and transcription factors [7, 65, 66] (Figure 1). Conversely, protein oxidation can be reversed by thiol donors such as glutathione [67].

Remarkably, the activation of redox signaling complexes at integrin-mediated cell-matrix adhesion sites and cadherin-mediated cell-cell junctions induces opposite effects, leading to the assembly of focal adhesions and the disassembly of adherens junctions, respectively [7].

3. ROS and Integrins

It is now well established that ROS are implicated in regulating many integrin-mediated cellular responses, including adhesion, cytoskeleton organization, migration, proliferation, differentiation, and survival. Indeed, a large body of evidence demonstrates that integrin activation triggers a transient and localized burst of ROS, either independently or in cooperation with growth factor receptors, which is essential to the proper transduction of outside-in signaling pathways [7, 47, 68]. Specifically, although the underlying molecular mechanisms remain to be precisely defined, there is clear evidence that integrin engagement with antibodies or extracellular matrix proteins triggers ROS production by promoting changes in mitochondrial metabolic/redox function [69–71], and activation of distinct oxidases, including NADPH-oxidases [47, 72, 73], and the AA-metabolizing enzymes 5-lipoxygenase (5-LOX) [70, 72] and cyclooxygenase-2 (COX-2) [74]. Conversely, there is evidence that ROS can also influence integrin-mediated inside-out signaling by inducing the conformational change required for integrin activation [64]. Remarkably, the small GTPase Rac1 has emerged as a crucial, common upstream mediator of ROS production in integrin-mediated outside-in signaling [64, 69–72]. Consistently, Rac1 acts upstream of both NADPH oxidase [7] and AA-metabolizing enzymes, such as PLA_2 [75, 76], 5-LOX [70, 72, 76], and COX-2 [77], whereas many reports show that AA metabolism modulates NADPH oxidase and mitochondrial ROS production, as well as the existence of a bidirectional signaling crosstalk between mitochondria, and NADPH oxidase, suggesting that Rac1 can orchestrate a complex web of regulation for ROS production [64, 78] (Figure 2). In addition, it is becoming evident that the formation of focal adhesions promotes the assembly of redox signaling platforms, involving integrins, growth factor receptors, and NADPH oxidases, which are essential for localized ROS production and activation of specific redox signaling pathways that mediate adhesion-dependent cell functions [7]. Furthermore, there is evidence that fine-tuned sequential compartmentalization and kinetics of ROS production can account for the modulation of distinct subsets of redox-sensitive signaling molecules involved in early and late phases of cell adhesion, leading to distinct outcomes [70, 79].

The signaling properties of integrin-induced ROS are largely due to the reversible oxidation of target proteins, and especially of PTPs, as the activity of these proteins is dependent on reactive cysteine residues (Cys-SH) at their active site that are readily susceptible to reversible oxidation [7, 65]. Indeed, ROS produced locally by the synergistic action of integrins and growth factor receptors on NADPH oxidase, as well as on mitochondria and 5-LOX, have been shown to induce oxidative inactivation of distinct PTPs, including the low-molecular-weight protein tyrosine phosphatase (LMW-PTP), PTP1B, PTEN, and SHP2, preventing these enzymes from dephosphorylating and inactivating specific targets, and thereby promoting downstream adhesion-related signaling events (Figure 2). Consistently, integrin-mediated adhesive and signaling functions are significantly mimicked by PTP inhibition [80]. However, ROS generated by integrin activation can also activate PTKs and RPTKs through either direct oxidation of susceptible cysteine residues or indirect inhibition of negative regulatory PTPs [68], whereas the synergistic cooperation between integrins and growth factor receptors expands enormously the plethora of ROS-regulated target proteins to include redox-sensitive small GTPases of the Ras superfamily and transcription factors such as AP-1 and NF-κB [7, 81–83] (Figure 2).

Remarkably, ROS production has often a dual role in small GTPase regulation, leading to either inhibition or activation under different conditions [83–88]. In particular, the inactivation of RhoA has been shown to occur indirectly through a signaling cascade involving the Rac-stimulated release of $O_2^{\bullet -}$ from NADPH oxidase, which in turn inhibits LMW-PTP. Because p190Rho-GTPase-activating protein (p190RhoGAP) is a substrate of LMW-PTP, inactivation of LMW-PTP results in accumulation of the active phosphorylated form of p190RhoGAP, which stimulates the hydrolysis of bound GTP to produce inactive GDP-bound RhoA, thereby determining well-characterized readouts, including decreased cell contractility and stabilization of cell-cell junctions [85, 88]. Conversely, RhoA can be directly activated by ROS-mediated reversible oxidation of two critical cysteine residues located in a unique redox-sensitive motif within the phosphoryl binding loop, leading to characteristic outcomes, including stress fiber and focal adhesion formation and cell-cell junction weakening [84, 89, 90]. On the other hand, there is evidence that ROS can activate Rap1 [91], whose signaling is in turn required for suppression of Ras-generated ROS and protection against oxidative stress and consequent cell dysfunctions [92–94].

4. ROS and Cadherins

Growing evidence demonstrates that ROS play a major role in either stabilization or destabilization of cell-cell junctions mediated by distinct cadherins, including E-, N-, and VE-cadherin [81, 95–99]. In particular, it has been reported that Rac1-mediated ROS production is an essential component in signaling cascades that promote p190RhoGAP translocation to the AJs and the consequent inhibition of

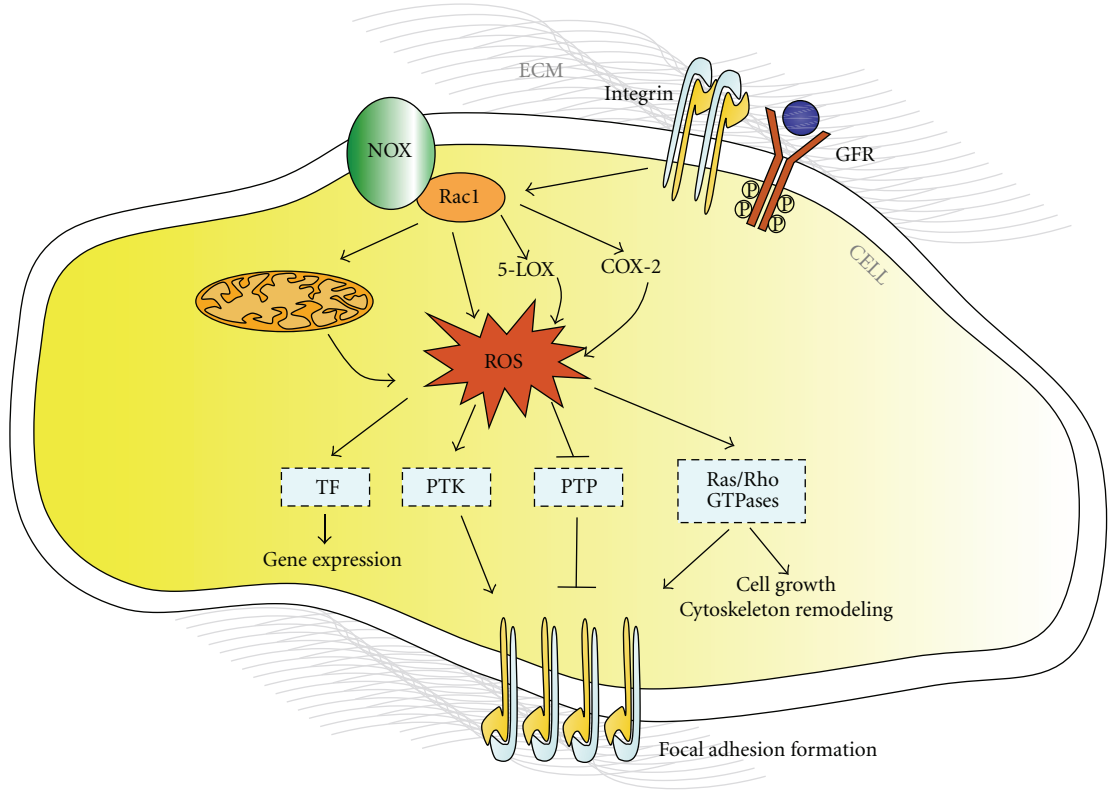

Figure 2: ROS mediate integrin outside-in signaling. Integrin engagement with extracellular matrix (ECM) proteins triggers a transient and localized burst of ROS, either independently or in cooperation with growth factor receptors (GFR), which is essential to the proper transduction of outside-in signaling pathways. The small GTPase Rac1 acts as a crucial upstream regulator of ROS production, orchestrating integrin outside-in signaling-mediated changes in mitochondrial metabolic/redox function, and activation of distinct oxidases, including NADPH-oxidases (NOX), 5-lipoxygenase (5-LOX), and cyclooxygenase-2 (COX-2). The signaling properties of integrin-induced ROS are largely due to the reversible oxidation of specific subsets of redox-sensitive proteins, including oxidative inhibition of PTPs, and activation of PTKs, RPTKs, small GTPases of the Ras and Rho families, and transcription factors (TF) such as AP-1 and NF-κB.

local RhoA activity, thus favoring the stabilization of cell-cell contacts [99]. Conversely, clear evidence shows that Rac1-induced ROS function as signaling molecules to disrupt VE-cadherin-based cell-cell adhesion leading to various biological responses, including endothelial barrier dysfunction, enhanced microvascular permeability, and endothelial migration and proliferation involved in angiogenesis [7, 96, 98, 100, 101] (Figure 3). Intriguingly, the apparent contrast between the positive and negative roles of ROS in the maintenance of cadherin-mediated cell-cell junctions correlates with similar features of small GTPases involved in this process, including Rac1, RhoA, and Rap1. Indeed, depending on the extracellular and intracellular context, the activities of Rac1, RhoA, and Rap1 may be not only involved in regulating AJs and endothelial barrier maintenance, but also in active enforcement or disruption of AJs and endothelial barrier integrity, suggesting that the location and duration of the activities of these small GTPases affect the choice of downstream targets, thereby determining distinct biological outcomes [81]. Indeed, while under basal conditions Rac1 enforces the junctions that form the endothelial barrier by promoting ROS-mediated p190RhoGAP recruitment to AJs and the consequent inhibition of local RhoA activity, upon certain growth factor stimuli, including VEGF, it becomes part of a barrier-disturbing mechanism by inducing ROS-mediated phosphorylation of VE-cadherin at Tyr^{658} and Tyr^{731}, and β-catenin at Tyr^{654}, which lead to the disassembly of AJs [81, 96]. Whether ROS act directly on growth factor receptor kinase activity or, more likely, inhibit VE-cadherin-associated tyrosine phosphatases has still to be clarified. In addition, there is evidence for the involvement of the Pyk2 and Src redox-sensitive kinases in the phosphorylation of AJ proteins, including β-catenin and $p120^{ctn}$, and the resulting loss of cell-cell adhesion mediated by the Rac1-ROS signaling pathway [95, 100, 102] (Figure 3). Notably, it has been reported that antioxidant compounds can inhibit VEGF-induced angiogenesis through disruption of ROS-dependent Src kinase activation and the subsequent VE-cadherin tyrosine phosphorylation, resulting in the retention of VE-cadherin at cell-cell contacts [100]. Conversely, the cell-cell contact-dependent inhibition of cell growth and stimulation of PTP activity [103] have been associated with a decrease in the steady-state levels of intracellular ROS and the consequent impairment of redox signaling mediated by growth factor receptors [104].

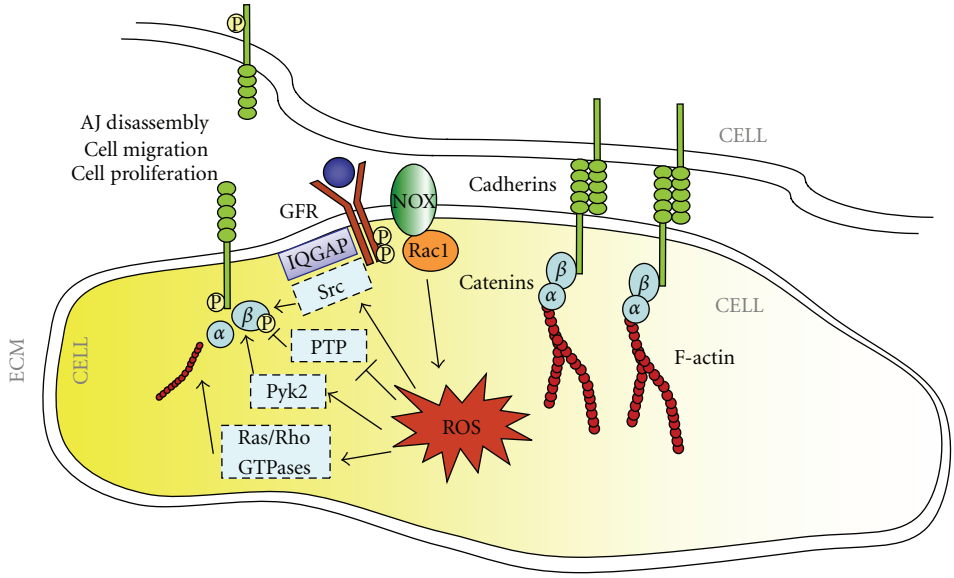

Figure 3: ROS modulate cadherin-mediated cell-cell junctions. Rac1-induced ROS may function as signaling molecules to disrupt cadherin-based cell-cell adhesion through either inhibition or activation of regulatory tyrosine phosphatases and kinases, respectively, as well as by localized activation of IQGAP and small GTPases, leading to various biological responses, including cell migration and proliferation.

Another component of the Rac1-ROS signaling pathway that plays an important role in the regulation of cadherin adhesive functions is IQGAP, a scaffold protein involved in cellular motility and morphogenesis [105]. IQGAP has been shown to be required for the establishment of VE-cadherin-based cell-cell contacts, and to colocalize and form a complex with VE-cadherin at cell-cell contact sites in quiescent endothelial cells [105]. It may act as a downstream effector of Rac1, as well as an inhibitor of its GTPase activity through a RasGAP-related domain [106–108]. Furthermore, it can facilitate localized ROS production through compartmentalization of Nox2 [109]. Indeed, there is evidence that IQGAP1 plays an essential role in VEGF-stimulated ROS production and VEGFR2-mediated endothelial cell migration and proliferation, suggesting that IQGAP1 may function as a scaffold protein to link VEGFR2 to the VE-cadherin/β-catenin complex at AJs, thereby promoting VEGF-stimulated ROS-dependent tyrosine phosphorylation of VE-cadherin, which may contribute to AJ weakening and angiogenesis [105, 110] (Figure 3).

Besides biochemical modification of AJ molecules, the ROS-dependent regulation of cadherins may be also driven by epigenetic events, as a ROS-induced hypermethylation of E-cadherin promoter, due to the upregulation of the transcriptional factor Snail and the recruitment of the DNA methyltransferase-1, and the consequent downregulation of cadherin expression have been reported [111].

5. ROS as Potential Pivotal Players in the Crosstalk between Integrins and Cadherins

A number of experimental reports have shown that the engagement of integrins with ECM proteins can affect cadherin-containing adherens junctions via multiple mechanisms, including the activation of signaling pathways mediated by small GTPases [10, 17, 42–45], cell surface receptors [48–50], and nonreceptor kinases [22, 27, 34, 47, 112], and the modulation of the actin network [26, 34, 51, 112]. Conversely, there are relatively fewer examples where cadherins have been shown to regulate integrin function [40, 113], but this may be due to the fact that crosstalk in this direction has been explored less extensively. In this context, we have previously reported that the small GTPase Rap1 plays a pivotal role in regulating the crosstalk between cadherins and integrins, suggesting a model where Rap1 acts as a turnabout for endosome signaling and membrane trafficking pathways to orchestrate the control of cadherin and integrin adhesive and signaling functions [10, 17].

Intriguingly, despite the lack of direct experimental evidence, the large number of studies implicating ROS as major modulators of integrin and cadherin adhesive and signaling functions strikingly supports the thought-provoking hypothesis that ROS play a crucial role in the crosstalk between integrins and cadherins (Figure 4). In particular, there is clear evidence that the assembly of integrin-mediated focal adhesions and the disassembly of cadherin-mediated adherens junctions require the activation of redox signaling complexes involving common regulatory proteins and mechanisms, including redox-sensitive small GTPases and the oxidative inactivation of PTPs [7]. Consistently, both focal adhesions assembly and adherens junctions disassembly are significantly mimicked by oxidative inhibitors of PTPs [10, 80], and prevented by ROS scavengers [95, 96].

Remarkably, both integrin- and cadherin-related redox signaling pathways involve Rac1 as a key mediator, which is in turn implicated in intimately intertwined functional

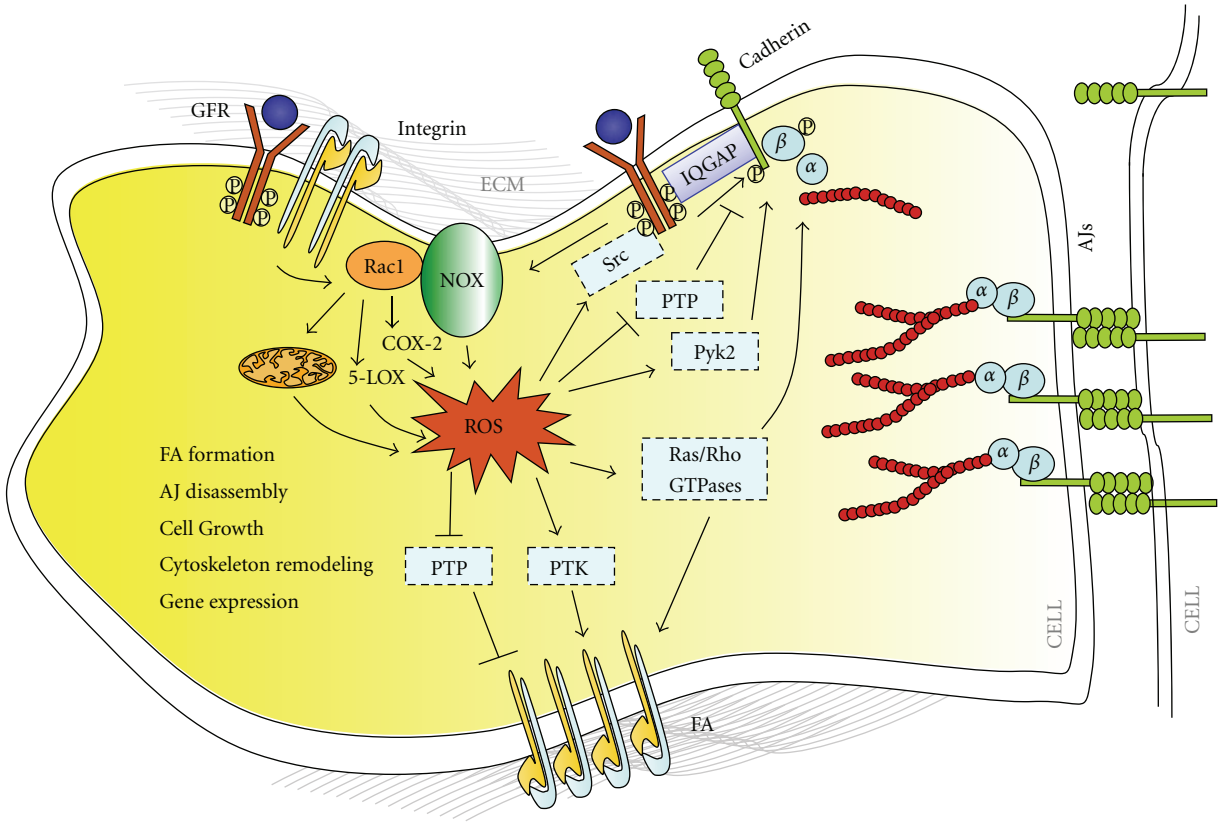

Figure 4: ROS in the crosstalk between integrins and cadherins. ROS generated by integrin activation may influence cadherin adhesive functions by various mechanisms, including inhibition of PTPs and/or activation of PTKs, RPTKs, and IQGAP acting at adherens junctions, as well as spatiotemporal modulation of the activity of redox-sensitive small GTPases and signaling endosomes.

relationships with other small GTPases, including Ras, RhoA, and Rap1 [7, 64, 69–72, 96, 98, 100, 101, 114].

Furthermore and importantly, recent evidence shows that Rap1 activation by Epac1, a Rap1-GEF involved in the Rap1-dependent regulation of cadherins, may be stimulated by ROS and inhibited by ROS scavengers, indicating that ROS production can trigger Rap1 activation [91]. Conversely, Rap1 signaling has been shown to be required for suppression of Ras-generated ROS and protection against oxidative stress and consequent cell dysfunctions [92–94]. Taking together, these data suggest that the role of Rap1 as pivotal regulator in the crosstalk between cadherins and integrins [10, 17] may underlie feedback mechanisms involving spatially and temporally regulated ROS production and scavenging. Consistently, KRIT1, a Rap1 effector whose loss-of-function mutations are implicated in endothelial cell-cell junction dysfunction and enhanced microvascular permeability underlying the Cerebral Cavernous Malformation disease, has been recently shown to play a role in molecular mechanisms involved in the maintenance of the intracellular ROS homeostasis to prevent oxidative cellular damage [115].

Finally, ROS generated by integrin activation could influence cadherin adhesive functions through the activation of either PTKs and RPTKs, including Src and growth factor receptors [68], or IQGAP, a component of the Rac1-ROS signaling pathway implicated in the modulation of AJ dynamics [105, 110] as well as in signaling downstream from both integrins and RPTKs [116], suggesting a further crosstalk mechanisms (Figure 4).

6. Concluding Remarks

It is well established that, besides their structural roles, both integrins and cadherins can provide bidirectional transmission of signals across topographically discrete regions of the plasma membrane. In addition, there is growing evidence supporting the existence of a fine-tuned, bidirectional crosstalk between these adhesion molecules, which may enhance or suppress their adhesive and signaling functions depending on the cellular and environmental context. Indeed, the integrin-cadherin crosstalk is involved in the epithelial-mesenchymal transition (EMT) underlying fundamental physiological and pathological processes, including embryonic development and cancer [22, 25–27, 33, 39].

This paper highlights recent growing evidence supporting a major role of reactive oxygen species (ROS) in both outside-in and inside-out signaling of integrins and cadherins, raising the possibility that ROS constitute master regulators of the crosstalk between these fundamental cell adhesion receptors.

Indeed, over the past few years, it has clearly emerged that outside-in integrin signaling triggers ROS production

by several distinct mechanisms, such as changes in mitochondrial metabolic/redox function [69–71] and activation of distinct oxidases, including NADPH oxidase [47, 70, 72–74]. On the other hand, growing evidence demonstrates that ROS play a major role in the regulation of cadherin adhesive and signaling functions by mechanisms involving either biochemical modifications (e.g., phosphorylation) of AJ proteins, including cadherins and catenins, epigenetic modifications of the cadherin promoter, or modulation of small GTPases regulating cadherin-dependent cell-cell adhesion [7, 81, 95–101].

Remarkably, whereas emerging data show that integrin and cadherin redox signaling involves shared regulatory proteins, accumulating evidence suggests that discrete subcellular compartmentalization of ROS constitutes a major mechanism of localizing activation of downstream redox signaling events, thereby playing a critical role in transmitting cell signals in response to various environmental stimuli to regulate distinct cell functions, including cell-matrix and cell-cell adhesion [7, 117]. In particular, ROS production may be localized through interactions of NADPH oxidase with signaling platforms associated with lipid rafts and caveolae, as well as with endosomes [7, 118]. Furthermore, there is evidence that growth factor receptors mediate signaling through a subset of signaling endosomes termed redoxosomes (redox-active endosomes), which are uniquely equipped with redox-processing proteins capable of transmitting ROS signals from the endosome interior to redox-sensitive effectors on the endosomal surface, thereby controlling redox-dependent effector functions through the spatial and temporal regulation of ROS as second messengers [117].

Taken together with the well-established roles of growth factor receptors, small GTPases and endosome signaling in the functional relationship between integrins and cadherins [17], the experimental evidence and observation discussed in this paper point to a novel hypothetical mechanism whereby the spatial and temporal regulation of ROS may contribute significantly to the modulation of the molecular crosstalk between these cell adhesion receptors, thus opening a novel research avenue.

Furthermore, as the impairment of the integrin-cadherin crosstalk is involved in the development of serious pathological processes, including abnormal angiogenesis, tumor invasion, and metastasis, strategies aimed at controlling ROS homeostasis to preserve the coordinated adhesive and signaling functions of integrins and cadherins might harbor important therapeutic potential for human health.

Acknowledgments

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Review Article

Reactive Oxygen Species in Skeletal Muscle Signaling

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Generation of reactive oxygen species (ROS) is a ubiquitous phenomenon in eukaryotic cells' life. Up to the 1990s of the past century, ROS have been solely considered as toxic species resulting in oxidative stress, pathogenesis and aging. However, there is now clear evidence that ROS are not merely toxic species but also—within certain concentrations—useful signaling molecules regulating physiological processes. During intense skeletal muscle contractile activity myotubes' mitochondria generate high ROS flows: this renders skeletal muscle a tissue where ROS hold a particular relevance. According to their hormetic nature, in muscles ROS may trigger different signaling pathways leading to diverging responses, from adaptation to cell death. Whether a "positive" or "negative" response will prevail depends on many variables such as, among others, the site of ROS production, the persistence of ROS flow or target cells' antioxidant status. In this light, a specific threshold of physiological ROS concentrations above which ROS exert negative, toxic effects is hard to determine, and the concept of "physiologically compatible" levels of ROS would better fit with such a dynamic scenario. In this review these concepts will be discussed along with the most relevant signaling pathways triggered and/or affected by ROS in skeletal muscle.

1. Introduction

Oxidative stressors, such as reactive oxygen species (ROS), have been initially and long considered as merely deleterious species to skeletal muscle tissue. Indeed, since the 1980s abundant evidence clearly indicated that ROS play a pathogenic role in inherited muscular dystrophies [1] and have then been identified as concausal factors in various muscular diseases [2–5]. However, and thereafter, accumulating evidence indicated that ROS, at least within concentrations emerging from physiological conditions, could also play a positive role in physiologically relevant processes in muscle cells. As an example, inflammation-derived ROS play a contradictory role in muscle repair [2]: in combination with other actors such as growth factors and chemokines, ROS participate in a cascade of events leading to muscle regeneration and repair; on the contrary, the local persistence of ROS sustained by infiltrated neutrophils may cause further injury by oxidatively damaging differentiating myoblasts and myotubes thus delaying the *restitutio ad integrum*. Similarly, ROS generated during exercise promote mitochondriogenesis (a key factor in muscle differentiation) *via* peroxisome proliferator-activated-receptor-gamma-coactivator-1α-(PGC-1α) activated signal transduction pathway [3] but, at higher and persistent levels, they might target mitochondria and mitochondrial DNA (mtDNA) turning into blockers of myogenic differentiation [4, 5]. Other examples of such diverging capacities—which strengthen the generally accepted notion that ROS act in a hormetic fashion—will be discussed thereafter. The prevalence of each of the two actions, that is, beneficial or detrimental, depends on the coincidence of various intrinsic and extrinsic factors among which the most prominent is the level and the duration of ROS targeting muscle cells; other variables are the source or the site of ROS generation, the antioxidant status of target cells, and their DNA repair capacity. The differentiation stage of muscle cells (satellite cell, differentiating myoblast or mature myotube) is also capable of redirecting the cell through different signaling pathways and of further modulating the ensuing cell response. Today ROS are known to trigger and/or affect many signaling pathways relevant to skeletal muscle cells'

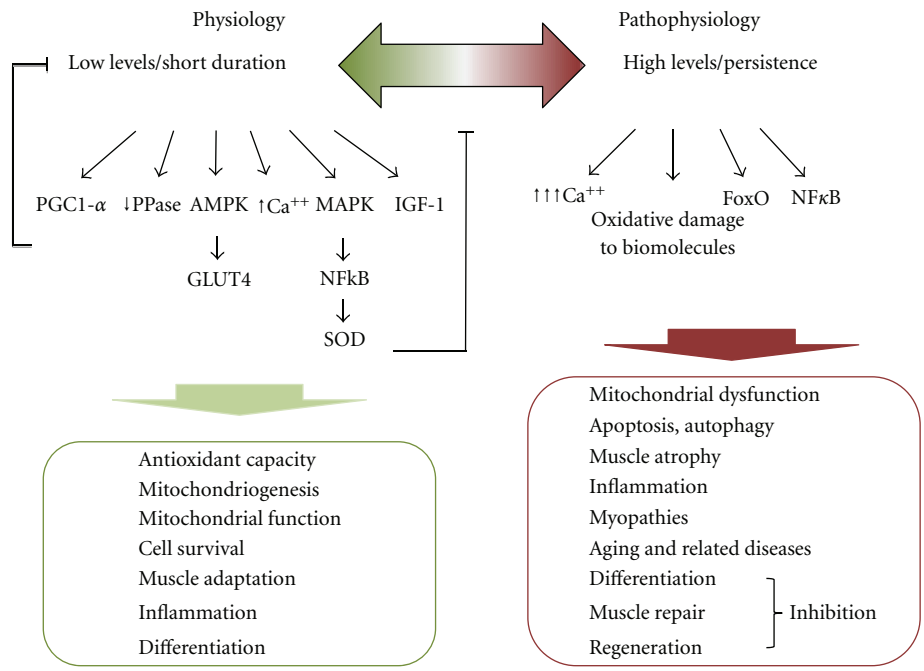

Figure 1: Major signaling pathways triggered and/or affected by ROS in skeletal muscle. Low levels of ROS activate specific key signaling molecules such as PGC-1α, AMPK, and MAPK, which control cellular mechanisms for muscle adaptation (oxidative metabolism, mitochondrial biogenesis, and mitochondrial functionality) as well as antioxidant enzymes that function as backregulators of intracellular ROS levels. Slight ROS accumulation also inhibits PPases and promotes the phosphorylation state of many proteins involved in the muscle signaling responses. Moreover, low levels of ROS play an important role in inducing upregulation of growth factors such as IGF-1, which has beneficial effects in muscle protein balance, supports oxidative metabolism, and contributes to the development of an oxidant-resistant phenotype, therefore preventing oxidative damage and chronic diseases. Thus, low levels of ROS elicit positive effects on physiological muscle responses. By contrast high levels of ROS cause functional oxidative damages of proteins, lipids, nucleic acids and cell components, induce a significant rise of intracellular [Ca^{2+}], and promote signaling cascades for apoptosis or autophagy *via* NF-κB or FoxO paths. For these reasons high ROS levels are reputed to act as etiological, or at least exacerbating factors in muscle atrophy, sarcopenia, wasting, and chronic-/aging-related muscle diseases and myopathies. Depending on their level/persistence, ROS may also turn the same process from "physiologic" into "pathologic", as in the case of inflammation.

homeostasis and adaptation: here we will illustrate some of the signaling pathways triggered/affected by ROS in muscle tissue and their physiopathological implications (see Figure 1 for a visual summary).

2. Generation of ROS in Skeletal Muscle Cells

Mitochondria are commonly considered as the predominant source of ROS in skeletal muscle cells [6, 7]. Increased mitochondrial ROS generation occurs during various and different situations, such as in the course of intense contractile activity [8] or in response to cytokines such as tumor necrosis factor-α (TNF-α) [9]. Early reports assumed that 2–5% of the total oxygen consumed by mitochondria may undergo one electron reduction with the generation of superoxide [10, 11]. More recent studies indicated that complexes I and III of the electron transport chain are the main sites of mitochondrial superoxide production [12, 13]. During exercise, it is assumed that the increased ROS generation in the course of contractile activity is due to the high oxygen consumption that takes place during increased mitochondrial activity. Indeed superoxide generation in skeletal muscle increases to about a 50- or 100-fold during aerobic contractions [14, 15].

However, recent evidence demonstrates that mitochondria may not be the prevalent source of ROS during exercise [8], and future studies are required to better elucidate the mitochondrial role in contraction-induced production of ROS in skeletal muscle. In 2002 St. Pierre and colleagues [16] reexamined the rate of mitochondrial ROS production and concluded that the total fraction of oxygen converted into superoxide was equal to 0.15%; this value is significantly lower than that (2–5%) estimated by other authors (see for example [17]). This lower rate of superoxide production takes account of the uncoupling proteins role (specifically UCP3 in skeletal muscle) as regulators of mitochondrial ROS production [18, 19] acting to prevent oxidative damage to mitochondria. In addition, growing evidence highlights that mitochondria produce more ROS during the basal state 4 of respiration as compared to state 3 (maximal ADP-stimulated respiration) [20–23]. Thus, since skeletal muscle mitochondria, during aerobic contractile activity, are predominantly in state 3, this limits their capacity of generating ROS during contractions [21–23].

Mitochondria are not the main and only source of ROS production in skeletal muscle during exercise. Indeed, other relevant sources of ROS production within muscle cells are nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOXs) located within the sarcoplasmic reticulum, transverse tubules, and the sarcolemma [24, 25].

Phospholipase A2 (PLA2) is another known source of intracellular ROS production [17]. Arachidonic acid released from cell membranes by PLA2 is a substrate for ROS-generating enzyme systems such as the lipoxygenases [26]. Activation of PLA2 can stimulate NOXs [27] and increased PLA2 activity can promote ROS production in muscle mitochondria [28] and cytosol [29] and release ROS into the extracellular space [26]. Both calcium-dependent and -independent forms of PLA2 exist in skeletal muscle and both contribute to muscle ROS generation [17]. In particular, the calcium-independent isoforms are likely to be involved in cytosolic oxidant activity in skeletal muscle cells [29], whereas a 14-kDa calcium-dependent isoform located within mitochondria is reputed to stimulate mitochondrial ROS generation during contractile activity [30]. In this light it has been proposed [29] that the calcium-independent PLA2 is a major determinant of ROS activity under resting conditions, whereas during processes or stress elevating intracellular calcium concentration (contraction, inflammation, heat stress, etc.) the calcium-dependent PLA2 is activated to promote ROS production.

Finally, superoxide anion is known to be generated by xanthine oxidase (XO) in the cytosol of contracting rat skeletal muscles cells [31]. However, human skeletal muscles contain lower levels of XO than rat muscle cells, and the question whether XO plays an important role in superoxide production in human skeletal muscle is still open [31, 32]. More ROS-generating mechanisms may be operative at the same time, as in the case of prolonged muscle ischemia where mitochondrial and cytoplasmic (*via* XO) ROS production has been simultaneously scored [33].

ROS can be generated through the above mechanisms not only within muscle cells but also in their proximity. For instance, during inflammation (a pathophysiological state that substantially alters cellular oxidative/antioxidant homeostasis) infiltrated polymorphoneutrophils activate NOX producing ROS *via* the respiratory burst and many cytokines, which amplify in a feedforward cycle ROS production, are secreted within muscles [34]. For instance, during the early phase of muscle injury, inflammatory cytokines can bind to membrane receptors and activate specific ROS-generating enzymes, such as cyclooxygenase-2, NOX, and XO [35]. Endothelial cells from injured muscle are known to secrete TNF-α, interleukine (IL)-1, IL-6, and IL-8, providing a positive feedforward cycle [36]. Whereas a transient oxidative stress is necessary in inflamed muscle cells to exert an antiseptic function and to activate various signal transduction pathways relevant to the *restitutio ad integrum*, prolonged severe oxidative stress may imbalance intracellular antioxidant homeostasis and hence long-term muscle welfare.

The oxygen-centered species that mostly arises from the processes described so far is superoxide anion, but its significance in ROS signaling pathways seems to be limited to a role as a precursor signaling molecule. Indeed superoxide undergoes enzymatic or spontaneous dismutation, a process generating H_2O_2. H_2O_2 is a nonradical, is a weak oxidant with a relatively long half-life allowing its diffusion within cells and across cell membranes [37], reacts with many different cellular molecules, and activates a wide number of signaling pathways. These properties render H_2O_2 the most relevant ROS signaling molecule in cells [38]. In contrast, H_2O_2 undergoing Fenton chemistry in the presence of redox active free iron ions or other transition metals can give rise to hydroxyl radicals, which react immediately with any surrounding biomolecules exerting most of the deleterious effects associated with oxidative stress. In this light, iron homeostasis can be considered as a comodulator of ROS signaling and effects. In particular, since skeletal muscle contains 10 to 15% of body iron—mainly in myoglobin and mitochondria—it could be particularly sensitive to alterations of iron homeostasis: accordingly it has been recently reported that levels of muscle nonheme iron and the iron transport protein, transferrin were elevated in senescence, suggesting that iron load is a significant component of sarcopenia [39].

3. Antioxidants and Modulation of Muscle Cells' Sensitivity to ROS

As it will be discussed throughout this review, the net effect of ROS on cells' signaling pathways and fate depends also on the cellular antioxidant capacity. The antioxidant network consists of enzymes, such as catalase, glutathione peroxidase (GPx), thioredoxin reductases (TRxs), superoxide dismutases (SODs), and soluble antioxidants such as glutathione (GSH) and vitamin E. Depending on its efficacy, the antioxidant cellular network plays a primary role in maintaining ROS below a physiologically compatible threshold level, thus allowing ROS to serve, theoretically, as signaling molecules and avoiding them to exert direct toxic effects. Many antioxidant enzymes are known to be induced in response to increased ROS generation. The increased ROS flux occurring in the course of strenuous exercise, through redox-sensitive mechanisms, induces the expression of γ-glutamylcysteinyl synthetase, the rate-limiting enzyme of GSH synthesis, of GPx, and of MnSOD [40]. Nuclear Factor KappaB (NF-κB), activator protein 1 (AP-1), and mitogen-activated protein kinases (MAPKs) have been identified as the major signaling pathways that can be activated by exercise-derived ROS and directly involved in the induction of the above antioxidant enzymes [40]. For instance, in the signaling path of MnSOD gene expression, NF-κB and AP-1 play an important role [41]: both NF-κB and AP-1 binding sites are present in the promoter of the mammalian MnSOD gene, and ROS have been shown to activate their binding.

The above responses, whose extent might be genetically predetermined, represent a fundamental adaptive countermeasure to conditions potentially resulting in frank oxidative stress. A specific modulator of ROS activity, working in an "antioxidant-like" fashion in ROS-mediated autophagy and apoptosis (see also "*ROS mediate autophagy and apoptosis*"),

is the p53-inducible glycolysis and apoptosis regulator (TIGAR), a p53-target gene. Indeed, TIGAR can reduce ROS levels in response to nutrient starvation or metabolic stress, thus inhibiting autophagy and apoptosis independently of mammalian target of rapamycin (mTOR) or p53 modulation [42]. The TIGAR protein functions as a fructose-2,6-bisphosphatase and contributes to the regulation of intracellular ROS levels by modulation of the glycolytic pathway [43]. By decreasing glycolytic rate and redirecting glycolytic intermediates to the oxidative branch of the pentose phosphate pathway, TIGAR causes an increase in NADPH production, which favours ROS scavenging through GPx/glutathione reductase and GSH cycle. In this manner, TIGAR lowers the sensitivity of cells to ROS-induced p53-dependent apoptosis [43].

As to the influence of physical exercise, it caused an activation of MAP kinases in gastrocnemius muscle from rats; this in turn activated the NF-κB pathway and consequently the expression of SOD and adaptation to exercise through increased expression of endothelial and inducible nitric oxide synthase [40]. All these responses were silenced when ROS production was prevented by allopurinol [40]. Thus ROS act as signals in exercise because their scavenging prevents activation of important signaling pathways promoting useful adaptations in cells. Because these signals result in an upregulation of powerful antioxidant enzymes, exercise itself can be considered an antioxidant.

Besides the established soluble cellular antioxidants, creatine (Cr) is emerging as a pleiotropic molecule capable of influencing muscle cell's trophism, differentiation, and sensitivity to ROS [44, 45]. Cr has a high tropism for skeletal muscles, where most of body Cr is stored and has been shown to exert direct and indirect antioxidant activity in proliferating and differentiating C2C12 myoblasts [44]. A recent article by Young et al. [46] showed that two TRxs situated in the mitochondria and cytoplasm, respectively, were increased in Cr-treated C2C12 myoblasts: peroxiredoxin-4, a type 2 peroxiredoxin, and thioredoxin-dependent peroxide reductase.

As it will be discussed below, the 5′-adenosine monophosphate-activated protein kinase (AMPK) signaling is critical in regulating mitochondrial content and function in a PGC-1α -dependent pathway in different tissues and in response to various stimuli [47]; furthermore AMPK signaling is important in preventing the mitochondrial dysfunction/impairment increasing ROS leakage and accompanying sarcopenia, disuse muscle atrophy, and other degenerative muscle disorders, in such a way that it can be considered as an indirect antioxidant cellular setting. Ceddia and Sweeney [48] firstly demonstrated that Cr supplementation may improve cellular bioenergetics by activating AMPK to improve overall mitochondrial content and/or function. It is currently unknown whether Cr supplementation exerts similar AMPK effects in oxidatively injured muscle tissue. At this regard, we recently observed in either control or oxidatively challenged differentiating myoblasts that a 24 h Cr preloading [0.3 mM] is an adequate stimulus to activate the AMPK pathway (unpublished observation).

This observation, along with others showing that Cr also acts as a direct antioxidant [5, 44, 49, 50] and protects differentiating myoblasts from H_2O_2-dependent differentiative arrest [5], suggests that in oxidative stressing conditions Cr treatment might confer myoblasts an enhanced adaptive capacity resulting in increased mitochondrial functionality and biogenesis and reduction of oxidative damage during myogenesis. Thus, besides established antioxidants, Cr might represent a skeletal-muscle-directed endogenous molecule capable of exerting multiple, pleiotropic actions which collectively help counteracting excessive ROS pressure. Consistently, beneficial effects of Cr supplementation have been reported for a large number of muscular, neurological, and cardiovascular diseases as well as in sarcopenia and aging [44, 51–56]. On the contrary, although great attention has been—and is still—paid to the administration of established antioxidants including polyphenols and vitamins in order to reduce the potential risk of the sustained and persistent action of ROS on skeletal muscle, there is no clear consensus on the benefits of these supplements [40, 57–59]. Thus, exploiting the efficacy of "atypical" and pleiotropic antioxidants such as Cr deserves consideration.

The integrity of the antioxidant network is particularly important in aging. Indeed, it is well-established that aging is associated with increased free radical generation and the resulting oxidative damage accumulated in organisms are likely to be involved, at least at a concausal level, in the progression of numerous diseases [60, 61]. It has long been suspected that senescent skeletal muscle may progressively loose its ability to adapt to oxidative stress [62, 63]. However, at the present, there is no clear consensus about how and whether senescent skeletal muscle becomes more susceptible to ROS pressure. For example, although in skeletal muscle antioxidant enzyme activities are increased with old age [64, 65], protein and mRNA levels of CuZnSOD, MnSOD, and GPx were found to be decreased or unaltered in the aged muscle [66, 67]. More importantly, aged muscle exhibited reduced antioxidant adaptation compared to training young muscle [62]. The reduced ability to rapidly activate an antioxidant adaptation program may render the senescent muscle more prone to oxidative damage. Notably, it has been hypothesized that the lack of adaptive capacity in aging muscle may depend on the impairment of signal transduction of antioxidant gene expression in response to oxidative stress [68]. At this regard, as discussed above, NF-κB and AP-1 are known to play an important role in MnSOD gene expression [41]. The decreased binding of these nuclear factors, despite increased ROS generation found in aged muscles, would suggest that aging slows down molecular signaling of antioxidant gene expression. Thus aging seems to decrease the ability of aged muscle to express at least MnSOD as demonstrated by lower nuclear protein binding, mRNA levels, and unaltered enzyme protein [62]. The observed increase in MnSOD activity in the same setting might depend on a posttranslational modification (activation) of the enzyme molecules in aged muscle. In contrast to MnSOD, CuZnSOD showed increased protein content and activity with age in type II muscle in the absence of mRNA changes [66]. On the whole, these data

suggest that the widely reported increase in antioxidant enzyme activities in aging skeletal muscle do not depend on enhanced gene transcription, but can rather derive from translational and/or posttranslational mechanisms. Since aged skeletal muscles are affected by augmented levels of lipid peroxidation, protein oxidation, and DNA damage, these compensatory increases in antioxidant enzyme activity are ineffective in counteracting increased ROS generation.

4. ROS, Mitochondrial Biogenesis and Function

It is well known that oxidative signals affect mitochondrial biogenesis, morphology, and function in skeletal muscle cells [69–71]: again, the effect of ROS seems to be bifaceted and controversial. Indeed, ROS may be important either in eliciting pathological effects leading to mitochondrial dysfunction and cell death ([72, 73], see also below "*Mitochondrial ROS Mediate Autophagy and Apoptosis*"), or play physiological roles promoting positive responses in mitochondrial biogenesis and function. Mitochondrial biogenesis is dependent on the expression of the mitochondrial genome and the nuclear genes that encode mitochondrial proteins [74]. An important pathway triggered by ROS is that leading to the upregulation of the mitochondrial biogenesis master gene PGC-1α. The PGC-1α transcriptional coactivator is a major regulator of energy metabolism [75]. It controls many aspects of oxidative metabolism, including mitochondrial adaptations, insulin-sensitizing *via* the upregulation of selected genes involved in fatty acid β-oxidation, glucose transport, and oxidative phosphorilation [76–79]. The mitochondrial biogenesis signaling activated by PGC-1 members family involves the transcription factors that regulate expression of nuclear genes such as nuclear respiratory factor (NRF) 1/2 and estrogen-related receptor-α (ERR-α). These three latter genes control the expression of nuclear genes encoding mitochondrial proteins and induce expression of mitochondrial transcription factor A (T-fam), which regulates mtDNA replication and transcription, thus activating the coordinated expression of mitochondrial proteins [80, 81].

Several signaling kinases have been involved in mediating PGC-1α transcriptional activation in response to a variety of stimuli among which the most important are calcium/calmodulin-dependent protein kinase (CaMK) type IV [82], AMPK [83], and p38 mitogen-activated protein kinase [84]. Their activation induces the PGC-1α promoter transcriptional regulation [69]. Recently, it has been demonstrated that mitochondrial biogenesis in skeletal muscle is controlled, at least in part, by a redox-sensitive mechanism and that physical exercise, increasing the ROS production over the physiological level, stimulates the muscle PGC-1α/NRF-1/T-fam signaling [85]. Irrcher and colleagues have evaluated the link between ROS levels and PGC-1α gene expression [69] in C2C12 cells. They found that endogenously produced ROS, at least within skeletal muscle cells, are important for the maintenance of PGC-1α expression levels within a normal physiological range. Indeed, quenching basal endogenous ROS with N-acetylcysteine (NAC) results in reduced PGC-1α mRNA expression, an effect which is unrelated to any inhibition of PGC-1α promoter activity, but probably dependent on the enhanced instability of PGC-1α mRNA occurring in a low ROS environment. On the contrary, increasing ROS levels with exogenous H_2O_2 augments PGC-1α transcription indirectly *via* the AMPK activation caused by the oxidatively-induced ATP depletion. This stimulates the binding of USF-1 to an Ebox within the PGC-1α promoter, increases transcription and results in the induction of PGC-1α mRNA expression, whose stability would also be restored in a more ROS-rich environment. The interplay of PGC-1α and ROS is further strengthened by the fact that, besides being a key modulator of mitochondrial biogenesis, it is important in regulating the expression level of protective enzymes acting against ROS generation and damage [86]. Indeed, experiments with either genetic knockouts (KOs) or using RNA interference for PGC1α show that the ability of ROS to induce a ROS-scavenging program depends largely on PGC-1α activity [86]. This response includes genes encoding for antioxidant enzymes localized either within mitochondria (MnSOD) or cytosol (catalase and GPxs). Indeed, cells lacking PGC-1α are more susceptible to the toxicity induced by oxidative stress caused by H_2O_2 [86]. These latter effects of PGC-1α are likely to represent a compensatory response where it plays a central role in the adaptation of cellular energy metabolism, mitochondrial biogenesis and antioxidant capacity in response to oxidative challenge. At this regard and extending previous research from our group [5], we have recently addressed the problem of the role of PGC-1α in C2C12 myoblasts subjected to oxidative stress during the early stages of differentiation. In particular, we examined the effect of a mildly toxic concentration of exogenously added H_2O_2 [0.3 mM] on the regulation of PGC-1α expression and its relationship with AMPK activation (unpublished observations). According to Kang and Irrcher [69, 85], we found that 1 h treatment with H_2O_2 markedly increased PGC-1α mRNA expression. It is of worth that, concurrently, we also found an increased phosphorylation of AMPK as compared to untreated cells, suggesting that oxidative stress induces PGC-1α through the AMPK signaling pathway. However, despite the fact that challenged C2C12 myoblasts rapidly activate a defense-oriented signaling cascade, they displayed a 30–40% reduction of their viability as well as a survivors' reduced differentiative efficiency during the post-challenge incubation stage (up to 7 days of culture). This observation would imply that, besides probably being an obligatory and physiological response to ROS, activation of AMPK and of PGC-1α may not be sufficient to afford a complete protection to cells against an overwhelming oxidative stress. Accumulating or excessive oxidative stress is known to be detrimental for mitochondria: for instance mtDNA represents a critical target for oxidative damage [49]. Indeed, mtDNA mutations are known as being an etiological factor in oxidative stress-related disorders including cardiovascular diseases and inherited or acquired neurodegenerative disorders, mitochondrial myopathies, and the normal aging process.

5. ROS Mediate Autophagy and Apoptosis

ROS may trigger either autophagy or apoptosis: whether these two pathways will be activated depends on the cell context and on the availability of specific modulators of ROS activity [87]. Autophagy is one of the cellular defense mechanisms activated in response to an excessive ROS production. Indeed, ROS act as signaling molecules in the early events of autophagy induction [87]. Phosphoinositide 3 kinase (PI3K) is known to mediate, at least in part, ROS effects. If the prosurvival effort fails, ROS induce cell death which may involve either the autophagic or the apoptotic pathway, or both [72, 88].

ROS signaling pathways play an important role in the induction of autophagy under physio- and pathological conditions. In healthy cells, autophagy is routinely involved in organelles and proteins turnover as well as in cellular energetic balance [89]. One of its strongest and better-characterized stimuli is starvation, where mitochondrial ROS production is enhanced and autophagy increased [87]. Increased ROS generation in the mitochondria under starvation is known to depend, at least in part, by class III PI3K: this event is essential for the induction of autophagy [90]. Indeed, upon starvation, ROS, and in particular H_2O_2, oxidize and inhibit Atg4, a protease responsible for microtubule-associated protein (MAP) light chain 3 (LC3) delipidation, that is, a condition resulting in the stabilization of the lipidated forms of LC3 and promoting the autophagosome maturation [87]. Notably, the same authors reported that addition of antioxidants inhibits these effects, preventing autophagosome biogenesis [91].

Thus, autophagy induced by starvation, where ROS participate in a feedforward manner, plays a prosurvival role since it contributes to the mobilization and reutilization of diverse cellular energy stores [89].

In a different direction, it is also known that when autophagy is prolonged, it could lead to cell death independently from apoptosis [92]. Indeed in nonmuscle tissues and in specific pathological conditions, ROS-induced autophagy was often linked to cell demise and death. As to skeletal muscle, ROS have been implicated in the induction of autophagy in muscle atrophy, disuse, and aging [72, 93]. Important new evidence on the wasting effect induced by increased oxidative stress on muscle phenotype was obtained by targeting a mutant SOD variant found in human amyotrophic lateral sclerosis myopathy [93, 94]. Indeed, these authors created a mouse model with a G93A mutation of SOD1 restricted to skeletal muscle [93]: accumulation of ROS in the muscles of these mice induced progressive atrophy associated with increased autophagy and forkhead transcription factors O (FoxO3) expression, a transcription factor which controls the transcription of autophagy-related genes and is required for the induction of autophagy through the lysosomal pathway in skeletal muscle in the absence of AKT repression [95–97]. In addition, NF-κB signaling has been proposed as an alternative pathway linked to ROS-mediated skeletal muscle atrophy [98]: indeed NF-κB was found to induce muscle atrophy and wasting *via* the lysosomal enzyme cathepsin L [93, 99] upregulation. Since cathepsin L is typically upregulated by FoxO3, it might be speculated that ROS-induced NF-κB converges on the FoxO3 autophagic pathway.

Increasing evidence suggests that autophagy of mitochondria is a selective and defense-oriented response against ROS, mitochondrial dysfunction and the accumulation of somatic mutations of mtDNA with aging [72, 100]. For this reason it has been recently proposed the term "mitophagy" to emphasize the nonrandom nature of this process [100]. Damaged mitochondria are removed by mitophagy by Binp3, a BH3 proapoptotic member of the Bcl-2 family and fis 1, a pro-fission mitochondrial protein that induces mitochondrial fragmentation and enhances the extent of mitophagy. Notably, inhibition/alteration of mitophagy can contribute to myofiber degeneration and weakness in muscle disorders characterized by accumulation of abnormal mitochondria and inclusions [101, 102].

ROS may have various and important roles in apoptotic cell death: direct actions such as oxidation of cellular proteins and lipids, damage of nucleic acids and functional alteration of organelles; ROS may also modulate cell death processes affecting various signaling cascades [103]. Indeed, ROS participate in early and late steps of the regulation of apoptosis, affecting different apoptotic signaling cascades in both intrinsic or extrinsic pathways.

The extrinsic path, which involves stimulation of receptor-mediated apoptotic pathways, can be initiated by ligand-induced (e.g., TNFα and Fas-L and TNF-related apoptosis-inducing ligand, TRAIL) binding, which promotes the activation of caspase-3 and subsequent degradation of genomic DNA [103]. Recent evidence suggests possible direct roles for ROS in mediating death receptors activation and subsequent induction of apoptosis [104]. Indeed, apoptotic signaling is induced by NOX-derived ROS at the plasma membrane level, which lead to lipid raft formation and death receptor clustering activation [104]. The physiological relevance and significance of ROS-dependent receptor-mediated apoptosis as compared to the classical receptor/ligand-induced apoptotic signaling is, at present, incompletely understood and warrants further investigation.

ROS may act as intracellular intermediates directly dysregulating the sarcoplasmic reticulum Ca^{++} flux and handling, which results in caspase-7 and calpain activation. Furthermore, ROS may cause mitochondrial swelling and fragmentation, and/or alter the conformation of the mitochondrial permeability transition pores (MPTPs), thus facilitating their opening and the release of proapoptotic proteins such as cytochrome c (Cyt C). Independently of caspase activity, apoptosis may follow the intrinsic path, where ROS may directly cause the release of mitochondrial endonuclease G (Endo G), and/or of apoptosis inducing factor (AIF), which is capable of promoting DNA fragmentation in skeletal muscle myonuclei [105].

Another protein coupled with ROS-induced apoptosis is the voltage-dependent anion selective channel protein 1 (VDAC1). This transmembrane protein has been defined a ROS sensor [106] that triggers opening of the MPTP complex under conditions of oxidative stress. Indeed VDAC1 is the main channel within the mitochondrial outer membrane and

upon ROS accumulation exhibits an increased conductance associated with MPTP opening and dissipation of $\Delta\Psi$, thus favouring the efflux of apoptotic proteins located in the intermembrane space and finally cell death [107]. Notably, the pro- and antiapoptotic Bcl2-family proteins are released *via* VDAC1 action and ROS may further affect these responses as they are known, in nonmuscle cells, to down-regulate the endogenous levels of the antiapoptotic protein Bcl-2 [108]. The mechanism through which Bcl-2 levels are affected by ROS has been studied by Azad et al. in nonmuscle cell types and seems to depend on superoxide anion-related degradation of Bcl-2 protein through the ubiquitin-proteasomal pathway [109].

Furthermore, under oxidative stressing conditions, ROS activate a signaling cascade involving the protein kinase C (PKC) b-dependent phosphorylation of the Shc adaptor protein p66shc and its translocation to the mitochondrial matrix. In particular, the mitochondrially translocated fraction of p66shc behaves as redox enzyme that utilizes reducing equivalents derived from the mitochondrial electron transport chain to produce H_2O_2 in the intermembrane space, an event which is known to trigger apoptosis [110, 111].

The accumulation of ROS within the mitochondrial matrix, as well as their capacity of triggering apoptosis, is counteracted/regulated by mitochondrial antioxidant enzymes, namely, phospholipids hydroperoxide glutathione peroxidase, GPx, and Mn-SOD [3, 112].

Thus increased mitochondrial production of ROS is involved at multiple levels in promoting apoptosis in skeletal muscle cells, an event which participates in the aetiology and progression of numerous pathologies including sarcopenia and disuse muscle atrophy as well as in aging [71, 113].

Physical training and exercise are known to increase mitochondrial biogenesis and density as well as mitochondrial ROS production especially during repeated contractions [85]. Therefore and unless other determinants are considered, it might appear paradoxical that although a routine of regular exercise is associated with numerous health benefits, physical exercise might potentially promote oxidative stress and ROS-associated apoptosis of skeletal muscle cells [17]. Indeed, chronic contractile activity (CCA) and endurance training induce an adaptive response in skeletal muscle cells leading to increased mitochondrial biogenesis [114] and—theoretically—an obligatory increase in a number of proapoptotic mitochondrial proteins and byproducts such as ROS. However, as a matter of fact recent evidence indicates that mitochondria isolated from rat skeletal muscle subjected to CCA seem to acquire an antiapoptotic, rather than proapoptotic, behaviour [114]. The study also addressed the problem of the relative antiapoptotic role acquired by different mitochondrial subpopulations from CCA-trained muscles, namely, the intramyofibrillar (IMF) and the subsarcolemmal (SS) mitochondria. The release of both Cyt C and AIF caused by exogenous H_2O_2 from CCA-isolated IMF and SS mitochondria was decreased; CCA augmented the expression of antiapoptotic HSP70 and caspase recruitment domain protein in either SS or IMF and caused a decreased ROS generation in IMF mitochondria. On the contrary, states III and IV respiring SS mitochondria showed a modestly increased rate of ROS generation as well as an increased resilience of MPTP opening. It was then hypothesized that these effects might collectively reflect the overall reduced apoptogenic capacity acquired by mitochondria following CCA training of skeletal muscles and that, in particular, the slight increase of ROS generated by SS would contribute to the activation of redox-sensitive transcription factors promoting muscle fiber plasticity and adaptation, rather than to function as proapoptotic triggers. Again, such a scenario is indicative of the diverging effects that ROS may assume depending on specific situations of cells' life, rather than on their net concentration and site of generation.

6. ROS Signaling and Myogenic Differentiation

Increasing evidence indicates that ROS are capable of affecting—mostly reducing—the efficiency of myogenic differentiation. The integrity/alteration of myogenic differentiation is central to many physiological and pathological processes. Successful differentiation of satellite-derived myoblasts into functioning and integrated myotubes is a fundamental prerequisite for muscle regeneration, a repair process which is of primary importance in maintaining muscle function [115]. Notably, oxidative stress is known to play a concausal and detrimental role in a variety of multifactorial muscular pathologies characterized by proliferation/differentiation imbalance such as Duchenne dystrophy [116], myotonic dystrophy [117], sarcopenia [118], and cachexia [119].

The role of ROS in this context has been extensively documented. Ardite et al. [120] showed that ROS induced a strong depletion of the intracellular GSH pool: notably depletion of GSH causes further intracellular accumulation of ROS which favors NF-κB activation, thus contributing to the lower expression of MyoD and impaired myogenesis (see below).

According to Ardite et al. [120], data from our group [5] indicate that a mildly toxic H_2O_2 treatment during the early stages of C2C12 myoblast differentiation results in GSH depletion and strongly impairs the differentiative outcome. This effect is unlikely to be a mere result of ROS-induced cell demise: indeed, the cells surviving H_2O_2, although exhibiting a partial and late recovery of protein synthesis and of viability, were unable to continue and execute the differentiative task. These cells also displayed a strong and long-lasting reduction of the mRNA levels of MyoD, which is involved in early stem cell commitment, and of myogenin and MRF4, both recruited at later differentiation times [121, 122]. Whether the transcription of these muscle regulatory factors (MRFs) is a result of a specific signaling promoted by ROS or of a cell suffering is still to be understood. Under the same conditions depressing these MRFs, insulin-like growth factor 1 (IGF-1) which plays a pivotal role in controlling muscle growth [123], was inhibited to an even greater extent (see also "ROS and IGF-1 signaling"). Interestingly, H_2O_2-injured cells showed signs of extensive mitochondrial degeneration (swelling and disruption) and lower mitochondrial density, suggesting that these organelles

are specifically targeted by—or particularly sensitive to—exogenous ROS. Loss of mitochondria is a clearly detrimental event in a process typically requiring active mitochondriogenesis such as muscle differentiation [4, 124].

ROS generated by the inflammatory cytokine TNFα are known to inhibit myogenesis, and this effect is widely attributed to oxidative activation of NF-κB and subsequent gene expression [125–127]. However, the effect of TNFα is likely to be more complex since Langen et al. [9] showed that TNFα causes loss of myogenic capacity of C2C12 cells *via* NF-κB-dependent and-independent and oxidative-sensitive and-insensitive pathways. In particular they hypothesized that an oxidative-sensitive, NF-κB-independent mechanism might involve the blockage of the formation of functional catenin-adherin complexes proximate to the cell membrane [128]. Potentially, disruption of these complexes and the resulting alteration of cell-matrix and cell-cell interactions, might be responsible for the inhibition of myotube formation independently of NF-κB.

The redox regulation of the NF-κB family of transcriptional activators plays a central role in differentiation, adaptation, and death of muscle cells. This role is extremely complex: indeed the effects promoted by NF-κB are sometimes contradicting. As an example, although ROS can directly stimulate NF-κB, oxidized NF-κB has a diminished DNA-binding activity [17]. NF-κB has been mostly associated with a negative regulation of skeletal muscle differentiation [119, 129, 130]. NF-κB is constitutively active in proliferating myoblasts and can inhibit myogenesis by promoting a mitogenic activity *via* cyclin D1 or by inhibiting the synthesis of MyoD, a muscle-specific helix-loop-helix transcription factor operating in muscle development and repair [131–133]. More recently, NF-κB was shown to suppress myofibrillar gene expression through the regulation of the myogenic transcriptional repressor Yin Yiang 1 [134]. Moreover, treatment of primary myoblasts with the NF-κB inhibitor curcumin stimulates myoblast fusion thereby enhancing myogenesis and repair [125]. In line with these *in vitro* findings, activation of the TNFα pathway by muscle gene transfer inhibits regeneration *in vivo*, while muscle-specific deletion of the heteromeric kinase complex IKK was recently described to promote secondary myogenesis in response to acute injury signals [126, 127]. Activation of NF-κB downstream ROS formation is also capable of stimulating the activity of inducible nitric oxide synthase (iNOS), whose role in myogenic process is controversial [24]. Some authors found that iNOS activity suppresses muscle differentiation, whereas others reported that stimulation of iNOS *via* NF-κB represents a positive and necessary stimulus for muscle differentiation, that iNOS activity paralleled myogenesis from the early to later stages in H9C cells and that ROS formed by NOX 2 were the basic trigger leading to iNOS stimulation *via* NF-κB recruitment [24, 135, 136]. Blockage of this pathway, or inhibition of iNOS with specific inhibitors, led to differentiative arrest. Also, a recent article by Lee et al. [137] indicates that complex-I-derived superoxide anions, produced through reverse electron transport, were dismutated into H_2O_2 by MnSOD induced *via* NF-κB activation and that H_2O_2 stimulated muscle differentiation as a signaling messenger. Thus the scenario arising from these results would indicate that ROS negatively or positively regulate muscle differentiation *via* the signaling pathways involving NF-κB activation.

Another evidence which lends support to the detrimental role of ROS in muscle differentiation comes from the studies on the role of p66Shc in skeletal muscle ischemic injury. p66Shc, along with its isoforms p46 and p52, constitutes the mammalian Shc adaptor protein group. The three isoforms share a common structure, but p66ShcA has the unique feature of an additional domain at the N terminus which contains a serine residue at position 36 (Ser-36) that is phosphorylated in response to several stimuli, including H_2O_2. Due to this feature p66 isoform regulates ROS metabolism and apoptosis [138, 139]; indeed, a fraction of p66ShcA is localized in the mitochondria where, as discussed above, it produces mitochondrial ROS as signaling molecules for apoptosis [110, 111]. Interestingly, both p66ShcA KO cells and mice display lower levels of intracellular ROS [139–141] and are less prone to apoptosis induced by an array of different stimuli. Also, p66Shc KO mice are resistant to ischemia-induced apoptosis and show decreased muscle damage in response to hind limb ischemia [142]. More recently, Zaccagnini et al. [143] unravelled the role of p66Shc and ROS in muscular damage and regeneration following acute hind limb ischemia in both WT and p66Shc KO mice. WT mice showed detectable levels of oxidative stress markers during the postischemic and regenerative stages; on the contrary, the same markers were undetectable in KO mice. More interestingly, although the initial ischemic damage was identical and no advantage in terms of muscle vascularization and perfusion was observed in KO mice, their regenerative capacity was significantly higher as compared to WT. Satellite cell populations were similar in both groups, but those from KO mice showed a higher proliferation rate at first and spontaneous differentiation when cultured under prodifferentiative conditions. Finally, p66Shc KO satellite cells were resistant to the myogenic inhibition induced by H_2O_2 acute challenge or hypoxia. The authors proposed different and possible explanations for the above effects. The first one involves the different availability of NO—whose promyogenic role has been discussed above—in KO mice: since active p66Shc generates superoxide anions, which consume available NO forming the toxic species peroxynitrite, p66Shc KO mice would benefit of higher NO availability and would not suffer of peroxynitrite toxicity, two effects favouring myogenesis and muscle regeneration. Another plausible mechanism involves the NAD^+-dependent histone deacetylase Sir2. Sir2 deacetylase activity is dependent on the fluctuation of cytosolic NAD^+/NADH ratio, that is, the cellular redox state [144]. Under conditions of high ROS concentrations, NAD levels increase and promote Sir2 activation, which in turn inhibits MyoD-dependent transcription. p66Shc KO mice are characterized by lower levels of ROS and, as a result, decreased Sir2 activity, that is a condition which affects MyoD functions to a lesser extent. Finally, since oxidative DNA damage may trigger a differentiation checkpoint and cause a reversible inhibition of myogenic differentiation targeting MyoD phosphorylation,

such a checkpoint activation may be attenuated by p66ShcA deletion, which results in decreased intracellular ROS levels.

With regard to prodifferentiative effects induced by ROS in this context, in addition to the already cited report by Lee et al. [137], it has been recently demonstrated that in a non skeletal-muscle cell, that is, vascular smooth muscle cells (VSMC), ROS increase their differentiation rate after quiescence through a p38 MAPK-dependent pathway [145]. Similarly, other studies focusing on ROS and muscle metabolism, differentiation, and growth unravelled some positive interactions with IGF-1 signaling (see below).

Again, the most likely explanation for these opposite effects is that cell fate may depend on the intracellular ROS type (i.e., which is the prevailing reactive species) and level. In fact, it is well known that ROS elicit a wide spectrum of cellular responses, depending on their intracellular level [146]. A low dose of ROS controls normal cellular signaling pathways while an intermediate dose results in either temporary or permanent growth arrest [147]. Obviously, a high dose of ROS causes cell death *via* either apoptotic or necrotic mechanisms [142].

7. ROS and IGF-1 Signaling

Growing evidence suggests that oxidative stress is responsible, as a causal or a concausal factor, for the pathogenesis of many muscle diseases and muscle wasting [148, 149]. In muscle cells, IGF-1 is known to promote muscle welfare inducing muscle hypertrophy and stimulate muscle-cell proliferation, differentiation, and survival [123]. IGF-1 has also been found to contribute to oxidative balance and to mediate protective responses against iron-induced-lipid oxidative stress *in vivo* [150]. Accordingly, Yang and colleagues [151] demonstrated that IGF-1 displayed protective effects on muscle cells after oxidative stress: indeed, pretreatment with IGF-1 protected muscle cells from H_2O_2-induced cell death and enhanced their survival through promotion of the antiapoptotic protein Bcl2. The same authors showed that protection was *via* an IGF-1 subpathway: PI3K/Akt and ERK1/2 MAPK pathways [151].

IGF-1 is a peptide hormone with a complex post-trascriptional regulation, generating distinct isoforms, namely, IGF-1Ea, IGF-1Eb, and IGF-1Ec (this latter also known as mechano growth factor, MGF) [152]. Mouse models have provided insights into the tissue-specific functions and responses to ROS of the different IGF-1 isoforms [152–155]. For example, in murine models, the local muscle isoform of IGF-1 (mIGF-1, the orthologue of human MGF) has been shown not only to activate proliferation of myoblasts [156], but also to protect cardiomyocytes from oxidative stress *via* the Sirtuin 1 deacetylase activity [157].

As to physical activity, although its role in regulating the expression of specific IGF-1 isoforms has been widely studied, data in the literature regarding humans are often contradictory and are affected by many uncontrolled variables such as the lack of dietary control, heterogeneity of subjects, their physical fitness, differences in proposed physical exercise, and time course of sampling [158–160].

Similarly to other pathways, ROS may regulate either positively or negatively IGF-1 signaling [161]. Low levels of endogenous ROS—due to their reversible oxidative inhibition of protein tyrosine phosphatases (see also "ROS as multipurpose local regulators of muscle cell functions")—induce the phosphorylation on specific tyrosine residues of insulin receptor (IR) and IR substrates (IRS) protein(s), thus facilitating the IGF-1 signaling. Indeed, the IRβ chain contains multiple sites for the phosphorylation of tyrosine that are sensitive targets of ROS such as H_2O_2 [162]. By contrast, higher ROS levels inhibit IGF-1 signaling cascades and recent evidence implicates ROS as downregulators of IGF-1 signaling and inducers of insulin resistance and its pathological sequelae [162].

However, ROS may be also involved in the activation of "insulin-like" metabolic effects by activating other non-insulin-initiated signaling pathways: one of the most important examples is the stimulation of glucose transport in skeletal muscle during exercise [163, 164]. Skeletal muscle contraction stimulates, as well as insulin, glucose transport by up to 50-fold during maximal exercise in humans [165]. Adding exogenous ROS to skeletal muscle *in vitro* stimulates glucose transport [166] whereas NAC, a potent antioxidant, reduces contraction-mediated glucose uptake by about 50% [167]. This effect of NAC was associated with a similar degree of inhibition of contraction-induced activation of AMPK. This kinase is a fundamental signaling kinase which, besides being involved in mitochondrial biogenesis (see "*ROS signaling and myogenic differentiation*"), is also known to upregulate the glucose uptake in muscle under conditions of high AMP/ATP ratio, like hypoxia and muscle contraction, forming a non-insulin-dependent pathway to increase muscle glucose utilization [158, 168–172]. Thus the proposed role of ROS in mediating the stimulation of glucose transport is related to skeletal muscle contraction, that increases superoxide anion production *via* mitochondrial respiration. Superoxide anion is rapidly converted to H_2O_2 by SOD, resulting in direct activation of AMPK, Glucose transporter 4 (Glut4) translocation to the plasma membrane, and an increase in glucose transport [173]. Moreover, in muscle cells, NAC antagonized ROS-mediated increase in glucose uptake in response to contraction, but not to insulin. Activation of AMPK in aerobic-exercise-induced glucose uptake is paradigmatic of ROS participation in physiologically-oriented signaling pathways relevant to the homeostasis of the entire organism.

It has also been demonstrated that ROS regulate IGF-1-induced myotube hypertrophy *in vitro*. It is well known that exercise-induced muscle hypertrophy mostly depends on the increased local production of IGF-1 *via* activation of the PI3K/Akt pathway [174, 175]; interestingly ROS, which are being overproduced during exercise, contribute in a feedforward manner to stimulate IGF1 net accumulation.

Previous reports show that there are positive and negative interactions between ROS and IGF-1 synthesis in both skeletal and VSMCs [176–178]. Treating VSMCs with H_2O_2 or XO augments both IGF-1 mRNA and IGF-1 protein secreted into the cultured medium, indicating that ROS enhance the IGF-1 autocrine system in VSMCs [176]. By

contrast, we and others [5, 178] found that toxicologically relevant concentrations of H_2O_2 negatively regulate the IGF-1 mRNA levels in differentiating C2C12 myoblasts. In our experience, oxidative insult significantly decreased IGF-1 mRNA expression levels [5]. Cr, notably, prevented its inhibition: moreover Cr is known to induce hypertrophy of differentiating myoblasts *via* IGF-1 pathway [123].

Taken collectively, these results suggest that—although ROS enhance IGF-1 signaling—there is a negative feedback regulation of IGF-1 mRNA levels occurring with mildly toxic ROS levels in C2C12 cells. Thus, ROS regulate IGF-1 action *via* a variety of mechanisms, and the effects are likely, again, to be cell type and dose dependent.

Thus, ROS play a crucial role in the IGF-1 signaling regulation and its biological action in muscle cells. However, additional studies are necessary to better explain the physiological significance of these interactions in humans, with particular regard to the identification of the distinct actions on the IGF-1 propeptide isoforms.

8. ROS as Multipurpose Local Regulators of Muscle Cell Functions

Similarly to other noninflammatory cells, skeletal muscle cells produce transient fluxes of ROS in response to an array of diverse stimuli, such as intense contractile activity [179, 180], heat stress [181], short-term disuse atrophy [182], acute hypoxia [143, 183], acute osmotic stress [184], and stretch [185]. Furthermore, locally produced waves of ROS are also released by skeletal muscle in response to cell surface receptor activation *via* cytokines, hormones, growth factors [186–188], or nuclear receptor activation [189, 190]. Considering the large variety of different stimuli converging to ROS production along with their lack of chemical specificity, it is hard to formulate a unitary explanation of the physiological significance of ROS in the responses triggered by such divergent signals [188]. At this regard, data published by Wright et al. [191] prompted these authors to draw an attractive hypothesis which involves the regulation of the protein phosphatases (PPases) "tone" in muscle cells and tissue. PPases belong to two broad families, the protein tyrosine PPases (about 112 human proteins) and the serine/threonine PPases (about 31 proteins). These two families are divided into further subclasses according to their specificity (only tyrosine targets or tyrosine plus serine/threonine targets) or, with regard to the second family, the subclasses characterized by a Zn^{2+}/Fe^{2+} complex at the catalytic site or by the Mn^{2+}/Mg^{2+} dependence [191].

The redox sensitivity of the protein tyrosine PPases and its potential biological importance is well documented *in vitro* and in cell culture systems since the early 1990s [192, 193]. As to Ser/Thr PPases, their sensitivity to oxidants is more controversial: calcineurin is the first whose sensitivity to oxidants has been clearly identified [194–196].

Interestingly, Wright et al. [191] found that not only protein tyrosine PPases, but also Ser/Threo PPases are inhibited by exposure to ROS or ROS generating agents (namely, H_2O_2 and DMNQ, resp.). The relative sensitivity of different PPases to oxidation in the above scenario has not yet been addressed. The mechanism by which PPases are oxidized is likely to involve the vulnerability of their ubiquitous and conserved cysteine-based active site; more surprising and still unexplained is the observed inhibition of ser/threo PPases which—with the exception of calcineurin—are best known in literature as "relatively immune to oxidation".

Indeed, the same study by Wright et al. [191] shows that in muscle tissue even minimal, physiologically relevant concentrations of oxidants, lead to an overall inhibition of PPases' activity. Notably, the concentrations used neither affected contractile function nor resulted in clear oxidative stress. Consistently, the level of net phosphorylation of a wide range of functionally diverging proteins was correspondingly higher in treated muscle preparations. This latter data suggests that oxidants are capable of affecting a broad range of PPases. Interestingly the majority of kinases are equally sensitive to oxidants but, contrary to PPases, oxidants promote their activation. Since kinases operate sequentially as amplification chains, it is likely that the observed increase in the net protein phosphorylation level under low-oxidative stressing conditions is the result of a lower PPases activity along with an increased kinases activity. These two combined events promoted by ROS would obviously trigger and/or affect many different signaling pathways, contributing to orchestrate the final cellular responses.

In summary, oxidants could function to regulate *in vivo* global "phosphatase and kinase tone" and thus influence the kinetics and amplification of many kinase signaling pathways. With respect to skeletal muscle, such a scenario would be of great biological and physiopathological relevance, since muscle cells typically and continually produce ROS fluxes of different duration, intensity, and localization, depending on either intrinsic and extrinsic variables. Notably, such a hypothesis fits well with the hormetic nature of ROS.

9. Conclusion

The picture arising from this review indicates that ROS activate and/or participate in many signaling pathways promoting complex and diverging effects in skeletal muscle cells, ranging from positive to detrimental. As an example, many studies have concluded that inactivity-induced ROS production in skeletal muscle contributes to disuse muscle atrophy [197, 198]. On the contrary, growing evidence also suggests that intracellular ROS production is a required signal for the normal remodelling that occurs in skeletal muscle in response to repeated bouts of endurance exercise [40, 199, 200]. How can the same trigger promote such opposite effects? Based upon current knowledge, it appears that the mode and the situation characterizing skeletal muscle cells exposure to ROS may account, at least in part, for this apparent paradox. Transiently increased, moderate levels of oxidative stress might represent a potentially health-promoting process, whereas its uncontrolled persistence and/or propagation might result in overwhelming cell damage thus turning into a pathological event: for instance, the role of ROS in inflammation fits well with this model. In addition, the complexity, the variety, the interplay, and the functionally diverging roles of the signaling pathways

activated or modulated by ROS contribute to further complicate this scenario. Thus, a gradual and variable, rather than a sharp, boundary is likely to characterize the transition between the two types of ROS actions. Such a variable "greyscaling" of ROS effects may depend on extrinsic and intrinsic situations such as, at least, (i) the concentration of ROS, (ii) the type of reactive species involved, (iii) the persistence of ROS activity, (iv) the localization of ROS source, (v) the antioxidant capacity and the energy status of muscle cells, (vi) their ability to adapt to oxidative stress (which *in vivo* also depends on ageing and/or physical training), (vii) the differentiative status, for example, myoblasts versus integrated myotubes, (viii) the absence/presence of an inflammatory process, and (ix) the plasticity of the signaling pathways triggered/affected by ROS. The balance between these factors will ultimately determine which type of signal(s) and effect(s) will prevail within the cell. Again, the hormetic nature of ROS emerges as the key feature of these species in many tissues, including skeletal muscle. Careful titration of ROS levels within skeletal muscle cell may therefore lie at the cross between the initiation and progression of disease and cell death, the induction of mitochondrial biogenesis, repair, and more generally cellular metabolic health. Supplementation with exogenous antioxidants is being widely studied to attain and maintain an "ideal titration" of ROS within skeletal muscle: unfortunately, at the present, no clear indication of the benefits arising from supplemental antioxidant intake emerges from literature. This reflects the need for further studies aimed at clarifying how to regulate ROS levels to exploit their physiological effects and avoid their damages.

Abbreviations

AMPK: Adenosine monophosphate-activated protein kinase
Bcl-2: B-cell lymphoma 2
FoxO: Forkhead box O
GLUT4: Glucose transporter type 4
MAPK: Mitogen-activated protein kinase
NF-κB: Nuclear factor kappa B
PGC-1α: Peroxisome proliferator-activated receptor gamma coactivator 1 alpha
PPases: Phosphatases
ROS: Reactive oxygen species
SOD: Superoxide dismutase.

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Review Article

The Interplay between ROS and Ras GTPases: Physiological and Pathological Implications

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The members of the RasGTPase superfamily are involved in various signaling networks responsible for fundamental cellular processes. Their activity is determined by their guanine nucleotide-bound state. Recent evidence indicates that some of these proteins may be regulated by redox agents. Reactive oxygen species (ROSs) and reactive nitrogen species (RNSs) have been historically considered pathological agents which can react with and damage many biological macromolecules including DNA, proteins, and lipids. However, a growing number of reports have suggested that the intracellular production of ROS is tightly regulated and that these redox agents serve as signaling molecules being involved in a variety of cell signaling pathways. Numerous observations have suggested that some Ras GTPases appear to regulate ROS production and that oxidants function as effector molecules for the small GTPases, thus contributing to their overall biological function. Thus, redox agents may act both as upstream regulators and as downstream effectors of Ras GTPases. Here we discuss current understanding concerning mechanisms and physiopathological implications of the interplay between GTPases and redox agents.

1. Introduction

The Ras GTPase superfamily includes low molecular weight GTP-binding and hydrolyzing (GTPases) proteins that act as molecular switches by coupling extracellular signals to different cellular responses, thus controlling cellular signaling pathways responsible for growth, migration, adhesion, cytoskeletal integrity, survival, and differentiation. The three human Ras proteins, H-Ras, N-Ras, and K-Ras, are the founding members of this large superfamily of small GTPases comprising over 150 human members with evolutionarily conserved orthologs found in *Drosophila, C. elegans, S. cerevisiae, S. pombe, Dictyostelium,* and plants. This superfamily is divided into families and subfamilies on the basis of sequence and functional similarities (Table 1). The five major families are Ras, Rho, Rab, Arf, and Ran [1]. In addition to the different Ras isoforms, the Ras family includes Rap, R-Ras, Ral, and Rheb proteins, also regulating signaling networks. Rho GTPase family includes the well-characterized family members Rac1, RhoA, and Cdc42, each of which is associated with unique phenotypes and functions [2–4]. Rab proteins comprise the largest branch of superfamily and regulate intracellular vesicular transport and trafficking of proteins. Like the Rab proteins, Arf family proteins are involved in regulation of vesicular transport. The Ran protein is the most abundant small GTPase in the cell and is best known for its function in nucleocytoplasmic transport of both RNA and proteins [1].

Although being similar to the heterotrimeric G protein α subunit in biochemical mechanism and function, Ras GTPases function as monomeric GTP-binding proteins. The functional diversity of these proteins is based on variations in structure, posttranslational modifications that dictate specific subcellular localizations, and proteins that act as regulators and effectors [1, 5].

Signal transduction through Ras proteins occurs by reversible binding of GTP, while the inactive form is bound to GDP. Switching between these two states is regulated by three distinct types of protein modulator agents: Guanine nucleotide Exchange Factors (GEFs) catalyze the exchange of GDP with GTP to promote Ras activation, whereas GTPase-Activating Proteins (GAPs) deactivate the Ras protein by

Table 1: The Ras superfamily of small GTPases. The RasGTPase superfamily is divided into 9 families of small GTP-binding proteins on the basis of sequence and functional similarities (modified from [7]).

Ras	Rab	Rho	Sec	Arf	Rad	Ran	RheS	Rit
H-Ras	Rab1A	RhoA	N-Sec1	Arf1	Rad	Ran/TC4	Rhes	Rit
K-Ras	Rab1B	RhoB	S-Sec1	Arf2	Gem	Dexras1	Others	Rin
N-Ras	Rab2	RhoC	Sec4	Arf3	Kir	Others		Ric
E-Ras	Rab3A	RhoD	Sly1p	Arf4	Rem1			Others
TC21	Rab3B	RhoE	Others	Arf5	Rem2			
R-Ras	Rab4	Rnd1		Arf6	Others			
M-Ras	Rab5A	Rnd2		Arf7				
Rap1A	Rab5B	RhoG		Others				
Rap1B	Rab6	Rac1						
Rap2A	Rab7	Rac2						
Rap2B	Rab8	Rac3						
RalA	Rab9	Cdc42						
RalB	Rab10	TC10						
Others	Others	TTF						
		Others						

stimulating hydrolysis of bound GTP to GDP. Deactivation can also be achieved by association with Guanine Nucleotide Dissociation Inhibitors (GDIs), which prevent membrane association, and GDP dissociation. All of these regulatory proteins are themselves affected by diverse upstream signals, which serve to activate or inactivate Ras GTPase signaling pathways. The transition of Ras proteins between the GDP- and GTP-bound states is accompanied by a conformational change that greatly enhances their affinity for downstream effectors [6]. The interaction between the active GTP-bound GTPase and the effector molecule leads to activation of downstream signal transduction pathways.

In addition to these protein regulatory factors, many of the Ras superfamily small GTPases have been shown to be redox sensitive, and their known conserved redox-sensitive sequences have been termed the NKCD, GXXXXGK(S/T)C, and CGNKXD motifs. The action of redox agents on these redox-sensitive GTPases is similar to that of guanine nucleotide exchange factors in that they perturb GTPase nucleotide-binding interactions that result in the enhancement of the guanine nucleotide exchange of small GTPases [7].

For many years, the generation of intracellular redox agents such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) was viewed solely as the unregulated by-product of aerobic metabolism and other enzymatic processes, and redox agents have been historically considered pathological agents which can react with and damage many biological macromolecules including DNA, proteins, and lipids. However, over the last years a growing number of reports have suggested that mammalian cells can rapidly respond to ligand stimulation with a change in intracellular ROS thus indicating that the production of intracellular ROS is tightly regulated and that these redox agents serve as intracellular signaling molecules being involved in a variety of cell signaling pathways, including growth factor signaling [8, 9], inflammation [10], engagement of integrins [11, 12], and adhesion to extracellular matrix [13]. The precise means of regulation is not completely understood. However, numerous observations have suggested that the Ras GTPases appear to regulate ROS production and that oxidants function as effector molecules for the small GTPases, thus contributing to their overall biological function [14].

Here we discuss current understanding concerning the interplay between GTPases and redox agents. The discussion also takes into account pathological implications of alterations of both ROS regulation by small GTPases and small GTPases regulation by ROS.

2. ROS Regulation by RasGTPases

Among the major source of ROS, NADPH oxidases have been demonstrated to play a fundamental role in the compartmentalization of ROS production and redox signaling [15]. Besides NADPH oxidase, an important role in the spatio-temporal regulation of ROS production is also played by enzymes involved in arachidonic acid (AA) metabolism, such as phospholipase A_2 (PLA_2), lipooxygenases (LOXs), and cyclooxygenases (COXs), suggesting that a complex regulatory network may take place for proper modulation of redox signaling [16].

The NADPH oxidase (NOX) complex was originally identified in phagocytic leukocytes as an enzymatic defense system against infections required for the oxidative burst-dependent microbial killing [17–19]. It is composed of membrane-associated and cytosolic components, which assembly to form the active NOX enzymatic complex in response to appropriate stimuli. Specifically, this complex consists of membrane-associated cytochrome b558, comprising the catalytic $gp91^{phox}$ (also known as NOX2) and regulatory $p22^{phox}$ subunits, and four cytosolic regulatory components, including $p40^{phox}$, $p47^{phox}$, $p67^{phox}$, and the small GTPase Rac1 [17]. The neutrophil expresses two different Rac isoforms, including the phagocyte-specific Rac2 and the more ubiquitously expressed Rac1. Detailed

molecular analysis has revealed that Rac proteins function as a necessary switch for ROS generation and that the protein is recruited to the membrane following neutrophil activation where it can bind to both $p67^{phox}$ and $gp91^{phox}$ [20].

Many evidences suggest that certain aspects of neutrophil biology appear to be conserved in the ROS signaling of nonphagocytic cells. In particular, homologues of the NADPH oxidase were found in vascular endothelial cells and smooth muscle cells, as well as in other normal or transformed cells such as colon cancer or melanoma [21]. Several isoforms of the catalytic NOX2 protein were identified, including NOX1, NOX3, NOX4, and NOX5, and shown to localize in proximity of specific redox-sensitive molecular targets within discrete subcellular compartments, thereby facilitating the compartmentalization of redox signaling [15]. In addition, the expression of a constitutively activated form of Rac1 was noted to increase the basal level of hydrogen peroxide in immortalized fibroblasts [22] as well as in certain transformed cell lines [23], while the expression of a dominant negative form of Rac1 was shown to inhibit the production of ROS following addition of various ligands [22]. These data suggest that a Rac-regulated oxidase exists in a wide range of cell types and participates in normal signal transduction.

It has been shown that NOX1 constitutively binds the RacGEF βPIX, and the interaction is caused by growth factor stimulation [24]. This and previous studies [25] also support a pathway where ligand addition results in the sequential activation of phosphatidylinositol 3-kinase (PI3K), which in turn generates lipid products that can activate GEFs through the PH (pleckstin homology) domain present within the exchange factors. Activation of the GEF leads to increased Rac activity that is presumed to directly stimulate NOX [14].

The hypothesis that ROS generation is regulated by Rac and the role of ROS as specific effector molecules that act downstream of Rac is supported by several evidences. In a recent paper there has been shown a role of Rac-regulated ROS in the crosstalk between G-protein-coupled receptors (GPCRs) and the JAK/STAT pathway [26], while different studies support a role of Rac1 as a crucial, common upstream mediator of ROS production in integrin-mediated outside-in signaling [11–13, 16, 27].

Several evidences have implicated ROS in the integration of signals from VEGF and Rac to regulate the integrity of the endothelial barrier [22, 28–32]. Further studies demonstrated that the VEGF-dependent phosphorylation of VE-cadherin and β-catenin are dependent on Rac and ROS and result in decreased junctional integrity and enhanced vascular permeability [33, 34].

In addition to NADPH oxidase, Rac1 has been demonstrated to act upstream of AA-metabolizing enzymes, such as PLA_2 [35, 36], 5-LOX [13, 26, 27], and COX-2 [37], whereas many reports show that AA metabolism modulates NADPH oxidase and mitochondrial ROS production [16].

Another aspect of oxidant signaling derived from the initial observation that Rac proteins regulate ROS levels is the demonstration of redox-dependent crosstalk between different small GTPase family members.

ROS production is apparently an essential component in signaling cascades that mediate Rac1/p190RhoGAP-induced downregulation of RhoA and concomitant formation of membrane ruffles and integrin-mediated cell spreading. The pathway linking generation of ROS to downregulation of Rho involves inhibition of the low-molecular-weight protein tyrosine phosphatase (LMW-PTP) and a consequent increase in the activation by phosphorylation of the Rho inhibitor p190Rho-GAP [38]. It has been shown that ROS production causes p190RhoGAP translocation to the adherens junctions (AJs), where it binds p120ctn, and subsequently inhibits local Rho activity [39]. It thus plays a role in the stabilization of cell-cell contacts [34].

These findings suggest that Rac1 downregulates Rho and stress fiber formation in a redox-dependent manner and define a mechanism for the coupling of changes in cellular redox state to the control of actin cytoskeleton rearrangements by Rho GTPases.

In addition to Rac, the production of ROS by nonphagocytic cell types stimulated by growth factors or cytokines includes the participation of p21Ras [22]. Fibroblasts expressing constitutively active mutants of both Rac and Ras produce high levels of ROS associated with a high rate of proliferation. In the same study experimental evidence was provided suggesting that Rac is positioned downstream to Ras. Similar overexpression of Ras in other cell types such as keratinocytes [40] and epithelial cells [41] also demonstrated an increase in basal ROS levels. The pathway by which Ras regulates the levels of ROS remains incompletely understood. It has been shown that in some cells it proceeds through a PI3K and Rac-dependent pathway [25] leading to the regulation of a NOX-dependent oxidase. In other cell types the source of Ras-induced ROS appears to be linked to the mitochondria [42].

Mitochondria have the highest levels of antioxidants in the cell and play an important role in the maintenance of cellular redox status, thereby acting as a ROS and redox sink and limiting NADPH oxidase activity. However, mitochondria are not only a target for ROS produced by NADPH oxidase but also a significant source of ROS, which under certain conditions may stimulate NADPH oxidases. Many findings indicate the existence of a bidirectional signaling crosstalk between mitochondria and NADPH oxidase, where small GTPases can orchestrate a complex web of regulation for ROS production [43–45].

Indeed, in integrin signaling, the regulation of mitochondria by both Rac and RhoA appears to be related to their ability to alter intracellular ROS [12].

It has been shown that Nerve Growth Factor- (NGF-) induced differentiation of PC12 cells is mediated by significant alteration of mitochondrial metabolism by reducing mitochondrial-produced ROS and stabilizing the electrochemical gradient. This is accomplished by stimulation of mitochondrial manganese superoxide dismutase (MnSOD) via Ki-Ras and ERK1/2 [46].

Thus ROS produced by small GTPases could regulate mitochondrial properties, including the overall metabolic rate and the generation of mitochondrial oxidants with important signaling functions within the cell [14].

3. RasGTPase Regulation by ROS

Although several studies implicate RasGTPases in the production and regulation of intracellular ROS, many evidences indicate that Ras proteins can also be direct targets of ROS. Similar to the action of GEFs, various redox agents, including both ROS and RNS, have been shown to stimulate Ras guanine nucleotide dissociation *in vitro* and upregulate Ras function *in vivo*.

Lander and coworkers showed for the first time that NO is able to activate Ras by promoting RasGDP dissociation *in vitro*, GTP binding to Ras *in vivo*, and stimulation of pathways downstream to Ras [47–54]. The target site of NO-mediated guanine nucleotide dissociation on Ras is Cys^{118}, which is located in the nucleotide-binding NKCD motif [49, 50, 54, 55]. Further studies indicated that $\bullet NO_2$, a reaction product of NO with O_2, reacts with the Ras Cys^{118} thiol to induce a radical-based process leading to stimulation of nucleotide exchange on Ras [56, 57]. In addition to NO, $O_2^{\bullet -}$ showed to be able to facilitate guanine nucleotide dissociation from Ras as well as the Ras-related GTPase Rap1A. The molecular mechanism of $O_2^{\bullet -}$-mediated guanine nucleotide dissociation is similar to that of the NO/O_2-mediated guanine nucleotide dissociation [58]. The redox-sensitive NKCD motif has been found within the Ras subfamily of GTPases such as H, N, K, and E-Ras as well as in Rap1A [7].

Redox-active motifs were afterwards found to be present in other Ras superfamily GTPases, suggesting that redox regulation of GTPase signaling is more widespread that previously envisioned [59].

The GXXXXGK(S/T)C redox-sensitive motif, located in the phosphoryl-binding loop important for redox-mediated regulation of guanine nucleotide exchange activity *in vitro,* was identified and characterized in the Rho family GTPases. This motif contains a redox-sensitive cysteine (Cys^{18}, Rac1 numbering) at the C-terminus and it is conserved in almost half of Rho family GTPases such as Rac1 (and its isoforms Rac2 and 3), Cdc42, and RhoA (and its isoforms RhoB and C) [59, 60]. The radical-based molecular mechanism of Rho GTPase guanine nucleotide exchange appears similar in nature to the mechanism characterized for Ras GTPases.

An *in vivo* study aimed to analyze the effect of exogenous and endogenous ROS on the activation of RhoA in fibroblasts was performed by Aghajanian and coworkers [61]. This study showed that RhoA can be directly activated by ROS in cells by oxidative modification of critical Cys residues within the redox-active motif, and that ROS-mediated activation of RhoA can induce cytoskeletal rearrangement, thus supporting the existence of a novel mechanism of regulating GTPase signaling cascades, independent to classical regulation by GEFs and GAPs, that can affect cytoskeletal dynamics [61].

A number of Rab proteins also have the GXXXXGK(S/T)C motif (Rab1B, Rab2A/B, Rab4A/B, Rab14, Rab15, Rab19, and Sec4). Intriguingly, many Rab GTPases (Rab1A, Rab8A/B, Rab10, and RAb13) possess both the NKCD and GXXXXGK(S/T)C motifs, whereas some Rab proteins (Rab3A/B/C/D, Rab 7, Rab22, and Rab38) possess only the NKCD motif [7].

A CGNKXD redox-sensitive motif was found in Ran protein [62]; this motif contains a redox-sensitive cysteine, Cys^{120}, at the N-terminal. In addition to this CGNKXD motif, Ran possesses an additional redox-sensitive cysteine Cys^{85} (Ran numbering). This type of redox center is also conserved in Dexras1 and Rhe proteins as well as in some Rab GTPases [62].

Although redox regulation of the members of Rab and Ran families has been recently discovered, its physiological relevance and pathological consequences linked to the misregulation of redox signaling associated with these redox sensitive small GTPases have not yet been explored [7].

4. Pathological Implications of the Interplay between Small GTPases and ROS

Over the past several years, it has become clear that ROSs play an important role in physiological processes like cell differentiation, proliferation, migration, and vasodilatation. On the other hand, production of ROS "in the wrong place at the wrong time" results in oxidative stress leading to cellular dysfunction and apoptosis, which contributes to different pathologies like atherosclerosis, heart failure, hypertension, ischemia/reperfusion injury, cancer, aging, and neurodegeneration [40].

There is a vast body of literature that links vascular ROS production to cardiovascular disease [63]. Vascular ROS production as well as Rac1 activation has been associated with hypertrophy and smooth muscle cell proliferation, endothelial dysfunction as well as endothelial cell migration, hypertension inflammation, and atherosclerosis [64–67]. Vascular hypertrophy has been ascribed to the effects of various receptor agonists, including Angiotensin II (Ang II), which induces ROS production in VSMCs in a Rac1-dependent fashion [68]. Recent studies showed that this Ang II-induced ROS production also requires the membrane adapter caveolin, which is involved in Rac1 activation [69, 70], and the lipid kinase PI3K-γ [68, 71].

Ischemia/reperfusion (I/R) injury is also associated with ROS production. This is a clinically relevant problem occurring as damage to the myocardium following blood restoration after a critical period of coronary occlusion. It is well known that immediately following the reinstitution of oxygenated blood into ischemic tissue, there is a rapid burst of ROS, but the molecular basis and source of this process are not yet convincingly identified [14, 72]. However, both *in vitro* and *in vivo* experiments [73, 74] have suggested that Rac1 plays a dominant role in ROS generation after I/R, and it activates the nuclear factor NF-κB and stimulates mRNA expression of several inflammatory genes, such as TNF-α and iNOS in the liver, leading to massive hepatocyte necrosis. Thus, efforts aimed at inhibiting Rac protein function could be useful therapeutic strategies in a variety of clinical settings in which there is concern about the potential harmful effects of I/R injury [73, 74].

Data from the literature suggest that ROS and RhoA activation are associated to airway smooth muscle contractility [75–77]; it has been shown that oxidative stress with

H_2O_2 leads to airway smooth muscle contraction mediated by increases in intracellular Ca^{2+} concentration and the Rho/Rho kinase pathway [77].

Both ROS and Rho/Rho kinase have been suggested to play important roles in vasoconstriction and may contribute to the pathogenesis of hypertension in experimental animals and humans. Jin and coworkers demonstrated the direct activation of the Rho/Rho kinase signaling pathway by ROS in rat aorta, suggesting an important role for ROS-mediated Rho/Rho kinase activation in vasoconstriction [78].

As previously discussed, Aghajanian and coworkers proposed a novel mechanism for the regulation of RhoA in cells by ROS that allows predicting that ROS may directly activate Rho signaling in smooth muscle and in the endothelium thus affecting vascular permeability. This mechanism of regulation, which is independent of classical regulatory proteins, may be particularly relevant in pathological conditions where ROSs are generated and the cellular redox-balance altered, such as in asthma and I/R injury [62].

It is well known that activated Ras signaling contributes to oncogenic transformation by providing molecular signals that promote cell proliferation, obstruct cell death, inhibit cellular differentiation, and induce angiogenesis [79]. Signaling pathways starting from activated Ras and resulting in mitochondrial ROS production and downstream signaling regulation have been the subject of several recent interesting studies, and different mechanisms have been proposed to elucidate the role of mitochondrial respiration in cancer.

It has been shown that the activation of K-Ras(G12V) causes modifications in mitochondrial metabolism finalized to support growth under hypoxic conditions, and leading to increased generation of ROS [80]. The major source of ROS generation required for KRas-induced anchorage-independent growth is the Q_o site of mitochondrial complex III [81]. Thus mitochondrial dysfunction appears to be an important mechanism by which K-Ras(G12V) causes metabolic changes and ROS stress in cancer cells and promotes tumor development [80].

Mitochondrial dysfunction and ROS production mediated by activation of Ras, Myc, and p53 produce downstream signaling (e.g., NFκB, STAT3, etc.) that are crucial in cancer-related inflammation. Different inflammation-associated cancers resulting from signaling pathways coordinated at the mitochondrial level have been identified that may prove useful for developing innovative strategies for both cancer prevention and cancer treatment [82].

Several studies suggest that autophagy may be important in the regulation of cancer development and progression and in determining the response of tumor cells to anticancer therapy [83]. A recent paper shows that autophagy is associated with the malignant transformation of mammalian cells induced by K-Ras and that ROSs are involved as signaling molecules in K-Ras(G12V)-induced autophagy. The increase in intracellular ROS produced in response to oncogenic K-Ras involves p38 MAPK signaling and leads to JNK activation. JNK acts downstream of ROS and plays a causal role in autophagy induction through upregulation of autophagy-specific genes 5 and 7 (ATG5 and ATG7) [84]. As mitochondria sustain viability of Ras-expressing cells in starvation, autophagy is required to maintain the pool of functional mitochondria necessary to support growth of Ras-driven tumors [85]. These findings provide new insights into the relationship between autophagy and oncogenesis and suggest that targeting autophagy and mitochondrial metabolism are valuable new approaches to treat cancers with Ras mutations.

Oncogenic activation of the *H-Ras* gene has been found in more than 35% of patients with urothelial carcinomas [86]. It has been recently shown that in addition to tumorigenic ability, oncogenic H-Ras possesses a novel proapoptotic activity to facilitate the induction of apoptosis by histone deacetilase inhibitors (HDACIs), a new class of anticancer agents characterized by high cytotoxicity toward transformed cells [87]. Expression of oncogenic H-Ras in human bladder tumor J82 cells and treatment of cells with the HDACI, FK228, sinergistically induce the ERK pathway, resulting in differentially increased NOX-1 elevation and ROS production, leading to differential activation of caspases and cell death [88–90]. Thus, in addition to its well-known role in mediating mitogenic signals for cell proliferation and transformation, the ERK pathway plays an essential role in mediating apoptotic signals induced by HDACIs through induction of NOX-1 elevation to ROS production and caspase activation for inducing cell death. In addition, expression of oncogenic H-Ras in J82 cells also results in an increased susceptibility to exogenous H_2O_2 for inducing caspase activation and apoptosis [88]. Further studies revealed that FK228 combined with exogenous H_2O_2 cooperatively induces activation of MEK1/2 and ERK1/2 to increase NOX-1 elevation, intracellular ROS production, caspase activation, and cell death. Expression of oncogenic H-Ras significantly increases these FK288- and exogenous H_2O_2-induced effects. Oncogenic H-Ras-increased susceptibility to FK228 could be alternatively achieved by additional treatment with exogenous H_2O_2. These findings have important and useful implications as combined use of HDACIs with ROS-generating agents may apply to therapeutic strategies to preferentially kill malignant cells with or without oncogenic H-Ras activation [91].

Due to the crucial role played by Ras in many cellular signaling cascades, diseases relevant to dysregulation of redox signaling often result in deregulation of Ras-dependent cellular signaling events. Since the first identification of the redox-sensitive NKCD motif of Ras [51], considerable pathophysiological data are available, including some bearing directly on the relevance of redox-mediated misregulation of the Ras NKCD motif to certain diseases [7]. Rap1A, another reprehensive protein that possesses the NKCD-motif, is a regulator of NAD(P)H oxidase. However, a pathophysiological outcome associated with the misregulation of Rap1A redox signaling has not been clearly investigated [7].

Cancer is one of the most prevalent disorders caused by misregulation of Ras activity by a redox agent. Numerous studies show that cancers, to a large extent, are induced by misregulation of Ras redox signaling combined with an alteration of Ras downstream cellular transduction cascades. As with cancers, many cardiovascular and neuronal disorders

appear to be the result of dysregulation of various cellular signaling events via the redox-sensitive Ras (for a deeper investigation see [7]).

The misregulation of the redox signaling of Ras with its downstream cascades also has been linked to various disorders linked with immune and embryo developments. The Ras-dependent activation of Raf also leads to stimulation of a phosphorylation of Ets-like protein-1 and tumor necrosis factor-α messenger RNA induction; both actions suggest that NO, through the Ras-dependent Raf-MEK1/2-ERK1/2 pathway, modulates a host's defenses and the inflammation of T lymphocytes [92]. ROS-mediated signaling via Ras, NF-κB, and related transducers may link to embryopathies [93].

5. Concluding Remarks

Although, for many years, the generation of intracellular redox agents was viewed solely as the unregulated by-product of aerobic metabolism and other enzymatic processes, over the last years a growing number of reports have suggested that the production of intracellular ROS is tightly regulated and that these redox agents serve as intracellular signaling molecules being involved in a variety of cell signaling pathways. Here we have reviewed studies reporting that members of the RasGTPase superfamily are able to regulate intracellular ROS production, and that the production of ROS by small GTPases is an important aspect of the function of these monomeric G-proteins. In addition, the functional cross-talk between some different RasGTPase family members (see Rac1 and RhoA) appears strictly related to redox signaling. Finally, due to the presence of conserved redox-sensitive sequences, many of the Ras superfamily small GTPases have been shown to be targets of ROS regulation.

Thus, redox agents, as upstream regulators and/or downstream effectors of redox-sensitive RasGTPases, strongly contribute to their overall biological function playing a key role in various cellular signaling events. Dysregulation of small GTPases by redox agents or dysregulation of redox signaling by small GTPases may significantly alter cellular signaling pathways and lead to the pathological state.

Given the prominent role the RasGTPase family members play in regulating fundamental cell processes like growth, migration, adhesion, cytoskeletal integrity, survival, and differentiation, the comprehension of molecular mechanisms of the interplay between small GTPases and ROS may strongly help to clarify how redox agents contribute to physiological and pathological cellular events and provide novel strategies for treatment of many pathological conditions where both RasGTPases and oxidative stress play a role.

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Review Article

Mitochondria-Ros Crosstalk in the Control of Cell Death and Aging

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Reactive oxygen species (ROS) are highly reactive molecules, mainly generated inside mitochondria that can oxidize DNA, proteins, and lipids. At physiological levels, ROS function as "redox messengers" in intracellular signalling and regulation, whereas excess ROS induce cell death by promoting the intrinsic apoptotic pathway. Recent work has pointed to a further role of ROS in activation of autophagy and their importance in the regulation of aging. This review will focus on mitochondria as producers and targets of ROS and will summarize different proteins that modulate the redox state of the cell. Moreover, the involvement of ROS and mitochondria in different molecular pathways controlling lifespan will be reported, pointing out the role of ROS as a "balance of power," directing the cell towards life or death.

1. Introduction

From the first observation of the real mitochondrial structure in 1950 [1], it was clear that this particular composition reflected the different functions of this organelle. Mitochondria are delimitated by double-membrane architecture, an outer membrane of eukaryotic origin and an inner membrane characterized by the absence of cholesterol and the presence of cardiolipin, typical elements of bacterial membranes. The inner membrane is organized in characteristic folds, termed cristae, which protrude into the matrix and accommodate the respiratory chain complexes. In healthy cells, the inner membrane is impermeable to ions [2], which allows the electrons transport chain (ETC) to actively build up the proton gradient. The mitochondrial membrane potential ($\Delta\Psi m$) results from the difference in electrical potential generated by the electrochemical gradient across the inner membrane. Through oxidative phosphorylation, mitochondria play their essential role to supply the cell with metabolic energy in the form of ATP. As a consequence, they are also the primary source of cellular reactive oxygen species (ROS), especially at level of the respiratory chain complexes I and III, where electrons derived from NADH and ubiquinone can directly react with oxygen or other electron acceptors and generate free radicals [3, 4]. Therefore, ROS are a normal side product of the respiration process, and they react with lipids, protein, and DNA, generating oxidative damage. Indeed, mitochondria are the major site of ROS production, but also the major targets of their detrimental effects, representing the trigger for several mitochondrial dysfunctions. In this review, we will focus on this deadly liaison, with particular attention on ROS production, mitochondrial ROS targets, and their role in apoptosis, autophagy, and aging.

2. Mitochondrial ROS Production

Mitochondria are responsible for 90% of the energy that cells, and thus tissues, organs, and the body as a whole need

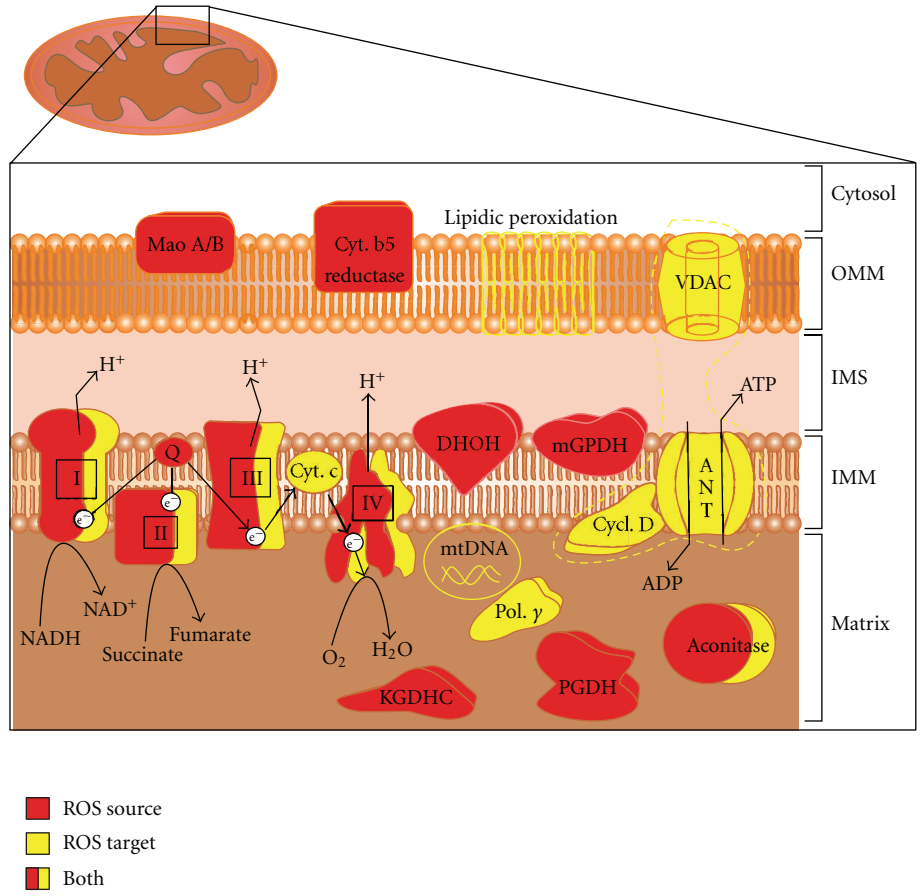

Figure 1: Mitochondrial sources of ROS and mitochondrial ROS targets. ROS generators (red) and ROS targets (yellow) are shown in their precise localizations inside mitochondria. Dotted yellow trace encloses the permeability transition pore components. See text, in particular sections 2 and 3, for further details. Abbreviations: OMM: outer mitochondrial membrane; IMS: intermembrane space; IMM: inner mitochondrial membrane; MAO A/B: monoamine oxidases A and B; Cyt. b5 reduct.: cytochrome b5 reductase; DHOH: dihydroorotate dehydrogenase; mGPDH: glycerol-3-phosphate dehydrogenase; I, II, III, and IV: Complex I to IV of the respiratory chain; Q: coenzyme Q; Cyt. c: cytochrome c; KGDHC: α-ketoglutarate dehydrogenase complex; PGDH: pyruvate dehydrogenase complex; e^-: electrons; VDAC, voltage-dependent anion channel, Cycl. D: cyclophilin D; ANT: adenine nucleotide translocase; Pol. Γ: polymerase γ; mtDNA: mitochondrial DNA.

to function. Hence, they are known as the "cells powerhouse," the core of cellular energy metabolism, being the site of most ATP generation through mitochondrial oxidative phosphorylation (OXPHOS) [5].

In this process, electrons liberated from reducing substrates are delivered to O_2 establishing an electrochemical gradient used to drive ATP synthesis. During the OXPHOS, the reduction of oxygen by one electron at a time ($O_2 \rightarrow O_2^{-}\bullet \rightarrow H_2O_2 \rightarrow \bullet OH \rightarrow H_2O$) produces ROS, relatively stable intermediates with one unpaired electron [6–8]. Although Brown and Borutaite in their recent review have presented a number of examples supporting the point of view that mitochondria are not a major source of ROS in the cell [9], the fact that oxidative phosphorylation accounts for 90% to 95% of cellular oxygen consumption and that 3% from that pool can be converted to superoxide is a very strong argument in favour of mitochondria as a main source of this oxygen radical. Hence, it is a firm paradigm that mitochondria are also the major source of ROS in mammalian cells (Figure 1).

The primary ROS generated by mitochondria, as a result of monoelectronic reduction of O_2, is superoxide anion ($O_2^{-}\bullet$) that is the precursor of most ROS and a mediator in oxidative chain reactions. In vivo, $O_2^{-}\bullet$ is produced both enzymatically by NADPH oxidase, cytochrome P450-dependent oxygenases, and xanthine oxidase and non-enzymatically, when a single electron is directly transferred to O_2 [8]. In addition, $O_2^{-}\bullet$ may react with other radicals including nitric oxide (NO•) producing reactive nitrogen species (RNS) [10]. Dismutation of $O_2^{-}\bullet$, either spontaneously [11], or through a reaction catalyzed by superoxide dismutases (SODs) [12], produces hydrogen peroxide (H_2O_2) [13]. H_2O_2 generated in mitochondria has many possible fates. Because H_2O_2 is relatively stable and membrane permeable, it can diffuse within the cell and be eliminated by cytosolic or mitochondrial antioxidant systems

such as catalase, glutathione peroxidase, and thioredoxin peroxidase [14]. Mitochondrially generated H_2O_2 can also act as a signaling molecule in the cytosol, affecting multiple networks that control, for example, cell cycle, stress response, energy metabolism, and redox balance [15–17].

When not metabolized, H_2O_2 can be further transformed to hydroxyl radical (•OH), in the presence of metal ions, by Fenton reaction [18]. •OH is one of the strongest oxidants in nature, is highly reactive, and generally acts essentially as a damaging molecule. For this reason, mitochondria are believed to have developed efficient H_2O_2 removal systems, as well as metal-chelating mechanisms, such as chaperone proteins, preventing the formation of this radical.

At least, ten mammalian mitochondrial enzymes contribute to ROS production [17, 19] even if their capacity of ROS producing greatly differ in a tissues-specific manner [20]. *In vitro* experiments demonstrate that mitochondria isolated from mouse heart, brain, and kidney have selective substrate and inhibitor preferences for H_2O_2 generation and that the apparent sites of H_2O_2 generation are both substrate and organ specific [21].

The major bulk of mitochondrial ROS generation occurs at the electron transport chain, as a byproduct of respiration [7, 8, 22]. Cytochrome c, oxidase (Complex IV) is the terminal component of the ETC, receives four electrons from cytochrome c and reduces one O_2 molecule to two H_2O. It retains all partially reduced intermediates until full reduction is achieved and does not seem to release these intermediates in measurable quantities [6].

Historically, the first mitochondrial site producing ROS was identified at the Complex III located at the inner side of inner mitochondrial membrane (bc1 complex, ubiquinone: cytochrome c reductase). The primary ROS produced at this site is O_2^-•, through the referred Q-cycle [23]. Despite the recent advances in understanding the structure of the bc1 complex, a mechanism of O_2^-• production is not yet known [24–26].

Succinate dehydrogenase (Complex II, SDH) is a flavoprotein located at the inner surface of inner mitochondrial membrane. Although oxidation of succinate can theoretically produce ROS with a high rate, significant O_2^-• formation from this enzyme has not been measured so it is still unclear whether SDH produces ROS *in situ*, in mitochondria. Despite the lack of ROS formation by Complex II itself, succinate is an important source of ROS in many tissues through a mechanism involving reverse electron transfer. This particular phenomenon may result from high mitochondrial membrane potential, which thermodynamically favourites electron donor from Complex II to I. Because of this, succinate may promote ROS generation at Complex I level [6, 7].

Mitochondrial Complex I, "NADH dehydrogenase," in the inner face of inner membrane provides a major entry point for electrons into respiratory chain. It is a significant source of ROS [27] in particular O_2^-• [28] and H_2O_2 although whether or not Complex I is the major site of ROS production in intact mitochondria *in vivo* is a complicated issue. Indeed all the evidence on ROS production by Complex I was obtained *in vitro* with isolated mitochondria [29].

There are other mitochondrial nonrespiratory chain enzymes that produce ROS although pinpointing their specific contribution to total mitochondrial ROS production remains unclear.

Mitochondrial cytochrome b5 reductase is located in the outer mitochondrial membrane [30]. The enzyme is widely distributed in mammalian tissues and may be involved in O_2^-• generation with a very high rate ~300 nmoL per min per mg protein [31].

Monoamine oxidases (MAO-A and MAO-B) are also located in the outer mitochondrial membrane and ubiquitously expressed in various mammalian tissues. These enzymes catalyze the oxidation of biogenic amines accompanied by the release of H_2O_2 [32].

Dihydroorotate dehydrogenase (DHOH) is located at the outer surface of inner mitochondrial membrane. It catalyzes the conversion of dihydroorotate to the pyrimidine base, orotate, which is a step in the de novo synthesis of uridine monophosphate. The DHOH is ubiquitously distributed in mammalian tissues, and it is considered a mitochondrial source of O_2^-• and H_2O_2 although the capacity of DHOH to produce O_2^-• requires further clarification [33].

Mitochondrial dehydrogenase of α-glycerophosphate (Glycerol-3-Phosphate Dehydrogenase, mGPDH) is located at the outer surface of inner mitochondrial membrane. The mGPDH is involved in lipid metabolism and in the so-called glycerol phosphate shuttle capable of regenerating cytosolic NAD+ from the NADH formed in glycolysis. It is ubiquitously but unequally expressed in various mouse tissues and mediate the production of H_2O_2 [34, 35].

Mitochondrial aconitase is an enzyme localized to the matrix space of mitochondria; it participates in tricarboxylic acid cycle catalyzing a conversion of citrate to isocitrate. The enzyme contains an iron-sulfur cluster that can be oxidized by O_2^-• or H_2O_2 producing •OH [36].

Ketoglutarate dehydrogenase complex (KGDHC, 2-oxoglutarate dehydrogenase) is an integral mitochondrial enzyme tightly bound to the inner mitochondrial membrane on the matrix side [37]. It catalyzes the oxidation of α-ketoglutarate to succinyl-CoA in the tricarboxylic acid cycle. Structurally, KGDHC is composed of three enzymes: α-ketoglutarate dehydrogenase (E1 subunit), dihydrolipoamide succinyltransferase (E2 subunit), and lipoamide dehydrogenase (E3 subunit). The E3 component appears to be the principal ROS source generating O_2^-• and H_2O_2 [38, 39].

Pyruvate dehydrogenase complex (PGDH) localized into mitochondrial matrix contains flavoenzyme dihydrolipoyl dehydrogenase subunits like KGDHC, which are very important ROS sources [38].

3. Mitochondrial Targets of ROS

A wide range of mitochondrial ROS-induced damages has been described, including protein carbonylation, lipid peroxidation, or mtDNA damage. These damages, either individually or collectively can lead to a cellular energetic catastrophe. In this section, we are going to treat the most

important mitochondrial targets of ROS action, also summarized, with their mitochondrial localization, in Figure 1.

3.1. Oxidative Damage of mtDNA. The sequence and organization of the human mitochondrial genome has been presented for the first time by Anderson et al. in the landmark paper in 1981 [40]. Mitochondrial DNA (mtDNA) was identified as a 16.5 kb circular molecule encoding 13 polypeptides, 22 tRNAs, and 2 rRNAs. All of the above mentioned are components of mitochondrial respiratory machinery. MtDNA is attached to the IMM on the matrix side, rendering it particularly prone to oxidative damage, as the aforesaid localization is in immediate proximity to the cells' major source of ROS, that is to the mitochondrial respiratory chain. ROS accumulating within the matrix are in such a case explicitly deleterious. Additionally, the lack of introns, histones, and other DNA-protecting proteins as well as DNA repair systems inferior to those observed in nDNA allows increased deterioration [41, 42]. However, mitochondria possess a number of DNA repair enzymes such as MYH (2-OH-A/adenine DNA glycosylase) and OGG1 (8-oxoG DNA glycosylase) [43]. In nucleic acids, both the bases as well as the sugar are prone to ROS attacks. This may lead to modifications of base and abasic sites and result in strand breaks [44]. A good example that may be the addition of the hydroxyl radical to the double bonds may occur. In this case the reaction rate-limiting factor is diffusion of approximately 4.5×10^9 to $9 \times 10^9\,M^{-1}s^{-1}$ [45]. The main outcome of the above-mentioned attacks is the hydroxylation of pyrimidines at C5 and C6 as well as purines at C4, C5, and C8. So far over 80 different oxidized bases in DNA exposed to oxidant factors, such as H_2O_2 and singlet oxygen, have been identified [45, 46]. Experiments from the mid 1980s led to the establishment of a dependable marker of these alterations—8-hydroxy-deoxyguanosine (8-OHDG) [47–51]. An increase in 8-OHDG is a sign of mtDNA fragmentation and is observed in events involving oxidative stress [52].

Alterations in the mitochondrial genome are reflected in a number of disorders, most of which involve impaired respiration and oxidative phosphorylation. As a matter of fact, over 150 pathogenic mutations have been identified in the mtDNA, which cause human diseases [53]. The scope of these disorders has been positively correlated with increasing age and tissues with high-energy demands [54–58]. Individuals may develop a number of symptoms including ataxia, dystonia, epilepsy, dementia, peripheral neuropathy, optic atrophy, and deafness [59]. A number of authors report associations between mtDNA point mutations and neurodegenerative diseases [60–63]. However, some of the mutations are yet to be confirmed and therefore are under dispute. On the other hand, in some cases, such as Parkinson's disease (PD), there seems to be a consistent PD-associated haplogroup [64–66]. Brains of aged individuals as well as those with neurodegenerative diseases exhibit increased levels of mutated mtDNA, particularly in substantia nigra neurons, as compared to hippocampal neurons [67]. It is not clear, whether these mutations are an effect of oxidative stress. Nevertheless, such explanation seems reasonable.

3.2. Oxidative Damage of Mitochondrial Proteins. Oxidative modification of proteins (oxidation of arginine, lysine, threonine, and proline residues) induces the formation of protein carbonyls, commonly detected under oxidative conditions, in aged cells and in aged animal tissues. On the other hand, peroxynitrite oxidizes tryptophan, cysteine, and tyrosine protein residues as well as unsaturated fatty acids and low molecular mass components of the antioxidant defense system like glutathione, α-tocopherol, and ascorbate. Additionally, oxidative damage can affect carbohydrates, which, when oxidized, may be used in glycation process causing the formation of glycation end products. It has been repeatedly shown that the accumulation rate of such oxidatively modified proteins (under oxidative stress or in aging) is tissue specific and depends on protein type [68].

3.2.1. Mitochondrial Respiratory Chain. The mitochondrial respiratory chain, due to its natural imperfections, can be considered a peculiar superoxide generator. In such a case, complexes of the respiratory chain are in the firsthand exposition to oxidative modification because they are present at the sites of ROS production. The high sensitivity of the respiratory chain complexes to oxidizing agents results from the fact that they contain iron-sulfur clusters (Complexes I, II, and III), heme groups (Complexes II, III, and IV) and copper centers in complex IV, all of which can be a site of direct ROS attack, and their oxidative modifications can manifest with a decrease of their enzymatic activity and dysfunction of the whole respiratory chain [69–71]. Interestingly, three mechanisms have been reported in the NO• inhibition of Complex I: S-nitrosation, tyrosine nitration and damage to FeS centres [72]. The inhibition of mitochondrial Complex I activity is consistently detected in Parkinson's disease patients as well as in mitochondrial toxin models of the disorder [73].

Cytochrome c (a component of OXPHOS) can be modified by nitration of its tyrosine residues, which also affects electron flow through the respiratory chain [74]. Nitration of a single tyrosine residue in cytochrome c by a relatively low dose of peroxynitrite resulted in the upregulation of its peroxidase activity for H_2O_2 and in the impairment of the membrane potential formation [75]. Oxidative damage of individual respiratory chain complexes results not only in a decreased efficiency of ATP production but can also increase ROS production by oxidatively affected OXPHOS subunits, contributing in this way to the intensification of oxidative stress.

3.2.2. Mitochondrial Carriers. The effect of oxidative stress (i.e., caused by site-specific metal-catalyzed oxidation) on mitochondrial carriers was studied intensively in a model exploiting components of the mitochondrial permeability pore: the adenine nucleotide translocase (ANT), porin (VDAC), and creatine kinase. ANT contains many proline, arginine, and lysine residues, which are very sensible for such type oxidation (systems: vanadyl/H_2O_2-generating hydroxyl free radicals or Cu^{2+}/tert-butyl hydroperoxide).

Moreover, ANT is localized in the IMM in cardiolipin-rich (containing unsaturated fatty acids) domains. This can explain its sensibility to oxidation also by lipid peroxidation products. In 1998, interesting studies of Sohal's group revealed that ANT in mitochondria of flight muscles is significantly carbonylated in senescent flies. Surprisingly, ANT was the only mitochondrial protein demonstrating age-related increase of carbonylation in this experimental model. In vitro experiments basing on the exposure of isolated mitochondria to the hydroxyl free radicals showed an increased level of carbonylated ANT exhibiting lower nucleotide exchange activity [76].

In addition to controlling the release of superoxide from mitochondria [77], VDAC, which accounts for about 10% of outer mitochondrial membrane (OMM) proteins, can also be a target for intracellular $O_2^{-}\bullet$. Madesh and Hajnoczky observed that VDAC-dependent permeabilization of the OMM and cytochrome c release can be caused by superoxide but not by H_2O_2. Moreover, the observed effect of superoxide does not require the contribution of the proapoptotic proteins like Bak or Bax [78]. On the other hand, another component of the mitochondrial permeability transition pore (mPTP), mitochondrial creatine kinase, present in the intermembrane space (IMS) is affected by H_2O_2 and peroxynitrite. The drop in the creatine kinase activity is caused by oxidative modifications of its sulfhydryl groups and aromatic aminoacids [79, 80].

3.2.3. Permeability Transition Pore as a Ros Target. Based on the literature, permeability transition pore (mPTP) seems to be a multiprotein complex with variable composition. The main core of mPTP consists of the voltage-dependent anion channel (VDAC), ANT, and cyclophilin D localized in the outer-, inner mitochondrial membrane, and mitochondrial matrix, respectively [81–83]. Still there is no consensus concerning exact composition of mPTP. Recent studies with the use of transgenic VDAC1 or ANT1 knockout mice seem to contradict the stereotype model of PTP. To find more about the presence of VDAC, ANT, or cyclophilin D in the mPTP, refer to [84–86]. The other components, such as hexokinase, benzodiazepine receptor, and creatine kinase seem to play a regulatory role rather than being structural components of the mPTP. However, regardless of the composition of mPTP, it has been repeatedly described already in 90ties that oxidation of critical thiol residues of ANT induces opening of the mPTP and collapse of mitochondrial membrane potential, mitochondrial swelling, and cytochrome c release. When the thiol groups of ANT are in reduced state, probability of mPTP opening is much lower than when they are oxidised and cross-linkage of ANT thiol groups can occur. Especially oxidation of the Cys^{160} and Cys^{257} of ANT promotes PTP opening [87]. Moreover, Cys^{56} oxidation alters the conformation of ANT inducing it to become a nonspecific pore, thus mimicking the effect of Ca^{2+} which converts ANT into a large channel [88]. Sensitivity of ANT to the oxidizing agents makes mPTP one of the direct sites of ROS action in mitochondria.

3.2.4. Aconitase. In Section 2, we have argued m-aconitase as a source of ROS, but, at the same time, it can be considered a well-established example of oxidative damage targeting a mitochondrial enzyme. In fact, its susceptibility to oxidative damage mainly from superoxide is related to the iron-sulfur cluster present in the active site of the enzyme [89].

3.2.5. Polymerase γ. Nearly 10 years ago, the studies of Copeland's group confirmed that polymerase γ present in the mitochondrial matrix responsible for mtDNA replication can also be considered as an intrinsic target for ROS. The group found that the catalytic subunit of polymerase γ is one of the proteins most abundantly oxidized in the mitochondrial matrix and correlated oxidative modification of polymerase γ with the drop of its enzymatic activity. This in turn can result in the reduction of mtDNA repair and replication [90].

3.3. Peroxidation of Mitochondrial Lipids. In mitochondrial membranes, unsaturated fatty acids, being components of phospholipids, are very susceptible to oxidation by the hydroxyl radical. Lipid peroxidation products, lipid hydroperoxides, generate very reactive unsaturated aldehydes like 4-hydroxy 2-nonenal (4-HNE), malondialdehyde (MDA), and acrolein. Moreover, the generation of lipid radicals can induce a chain reaction leading to the generation of new radicals which intensifies lipid peroxidation. Lipid peroxides cause various effects in the cell. It has been reported that oxidatively modified lipids affect membrane fluidity; this is the case of lipid peroxidation at inner mitochondrial membrane level, leading to increased permeability to protons and uncoupling of oxidative phosphorylation [91]. Moreover, lipid radicals diffuse easily in the membranes and can covalently modify membrane proteins [92], as well as cause "lipoxidative" damage to the mtDNA. For example, 4-HNE inhibits ANT activity in isolated mitochondria due to the modification of critical sulfhydryl groups in ANT [93]. It has also been suggested that the protein carbonylation process is more often related to the lipid-protein oxidation than the direct protein oxidation by reactive oxygen species [94, 95].

4. Mitochondrial ROS Measurements

Direct imaging of ROS in biological samples has proven to be extremely challenging. A large amount of methods for ROS monitoring has been developed in the last years, including fluorimetric, spectrophotometric, and chemiluminescent assays [96].

Among different fluorescent assays, MitoSOX is largely used to visualize superoxide ions inside mitochondria [97]. This reagent is a cell permeant dye that once in mitochondria is oxidized by superoxide and trapped inside organelle, becoming weakly fluorescent. Subsequent binding with nucleic acid permit to the dye to show high fluorescence, with excitation at 510 nm and emission at 580 nm. It can be considered a superoxide-specific probe, because its reactivity for hydrogen peroxide or reactive nitrogen

species is relatively low. However, several caveats exist in using MitoSOX probe [98, 99]; one of the mostly frequent is, without doubts, the photochemical oxidation of the dye, an aspect which has to be taken into consideration for proper interpretation of results.

One of the widespread methods to detect H_2O_2 production by mitochondria is based on 2′,7′ dichlorofluorescein (DCF-H_2) oxidation. This dye is commercialized as a diacetate chemical probe, in order to be cell permeant, and, once deacetylated from cellular diesterases, it becomes impermeable and weakly fluorescent. DCF-H_2, when oxidized by H_2O_2, is converted into 2′,7′-dichlorofluorescein (DCF), a high fluorescent component (λ_{ex} 500 nm $-\lambda_{em}$ 520 nm) [100]. DCF formation is directly proportional to H_2O_2 production; therefore, for data analysis is preferable to calculate variation in fluorophore accumulation speed. One of the most relevant limitations of DCF is that it cannot be used to measure H_2O_2 production exclusively inside mitochondria. Mitochondria peroxy yellow 1 (MitoPY1), a new type of fluorophore for imaging mitochondrial H_2O_2 in living cells with ROS and spatial specificity [101], may represent the answer to this problem, but further studies have to be performed to clarify its real efficacy.

Recently, a new highly specific fluorescent probe has been introduced in methods for ROS measurements. HyPer (distribuited by Evrogen) is a genetically encoded fluorescent sensor specific for H_2O_2 and consists in a circularly permuted yellow fluorescent protein (cpYFP), inserted into the regulatory domain of OxyR, a hydrogen peroxide-sensitive transcription factor isolated from *E. coli*. HyPer is a ratiometric indicator; the excitation wavelength in normal condition is 420 nm, and, in the presence of H_2O_2, a red-shift moving excitation to 500 nm occurs. Emission spectrum has a maximum at 516 nm, independently from oxidants activity. The most palatable feature is the possibility to direct HyPer to mitochondrial matrix using COX-targeting sequence, permitting directing fast measurements of mitochondrial ROS production [102]. HyPer is not the first ratiometric probe used for ROS assessment. In 2004, Tsien and coworkers developed ratiometric redox-sensitive versions of GFP, termed redox-sensitive green fluorescent proteins (roGFPs) [103, 104]. When a population of roGFP is oxidized, excitation increases at the 400 nm peak while diminishing at the 480 nm peak; the emission is measured at 510 nm. Because the indicator is genetically encoded, it can be targeted to specific proteins or organelles of interest and expressed in a wide variety of cells and organisms (for an exhaustive discussion of the biophysical properties and development of these probes, see [105]).

Fluorescent dyes show the high advantage to be easy to use, fast, and weakly invasive, leading to a quite accurate calibration using peroxide (or nitric oxide) donors. However, they are fast degradable and easily produce artefacts; in fact, as cited above, high light intensity could induce the activation of the dye, with consequent artefactual ROS generation, resulting in nonspecific signal amplification.

5. Deadly Liaisons: ROS and Mitochondria in the Control of Cell Death

As mentioned in the introduction, mitochondria are often targets of high ROS exposure with deleterious consequences, such as oxidative damage to mitochondrial DNA. This is certainly true, but recent evidence has suggested a deep involvement of ROS also in the extrinsic pathway of apoptosis.

The extrinsic receptor-mediated death pathway requires effective engagement between the death receptors found on the surface of the cell membranes and their respective ligands [106]. The receptor-mediated pathway involves death receptors from the tumor necrosis factor (TNF) superfamily such as TNF, CD95 (Fas), and TNF-related apoptosis-inducing ligand (TRAIL) receptors. Fas and TNFR1 activation is known to generate ROS in response to stimulation, which has been hypothesized to be due to the production of superoxide ($O_2^{\bullet -}$) because of the formation of lipid raft-derived NADPH oxidase platforms. This lipid raft-associated ROS downstream generation may be of high importance in induction of apoptosis or necrosis [107, 108]. Moreover, ROS are required for apoptosis induction by Fas ligand (FasL) in primary lung epithelial cells. ROS mediate the downregulation of FLIP (FLICE inhibitory protein, a strong inhibitor of apoptosis) by ubiquitination and subsequent degradation by proteasome or through nitric oxide (NO) scavenging that prevents FLIP S-nitrosation and cytoprotection [109].

Other evidences underline how ROS generation may influence the intracellular milieu, favouring the effective execution of the downstream events leading to extrinsic apoptosis. ROS has been shown to sensitize cancer cells to TRAIL-induced apoptosis [110], and massive upregulation of CD95 and TRAIL death receptors have also been observed in response to hydrogen peroxide, through a mechanism involving the activation of NF-kappaB [111]. Interestingly, the ability of H_2O_2 to promote apoptosis, through the activation of JNK, seems inhibited in lung fibroblasts from TNFR1-deficient mice [112]. ROS activation of JNK can induce extrinsic or intrinsic apoptotic signaling. TNFα is a potent activator of the MAPK cascade, and TNFα-induced ROS cause oxidation and inhibition of JNK-inactivating phosphatases, by converting their catalytic cysteine to sulfenic acid, with a consequent prolonged JNK activation, which is required for cytochrome c release and caspase 3 cleavage, as well as necrotic cell death [113].

Upstream of JNK is the redox-sensitive MAPK kinase kinase, ASK1. Under non-oxidizing conditions, reduced thioredoxin1 (Trx1) binds ASK1; the resultant Trx1/ASK1 complex, called "ASK1 signalosome," functions as a perfect redox switch. Robust and sustained cellular ROS induce the dissociation of oxidized Trx1 from the complex and let to complete the activation of ASK1, also through the recruitment of TRAF2/6 [114]. Moreover, ASK2, another member of the ASK family, binds and stabilizes ASK1 not only in the cytosol, but also in nucleus and mitochondria. Recently, Saxena et al. have shown how the thioredoxin-interacting protein TXNIP, a ubiquitously expressed redox protein that promotes apoptosis, could shuttle from nucleus to mitochondria under oxidative stress, forming a complex

with mitochondrial Trx2 and removing it from ASK1. This alleviation of Trx2-mediated inhibition results in phosphorylation of ASK1 and consequent induction of the mitochondrial pathway of apoptosis, with cytochrome c release and caspase-3 cleavage [115].

The major ROS target inside mitochondria is undoubtedly the permeability transition pore (see Section 3). Oxidative modifications of mPTP proteins will significantly impact mitochondrial anion fluxes [116]. In response to proapoptotic stimuli, including ROS and Ca^{2+} overload, the mPTP assumes a high-conductance state that allows the deregulated entry of small solutes into the mitochondrial matrix along their electrochemical gradient. This phenomenon, which is known as mitochondrial permeability transition (MPT), results in immediate dissipation of the mitochondrial membrane potential and osmotic swelling of the mitochondrial matrix [2]. Mitochondrial swelling is defined as an "increase in the volume of mitochondria due to an influx of fluid." The mechanism concerns an early phase of mitochondrial swelling, which involves movement of water from the intercristal spaces into the matrix; when this water movement continues, the pressure applied to the outer membrane from the increased matrix volume leads to the opening of mPTP and/or rupture of the mitochondrial outer membrane, allowing further matrix expansion [117]. This leads to the release of cytochrome c and the subsequent engagement of the Apaf-1-pro-caspase 9 apoptosome complex, which activates downstream effector caspases. Cytochrome c is present as loosely and tightly bound pools attached to the inner membrane by its association with cardiolipin; this interaction must first be disrupted to generate a soluble pool of this protein. In 2002, the group of Orrenius elegantly showed how oxidative modification of mitochondrial lipids, specifically cardiolipin, is fundamental for tightly bound pool of cytochrome c mobilization, that is so detached because of disturbances in membrane structure [118]. The cardiolipin-bound cytochrome c assumes the role of a membrane-bound peroxidase that can effectively catalyze oxidative stress and cause oxidation of cardiolipin if a source of oxidizing equivalents, such as H_2O_2, is activated. This can lead to a putative "oxidative wave propagation:" membrane-bound cytochrome c may be seen as a mitochondrial death receptor transducing proapoptotic signals into executing oxidative cascades, with a consequent overload of oxidized cardiolipin species, detachment of cytochrome c from the membrane and formation of mPTP (reviewed in [119]). In this regard, it has been recently shown that overexpression of human telomerase reverse transcriptase (hTERT), the catalytic subunit of the telomerase holoenzyme, alleviates cellular ROS levels also through its mitochondrial localization, improving activity of cytochrome c oxidase, blocking cytochrome c release, mitochondrial membrane permeabilization, and inhibiting apoptosis in cancer cells [120].

The pathophysiological importance of intracellular redox balance and apoptosis regulation was also taken in to consideration during the study of cerebral cavernous malformations (CCMs) [121]. Ablation of Krit1, a protein whose loss of function has been associated to this particular pathogenesis, leads to a significant increase in intracellular ROS levels, due to the modulation of the expression of the antioxidant protein SOD2 and to a drastic decrease of mitochondrial energy metabolism, with consequent increased susceptibility to oxidative damage [121]. In the same way, MUC1, an oncoprotein aberrantly expressed in acute myeloid leukemia (AML) cells, regulates ROS levels and the differentiation of hematopoietic cells [122]. MUC1 expression is associated with attenuation of endogenous and H_2O_2-induced intracellular levels of reactive oxygen species [123], and, inhibition of MUC1 results in the disruption of redox balance and thereby AML cell death [122].

Redox regulation of proteins by moderate levels of ROS is observed in various signaling pathways, including autophagy, a catabolic pathway for degradation of intracellular proteins and organelles via the lysosome [124]. Autophagy is activated mainly by nutrient starvation, and it plays a dual role; it is primarily a surviving mechanism, but it also leads to cell death (called type III cell death) thus possibly acting as an alternative to apoptosis. It is generally accepted that ROS induce autophagy [125, 126], and that autophagy, in turn, serves to reduce oxidative damage [124]. Given that, antioxidants may represent natural inhibitors of this process. As a matter of fact, the p53-target gene TIGAR contributes to the regulation of intracellular ROS levels by modulation of the glycolytic pathway, increasing NADPH production (with a consequent decrease in intracellular ROS levels) and lowering the sensitivity of cells to p53-dependent apoptosis induced by oxidative stress [127]. In this manner, TIGAR inhibits autophagy induced by nutrient starvation and metabolic stress, in a p53-indipendent way [128]. Moreover, a mutant form of SOD1 ($SOD1^{G93A}$, associated with one-fifth of familial amyotrophic lateral sclerosis cases [129]) promotes ROS accumulation and autophagy [130, 131], despite its role in muscular atrophy induction has not been fully understood. In transgenic mice spinal cord, $SOD1^{G93A}$ interacts with p62, an LC3-binding partner known to target protein aggregates for autophagic degradation, suggesting that p62 may direct mutant SOD1 aggregates to autophagy [132].

Because mitochondria are, at the same time, primary source and target of ROS, they play a fundamental role in ROS-mediated autophagy regulation. Indeed, cells use a specialized form of autophagy, called mitophagy, to selectively eliminate defective mitochondria. The term, coined by Lemasters in 2007 [133], indicates a selective degradating process to maintain a healthy population of mitochondria. Increases in cellular ROS lead to loss of mitochondrial membrane potential ($\Delta\Psi m$), which is considered a trigger for mitophagy [133]. Under serum deprivation, a typical decrease in mitochondrial membrane potential is observed in hepatocytes prior to engulfment by autophagosomes, whereas Cyclosporin A (CsA), a mPTP inhibitor, prevented this depolarization and the autophagosomal proliferation [134, 135]. However, in different setups represented by rat pituitary GH3 cells [136] and muscles [137], Cyclosporin A works as a strong autophagy-inducer: in particular, CsA treatment in skeletal muscles of collagen VI knockout mice (Col6a1 $-/-$), characterized by impaired autophagy, presence of abnormal mitochondria, and myofiber degeneration, promotes autophagy with a concomitant block of apoptotic

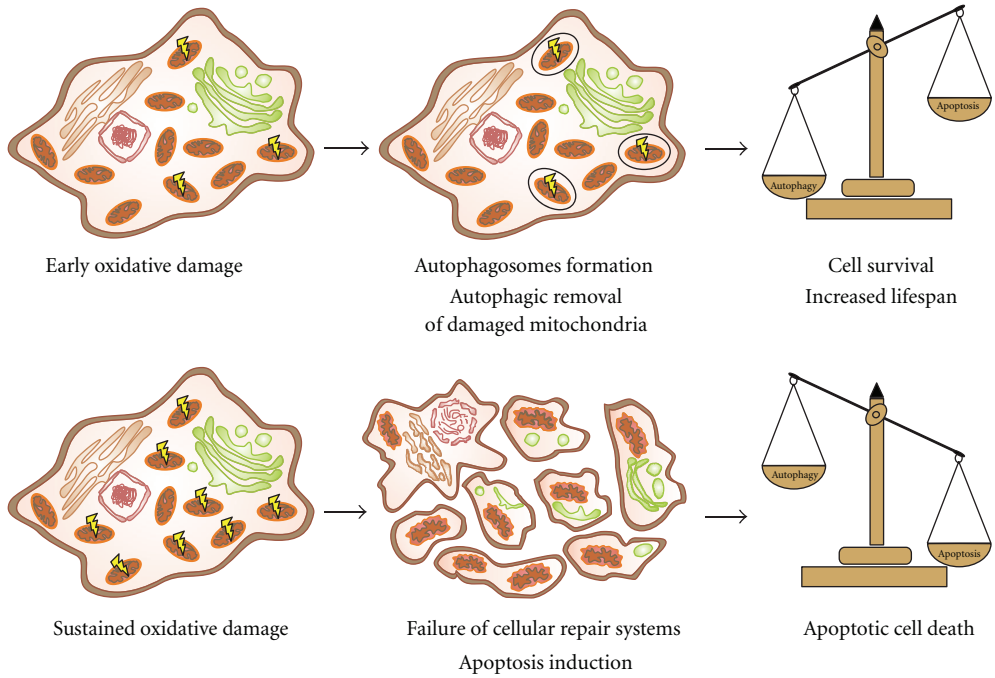

Figure 2: ROS levels control cell fate. Low production of ROS works as trigger of autophagic/mitophagic process, with consequent removal of damaged mitochondria and in turn cellular survival (upper panel). On the other hand, high levels of ROS lead to cell death promoting the apoptotic pathway when prosurvival attempt fails (lower panel).

degeneration and recovery of muscle strength [137]. This discrepancy suggests that mitochondrial depolarization is not general, leading event to rage the mitophagic process.

Abnormalities in mitochondrial respiration and increased oxidative stress are observed in cells and tissues from parkinsonian patients [138], which also exhibit increased mitochondrial autophagy [139]. Two genes, which encode the OMM kinase PINK1 and the E3 ubiquitin ligase parkin, are mutated in autosomal recessive Parkinson's disease [140]. Parkin is able to induce mitophagy through translocation from cytosol to mitochondria in many different cellular settings, after stress induction by mitochondrial uncouplers, which mimic damage by decreasing the mitochondrial membrane potential [141], or by the administration of oxidative stress inducers, such as Paraquat [142]. Moreover, Parkin may signal the selective removal of defective mitochondria within the cell. Overexpression of Parkin can eliminate mitochondria with deleterious COXI mutations in heteroplasmic cybrid cells, thereby enriching cells for wildtype mtDNA and restoring cytochrome c oxidase activity [143]. PINK1 (PTEN-induced kinase 1) mediates recruitment of parkin to the damaged mitochondria and activates parkin's ligase activity [144], thereby promoting autophagy. Recently, it has been shown that knockdown of PINK1 expression also leads to mitophagy in SH-SY 5Y cells, due to mitochondrial fragmentation and mitochondrial ROS production [145]. PINK1 knockdown led to decreased transmembrane potential, which was ameliorated by antioxidant MnTBAP treatment, suggesting that ROS production is upstream of mitochondrial depolarization [146].

Based on these observations, ROS act as signaling molecules influencing cell fate. Undoubtedly, redox regulation can promote both survival, during starvation for example, as well as cell death, during oxidative stress. ROS play the role of trigger in the early phase of autophagic process, inducing cytoprotection by eliminating potential sources of proapoptotic stimuli. On the other hand, if the prosurvival attempt fails, ROS cause cell death which involves either the autophagic or the apoptotic pathway, or both (Figure 2).

6. ROS as Key Regulators of Aging

The decline associated with aging is caused by the accumulation of ROS, as supported by cellular and biological data from different model systems and organisms [147]. Indeed defects in antioxidant defense mechanisms fail to protect against oxidative damage, reducing lifespan [148, 149] and causing cardiomyophaty, neurodegeneration [150], and cancer [151].

The relationship between mitochondria dysfunctions observed during aging and ROS production is still debated. However, it is clear that the decline of the integrity of mitochondria as a function of age is implicated in aging and age-related diseases [147].

Given this wide scientific evidence, many studies were aimed to identify the molecular mechanisms responsible for ROS deleterious effects on the aging process. Genes that extend lifespan partially included those involved in oxidative stress response, while partially were clustered in the IGF-1/insulin-like signaling pathway [152, 153].

A key mitochondrial effector is the adapter protein p66shc, which directly mediate, the production of ROS within the mitochondria [154], participating in intracellular pathways that control oxidative stress, apoptosis, and lifespan determination [155, 156]. Accordingly, p66shc knockout mice are one of the best characterized genetic model of longevity, is being more resistant to oxidative stress [157] and protected against age-dependent, ROS-mediated cardiovascular complications induced by diabetes [158–160]. On the contrary, overexpression of p66shc causes alterations of mitochondrial Ca^{2+} responses (which is an early event of mitochondrial damage) and fragmentation of mitochondrial network [161], leading to cytochrome c release and apoptosis [162]. Orsini and coworkers showed, for the first time, that oxidative stress promotes a translocation of part of the cytosolic pool of p66Shc to mitochondria, binding mtHsp70 in mitochondrial matrix [163]. The same group in 2005 claimed that p66Shc is rather present in the intermembrane space, where it interacts with cytochrome c. This interaction, which results in H_2O_2 production [162], can be supported by an earlier report showing that p66Shc has internally located mitochondrial targeting sequence [164]. In order to explain its activity, p66Shc has to be phosphorylated at serine 36 by PKCβ, and this event leads to a consequent recognition by the prolyl isomerase Pin1, allowing p66Shc entrance into mitochondria [156]. Our recent data have indicated that p66Shc is also present in MAM (mitochondria-associate membranes) fraction, which consists in membranes interacting with mitochondria [165, 166]. Interestingly, the level of p66Shc in MAM fraction changes in an age-dependent manner (MAM fraction isolated from livers of old animals contained more p66Shc than MAM isolated from young individuals) [167]. In response to the oxidative stress, high p66Shc Ser36-phosphorylation status promotes both an additional intracellular ROS generation and reduction of the antioxidant defence system efficiency. The disturbance of the antioxidant enzymes level is connected with the recruitment of Akt to the p66Shc-FOXO3a complex, resulting in a direct inactivation of FOXO transcription factors by their phosphorylation [168, 169]. In this way, genes encoding antioxidant enzymes controlled by FOXO transcription factors are downregulated, with consequent increased ROS production and oxidative stress. Accordingly, decrease of phosphorylated p66Shc is accompanied by positive modulation of the antioxidant defence system [170].

Many alterations that extend lifespan affect not only stress response proteins, such as p66shc, but also nutrient sensors, such as insulin growth factor (IGF-1), target of rapamycin (TOR) protein kinase, AMP kinase (AMPK), sirtuins, and PGC-1α [171]. Caloric restriction (CR) is the best example of signals that modulate the activity of nutrients sensors, and it is the most robust intervention to extend lifespan and ameliorate various diseases in mammals, reducing oxidative stress and damage [172, 173]. Despite CR was historically considered a nongenetic process regulating lifespan, many recent lines of evidence have suggested its role in several signaling pathways, causing ROS reduction and mitochondrial biogenesis [174].

Nutrient sensor IGF-1 signaling pathway was the first to be associated to CR-mediated regulation of lifespan in animals. Indeed, heterozygous knockout mice for the IGF-1 receptor (Igfr +/−) live longer, show a delay in the onset of pathologies, and display greater resistance to oxidative stress [175, 176], suggesting that the effects of the IGF-1 pathway on longevity are closely related to mitochondrial protection from oxidative damage. Accordingly in *C. elegans*, mutations of downstream components of this pathway, such as age-1 (the homologue of PI3K) and akt-1 and akt-2 (the homologue of AKT1 and AKT2), result in extension of lifespan [153].

The most important targets regulated by IGF-1-signaling pathway are the transcription factors FoxO [177] and TOR protein kinase [178]. FoxO is required for the antiaging effects of the IGF-1 pathway [179, 180], once downregulated by CR, since it induces the expression of several antioxidative enzymes [181, 182], mediating the production of secondary signals that regulates lifespan.

TOR is highly conserved during evolution and functions as the major amino acid and nutrient sensor in the cell [183]. Much evidence points out the central role of this kinase in lifespan extension by CR. In yeast, *C. elegans,* and *Drosophila*, TOR is required for the effects of dietary restriction, and, importantly, its downregulation extends lifespan of all these models [184] and in mice too [185].

Recently, other signaling proteins have been shown to converge and regulate the FoxO transcription factors and thus oxidative stress and aging. Specifically, the proteins known as sirtuins, NAD-dependent histone deacetylases [186], are activated during CR, when the NAD/NADH ratio is elevated [187]. This group of proteins increases lifespan in yeast, *C. elegans*, and *Drosophila*, and it is thought to act similarly in mammals [188]. In this respect, aging is often associated with reduced sirtuins levels. Sirtuins are mainly antiaging genes via the promotion of mitochondrial function and autophagy and inhibition of apoptosis. They also have an inhibitory activity on ROS. Sirtuin function may be enhanced by restricting caloric intake or increasing physical activity, thereby, extending lifespan. Importantly, three of seven mammalian sirtuins (SIRT3, 4, and 5) are targeted to mitochondria, and SIRT1 is a regulator of mitochondrial biogenesis [189]. SIRT3 is required for the maintenance of mitochondrial integrity and metabolism during stress [190]. The protective effects of CR on oxidative stress and damage are diminished in mice lacking SIRT3. SIRT3 reduces cellular ROS levels dependent on SOD2. SIRT3 deacetylates two critical lysine residues on SOD2 ($Lys^{53/89}$) and promotes its antioxidative activity. Importantly, the ability of SOD2 to reduce cellular ROS and promote oxidative stress resistance is greatly enhanced by SIRT3 [191]. In a very recent paper, Chen et al. have reported that SOD2 is also acetylated at Lys^{68} and that this acetylation decreases its activity. SIRT3 binds to, deacetylates, and activates SOD2. Increase of ROS levels stimulates SIRT3 transcription, leading to SOD2 deacetylation and activation [192].

Other studies confirmed that SIRT1 is part of the insulin/IGF-1 pathway, being able to deacetylate and, thus, activate FoxO transcription factors [193]. Moreover, SIRT1 can

activate PGC-1α (peroxisome-proliferator-activated receptor γ coactivator-1α). Several data linked PGC-1α and aging. First, PGC-1α is progressively downregulated during aging, and this event is prevented by CR [194]. Furthermore, oxidative stress is known to induce the expression of this gene [195]. In general, PGC-1α improves mitochondrial dysfunction, the major phenotype of aging and age-related diseases, in a tissue-dependent fashion [196] and during CR promotes mitochondrial biogenesis [197], thereby lowering ROS production [198]. PGC-1α is also directly activated by phosphorylation by AMPK that is the master nutrient sensor in the cell [199]. The same kinase is able to phosphorylate FoxO factors too [200].

Recently another protein, named Bmi1, has demonstrated to increase the expression of a collection of gene products involved in mitochondrial function and ROS production in aging. Cells defective in Bmi1 have significant mitochondrial dysfunction with a sustained increase in ROS that is sufficient to cause a marked increase in the intracellular levels of reactive oxygen species and subsequent engagement of the DNA damage response pathway. Also mice Bmi1 $-/-$ present numerous abnormalities including a severe defect in stem cell self-renewal, alterations in thymocyte maturation, and a shortened lifespan [201].

Aging is characterized also by the decline of the autophagic pathway [202]. As described in the previous section, autophagy is required in order to remove compromised mitochondria suffering from oxidative stress. Moreover, autophagy modulation in different model organisms has yielded very promising results suggesting that the maintenance of a proper autophagic activity contributes to extend longevity [203]. During aging, the number of mitochondria suffering from oxidative stress may increase, while their cleanup by the autophagic system may become limiting, leading to the accumulation of damaged mitochondria [204]. It is important to point out that a greater pool of functional mitochondria could ameliorate tissue damage by opposing cells against the gradual energetic decline occurring in cells as mitochondria become damaged during aging [171].

In this respect, the efficient and selective removal of damaged mitochondria by autophagy is a crucial element in the maintenance of cellular health since the accumulation of ROS from dysfunctional mitochondria and eventual cell death via apoptosis is avoided [204]. At the level of the organism, apoptosis will be the ultimate resort to remove seriously damaged cells. This will particularly affect the lifespan of nondividing cells, like neurons, thereby, affecting the lifespan of the whole organism. Indeed, the degenerative processes that arise in old organisms are partly caused by increased cell death of nondividing cells [205]. In any case, the accumulation of damaged mitochondria and their impaired removal is a hallmark of aging and will contribute to decreased cell viability. Interestingly, autophagy is regulated by the same signaling pathways that determine lifespan, acting as one of the downstream effectors of these signaling cascades. Autophagy is essential for the effects of the IGF-1/insulin pathway on longevity. The insulin/IGF-1 pathway regulates autophagy through TOR and FoxO, with opposite effects; TOR was shown to inhibit autophagy [206, 207], while FoxO induces autophagy [208, 209]. Moreover, SIRT1 seems to be, at least in part, responsible for the direct induction of autophagy [210], as it forms complexes with several autophagy-related genes (ATG) proteins, causing their deacetylation and activation [211]. Autophagy is suggested to be crucial also for the onset of age-related diseases since knockout animals for ATG5 and ATG7 in the nervous system show the accumulation of abnormal proteins and massive neuronal loss leading to neurodegeneration with decreased lifespan [212, 213]. Altogether, these data suggest the presence in the cell of a redundant control of autophagy that allows to finely tune this process under a wide array of physiological and pathological conditions.

7. Concluding Remarks

Mitochondria and ROS signalling tightly control cellular homeostasis by regulating fundamental cell-death and cell-survival processes like apoptosis and autophagy. Therefore, ROS production may be considered a "balance of power," directing the cell towards life or death. It is clear that many proteins that mediate apoptosis and autophagy directly affect ROS signalling, through translocation to the mitochondrial compartment and/or modulation of pro/antioxidant proteins. Although correlation between mitochondrial ROS and aging has been the subject of debate for over forty years, recent discoveries about the role of autophagy in cleaning up damaged mitochondria, prolonging lifespan, underline the importance of studying ROS dynamics and show that they continue to be extremely fashionable research targets.

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Review Article

Neurospora crassa Light Signal Transduction Is Affected by ROS

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In the ascomycete fungus *Neurospora crassa* blue-violet light controls the expression of genes responsible for differentiation of reproductive structures, synthesis of secondary metabolites, and the circadian oscillator activity. A major photoreceptor in *Neurospora* cells is WCC, a heterodimeric complex formed by the PAS-domain-containing polypeptides WC-1 and WC-2, the products of genes *white collar-1* and *white collar-2*. The photosignal transduction is started by photochemical activity of an excited FAD molecule noncovalently bound by the LOV domain (a specialized variant of the PAS domain). The presence of zinc fingers (the GATA-recognizing sequences) in both WC-1 and WC-2 proteins suggests that they might function as transcription factors. However, a critical analysis of the phototransduction mechanism considers the existence of residual light responses upon absence of WCC or its homologs in fungi. The data presented point at endogenous ROS generated by a photon stimulus as an alternative input to pass on light signals to downstream targets.

1. Introduction

The light perception of fungi is a part of the complex sensory system responding also to changes in the concentrations of nutrient substrates, hormones, temperature shifts, mechanical damage, and so forth, which allows the fungus to adapt its vital functions to environmental changes [1–3]. Fungi use light as a source of information but not as a source of energy.

Light, as all stress agents, increases intracellular concentration of reactive oxygen species (ROS) in fungi [1, 4]. Experimentally detected relationship of developmental processes with the action of factors increasing intracellular ROS concentration indicated that ROS act as signaling molecules regulating physiological responses and developmental processes in fungi [3, 5–7].

Considerable recent attention is focused to molecular mechanisms of ROS signal reception and transduction and modification of gene activity in response to stress factors.

Absence of biological motility and lack of behavioral responses in fungi led to induction of the synthesis of compounds (especially carotenoids and melanins in the case of light action) that ensure increased resistance to detrimental effects. Another adaptive response is the differentiation of survival structures such as sclerotia and, of course, spores—the copies of genetic material of the organism, well protected from damaging environmental influences.

Neurospora crassa has served as a model organism to study light responses in eukaryotic cells for several decades [2, 8–11]. In this organism, various processes of differentiation such as the induction of carotenoid production in mycelia [12], protoperithecial formation [13], phototropism of perithecial beaks [14], perithecial polarity [15, 16], and circadian rhythm [17, 18] are controlled by blue light, which is associated with the generation of ROS [4, 19–21]. Underlying these biological phenomena is the regulation of many *Neurospora* genes by light. Recently, of the 5600 detected genes on a whole genome microarray, approximately 5.6% or 314 responded to a light stimulus by a relatively rapid increase in transcript amount [22].

Neurospora crassa uses blue light (350–500 nm) as the primary signal for photoreception. The primary photoreceptor system for blue light in the fungus is the white collar (WCC) complex, a protein complex formed by two proteins WC-1 and WC-2. WC-1 is a protein with a flavin-binding domain and a zinc-finger domain and interacts with WC-2, another zinc-finger domain protein. The WCC complex

operates as a photoreceptor and a transcription factor for blue-light responses in *Neurospora*. It represents also a key transcription factor for circadian oscillator [10, 23].

On the other hand it has been shown that manipulation of ROS was a strategy to regulate cell differentiation in *Neurospora crassa* [5, 7, 24, 25]. In order to take a step closer to understanding ROS functions in *Neurospora* differentiation, the present review considers participance of ROS in blue light signal transduction through *N. crassa* WCC complex.

2. Light in *Neurospora* Development and Differentiation

After the classic studies performed by Beadle and Tatum in the 1940s, *Neurospora* became a recognized model in genetic and biochemical studies. *Neurospora* is multicellular and produces at least 28 morphologically distinct cell types, many of which are derived from hyphae [26, 27]. The mycelium of *N. crassa* is composed of multinuclear branched hyphae which show apical polar growth. The hyphae are divided into compartments (100–200 μm) by septa, each having a central pore up to 0.5 μm in diameter. The pore is permeable to cytoplasm, nuclei, and mitochondria. The septal pores of *N. crassa* are considered to be functional analogues of gap junctions of animal cells, plasmodesmata of plants, and microplasmodesmata of filamentous cyanobacteria [28]. The diffusional and electric relationships between hyphal cells are local, as it is in other organisms, and involve three or four compartments along the hypha. These relationships appear to be genetically determined and controlled by the gradient of membrane potential between hyphal compartments. They are also controlled by light of the blue-violet spectral area [1, 28].

Frequent fusion among hyphal filaments produces a complex hyphal network (the mycelium) [29] and promotes the formation of heterokaryons in which multiple genomes can contribute to the metabolism of a single mycelium. Specialized aerial hyphae are differentiated from vegetative hyphae in response to nutrient deprivation, desiccation, or various stresses, and these form chains of asexual spores (the multinucleate macroconidia) for dispersal [30] (Figure 1). The timing of macroconidiation is controlled by a circadian rhythm, which in turn is modulated by exposure to blue light. Another type of asexual spore, the uninucleate microconidium, is differentiated from microconidiophores or directly from the vegetative hypha [27, 30–32]. Limiting nitrogen induces a type of hyphal aggregation that leads to generation of multicellular female sexual organs (protoperithecia) [32, 33]. Mating is accomplished by chemotropic growth of a specialized female hypha from the protoperithecium toward the male cell (typically a conidium) in a process involving pheromones [34]. Once fertilized, protoperithecia increase in size, darken, and transform into perithecia. The sexual process is followed by a short-term diploid phase. In the perithecia, a fruiting body, black (melanin-containing) ascospores (haploid spores of the sexual cycle) mature for several days after meiosis. Each perithecium comprises 200–400 asci, each containing eight

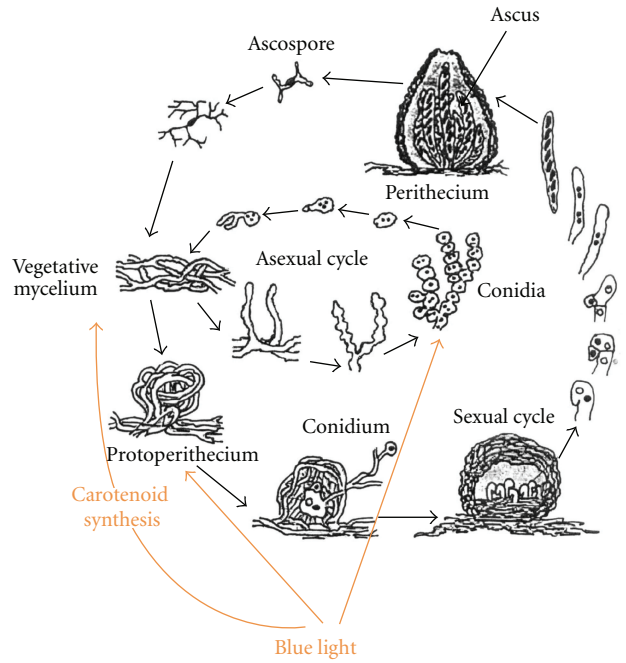

Figure 1: Life cycle of *Neurospora crassa*. Depending on environmental conditions, the vegetative mycelium can undergo the asexual sporulation processes (macroconidiation and microconidiation). It can enter the sexual cycle by forming protoperithecia. Upon fertilization, they initiate development leading to the production of meiotically derived ascospores. Blue light inputs are shown by arrows.

oval mononuclear haploid ascospores. During germination of ascospores, hyphae of vegetative mycelium develop, as in the case of conidia (Figure 1).

The genome of *Neurospora*, comprising 42.9 million bp, has been decoded [35]. The network of the fungus chromosomes includes 527 multigene families containing approximately 10,000 genes. Consistent with the greater biological complexity of filamentous fungi compared to both fission and budding yeast, *Neurospora* possesses nearly twice as many genes as *Schizosaccharomyces pombe* (4,800) and *S. cerevisiae* (6,300). *Neurospora* contains almost as many genes as *Drosophila melanogaster* (14,300), despite the relative developmental complexity of the latter [35]. The *Neurospora* gene complement also displays greater structure complexity than that of the two yeasts.

Neurospora can be easily cultured on media of a specific chemical composition. Its development cycle takes one to two weeks. A change of morphologically distinct development phases is easily induced by a change in the composition of the culture medium or other related factors. Quiescent spores germinate to form a haploid vegetative mycelium with hyphae spreading over the substrate at a rate of up to 10 cm/day. Filamentous branching hyphae of the mycelium are approximately 10–20 μm in diameter.

The effect of light is manifested at different stages of the *Neurospora* life cycle (Figures 1 and 2). Light promotes changes in the electrophysiological parameters of hyphae: the input resistance increases, followed by hyperpolarization of

the cytoplasmic membrane [36]. The last phenomenon may be accounted for by regulation of activity of H^+-ATPase, a plasma membrane proton pump [37].

Changes in these parameters are transient, and their values subsequently return to the initial level. Illumination also affects the intercellular communication mechanism (electric-bond coefficient) or, in other words, the rate of diffusion of ions between interseptal hyphal areas. It can be assumed that light-dependent changes in electrophysiological parameters are part of the energy cooperation system in interseptal hyphal areas, which allows the fungus to more effectively supply energy in the form of membrane potential for membrane transport in the apical compartment of growing hyphae [28]. The photoreceptor mutant *white collar* 1 (*wc-1*) has a lower constitutive membrane potential, disrupted intrahyphal communication mechanisms, and it lost all the blue-light induced electrical reactions: a transitive increase of input resistance and membrane potential [28]. Thus changes in the electrical properties of the *N. crassa* plasma membranes upon the light action appear to be controlled via WCC complex (Figure 2).

Light induces the expression of genes *albino* (*al*-1, *al*-2, and *al*-3) involved in carotenogenesis in hyphae and, as a result, the accumulation of neurosporaxanthine and other pigments imparting orange color to the mycelium (Figure 3) [38, 39]. Carotenogenesis in conidia, in contrast to mycelium, has a constitutive nature. The synthesis of carotenoids—the quenchers of oxygen-excited states and the inhibitors of free radical processes—is regarded as a means of cell defense against light-induced damage.

Two light-regulated phenomena, the electrogenic transport function of membrane and accumulation of carotenoids in the cell, are apparently physiologically related. In the *nap* mutant, damage of the proton pump which consumes as much as 50% of intracellular ATP caused an increase in the content of ATP and utilization of its energy in other metabolic processes (including the synthesis of precursors of carotenoids); as a result, the synthesis of pigments increased [40].

Light also affects some mycelial enzymes. For example, illumination increases the degree of phosphorylation of nucleoside diphosphate kinase [16], activates cAMP phosphodiesterase [41], and changes the activity of molecular forms of NAD^+-kinase [42]. In addition, light changes the inactive (reduced) form of nitrate reductase into the active (oxidized) state [43]. Photoreactivation with near ultraviolet light (UV-A) of DNA molecules damaged by more shortwave radiation occupies a special place. This phenomenon is based on DNA photolyase-catalyzed cleavage of C–C bonds between neighboring pyrimidine bases [44].

As mentioned above, differentiation of reproductive structures is controlled by a complex of external signals whose effect is regulated by the cell, with light playing a key role in this mechanism [45]. Exhaustion of a nutritive substrate is a necessary condition of differentiation. Some effects (e.g., carbon starvation or mycelium drying) promote rapid conidiation, with light additionally stimulating this process [45]. Nitrogen starvation induces the formation of protoperithecia and simultaneous (yet less active than in carbon starvation) conidiation. Unlike carbon starvation, in nitrogen starvation light inhibits conidiation and simultaneously stimulates the formation of protoperithecia [46]. In other words, under these conditions light determines the selection of either the sexual or asexual development pathway (Figure 1). Perithecia occurring during the sexual cycle are also sensitive to light, which induces their polarity (i.e., formation of a so-called beak at one end of the perithecium, which, in turns, exhibits positive phototropism) [14] (Figure 2).

Light also affects the circadian rhythm endogenous sensor function. Conidia are formed with a certain periodicity on the mycelium that spreads over the substrate surface, which leads to the occurrence of spatially separated sporulation zones. Pulse illumination changes rhythm parameters, and constant illumination suppresses manifestations of rhythmicity [11].

3. Light Effects Are Accompanied by Formation of ROS

All of the environmental stresses triggering *N. crassa* differentiation are apparently sources of ROS [24, 25, 47]. Among these factors are ionizing radiation (alpha, beta, gamma, and X-ray beams), UV radiation (far 200–290 nm, medium 290–320 nm, and near 320–420 nm), and visible light. ROS appear to mediate blue light effects in cells, but the sources of ROS and their respective roles in the cellular response to blue light are not completely understood. Direct evidence of ROS formation under light on various objects is given hereinafter.

High-fluence blue light can induce H_2O_2 generation at both the plasma membrane and the chloroplast of *Arabidopsis*. The high-fluence blue light-induced H_2O_2 generation can be abolished by the administration of the H_2O_2-specific scavenger catalase and other antioxidants or by the addition of diphenyleneiodonium, which is an NADPH oxidase inhibitor, and the blocker of electron transport chain dichlorophenyl dimethylurea [48]. The generation of $O_2^{-\bullet}$—(by the coleoptile tip of Sorghum bicolor and wheat (*Triticum vulgare*) was augmented upon illumination with blue light. Various thiol blockers caused powerful inhibition of blue light induced $O_2^{-\bullet}$ generation [49]. Blue light increased intracellular ROS equally in both normal human epidermal keratinocytes and oral squamous cell carcinoma. Blue light-generated ROS suppress cellular mitochondrial activity. However, the identity of blue light targets that mediate these changes remains unclear [21]. In addition, it was found that acute exposure of keratinocytes to both UVA and UVB results in activation of NOX and generation of ROS [50–52]. These studies suggest that a rapid activation of NOX by UV irradiation in these cells may have a distinct physiological importance. How irradiation activates NOX is not totally understood [47]. Thus UV-blue light effects on various organisms provide ROS formation inside the cells.

4. Intracellular Sources of ROS in Fungi

ROS are formed in fungi in the course of metabolic activity. The involvement of oxygen in metabolic processes in fungi is

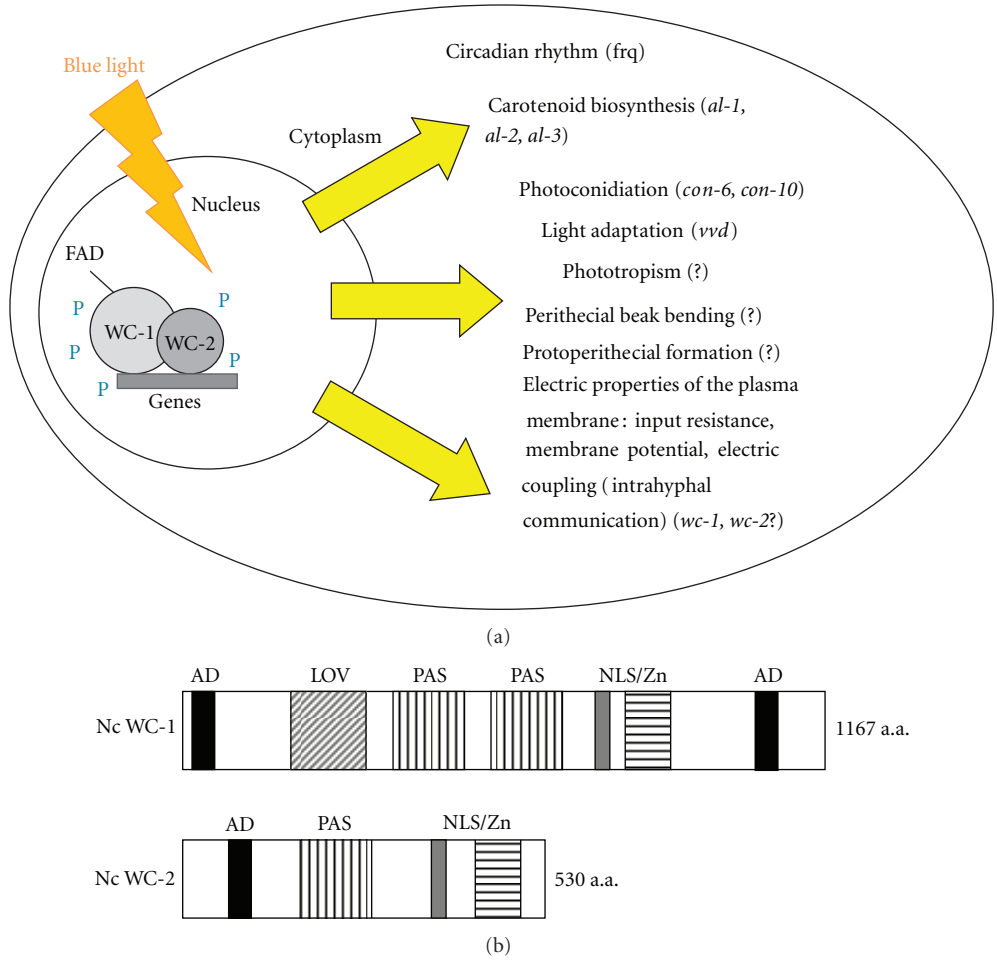

Figure 2: Blue light reception through WCC complex. (a) WCC-mediated gene expression and various light responses in *Neurospora crassa*. (b) Photoreceptor proteins in *Neurospora crassa*. The figure shows two multidomain proteins WC-1 and WC-2 forming photoresponsive WCC complex. WC-1 interacts with WC-2 through PAS (protein-protein interaction) domains. LOV-domain (a specialized variant of the PAS domain) in photoreceptor WC-1 noncovalently binds FAD. The two proteins contain activation domains (ADs), DNA-binding Zn-finger domains, and nuclear localization domains (NLSs).

coupled to its activation and formation of number of highly reactive compounds such as ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2), and $OH^\bullet$. In addition to the respiratory chain, as an intermediate product ROS are generated in reactions with involvement of xanthine oxidase, microsomal monooxygenases, lipoxygenase, and, as a result, of autooxidation of thiols, flavins, quinones, and catecholamines, as well as the reduction of xenobiotics [53].

Certain intracellular enzymes producing ROS cannot be ruled out. These include, first of all, NADPH-oxidases (NOX), specifically producing ROS and playing a significant role in growth and differentiation of *Neurospora crassa* [54–56]. It is known that specific enzymes, such as NOX, produce ROS to regulate different cellular functions, including growth, cell differentiation, development, and redox-dependent signaling [47, 54–56]. The fact that NOX regulate developmental processes in different microbial eukaryotes suggests that ROS regulate cell differentiation, and that this is a ROS ancestral role conserved throughout the eukaryotes [47]. Enzymes belonging to the NOX family produce $O_2^{-\bullet}$ in a regulated manner. It has been shown in *N. crassa* that NOX-1 and NOX-2 are both involved in different aspects of growth and development; a single regulatory subunit, NOR-1, an ortholog of the mammalian NOX-2 regulatory subunit gp67 (phox), is regarded for the function of both NOX. *N. crassa* NOX-1 elimination results in complete female sterility, decreased asexual development, and reduction of hyphal growth. The lack of NOX-2 did not affect any of these processes but led instead to the production of sexual spores that failed to germinate, even in the presence of exogenous oxidants. These results indicate a link between NOX-generated ROS and the regulation of growth [55].

It was revealed that $NO^\bullet$ synthase participated in asexual spore development of *N. crassa* and in differentiation of other fungi [57]. Glyoxal oxidase appeared to be involved in differentiation of phytopathogenic fungi [58, 59].

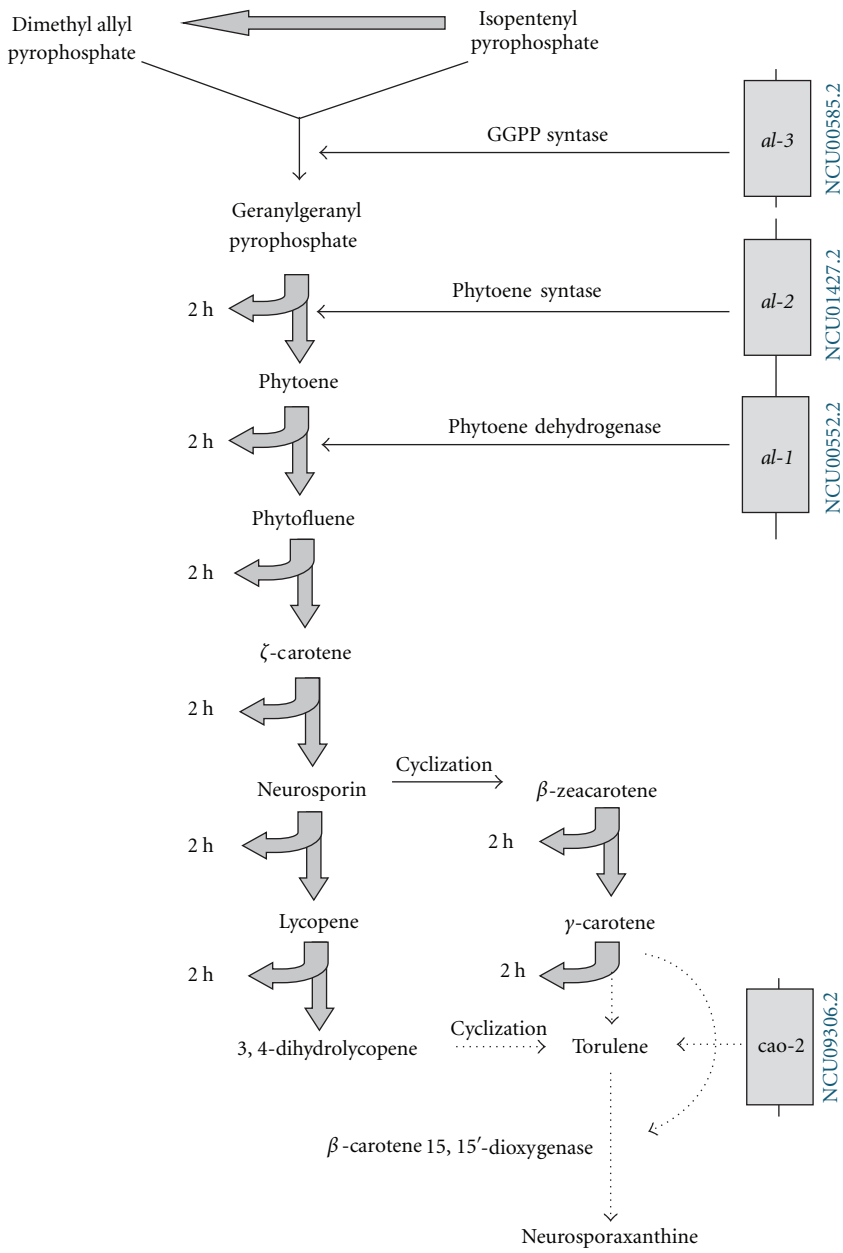

Figure 3: The enzyme pathway of carotenoid biosynthesis in *Neurospora crassa*. NCU numbers of light-regulated genes are shown. The figure is modified from Uspekhi Biologicheskoi Khimii [1].

5. ROS in *Neurospora* Development and Differentiation

5.1. Changes in ROS Concentration and Differentiation in Fungi. High reactivity of ROS is responsible for oxidation of proteins, lipids, and nucleic acids. Consequently, systems defending against ROS by repair or resynthesis of damaged molecules are present in the cell. Nevertheless, impairment of intracellular redox status, as a result of an increase in generation of oxygen radicals exceeding the cellular capacity to neutralize them, can generate a hyperoxidation state (oxidative stress). As distinct from growth and differentiation state, oxidative stress is an unstable one, and elimination or partial inhibition of intracellular antioxidant systems may cause cell death [5, 7]. Intracellular ROS increase is accompanied by the cessation of growth, and it provokes morphological changes leading to cell adaptation to changes in life conditions as well as the decrease in intracellular oxidants. Numerous experimental data support the relationship of differentiation triggering processes with an increase in intracellular ROS [5, 60–63]. Just so, in the myxomycete *D. discoideum* an increase in intracellular $O_2^{-\bullet}$ as well as extracellular one provoked aggregation of myxamoebae and subsequent differentiation, and the aggregation process was

prevented by $O_2^{-\bullet}$ scavengers together with an increase in expression of genes controlling antioxidant defense systems (ADS) [62].

H_2O_2 is considered as one of the most important metabolites in all respiring cells. H_2O_2 provoked global changes of gene transcription, including the ADS genes, in *A. nidulans* [60], as well as sclerotial differentiation in *Sclerotium rolfsii* [63], increased expression of genes of carotenogenesis in *N. crassa* [64], and promoted transition to filamentous growth in *U. maydis* and development of its pathogenicity [59]. It is known that sclerotial differentiation in *S. rolfsii* is coupled to H_2O_2 generation inside the cell. Its concentration increased under the action of light and iron ions [63].

$OH^{\bullet}$ formed on the interaction of transition metals with H_2O_2 was inhibited by such scavengers as dimethylsulfoxide, phenylthiourea, p-nitrosodimethylaniline, ethanol, and benzoate, which suppress sclerotial differentiation in *S. rolfsii* [65]. Sclerotial differentiation was similarly inhibited by antioxidants (ascorbic acid, β carotene) [66, 67]. It was shown that $O_2^{-\bullet}$ increased cleistothecium differentiation in *A. nidulans* [68], while $NO^{\bullet}$ promoted fruit body development in *F. velutipes* [69].

At the onset of different stages of *N. crassa* macroconidia differentiation (aggregation of hyphae, aerial hyphae formation, differentiation of macroconidium), a spontaneous, low-level chemiluminescence was detected enhanced by lucigenin and/or luminol, indicative of an increase in level of intracellular oxygen radicals. Antioxidants abolished chemiluminescence and stopped differentiation, which supports the formation of ROS ahead of every stage of fungal development [70]. Thus ROS formation is essential for differentiation of *N. crassa* as well as development of other fungi.

5.2. Changes in Fungal Cell Metabolism under ROS Action. An increase of oxidant level inside the cell inevitably causes the oxidation of organic molecules. It has been shown that differentiation of sclerotia on the mycelium of *S. rolfsii* was accompanied by lipid peroxidation [70]. Light and Fe^{2+} enhanced lipid peroxidation as well as the intensity of sclerotium formation [63], and lipid peroxides and aldehyde degradation products inhibited many proteins, affected cell differentiation and proliferation, and might promote apoptosis [71].

Oxidation of sulfhydryl groups in proteins upon ROS action promotes a change in activity of some enzymes. As an example, decrease in glycolytic enzymes and decline of protein synthesis enzymes have been observed, coupled to cessation of growth [72, 73].

Oxidative stress was accompanied by cessation of growth and severe metabolic changes directed towards decrease in primary metabolites (acetate, glucose) and synthesis of compounds participating in cell protection, for example, carotenoids, melanins, proline, and polyols [3]. Trehalose is of fundamental importance in defending yeast cells in oxidative stress [74]. At the start of separate steps of macroconidium differentiation in *N. crassa*, mass protein oxidation and their subsequent degradation [75], release of iron ions upon oxidation of [Fe-S] clusters of enzymes, oxidation of intracellular NADP and NADPH, glutathione oxidation, glutathione disulfide excretion to the extracellular medium [76], synthesis of antioxidant enzymes [7, 77], and ROS-dependent chemiluminescence [24] were the experimental evidence of hyperoxidant state. An increase in protein carbonylation by ROS has been observed in different species of mycelial fungi: *Mucor racemosus*, *Humicola lutea*, *F. oxisporum*, *A. solani*, *Cladosporium elatum*, *Penicillium chrysogenum*, *P. brevicompactum*, *P. claviforme*, *P. roquefortii, A. niger, A. argilacceum, A. oryzae,* and *N. crassa* [78].

A comparative study of the changes in the components of the ADS, the activity of superoxide dismutase (SOD) and catalase and the level of extractable SH-groups, during the growth of wild-type and *N. crassa* mutants (*white collar*-1 and *white colar*-2) showed that oxidative stress developing during spore germination and upon the transition to a stationary growth phase was accompanied by an increase in the level of extractable SH-groups and SOD activity in all the strains, whereas the total catalase activity decreased during growth. However, in contrast to the wild-type strain, the activity of the catalase in the mutant strains *wc*-1 and *wc*-2 slightly increased upon the transition to the stationary phase. In the *wc*-2 mutant, SOD activity and the level of extractable SH-groups in the exponential growth phase were always lower than those in the wild-type and *wc*-1 strains [79]. As in previous works [5, 7, 75, 76], our data pointed to formation of ROS upon transition to interchangeable phases of development. Moreover, the data revealed that mechanisms of inactivation of increased intracellular ROS, developing during spore germination and entry into the stationary growth phase, distinguished *wc*-1 and *wc*-2 mutants from the wild strain [79]. These data prompted us to pay a closer attention to mechanisms of blue light signal transduction through WCC and to the role of ROS in this process.

6. Photoreceptor Complex, Other Photoreceptors, and Other Signal Transduction Pathways

6.1. Photoreceptor Complex WCC. The main blue-light responses in *Neurospora* include induction of sporulation and sexual development, induction of carotenoid synthesis in mycelium, and the regulation of circadian clock. All of the mentioned processes require the products of *white collar* 1 (*wc*-1) and *white collar* 2 (*wc*-2) genes-GATA zink finger family members [80]. WC-1 is the product of the *wc*-1 gene, a protein with a Zn-finger, two PAS domains involved in protein-protein interactions, a putative transcriptional activation domain, a nuclear localization signal, and a chromophore-binding domain [81] (Figure 2). The chromophore binding domain binds the flavin chromophore FAD allowing WC-1 to act as a photoreceptor [82, 83]. The WC-1 flavin-binding domain (LOV-light, oxygen, voltage) has been described in other photoreceptor proteins, most notably in plant phototropins [84]. The primary photochemical event in phototropins is the formation of

a flavin-cysteinyl adduct at a cysteine of the LOV domain [85], suggesting that WC-1 activation occurs through the formation of a light-dependent flavin-cysteinyl adduct [86].

WC-2 is the product of the *wc*-2 gene, a protein with a zink finger, a single PAS domain, a putative transcriptional activation domain, and a nuclear localization signal [87] (Figure 2). WC-1 and WC-2 interact through the PAS domains to form a WCC complex [88–93]. In the WCC complex, WC-1 is the limiting factor while WC-2 is in excess [89, 93]. WCC binds the promoter of light inducible genes [81, 82, 94–96]. Light causes a decrease in the mobility of the WCC complex bound to the promoter, suggesting a light-dependent aggregation of WCC complexes [82, 96].

The WCC proteins are present in the dark [92, 93, 97] and are preferentially located in the nucleus although WC-2 is also observed in the cytoplasm and is more abundant than WC-1 [92, 95, 97, 98]. Nuclear localization of either WC-1 or WC-2 is not affected by light and is not altered by mutations in *wc*-2 or *wc*-1, respectively, indicating that nuclear localization does not require a complete WCC complex [97].

Microarray analysis showed that the expression of 314 genes responded to the light stimulus by increasing transcript levels [22]. Most of the identified genes (92%) were either early (45%), with peak expression between 15 and 45 minutes, or late (55%), with the induced expression peaking between 45 and 90 minutes after lights on [1, 99]. Genes related to the synthesis of photoprotective pigments (7.1%), vitamins, cofactors, and prosthetic groups (4.7%), secondary metabolism (4.7%), DNA processing (6.3%), cellular signaling (5.5%), and environmental sensing and response (1.6%) were found enriched in the early light response. In contrast, genes involved in carbohydrate metabolism (20%), oxidation of fatty acids (1.9%), and oxygen detoxification reaction (2.5%) were found enriched in the late light response. Within the early group were several transcription factors most of which show mutant phenotypes during development. Transcription factor SUB1 is required for efficient transduction of light signals to the most of late light response genes [22, 99].

Gene photoactivation is transient. After further light exposure, WC-1 is phosphorylated [93, 96, 97, 100] leading to exclusion of the WCC complex from the promoter and the end of gene transcription.

The protein VIVID, an additional *N. crassa* photoreceptor, is a flavoprotein and serves as a fungal blue light photoreceptor for photoadaptation [22]. VIVID (VVD) is a small protein (186 amino acids) with a single LOV domain functioning downstream of the WCC to regulate negatively the light responses initiated by the WCC [22, 86, 101–104]. The induced VVD protein accumulates in the nucleus and physically interacts with WCC to regulate photoadaptation by repressing WCC activity in constant light. The kinetics of photoadaptation is predominantly regulated by the amount of VVD protein in the system [105].

The excluded WCC complex is dephosphorylated and partially degraded, probably through an interaction with the protein kinase C (PKC). Since protein phosphatase 2A participates in the dephosphorylation and activation of the WCC complex in vivo [95], it is possible that this enzyme is also involved in the dephosphorylation of the WCC complex after light exposure. After a certain period in the dark the WCC complex, probably with the addition of newly synthesized WC-1 and WC-2, is ready for gene photoactivation again.

The amount of WC-1 and the kinetics of the light-dependent phosphorylation is altered by the presence of a mutant form of WC-2 suggesting that WC-2 is necessary to sustain the transiency and magnitude of WC-1 phosphorylation [93, 97, 106].

In *Neurospora*, the photoreceptor complex WCC serves as an exogenous regulator of the circadian clock which is an important recipient of light information (Figure 2). The circadian clock controls the program of *Neurospora* development [107, 108]. When cultured in the dark in the absence of external signals, the fungus periodically (with an approximately 21.5-hour period in the case of wild-type cells) switches from mycelial growth to conidiation. VVD has been shown to take part in regulating various circadian clock properties, most likely through its effects on the WCC, including gating of light input to the clock [101], maintenance of the clock during the light phase [102, 103], and temperature compensation of the circadian phase [104].

Thus, the first fungal photosensor identified was White collar-1 (WC-1) of *N. crassa* [108], and this system has been extensively studied with emphasis on the circadian clock of this fungus and how it is regulated by the WC-1 and WC-2 proteins, the clock protein FRQ and interacting factors [109, 110]. More recently, WC-1 homologues have been identified in basidiomycetes [111, 112] and zygomycetes [113, 114] as well as other ascomycetes [115, 116]. This information extends the function of WC-1 homologues in photosensing across the fungal kingdom.

6.2. Other Photoreceptors. The *Neurospora* genome contains genes for additional photoreceptors, including a cryptochrome gene (*cry*), an opsin gene (*nop* 1), and two phytochrome genes (*phy* 1 and *phy* 2), but their function in *Neurospora* photobiology remains mostly unknown [26, 35].

It has been shown recently that the activity of the WCC is negatively regulated by the photoreceptors CRY-1, NOP-1, and PHY-2, presumably through the light-dependent activation of a putative repressor of the WCC. It is possible that each photoreceptor may activate an independent repressor of the WCC [117].

The regulation by secondary photoreceptors of the WCC may modify the activity of some genes, as it was observed for *con*-6, *al*-1, and *vvd*. It has been suggested that a light-dependent repressor of the WCC may be a general feature of light reception in *N. crassa* [117].

A major regulator of conidiation in *Aspergillus nidulans* is the product of the gene *veA*. VeA is preferentially located in the nucleus in cells grown in the dark, which is consistent with the role of VeA as a repressor of light-dependent processes [118], and the VeA protein interacts in a complex with other regulatory proteins for the regulation by light of development and secondary metabolism [119]. The *Neurospora* ortolog *ve-1* encodes a protein Ve-1. A pronounced reduction in light-dependent carotenoid

accumulation (threefold) was observed in the *ve*-1 strain suggesting that the putative regulatory Ve-1 protein is required for full photocarotenogenesis in *Neurospora* [117].

6.3. The Complexity of Neurospora Light Sensing Cascade. It cannot be ruled out that there are possibly multiple intertwined pathways in the mechanism of photosignal transduction. The analysis of promoters of approximately 20 light-inducible genes did not reveal any common cis-acting elements (i.e., DNA sequences that are recognized by light-dependent transcription factors). The situation is additionally complicated by the fact that there are light-dependent genes whose expression is not mediated by the functional proteins WC-1 and WC-2 [87, 120].

An additional complicating factor may be chemical modification (enzymatic methylation) of these sequences. Although the genome of *Neurospora* is methylated fairly weakly, it is known that the level of methylation may undergo changes in the course of ontogeny [121]. It should be noted that methylation plays an important role in ontogenetic photoregulation. There are grounds to believe that the WCC complex is involved in the regulation of DNA methylation, the level of which determines the light-dependent selection of either sexual or asexual development by the fungus. It was shown that 5-azacitidine, an inhibitor of DNA methylation, suppressed photoinduced formation of sexual structures (protoperithecia) and simultaneously abolished the inhibitory effect of light on conidiogenesis [46, 122].

It should be noted that illumination induced a rapid and transient (30–600 s) decrease in the cAMP content in *Neurospora* mycelium [123, 124] as a result of increase in the activity of cAMP phosphodiesterase [41]. The treatment with 3-isobutyl-1-methylxanthine, an inhibitor of phosphodiesterase, as well as addition of exogenous cAMP, inhibited the cell response to illumination (expressed as the synthesis of carotenoids), whereas a decrease in the cAMP level, observed in some mutants, was accompanied by induction of carotenogenesis in the dark. Exogenous cAMP completely inhibited photoinduction of expression of the genes *al*-1, *al*-2, *bli*-3, *bli*-4, *ccg*-2, *con*-8, and *con*-10. It can be assumed that the effect of cAMP may be implemented at the transcriptional level via the cyclic AMP response element (CRE) in the promoters of photoinducible genes [125]. In addition, it cannot be excluded that photoregulation influences the change in phosphorylation of WC proteins that is catalyzed by cAMP-dependent protein kinase. The involvement of cAMP pathway in blue light signal transduction, possibly with the involvement of RAS protein, was mentioned in several investigations on *N. crassa* [22, 126, 127] and *Trichoderma atroviride* [128]. Possibility of ROS participation in blue-light signal transduction makes the regulatory networks of the *Neurospora* light-sensing cascade far more complicated.

7. ROS Affected WCC Signal Transduction

According to previous studies with various fungi an assumption was produced that intracellular redox state and light-induced carotenogenesis were related processes [129, 130].

Sure enough, some experimental data consider participance of ROS in blue light signal transduction through WCC complex in *N. crassa*. It was shown that illumination of *Neurospora* mycelium under O_2-enriched air increased transcript level of *al*-1 encoding phytoene dehydrogenase. It also highly enhanced carotenoid production in the mycelium [131]. These results suggest that increased ROS, under oxygen enriched air, could increase light-induced carotenoid production and might act as a controlling factor in the WCC-signaling cascade, because the light-induced expression of *al*-1 mRNA depends on WCC complex function [64]. This assumption was supported by the fact that *sod*-1 mutant, with a defective Cu, Zn-SOD showed accelerated light-dependent induction of carotenoid biosynthesis in the mycelium compared to the wild type [131]. In *N. crassa*, catalase-3-deficient mutants showed increased carotenoid production in colonies under illumination [25]. It was found that menadione treatment of *Neurospora wc* mutants restored circadian conidiation in *N. crassa* [127].

Thus intracellular ROS apparently enhance several light-induced responses in *N. crassa*. They increase the blue-light action but apparently do not trigger WCC-induced blue-light responses.

It is well known at present that increase in intracellular ROS is accompanied by activation of intracellular ADS specific to developing ROS [132]. Antioxidant enzymes (SOD and catalase) in wild type and *wc*-1 and *wc*-2 mutants of *N. crassa* responded differently to various stress factors (oxygen, light, temperature increase) which rise intracellular ROS in cells. Menadione treatment provided SOD increase in the wild type. The enzyme activity did not change in WCC-mutants (*wc*-1 and *wc*-2) [133]. Protein carbonyls (oxidative stress marker) showed a double increase in the wild type (but not in the mutants) as a result of menadione treatment [133]. No increase in SOD activity as well as no rise in protein carbonyls was revealed in WCC mutants thus demonstrating high resistance of the mutant strains to menadione.

It should be noted that a high increase in catalase activity was found only in WCC mutants (*wc*-1 and *wc*-2) upon action of stress agents increasing intracellular ROS [134]. Increase in catalase activity in the mutants upon H_2O_2 treatment points to H_2O_2 signal transduction independent of WCC. Thus WCC apparently participates in environment signal transduction forming intracellular $O_2^{-\bullet}$ in the wild type. Lack of SOD activity increase upon stress agents, including light, and high resistance of WCC mutants to menadione, points apparently to some other systems preventing intracellular $O_2^{-\bullet}$ formation in *wc*-1 and *wc*-2 [134].

As it has been mentioned before, each morphogenetic step of *N. crassa* conidiation was preceded by NAD(P)(H)/NAD(P) and GSH/GSSG redox imbalance [76]. Generation of singlet oxygen was observed during germination of *N. crassa* conidia [135]. Exposure of fungal cells to oxidative stress results in the modulation of various signaling pathways. Oxidation and reduction of protein thiols are thought to be the major mechanisms of ROS integration into cellular signaling pathways. It has been shown that incubation in air (increased intracellular ROS) provided

a significant protein disulfide increase only in the *N. crassa* wild type mycelium particularly under light treatment. A decrease in the formation of disulfide bonds in the proteins of *wc*-1 and *wc*-2 mutants (as compared with the wild type strain) was recorded [134]. It can be assumed that at least one ROS signal transduction pathway may be controlled by the WCC.

The main intracellular source of ROS is the mitochondria respiratory chain. Comparative analysis of respiratory activity in the *N. crassa* wild type and its photorecepror complex mutants (*wc*-1 and *wc*-2) revealed high cyanide-resistant respiration in the mutant strains under glucose oxidation pointing to the increased activity of alternative oxidase in the mutant strains. This fact was confirmed by inhibitory analysis [136]. Transfer of electrons through alternative oxidase is not coupled with ATP synthesis. Alternative oxidase prevents autooxidation of electron carriers under ROS increase [137]. It can be assumed that antioxidant defence in WCC mutants is performed using catalase and alternative oxidase.

The data presented show that signal transduction via WCC complex enhances oxidative stress in *Neurospora* cells. In the WCC mutants—*wc*-1 and *wc*-2—no experimental evidence of oxidative stress was revealed. On the other hand, alternative signal transduction pathways apparently functioned. The fact is confirmed by increase in catalase and alternative oxidase levels in *wc*-1 and *wc*-2 mutants. It can be assumed that accomplishment of blue-light responses through WCC-complex in *Neurospora* cells is coupled to oxidative stress.

8. Conclusion

Light signaling pathways and circadian clock have profound effects on behavior in most organisms. *N. crassa* is eukaryotic model for light responses and circadian clock. Sequence and functional orthologs of WC-1 and WC-2 and most of the other light signaling components are widespread among the fungal kingdom. Recent studies have demonstrated that WC-1- and WC-2-like molecules in various fungal species play an essential role in mediating light signals from the Ascomycota, Basidiomycota, and Zygomycota phyla [8–10, 138, 139].

Successful work on the WCC in *Neurospora* has led to fundamental breakthroughs in understanding photobiology in other fungi. While many of the downstream genes regulated by the WCC are not well studied or are uncharacterized, most of them have homologs in plants and mammals. Unfortunately even after extensive research, little is known about mechanisms that directly link photoreceptor activation to signaling pathways eliciting light responses. Proteomic analysis across human, yeast, and bacterium has raised that the cellular stress response can be characterized by the induction of a limited number (300) of highly conserved proteins [140]. It is noteworthy that among the 44 proteins with known functions, 40% of them are related to regulation of the intracellular redox status. It is noticed that increased reactive oxygen species (ROS) generation seems to be a common response in fungal organisms exposed to stresses; thus, redox regulation in fungal cells may represent a second messenger system that is upstream of the fungal stress signaling network. It cannot be excluded that reactive oxygen species generated by a photon stimulus might provide a transcriptional response through redox signaling pathways or serve as an input pass on some extra signal transduction systems to downstream targets correcting for the complexity of *Neurospora* light sensing cascade.

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Review Article

Nuclear Transport: A Switch for the Oxidative Stress—Signaling Circuit?

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Imbalances in the formation and clearance of reactive oxygen species (ROS) can lead to oxidative stress and subsequent changes that affect all aspects of physiology. To limit and repair the damage generated by ROS, cells have developed a multitude of responses. A hallmark of these responses is the activation of signaling pathways that modulate the function of downstream targets in different cellular locations. To this end, critical steps of the stress response that occur in the nucleus and cytoplasm have to be coordinated, which makes the proper communication between both compartments mandatory. Here, we discuss the interdependence of ROS-mediated signaling and the transport of macromolecules across the nuclear envelope. We highlight examples of oxidant-dependent nuclear trafficking and describe the impact of oxidative stress on the transport apparatus. Our paper concludes by proposing a cellular circuit of ROS-induced signaling, nuclear transport and repair.

1. Introduction

1.1. Reactive Oxygen Species. Oxidative stress is generated by an increase in reactive oxygen species (ROS), either in the form of free radicals or nonradical oxidants [1, 2]. Although elevated levels of ROS can damage a wide variety of molecules, ROS production is essential to normal cell physiology [3–12]. As such, ROS participate in cell-signaling events and can function as second messengers. Moreover, ROS are generated at sites of inflammation, where they fend off microbial infections [13–16]. On the other hand, ROS are believed to contribute to aging [3–9, 12]; they are also produced in response to environmental insults, such as X-rays, UV light, ultrasound, or microwave radiation [17–19]. At the cellular level, ROS are generated as metabolic byproducts of normal biological processes, with oxidative phosphorylation in mitochondria as the primary source in eukaryotic cells [20]. Aside from the mitochondrial electron transport chain, NADPH oxidases, cyclooxygenases, lipoxygenases, xanthine oxidase, and other cellular enzymes make also important contributions to cellular ROS production [21–25].

The different types of ROS and their mode of action have been discussed in detail [1, 11, 26–30]. ROS that are particularly important to cell physiology include the hydroxyl radical •OH, superoxide anion $\bullet O_2^-$, the nonradical hydrogen peroxide (H_2O_2), alkoxy and peroxy radicals, hypochlorous acid or peroxynitrite, and reactive sulfur species [1, 29, 31, 32]. Here, we recapitulate the properties of those ROS only that are relevant to the experiments discussed in this review.

The hydroxyl radical •OH is highly reactive and causes damage to nucleic acids and proteins, this radical also promotes lipid peroxidation [2, 12, 33]. Due to their high reactivity, hydroxyl radicals are especially harmful and considered a major cause of oxidant-induced damage [34]. The superoxide free radical $\bullet O_2^-$ can interfere with the proper function of enzymes by damaging their active sites, with cysteine residues being particularly susceptible [32]. In an experimental setting, superoxide radicals can be generated by providing xanthine oxidase with the appropriate substrates [35].

There is some debate about the impact of H_2O_2 on the cellular redox homeostasis. On one hand, H_2O_2 is not deemed a major direct threat for the cellular redox homeostasis due to its poor reactivity towards biomolecules [36]. However, H_2O_2 rapidly translocates through lipid bilayers

and is a potential precursor for •OH radicals [32, 37]. Thus, high concentrations of H_2O_2 can release iron from heme proteins and catalyze the conversion of H_2O_2 to hydroxyl radicals [37]. It was also proposed that the nonradical oxidant H_2O_2 may have profound effects on redox signaling in living cells, where it alters the function of redox circuits that are composed of redox-sensitive building blocks [1]. Despite these different views on how H_2O_2 contributes to oxidant-induced damage, we and others [38–42] have used this compound extensively to examine the impact of oxidative stress on nuclear transport (see below).

1.2. Oxidative Stress and Cellular Defense Mechanisms. The appropriate response to stress is fundamental to cell survival and the recovery from disease-related or environmental damage [3, 5, 6, 9, 11]. Thus, in order to maintain redox homeostasis, the balance between production and clearance of ROS is essential. Imbalances in ROS concentration, if left without proper intervention, can interfere with a wide variety of cellular processes, leading to serious injuries and possibly cell death, either by apoptosis or necrosis [28, 43].

Upon accumulation, ROS can interact inappropriately with a large number of biomolecules, including lipids, proteins, and DNA, thereby interfering with numerous cellular functions [28, 37]. For instance, ROS may induce damage to various enzymes, leading to the partial or complete loss of their function. Notably, ROS-damaged proteins can form toxic aggregates that cause cell injury and ultimately cell death [16]. Furthermore, ROS-induced lipid peroxidation may alter the permeability of cellular membranes, potentially destroying the membrane integrity and triggering cell death [33, 44]. In addition, ROS-induced modifications of DNA can be mutagenic, possibly initiating cell transformation and promoting cancer [45].

In line with the complex pattern of damage triggered by oxidative stress, ROS accumulation contributes to the pathophysiologies of many human diseases and syndromes. In particular, oxidative stress plays a critical role in the onset and the progression of neurodegenerative disorders, diabetes, cardiovascular diseases, and nephropathy [27, 46–58].

To counteract the potential damage of elevated ROS concentrations, cells have developed different strategies that limit the action of reactive compounds and prevent their accumulation. To this end, eukaryotic cells are equipped with multiple defense mechanisms that promote the removal and inactivation of ROS in different cellular compartments [59–62]. These mechanisms rely on the coordinated action of several enzymatic systems that are able to react with and neutralize different ROS. For example, the superoxide dismutase (SOD) system is essential to redox homeostasis [11, 63–65], as it catalyzes the conversion of $\bullet O_2^-$ to H_2O_2. H_2O_2 produced by SOD can then be eliminated by the enzymatic action of catalases.

The glutathione/glutathione disulfide system (GSH/GSSG) is one of the major contributors to redox homeostasis and of particular importance to the intracellular redox state. Accordingly, glutathione is believed to be the primary defense when cells are injured by oxidative stress during ischemia/reperfusion [66, 67]. Moreover, changes in the GSH/GSSG ratio affect the intracellular redox state, and depletion of intracellular glutathione generates oxidative stress [61]. Owing to its pivotal importance to redox homeostasis, imbalance of the GSH/GSSG system has been linked to many human diseases, pathologies, and aging [11, 66, 68]. The GSH/GSSG system can be modulated experimentally, and diethyl maleate is one of compounds that deplete glutathione, thereby causing oxidative stress [38, 69]. Furthermore, the cellular redox homeostasis can also be altered by changing the activity of glutathione peroxidase, glutathione, or thioredoxin reductase.

2. Oxidative Stress and Nucleocytoplasmic Transport

2.1. Nuclear Transport of Macromolecules. Nucleocytoplasmic transport is central to the cellular homeostasis, as the proper and timely response to endogenous and environmental stimuli relies on the communication between the nucleus and cytoplasm. This applies in particular to kinases and phosphatases, many of which move in and out of the nucleus in response to oxidants or other stressors (see below). The nuclear envelope provides the barrier between these two compartments [70, 71], and macromolecules traverse the nuclear envelope via nuclear pore complexes (NPCs). Trafficking in and out of the nucleus controls signal transduction, gene expression, cell-cycle progression, and apoptosis; regulated nuclear transport is also essential for development and required for the proper response to stress [72–75]. The separation of nucleus and cytoplasm is ideal to divide signaling and other events. However, this compartmentalization can impede the intracellular communication if components of the nuclear transport apparatus are affected by ROS. This is indeed the case, as nuclear transport factors are primary cellular targets for oxidants. Before describing the impact of oxidative stress on nuclear transport, we briefly summarize those mechanisms of nuclear trafficking that are relevant to our review (Figure 1).

Although diffusion across the NPC is not simply a function of the molecular mass, most proteins that are larger than 40 kD do not efficiently diffuse across the nuclear envelope. Nevertheless, molecules exceeding the diffusion channel of the NPC can move in or out of the nucleus if they carry specialized transport signals. Nuclear localization (NLS), nuclear export (NES), or shuttling sequences serve as permanent signals that mediate targeting to the proper location. Classical NLSs are characterized by clusters of basic amino acid residues, whereas NESs are frequently enriched for leucine or isoleucine residues. However, the final destination of a macromolecule not only depends on such transport signals; the steady-state distribution is also controlled by its retention in the nuclear or cytoplasmic compartment.

Nuclear Carriers. Nuclear transport of most proteins depends on transporters of the importin-β group (also called karyopherin-β). Importin-β family members interact with their cargo either directly or through an adaptor. The latter

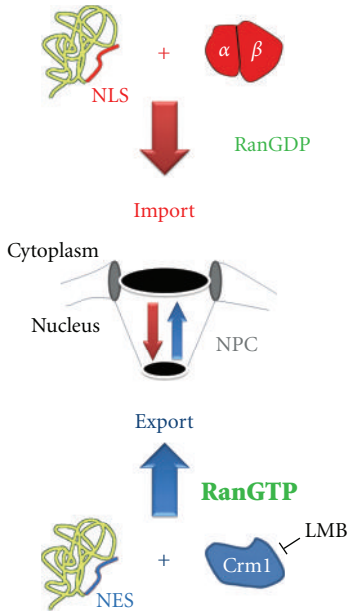


Figure 1: Simplified model for classical nuclear import and Crm1-mediated export, two essential transport pathways. Classical nuclear import depends on the carrier importin-β and the adaptor protein importin-α. Together, importin-α/β move NLS-containing cargos to the nucleus. The absence of RanGTP from the cytoplasm permits the assembly of import complexes in the cytoplasm. Conversely, the high RanGTP concentration in the nucleus promotes the dissociation of classical import complexes after they translocate across the NPC. RanGTP in the nucleus is also necessary to generate export complexes that contain Crm1 and NES-containing cargo. The function of Crm1 is inhibited by leptomycin B (LMB).

applies to classical nuclear import, which relies on the carrier importin-β1 and its adaptor importin-α (Figure 1). Multiple isoforms of importin-α exist in higher eukaryotes, where they recognize classical NLSs in endogenous and fluorescent cargos such as NLS-mCherry (Figure 2(a)). Crm1/exportin-1 [76], another importin-β family member, moves NES-containing proteins like mCit-NES to the cytoplasm (Figure 2(b)). This transport route can be inhibited specifically with leptomycin B, a compound that covalently modifies a cysteine residue of Crm1 [77].

The RanGTPase System. Carriers of the importin-β family require the small GTPase Ran and factors that modulate Ran activity. These factors include in the cytoplasm RanBP1 (Ran-binding protein 1) and the GTPase activating protein RanGAP1, with RanGAP1 binding to Nup358 at the cytoplasmic side of the NPC. By contrast, the RanGTP-binding protein RanBP3 and the guanine nucleotide exchange factor RCC1 (RanGEF) are located in the nucleus, where RCC1 binds to chromatin. The asymmetric distribution of Ran modulators generates a gradient across the nuclear envelope, with RanGTP in nuclei and RanGDP in the cytoplasm (Figure 1). This gradient provides the driving force for all importin-β dependent transport [70, 71].

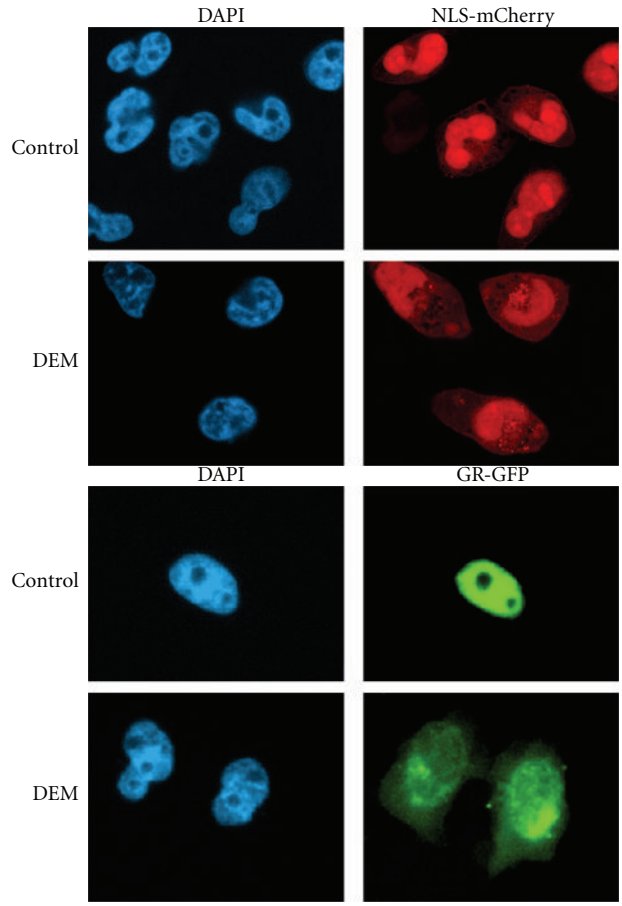
Regulation of Nuclear Transport. Control of nuclear trafficking is crucial under normal, stress, and disease conditions, and it occurs on multiple levels [72, 73]. For instance, phosphorylation and other posttranslational modifications can change the transport of individual cargos [73, 78]. A more general regulation that affects multiple transport cargos is achieved by targeting components of the nuclear transport machinery. This can be accomplished by altering the localization or posttranslational modification of transport factors, and such changes are observed in response to oxidative stress [72].

The following sections summarize the effects of oxidative stress on specific cargos that are relevant to human health, the nucleocytoplasmic transport apparatus, and important signaling components. We will then build on this information to propose that the interdependence of oxidative stress, nucleocytoplasmic transport, and signaling provides a circuit that controls cell survival.

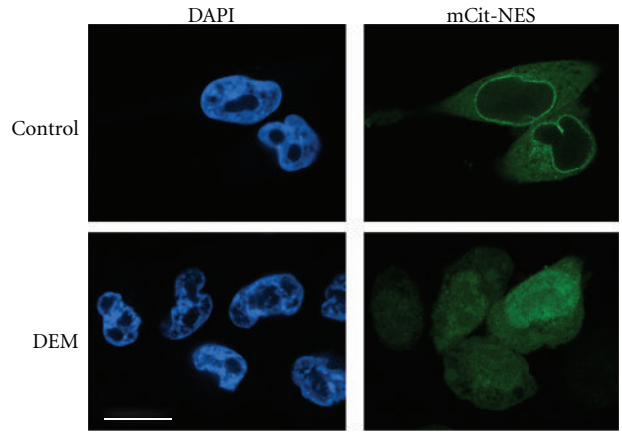
2.2. Oxidative Stress Impinges on Multiple Nuclear Cargos. As discussed above, oxidative stress causes the modification of targets in the nucleus and cytoplasm. Together, ROS-dependent modifications of cargos and the nuclear transport apparatus regulate the intracellular distribution of many of these targets. Among the oxidant-sensitive targets that translocate through NPCs are transcription factors, some of which are also implicated in the stress response. Prominent examples of transcription factors that relocate in response to oxidative stress are NF-κB and Nrf2 (NF-E2-related factor 2). The ROS-mediated redistribution of NF-κB and Nrf2 has been described extensively [80–83] and the relevant data will only be summarized here. Our discussion will focus on high-mobility group box 1 protein (HMGB1) and glycerolaldehyde-3-phosphate dehydrogenase (GAPDH) to illustrate the link between ROS, nuclear trafficking and signalling.

The role of NF-κB in immunity and inflammation is well established; however, this transcription factor is also critical for the synthesis of antioxidant proteins [80, 81]. The genes upregulated by NF-κB include MnSOD, Cu,ZnSOD, and HO-1 (heme oxygenase 1), all of which participate in antioxidant defense processes. ROS and numerous other stimuli control the intracellular distribution of NF-κB. In the absence of these stimuli, NF-κB is retained in the cytoplasm due to its association with I-κB. ROS trigger the degradation of I-κB, thereby promoting the nuclear accumulation of NF-κB and the subsequent transcription of genes that contain NF-κB response elements [81].

Nrf2 is another key player in the antioxidant response that relocates upon oxidant exposure. Under nonstress conditions, concentrations of the transcription factor Nrf2 are low, and the protein is retained in the cytoplasm owing to its association with Keap1 [82, 83]. In response to oxidative stress, a complex series of events leads to the stabilization of Nrf2 and its translocation into the nucleus. In the nucleus, Nrf2 upregulates the expression of several genes that are implicated in the antioxidant response [84]. The oxidant-induced nuclear accumulation of Nrf2 can be mediated by



(a) Nuclear import



(b) Nuclear export

Figure 2: Oxidative stress inhibits classical nuclear import and Crm1-mediated export. (a) Nuclear import. HeLa cells transiently synthesizing the import substrates NLS-mCherry or GFP-tagged glucocorticoid receptor (GR-GFP) were incubated under nonstress conditions (control) or with DEM as described [69]. Note that a significant amount of the reporter proteins relocated to the cytoplasm upon oxidant treatment, indicating that classical nuclear import was inhibited. (b) Nuclear export. HeLa cells synthesizing the fluorescent reporter protein mCit-NES, a Crm1 cargo, were exposed to DEM and processed as in part a. The Crm1 export cargo was excluded from the nucleus under control conditions, but relocated to nuclei upon oxidative stress. Size bar is 20 μm.

importin-α5/importin-β1 [85], whereas Nrf2 nuclear export is promoted by Crm1 [86]. Phosphorylation of Nrf2 likely plays a role in its nuclear import and export, with PI3 kinase possibly stimulating Nrf2 nuclear accumulation [83, 84].

More recent studies identified HMGB1 and GAPDH as redox sensitive proteins whose nucleocytoplasmic distribution is regulated by ROS and signaling [87, 88]. Like Keap1/Nrf2, HMGB1 functions as a redox sensor [87]. In nuclei, HMGB1 serves as a DNA chaperone and participates in replication, transcription, as well as DNA repair. However, HMGB1 also contributes to a variety of signaling processes, which involve HMGB1 export to the cytoplasm and its subsequent secretion. At steady-state HMGB1 shuttles between the nucleus and cytoplasm, but hyperacetylation triggers its relocation to the cytosol [89]. It was speculated that lysine acetylation reduces the number of positive charges and thus interferes with nuclear import of the protein [89]. Karyopherin-α1, a member of the importin-α family, was identified as a binding partner that supports *in vitro* nuclear import of HMGB1, most likely in conjunction with importin-β1 [90]. The interaction of HMGB1 with karyopherin-α1 can be abrogated by phosphorylation, and modification of two NLS segments is necessary to relocate HMGB1 to the cytoplasm [90]. Taken together, a combination of acetylation and phosphorylation controls HMBG1 nuclear accumulation. These posttranslational modifications likely prevent the recognition of HMBG1 by the classical import apparatus.

Nuclear export of HMGB1 is at least in part mediated by Crm1, as leptomycin B drastically reduced HMGB1 exit from the nucleus [89]. Treatment with H_2O_2 upregulated the interaction Crm1/HMBG1 and relocated HMBG1 to the cytoplasm for secretion [91]. This oxidant-dependent secretion was sensitive to JNK and MEK inhibitors, in line with the idea that several members of the MAP kinase families control HMBG1 movement from the nucleus to the cytoplasm and its subsequent release. In other studies, IL-1β-dependent ERK1/2 activation increased the concentration of Crm1 and led to HMBG1 accumulation in the cytoplasm [92]. Whether H_2O_2 treatment, which activates ERK1/2, has the same effect on Crm1 levels is an exciting question that has to be answered in the future.

In recent years GAPDH has emerged as an enzyme that is involved in diverse cellular processes [88, 93]. Thus, GAPDH not only functions in glycolysis in the cytoplasm, but also plays additional important roles in other compartments of the cell, including the nucleus [88, 94–101]. The nuclear accumulation of GAPDH is controlled by posttranslational modifications and the interaction with different binding partners in the cytoplasm and nucleus. In response to oxidative stress, GAPDH undergoes S-nitrosylation and subsequent association with Siah. The GAPDH-Siah complex then moves into the nucleus, where it participates in the regulation of gene expression and apoptosis [88]. GAPDH nuclear accumulation depends on the acetylation of three lysine residues by the acetyltransferase p300 [101]. Furthermore, *O*-GlcNAc glycosylation of GAPDH occurs close to the Siah-binding site, and this modification promotes GAPDH nuclear accumulation [100]. Although not

tested by the authors, *O*-GlcNAc modifications rise in response to oxidative stress [102] and could therefore assist in the stress-induced nuclear accumulation of GAPDH. Interestingly, the nucleocytoplasmic trafficking of GAPDH has been linked to several signaling pathways. In particular, activation of AMPK promoted the nuclear accumulation of GAPDH, whereas signaling through the PI3 kinase → Akt module is required for Crm1-dependent nuclear export [96].

The intracellular location of GAPDH is directly relevant to human health (see below). For example, when in the nucleus GAPDH might contribute to the initiation of apoptosis in brain cells. Moreover, the oxidant-induced changes in GAPDH subcellular localization probably play a role in the pathology of Alzheimer disease [93]. GAPDH is also critical to the development of diabetic complications, and changes in its nuclear accumulation might aggravate diabetic retinopathy [97].

Taken together, there is a growing list of proteins whose nucleocytoplasmic distribution is controlled by the intracellular redox homeostasis. This regulation frequently relies on posttranslational modifications, which can alter the interaction of a particular cargo with its carrier or the retention in nuclear and cytoplasmic compartments.

2.3. Oxidative Stress as a Key Player in Human Health. The cellular damage caused by oxidative stress promotes the onset as well as progression of several diseases and pathophysiologies. Thus, oxidative stress plays a critical role in neurodegenerative disorders, cardiovascular and metabolic diseases, as well as the complications associated with diabetes. Here, we focus on some examples that highlight the adverse effects of oxidative stress on human health.

Oxidative Stress and Neurodegenerative Diseases. The human brain is particularly vulnerable to oxidant-induced damage owing to high oxygen consumption, lipids rich in polyunsaturated fatty acids, high amounts of redox-active transition metals, and relatively poor defense against oxidative stress [30, 48, 103]. Several lines of evidence implicate oxidative stress in the neuronal damage that accompanies neurodegenerative disorders [25, 30, 34, 103, 104]. For instance, analysis of cerebrospinal fluid, plasma, and urine samples or postmortem brain specimens demonstrated the increase in oxidative damage in patients suffering from amyotrophic lateral sclerosis [105], Friedreich ataxia, Parkinson, Alzheimer, and Huntington diseases [30, 48, 103]. Oxidant-induced injury is elevated in the brain at early stages of these diseases, supporting the model that oxidative stress contributes to the etiology of neurodegeneration. In line with this hypothesis, mitochondrial dysfunction and oxidative damage to mitochondrial proteins are shared features of different neurodegenerative diseases [25, 30, 34, 103]. Animal models further support this idea, as inhibitors of mitochondrial function can induce some of the pathologies associated with Parkinson disease [34]. In addition, proteomics identified a large number of proteins that show increased oxidative damage in patients suffering from various forms of neurodegeneration. These proteins include several enzymes that are critical to oxidative phosphorylation and glycolysis. Notably, when compared to control subjects GAPDH oxidation was increased in Alzheimer and Parkinson patients; GAPDH was also affected in ALS mouse models [103]. This is significant, because GAPDH and its subcellular trafficking are of particular importance to human metabolism and the pathologies associated with neurodegenerative diseases. As such, oxidative damage not only reduces the enzymatic activity of GAPDH in Alzheimer disease, but also supports the association with Siah and the subsequent translocation of the GAPDH-Siah complex to the nucleus (see above). In Alzheimer disease, both GAPDH expression and nitrosylation are increased, probably leading to elevated concentrations of GAPDH-Siah in the nucleus, which in turn promotes apoptosis [93]. Taken together, the oxidant-induced changes in GAPDH enzyme activity and intracellular distribution will reduce the energy supply and advance apoptosis in the brain of Alzheimer patients. Since GAPDH is an established target of oxidative damage in several neurodegenerative diseases [103], it is possible that its oxidant-dependent change in nuclear transport and the subsequent increase in cell death are common to multiple forms of neurodegeneration. Interestingly, GAPDH also plays a critical role in the development of diabetic complications.

Oxidative Stress and Diabetes. Oxidative stress is crucial to the etiology of diabetes mellitus and the ensuing damage to different tissues and organs [27, 49, 55, 106, 107]. Thus, oxidative stress can alter insulin signaling by targeting insulin receptor and insulin receptor substrates or through the activation of ser/thr kinases that regulate insulin signaling [55]. In this scenario, the ROS-induced changes to the insulin signaling pathway will advance insulin resistance and the subsequent development of diabetes. PI3 kinase and the MAP kinases ERK1/2 are major components of insulin-mediated signaling. Interestingly, signaling through these kinases is also modulated by oxidative stress and regulates nuclear trafficking (see below).

Oxidative stress not only promotes the development of diabetes, but diabetes also triggers the increase in oxidative stress due to elevated blood glucose and free fatty acids. Such disease-induced ROS production further exacerbates cellular damage and contributes to diabetic complications. In the following, we discuss some of the routes that generate oxidative stress in the diabetic patient [49, 55, 106–108].

Hyperglycemia rises intracellular glucose concentrations and the subsequent production of pyruvate, which is ultimately metabolized via the tricarboxylic acid cycle. As a result of the high abundance of pyruvate, increased amounts of NADH and $FADH_2$ are generated by the tricarboxylic acid cycle. Both NADH and $FADH_2$ enter into the mitochondrial electron transport chain, but their excess interferes eventually with the proper transfer of electrons. As a consequence of this overload, superoxide production by mitochondria increases and promotes cellular damage, especially in the diabetic vasculature [109, 110]. The importance of mitochondria in hyperglycemia-induced injuries was demonstrated experimentally, as inhibitors of

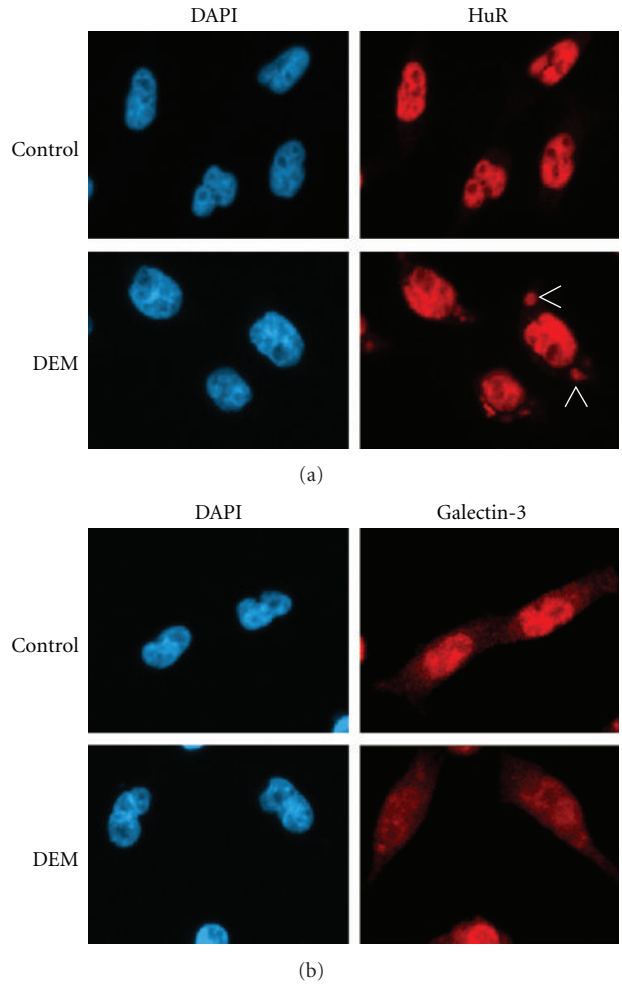


Figure 3: Oxidative stress interferes with importin-α1-dependent import of endogenous cargos. The import of two endogenous proteins, the RNA-binding protein HuR and galectin-3, was monitored in HeLa cells under the conditions described for Figure 2(a). Importin-α1 promotes nuclear import of both proteins. HuR and galectin-3 were visualized by indirect immunofluorescence and nuclei were stained with DAPI [69]. HuR was nuclear in control cells and redistributed to the cytoplasm of stressed cells, where it accumulated in stress granules (SGs). Similarly, galectin-3 was more concentrated in the nuclei of control cells and relocated to the cytoplasm upon DEM treatment. Arrows indicate the position of some of the SGs.

the electron transport chain, upregulation of the uncoupling protein UCP1, or mitochondrial SOD ameliorated some of the damage [49, 106].

The excess of mitochondrial superoxide, combined with other hyperglycemia-induced changes, culminates in secondary diabetic complications. In particular, diabetic nephropathy, retinopathy, neuropathy, and cardiomyopathy arise from the modulation of multiple biochemical pathways, some of which alter the cellular redox homeostasis [27, 49, 107]. For example, upon diabetes, the abundance of intracellular glucose and glycolytic metabolites leads to the increased production of sorbitol and other sugar alcohols by the polyol pathway. This generation of sugar alcohols mediated by members of the aldo-keto reductase family relies on the conversion of NADPH to $NADP^+$ [49]. Since NADPH is necessary to generate GSH from GSSG, excessive NADPH consumption will compromise the antioxidant defense and promote ROS-induced damage.

Moreover, ROS concentrations can also be elevated by hyperglycemia-dependent changes in cell signaling. As described above, GAPDH is sensitive to oxidative stress, and the inhibition of GAPDH by ROS increases intracellular concentrations of triose phosphate, a precursor of the PKC activator diacylglycerol. Hence, hyperglycemia triggers PKC activation, thereby changing the signaling events in the diabetic retina, heart, and endothelial cells [49, 106]. Moreover, this hyperglycemia-induced PKC activation is particularly detrimental to the kidney, as it stimulates ROS production by NAD(P)H oxidases and advances diabetic nephropathy [106, 111].

Like other forms of stress, diabetes modulates the nucleocytoplasmic distribution of transcription factors, with NF-κB as a prominent example [112]. Similarly, high glucose concentrations accumulated GAPDH in the nucleus of bovine retinal endothelial cells [97], where it could contribute to the progression of diabetic retinopathy.

The downstream effects of hyperglycemia further include changes in the posttranslational modification of proteins. Thus, elevated glucose concentrations raise the amount of fructose-6-phosphate that enters the hexosamine pathway [27, 106], which in turn increases the production of UDP-*N*-Acetylglucosamine and the subsequent *O*-GlcNAc modification of proteins. These changes are important to nuclear transport, because nucleoporins are well established targets for *O*-GlcNAc-glycosylation.

In summary, oxidative stress is implicated in different pathophysiological conditions, some of which alter the proper coordination of nuclear and cytoplasmic events. As discussed in the following section, ROS impinge on the nuclear transport apparatus and thereby modify the communication between nucleus and cytoplasm.

2.4. Nuclear Transport and Redox Homeostasis. Changes in cell physiology affect nucleocytoplasmic trafficking in a wide variety of eukaryotes, and the effects of oxidative stress on the nuclear transport apparatus have been analyzed during the past years. We have shown for the yeast *S. cerevisiae* and mammalian culture cells that different forms of stress, including oxidants, heat, and nutrient deprivation inhibit classical nuclear import and export [38, 39, 69, 72, 79, 113–121]. Our previous studies examined the impact of severe and mild oxidative stress. While severe oxidative stress was produced with high concentrations of H_2O_2 [39], mild oxidative stress was generated by the oxidant diethyl maleate, DEM [69]. Under severe stress conditions, cells underwent apoptosis, but a large fraction of cells survived the milder stress inflicted with DEM [69]. Nevertheless, Figures 2 and 3 show that DEM treatment diminished nuclear transport of both fluorescent reporter proteins and endogenous cargos [69, 79, 117]. This is not simply a consequence of stress-induced permeabilization of nuclear envelopes, because the barrier function of nuclear membranes was preserved under

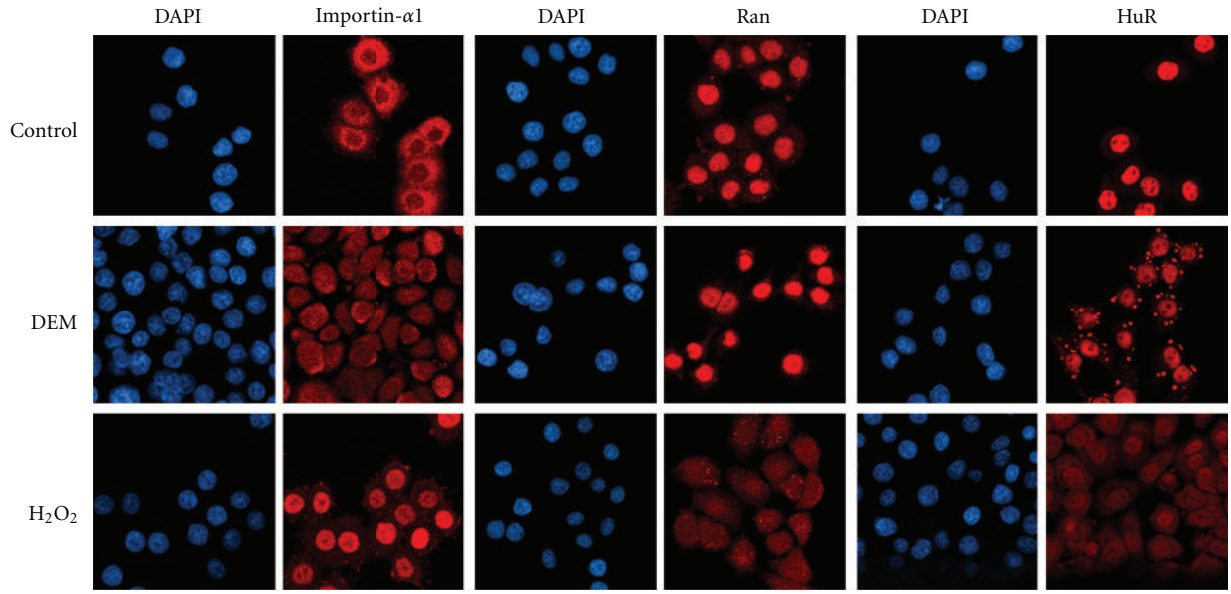


Figure 4: Mild and severe oxidative stress have different effects on nuclear transport factors. The effects of mild (2 mM DEM) and severe oxidative stress (10 mM H_2O_2) on the subcellular distribution of importin-α1, Ran, and HuR were analyzed in HeLa cells. Proteins were located by indirect immunofluorescence [39, 69, 79]. DEM treatment accumulated importin-α1 in nuclei but did not drastically affect the distribution of Ran. By contrast, severe oxidative stress induced by H_2O_2 caused a pronounced nuclear accumulation of importin-α1 and collapsed the nucleocytoplasmic Ran gradient. Both treatments relocated HuR to the cytoplasm. However, DEM triggered the assembly of HuR-containing SGs, which were rare or absent upon incubation with H_2O_2.

these conditions [122]. Since fluorescent reporter proteins like NLS-mCherry or mCit-NES do not contain nuclear or cytoplasmic retention signals, it was reasonable to assume that their stress-induced redistribution reflected changes to the transport apparatus. As described below, such changes were indeed reported by different laboratories, both for severe and mild forms of oxidative stress.

A common consequence of H_2O_2-induced severe oxidative stress is the collapse of the nucleocytoplasmic Ran GTPase gradient in growing cells (Figure 4); this collapse contributes to classical import inhibition [35, 38, 39, 121]. In addition, three key components of the transport apparatus, nucleoporin Nup153, the carrier importin-β1, and importin-α1 (Figure 4), redistributed when cells were treated with H_2O_2 [39]. Aside from transport factor redistribution, H_2O_2 also caused the degradation of Ran, Nup153 and importin-β1, both by proteasome and caspase-dependent mechanisms. In addition to growing cells, the consequences of H_2O_2 incubation were also examined *in vitro*. In these experiments, oxidant treatment led to a significant reduction of the docking step of nuclear import, as it diminished the binding of importin-α1/β1/cargo complexes at the nuclear envelope [39].

Our more recent studies investigated how nonlethal oxidative stress affects the transport apparatus. To this end, intracellular glutathione concentrations were depleted with DEM. Unlike severe oxidative stress, DEM incubation caused neither a dissipation of the Ran gradient (Figure 4) nor the degradation of transport receptors. However, DEM treatment mislocalized several transport components, including importin-α1, its nuclear exporter CAS as well as nucleoporins Nup153, Nup88, and Nup50 [69]. Nuclear retention was one of the mechanisms that contributed to the oxidant-induced nuclear accumulation of these proteins. Concomitant with nuclear retention, high molecular mass complexes were formed in nuclei that contained importin-α1, Nup153, and Nup88. A second mechanism promoting the redistribution of transport factors was the increase in nuclear import for importin-α1 and CAS [69]. Notably, the subcellular redistribution of importin-α1, CAS, Nup153, and Nup88 was accompanied by changes in their posttranslational modification. For example, DEM augmented the phosphorylation for each factor and increased the *O*-GlcNAc modification of Nup153 [117]. All of these events are possibly linked to oxidant-induced signaling, as the relocation of importin-α1, CAS, Nup153 and Nup88 was modulated by MEK → ERK1/2 and PI3K → Akt pathways [117].

Oxidative stress not only inhibits nuclear import, the Crm1 export pathway is sensitive to oxidants as well [79], and our group demonstrated that Crm1-mediated export was inhibited by DEM. Consequently, mCit-NES, a Crm1 cargo predominantly in the cytoplasm of unstressed cells, relocated to nuclei in DEM-treated samples (Figure 2). Several mechanisms participated in the oxidant-induced inhibition of Crm1-dependent export [79]. First, oxidative stress changed the association of Nup358, Nup214, Nup62, and Crm1 with the nuclear envelope and redistributed Nup98. Second, the interaction among these nucleoporins was altered. Third, oxidant treatment impaired Crm1 exit from the nucleus and increased its binding to Ran.

Taken together, these studies revealed that oxidative stress alters several steps of classical nuclear import and export

and substantiated the hypothesis that the nuclear transport apparatus is an important target for oxidants. Some of the oxidant-sensitive components are shared by import and export pathways, which might explain why both transport routes are affected in stressed cells.

Work by other groups identified additional transport factors that are likely controlled by ROS homeostasis [72]. For instance, ceramide inhibited nuclear import through a pathway that relied on the MAPK p38 [123]. As ceramide is believed to cause oxidative stress [124, 125], these experiments provide another link between ROS imbalance and changes in nuclear trafficking. This idea is further supported by experiments in smooth muscle cells, where lysophosphatidylcholine modulated RanGAP1 activity [126]. Since lysophosphatidylcholine can induce ROS production [127], RanGAP1 and thereby the generation of RanGDP in the cytoplasm are potential candidates for ROS-dependent regulation. The role of RanGAP1 as an oxidant-sensitive target in the cytoplasm is significant, because RanGAP1 promotes the termination of protein export for all importin-β like carriers. Furthermore, RanGAP1 has emerged as target for several MAP kinases [128], emphasizing its potential to serve as a redox-sensitive transport regulator at the NPC.

The idea of redox-dependent control at the nuclear pore is consistent with a recent publication that detected the MAP kinases ERK, p38, and JNK at the NPC [129]. Importantly, all of these kinases are activated and/or redistributed by ROS (Table 1). Moreover Nup50, Nup153, and Nup214 are established ERK targets [130], and their phosphorylation changed several interactions that are important for nuclear transport. Specifically, ERK-dependent modification of Nup50 interfered with its binding to importin-β and transportin, which are both carriers of the importin-β family. Similarly, when Nup153 and Nup214 were phosphorylated by ERK, their association with importin-β was reduced.

In summary, multiple signaling pathways are activated by oxidants, MAP kinases reside at the NPC or relocate upon stress (see below), and several transport factors are targeted by these kinases. Hence, it is reasonable to propose a simplified chain of events: oxidative stress → signaling → transport factor modification and/or relocation → changes in nuclear trafficking → altered distribution of cargos. This is by no means a one-way street, as nuclear transport factors also play a critical role in modifying signaling events.

An example for the interdependence of signaling and nuclear transport is provided by RanBP3. This transport factor is not only regulated by multiple kinase modules, it also controls signaling [131, 132]. RanBP3 is predominantly located in the nucleus and a binding partner for Ran, RCC1, and Crm1. Aside from participating in Ran translocation to the cytoplasm, RanBP3 may also sequester Ran in the nucleus [131]. Phosphorylation by RSK and Akt can modulate RanBP3 function. In particular, RanBP3 modification is believed to stimulate nuclear import by regulating its interaction with RCC1. In support of this model, nonphosphorylatable mutants of RanBP3 displayed a reduced ability to stimulate RCC1 *in vitro* and caused a partial dissipation of the Ran gradient in growing cells [131]. The emerging scenario is that signaling through Ras → MEK1/2 → ERK1/2 → RSK and PI3 kinase → Akt leads to RanBP3 phosphorylation, thereby maintaining the Ran gradient. Since both signaling pathways are modulated by ROS, it is tempting to speculate that their activation by oxidants will help to preserve or re-establish the Ran gradient in stressed cells.

Besides being a downstream target of several signaling pathways, RanBP3 has a critical role in controlling TGF-β signaling [132]. Signaling through TGF-β and its receptors have multiple links to oxidative stress [133–136], and many effects of TGF-β-like ligands are exerted by the downstream transcriptional regulators Smad2/3. Smad2/3 are shuttling proteins, and their transport to the nucleus relies on direct binding to importin-β, without involvement of the adaptor importin-α [137]. Following activation of TGF-β, Smad2/3 are phosphorylated and accumulate in nuclei, where they regulate the expression of target genes. The termination of TGF-β signaling involves the dephosphorylation of Smad2/3 and their export to the cytoplasm. Notably, Smad2/3 nuclear export is not sensitive to leptomycin B, suggesting that Crm1 is not required for exit from the nucleus. Indeed, RanBP3 was identified as a possible carrier that helps to move Smad2/3 to the cytoplasm [132]. Several lines of evidence support this idea; RanBP3 bound nonphosphorylated Smad2/3, interacted with Smad2/3 in the nucleus and promoted Smad2/3 nuclear export in a Ran-dependent fashion. Together, these studies established an essential role for RanBP3 as a negative regulator of Smad2/3 signaling, which relies on its ability to transport Smad2/3 to the cytoplasm.

The impact of ROS on nuclear transport is not limited to signaling-dependent effects, since ROS can directly induce the modification of nuclear transport components. Protein carbonylation is one of the consequences of oxidative stress, and it occurs in an age-dependent fashion for nucleoporins Nup153 and Nup93. Nucleoporin carbonylation correlated with the "leakiness" of NPCs [138], and could be particularly harmful to postmitotic cells, in which some nucleoporins are replaced only slowly. In the context of signaling, it will be interesting to determine whether the age-dependent nucleoporin carbonylation alters the NPC association of MAP kinases or nucleoporin phosphorylation.

In summary, experiments described above suggest that the stress-induced modulation of nuclear trafficking is caused by changes in the concentration, distribution, and posttranslational modification of transport factors [72, 82]. This process is further complicated by the fact that oxidant-dependent relocation of transport factors can be compartmentalized even within the nucleus or cytoplasm, as shown by the formation of cytoplasmic stress granules.

2.5. Oxidative Stress, Stress Granule Assembly, and Nuclear Transport. One of the possible consequences of oxidative stress is the formation of cytoplasmic stress granules (SGs). SGs are generated in response to stress that leads to the accumulation of stalled translation initiation complexes [139, 140]. SG assembly is part of a stress defense mechanism that helps to retain and protect mRNAs from degradation. One of the signaling events crucial for the formation of most SGs

Table 1: Redox-sensitive cellular targets in eukaryotic cells. Components that alter their activity and/or nucleocytoplasmic distribution when ROS concentrations increase are listed. See text for details.

Component or process	Effect of ROS
Signaling proteins, transcriptional regulators	
JNK, MAPK	Activation
p38, MAPK	Activation, nuclear translocation
ERK1/2, MAPK	Activation, nuclear accumulation
PI3 kinase (some isoforms)	Activation, changes in nucleocytoplasmic distribution
5′-AMP activated kinase	Inhibition, nuclear accumulation; possibly by reduced nuclear export *via* Crm1
Human insulin receptor kinase activity	Activation
Src family kinases	Activation
EGFR	Nuclear translocation; DNA repair
Protein tyrosine phosphatases	Inactivation
PTEN	Nuclear accumulation; association with p53
STAT3	Nuclear translocation
NF-κB, transcription factor	Nuclear accumulation; transcription
FoxO transcription factors	Nuclear translocation (i.e., FOXO1, FOXO3a, and FOXO4)
yAP-1, yeast transcription factor	Nuclear translocation
Msn2p, Msn4p, yeast transcription factors	Nuclear translocation, transcription
CREB	Phosphorylation, nuclear translocation
Nrf2	Nuclear accumulation
HMGB1	Cytoplasmic translocation
HuR, RNA-binding protein	Relocation to cytoplasm, accumulation in stress granules
Nuclear transport apparatus	
Classical nuclear import	Inhibition
Crm1-dependent nuclear export	Inhibition
Ran, small GTPase; Gsp1 in *S. cerevisiae*	Relocation to cytoplasm upon severe oxidative stress
Importin-α1, adaptor for classical nuclear import	Accumulation in nuclei, accumulation in cytoplasmic stress granules
Crm1, nuclear exporter	Accumulation at nuclear envelope
CAS, exporter for importin-α	Nuclear accumulation
Multiple nucleoporins located at different positions within the nuclear pore complex: Nup358, Nup214, Nup88, Nup62, Nup153, Nup50, Nup98, and others	Changes in the association with nuclear envelope; altered nucleocytoplasmic distribution; degradation upon severe stress, in some cases mediated by caspases.

is Ser51 phosphorylation on eIF2α (eukaryotic translation initiation factor 2) [139–141]. Ser51 can be modified by four different upstream kinases, PKR, PERK, GCN2, and HRI (heme-regulated initiation factor 2 kinase), which are activated by various stressors, including the oxidant arsenite. Other signaling events are relevant to SG biogenesis and disassembly; for instance, arsenite promotes the sequestration of Rho and ROCK1 in SGs, possibly to limit the activation of the downstream target JNK [142]. Moreover, focal adhesion kinase (FAK) controls the disassembly of SGs and can be stimulated with H_2O_2 [143, 144].

In addition to components of the small ribosomal subunit and RNA-binding proteins, arsenite-induced SGs contain importin-α1 [145]. Notably, importin-α1 knockdown delays SG formation, suggesting a role in the dynamics of SG assembly. These are important data which further substantiate the contribution of nuclear protein transport factors to the stress response. At present, we do not fully understand these events; however, it is conceivable that SGs are one of the "hubs", where ROS-mediated signaling and nuclear transport components come together in the cytoplasm. Results for the mRNA-binding protein HuR support this idea. HuR shuttles between the nucleus and cytoplasm and relies on importin-α1 for nuclear import. Under normal growth conditions, HuR is predominantly in the nucleus, but a 4-hour DEM treatment concentrated HuR in SGs (Figures 3 and 4). At the same time, importin-α1 accumulated in nuclei, but it was still detectable in the cytoplasm [69, 117]. It should be emphasized that the association of macromolecules with SGs is dynamic. Proteins as well as RNA can shuttle between SGs and the surrounding cytoplasm [141, 146], and this may also apply to importin-α1.

What are the possible mechanisms that promote the ROS-dependent changes in importin-α1 and HuR distribution and how are these events linked to SG assembly?

The DEM-induced relocation of HuR is likely driven by the combination of importin-α1 nuclear accumulation and HuR association with SGs. In particular, concentrating importin-α1 in nuclei of stressed cells could diminish nuclear import of HuR. At the same time, importin-α1 has a role in SG biogenesis. Although details of this process have yet to be defined, importin-α1 may recruit components to cytoplasmic foci that are destined to form SGs. Given that importin-α1 binds and transports a variety of cargos, importin-α1 shuttling between SG foci and the cytoplasm could accomplish this task. If our model is correct, it could help explain the lack of SG formation in cells incubated with H_2O_2 [147, 148]. As shown in Figure 4, H_2O_2 did not induce SGs, and importin-α1 became highly concentrated in the nucleus, with little of the protein remaining in the cytoplasm. Moreover, stress can also increase nuclear retention and import of importin-α1 [113]. As a result of these events, the concentration of importin-α1 in the cytoplasm will be low when cells are treated with H_2O_2, which in turn could limit the formation of SGs.

The potential contribution of nuclear transport factors to SG assembly or function is not restricted to importin-α1. Support for this notion comes from importin-β family members importin 8 and transportin which localize to SGs upon arsenite treatment [149, 150]. At this point, we have only few examples that connect nuclear transport components with SGs, and future studies will have to unravel how nuclear trafficking, SG assembly, and ROS-dependent signaling are integrated.

2.6. Oxidative Stress and the Subcellular Distribution of Key Signaling Molecules. Elevated levels of ROS modify the activity of redox sensitive components that participate in signaling or other essential biological processes [1, 6, 9, 39, 69, 79, 87, 88, 114, 116, 151–153]. Notably, such ROS-dependent changes in activity are frequently accompanied by the intracellular relocation of the redox-sensitive factors. This scenario applies to a growing list of protein kinases, phosphatases, transcription factors, and components of the nuclear transport apparatus (Table 1). Several of the kinases and phosphatases that redistribute under oxidative stress conditions are key players in signaling circuits, where they control cell survival, growth, proliferation, or death. The interdependence of the activation status and intracellular distribution is crucial for these enzymes, as it determines the specificity and duration of signaling events [152, 154–156]. In the following, we discuss some of the kinases and phosphatases for which oxidant-dependent relocation has been established.

The activity and location of several members of the MAPK and PI3 kinase families are modulated by ROS. Such spatiotemporal control is particularly important for the response to stress, where the repair of stress-induced damage and cell survival relies on the outcome of compartment-specific signaling events. Multiple signaling modules that respond to ROS, both by activation and relocation, have been analyzed in our group [114, 116]. We focused on Akt and ERK1/2, kinases that are essential for signal transduction through PI3 → Akt and MEK → ERK1/2 modules. The stressor DEM elevated the phosphorylation of Akt on Thr308 and Ser473, which leads to Akt activation. At the same time, DEM induced the dual phosphorylation of ERK1/2, thereby activating the MAP kinases. Importantly, DEM not only activates Akt and ERK1/2, but also increased significantly the nuclear/cytoplasmic ratio of phospho-Akt(Ser473) and dually phosphorylated-ERK1/2 [114]. A possible outcome of this shift is a change in the phosphorylation profiles of nuclear and cytoplasmic targets. Notably, the compartmentalization of Akt and ERK1/2-dependent signaling events is even more complex [114], as we demonstrated in the nucleus a direct correlation between the levels of phospho-Akt(Ser473) and phospho-ERK1/2. Our studies suggested that the nuclear concentration of phospho-Akt(Ser473) is dependent on nuclear phospho-ERK1/2 and *vice versa.* Accordingly, crosstalk occurs between phospho-Akt(Ser473) and ERK1/2 in response to oxidative stress; this crosstalk is specific for the nuclear compartment.

More recent work on PI3 kinase by others further emphasizes the importance of the localized action of signaling molecules. The PI3 kinase catalytic subunit p110β carries a nuclear localization signal in its C-terminal domain, while the regulatory subunit p85β harbors a nuclear export signal. The analysis of a p110β transport mutant showed that the ability of the p85β/p110β complex to regulate cell survival was strictly dependent on its nuclear localization [157]. Although the effect of oxidative stress on the distribution of this kinase has yet to be determined, these findings provide compelling evidence for the control of cell signaling by nuclear transport.

Another example that illustrates the ROS-dependent activation and distribution of protein kinases is the heterotrimeric enzyme 5′-AMP activated kinase (AMPK). AMPK is an energy sensor which plays a pivotal role in the regulation of metabolic homeostasis by phosphorylating targets that are involved in glucose, carbohydrate, lipid, and protein metabolism [158–161]. In unstressed cells, AMPK shuttles between the nucleus and cytoplasm and this shuttling relies on the nuclear exporter Crm1 [116, 162]. However, in response to oxidative stress, AMPK α- and β-subunits concentrated in the nucleus. This could be accomplished—at least in part—by ROS-induced changes to the nuclear export apparatus, as Crm1 is one of the transport components that are affected by ROS (see above). Interestingly, the link between AMPK activity, subcellular distribution, and nuclear trafficking is even more intricate, as importin-α1, a component of the nuclear transport apparatus, is also modified by AMPK [163].

Epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase that is especially important to human health, because signaling through EGFR is linked to tumorigenesis, metastasis and radioresistance. EGFR is located in the plasma membrane, but it also entered the nucleus in response to oxidative stress, heat, or radiation [164]. Moreover, incubation of cultured cells with hydroxy-nonenal, a compound generated by lipid peroxidation, promoted the nuclear accumulation of EGFR [19]. When in the nucleus, EGFR stimulated DNA repair, a process that contributes

to radioresistance and potentially limits the success of radiotherapy. Since EGFR is membrane bound, details of its nuclear transport are likely to differ from soluble cargos. Nevertheless, importin-β1 and Crm1 (Figure 1) were identified as nuclear carriers that participate in EGFR trafficking [165, 166].

The link between oxidative stress and the localization of key signaling components is not limited to protein kinases. For instance, the lipid and protein phosphatase PTEN has functions in the nucleus and cytoplasm, and oxidative stress promotes PTEN nuclear accumulation [167]. In cells treated with H_2O_2, PTEN concentrated in nuclei, where it stabilizes the tumor suppressor p53. Under normal conditions, PTEN is exported from the nucleus by the carrier Crm1 in a cell-cycle dependent fashion, and this export relied on signaling through PI3 kinase [168]. However, incubation with H_2O_2 induced PTEN phosphorylation on Ser380, which inhibited its nuclear export [167]. The control of PTEN shuttling upon oxidative stress probably goes beyond the oxidant-induced phosphorylation of the enzyme. As such, the exporter Crm1 is one of the cellular targets that are sensitive to ROS, and signaling through the PI3 kinase → Akt module regulates several components of the nuclear transport apparatus [79, 117]. This interdependence of nuclear transport and signaling is further complicated by the fact that the enzymatic activity of PTEN is regulated by oxidants (see below).

For the examples discussed here, ROS-mediated changes in the nucleocytoplasmic distribution of kinases and phosphatases could reflect the requirement to modify selected substrates in specific subcellular compartments. To this end, the ROS-induced nuclear accumulation of ERK1/2, PI3 kinase, AMPK, EGFR, or PTEN will alter the phosphorylation and activity of nuclear substrates such as transcription factors and other regulators of gene expression. However, such redistribution will also impact other compartments, because the sequestration of kinases or phosphatases in the nucleus can change the phosphoproteome in the cytoplasm as well.

2.7. What Is the Interface between the Initial Oxidant Exposure and Changes in the Nuclear Transport Apparatus? As discussed in previous sections, oxidative stress targets components of the nuclear transport machinery. Moreover, different signaling cascades are implicated in the control of trafficking across the NPC, in part by regulating the posttranslational modification of nuclear transport factors. A complete mechanistic understanding of these events requires that the initial impact of the oxidant can be connected to functional changes of the nuclear transport apparatus. For many of the processes described here, the interface between the primary oxidant-induced event and changes in the posttranslational modification or function of transport factors is not fully defined. In the following, we will, therefore, speculate on some of the possible links.

In principle, two distinct mechanisms can underlie the effect of ROS on nuclear transport factors. First, ROS might react directly with the nuclear transport apparatus, leading to the covalent modification of individual components. Second, oxidative stress could activate signaling cascades that ultimately trigger the phosphorylation and/or *O*-GlcNAc glycosylation of the transport machinery. In the second scenario, signaling begins with a redox-sensitive target that induces a chain of events which conclude with the post-translational modification of one or more nuclear transport factors.

Direct Modification of the Nuclear Transport Apparatus by ROS. In line with what is known about redox-sensitive residues in proteins, we expect that for nuclear transport components cysteine, methionine, lysine, arginine, and histidine residues are among the side chains that are particularly prone to direct oxidation or other ROS-dependent modifications [169]. This idea is supported by a study describing the S-nitrosylation of Crm1 on two cysteine residues and the concomitant inhibition of Crm1-mediated nuclear export [170]. Besides Crm1, nucleoporins are other candidates for a direct modification by ROS or compounds generated upon oxidative stress. Our hypothesis is supported by the increase in nucleoporin carbonylation when cells encounter oxidative stress [138].

Signaling as Possible Interface between Oxidant Exposure and Nuclear Transport Modification. Although many of the enzymes that mediate the posttranslational modification of transport factors are known, upstream events regulating these enzymes are less well understood. This applies in particular to the first step of the process, that is, the impact of ROS on its primary target. We propose that protein kinases, phosphatases, or small GTPases that are redox-sensitive [171–174] could fill this gap, as they activate signaling pathways that culminate in transport factor modification. A particularly interesting candidate in this respect is the protein kinase Src, which contains a cysteine switch that is oxidized in order to achieve full kinase activation. Moreover, the redox-dependent stimulation of Src promotes the ligand-independent transphosphorylation of EGFR and subsequent activation of PI3 and ERK kinases [175]. In line with this order of events, it is possible that the ROS-induced formation of disulfide bonds in Src will stimulate the PI3 and ERK-dependent effects on nuclear transport factors as they are discussed here.

The same reasoning applies to several phosphatases [174], including PTEN and low molecular weight protein tyrosine phosphatase (LMW-PTP). PTEN is crucial for the downregulation of PI3 kinase signaling. However, oxidant-induced thiol modification of PTEN inactivates the phosphatase, and thereby promotes signaling through the PI3 kinase → Akt module [174]. With respect to nuclear transport, ROS-induced PTEN inactivation would increase the impact of PI3 kinase on trafficking. In a similar fashion, the redox-dependent inactivation of LMW-PTP leads to sustained ERK activation [176]. This could elevate the ERK-dependent phosphorylation of soluble transport factors and nucleoporins, thus altering their function.

Aside from phosphorylation, *O*-GlcNAc glycosylation of nucleoporins is induced by oxidative stress. The oxidant-dependent increase in *O*-GlcNAc modification is possibly achieved by the complex regulation of *O*-GlcNAc transferase and *β*-*N*-acetylglucosaminidase. At present, these events are not fully understood [177].

Taken together, we propose that changes in the cellular redox homeostasis impact nucleocytoplasmic trafficking by two general mechanisms that are likely to operate in parallel. First, ROS or ROS-generated compounds directly modify redox-sensitive transport factors, this can alter their function. Second, the impact of ROS on redox-sensitive signaling proteins will ultimately modulate the posttranslational modification and activity of nuclear transport components.

2.8. Antioxidant Defenses Occur in a Compartmentalized Fashion. In addition to the compartmentalized activation and action of kinases and phosphatases, components of the antioxidant defense apparatus are also unequally distributed within the cell [63, 178]. This is illustrated by catalase, an enzyme concentrated in peroxisomes, and the different forms of superoxide dismutase (SOD) [64, 65, 179, 180]. While manganese-containing SOD (MnSOD) is in the mitochondrial matrix, copper- and zinc-containing SOD (Cu,ZnSOD) can be found preferentially in the cytoplasm and extracellular SOD (EC-SOD) on the cell surface. Moreover, the unequal distribution of GSH and enzymes involved in GSH metabolism will also contribute to subcellular differences in the response to ROS [59, 181–183]. Aside from these enzymes and antioxidants, the localized action of chaperones, critical factors for the repair of stress-induced damage, is well established [115, 184–186]. Since chaperone function is essential for proper signaling and also required for nuclear transport, the nucleocytoplasmic localization and function of heat shock proteins and other chaperones will have significant impact when cells experience ROS imbalances.

We propose that the unequal distribution of antioxidant defense and repair components will impact both cargos and transport factors in a compartment-specific fashion. Accordingly, the prevention and repair of oxidant-induced damage will be different in the nucleus and cytoplasm. Depending on its subcellular location, this could have differential effects on the movement and function of a shuttling protein. For example, nuclear cargos that encounter higher levels of ROS in the cytoplasm could be immobilized in this compartment. The same model can be applied to nuclear transport factors. Thus, nucleoporins on the nuclear and cytoplasmic side of the NPC could be exposed to different levels of ROS and repair. Since nuclear and cytoplasmic nucleoporins participate in different steps of trafficking, damage on either side of the nuclear pore could have unique consequences for nuclear transport.

3. Conclusions

The impact of ROS on human health is well established, and links between oxidative stress, nuclear transport, and

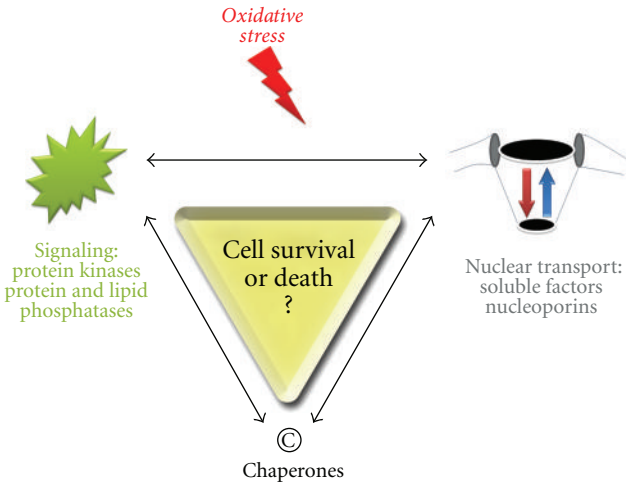


Figure 5: Simplified model for the crosstalk between signaling and nuclear transport in response to oxidative stress. Oxidative stress impinges on signaling molecules and the nuclear transport apparatus, with chaperones modulating both processes. Different scenarios can explain the communication between nuclear transport and signaling pathways in oxidant-treated cells. In one case, oxidative stress alters the localization and activity of transport factors. This will change the subcellular distribution of key signaling molecules, which in turn affects the modification of downstream targets. Alternatively, the signaling pathways activated by oxidative stress cause the modification and redistribution of transport factors. Both scenarios are likely to take place side-by-side, and the balance of these events will ultimately determine cell fate.

disease have been defined. For instance, oxidative stress plays a pivotal role in the hyperglycemia-induced damage of multiple tissues and organs [47, 49, 51–53, 55, 187]. GAPDH nucleocytoplasmic shuttling not only participates in these processes, but has also been connected to cancer and neurodegenerative disorders, such as ALS, Alzheimer, or Parkinson disease [88]. Hence, it is conceivable that the oxidant-induced relocation of GAPDH is common to diabetes, cancer, and some forms of neurodegeneration. This shared feature can be extended to the stress-induced nuclear trafficking of the transcriptional regulators NF-*κ*B and Nrf2 and may include other diseases, such as Friedreich ataxia [56, 188, 189].

The examples highlight how the compartment-specific action of signaling molecules, defense and repair reactions provide sophisticated tools to regulate cell physiology. Thus, confining these processes to specific locations will limit the access to downstream targets and clients. In the context of this review, the nucleocytoplasmic distribution of kinases, phosphatases, and other factors involved in posttranslational modification or folding can be expected to directly affect the communication between cytoplasmic and nuclear compartments. This is emphasized by the fact that many of the nuclear transport components and their cargos are modified in an ROS-dependent fashion by phosphorylation, *O*-GlcNAc glycosylation, acetylation, or sumoylation.

Our current understanding of ROS, signaling, and nucleocytoplasmic transport supports the notion that these

processes are intricately connected. Although many of the details are still to be discovered, the findings from different fields can be merged into a simplified model. Here, we propose that crosstalk and feedback between different components of this signaling circuit will determine how cells respond to oxidative stress (Figure 5). In one scenario, the activation of signaling pathways promotes the posttranslational modification of nuclear transport factors. This triggers the redistribution of transport factors and alters the movement of cargo across the nuclear envelope. Alternatively, oxidant-induced damage to the transport apparatus could modulate the nucleocytoplasmic localization of kinases or phosphatases, thereby changing the spatiotemporal pattern of signaling. We believe that the two scenarios will take place side by side, affecting different signaling modules and targets in the nucleus and cytoplasm. Both scenarios are further shaped by the localized action of chaperones, which impact both signaling and nuclear transport. The input from signaling, trafficking, and repair will culminate in the decision on cell survival or death.

As outlined in this review, the dynamic organization of signaling cascades and the nuclear transport apparatus are ideal to respond to internal and external cues. In this context, nucleocytoplasmic trafficking provides the switch to direct events to the nucleus or cytoplasm. The interdependence of signaling and transport pathways provides the flexibility to adjust to a wide variety of changes in cell physiology.

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Review Article

Oxidative Stress Induced by MnSOD-p53 Interaction: Pro- or Anti-Tumorigenic?

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The formation of reactive oxygen species (ROS) is a result of incomplete reduction of molecular oxygen during cellular metabolism. Although ROS has been shown to act as signaling molecules, it is known that these reactive molecules can act as prooxidants causing damage to DNA, proteins, and lipids, which over time can lead to disease propagation and ultimately cell death. Thus, restoring the protective antioxidant capacity of the cell has become an important target in therapeutic intervention. In addition, a clearer understanding of the disease stage and molecular events that contribute to ROS generation during tumor promotion can lead to novel approaches to enhance target specificity in cancer progression. This paper will focus on not only the traditional routes of ROS generation, but also on new mechanisms via the tumor suppressor p53 and the interaction between p53 and MnSOD, the primary antioxidant enzyme in mitochondria. In addition, the potential consequences of the p53-MnSOD interaction have also been discussed. Lastly, we have highlighted clinical implications of targeting the p53-MnSOD interaction and discussed recent therapeutic mechanisms utilized to modulate both p53 and MnSOD as a method of tumor suppression.

1. Introduction

Oxidative stress has been defined as the cellular imbalance of prooxidants versus antioxidants that overwhelms the cell's capacity to scavenge the oxidative load and contributes to the pathogenesis of various diseases. Reactive oxygen species (ROS) are free radicals derived from molecular oxygen that play a key role in promoting oxidative stress. These radicals result from the incomplete reduction of oxygen mainly during mitochondrial respiration. There are several products of oxygen metabolism, both nonradicals and radicals that form ROS such as hydrogen peroxide (H_2O_2) and superoxide anions ($O_2^{\cdot -}$). Contributors of ROS can modify the intracellular redox status through unfavorable interactions with endogenous regulators of oxidative stress. Superoxide radicals can interact with mitochondrial nitric oxide to form peroxynitrite which can alter antioxidant enzymes such as aconitase and the mitochondrial complexes of the electron transport chain [1]. On the other hand, the presence of oxidative stress can alter normal cellular homeostasis by modifying proteins involved in DNA repair; activating signal transduction pathways involved in cell survival and inflammation; as well as, inducing cellular apoptotic pathways that are detrimental to the cell. For many years, scientists have tried to combat free radical generation and superoxide production through the utilization of the exogenous antioxidant supplementation, such as ascorbate, vitamin E, as well as linoleic acid. However, many of these trials have failed showing no significant decrease in cancer incidence, death, or major cardiovascular events [2]. Herein, we will focus on several novel signaling pathways affecting ROS generation, such as p53 signaling and the interaction between p53 and manganese superoxide dismutase (MnSOD) and how to potentially target these pathways for cancer therapy.

2. Oxidative Stress

Oxidative stress has been repeatedly shown to contribute to the progression of multiple diseases, such as cancer [3], diabetes [4], ulcerative colitis [5], cardiovascular disease [6],

pulmonary disease [7] as well as neurodegenerative diseases [8]. Nevertheless, the biological significance of oxidative stress can be beneficial or detrimental depending on certain parameters such as concentration, duration of action, cell type exposed, the type of free radicals and reactive metabolites involved, and the activities of the associated signal transduction pathways.

The mitochondrial electron transport chain remains to be one of the main sources of intracellular oxidative stress [9]. During mitochondrial respiration, electrons flow through four integral membrane protein complexes to finally reduce molecular oxygen to water. However, approximately 1-2% of molecular oxygen undergoes incomplete reduction, resulting in the formation of superoxide anions and mitochondria-mediated ROS generation [10]. Though mainly produced from mitochondrial respiration, superoxide anions can be detoxified via endogenous antioxidant enzymes such as manganese superoxide dismutase (MnSOD) to hydrogen peroxide, which is further converted to water via the enzymatic actions of various antioxidant enzymes including glutathione reductases, peroxiredoxins, glutathione transferases, as well as catalase which all function in the removal of hydrogen peroxide.

Nevertheless, it is common for cells in response to stress to enhance ROS generation. Oxidoreductases are enzymes that are often activated during the cellular stress response and catalyze the transfer of electrons from the electron donor (reductant) to the electron acceptor (oxidant) [11] with associated formation of superoxide anions and ROS as byproducts. There are several enzymes that act as oxidoreductases and contribute to intracellular ROS generation, such as cyclooxygenase [12], lipoxygenase [12, 13], cytochrome P450 enzymes [14], nitric-oxide synthase [15], xanthine oxidase [16], and mitochondrial NADH: ubiquinone oxidoreductase (complex I) [17]. NAPDH oxidases of the Nox family are also oxidoreductases that produce superoxide anions as a primary product and one of the key sources of intracellular ROS formation. NADPH oxidases (Nox) are endogenous enzymatic heterogenic complexes that reduce molecular oxygen to superoxide, in conjunction with NADPH oxidation, which can be converted to various ROS. Nox can be activated by a myriad of cellular stress stimuli such as heavy metals [18, 19], organic solvents [20], UV and ionizing irradiation [21, 22]. Once the cellular stress response is initiated two cytosolic regulatory units of Nox, $p47^{phox}$, $p67^{phox}$, and the small G protein Rac translocate to the membrane and associate with cytochrome *b558* (consisting of two subunits gp91phox (Nox2) and p22phox), which acts as a central docking site for complex formation [23]. Emerging evidence has linked Nox enzymes to oxidative stress that may contribute to disease progression [11, 17, 24, 25]. The radicals generated from Nox activation are capable of modulating various redox-sensitive signaling pathways involved in the activation of mitogen-activated protein kinases (MAPKs) and transcription factors (NF-κB) [26–28] causing regulation of Nox activation to be complex.

Oxidative stress can be generated endogenously, as well as promoted exogenously by multiple environmental factors. Ultraviolet irradiation (UV) is an environmental promoter of oxidative stress. UV is known to damage DNA and other intracellular proteins through direct and indirect mechanisms. UV exists in three forms UVA (400–320 nm), UVB (320–290 nm), and UVC (290–100 nm). UVA and UVB are the most biologically significant, with UVC being most absorbed by ozone [29]. UV is known to directly induce the cross-linking of neighboring pyrimidines to form pyrimidine dimers in DNA that result in mutagenic DNA lesions [30–35]. However, UV is known to promote ROS generation that can damage a large number of intracellular proteins and can indirectly damage DNA.

Associated with oxidative damage is lipid peroxidation. High levels of ROS are detrimental and can cause damage to various biomolecules, which include the fatty acid side chains of membrane lipids that form reactive organic products such as malondialdehyde and 4-hydroxynonenal, both of which can generate DNA adducts and point mutations [36]. Lipid peroxidation not only affects DNA stability, but can also alter lipid membrane proteins that are involved in signal transduction pathways to promote constitutive activation and downstream cellular proliferation. Furthermore, previous studies have shown that products of lipid peroxidation served as intermediates in the activation of signaling pathways that involved phospholipase A2 and the MAPK pathway, both associated with UV-induced carcinogenesis [37–39].

Although there are various sources of endogenous oxidative stress, mitochondria are the major cellular organelles that contribute to intracellular ROS generation. Mitochondria consume approximately 80–90% of the cell's oxygen for ATP synthesis via oxidative phosphorylation. In the early 1920s Otto Warburg and colleagues theorized that defective oxidative phosphorylation during cancer progression caused tumor cells to undergo a metabolic shift requiring high rates of glycolysis that promoted lactate production in the presence of oxygen. This phenomenon became known as aerobic glycolysis and later coined "The Warburg Effect." Some of the metabolic enzymes that are altered during cancer progression are involved in the mitochondrial electron transport chain [40, 41]. The electron transport chain consists of a constant flow of electrons through mitochondrial intermembrane complexes with molecular oxygen being the ultimate electron acceptor. The process of the electron transport chain is used to pump protons into the mitochondrial inner membrane creating an electrochemical gradient. The gradient that is created is coupled to ATP synthesis. However, leaking electrons contribute to the incomplete reduction of molecular oxygen, resulting in superoxide anion formation. Mitochondria are readily susceptible to oxidative damage for various reasons: (1) lack of effective base excision repair mechanisms; (2) the close proximity of mitochondrial DNA to ROS generation; (3) lack of mitochondrial DNA protective histones [42]. Therefore, alterations in mitochondrial ROS generation and protection via antioxidant expression are key in the detrimental effects of disease progression.

3. Manganese Superoxide Dismutase (MnSOD)

Maintaining a balance between free radicals and antioxidants is required for cellular homeostasis. However, when this

balance is altered in favor of free radical generation, normal physiology is altered and the pathogenesis of disease is promoted. Antioxidants are endogenous defense mechanisms utilized by the cell to fight against fluctuations in free radical generation, which include both enzymatic and nonenzymatic contributors. Ascorbic acid (Vitamin C) and α-tocopherol (Vitamin E) are nonenzymatic antioxidants that have been previously shown to effectively scavenge free radicals. On the other hand, antioxidants such as glutathione peroxidase and superoxide dismutase are enzymatic antioxidants that catalyze the neutralization of free radicals into products that are nontoxic to the cell. Superoxide dismutase catalyzes the dismutation of superoxide anions leading to the formation of hydrogen peroxide and molecular oxygen. Hydrogen peroxide is further detoxified to water via catalase and other endogenous antioxidant enzymes. The superoxide dismutase family consists of metalloenzymes. Currently, there are three major superoxide dismutase enzymes within the human cell: manganese superoxide dismutase (MnSOD), copper-zinc superoxide dismutase (Cu, ZnSOD), and extracellular superoxide dismutase (ECSOD). MnSOD is localized in the mitochondrial matrix [43], Cu, ZnSOD is found primarily in the cytosol [44] and can be detected in the mitochondrial intermembrane space [45], and ECSOD is a homotetrameric glycosylated form of Cu, ZnSOD found in the extracellular space [46].

MnSOD is ubiquitously found in both prokaryotes and eukaryotes, and its increased activity is often associated with cytoprotection against oxidants. MnSOD can be induced by various mediators of oxidative stress such as tumor necrosis factor, lipopolysaccharide, and interleukin-1 [47]. This antioxidant enzyme is nuclear encoded by a gene localized to chromosome 6q25 [48], a region often lost in cancers such as melanoma [49]. MnSOD is synthesized in the cytosol as a larger precursor with a transit peptide on the N-terminus and imported to the mitochondrial matrix via proteolytic processing to the mature form [50]. Most cancer cells and *in vitro* transformed cell lines have diminished MnSOD activity compared to normal counterparts [51]. In addition, deficiencies in MnSOD may contribute to oxidative stress generation that promotes neoplastic transformation and/or maintenance of the malignant phenotype. In looking at the correlation between MnSOD expression and cancer progression, mutations within the MnSOD gene and its regulatory sequence have been observed in several types of human cancers [52, 53]. However, antioxidants can suppress carcinogenesis, particularly during the promotion phase. In addition, our laboratory as well as others has shown that overexpression of MnSOD reduces tumor multiplicity, incidence, and metastatic ability in various *in vitro* and *in vivo* models [54–57].

4. The Tumor Suppressor p53

p53 is a well-characterized transcription factor known to induce its tumor suppressor activity by activating genes known to play a role in cell cycle arrest, such as $p21^{CIP1}$ and *GADD45*. These genes, once activated, arrest the cell cycle to allow for adequate DNA repair to restore normal cell proliferation. However, if the cell becomes overwhelmed by the stressor or the DNA damage cannot be repaired, p53 can ultimately induce apoptosis. The tumor suppressive activities of p53 can also be defined by the induction senescence. Senescence is characterized by irreversible loss of proliferative potential, acquisition of characteristic morphology, and expression of specific biomarkers such as senescence-associated β-galactosidase [58]. Nevertheless, how p53 regulates senescence is often contradictory and dependent on ROS generation. p53 can mediate cellular senescence via the transactivation of $p21^{CIP1}$. Nonetheless, emerging evidence suggests ROS as a common mediator of senescence with the involvement of superoxide dismutase and p53. Blander et al. reported that RNAi-mediated knockdown of SOD1 in primary human fibroblasts induced cellular senescence mediated by p53. However, senescence was not induced in p53-deficient human fibroblasts [59]. Furthermore overexpression of MnSOD induced growth arrest in the human colorectal cancer cell line HCT116 and increased senescence which required the induction of p53 [60]. On the contrary, p53 can suppress senescence through the inhibition of the mTOR pathway via multiple mechanisms [61–63]. Nevertheless, this diverse biological spectrum of p53 regulation of cellular function remains complex and is dependent on the source of activation and cell type.

There are various sources of p53 activators, which include nucleotide depletion, hypoxia, ultraviolet radiation, ionizing radiation, as well as many chemotherapeutic drugs can act as activators of p53 (i.e., Doxorubicin). In normal cells, p53 remains at a low level and is under strict control by its negative regulator Mdm2. p53 induces autoregulation via Mdm2. As a transcription factor, p53 can bind to the promoter region of the *mdm2* gene to promote transcription of Mdm2 mRNA [64, 65]. Following proper translation into a functional protein, Mdm2 acts as an E3 ligase during p53 activation. Mdm2 can polyubiquitinate p53 leading to proteasomal degradation [66]. However, Mdm2 can also monoubiquinate p53 leading to intracellular trafficking [67]. The decisive role of p53 to induce cell cycle arrest, senescence, or apoptosis involves intricate posttranslational, as well as, transcription-dependent and transcription-independent mechanisms. The tumor suppressor p53 is a well-characterized transcription factor known to induce the transactivation of proapoptotic genes such as Bax, Puma, Noxa, Bid and represses the transcription of anti-apoptotic genes such as Bcl-2, Bcl-xL, and survivin [68, 69]. Nevertheless, p53 can induce apoptosis independent of its transcriptional activity. Many of the transcription-independent mechanisms of p53 were discovered through the use of inhibitors of transcription/translation, as well as p53 truncated mutants with altered subcellular localization, DNA binding, and cofactor recruitment. The p53 monomer consists of various multifunctional domains including the N-terminal transactivation domain (residues 1–73), a proline-rich region (residues 63–97), the highly conserved DNA-binding core domain (residues 94–312), a tetramerization domain located within the C-terminus (residues 324–355), and an unstructured basic domain (residues 360–393) [70] (Figure 1). There are multiple polymorphisms that occur within the *TP3*

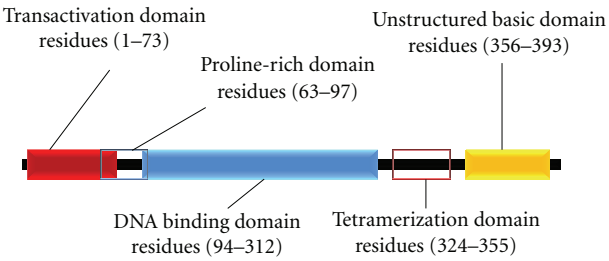

Figure 1: p53 Multifunctional domains. The p53 monomer consists of various multifunctional domains including the N-terminal transactivation domain (residues 1–73), a proline-rich region (residues 63–97), the highly conserved DNA-binding core domain (residues 94–312), a tetramerization domain located within the C-terminus (residues 324–355), and an unstructured basic domain (residues 360–393).

gene that may enhance or alter p53 functionality. Dumont et al. discovered functional differences in polymorphic variants that enhanced p53-mediated apoptosis independent of its transactivation abilities [71]. A common sequence polymorphism that occurs within the proline-rich domain encoding arginine at position 72 exhibited a fivefold increase in inducing apoptosis compared to the common proline (Pro72) variant. These results suggested two mechanisms of Arg 72 apoptotic enhancement: (1) increased mitochondrial localization; (2) enhanced binding of the Arg 72 variant to the negative p53 regulator E3 ligase, Mdm2. Although increased binding to Mdm2 did not augment p53 degradation, it was suggested that the altered confirmation of the p53 Arg 72 variant enhanced the binding ability and facilitated greater nuclear export [71]. This suggests the importance of understanding the regulation of structure-activity relationships in polymorphic forms of p53 in transcription-independent apoptosis.

During p53-mediated apoptosis, a distinct cytoplasmic pool of p53 translocates to the mitochondria. To promote mitochondrial translocation, the E3 ligase, Mdm2 monoubiquitinates p53 [72]. Since the p53 protein lacks a mitochondrial localization sequence, p53 interacts with Bcl-2 family proteins via Bcl-2 homology (BH) domains. The presence of the BH domain allows proteins to regulate and interact with other Bcl-2 members that consist of multiple BH domains [73]. Once p53 arrives at the mitochondrial outer membrane, p53 binds to Bak inducing a conformational change and Bak homo-oligomerization that results in mitochondrial outer membrane permeabilization (MOMP). MOMP allows for the release of pro-apoptotic signaling molecules from the outer and inner mitochondrial membranes into the cytosol triggering the intrinsic apoptotic signaling cascade. ROS generation has been suggested as an alternative p53 apoptotic target independent of cytochrome *c* release. Li et al. found that ROS generation regulated the mitochondrial membrane potential ($\Delta\psi$), which was found to be a key constituent in the induction of p53-mediated apoptosis [74]. Interestingly, during ROS generation, apoptosis occurred in the absence of Bax mitochondrial translocation, Bid activation, as well as cytochrome *c* release. Several studies have suggested that the downstream effects of p53-mediated apoptosis are regulated by Bax expression. It has been shown that the introduction of recombinant Bax protein into isolated mitochondria induced cytochrome *c* release. The ability of Bax to initiate pore formation in synthetic membranes has been shown to regulate cytochrome *c* release resulting in the induction of apoptosis [75, 76]. However, discrepancies exist with *in vivo* studies showing Bax being localized in the cytosol, rather than within the mitochondrial membrane at physiological conditions [77].

Herein, we show how p53 has been shown to play a dual role in early-versus late-stage cancer progression. During the process of carcinogenesis, mutations can occur both upstream and downstream of p53 activation. For example, loss of upstream activators of p53, for example, ATM and Chk2, can prevent p53 activation, contributing to unregulated cell cycling and promoting tumorigenesis [78]. In addition, mutations within the p53 protein can alter necessary structure conformational changes and DNA binding properties needed for efficient p53 activation. Lastly, many of these mutations lead to loss of downstream genes such as Bax or NOXA which are pro-apoptotic and necessary for regulation of cellular proliferation and death signaling.

The process of tumor formation is a multistage process that involves both the activation of protooncogenes, and the inactivation of tumor suppressor genes, such as PTEN and p53. The multistage carcinogenesis paradigm consists of three well-characterized stages: initiation, promotion, and progression. During the initiation stage, there is the induction of mutations within critical target genes of stem cells, for example, H-ras; however in the skin carcinogenesis model, the epidermal layer remains phenotypically normal. During the tumor promotion stage, a noncarcinogenic agent such as a phorbol ester can be used to induce the clonal expansion of the initiated stem cells through epigenetic mechanisms. This stage is often used by investigators to identify potential therapeutic targets due to its reversibility. During the tumor progression stage, malignancy takes place, being characterized by enhanced invasiveness via the activation of proteases, and metastasizes via tumor cells entering into the lymphatics and loss of tumor suppressor activity (e.g., p53).

The two-stage skin carcinogenesis mouse model has been well characterized and used in numerous studies to screen anti cancer agents. An initiator, such as dimethylbenz[a]anthracene (DMBA), is applied to the skin to initiate DNA damage within skin cells. Following DMBA treatment, a tumor promoter such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA) is applied topically to the same area repeatedly for the duration of the study to promote the clonal expansion of mutated cells during the promotion stage. Interestingly, during the early stages of DMBA/TPA-mediated tumor promotion both oncogenes and tumor suppressor genes are activated, resulting in increased cell proliferation being accompanied by increased cell death [79] (Figure 2). Both processes exist throughout skin tumor formation. Not surprising, these two opposing events are closely related.

Many of the tumor-promoting mechanisms utilized by phorbol esters are directly linked to the involvement of cell surface membranes [80, 81]. TPA can mediate its pleiotropic

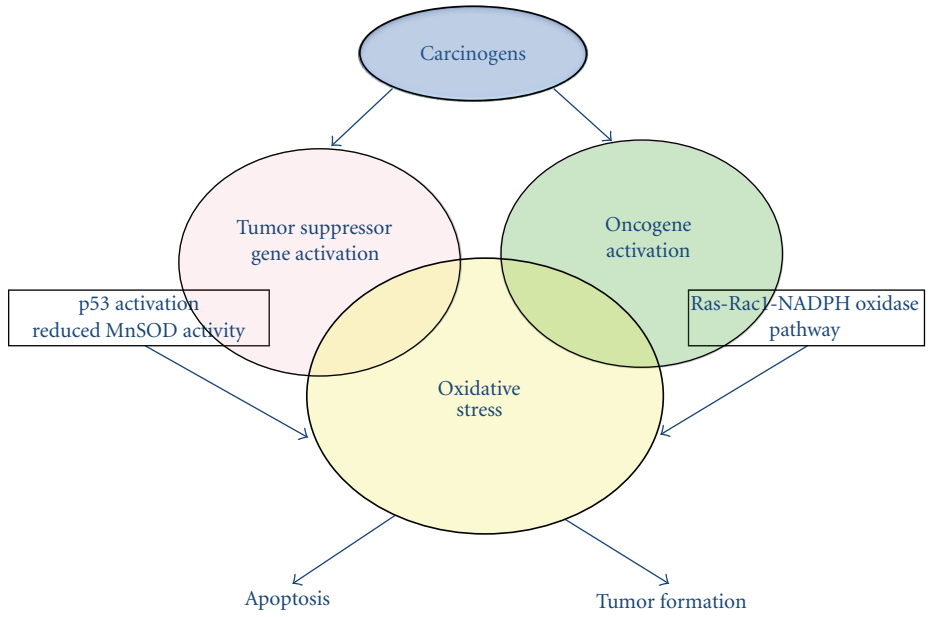

Figure 2: Mechanisms of carcinogens in early stage carcinogenesis. During the early stages of tumor promotion both oncogenes and tumor suppressor genes are activated, resulting in increased cell proliferation being accompanied by increased cell death.

actions through intercalating into the cellular membrane and inducing the activation of the Ca^{2+}-activated phospholipid-dependent protein kinase, protein kinase C (PKC) both *in vitro* and *in vivo*. TPA can directly activate PKC via molecular mimicry by substituting for diacylglycerol, the endogenous substrate, increasing the affinity of PKC for Ca^{2+} which leads to the activation of numerous downstream signaling pathways involved in a variety of cellular functions including proliferation and neoplastic transformation [82]. In addition, it is known that a direct correlation exists between phorbol ester-mediated tumor promotion and enzymatic activation of PKC [82, 83]. The PKC family consists of various highly conserved serine/threonine kinases. PKCs are involved in numerous cellular processes including cell differentiation, tumorigenesis, cell death, aging, and neurodegeneration [84]; however the induction of the signaling pathway is determined by the intracellular redox status and the isoform that is activated. The PKC family consists of a myriad of isoforms that have been divided into three classes: (a) classical or conventional PKCs (cPKC: α, βI, βII, and γ); (b) novel PKCs (nPKC: δ, ε, η, and θ); (c) the atypical PKCs (aPKC: λ, ι, and ζ) which are classified based on sensitivity to Ca^{2+} and diacylglycerol (DAG) [84]. In various types of cancers PKCε has been shown to be upregulated while PKCα and PKCδ are downregulated. Interestingly, TPA activates the PKCε isoform in mouse skin tissues [85]. Furthermore, overexpression of PKCε has been shown to enhance the formation of skin carcinomas [86]. Moreover, TPA treatment leads to the concomitant activation of the redox-sensitive transcription factor activator protein-1 (AP-1) [85]. The AP-1 complex consists of both Jun and Fos oncoproteins. There are 3 jun isoforms (c-jun, jun-B, and jun-D) and 4 fos family members (c-fos, fra-1, fra-2, and fos-B) [87] whose activation is modulated by oxidants such as superoxide and hydrogen peroxide, while DNA binding activities are modulated by the intracellular redox status [88–90]. Kiningham and Clair reported a reduction in tumorigenicity and AP-1 DNA binding activity following overexpression of MnSOD in transfected fibrosarcoma cells [91]. Furthermore, the protein expression of Bcl-xl, an antiapoptotic AP-1 target gene, was decreased, as well. In addition, PKCε activation was reduced in MnSOD transgenic mice treated with DMBA/TPA compared to their nontransgenic counterparts [85]. These results suggest a mechanistic linkage between MnSOD expression, mitogenic activation, and AP-1 binding activity.

5. MnSOD-p53 Mitochondrial Interaction

Another activated signaling pathway that has been defined following DMBA/TPA treatment is the Ras-Rac1-NADPH oxidase pathway, which leads to p53 mitochondrial translocation and apoptosis [92]. NADPH oxidase forms a stable heterodimer with the membrane protein $p22^{phox}$, which serves as a docking site for the SH3 domain-containing regulatory proteins $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$. Upon TPA treatment, Rac, a small GTPase, binds to $p67^{phox}$ which induces NADPH oxidase activation [11] and superoxide production. Mitochondrial p53 has been shown to interact with MnSOD, resulting in decreased enzymatic activity and promoting oxidative stress propagation [93].

The primary role of MnSOD is to protect mitochondria from oxidative damage. In 2005, Zhao et al. found that TPA treatment, both *in vitro* and *in vivo*, can induce p53 mitochondrial translocation [93]. In addition, p53 not only came in contact with the outer mitochondrial membrane but was able to localize to the mitochondrial matrix. Interestingly, following p53 mitochondrial translocation and

matrix localization, p53 interacted with the mitochondrial antioxidant enzyme MnSOD that resulted in a reduction in MnSOD activity and propagation of oxidative stress [93]. However, the question remains: does mitochondrial p53 contribute to or suppress tumor promotion during the early stages of skin carcinogenesis? We addressed this question by utilizing the JB6 mouse skin epidermal cells. JB6 cells were originally derived from primary BALB/c mouse epidermal cell culture [94]. Through nonselective cloning, it was discovered that clonal variants existed within the JB6 cell lineage that were either stably sensitive (P+) or resistant (P−) to tumor promoter-induced neoplastic transformation [95–97]. In addition, JB6 cells remain the only well-characterized skin keratinocytes for studying tumor promotion and screening anti-cancer agents. In 2010, we utilized the JB6 P+ and P− clonal variants to determine if a relationship existed between tumor promotion and early-stage TPA-induced p53 activation [98]. Surprisingly, we found that p53 was only induced in promotion-sensitive P+ cells and not promotion resistant (P−) cells, therefore suggesting that p53 expression is highly associated with early stage tumor promotion. We then assessed Bax protein expression levels, as a marker for p53 transcriptional activity, and found that Bax expression is only induced in JB6 P+ cells and not P− cells, suggesting that p53 expression, as well as transcriptional activity, is highly associated with early-stage tumor promotion following TPA treatment. MnSOD expression was also measured in both JB6 P+ and P− cells and was found to be highly expressed in promotion-resistant P− cells compared to promotable P+ cells. TPA-mediated ROS generation was measured in P+ and P− cells (unpublished data), and promotion resistant cells contained significantly lower levels of ROS following TPA treatment when compared to their promotable counterparts. It is known that reduced MnSOD expression contributes to increased DNA damage, cancer incidence, and radical-caused diseases [99, 100]. Consistent with that, an increase of several markers of oxidative damage such as 4-HNE, 8-oxoDG, and lipid peroxidation has been seen in both *in vitro* and *in vivo* studies following TPA treatment [57, 85, 101, 102], suggesting the involvement of oxidative stress in the promotion of tumorigenesis. These results imply the importance of redox regulation in modulating cellular functions during the early stage of tumor promotion. We questioned whether the ROS generated from the MnSOD-p53 mitochondrial interaction was sufficient to promote tumorigenicity. Therefore, we utilized promotion-resistant JB6 P− cells that exhibited no p53 protein expression or transactivation following TPA treatment to address this question. Interestingly, we found that when JB6 promotion-resistant cells were transfected with wild-type p53, these cells were able to transform and form colonies in soft agar, in comparison to their control counterparts [98]. These results suggest a dual role of p53-mediated ROS generation during the early stages of skin carcinogenesis and how the presence of p53 is necessary for tumor promotion in skin (Figure 3).

The contradictory role of p53 in promoting cell survival or death is the result of the ability to regulate the expression of both pro- and antioxidant genes. For example, p53 can promote the generation of ROS through the induction of genes involved in mitochondrial injury and cell death which include Bax, Puma, and $p66^{SHC}$ and ROS-generating enzymes such as quinine oxidoreductase (NQO1) and proline oxidase [103]. However, p53 can upregulate the expression of various antioxidant enzymes to modulate ROS levels and promote cell survival such as aldehyde dehydrogenase 4 and mammalian sestrin homologues that encode peroxiredoxins and GPX1, which are major enzymatic removers of peroxide [103].

Dhar et al. suggested that p53 possessed "bidirectional" regulation of the antioxidant MnSOD gene. Previous reports suggest the presence of a p53 binding region at 328 bp and 2032 bp upstream of the transcriptional start site of the MnSOD gene [104, 105]. Others suggest that p53 represses MnSOD gene expression by interfering with transcription initiation [106], inhibiting gene activators at the promoter level by forming an inhibitory complex suppressing gene transcription [107] and protein-protein interactions [108]. Nevertheless, p53 can induce the gene expression of MnSOD [104]. p53-mediated MnSOD expression is regulated in conjunction with other cell proliferative transcription factors such as NF-κB. Kiningham and Clair demonstrated the presence of an NF-κB binding site within the intronic enhancer element of the MnSOD gene [91]. It was later shown that mutation of the NF-κB site within the enhancer element abrogated p53 induced MnSOD gene transcription. In addition, knockdown of p65 via siRNA reduced MnSOD gene transcription via p53 as well. Overall the effects of p53 on MnSOD gene expression have been suggested to be concentration dependent, with low concentrations of p53 increasing MnSOD expression via corroborative NF-κB binding promoting cell survival and high concentrations of p53 suppressing MnSOD expression by interfering with important transcriptional binding elements such as SP1.

6. Clinical Implications of the MnSOD-p53 Interaction

p53 is mutated in 50% of human cancers. However, the remaining human tumors contain wild-type p53 with defects in the downstream mediated p53-signaling pathways. This, in turn, provides novel areas of discovery in stabilization and restoration of wild-type p53 activity. Currently, many drug companies are focused on utilizing p53 interactions as targets for pharmacological intervention [78]. There are various protein-protein interactions that occur within the cell that positively or negatively regulate p53 expression and function. For example, Mdm2 is an E3 ligase of p53 that polyubiquitinates p53, priming the tumor suppressor for proteasomal degradation. Many have found that, by blocking this interaction through peptides or transcriptional inhibitors, longer durations of p53 activation have resulted. Some of the therapeutic strategies that are currently being utilized are peptides that increase p53 activation through inhibition of Mdm2 function [109]. Three-dimensional structural models [110] of the hdm2-p53 interaction along with biochemical data [111, 112] have identified three residues that are important to this interaction, Phe19, Trp23, and Leu26

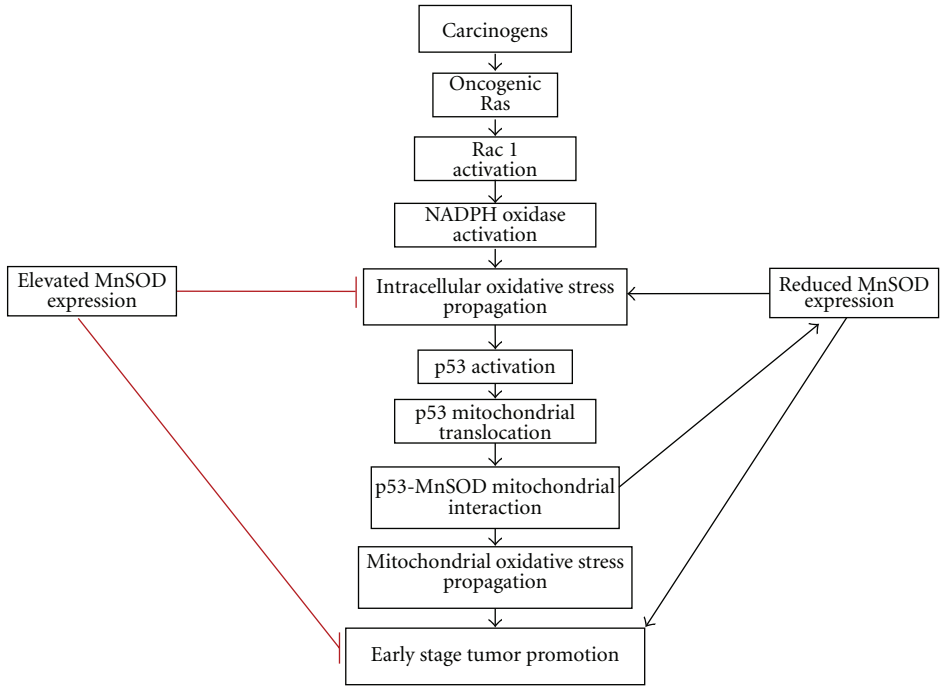

Figure 3: Mechanism involving the p53-MnSOD interaction during the early stage of tumor promotion. Following exposure to a carcinogen the Ras-Rac1-NADPH oxidase pathway is activated, which leads to p53 mitochondrial translocation. Mitochondrial p53 has been shown to interact with MnSOD, resulting in decreased enzymatic activity and promoting oxidative stress propagation contributing to the early stage of skin tumorigenicity. Elevated levels of MnSOD reduced oxidative stress propagation, suppressed p53 mitochondrial translocation, and decreased downstream skin tumor formation. Reduced levels of MnSOD have been shown to contribute to oxidative stress propagation and promote early-stage skin tumorigenicity.

[111, 112]. From this data, an 8-mer peptide was generated [113] and showed promising results in inducing apoptosis in tumor cells that overexpressed hdm2 [112]. However, these conditions were difficult to optimize with a smaller molecule therefore causing this peptide to be therapeutically inefficient. Also nutlins have been utilized to disrupt the mdm2-p53 interaction resulting in reactivation of the p53 response [114, 115]. Others have used antisense and transcription inhibitors to prevent the expression of Mdm2 [116].

Gene replacement therapy is another therapeutic modality that has been explored in treating tumors lacking or containing mutant p53. This technique utilizes adenoviruses, as well as retroviruses to achieve high expression of p53 in tumor cells. Promising results have been seen with retroviral vectors in patients with nonsmall cell lung cancers [117]. On the contrary, although we have seen the enhancement of tumorigenicity in our *in vitro* p53 transfection studies [98], we have not tested stably transfected cells in *in vivo* xenograft mouse models, nor have we tried other tissue types. Therefore, the reintroduction of the p53 gene into tumors may have contradictory outcomes depending on the cell type and tissue microenvironment. This concern has echoed through various studies, persuading investigators to opt to combine gene therapy with chemotherapy and radiotherapy [118–121].

For decades, it has been shown that p53 functions only as a tumor suppressor. In addition, p53-mediated ROS generation has been limited to the induction of apoptosis. Currently, the ability of wild-type p53 to contribute to tumor promotion has received considerable attention. We have shown that the p53-MnSOD interaction contributes to the early stage of tumor promotion. In addition, it has been consistently shown that MnSOD activity is altered in human tumors. Therefore, designing diagnostic tools to assess MnSOD activity, as well as p53 activation, can be used to effectively design individualized treatments for cancer patients. For example, following chemotherapeutic treatment, patients that have higher levels of p53 expression and exhibit lower levels of MnSOD can receive an SOD mimetic that can upregulate MnSOD or synthetic compounds that can downregulate p53 activity to decrease ROS-mediated apoptosis and potential relapse within these patients.

Gene therapy has also been utilized to modulate MnSOD activity during cancer progression. Overexpression of MnSOD through gene therapy introducing genetically engineered DNA/liposomes containing the human MnSOD transgene into preclinical and clinical models has been shown to be protective in normal tissues against ionizing irradiation. The final product (VLTS-582) is a DNA/liposome formulation that consists of a double-stranded DNA bacterial plasmid containing human MnSOD cDNA in conjunction with two lipids {cholesterol and DOTIM (1-[2-[9-(2)-octadecenoyloxy]]-2-[8-(2)-heptadecenyl]-3-[hydroxyethyl] imidazolinium chloride)} [122]. Recent studies suggest that this formulation has been successful in murine

models and has been administered orally to patients concurrently with a weekly chemotherapy regime exhibiting no dose-limiting toxicities. Although proven therapeutically efficacious, more studies are needed to improve (1) delivery of the transgene to the targeted tissue; (2) reducing rapid elimination of the transgene; (3) control of the expression of the transgene within targeted tissues.

On the other hand, a topical application of an SOD mimetic has also been described [123]. The Mn (III) porphyrin Mn^{III} TE-2-Pyp^{5+} possesses highly potent SOD activity as facilitated by the redox properties of the metal center and the positive charge to the ortho-N-ethylpyridyl nitrogens [124]. Mn^{III} TE-2-Pyp^{5+} has been proven effective *in vitro* and in various human diseases such as stroke [125, 126], diabetes [127, 128], and cancer and radiation-related treatment [129–132]. In preclinical animal models, topical application of Mn^{III} TE-2-Pyp^{5+} was shown to reduce levels of oxidative damage and reduced cell proliferation without interfering with p53-mediated apoptosis when applied prior to TPA treatment [129]. These data support the concept that overexpression of MnSOD when applied in conjunction with standard chemotherapeutics or during the tumor promotion stage is protective in both preclinical and clinical models.

Nevertheless, both p53 and MnSOD have been shown to posses reduced activity and/or mutated in most human diseases including cancer. Therefore, more therapeutic quests are needed to detect and restore both MnSOD and wild-type p53 activity. However, future therapeutic optimization strategies should have minimal nonspecific drug-related toxicities and be based on the stage of cancer progression which may reveal a therapeutic window for treatment intervention.

7. Concluding Remarks

In summary, reactive oxygen species have been implicated in the pathogenesis of various hyperproliferative and inflammatory diseases [133]. In addition, the tumor suppressor p53 has been shown to be activated during the early stage of skin carcinogenesis and contributed to the propagation of oxidative stress. Recent studies demonstrate a novel role of mitochondrial p53 activation. Once in the mitochondria, p53 physically interacts with MnSOD. As a result, this interaction reduces the free radical scavenging abilities of MnSOD, promoting enhanced ROS generation which has been shown to act as a tumorigenic stimulus during cancer progression. This suggests that wild-type p53 may play a direct role in promoting oxidative stress and contributing to the ROS-mediated tumor-proliferative stimuli. In addition, others have shown that mutant p53 can, in fact, translocate to the mitochondria and interact with MnSOD [134]. However, Lontz et al. observed following doxorubicin treatment of lymphoma cell lines with varying wt or mutant p53 levels, mitochondrial function, as evidenced by Complex I/II activities, was only compromised in lymphoma cells expressing wild-type and not mutant p53 [134]. Therefore, the continuation of deciphering mechanistic differences in tumors containing wild-type or mutant p53 can lead to the development of therapeutic p53-mediated interventions and a clearer understanding of chemoresistance in both wild-type and mutant p53 human tumors.

Several studies have suggested that MnSOD may play a primary protective role against tissue injury. MnSOD has been found to be depleted in a variety of tumor cells, as well as *in vitro* transformed cell lines, suggesting that MnSOD may act as a novel tumor suppressor, protecting cells from oxidant-induced carcinogenesis [135]. Nevertheless, overexpression of MnSOD decreases the pathogenesis of human diseases such as cancer. Consistent with that, accumulating evidence suggests that a number of antioxidants or drugs with antioxidant properties can reduce mediators of tumor promotion [136]. Clair et al. showed that transfecting mouse 10T 1/2 cells with human MnSOD cDNA promoted differentiation with 5-azacytidine treatment and protected against neoplastic transformation [137]. In addition, transfecting human MnSOD cDNA into MCF-7 breast cancer cells and UACC-903melanoma cells suppressed their malignant phenotype and suppressed growth in nude mice [54, 138]. We have shown that the cumulative induction of endogenous antioxidant enzymes (i.e., catalase, total SOD and MnSOD) is efficient in reducing tumor incidence and multiplicity [57]. In addition, the induction of endogenous antioxidant enzymes via dietary administration can suppress p53 mitochondrial translocation [98]. TPA can induce p53 mitochondrial translocation; however, this phorbol ester also decreases the mitochondrial membrane potential, as well as mitochondrial complex activities and respiration. Other studies have shown that MnSOD overexpression in mice protects complex I from adriamycin-induced deactivation in cardiac tissue [139]. These results suggest that antioxidant expression protects against fluctuations in mitochondrial functions which suppress p53 mitochondrial translocation, p53-mediated ROS, and both downstream apoptotic and cell proliferation signaling pathways. On the contrary, Connor et al. suggest that overexpression of MnSOD in HT-1080 fibrosarcoma cells and 253J bladder tumor cells enhanced the migatory ability and invasiveness of tumor cells, through the upregulation of matrix metalloproteinases [140]. Although some tumors express higher levels of MnSOD, the downstream effects of enhanced antioxidant expression are dependent on the tumor type and susceptibility to oxidative damage, underlying oncogenic mutations and the stage of disease progression [140]. Nevertheless, these investigators stressed the need of refined regulation of H_2O_2 production. Therefore the question remains, are the effects of the p53-MnSOD interaction protumorigenic or anti-tumorigenic? To definitively answer this question further investigation of this interaction is needed. However, there are several factors that must be considered in determining the fate of the p53-MnSOD interaction, which include the stage of disease progression as well as tumor microenvironment. It has been shown that p53 activation is required in tumor promotion and can mediate ROS generation. However, the duration of enhanced ROS generation, severity of oxidative damage, and the status of the cellular antioxidant capacity can all contribute to the proliferative/apoptotic switch that occurs during the response to cellular stress. Overall, further studies are needed to clearly assess the status of MnSOD during the various

stages of carcinogenesis to enhance the efficacy of standard treatment regimens currently being used.

Consistent with that, defining the downstream effects of the p53-MnSOD complex formation can expand our knowledge of the molecular mechanisms that contribute to the early stage of tumorigenesis and how they may be altered during cancer progression. With further knowledge, modulators of MnSOD, p53 and their associated regulators can be therapeutically useful in the treatment of cancer and various stages of tumor progression.

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Review Article

Oxidative Stress, Mitochondrial Dysfunction, and Aging

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Aging is an intricate phenomenon characterized by progressive decline in physiological functions and increase in mortality that is often accompanied by many pathological diseases. Although aging is almost universally conserved among all organisms, the underlying molecular mechanisms of aging remain largely elusive. Many theories of aging have been proposed, including the free-radical and mitochondrial theories of aging. Both theories speculate that cumulative damage to mitochondria and mitochondrial DNA (mtDNA) caused by reactive oxygen species (ROS) is one of the causes of aging. Oxidative damage affects replication and transcription of mtDNA and results in a decline in mitochondrial function which in turn leads to enhanced ROS production and further damage to mtDNA. In this paper, we will present the current understanding of the interplay between ROS and mitochondria and will discuss their potential impact on aging and age-related diseases.

1. Introduction

The fundamental manifestation of the aging process is a progressive decline in the functional maintenance of tissue homeostasis and an increasing propensity to degenerative diseases and death [1]. It has attracted significant interest to study the underlying mechanisms of aging, and many theories have been put forward to explain the phenomenon of aging. There is an emerging consensus that aging is a multifactorial process, which is genetically determined and influenced epigenetically by environment [2]. Most aging theories postulate a single physiological cause of aging, and likely these theories are correct to a certain degree and in certain aspects of aging.

Reactive oxygen species (ROS) are highly reactive molecules that consist of a number of diverse chemical species including superoxide anion (O_2^-), hydroxyl radical ($^{\cdot}OH$), and hydrogen peroxide (H_2O_2). Because of their potential to cause oxidative deterioration of DNA, protein, and lipid, ROS have been implicated as one of the causative factors of aging [3]. As ROS are generated mainly as by-products of mitochondrial respiration, mitochondria are thought to be the primary target of oxidative damage and play an important role in aging. Emerging evidence has linked mitochondrial dysfunction to a variety of age-related diseases, including neurodegenerative diseases and cancer. Details of the precise relationship between ROS-induced damage, mitochondrial dysfunction, and aging remain to be elucidated.

2. ROS and Aging

2.1. ROS, Oxidative Damage, and Cellular Signaling. There are several sources of ROS within a cell. ROS are generated as by-products of aerobic respiration and various other catabolic and anabolic processes [4]. Mitochondria are the major producer of ROS in cells, and the bulk of mitochondrial ROS is generated at the electron transport chain [5, 6]. Electrons leak from the electron transport chain directly to oxygen, producing short-lived free radicals such as superoxide anion (O_2^-) [7, 8]. O_2^- can be converted to non-radical derivatives such as hydrogen peroxide (H_2O_2) either spontaneously or catalyzed by superoxide dismutase (SOD) [9–13]. H_2O_2 is relatively stable and membrane permeable. It can be diffused within the cell and be removed by cytosolic antioxidant systems such as catalase, glutathione peroxidase, and thioredoxin peroxidase [14, 15]. In addition to being generated during cellular metabolism in mitochondria, ROS can be produced in response to different environmental stimuli such as growth factors, inflammatory cytokines, ionizing radiation, UV, chemical oxidants, chemotherapeutics,

hyperoxia, toxins, and transition metals [16–26]. Other than mitochondrial respiration, a number of cytosolic enzymes are able to generate ROS [27]. The nicotinamide adenine dinucleotide phosphate (NADPH) oxidases are a group of plasma membrane-associated enzymes found in a variety of cell types [28]. The function of NADPH oxidases is to produce superoxide from oxygen using electrons from NADPH [29].

Once they are produced, ROS react with lipids, proteins, and nucleic acids causing oxidative damage to these macromolecules [30–34]. ROS readily attack DNA and generate a variety of DNA lesions, such as oxidized DNA bases, abasic sites, and DNA strand breaks, which ultimately lead to genomic instability [35]. 7,8-dihydro-8-oxodeoxyguanosine (8-oxo-dG) is one of the most abundant and well-characterized DNA lesions caused by ROS [36]. It is a highly mutagenic lesion that results in G : C to T : A transversions [37]. To limit the cellular damage caused by ROS, mammalian cells have evolved a number of sophisticated defense mechanisms. ROS-generated DNA lesions are repaired mainly by base excision repair as well as other DNA repair pathways including nucleotide excision repair, double-strand break repair, and mismatch repair [38–40]. In addition, the damaging effects of ROS can be neutralized via elevated antioxidant defense, which includes superoxide dismutase, catalase, and glutathione peroxidase to scavenge ROS to nontoxic forms [41].

Intracellular ROS are normally maintained at low but measurable level within a narrow range, which is regulated by the balance between the rate of production and the rate of scavenging by various antioxidants [42]. ROS, at low level under normal conditions, is found to act as signaling molecules in many physiological processes, including redox homeostasis and cellular signal transduction [7]. By activating proteins such as tyrosine kinases, mitogen-activated protein kinases, or Ras proteins, ROS are important mediators of signal transduction pathways [7]. Dependent on cell types, ROS have been found to function as signaling molecules in cell proliferation [43], cellular senescence [44], or cell death [45, 46]. The divergent effects of ROS on many cellular processes suggest that ROS are not merely detrimental byproducts, but also generated purposefully to mediate a variety of signaling pathways.

2.2. The Free Radical Theory of Aging. The free radical theory of aging proposed by Denham Harman more than fifty years ago postulates that aging results from the accumulation of deleterious effects caused by free radicals, and the ability of an organism to cope with cellular damage induced by ROS plays an important role in determining organismal lifespan [3]. In agreement with this theory, increased ROS production by mitochondria and increased 8-oxo-dG content in the mtDNA are frequently detected in aged tissues [40, 47–50], suggesting that progressive accumulation of oxidative DNA damage is a contributory factor to the aging process. Consistently, many studies have found that increased oxidative damage in cells is associated with aging [51–53]. Furthermore, genetic studies in worm, fly, and mouse have linked enhanced stress resistance or reduced free radical production with increased lifespan [27]. Mutant strains of *C. elegans* that are resistant to oxidative stress have extended lifespan, whereas those more susceptible to free radicals have shortened lifespan [54, 55]. Mice lacking the antioxidant enzyme superoxide dismutase 1 (SOD1) exhibit a 30% decrease in life expectancy [56]. Conversely, simultaneous overexpression of SOD1 and catalase extends lifespan in Drosophila [57]. Small synthetic mimetics of SOD/catalase increase lifespan in *C. elegans* [58], while treatment of antioxidant drugs in mice increases the median lifespan up to 25% [59, 60]. Further supporting this hypothesis, mice lacking Ogg1 and Myh, two enzymes of the base excision repair pathway that repairs oxidative DNA damage, show a 50% reduction in life expectancy [61]. Collectively, these studies demonstrate that interplay between ROS and protective antioxidant responses is an important factor in determining aging and lifespan.

Despite a large body of evidence supporting the role of ROS in aging, the free radical theory of aging faces some challenges [62]. Mice heterozygous for superoxide dismutase 2 ($Sod2^{+/-}$) have reduced manganese SOD (MnSOD) activity, increased oxidative damage, but normal lifespan [63]. Overexpression of antioxidant enzymes in mice, such as SOD1 or catalase, does not extend lifespan [64, 65]. The median lifespan of mice heterozygous of glutathione peroxidase 4 ($Gpx4^{+/-}$), an antioxidant defense enzyme that plays an important role in detoxification of oxidative damage to membrane lipids, is significantly longer than that of wild-type mice, even though $Gpx4^{+/-}$ mice show increased sensitivity to oxidative stress-induced apoptosis [66]. Studies of long-lived rodents also do not find a convincing correlation between level of oxidative damage and aging [67]. Furthermore, pharmacologic intervention with antioxidants in humans and mice has little effect on prolonging lifespan [68–70]. More investigations are clearly needed to clarify the discrepancy in the role of ROS and antioxidant enzymes in aging among different species and to understand the precise role that free radicals play in aging.

2.3. ROS and Senescence. Senescence, a process in which normal somatic cells enter an irreversible growth arrest after a finite number of cell divisions [71], is thought to contribute to organismal aging [72–74]. Senescent cells are associated with high level of intracellular ROS and accumulated oxidative damage to DNA and protein [75–77]. In contrast, immortal cells suffer less oxidative damage and are more resistant to the deleterious effects of H_2O_2 than primary cells [78]. Increasing intracellular oxidants by altering ambient oxygen concentrations or lowering antioxidant levels accelerates the onset of senescence, while lowering ambient oxygen or increasing ROS scavenging delays senescence [76, 78–81].

Telomere shortening is considered as the major cause of replicative senescence [82, 83]. It has been reported that the rate of telomere shortening is directly related to the cellular level of oxidative stress [84]. Telomere shortening is significantly increased under mild oxidative stress as compared to

that observed under normal conditions, whereas overexpression of the extracellular SOD in human fibroblasts decreases the peroxide content and the rate of telomere shortening [79]. ROS can affect telomere maintenance at multiple levels. The presence of 8-oxoguanine (8-oxoG), an oxidative derivative of guanine, in telomeric repeat-containing DNA oligonucleotides has been shown to impair the formation of intramolecular G quadruplexes and reduces the affinity of telomeric DNA for telomerase, thereby interfering with telomerase-mediated extension of single-stranded telomeric DNA [85]. ROS also affect telomeres indirectly through their interaction with the catalytic subunit of telomerase, telomerase reverse transcriptase (TERT). Increased intracellular ROS lead to loss of TERT activity, whereas ROS scavengers such as *N*-acetylcysteine (NAC) block ROS-mediated reduction of TERT activity and delay the onset of cellular senescence [86]. Furthermore, the presence of 8-oxoG in the telomeric sequence reduces the binding affinity of TRF1 and TRF2 to telomeres [87]. TRF1 and TRF2 are components of the telomere-capping shelterin complex that protects the integrity of telomeres [88]. In addition, ROS-induced DNA damage elicits a DNA damage response, leading to the activation of p53 [89], a critical regulator of senescence. It has been shown that p53 transactivates E3 ubiquitin ligase Siah1, which in turn mediates ubiquitination and degradation of TRF2. Consequently, knockdown of Siah1 expression stabilizes TRF2 and delays the onset of replicative senescence [90]. The p53-Siah1-TRF2 regulatory axis places p53 both downstream and upstream of DNA damage signaling initiated by telomere dysfunction. By regulating telomere maintenance or integrity directly or indirectly, ROS plays a critical role in senescence.

2.4. ROS and Stem Cell Aging. Tissue-specific or adult stem cells, which are capable of self-renewal and differentiation, are essential for the normal homeostatic maintenance and regenerative repair of tissues throughout the lifetime of an organism. The self-renewal ability of stem cells is known to decline with advancing age [91–94], suggesting that decline in stem cell function plays a central role in aging. Increasing evidence suggests that dysregulated formation of ROS may drive stem and progenitor cells into premature senescence and therefore impede normal tissue homeostasis.

Genetic studies of mice deficient in genes implicated in ROS regulation indicate that elevated level of ROS within the stem cell compartments leads to a rapid decline in stem cell self-renewal [95–98]. Deletion of Ataxia telangiectasia mutated (ATM) kinase results in increased ROS level in hematopoietic stem cell (HSC) population in aged mice, which correlates with a rapid decline in HSC number and function [95]. When $Atm^{-/-}$ mice are treated with antioxidants, the defect in stem cell self-renewal is rescued [95], suggesting that high level of ROS causes the decline in stem cell function. Furthermore, deficiency in telomerase reverse transcriptase (TERT) accelerates the progression of aging, resulting in an even shorter lifespan in $Atm^{-/-}$ mice accompanied by increased senescence in hematopoietic tissues and decreased stem cell activity [99]. These TERT-deficient HSCs are also sensitive to ROS-induced apoptosis, suggesting another possible cause of stem cell impairment during aging [99]. Similarly, defect in HSC number and activity accompanied by increased accumulation of ROS is observed in mice lacking three members of *Forkhead box O-class (FoxO)* [96–98]. Increased level of ROS in FoxO3-null myeloid progenitors leads to hyperproliferation through activation of the AKT/mTOR signaling pathway, and ultimately premature exhaustion of progenitors [100]. Mice carrying a mutation in *inner mitochondrial membrane peptidase 2-like* (*Immp2l*) gene, which is required to process signal peptide of mitochondrial cytochrome c1 and glycerol phosphate dehydrogenase 2, exhibit an early onset of aging phenotypes, including premature loss of fat [101]. Elevated mitochondrial ROS level in the *Immp2l* mutant mice leads to impaired self-renewal of adipose progenitor cells, suggesting that ROS-induced damage to adult stem cells is the driving force of accelerated aging in these mice [101]. Further supporting this notion, intracellular level of ROS is found to correlate with the long-term self-renewal ability of HSCs in mouse [102]. HSCs with high level of ROS show a decreased ability of long-term self-renewal, and treatment of antioxidant NAC is able to restore the functional activity of HSCs with high level of ROS [102]. Taken together, these studies suggest that ROS play an important role in stem cell aging.

ROS-generated DNA lesions are repaired by several DNA repair pathways including base excision repair, nucleotide excision repair, double-strand break repair, and mismatch repair [38–40]. Endogenous DNA damage accumulates with age in HSCs in mouse. HSCs in mice deficient in DNA repair pathways, including nucleotide excision repair, telomere maintenance, and nonhomologous end-joining, exhibit increased sensitivity to the detrimental effect of ROS, diminished self-renewal and functional exhaustion with age [103]. These data support the notion that accumulated DNA damage is one of the principal mechanisms underlying age-dependent stem cell decline.

3. Mitochondria and Aging

3.1. The Mitochondrial Theory of Aging. Because mitochondria are the major producer of ROS in mammalian cells, the close proximity to ROS places mitochondrial DNA (mtDNA) prone to oxidative damage [104]. Consistently, many studies have shown that 8-oxo-dG, one of the common oxidative lesions, is detected at higher level in mtDNA than nuclear DNA, suggesting that mtDNA is more susceptible to oxidative damage [52, 105–113]. As both the major producer and primary target of ROS, mitochondria are thought to play an important role in aging. The mitochondrial theory of aging, extended from the free radical theory, proposes that oxidative damage generated during oxidative phosphorylation of mitochondrial macromolecules such as mtDNA, proteins, or lipids is responsible for aging [114]. As mtDNA encodes essential components of oxidative phosphorylation

and protein synthesis machinery [115], oxidative damage-induced mtDNA mutations that impair either the assembly or the function of the respiratory chain will in turn trigger further accumulation of ROS, which results in a vicious cycle leading to energy depletion in the cell and ultimately cell death [104, 114, 116–118].

As mitochondria play a critical role in regulation of apoptosis, which is implicated in the aging process [119], age-related mitochondrial oxidative stress may contribute to apoptosis upon aging. The activation of the permeability transition pore in mitochondria, which is believed to play a critical role in cell necrosis and apoptosis, is enhanced in spleen, brain, and liver of aged mice [120, 121]. Moreover, mitochondrial adenine nucleotide translocase, a component of the permeability transition pore, exhibits an age-associated increase of oxidative modification in male houseflies [122]. Such selective oxidative modification may cause the cells more vulnerable to apoptotic inducers [123]. Thus, mitochondria appear to influence the aging process via modifying the regulatory machinery of apoptosis.

Mice expressing proof reading-deficient mitochondrial DNA polymerase show a consistent increase in mtDNA mutations, premature onset of the aging phenotypes and reduced lifespan [124, 125], suggesting a critical link between mitochondria and aging. Interestingly, ROS production in these mice is not increased [124, 125]. Similarly, mice expressing proof reading-deficient mitochondrial DNA polymerase specifically in heart show accumulation of mutations in mtDNA and develop cardiomyopathy, but oxidative stress in the transgenic heart is not increased, indicating that oxidative stress is not an obligate mediator of diseases provoked by mtDNA mutations [126]. More studies are required to further clarify the consequence of oxidative stress and mitochondrial dysfunction in aging.

3.2. Age-Associated Changes of Mitochondria. Mitochondrial genome encodes proteins required for oxidative phosphorylation and ATP synthesis, and RNAs needed for mitochondrial protein translation [115]. The mtDNA is densely packed with genes and only contains one noncoding region called the displacement loop (D-loop) [127]. The D-loop is important for mtDNA replication and transcription and has been extensively studied for the presence of age-related mutations [115]. Age-dependent accumulation of point mutations within the D-loop has been reported in various types of cells and tissues, including skin and muscle [128–132]. In addition to point mutations, deletions of mtDNA are detected at higher frequency in aged human and animal tissues [133–145]. Replication is thought to be the likely mechanism leading to the formation of mtDNA deletions [146–148], but recent studies suggest that mtDNA deletions may be generated during repair of damaged mtDNA rather than during replication [149]. It is thought that repair of oxidative damage to mtDNA accumulated during aging leads to generation of double-strand breaks [149], with single-strand regions free to anneal with microhomologous sequences on other single-stranded mtDNA or within the noncoding region [150]. Subsequent repair, ligation and degradation of the remaining exposed single strands would result in the formation of an intact mitochondrial genome harboring a deletion [149]. Whether and how exactly mutations and deletions of mtDNA cause the aging phenotypes are not clear. Among mtDNA deletions during aging, especially in postmitotic tissues like muscle and brain, the most common one is a 4977-bp deletion [151–153]. The frequency of this deletion increases in brain, heart, and skeletal muscle with age, although the increase varies in different tissues of the same individual [154], or even in different regions of the same tissue [134, 136, 137]. This deletion occurs in a region encoding subunits of the NADH dehydrogenase, cytochrome *c* oxidase, and ATP synthase [155]. Whether deletion of these genes plays a causative role in the development of aging phenotypes remains to be determined.

In addition to age-associated increase of mtDNA mutations and deletions, the abundance of mtDNA also declines with age in various tissues of human and rodent [156–158]. For instance, in a large group of healthy men and women aged from 18 to 89 years, mtDNA and mRNA abundance is found to decline with advancing age in the vastus lateralis muscle. Furthermore, abundance of mtDNA correlates with the rate of mitochondrial ATP production [158], suggesting that age-related mitochondrial dysfunction in muscle is related to reduced mtDNA abundance. However, age-associated change in mtDNA abundance seems to be tissue specific, as several studies have reported no change in mtDNA abundance with age in other tissues in human and mouse [159–161]. It is possible that tissue-specific effect of aging on mtDNA abundance is related to the status of aerobic activity [156, 158], as aerobic exercise has been shown to enhance muscle mtDNA abundance in both human and mouse [162–164]. Increased prevalence of mtDNA mutations/deletions and decreased mtDNA abundance offer attractive underlying causes of mitochondrial dysfunction in aging, which warrants further investigation.

3.3. Mitochondria Malfunction in Age-Associated Human Diseases. A heterogeneous class of disorders with a broad spectrum of complex clinical phenotypes has been linked to mitochondrial defect and oxidative stress [165, 166]. Particularly, mitochondria are thought to play an important role in the pathogenesis of age-associated neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, and Huntington's disease. This is not surprising as neurons are especially sensitive and vulnerable to any abnormality in mitochondrial function because of their high energy demand.

Alzheimer's disease (AD) is the most common form of dementia and often diagnosed in people over 65 years of age. AD is characterized by severe neurodegenerative changes, such as cerebral atrophy, loss of neurons and synapses, and selective depletion of neurotransmitter systems in cerebral cortex and certain subcortical region [167]. Mitochondria are significantly reduced in various types of cells obtained from patients with AD [168–170]. Dysfunction of mitochondrial electron transport chain has also been associated with

the pathophysiology of AD [170]. The most consistent defect in mitochondrial electron transport enzymes in AD is a deficiency in cytochrome *c* oxidase [171, 172], which leads to an increase in ROS production, a reduction in energy stores, and disturbance in energy metabolism [173].

Parkinson's disease (PD) is the second most common progressive disorder of the central nervous system, which is characterized prominently by loss of dopaminergic neurons in the substantia nigra and formation of intraneuronal protein aggregates [174]. The finding that exposure to environmental toxins, which inhibit mitochondrial respiration and increase production of ROS, causes loss of dopaminergic neurons in human and animal models leads to a hypothesis that oxidative stress and mitochondrial dysfunction are involved in PD pathogenesis [175]. Consistent with this notion, a significant decrease in the activity of complex I of the electron transport chain is observed in the substantia nigra from PD patients [176]. Furthermore, neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, which acts as an inhibitor of complex I, can induce parkinsonism in human, monkey, and rodent [177, 178]. Genetic studies of *PINK1* and *PARKIN* further support the role of mitochondrial dysfunction in pathogenesis of PD [179, 180]. Autosomal recessive mutations in *PINK1* and *PARKIN* are associated with juvenile Parkinsonism [181–183]. Studies in *Drosophila* have provided strong evidence that *PINK1* and *PARKIN* act in the same genetic pathway to control mitochondrial morphology in tissues with high energy demand and requirement of proper mitochondrial function, such as indirect flight muscle and dopaminergic neurons [184–186]. Consistent with the finding in *Drosophila*, primary fibroblasts derived from patients with *PINK1* mutations show similar abnormalities in mitochondrial morphology [187]. The morphologic changes of mitochondria can be rescued by expression of wild-type *PARKIN* but not pathogenic *PARKIN* mutants [187], suggesting that mitochondrial dynamics plays an important role in PD pathogenesis.

Huntington's disease (HD) is another hereditary neurodegenerative disorder that affects muscle coordination and leads to cognitive decline and dementia. HD is caused by an autosomal dominant mutation in the Huntingtin (HTT) gene [188]. Morphologic defects of mitochondria, such as reduced mitochondrial movement and alterations in mitochondrial ultrastructures, have been observed in patients with HD or transgenic HD mouse models [189, 190]. In addition, expression of mutant HTT leads to impaired energy metabolism, abnormal Ca^{2+} signaling and mitochondrial membrane potential, and drastic changes in mitochondrial ultrastructures [191, 192]. Although the underlying molecular mechanism remains to be determined, it is recently proposed that mutant HTT conveys its neurotoxicity by evoking defects in mitochondrial dynamics, mitochondrial fission and fusion, and organelle trafficking, which in turn result in bioenergetic failure and HD-associated neuronal dysfunction [189].

Mitochondrial dysfunction and increased oxidative damage are often associated with AD, PD, and HD, suggesting that oxidative stress may play an important role in the pathophysiology of these diseases [193]. Increased production of cellular ROS and oxidative stress have been reported to induce autophagy, a homeostatic process that enables cells to degrade cytoplasmic proteins and organelles [194–197]. The observation of increased autophagy in the brains of patients with AD, PD, and HD suggests that autophagy contributes to the pathogenesis of these neurodegenerative diseases, possibly by causing cell death [170, 198–202]. Consistently, oxidative stress-induced autophagy of accumulated amyloid β-protein in AD causes permeabilization of lysosomal membrane and leads to neuronal cell death [203]. Mitochondria damaged by significantly increased oxidative stress in pyramidal neurons of AD are subjected to autophagic degradation, ultimately leading to neurodegeneration [204]. Furthermore, overexpression of wild-type PINK1 increases mitochondrial interconnectivity and suppresses toxin-induced autophagy, whereas knockdown of PINK1 expression potentiates mitochondrial fragmentation and induces autophagy [197], suggesting that induced autophagy as a consequence of loss of function of *PINK1* may contribute to the pathogenesis of PD.

Interestingly, autophagy also serves as a protective mechanism in age-related neurodegenerative diseases. Several studies demonstrate that basal level of autophagy clears the deleterious protein aggregates that are associated with AD, PD, and HD [205–207], therefore playing a protective role in the maintenance of neural cells. For instance, autophagy is involved in degradation of HTT aggregates [198]. Administration of rapamycin induces autophagy and enhances the clearance of mutant HTT, improving cell viability and ameliorating HD phenotypes in cell and animal models [208]. Furthermore, PARKIN, whose loss of function mutation causes early onset PD, has been found to promote autophagy of depolarized mitochondria [209], suggesting that a failure to eliminate damaged mitochondria by mutant PARKIN is responsible for the pathogenesis of PD. It is not entirely clear why autophagy can exert protective or deleterious effects on pathogenesis of these neurodegenerative diseases. A better understanding of autophagy, mitochondrial dysfunction, and oxidative stress is necessary in order to dissect the pathogenesis of AD, PD, and HD.

Cancer is considered an age-associated disease, as the incidence of cancer increases exponentially with age. Warburg first discovered that cancer cells constitutively metabolize glucose and produce excessive lactic acid even in the presence of abundant oxygen, a phenomenon named "aerobic glycolysis" [210]. In contrast, normal cells generate energy mainly from oxidative breakdown of pyruvate, which is an end product of glycolysis and is oxidized in mitochondria. Conversion of glucose to lactate only takes place in the absence of oxygen (termed "Pasteur effect") in normal cells. He hypothesized that defect in mitochondrial respiration in tumor cells is the cause of cancer, and cancer should be interpreted as mitochondrial dysfunction [210]. A growing body of evidence has demonstrated the presence of both somatic and germline mutations in mtDNA in various types of human cancers [211–213]. The most direct evidence that mtDNA mutations may play an important role in neoplastic transformation comes from the study by introducing

a known pathogenic mtDNA mutation T8993G into the prostate cancer cell line PC3 through transmitochondrial cybrids [214]. The T8993G mutation derived from a mitochondrial disease patient causes a 70% reduction in ATP synthase activity and a significant increase in mitochondrial ROS production [215]. Tumor growth in the T8993G mutant cybrids is much faster than that in the wild-type control cybrids [214]. Moreover, staining of tumor sections confirms a dramatic increase in ROS production in T8993G mutant tumors, suggesting that mitochondrial dysfunction and ROS elevation contribute to tumor progression. Consistent with this notion, the $Sod2^{+/-}$ mice exhibit increased oxidative damage and enhanced susceptibility to cancer as compared to wild-type mice [63]. Collectively, these studies suggest that mtDNA mutations could contribute to cancer progression by increasing mitochondrial oxidative damage and changing cellular energy capacities.

3.4. Mouse Models of Oxidative Stress and Mitochondrial Dysfunction in Aging. Genetically engineered mouse models provide great systems to directly dissect the complex relationship between oxidative damage, mitochondrial dysfunction, and aging. Although it is difficult to manipulate mitochondrial genome, genetic engineering of nuclear genes that are involved in oxidative stress response and mitochondrial function has been utilized to study mitochondrial biology and aging.

Mammalian cells scavenge ROS to nontoxic forms through a sophisticated antioxidant defense that includes superoxide dismutase (SOD), catalase, and glutathione peroxidase. Genetic ablation of *SOD*2, which encodes a mitochondrial manganese SOD (MnSOD), leads to early postnatal death in mice accompanied by a dilated cardiomyopathy, metabolic acidosis, accumulation of lipid in liver and skeletal muscle, increased oxidative damage, and enzymatic abnormalities in mitochondria [216, 217]. Treatment of $Sod2^{-/-}$ mice with a synthetic SOD mimetic not only rescues their mitochondrial defects in the liver, but also dramatically prolongs their survival [218]. Furthermore, heterozygous $Sod2^{+/-}$ mice show evidence of decreased membrane potential, inhibition of respiration, and rapid accumulation of mitochondrial oxidative damage [219]. Mitochondrial oxidative stress induced by partial loss of *SOD*2 leads to an increase in proton leak, sensitization of the mitochondrial permeability transition pore and premature induction of apoptosis [219]. These studies clearly demonstrate that ROS generated in mitochondria play an important role in cell homeostasis and aging.

Conflicting results of the effect of increased *SOD2* expression on aging are obtained using different *SOD*2 transgenic mouse strains [220–222]. A transgenic line carrying a human SOD2 transgene under the control of a human β-actin promoter shows protection against hyperoxic lung injury [220], reduction in mitochondrial superoxide in hippocampal neurons, and extended lifespan as the result of increased activity of MnSOD [221]. Another transgenic line carrying a 13-kb mouse genomic fragment containing *SOD*2 [223] has a twofold increase in the activity of MnSOD [222]. Such level of *SOD*2 overexpression does not alter either lifespan or age-related pathology, even though these mice exhibit decreased lipid peroxidation, increased resistance against paraquat-induced oxidative stress, and decreased age-related decline in mitochondrial ATP production [222]. The reason behind the different outcomes of these two *SOD*2 transgenic mice on lifespan is not clear, but may be related to different levels of *SOD*2 expression. The precise role of SOD2 in aging needs further investigation.

An important function of mitochondria is to produce ATP. Targeting genes involved in ATP production offers a great opportunity to study the role of mitochondrial function in aging. An example is a mouse model with targeted inactivation of adenine nucleotide translocator (ANT), a transporter protein that imports ADP and exports ATP from the mitochondria. $Ant1^{-/-}$ mice exhibit classical physiological features of mitochondrial myopathy and hypertrophic cardiomyopathy in human, as evident of cardiac hypertrophy, an increase in succinate dehydrogenase and cytochrome *c* oxidase activities, a degeneration of the contractile muscle fibers, and a massive proliferation of abnormal mitochondria in skeletal muscle [224]. The increase in mitochondrial abundance and volume in muscle of $Ant1^{-/-}$ mice is accompanied by upregulation of genes that are known to be involved in oxidative phosphorylation [225]. Consistently, mitochondrial H_2O_2 production increases in skeletal muscle and heart of $Ant1^{-/-}$ mice [226]. The *Ant*1-deficient mouse model provides strong evidence that a defect in mitochondrial energy metabolism can result in pathological disease [224].

IMMP2L protein is a subunit of a heterodimer complex of inner mitochondrial membrane peptidase that cleaves signal peptide from precursor or intermediate polypeptides after they reach the inner membrane of mitochondria [227, 228]. Mammalian IMMP2L has two known substrates, cytochrome c1 and glycerol phosphate dehydrogenase 2, both of which are involved in superoxide generation [229]. The *Immp2l* mutant mice have impaired processing of signal peptide of cytochrome c1 and glycerol phosphate dehydrogenase 2 [230], and consequently show elevated level of superoxide ion, hyperpolarization of mitochondria, and increased oxidative stress in multiple organs. Furthermore, these *Immp2l* mutant mice exhibit multiple aging-related phenotypes, including wasting, sarcopenia, loss of subcutaneous fat, kyphosis, and ataxia [101]. These data provide a strong evidence that mitochondrial dysfunction is a driving force of accelerated aging.

4. Conclusion

Aging is a complex process involving a multitude of factors. Many studies have demonstrated that oxidative stress and mitochondrial dysfunction are two important factors contributing to the aging process. The importance of mitochondrial dynamics in aging is illustrated by its association with a growing number of age-associated pathogenesis. A better understanding of response to oxidative stress and mitochondrial dynamics will lead to new therapeutic

approaches for the prevention or amelioration of age-associated degenerative diseases.

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Review Article

ROS-Mediated Signalling in Bacteria: Zinc-Containing Cys-X-X-Cys Redox Centres and Iron-Based Oxidative Stress

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Bacteria are permanently in contact with reactive oxygen species (ROS), both over the course of their life cycle as well that present in their environment. These species cause damage to proteins, lipids, and nucleotides, negatively impacting the organism. To detect these ROS molecules and to stimulate the expression of proteins involved in antioxidative stress response, bacteria use a number of different protein-based regulatory and sensory systems. ROS-based stress detection mechanisms induce posttranslational modifications, resulting in overall conformational and structural changes within sensory proteins. The subsequent structural rearrangements result in changes of protein activity, which lead to regulated and appropriate response on the transcriptional level. Many bacterial enzymes and regulatory proteins possess a conserved signature, the zinc-containing redox centre Cys-X-X-Cys in which a disulfide bridge is formed upon oxidative stress. Other metal-dependent oxidative modifications of amino acid side-chains (dityrosines, 2-oxo-histidines, or carbonylation) also modulate the activity of redox-sensitive proteins. Using molecular biology, biochemistry, biophysical, and structure biology tools, molecular mechanisms involved in sensing and response to oxidative stress have been elucidated in detail. In this review, we analyze some examples of bacterial redox-sensing proteins involved in antioxidative stress response and focus further on the currently known molecular mechanism of function.

1. Introduction

Interference in the balance between the environmental production of reactive oxygen species (ROS), including hydroxyl radicals ($^{\bullet}OH$) and hydrogen peroxide (H_2O_2), and the ability of biological systems to readily detect and detoxify them, or repair the resulting damage, are defined as oxidative stress. Highly reactive radicals cause the oxidative damage of different macromolecules—proteins, DNA, and lipids—leading to loss of function, an increased rate of mutagenesis, and ultimately cell death. In humans, for example, oxidative stress is involved in many diseases, such as rheumatoid arthritis, autoinflammatory diseases, neurodegenerative diseases, and cancer [1, 2]. However, the production of some ROS (e.g., $^{\bullet}OH$) can also be beneficial, as they are used by the human immune system to attack and kill pathogens, such as the production of ROS by macrophages. Additionally, H_2O_2 is an important signalling molecule that participates in redox signalling [3].

Sensing of ROS-mediated signals also plays a crucial role in the biology of microorganisms. Bacteria, for example, are in continuous contact with ROS generated both endogenously, as a product of aerobic metabolism, or exogenously during ionizing (γ) and nonionizing (UV) irradiation leading to the production of a number of radical and peroxide species through the ionization of intracellular water. Industrial contaminants that are widespread in soils and on the surfaces of plants are also sources of ROS.

Iron is earth's fourth most abundant metal, after oxygen, silicon, and aluminium. Its relevance for bacterial cells is emphasized by the fact that it is involved in a wide range of biological processes, including photosynthesis, N_2 fixation,

H_2 production and consumption, respiration, oxygen transport, and gene regulation [4, 5]. However, in the presence of oxygen, ferrous ions frequently result in oxidative stress through the generation of hydroxyl radicals *via* the Fenton reaction ($Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + {}^{\bullet}OH + {}^{-}OH$). Therefore, bacteria have developed a variety of different mechanisms to ensure that iron is sufficiently accessible as well as being maintained in a nontoxic form [4]. They possess high-affinity iron transport systems (i.e., siderophores and membrane iron transporters) that enable iron to be scavenged. Intracellular iron can be stored in protein complexes (i.e., in Dps and ferritins). Thus, the homeostasis of these ions is tightly regulated so that their intracellular concentrations do not reach toxic levels. Previously, it has also been suggested that the production of hydroxyl radicals is induced by bactericidal antibiotics to kill bacteria, in which iron ions and the Fenton reaction play a role [6], or during a wide range of plant or human pathogen interactions. Moreover, ROS can also be produced during the degradation of natural existing biopolymers (cellulose, chitin, or xylan) by microorganisms [7]. To reduce the hazardous effects of iron-based production of ${}^{\bullet}OH$, bacteria produce proteins with an enzymatic activity to degrade ROS (i.e., superoxide dismutases, catalases, peroxidases, and alkylhydroperoxide reductases), other small redox proteins (thioredoxins and glutaredoxins) as well as low molecular-weight thiols (glutathione and mycothiol) [8, 9]. All these cell components contribute in maintaining a reducing environment both in the cell and in controlling the extent of the oxidative burst.

In analogy to Fe(II) ions, other transition metals ions [i.e., Cu(I), Co(II), Mn(II), Ti(III), or Cr(V)] are closely linked with the production of free radicals in cells [10]. Although these metal ions can be hazardous for living organisms, they also serve as signal mediators in signalling cascades. Another metal with high biological relevance is zinc. It is an essential trace element that is localized in the active center or in a structurally important site of many bacterial proteins [11]. Zinc is a cofactor for more than 300 enzymes (i.e., superoxide dismutase and alcohol dehydrogenase). It is also a structural element of at least 40 protein classes (i.e., RNA polymerase and tRNA synthetases). Additionally, zinc can protect sulfhydryl groups from free radicals and inhibits free radical formation by competing with redox-active metals such as iron [12, 13]. Binding of zinc as well as metal-catalyzed oxidation in proteins is closely related with the presence of redox-active cysteine residues (e.g., within Cys-X-X-Cys motifs) and other metal-sensing amino acids. Moreover, bacterial redox sensory proteins can act either as single transcription regulators (e.g., FurS or Irr) displaying a sensory and response domain within itself [14, 15], or as a part of multicomponent systems (e.g., HbpS or ChrS) in which sensing and response are distributed among each protein component [16–18].

In bacteria, there are a high number of redox sensory proteins that show different mechanism of function. Here, we will give an overview of them and focus further on signalling pathways in which redox-active cysteines as well as iron ions are involved.

2. Zinc-Containing Cys-X-X-Cys Motifs as Sensor Centres

Cysteine residues (Cys) in proteins are prominent targets for protein oxidation, as they easily react with H_2O_2 and free radicals. Oxidation of Cys by H_2O_2 involves nucleophilic attack of the cysteinyl thiol group on the electrophilic center of H_2O_2. Deprotonation of the Cys thiol group to generate the thiolate anion increases its nucleophilicity, and hence reactivity towards H_2O_2. These reactions are chemically highly complex and can lead to different sulfur oxidation states, including thiols, sulfenic and sulfinic acids, thiyl radicals, disulfide S-oxides, or disulfides [19]. Disulfides can be generated between two Cys either intra- or intermolecularly (Figure 1). The sensitivity of Cys thiol groups to oxidation provides them with redox sensitivity, and hence the ability to sense redox status. The molecular environment of the redox sensitive Cys also modulates the sensing mechanism. For example, the thiolate anion can be stabilised by proximity to hydrogen bond donors, basic residues, and metal ions [20].

In this review, we will focus on intramolecular disulfides that are formed within Cys-X-X-Cys motifs—(X: any amino acid)—a motif that is widespread in bacterial sensor proteins (Table 1). In addition to redox-sensing properties, these motifs are often involved in zinc binding (i.e., in FurS, Hsp33, RsrA, RslA, Trx2, and SbcC) (Table 1), and in the stabilization of protein domains that are crucial for function. It can be expected that the molecular environment of Cys is in this case also important for zinc binding.

Table 1 comprises two different types of Cys-X-X-Cys-containing proteins. Proteins belonging to the first group exhibit enzymatic function (Hsp33, ResA, DsbA, SbcC, cytochrome c, Trx2, AhpF/AhpC, CopA, and HypA). For example, under reducing conditions, the chaperone Hsp33 binds a single zinc ion through its C-terminal Cys-X-Cys-X_6-Cys-X-X-Cys motif. Upon oxidative stress, the four Cys residues form intramolecular disulfide bridges resulting in release of zinc accompanied by considerable conformational changes that lead to destabilization of its C-terminus [22]. As a consequence, Hsp33 dimerises and acquires a chaperone function to prevent protein aggregation [23]. Similarly, in the DNA repair protein SbcC, two monomers are linked *via* Cys-X-X-Cys motifs and a zinc ion, forming a functional zinc bridge that is important for SbcC exonuclease activity [24]. ResA—an extracellular low potential thiol-disulfide oxidoreductase—was shown to maintain Cys residues of cytochrome *c* in their reduced form. The thiolate species within the heme-binding motif (Cys-X-X-Cys-His) of apocytochrome *c* are required for covalent attachment of heme [25]. Contrary to the other Cys-X-X-Cys-containing proteins described here, ResA does not bind zinc through this motif. Other proteins with this signature and lacking zinc are listed in Table 1 but are not within the focus of this review.

The second group of Cys-X-X-Cys-containing proteins is involved in transcriptional regulation and can be subdivided into two subtypes: proteins that either directly control transcription (i.e., WhiB3, FurS, CatR, SoxR, and SurR) or require further regulatory components (i.e., Spx, RsrA, RslA, and RshA).

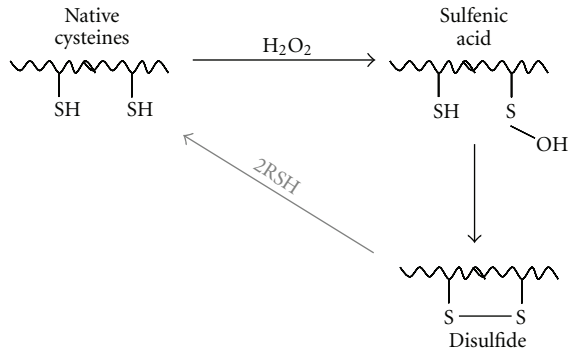

Figure 1: Formation of intramolecular disulfide bonds (S–S). Oxidation of a cysteine thiol by H_2O_2 yields a sulfenic acid residue that can undergo reaction with a neighbouring "back door" cysteine thiol to generate a disulfide linkage (S–S). S–S bonds can overtime be returned to the native SH state by reactions with biological thiols (RSH). This picture was adapted from [21].

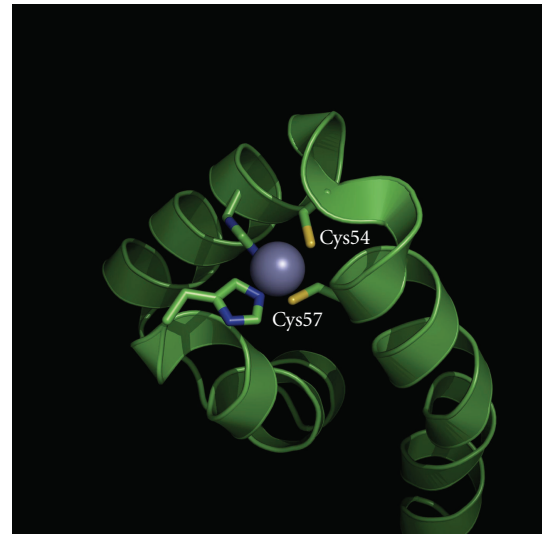

Figure 2: An image showing the redox-sensing cysteine residues (Cys54 and Cys57) within the crystal structure of RslA from *Mycobacterium tuberculosis* (PDB: 3HUG). The coordinated zinc ion (shown as a violet ball) strongly contributes to the overall fold stability.

The redox-dependent Cys-X-X-Cys-containing transcriptional regulator WhiB3, from *Mycobacterium tuberculosis,* has been shown to sense the intracellular redox state in the cell, and to be required for the production of virulence polyketides, including polyacyltrehaloses (PAT), sulfolipids (SL-1), *di-o-acyl-trehaloses* (DATs) and trehalose dimycolates (TDMs). WhiB3 directly regulates the expression of the following genes in a redox-dependent manner: *pks3* (encoding a polyketide *beta*-ketoacyl synthase involved in PAT and DAT anabolism), *pks2* (encoding a polyketide synthase involved in SL-1 anabolism), and *fbpA* (encoding a fibronectin-binding protein for TDMs production) [26]. WhiB3 contains an iron-sulfur cluster $[4Fe\text{-}4S]^{+}$ that under aerobic conditions is oxidized to $[4Fe\text{-}4S]^{2+}$. Further oxidative conversions result in complete loss of this cluster [27]. Unlike the superoxide stress sensor SoxR, the redox state of the WhiB3 iron-sulfur cluster does not modify the DNA binding affinity to target gene promoters, but rather the redox state of the cysteine residues is critical for DNA binding. It was shown that, while the reduction of Cys in WhiB3 abolishes its DNA binding activity, oxidation induces it. It is noteworthy to mention that reduction and oxidation in WhiB3 are reversible processes.

In the case of the zinc-containing transcriptional repressor FurS from *Streptomyces reticuli*, it was biochemically and spectrophotometrically shown that H_2O_2-mediated disulfide bond formation between Cys93 and Cys96 in FurS is accompanied by the release of the bound zinc ion. This leads to conformational changes in FurS, resulting in a loss of FurS binding to its own DNA operator sequence within the regulatory region of the target operon, *furS-cpeB*. As a result, the expression of this operon is no longer repressed, leading to an increase in production of FurS and the mycelium-associated catalase-peroxidase CpeB. This enhanced expression in turn detoxifies the surrounding environment of hazardous H_2O_2 [9, 14].

Proteins with Cys-X-X-Cys motifs are also indirectly involved in transcriptional regulation, by interacting with additional components. Reversible disulfide formation can regulate the activity of the sigma factor R (σ^R) of *Streptomyces coelicolor* A3(2). Here, the thiol-specific oxidant diamide induces σ^R-mediated transcription of >30 genes, including those encoding thioredoxins and thioredoxin reductases that are involved in antioxidative stress response. However, σ^R does not contain any Cys and *in vitro* studies have demonstrated that its transcriptional activity is not dependent on oxidative stress. This strongly suggests that an additional component must act as the oxidative stress sensor [28]. Indeed, researchers have characterised the protein RsrA, which interacts with σ^R and regulates its transcriptional activity in a oxidative stress-dependet manner [29]. RsrA is a zinc-containing and redox-sensitive antisigma factor that possesses seven Cys, two of which (Cys41 and Cys44) are located within a Cys-X-X-Cys motif that is important for activity. Under oxidising conditions, a disulfide bond is formed in RsrA, and this oxidized state cannot bind σ^R or inhibit its transcriptional activity. Oxidised RsrA can be re-reduced by thioredoxin and in the thiol-reduced state RsrA reassociates with σ^R and blocks its transcriptional activity. The redox sensing Cys in RsrA also coordinate zinc ions that provide the protein with a higher structural stability, strengthening its interaction with σ^R. Upon redox stress and induction of disulfide bond formation, zinc is released and RsrA undergoes conformational changes, generating a structure that does not bind σ^R[30]. Structural studies of an RsrA homolog, RslA (Table 1) from *Mycobacterium tuberculosis* revealed that the redox sensing and zinc binding Cys (Cys54 and Cys57) are closely located (~3, Å; Figure 2) to each other and are solvent exposed in the complex, thus, providing a structural basis for the redox sensitivity of RslA [31]. The solvent exposed state of Cys within the Cys-X-X-Cys motif is also an essential feature in redox sensing by the heat-shock protein Hsp33 (Table 1; PDB: 1VQ0) from *Escherichia coli* [22].

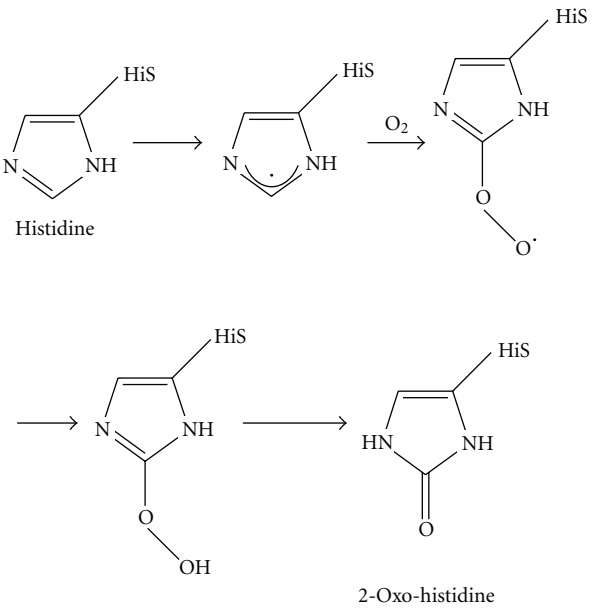

Figure 3: Metal-catalyzed oxidation of histidine. The exact chemical mechanism of the iron-mediated formation of 2-Oxo-histidine remains unclear. This picture was adapted from [34].

3. Iron-Based Oxidative Stress

To assure that iron is sufficiently accessible as well as being maintained in a nontoxic form, bacteria have developed a variety of protein-based protection mechanism. They employ iron-sensing regulatory proteins (i.e., PerR and HbpS-SenS-SenR; see also Table 2) that control the expression of proteins (i.e., H_2O_2-degrading enzymes such as catalases) blocking iron-dependent damage. Upon sensing of iron-based stress, these proteins undergo different oxidative modifications, including oxidation of histidines (Figure 3), dityrosine formation, or carbonylation.

Table 2 lists different iron-sensing proteins that mostly control redox stress resistance systems. They can directly regulate the transcription of target genes by DNA binding to regulatory regions (i.e., Fur, DtxR, RirA, Irr, PerR, DmdR1, IdeR, SirR, FNR, and TroR) or utilize further regulatory elements (HbpS-SenS-SenR; PmrA-PmrB, ChrS-ChrA, and FecA-FecR-FecI) that mediate the adaptive response.

Iron-dependent gene regulation has been demonstrated for the diphtheria toxin repressor (DtxR) from *Corynebacterium diphtheriae* and *Corynebacterium glutamicum* [35]. Binding of divalent iron ions to the global regulator DtxR results in the transcriptional repression of a number of genes required for iron uptake (i.e., *htaA* coding for a secreted iron acquisition and transport protein), as well as for usage and storage (i.e., *hmuO* coding for a heme oxygenase and *ftn* coding for a ferritin-like protein). Mutational analyses have additionally demonstrated that DtxR is also involved in the iron-dependent expression of DNA-protecting proteins, including Dps-like proteins that bind iron and DNA.

In *Bacillus subtilis,* the peroxide resistance regulator PerR has been shown to sense metal dependent as well as H_2O_2-based oxidative stress, and to regulate the corresponding adaptive response [36]. The metalloprotein PerR is a small dimeric protein that coordinates two metal ions per monomer. One binding site binds a zinc ion, whereas the second one a regulatory metal, either iron or manganese [37, 38]. *In vivo*, the regulatory metal is required for repression of the transcription of target genes, including those ones encoding a catalase, alkylhydroperoxide reductase, and the Dps-like DNA-binding protein MrgA. Under H_2O_2-based oxidative stress conditions, Fe^{2+} catalyzes the oxidation of histidines (either His37 or His91) in the regulatory binding site of PerR. This results in Fe^{2+} release and subsequent derepression of PerR target genes. Analysis of electron maps density in the crystal structure of the oxidised PerR protein (PerR-Zn-ox) showed the presence of a 2-oxo-histidine residue at position 37. Further, MALDI-TOF and tandem ESI-MS studies additionally revealed the oxidation of His91 within Per-Zn-ox [36, 38, 39]. It was concluded that the structural conformation of PerR is dependent on the oxidation state of the regulatory site.

Oxidative modifications have also been shown to regulate the activity of the redox sensor and heme-binding protein HbpS from *Streptomyces reticuli*. HbpS is extracellularly located and interacts with the membrane-embedded histidine autokinase SenS from the two-component system SenS SenR [40, 41]. Analyses of the crystal structure of HbpS combined with size exclusion chromatography and static light scattering [42–44] allowed the identification of HbpS as an octamer. Further studies demonstrated that the octameric assembly in HbpS is required for an efficient interaction with SenS [43]. Phosphorylation analyses also revealed that under nonoxidative stress conditions HbpS inhibits the autophosphorylation of SenS, whereas oxidative stress induces the HbpS-mediated activation of SenS [16]. After autophosphorylation at a conserved histidine, SenS transfers the phosphate group to its cognate response regulator SenR, resulting in SenR~P. SenS acts also as a phosphatase of SenR~P. The unphosphorylated form of SenR binds to specific sites upstream of the *furS-cpeB* operon (encoding for the redox regulator FurS and the mycelia-associated catalase-peroxidase CpeB), leading to its transcriptional repression. Once SenR has been phosphorylated, it loses the ability to bind to this operator, leading to a de-repression of the *furS-cpeB* transcription. In addition, SenR~P has been shown to activate the transcription of *hbpS*. Comparative physiological analyses have demonstrated that the presence of HbpS and SenS-SenR provides *S. reticuli* with a defence system against redox-stressing conditions [40, 41].

It was proposed that the switching of HbpS from its inhibitor to activator state of SenS autophosphorylation under oxidative stress conditions is controlled by conformational changes in HbpS. Indeed, CD spectroscopy as well as FRET analyses revealed that after iron-mediated oxidative stress HbpS undergoes secondary structure and overall intrinsic conformational changes, which are accompanied by oxidative modifications (i.e., carbonylation and dityrosine formation). While the sites of carbonylation have not yet been determined, the tyrosine residues participating in dityrosine formation have been identified as Tyr77. These residues are localized in the interface of HbpS subunits

Table 1: Examples of proteins containing the redox-sensing motif Cys-X-X-Cys. (*) The references given are for the protein from the strain listed. Sequence alignments demonstrate that highly homologous proteins are found across many different bacterial species.

	Name	Strain*	Function	Zn^{2+}-binding
Enzymatic function	Hsp33	*Escherichia coli*	The redox-regulated heat shock protein Hsp33 is a dual stress sensor responding to peroxide stress and increased temperature. Stress-mediated conformational changes result in zinc release and activation of Hsp33 chaperone function leading to suppression of protein aggregation [18].	Yes
	Trx2	*Escherichia coli*	Trx2 reductase activity is controlled by a redox switch within two CXXC motifs involved in zinc binding. Release of the bound zinc ion results in a conformational change leading to the reductase activity [23].	Yes
	ResA	*Bacillus subtilis*	Substrate selection of the membrane-bound thiol-disulfide oxidoreductase ResA is regulated by conformational changes determined by CXXC reduction or oxidation [24].	No
	DsbA	*Escherichia coli*	DsbA is a periplasmic protein oxidant for disulfide formation of extracellular proteins belonging to the Dsb family [25].	No
	SbcC	*Bacillus subtilis*	SbcC is a DNA repair protein with exonuclease activity [27].	Yes
	Cytochrome c	*Bacillus subtilis*	The covalent coordination of heme to apo-cytochrome c requires a reduced CXXC motif within the heme-binding motif [28].	No
	AhpF/AhpC	*Salmonella typhimurium*	AhpC and flavoprotein AhpF catalyze the pyridine nucleotide-dependent reduction of hydroperoxide substrates. AhpC, the peroxide-reducing component, is a scavenger of hydrogen peroxide in bacteria, whereas the disulfide reductase protein AhpF regenerates AhpC [29].	No
	CopA	*Thermotoga maritima*	CopA, a copper transport ATPase, sustains important roles in homeostasis of heavy metals and delivery of copper to metalloenzymes [30].	No
	HypA	*Escherichia coli*	HypA is required for nickel insertion into the hydrogenase precursor proteins [31].	No
Transcriptional regulator	FurS	*Streptomyces reticuli*	Oxidation of the transcriptional repressor FurS leads to derepression of the transcription of the gene *cpeB* coding for a catalase peroxidase [6].	Yes
	CatR	*Streptomyces coelicolor* A3 (2)	During peroxide stress, the Fur-like regulator CatR activates transcription of *catA* coding for catalase A [21].	No
	WhiB3	*Mycobacterium tuberculosis*	WhiB3 DNA binding to control the expression of genes coding for polyketide synthases is reversibly regulated by a thiol-disulfide redox switch. Reduction of the apo-WhiB3 Cys thiols of the CXXC motif suppresses genes regulating the synthesis of complex lipids, whereas oxidation stimulates it [22].	No
	SoxR	*Escherichia coli*	SoxR senses superoxide stress through a CXXC-coordinated [2Fe-2S]-cluster that results in transcriptional activation of a superoxide response regulon [32].	No
	SurR	*Pyrococcus furiosus (Archaea)*	A redox switch regulates the transcriptional regulator SurR. Oxidation with S^0 inhibits DNA binding by SurR, leading to repression of genes related to H_2 production and activation of genes involved in S^0 metabolism [33].	No
Regulatory element	Spx	*Bacillus subtilis*	Global oxidative stress regulator interacting with the α-subunit of RNA polymerase for transcriptional induction of genes involved in thiol homeostasis (*mrsA-mrsB* operon) [19].	No
	RsrA	*Streptomyces coelicolor* A3 (2)	Antisigma factor RsrA negatively regulates expression of the thioredoxin system in response to cytoplasmatic oxidative stress. Under reducing conditions, RsrA binds to σ^R resulting in inhibition of transcription [20].	Yes
	RslA	*Mycobacterium tuberculosis*	Membrane-associated RslA oxidation results in the release of bound Zn^{2+} through disulfide bond formation within the CXXC motif. The resulting conformational change leads to decreased σ^L binding. The released sigma factor regulates the expression of genes involved in cell-wall and polyketide synthesis [17].	Yes
	RshA	*Mycobacterium tuberculosis*	RshA is an antisigma factor of the central regulator SigH that responds to oxidative and heat stress; it functions as a negative regulator of the alternative sigma factor SigH activity under reducing conditions [26].	No

Table 2: Examples of iron-dependent redox sensor proteins in bacteria. (*) The references given are for the protein from the strain listed. Sequence alignments demonstrate that highly homologous proteins are found across many different bacterial species.

	Name	Strain*	Function
Transcriptional regulator	Fur	*Escherichia coli*	Regulator with iron-dependent DNA-binding affinity negatively regulates genes involved in ferric iron uptake [38].
	DtxR	*Corynebacterium glutamicum*	DtxR acts as a global iron-mediated regulator, activating genes involved in iron storage and DNA protection and repressing genes involved in iron uptake and utilization [39].
	RirA	*Rhizobium leguminosarum*	Transcriptional regulator RirA is involved in ferric uptake regulation by regulating genes coding for iron transport, siderophore biosynthesis, and iron-sulfur cluster assembly [40].
	Irr	*Bradyrhizobium japonicum*	Iron response regulator (Irr) senses iron through the status of heme biosynthesis to regulate gene expression involved in iron homeostasis [42].
	PerR	*Bacillus subtilis*	DNA binding by the regulator PerR in response to peroxide stress is iron dependent [51].
	DmdR1	*Streptomyces coelicolor*	The transcriptional regulator DmdR1 regulates genes involved in desferrioxamine production in response to iron availability [43].
	IdeR	*Mycobacterium smegmatis*	IdeR negatively regulates siderophore biosynthesis involved in iron acquisition [44].
	SirR	*Staphylococcus epidermidis*	SirR is a Fe^{2+}or Mn^{2+}-dependent transcriptional repressor regulating the *sit*ABC operon encoding an ATPase, a cytoplasmic membrane protein, and the 32-kDa lipoprotein involved in siderophore-mediated iron uptake [45].
	FNR	*Escherichia coli*	Transcription factor FNR regulates gene expression in response to oxygen deficiency by its redox-sensitive bound iron. Binding of an iron-sulfur cluster is required for a conformational change to enhance DNA binding [46].
	IscR	*Escherichia coli*	[2Fe-2S]-cluster assembly regulates activity in transcription factor IscR of genes coding for proteins involved in iron-sulfur cluster assembly [47].
	TroR	*Treponema denticola*	TroR is a Mn^{2+} and Fe^{2+}-dependent repressor of the ATP-binding cassette cation transport system (*tro*ABCD) regulating manganese and iron homeostasis [50].
Regulatory element	HbpS/SenS/SenR	*Streptomyces reticuli*	Iron-dependent activation/inhibition of the two-component system SenS-SenR involved in oxidative stress response through heme degradation and associated secondary structural changes [8].
	PmrA/PmrB	*Salmonella enterica*	The PmrA/PmrB two-component system senses iron and regulates the transcription of genes involved in iron resistance [41].
	ChrS/ChrA	*Corynebacterium diphteriae*	ChrS, the heme-sensing sensor kinase of the two-component system ChrS/ChrA, regulates genes involved in utilization of host heme as an iron source and in protecting the bacteria against the toxic effects of heme [48].
	FecA/FecR/FecI	*Escherichia coli*	The periplasmic protein FecR senses periplasmic iron dicitrate by the outer membrane protein FecA which is loaded with ferric citrate. FecR transmits the signal to the sigma factor FecI which results in transcriptional activation of the *fec*-operon for ferric citrate transport [52].
	AcnA/AcnB	*Escherichia coli*	The aconitases AcnA (induced by iron and oxidative stress) and AcnB posttranscriptionally regulate gene expression (i.e., *sodA*) by an iron-sulfur cluster-dependent switch [49].

and are located in close proximity to each other, over the monomer-monomer interface. Interestingly, Tyr77 is situated near to a postulated iron-binding site, containing Glu78 and Glu81 within an E-X-X-E motif (Figure 4) that has been previously characterized as an iron-binding motif [45–47].

The reported oxidative modifications in HbpS result in the degradation, either autonomously or protease dependent, of the oxidized protein [45]. A metal-catalyzed protein oxidation accompanied by cross-linking and degradation has been reported for the *Bradyrhizobium japonicum* iron

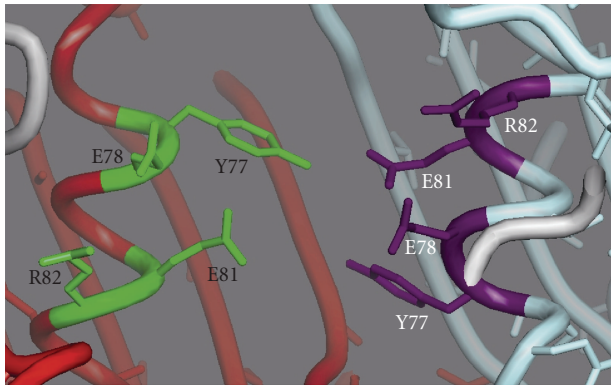

Figure 4: An image showing the arrangement of the internal EXXE motif between two subunits (red and turquoise chain, respectively) in the octameric HbpS (PDB: 3FPV). The amino acids Tyr77 (Y77), Glu78 (E78), Glu81 (E81), and Arg82 (R82) are indicated.

response regulator (Irr) protein, which is involved in the regulation of iron transport as well as of heme biosynthesis genes [15]. It is proposed that the iron-catalyzed oxidation with subsequent degradation of Irr is the molecular basis for the modulation of the activity of this regulator.

The membrane-embedded sensor kinase PmrB from the two-component system PmrA-PmrB of the pathogen *Salmonella enterica* serovar typhimurium exhibits two copies of the EXXE iron-binding motif. These are periplasmically located and have been proposed to be involved in sensing of extracellular ferric iron. Upon iron sensing and autophosphorylation, PmrB transfers the signal to its cognate response regulator PmrA, which in turn regulates Fe^{3+} resistance genes: *pbgP* and *ugd* both coding for enzymes that modify the lipid A region of lipopolysaccharide (LPS) with 4-aminoarabinose. The gene product of *pmrC* catalyzes the addition of phosphoethanolamine to lipid A, whereas the phosphatase encoded by *pmrG* targets the phosphate located in the core region of the LPS. These modifications result in a less-charged cell surface and diminished binding of ferric iron to the membrane [48]. In contrast to other iron-sensing systems (i.e., PerR and HbpS-SenS-SenR), PmrA-PmrB seems to protect the cell against iron toxicity independent of the simultaneous presence of oxygen.

Beside the posttranslational modifications (i.e., oxidation of histidines, carbonylation, dityrosine formation, or S–S bonding) that regulate iron-sensing proteins, posttranscriptional regulation of gene expression in response to oxidative stress has been reported for the aconitases AcnA and AcnB of *Escherichia coli* [49, 50]. In their holo form, both AcnA and AcnB contain an iron-sulfur cluster, [4Fe-4S], and exhibit enzymatic function. Under reducing conditions, they catalyze the isomerisation of citrate to isocitrate. Upon oxidative stress and iron depletion, the iron-sulfur cluster is released. The resulting apo-AcnA and apo-AcnB proteins act as RNA-binding proteins that stabilize *acnA*- and *acnB*-mRNA transcripts, leading to increased amount of the respective aconitases that subsequently complex iron-sulfur clusters [49]. Moreover, mutational analyses have revealed that the presence of AcnA provides *E. coli* with enhanced resistance against superoxide-mediated oxidative stress. Additionally, proteomics studies demonstrated that the expression of anti-oxidative stress-working proteins (i.e., SodA and TrxB) is dependent on AcnA.

4. Summary

As in humans, the exposure of bacteria to ROS causes damage to a variety of macromolecules, resulting in mutations and often in cell death. However, ROS may also be considered to be beneficial compounds, as they function as signalling molecules that lead to a coordinated response of bacteria under redox-stress conditions. These signals can be sensed by redox-active and zinc-coordinating Cys-X-X-Cys centres in proteins. Under reducing conditions, zinc stabilizes protein structure, but the presence of H_2O_2 provokes the release of zinc and the formation of S–S bridges that significantly alter the conformation and structure of the protein (i.e., FurS or RsrA). As a result, transcriptional activity is altered (by FurS), or the ability to interact with the partner DNA-binding protein is lost (by RsrA). In both cases, the transcription of genes involved in the anti-oxidative stress response is ultimately activated. The structural basis for the redox sensitivity is given by the location of S–S-forming cysteines in the protein, namely, their solvent exposition within the three-dimensional structure. Importantly, reduction and oxidation processes within Cys-X-X-Cys redox centres are reversible and provide bacteria with an elegant switch on/off mechanism.

Iron-dependent oxidative modifications (i.e., 2-oxo-histidine or dityrosine formation) are also involved in ROS-based signalling. Under H_2O_2-based stress, iron catalyzes the oxidation of histidines in the peroxide resistance regulator PerR, leading to release of iron and subsequently to derepression, and expression of target genes. In analogy, iron-based stress activates the HbpS-SenS-SenR-mediated signalling cascade. HbpS is a redox sensor that upon oxidative modifications undergoes conformational and structural changes, inducing the phosphorylation cascade between the sensor kinase SenS and the response regulator SenR. As known for other regulators, oxidative modifications lead to the building of covalent bonds within proteins. Such processes are irreversible and lead ultimately to degradation of oxidized proteins. Therefore, *de novo* protein biosynthesis is required to switch off the corresponding signal cascade.

The diversity of responsive protein elements among bacteria correlates with the diversity in: ecological niches, life cycles, pathogenic, and nonpathogenic character. Future efforts will undoubtedly demonstrate that additional further specialized systems exist.

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