IRIS AperTO

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IRIS AperTO

UNIVERSITÀ DEGLI STUDI DI TORINO

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AUTOPHAGY EXACERBATES MUSCLE WASTING IN CANCER CACHEXIA AND IMPAIRS MITOCHONDRIAL FUNCTION

Fabio Penna[1*], Riccardo Ballarò[1#], Paula Martinez-Cristobal[2,3,5#], David Sala[2], David Sebastian[2,3,5], Silvia Busquets[3], Maurizio Muscaritoli[4], Josep M. Argilés[3], Paola Costelli[1] and Antonio Zorzano[2,3,5*]

[1]*Dept. Of Clinical and Biological Sciences, University of Torino, Torino, Italy*

[2] *Institute for Research in Biomedicine (IRB Barcelona), The Barcelona Institute of Science and Technology, Barcelona, Spain*

[3]*Departament de Bioquímica i Biomedicina Molecular, Facultat de Biologia, Universitat de Barcelona, Barcelona, Spain*

[4]*Department of Clinical Medicine, Sapienza University, Rome, Italy*

[5]*Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM), Instituto de Salud Carlos III, Madrid, Spain*

#equal contribution

***** *Correspondence and requests for materials should be addressed to FP (fabio.penna@unito.it, phone: +390116707759 fax: +390116707753) and/or AZ (antonio.zorzano@irbbarcelona.org).*

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Abstract

Cancer cachexia is a multifactorial syndrome characterized by anorexia, weight loss and muscle wasting that impairs patients' quality of life and survival. Aim of this work was to evaluate the impact of either autophagy inhibition (knocking-down beclin-1) or promotion (overexpressing TP53INP2/DOR) on cancer-induced muscle wasting. In C26 tumor-bearing mice, stress-induced autophagy inhibition was unable to rescue the loss of muscle mass and worsened muscle morphology. Treating C26-bearing mice with formoterol, a selective β2-agonist, muscle sparing was paralleled by reduced static autophagy markers although the flux was maintained. Conversely, the stimulation of muscle autophagy exacerbated muscle atrophy in tumor-bearing mice. TP53INP2 further promoted atrogene expression and suppressed mitochondrial dynamics-related genes. Excessive autophagy might impair mitochondrial function through mitophagy. Consistently, tumor-induced mitochondrial dysfunction was detected by reduced ex vivo muscle fiber respiration. Overall, the results evoke a central role for muscle autophagy in cancer-induced muscle wasting.

Highlights

- Autophagy contribution to cancer-related muscle wasting is still debated.
- Blocking muscle stress-induced autophagy is unable to spare muscle mass.
- Formoterol counteracts muscle wasting without blocking autophagy flux.
- Muscle-specific autophagy induction via TP53INP2 overexpression exacerbates cachexia.

Introduction

Cancer-associated cachexia is a multifactorial syndrome characterized by anorexia and body weight loss, mainly due to muscle and fat wasting [1]. Cachexia and in particular skeletal muscle loss significantly impairs cancer patients' quality of life and tolerance to anti-neoplastic treatments, eventually reducing survival. Despite the relevance of this syndrome to cancer patient outcome, anti-cachexia treatments are still lacking. The currently available standard of care for cachectic cancer patients is limited to nutritional support, while specific treatments aimed at counteracting muscle wasting are under investigation and no clinical trial proved effective so far.

The mechanisms underlying muscle depletion have been extensively studied in the past, acceleration of protein turnover rates being the main reason for muscle mass depletion [2]. Specifically, myofibrillar protein catabolism occurs, although reduced protein synthesis might play a role as well [3]. As for protein degradation, two main proteolytic systems operate in the skeletal muscle: the ubiquitin-proteasome-dependent pathway (UPS) and macroautophagy (hereafter referred to as autophagy). UPS activation in cancer cachexia has been reported both in preclinical rodent models and in humans with some differences according to tumor stage and type ([4] and references within). However, pharmacological UPS inhibition (using bortezomib) is unable to prevent muscle wasting in two distinct experimental models of cancer cachexia [4] and no clinical trial has attempted to directly interfere with proteasomal degradation in cachectic patients.

Also excessive autophagic degradation has been proposed to play a role in the onset of muscle depletion in cancer cachexia, based on observations in both rodents [5] and cachectic cancer patients [6,7]. Autophagy modulation is a double edged sword, since basal activation is required to remove damaged proteins and organelles maintaining muscle quality, while enhanced autophagy flux favors muscle loss. As a consequence, both excessive and defective autophagy worsen muscle function and impair muscle mass [8], suggesting the importance of a finely tuned balance between protein degradation and synthesis. Even in cancer cachexia an impaired autophagy flux, likely due

to overload, has been proposed to occur, while the pharmacological reactivation rescues muscle homeostasis and counteracts cachexia [9]. Those data are in conflict with others showing that the promotion of autophagy worsens muscle wasting. Specifically, the overexpression of the autophagy-regulating TP53INP2/DOR protein triggers muscle wasting in diabetic (streptozotocin-induced) mice [10]. TP53INP2 activates basal autophagy in skeletal muscle directly interacting with LC3 and sustaining the degradation of ubiquitylated proteins. In this regard, muscle specific TP53INP2 gain- and loss-of-function have been shown to result in atrophy and hypertrophy, respectively [10].

When debating the role of autophagy in the regulation of muscle mass, a further consideration should be given to the cargo that is selectively degraded and might be different in healthy as compared to diseased conditions. Indeed, the degradation of damaged proteins and dysfunctional organelles is a premise for muscle quality while the uncontrolled targeting of myofibrillar proteins or mitochondria negatively impact muscle mass without any positive effect. Such dysregulation might be the consequence of specific signals depending on inflammation and tumor-related factors, such as the increased expression of ubiquitin-ligases [11] and mitophagy triggers [12], both sufficient to cause atrophy.

So far, only indirect evidences on the beneficial or detrimental effect of autophagic degradation in experimental cancer cachexia are available. The present work is aimed at overcoming such limitations by directly modulating (blocking or activating) autophagy in order to measure the resulting effects on muscle mass and quality.

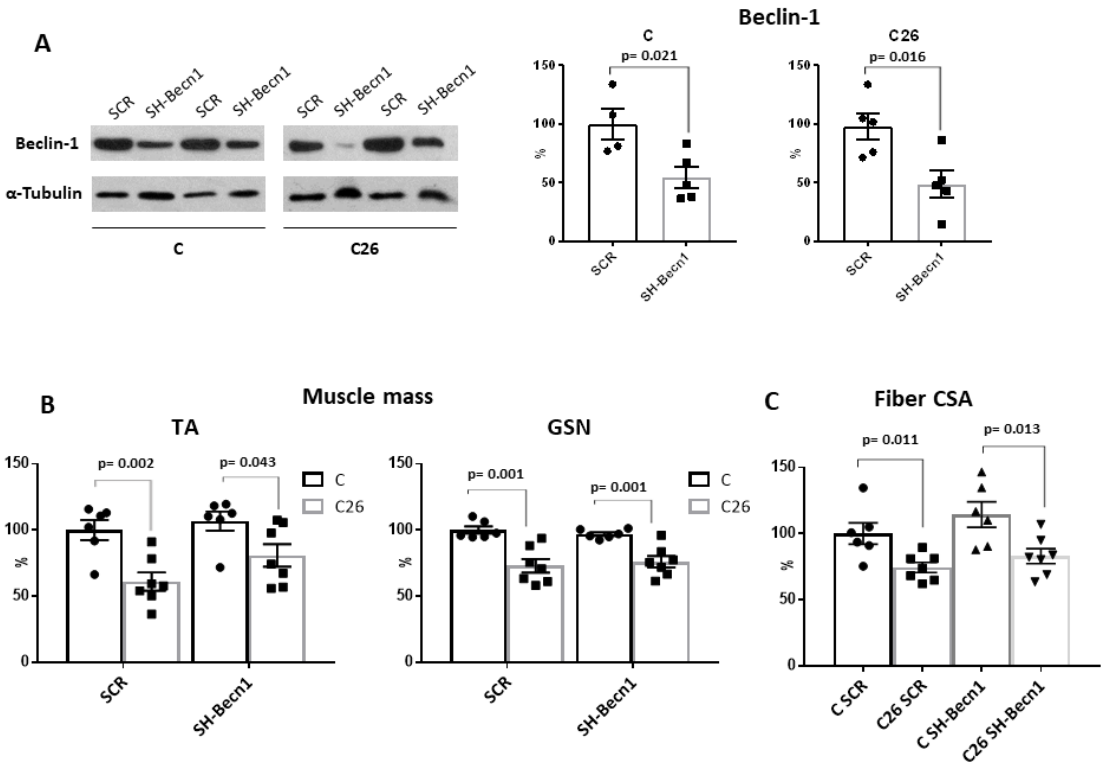
Results

Beclin-1 deficiency does not prevent muscle wasting in tumor-bearing mice

To understand whether muscle wasting in cancer cachexia could be prevented by blocking stress-induced (beclin-1-regulated) autophagy, beclin-1 was knocked-down in the *tibialis anterior* muscle

(TA) via electroporation of a specific shRNA, leading to a 50% reduction of the protein levels (Fig. 1A). As expected, in C26-bearing mice TA mass was reduced (39%) when electroporated with scramble sequence, while it was moderately protected against loss (24%) in condition of beclin-1 deficiency. Such a difference could not be appreciated in non-electroporated muscles such as the gastrocnemius (Fig. 1B).

Fig. 1



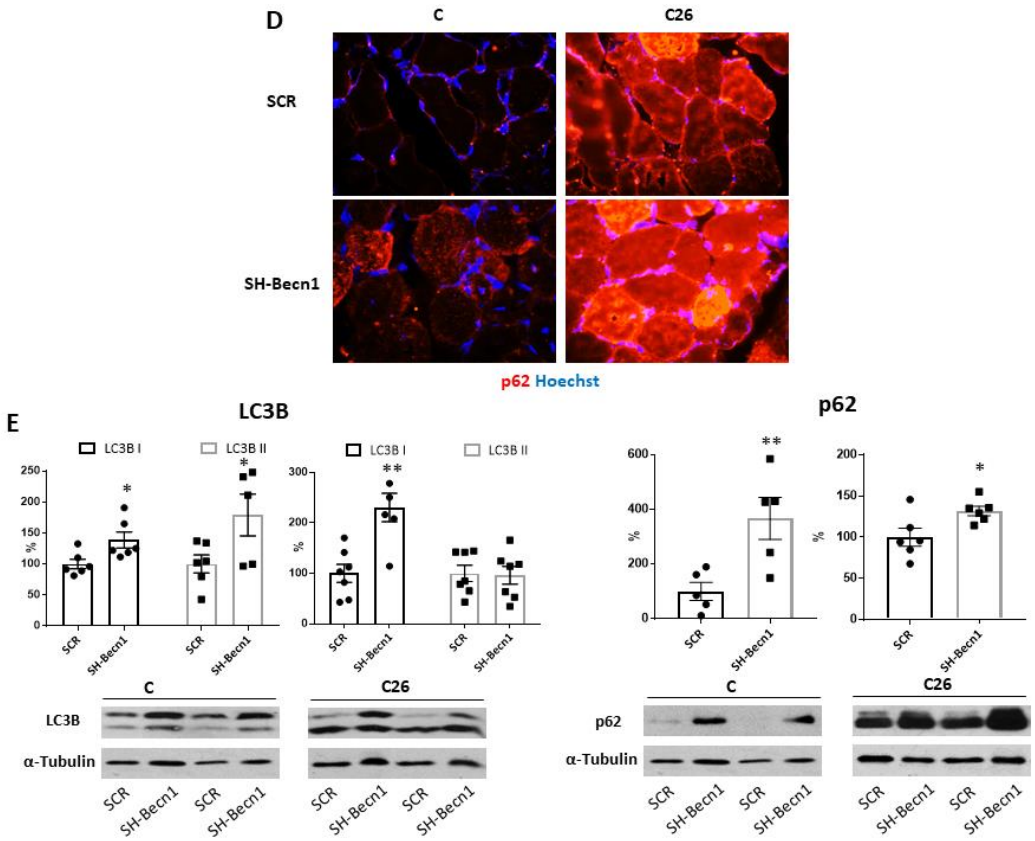


Fig. 1 *Muscle specific autophagy inhibition does not spare cancer-induced wasting. (**A**) Beclin-1 knock-down in the tibialis anterior (TA) muscle of control (C, n=6) and C26-bearing male mice (C26, n=7). (**B**) TA and gastrocnemius (GSN) mass and (**C**) TA fiber cross section area (CSA) in the above-mentioned groups. (**D**) Immunofluorescence images of TA muscle sections stained for p62 (red) and nuclei (blue). (**E**) LC3B and p62 western blotting analyses in TA homogenates. * $p < 0.05$ vs SCR; ** $p < 0.01$ vs SCR.*

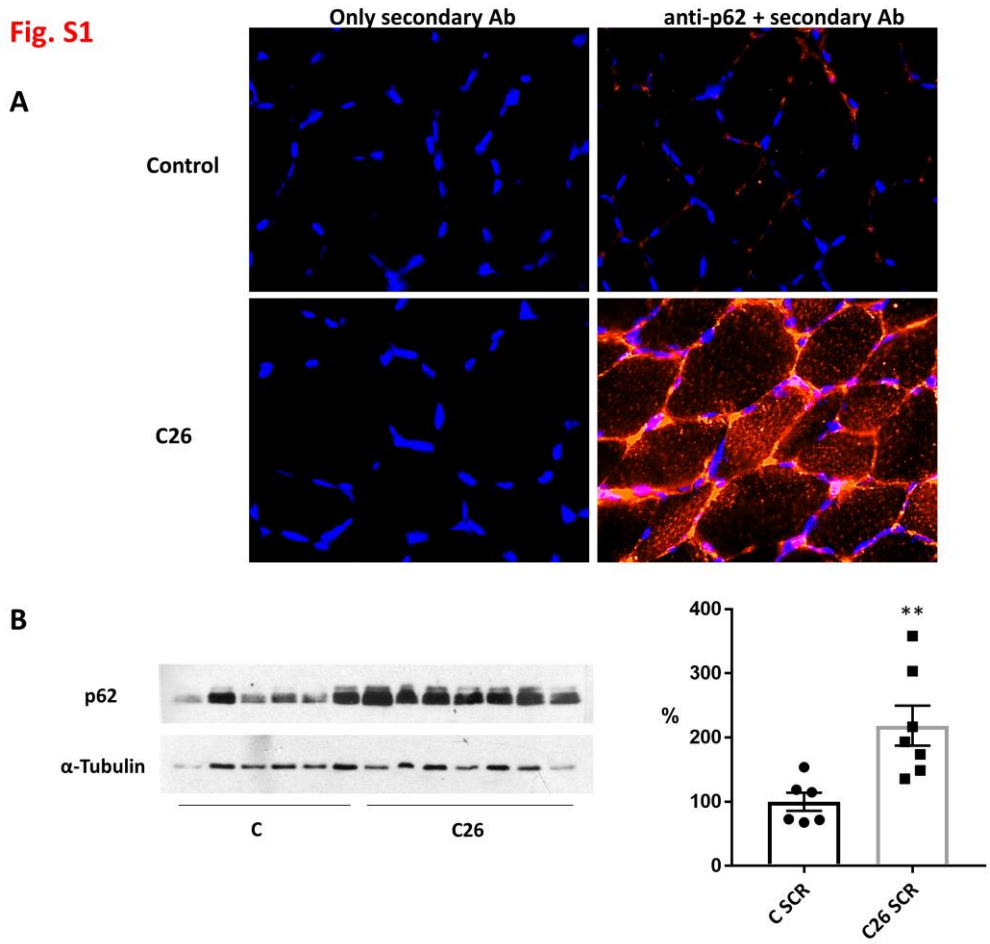


Fig. S1 *(**A**) Immunofluorescence images of TA muscle sections from Control and C26-bearing mice stained with the secondary fluorescent antibody (red) or primary anti-p62 antibody + secondary and nuclear stain (blue). (**E**) p62 western blotting analyses in scramble (SCR) TA homogenates. ** $p < 0.01$ vs C SCR.*

However, beclin-1 knockdown did not result in improved TA fiber cross section area (CSA; Fig. 1C) and induced p62 accumulation in the muscle of both control and tumor-bearing (TB) animals, in the latter going beyond the already increased expression (Fig.1D). Morphological data were confirmed biochemically (Fig. 1E), showing that beclin-1 loss-of-function prompted an abnormal p62 increase and beclin-1-independent LC3B accumulation, likely due to a reduced autophagy flux, as previously suggested [13]. Similar results were obtained in a different experimental setting, by blocking autophagy through electroporation of a vector harboring a non-phosphorylatable BCL2 AAA knock-in mutant (see [14] for details). In this experiment, TA mass was increased by BCL2

AAA expression in control mice (151 ± 12 vs 178 ± 20 mg % i.b.w., p= 0.018) whereas no effect was observed in TB mice (112 ± 27 vs 107 ± 31 mg % i.b.w.). Fiber CSA analysis showed no differences upon BCL2 AAA expression. On the whole, although cancer-induced muscle wasting is associated with increased autophagy, tissue-specific inhibition seems unable to counteract the depletion.

Muscle wasting is improved by formoterol while maintaining autophagy.

Changing perspective by focusing on anti-wasting treatments, autophagy was measured in TB mice treated with formoterol, a selective β2-agonist that effectively counteracted cachexia in both TB mice and cancer patients [15,16]. Consistently, in mice bearing the C26 tumor, formoterol counteracted the loss of body weight, muscle mass and strength, without protecting from adipose wasting (Fig. 2A). In agreement with previous observations (Penna et al., 2013), Beclin-1 and LC3B-II levels increased in C26 TB mice (Figure 2B) while treatment with formoterol only moderately reduced LC3B-II levels (Figure 2B), not allowing to draw conclusions on formoterol activity on the autophagy flux. An autophagy flux study was then performed in TB mice at early cachexia stages, treating systemically for 2 days with colchicine, in order to avoid premature animal death (see Penna et al., 2013). The results showed that the protection against muscle wasting exerted in the C26 hosts by formoterol was not associated with reduced autophagy flux (Fig. 2C).

Fig. 2

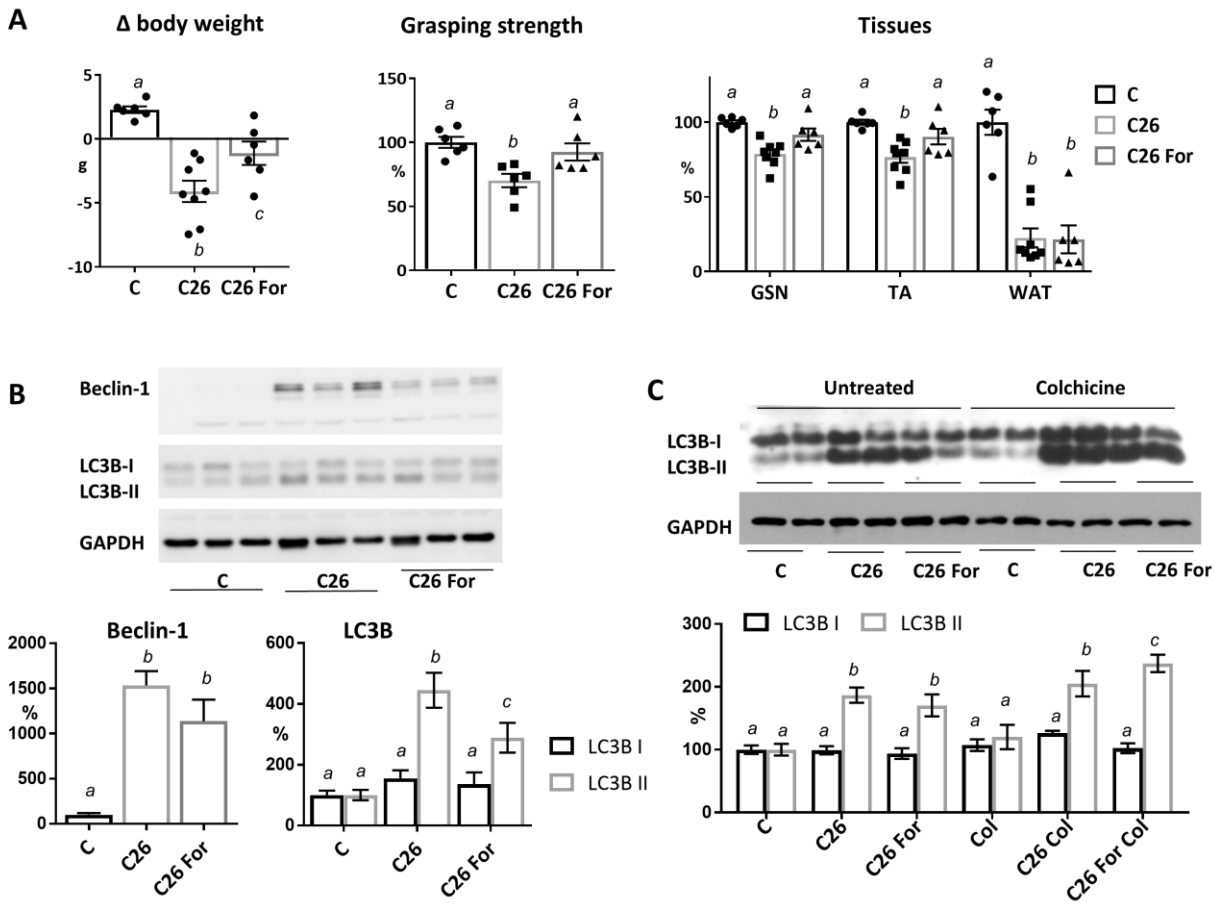


Fig. 2 *Formoterol counteracts muscle wasting while maintaining autophagy flux. (**A**) Body weight changes at the end of the experiment, voluntary grasping strength and tissue masses (GSN: gastrocnemius; TA: tibialis anterior; WAT: gonadal white adipose tissue) in controls (C), C26-bearing male mice (C26) and C26 receiving formoterol (C26 For), n = 6 for all groups. (**B**) Beclin-1 and LC3B western blotting analyses in GSN homogenates of the above-mentioned animals. (**C**) LC3B western blotting analyses in GSN homogenates of mice receiving colchicine (Col) or vehicle for 2 days before sacrifice. Different letters indicate statistically different results. Statistical significance is set at p<0.05.*

TP53INP2-mediated autophagy exacerbates cancer-induced muscle wasting.

Provided that cancer-induced muscle wasting could not be prevented by tissue-specific autophagy inhibition and that the formoterol-mediated muscle sparing effect was not achieved by reduced autophagy flux, the next question was whether increasing the autophagy flux would impact on muscle wasting in tumor-bearing mice. Along this line, Beclin-1 overexpression was excluded since the expression was already strongly increased in the muscle of tumor-bearing mice. The choice was

to modulate TP53INP2/DOR, a positive regulator of autophagy that however was previously reported to be down-regulated in muscle wasting conditions [17]. Muscle TP53INP2 transcript levels were reduced in both C26 and LLC hosts (Fig. 3A) as well as in cachectic cancer patients (Fig. 3B). In particular, TP53INP2 levels were unchanged in non-cachectic patients, suggesting the presence of an adaptive response to cope with an excessive stress-induced autophagy.

Fig. 3

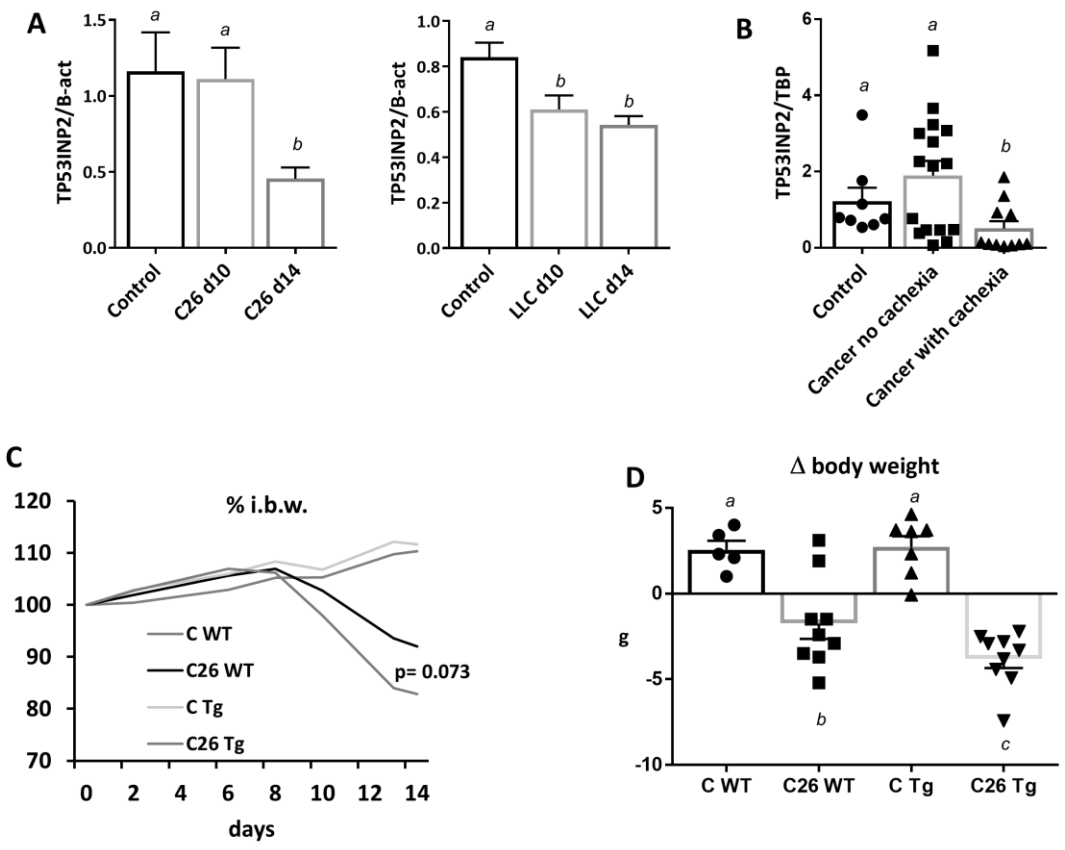


Fig. 3 *TP53INP2 is repressed in atrophic muscles and muscle overexpression exacerbates body weight loss. (**A**) TP53INP2 mRNA levels in the skeletal muscle of male mice bearing the C26 carcinoma or the LLC carcinoma (n = 6 for all groups). (**B**) TP53INP2 mRNA levels in muscle biopsies from healthy individuals (Control, n = 8), non-cachectic (n = 16) and cachectic (n = 11) cancer patients. (**C**) Body weight time course of controls (C) and C26-bearing male mice (C26) either WT (n = 5 for C and 9 for C26) or overexpressing TP53INP2 specifically in the skeletal muscle (Tg; n = 7 for C and 9 for C26). (**D**) Body weight changes at the end of the experiment in the above-mentioned animal groups. Different letters indicate statistically different results. Statistical significance is set at p<0.05.*

A gain of function experiment performed using transgenic mice overexpressing TP53INP2 in skeletal muscle (Tg mice, hereafter) showed that body weight loss was enhanced in Tg TB mice (Fig. 3 C, D), despite food intake and tumor burden remained comparable to wild-type (WT) tumor hosts (Fig. S2).

Fig. S2

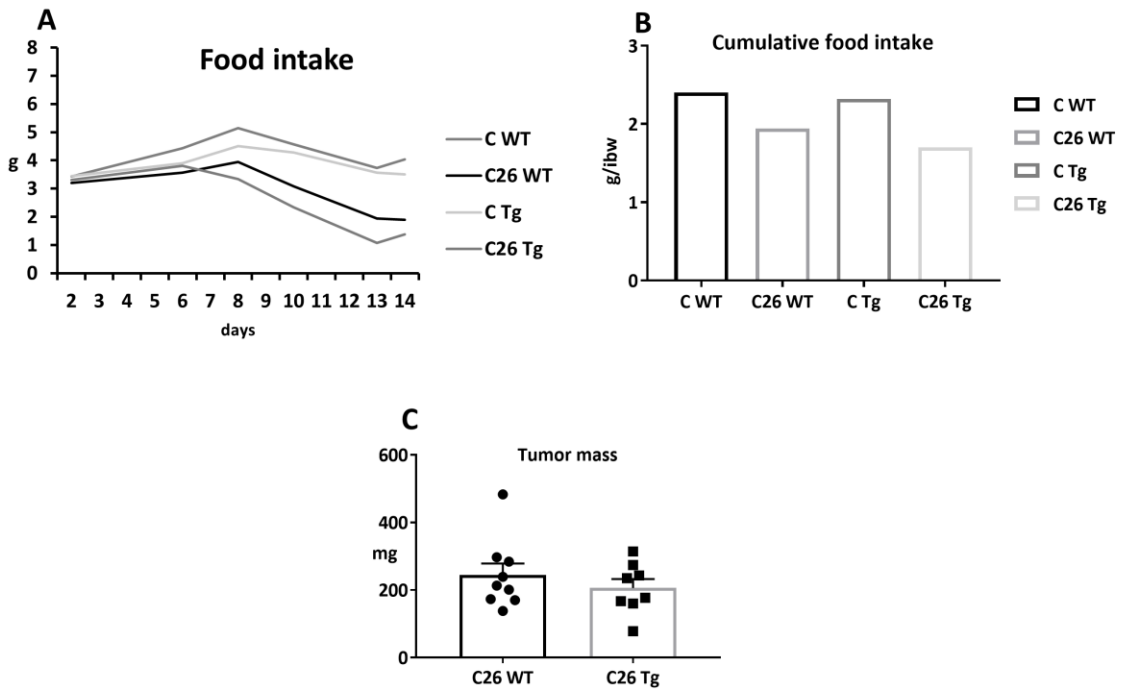


Fig. S2 *Food intake, time course (**A**) and cumulative (**B**), in controls (C) and C26-bearing mice (C26) either WT (n = 5 for C and 9 for C26) or overexpressing TP53INP2 specifically in the skeletal muscle (Tg; n = 7 for C and 9 for C26). (**C**) tumor mass in WT and Tg mice.*

The exacerbation of body wasting is likely due to excessive muscle protein catabolism. MRI analysis of body composition and the evaluation of tissue weight at necropsy showed a marked loss of lean and muscle mass, respectively, in wild-type (WT) TB mice, that worsened in Tg tumor hosts (Fig. 4 A, B). As for the fat mass, a biphasic trend was observed. In the early stages Tg TB mice

showed an accumulation of fat that was progressively lost reaching the same WT levels, confirmed at the endpoint by the WAT mass (Fig. 4 C, D).

Fig. 4

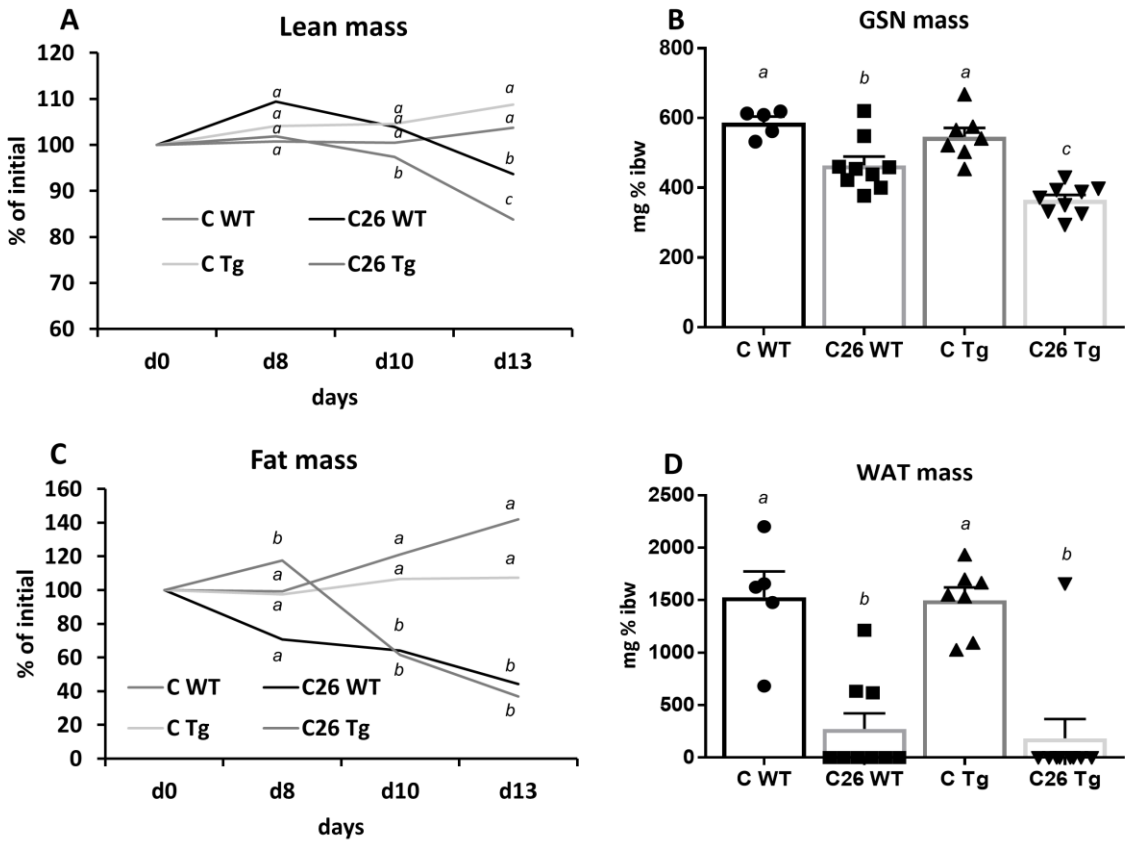


Fig. 4 *TP53INP2 overexpression worsens tumor-induced muscle though not adipose loss. Lean (**A**) and fat (**C**) % mass time course of controls (C) and C26-bearing mice (C26) either WT (n = 5 for C and 9 for C26) or overexpressing TP53INP2 specifically in the skeletal muscle (Tg; n = 7 for C and 9 for C26). Gastrocnemius (GSN; **B**) and gonadal white adipose tissue (WAT; **D**) mass expressed in mg x 100 g of initial body weight. Different letters indicate statistically different results. Statistical significance is set at p<0.05.*

TP53INP2 overexpression resulted in a trend towards increased cancer-induced muscle loss also in female mice, at both early and late stages of cachexia (Fig. S3).

Fig. S3

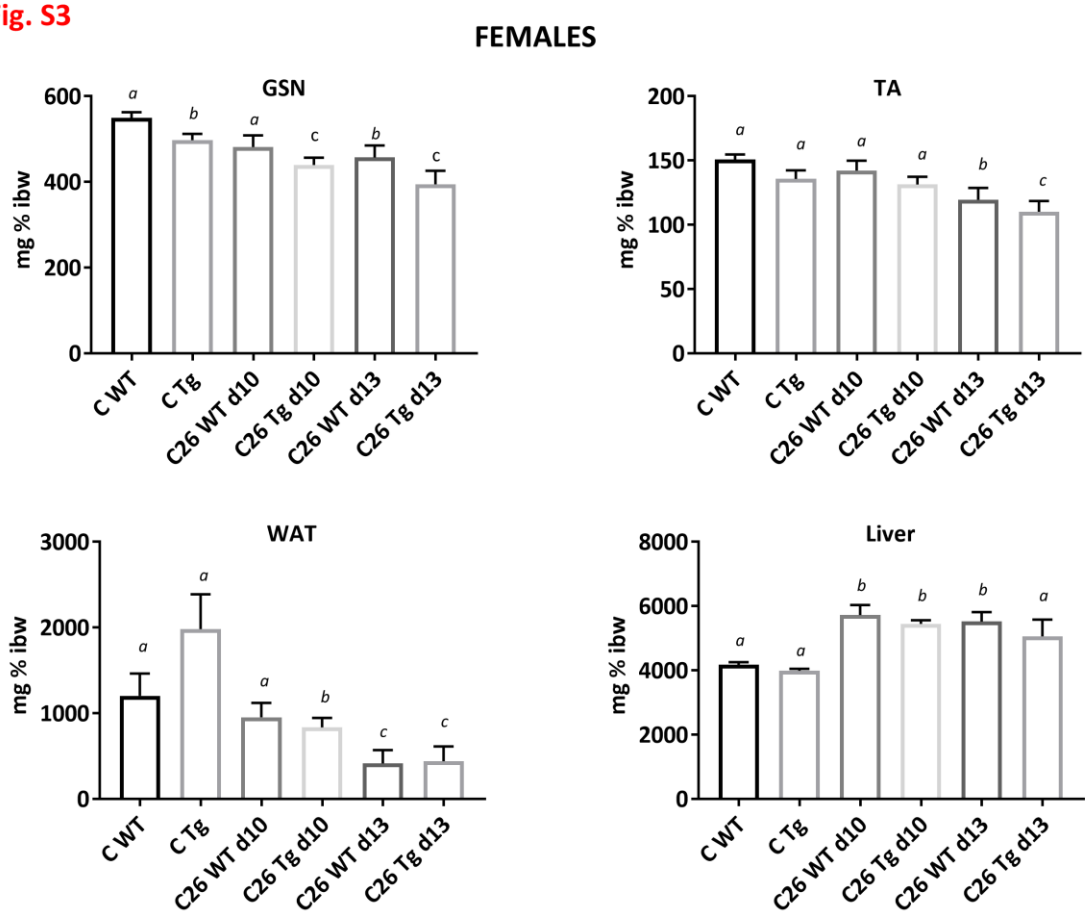


Fig. S3 *Gastrocnemius (GSN), tibialis anterior (TA), gonadal white adipose tissue (WAT) and liver mass expressed in mg x 100 g of initial body weight and TA fiber cross section area (CSA) in female (n= 6-7) controls (C) and C26-bearing mice (C26) either WT or overexpressing TP53INP2 specifically in the skeletal muscle. C26 mice were sacrificed either after 10 days (d10) or 13 days (d13) of tumor growth. Different letters indicate statistically different results. Statistical significance is set at p<0.05.*

To understand the molecular alterations associated with muscle wasting in WT and Tg TB-mice, the expression of relevant genes was assayed (Fig. 5).

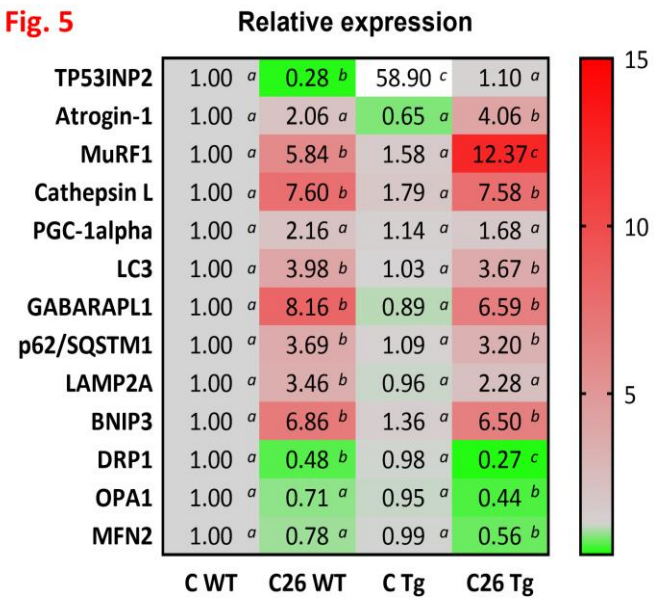


Fig. 5 *TP53INP2 overexpression exacerbates atrogene induction and mitochondrial dynamic suppression. Gene expression in the tibialis anterior of controls (C) and C26-bearing mice (C26) either WT (n = 5 for C and 9 for C26) or overexpressing TP53INP2 specifically in the skeletal muscle (Tg; n = 7 for C and 9 for C26).The expressed values are relative to C WT mice (set to 1 and grey color). Red denotes gene induction and green gene repression. Different letters indicate statistically different results. Statistical significance is set at p<0.05.*

Several atrogenes were induced in WT TB mice, atrogin-1 and MuRF1 being further increased in TP53INP2 TB mice, suggesting that both the proteasomal and the autophagic degradation contribute to the increased muscle protein catabolism. Autophagy was reported to be strongly induced in cachectic muscles [5] and in keeping with those data an enhanced expression of autophagy genes (LC3, GABARAPL1, p62, LAMP2A, BNIP3) was detected in the muscle of WT C26 hosts. In TP53INP2 TB mice transcript levels for all the assayed genes were comparable to WT C26 hosts, despite the role played by TP53INP2 in autophagic degradation, implying that TP53INP2-induced catabolism is not mediated by transcriptional regulation. Beyond bulk or selective autophagy, mitochondrial dynamics modulates muscle wasting as well [12,18]. In the present study, BNIP3 mRNA was similarly induced in WT and Tg TB mice, whereas both mitochondrial fission (DRP1) and fusion (OPA1 and MFN2) related gene expression was

suppressed in WT and even more severely in TP53INP2 TB mice. On the contrary, the mitochondrial biogenesis regulator PGC-1α transcript levels were unchanged.

Depression of muscle mitochondrial function in C26-bearing mice.

The association between autophagy and the repression of mitochondrial dynamics genes, prompted us to analyze mitochondrial function. Ex-vivo mitochondrial respiration in permeabilized EDL fiber bundles was severely impaired in TB mice irrespective of TP53INP2 overexpression (Fig. 6A). Respiratory complexes I and II showed a similar decrease in muscles from TB mice (Figure 6A). Such a reduction in mitochondrial respiration might be explained by mitophagy, as suggested by the increased BNIP3-mitochondria (SDHa) co-localization in both WT and TP53INP2 TB mice compared to control conditions (Fig. 6B and S4). Consistently with increased bulk autophagy, LC3B-II levels were increased in the cytosol from the muscle of TB mice (Fig. 6C), the more so in the mitochondrial fraction, suggesting a marked activation of mitophagy (Fig. 6C). SDHa protein content remained unaltered in wild-type TB mice with respect to control values, while a mild reduction was detected in the muscles of TP53INP2 TB mice, suggesting that TP53INP2 could promote an increased autophagy flux, allowing to speed mitochondrial clearance (Fig. 6D).

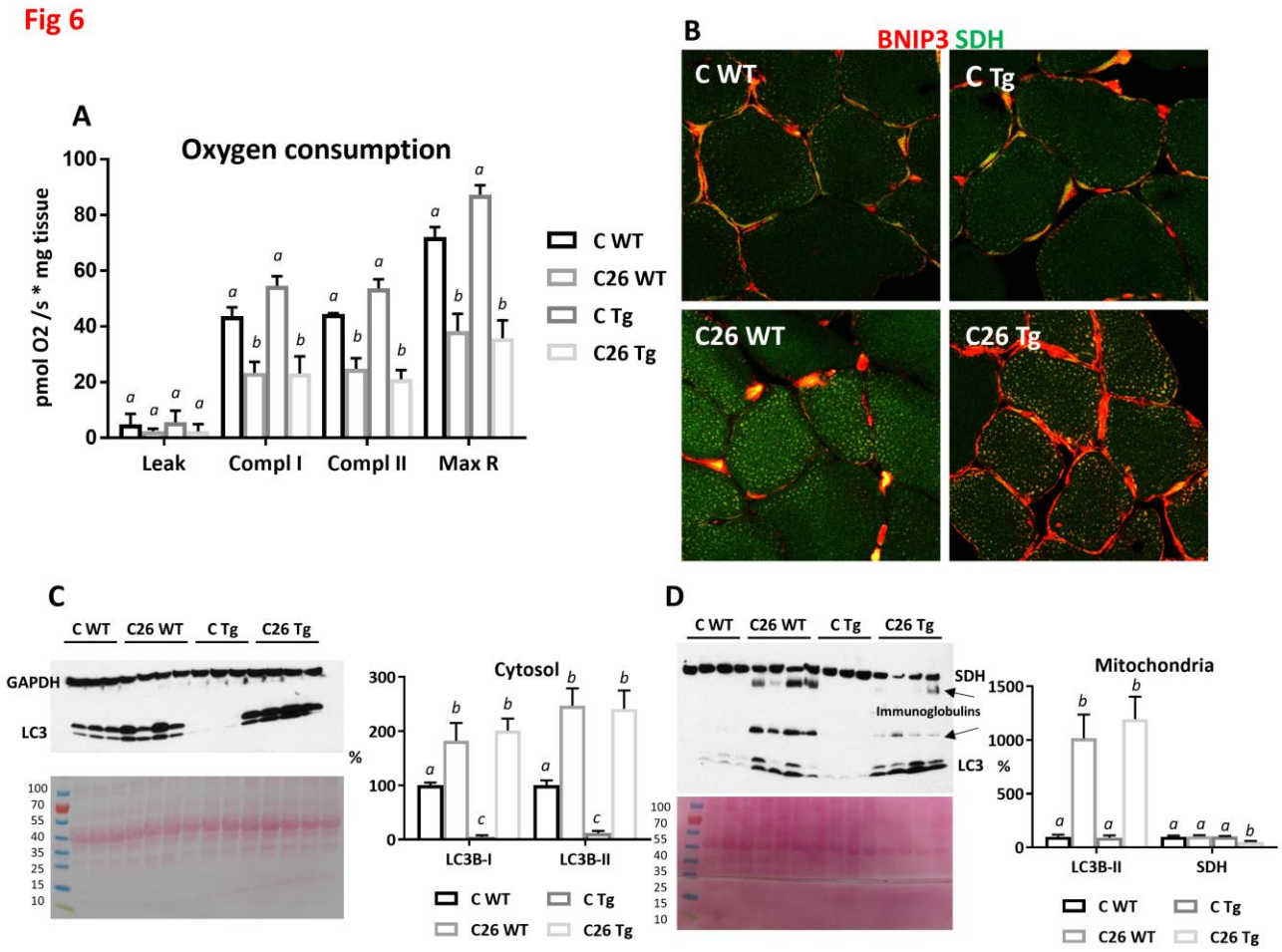


Fig. 6 *C26 tumor growth impairs muscle mitochondrial function through mitophagy. (**A**) Ex-vivo mitochondrial respiration in permeabilized extensor digitorum longus (EDL) fiber bundles of controls (C) and C26-bearing mice (C26) either WT (n = 5 for C and 9 for C26) or overexpressing TP53INP2 specifically in the skeletal muscle (Tg; n = 7 for C and 9 for C26). (**B**) Immunofluorescence images of TA muscle sections stained for BNIP3 (red) and SDH (green). Western blotting performed on cytosolic (**C**) or mitochondrial (**D**) enriched gastrocnemius (GSN) muscle fractions. Different letters indicate statistically different results. Statistical significance is set at p<0.05.*

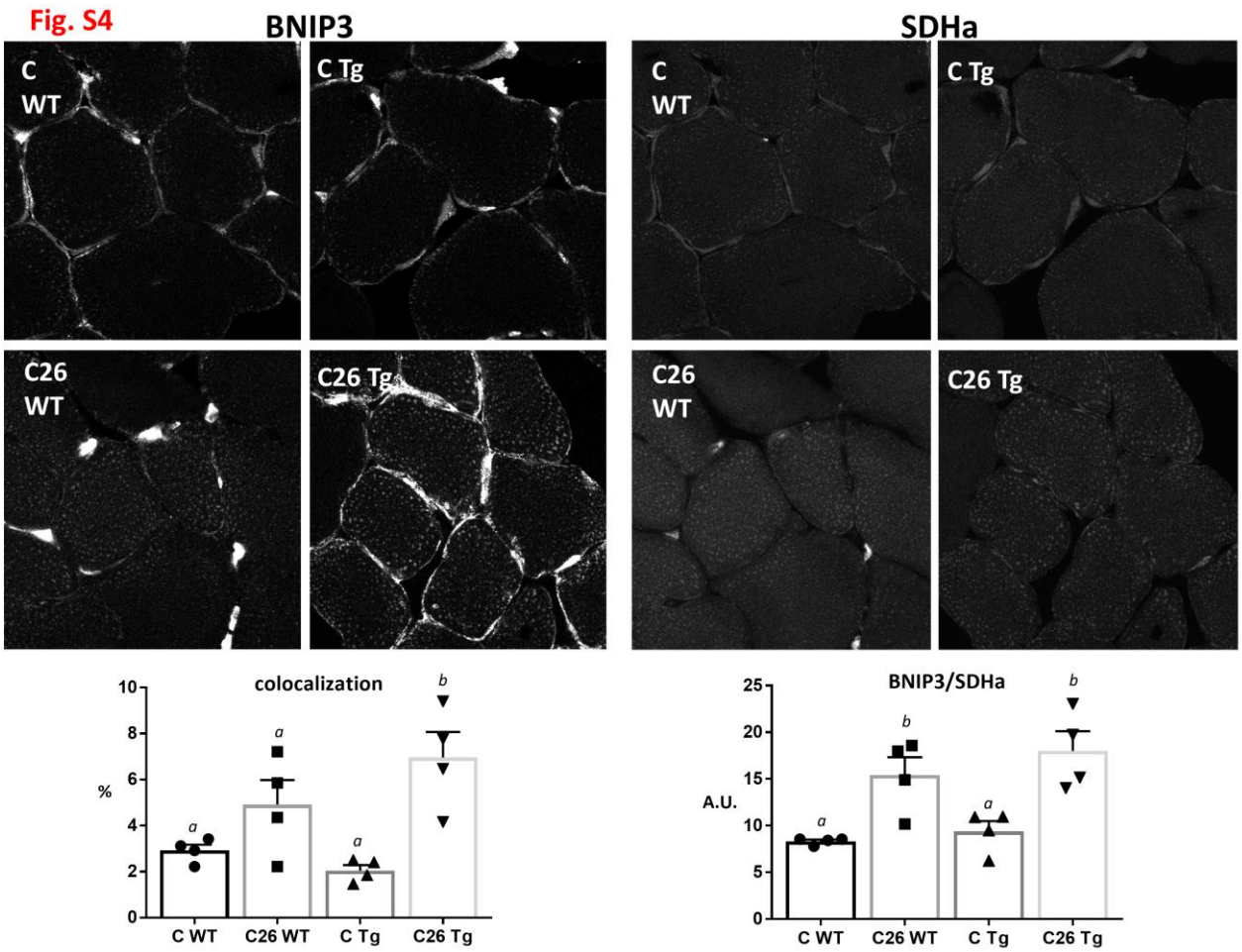


Fig. S4 *Single immunofluorescence image stacks of TA muscle sections stained for BNIP3 (left) or SDHa (right) reported in figure 6. Colocalization was quantified (n=4) using the ZEN software (Carl Zeiss MicroImaging) with fixed scatterplot crosshairs. The ratio between BNIP3 and SDHa signal intensity derives from the arbitrary fluorescence units measured with the ZEN software. Different letters indicate statistically different results. Statistical significance is set at $p<0.05$.*

Discussion

The present study was aimed to clarify the role of autophagy in cancer-induced muscle wasting, in the attempt to spare muscle mass through the modulation of muscle proteolysis. The results show that, despite autophagy is indubitably induced in the skeletal muscle of tumor hosts in a short experimental setting, its partial blockade by genetic manipulation does not improve tissue wasting. The explanations for such inefficacy might be various. First, given that beclin-1 knock-down was

not complete, the tendency to increased TA mass in tumor-bearing mouse suggests that a complete beclin-1 silencing/inhibition might turn in a consistent muscle sparing. Second, autophagy could be relevant in the short-term atrophy induction observed in the C26 model, followed by differential adaptations in the long term, vanishing the effects of blocking autophagy. However, we recently set-up a more 'chronic' model by treating C26-bearing mice with chemotherapy [19]. In that condition, although a flux experiment was not performed, the alterations of autophagy-related proteins are exacerbated, suggesting that the acute boost progresses into a chronic state of autophagy impairment. Third, autophagy is activated in the skeletal muscle upon wasting stimuli in parallel with other proteolytic systems, namely the calpains and the proteasome [2]. As for the latter, a tight crosstalk with autophagy is becoming evident in the last years [20] and a compensatory proteasome-mediated protein hypercatabolism potentially occurs as a consequence of autophagy blockade. Such a hypothesis was previously demonstrated in colon cancer cells [21], although convincing evidence in the skeletal muscle is lacking. In this regard, previous data reported that either proteasome [4] or calpain [22] inhibition do not improve cancer-induced muscle wasting, in agreement with the idea that targeting a specific proteolytic system likely leads to compensatory activation of the others, vanishing treatment effectiveness. The other way round, simultaneously blocking more than one proteolytic system likely results in potential cumulative side effects, considering the reported accumulation of p62 upon beclin-1 knock-down, although a phase 1 clinical trial using both proteasome and autophagy inhibitors was reported in patients with relapsed/refractory myeloma [23].

While blocking autophagy does not exert beneficial effect on cancer-induced muscle wasting, the restoration of normal muscle mass achieved by formoterol is associated with the preservation of the autophagy flux, supporting the idea that direct blockade of protein degradation should not be pursued. Few studies tried to uncover the mechanism underlying formoterol effect on muscle proteolysis in both healthy [24] and wasting (cancer-related) conditions [25,26]. The results of these

studies show that formoterol treatment was associated with proteasome inhibition in the skeletal muscle, with beneficial effect on autophagy static markers. The present data, demonstrating that autophagy flux is maintained upon formoterol administration suggest that autophagy is required to maintain muscle mass even in tumor-bearing animals. Indeed, given the positive effect exerted by formoterol on muscle mass and strength, we speculate that a preserved, although not exaggerated, autophagy flux is crucial in the maintenance of muscle function. Along this line, it is very likely that blocking protein catabolism could improve muscle mass but not necessarily muscle quality. Indeed, p62 accumulation in the muscle of TB mice upon autophagy blockade likely reflects an engulfment of proteins and organelles tagged for degradation, that arguably contributes to reduced muscle function. Moreover, colchicine, a highly toxic microtubule disorganizer that impairs autophagy, leads to sudden death of tumor-bearing animals [5]. This observation suggests that in parallel to the drug toxicicy, autophagy blockade in wasted anorectic individuals potentially results in a dangerous systemic energy deficit.

Consistently, survival of tumor-bearing mice is not impaired when autophagy is induced in the skeletal muscle by TP53INP2 overexpression, at least in the experimental settings adopted in this study, despite muscle protein depletion is exacerbated. Previous data suggested that TP53INP2 repression was part of an adaptive mechanism aimed at preserving muscle mass upon insulin deficiency or insulin resistance [10,17]. Merging these latter information with the present results, showing that TP53INP2 expression is reduced in both tumor-bearing mice and cancer patients, the existence of a regulatory mechanism that suppresses TP53INP2 when excessive proteolysis occurs can be proposed. The sensor for such mechanism if far from being identified and it is still unknown whether the trigger is the catabolic condition or the energy deficiency. The observation that muscle TP53INP2 expression increases when shifting from a diet providing the 18% to another providing the 80% of energy from fat [27] suggests the presence of a control based on nutrient availability, leading to TP53INP2 activation under substrate abundance and suppression upon nutrient shortage.

Since TP53INP2 is an important player in basal autophagy [28,29], that is in charge of degrading damaged organelles and aggregated and/or misfolded proteins, its down-regulation in wasted muscles might predict poor muscle quality despite excessive unselective stress-induced degradation. Cargo selectivity is an important feature of basal autophagy, a crucial quality control mechanism important for the maintenance of cell and tissue homeostasis [30]. From a speculative standpoint, TP53INP2 might be part of a protein/organelle quality control system, activated only in the presence of substrate abundance, shifting the balance towards improved quality in spite of reduced energy availability. Consistently, in fasted TP53INP2 Tg mice, muscle proteolysis is enhanced while high mitochondrial efficiency occurs in normal feeding conditions.

The present results highlight the marked impairment of mitochondrial function in the muscle of cachectic TB mice. Whether reduced mitochondrial respiration is a cause or a consequence of muscle protein hypercatabolism is still unclear. Both conditions potentially occur: modulation of mitochondrial dynamics triggers muscle wasting [12,18] while proteasome and autophagy activation might promote mitophagy [31]. A recent study demonstrates that mitochondrial damage and dysfunction precede the onset of muscle atrophy in LLC-bearing mice [32], suggesting that mitochondrial damage might be a driver of the wasting process in cancer cachexia. In the muscle of C26-bearing mice, the induction of genes pertaining to proteolysis, including those involved in autophagy and mitophagy, is associated with reduced expression of mitochondrial fusion-related genes, the more so in mice overexpressing TP53INP2. Both MFN2 and OPA1 deficiency were previously reported in sarcopenic muscles and the ablation of each single gene impaired mitochondrial quality control and triggered muscle atrophy [33,34]. Whether TP53INP2 directly regulates mitophagy and mitochondrial dynamics is still unknown, however, a sustained autophagy flux likely facilitates the removal of substrates targeted for degradation, including mitochondria. Future experiments stimulating mitochondrial fusion and preventing mitophagy will clarify the implications of such alterations in driving cancer-related muscle wasting.

In conclusion, the excessive autophagy induced in the muscle of TB mice in parallel to increased UPS activation causes the degradation of both proteins and mitochondria. Considering that bulk autophagy inhibition is not a viable strategy, targeting mitophagy and promoting mitochondrial quality/efficiency could represent a future therapeutic strategy aimed at supporting muscle function and possibly muscle mass, when coupled to anabolic/anticatabolic drugs such as formoterol.

Materials and methods

Animals and experimental design

All animal experiments were performed in compliance with guidelines established by the Barcelona Science Park's Committee on Animal Care, or with the Italian Ministry of Health Guidelines and the Policy on Humane Care and Use of Laboratory Animals (NRC 2011). Mice were maintained on a regular dark-light cycle (light from 8AM to 8PM), with free access to food and water during the whole experimental period.

Mouse strains

Cancer cachexia was induced in 6 week old males and females Balb/c mice (for C26 tumor) or 6 week old male C57BL/6 mice (for LLC tumor) obtained from Charles River Laboratories.

The transgenic mouse line (Tg) overexpressing TP53INP2 in skeletal muscle under the control of the Myosin-Light Chain 1 promoter/enhancer was generated in a C57BL/6 genetic background as described before [10]. For modeling cancer-induce cachexia, ~~beyond injecting LLC cells,~~ Tg mice were backcrossed with Balb/c mice in order to obtain successful C26 cell engraftment and a reproducible cachectic phenotype. Based on previously published data [35] the F2 generation of C57BL/6J mice backcrossed to Balb/c strain is permissive for C26 growth, and is comparable for induction of body weight loss, as compared to pure BALB/c mice in both male and female animals.

Cancer cachexia induction

For C26 experiments, mice were randomized and divided into two groups, namely controls and tumor bearers. Tumor bearing (C26) mice were inoculated subcutaneously in the back with $5x10^5$ Colon26 (C26) cells. C26 cells were maintained in vitro in DMEM (Invitrogen) supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 100 µg/ml sodium pyruvate, 2 mM L-glutamine, at 37°C in a humidified atmosphere of 5% CO2 in air. The day of tumor implantation, cells were trypsinized, resuspended in sterile saline, and injected. For LLC experiments, tumor-bearing mice were inoculated intramuscularly with 5×10^5 LLC cells obtained from exponential growth. Animal weight and food intake were recorded every other day at the beginning of the experiment or daily once the mice started to lose weight. Control and tumor-bearing mice were sacrificed under anesthesia 14 days after cell implantation. Several muscles and tissues were rapidly excised, weighed, frozen in melting isopentane cooled with liquid nitrogen and stored at -80°C.

Genetic autophagy inhibition

Four distinct short hairpin RNA sequences targeting mouse Becn1 gene were tested in C2C12 myoblasts for knock-down efficiency. The sequences were cloned in the pGFP-V-RS plasmid (Origene). The GI357932 sh-RNA showed the highest Beclin-1 knock-down efficiency (insert sequence: 5'-CACCATGCAGGTGAGCTTCGTGTGCCAGC-3'). A plasmid harboring a scrambled (SCR) sequence was used as control for the contralateral leg. As an alternative to Becn1 silencing, a BCL-2 triple mutant (Thr69Ala, Ser70Ala e Ser84Ala) was overexpressed in order to inhibit Beclin-1 activation (the plasmid was kindly gifted by Beth Levine, see [36]). In vivo transfection was performed by intramuscular injection of plasmids (20 µg) in the *tibialis anterior* muscles followed by electroporation using an ECM830 apparatus (BTX) as previously described [37].

Formoterol administration and autophagy flux

C26-bearing mice were subdivided in two groups, intraperitoneally treated daily either with saline or with formoterol at 1 µg/g of body weight, starting the day after C26 cell injection until day 14 of

tumor growth. For the subsequent in vivo autophagy flux experiment, the mice from each group were further subdivided in two and administered either 0.4 µg/g/day of colchicine or saline for 2 days before sacrifice (from day 10 to day 12 of tumor growth in order to avoid animal death in end-stage cachectic mice).

Body composition and grip strength

Body composition was evaluated in living awake, conscious (restrained but not anesthetized) animals at given time points using a magnetic resonance whole-body composition analyzer (EchoMRI, [38]). Lean mass, fat mass and total water mass were determined based on radio pulse emission properties able to differentiate between distinct tissue types.

Mouse skeletal muscle strength was assessed by the grip-strength test using a Panlab/Harvard Apparatus device [39]. At least three measurements were taken per mouse on both baseline and test days, and the results were averaged for analysis.

Human skeletal muscle biopsies

The cDNA used for TP53INP2 analysis in the rectus abdominis muscle from control and cancer (either cachectic or not) patients was obtained from a previous study performed by our group [7]. Patient charachteristics are reported in the above-mentioned study; they were considered cachectic according to the international consensus definition on cancer cachexia ([40]; body weight loss > 5% in the previous 6 months).

Protein extraction and western blotting

Total protein extracts were obtained by homogenization in RIPA buffer followed by clarification through centrifugation at 15.000 x *g*. Mitochondrial enriched fractions were obtained by homogenization in 250 mM sucrose, 50 mM KCl, 5 mM EDTA, 5 mM $MgCl_2$, 1 mM sodium pyrophosphate, pH 7.4 followed by two centrifugation steps at 740 x *g* in order to remove the unsoluble fraction. The mitochondria were pelletted by two centrifugation steps at 10,000 x *g* and the supernatant was collected as cytoplasmic fraction. Protein concentration was quantified using

the bicinchoninic acid (BCA) method (Thermo Scientific Pierce) with bovine serum albumin (BSA) as standard. The samples were resolved in 10%, 12.5% or 15% acrylamide gels for SDS–PAGE and were then transferred onto PVDF Transfer Membranes (Millipore) at 350 mA for 1 hour 30 minutes on ice. The following antibodies were used: Beclin-1, LC3B, p62 (Sigma) or SDHa (Santa Cruz). GAPDH or α-tubulin (Sigma) antibodies were used as a loading control. Proteins were detected by the enhanced chemiluminescence method and quantified by scanning densitometry.

Muscle histology

10 μm-thick transverse frozen sections of the *tibialis anterior* muscle were cut and stained with hematoxylin and eosin. Digital images were obtained for measuring myofiber cross sectional area (CSA) counting ~300–400 fibers per muscle section using the Image J software (http://rsb.info.nih.gov/ij/). For immunofluorescence, transverse sections were fixed in 4% paraformaldehyde and probed with the primary anti p62 (BD Bioscience), BNIP3 (Abcam) or SDHa (Santa Cruz) antibodies. Detection was performed using Alexa Fluor 555- or 488-conjugated secondary antibodies (Invitrogen). Nuclei were stained with the Hoechst 33342 fluorochrome and the images captured using an epiilluminated fluorescence microscope (Axiovert 35; Zeiss) or a confocal laser scanning microscope (LSM 800; Zeiss).

Gene expression analysis

Following the supplier's instruction, RNA from muscle was extracted using a protocol combining TRIzol reagent (Invitrogen) and RNAeasy® minikit columns (Qiagen), including DNAse I digestion to avoid potential contaminations of DNA. 1 μg of RNA was reverse-transcribed into cDNA with the SuperScript RTIII kit (Invitrogen). The expression of target genes of interest was quantified by quantitative real-time PCR (RT-PCR) and was performed using the ABI Prism 7900 HT real-time PCR device (Applied Biosystems) and the SYBR® Green PCR Master Mix or the Taqman Probes 20X (Applied Biosystems). Primers used for amplification are listed below. All

measurements were normalized to the expression of housekeeping genes ARP, β- actin and GAPDH.

Symbol	Forward	Reverse
ARP	AAGCGCGTCCTGGCATTGTCT	CCGCAGGGGCAGCAGTGGT
Atrogin1	GCAAACACTGCCACATTCTCTC	CTTGAGGGGAAAGTGAGACG
β-actin	GGTCATCACTATTGGCAACGA	GTCAGCAATGCCTGG
Bnip3	CACCTTTATCACTCTGCTGAATTCTCT	GATTTTGTTTTTCATTTCCAGTCTTTTAA
Cathepsin L	GCAGCAAGAACCTCGACCAT	GTTGTCCCGGTCTTTGGCTA
DRP-1	AGCCAATCCATCTCAAGGTTTTCTC	ATCGCCTACAGGTACTTTGGTCATTC
GABARAPL1	CACCATCCCTCCCACCAGT	GGCTTCTGCCTCATTTTCCAT
LAMP2A	CACATATGCAATGTTTTAAGGTCTGTC	TCAAGCAGTGTTTATTAATTCCAGTAAGAT
LC3B	AGCTCTTTGTTGG TGTGTAACTGTCT	TTGTCCTCACAGCTGACATGTATG
MFN2	AGAACTGGACCCGGTTACCA	CACTTCGCTGATACCCCTGA
MURF1	CATTGTGTGACTGGCGATTGT	TCTCTAGGCCACCGAGTGAGA
OPA1	TAQMAN Mm00453879_m1	
PGC-1α	GAAAGGGCCAAACAGAGAGA	GTAAATCACACGGCGCTCTT
p62/SQSTM1	CCCAGTGTCTTGGCATTCTT	AGGGAAAGCAGAGGAAGCTC

Mitochondrial respiration

Oxygen consumption was measured in permeabilized muscle fibers at 37°C by high-resolution respirometry with the Oxygraph-2k (Oroboros Instruments). A specific experiment on male mice, distinct from the previously described ones, was performed in order to process 4 mice per day (C, n=3; C26, n=5 for both WT and Tg mice). EDL muscles were excised and immediately placed in ice-cold biopsy containing preservation medium (2.77 mM CaK_2EGTA, 7.23 mM K_2EGTA, 20 mM imidazole, 20 mM taurine, 50 mM K-MES, 3 mM K_2HPO_4, 6.5 mM $MgCl_2$, 5.7 mM ATP, 15 mM phosphocreatine, and 0.5 mM DTT, pH 7.1. Muscle fiber bundles weighing around 3 mg were mechanically separated using thin tweezers and permeabilized with 50 µg/ml saponin for 30 minutes at 4°C according to the technique of Veksler et al. [41]. After rinsing of muscle bundles in respiration medium (EGTA 0.5 mM, $MgCl_2 \cdot 6H_2O$ 3mM, taurine 20 mM, KH_2PO_4 10 mM, HEPES 20 mM, BSA 1 g/l, K-lactobionate 60 mM, sucrose 110 mM, pH 7.1), the muscle fibers were transferred to the oxygraph to perform high-resolution respirometry corrected for wet weight. Resting respiration (state 4, absence of adenylates) was determined by the addition of 10 mM glutamate and 2 mM malate as the Complex I substrate supply, and state 3 respiration was then assessed by the addition of 2.5 mM ADP (Compl I). ADP control of coupled respiration and uncoupling control were examined through the addition of the protonophore carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone (FCCP; Max R); 0.5 µM rotenone and 2.5 µM antimycin A were added to inhibit Complexes I (allowing Compl II extrapolation) and III, respectively, to observe non-mitochondrial respiration (Leak). All respiration measurements were performed in duplicate for each muscle.

Expression of results and statistical analysis

Data are presented as mean ± SEM. Statistical significance was assessed by a two-tailed paired Student's t-test or One-way Anova analysis followed by Tukey's multiple comparisons test. A value of $p < 0.05$ was chosen as the limit of statistical significance.

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Competing interests

The authors declare no competing financial interests.

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