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# AUTOPHAGY AND SKELETAL MUSCLE WASTING: EFFECTS ON SATELLITE CELLS POPULATION

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# SUMMARY

Skeletal muscle represents the most abundant tissue in the human body and is one of the most important sites of metabolic control. Nitric oxide (NO), generated in skeletal muscle mostly by the neuronal NO synthases (nNOSμ), has several effects on muscle development and function [1], indeed nNOS signaling is defective in many skeletal muscle diseases contributing to muscle degeneration. Moreover NO in also relevant for mitochondria structure and function [2-3] and could be involved in the control of mitochondrial turnover [4]. Autophagy is constantly active in skeletal muscle and its role in tissue homeostasis is complex: at high levels, it can be detrimental and contribute to muscle wasting; at low levels, it can cause weakness and muscle degeneration, due to accumulation of damaged proteins and organelles and recently many studies have highlighted the importance of autophagy in the pathogenesis of muscle wasting in different types of inherited muscle disorders.

My phD project is divided in two sections: the formeris focused on the study of the relationship between the NO system, mitochondrial structure/activity and skeletal muscle wasting, paying attention on the role of autophagy and using a mouse model in which NOS1 (nNOSμ) is absent. The latter has examined the role of autophagy in myogenic precursor cells(satellite cells) and muscle development utilizing a model of skeletal muscle specific autophagy-knockout mice. The first important observation was that the deficit in NO signaling in skeletal muscle leads to alterations in mitochondrial morphology and network remodeling, associated with enhanced autophagy. NOS1-/- mice showed a disorganized mitochondrial network and. an active autophagic pathway, in tibialis anterior and diaphragm muscles of P120 NOS1-/- mice.

Then we evaluated the effects of the absence of NOS1 (nNOSμ) on skeletal muscle phenotype. The body weight and the visceral adipose tissue of NOS1-/- mice were significantly lower than wild-type control, moreover the same occurred with the relative muscle mass (normalized to body weight), demonstrated to be reduced compared with control. The overall morphology of the tibialis anterior and diaphragm muscle in P120 NOS1-/- mice was normal, without pathological features of necrosis, macrophage infiltration and centronucleated fibers. By contrast, laminin staining of tibialis anterior and diaphragm, used to identify individual muscle fibers, revealed a significant decrease in the mean CSA of tibialis anterior and diaphragm sections in P120 NOS1-/- mice when compared with control. The examination of multiple time points was then carried out in

order to establish a possible link between the changes in mitochondrial homeostasis and the reduction in muscle size. Furthermore, the observation that at different time point NOS1-/- and control mice expressed similar levels of transcripts encoding the classical atrogenes atrogin-1 and MuRF1, indicates that the atrophy pathways do not play a key role in the development of NOS1-/- muscles.

So far my data demonstrate that an altered NO signaling leads to mitochondrial dysfunction resulting in enhanced autophagy and reduced muscle growth, however not associated with atrophy induction. Also in my model the absence of NOS1 altered mitochondrial homeostasis and autophagic pathway in myogenic precursor cells with a decrease in the number of myonuclei per fibres and impaired muscle development at early stages of growth. An altered NO signaling in myogenic precursor cells led to mitochondrial network fragmentation and increased autophagy induction resulting in significant defects in myogenesis process both in vivo and in vitro, decreasing muscle development [5].

However, autophagy is essential to maintain muscle mass, but its role on regenerating population and its impacton muscle growth and development has not been evaluated yet. Starting form conclusion in the second part of my PhD project I focalized my attention on the role of autophagy specifically on satellite cells population, generating a transgenic mice in which autophagy is selectively inhibited in satellite cells and studying their impact on muscle growth.

To generate the mice we crossed Atg7-floxed mice with a transgenic line expressing Cre recombinase under the control of the Pax7 promoter in order to generate satellite cells lacking the autophagy gene Atg7 [6]. Measuring body weight after birth we detected very significant differences between control and Ag7 -/- mice with the last one smaller than control littermates. Normalizing all the organs weight to the body weight we demonstrated that only the skeletal muscle tissue was smaller out-of-proportion to the body weight in Atg7-/- mice, suggesting that a defective autophagy in satellite cells population causes a muscular phenotype more serious, but with some similarities withNOS1 -/- mice, in spite of opposing autophagy conditions.

To better understand the nature of the hypotrophic skeletal muscle observed in Atg7 -/- mice, skeletal muscle sections from male Atg7-/- and control mice at p21 days after birth were stained by hematoxylin and eosin (H&E) or immunostained with an anti-Laminin antibody. The overall morphology of the tibialis anterior of Atg7-/- mice was normal, without pathological features of necrosis, macrophage infiltration and centronucleated

fibers, conversely laminin staining revealed a significant decrease in the mean CSA in Atg7-/- mice when compared with control littermates. Also, we examined the mRNA levels of Myostatin and MuRF1 and Atrogin for atrophic pathway and none of them were significantly changed, demonstrating that loss autophagy does not affect this pathways. However skeletal muscle growth is dependent on the proliferative expansion of satellite cells and the balance between the proliferation of myogenic progenitors and their differentiation into muscle fibers, but was no significant difference in the average number of satellite cells in muscle. Thus, we evaluated whether loss of Atg7 could affect the proliferation of these cells and we shows a lower proliferation rate of Atg7-/- satellite cells**.**

To gain further insight into the role of autophagy on satellite cells we evaluated the myogenic cell determination on isolated myofibers after 48h of proliferation. Analyzing immunofluorescence for Pax7 and MyoD we observed that the number of MyoD+/Pax7- (cells committed to differentiated) decreased in Atg7-/- fibers. There was no significant differences in the number of MyoD+/Pax7+ (activated cells), whereas increased the number of $Pax7^{+}$/MyoD-(quiescent cells) cells was detected in Atg7-/- fibers, suggesting a delay of satellite cells to progress into myogenic lineage and the presence of more cells in quiescent state. Finally we analyzed the ability of satellite cells to fuse and differentiate. Satellite cells isolated from Atg7 -/- mice after 48h in differentiation medium showed a reduced ability to fuse and a sharp decrease in myotubes diameter associated with reduced expression of myogenic markers .

Taken together, our data suggest that autophagy is involved in the controlling of satellite cells functions. Autophagy loss of function in satellite cells impairs their proliferation rate as well as their capability to fuse and differentiate and these results explain, at least in part, because the muscles of the Atg7-/- mice are smaller than the muscles of control mice.

# INTRODUCTION

## Chapter 1 : Satellite cells and skeletal muscle

### 1.1 SKELETAL MUSCLE

Skeletal muscle tissue represents the most abundant tissue in the human body, amounting to 60% of the average weight. From a metabolic point of view it is very active and requires a constant flow of nutrients and metabolites, provided by an extensive capillary network forming an organized branching pattern throughout the fibers.

The structure of skeletal muscle is characterized by very well organized structure composed of parallel elements that can be divided in myofibrils, muscle fibers and fascicles (Fig 1).

The myofibrils are the cytoplasmic molecular machinery capable of performingmuscle contraction.They are bundled within the cytoplasm generating a multinucleated syncytium containing muscle cell or myofiber. The contraction of the myofibrils also depends on motoneuron connections that regulate the calcium required for contraction through the sarcoplasmic reticulum.

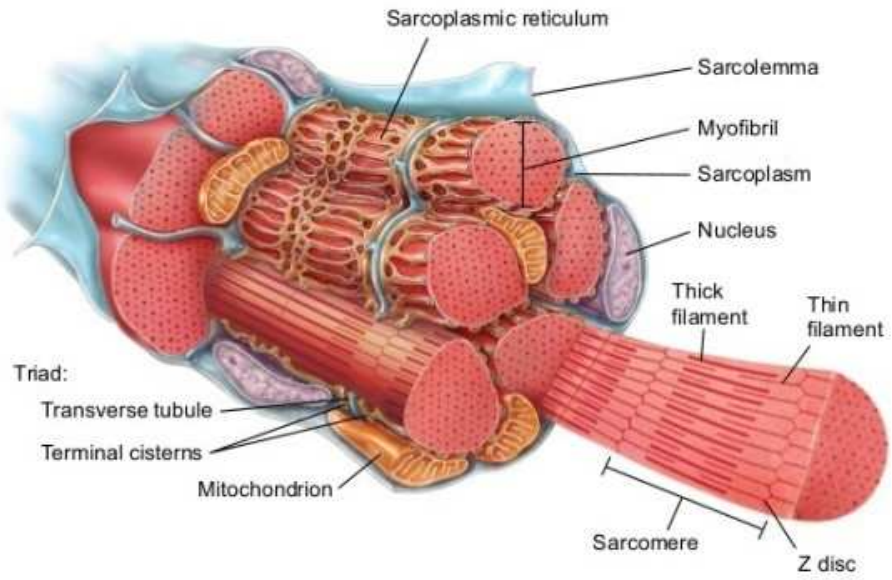



***Figure 1 Schematic representation of skeletal muscle structure***

Each myofibril is divided into segments calledsarcomeres, that are the contractile units of a muscle. Sarcomeres are composed ofthickfilamentsandthin filaments. The thin filaments are attached at one end to a Z disc, a dark stripe that marks the ends of one sarcomere and the beginning of the next and extend toward the center of the sarcomere. The thick filaments, by contrast, lie at the center of the sarcomere and overlap the thin filaments.

Myofibers constitute the parenchyma of skeletal muscle, and are bundled in a complex extracellular matrix structure which connects them to the muscle-tendon junction through three fibrous layers: the endomysium, surrounding individual myofibers, the perimysium, found over fascicles and the epimysium which cover the entire muscle.

Blood vessels are presentalong the fascicles in the perimysium, diffuseinto the endomysium, where they generatecapillaries that project around the myofibers andeach myofiber is connected to a capillary allowing the metabolic support of skeletal muscle.

Following blood vessels, also the axons of motorneurons, which transmit the synaptic signal to the myofibers terminate into neuromuscular junctions in the proximity of sarcolemma connections(triads)[7].

Skeletal muscles of adult mammalian species exhibit a remarkable capacity to adapt to physiological demands such as growth, training, and injury. The processes are largely attributed to asmall population of cells that are resident in adult skeletal muscle.

Satellite cells (SC) responsible for the maintenance and the continual production on myoblasts. Once activated, SCs will start dividing and producing muscle progenitor cells (MPCs) that will become myoblasts and fuse to the existing myofibers[8].SC pools can be replenished by muscular tissue pericytes (MPs), a population of cells that surround the endothelium of small vessels. They regulate numerous functions, such as vessel growth, stabilization, and permeability[9].They show the ability to undergo skeletal muscle myogenesis, on proper culture conditionsindeed it has been shown that cells from the vessel wall may receive signals instructing them to adopt the fate of the specific tissue, skeletal muscle in this case.

Finally, fibro/adipogenic progenitors (FAPs), presentin  a quiescence state near blood vessels, are activated in case of muscle injury. FAP cells are known to promote the differentiation of MPCs but do not have any capability to generate muscle tissue on their own; on the contrary, they appear to be the main source of the fibrotic and adipose tissues found in pathological muscles[10]. The interplay between these cell populations is

fundamental for the continuous renewal of skeletal muscle tissue, required to compensate the damage occurring in day-to-day activities; however, pathological states also require the activation of the same cells in different processes that can result in the regeneration of the tissue or the formation of a fibrotic scar.

## 1.2 SATELLITE CELLS

### 1.2.1 Morphology and location of satellite cells

Satellite cells (SCs) are the muscle stem cells responsible for longitudinal and cross-sectional postnatal growth and repair after injury providing new myonuclei when needed. Alexander Mauro [11]first discovered and namedthe satellite cell (SC) in frog skeletal muscle based on its peripheral location in the myofiber, identified between the sarcolemma and basal lamina. In terms of location, SCs have a similar position to skeletal muscle myonuclei, namely on the periphery of the myofiber but outside the sarcolemma, whilemyonuclei are just under the sarcolemma. In other words, SCs represent a differentcell population into themyofibers (figure 2).

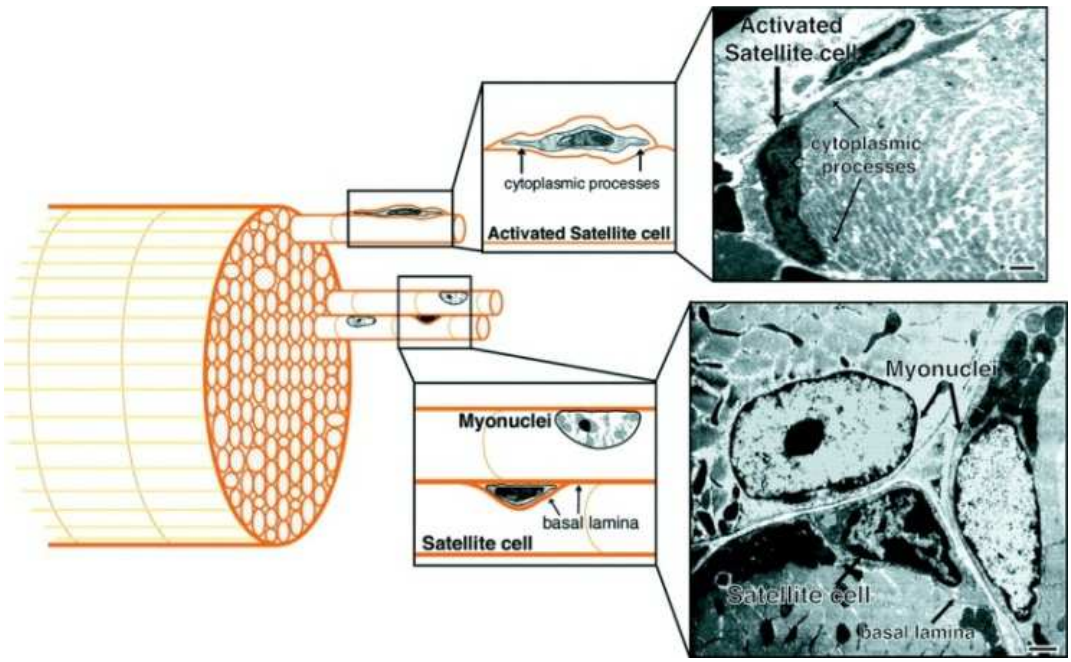



***Figure 2 Satellite cells oppupy a sublaminar position in adult skeletal muscle***

Satellite cell number depends on the species, age, and muscle fiber type . They constitute 30% of the muscle nuclei in the new bornand decrease with age to 4% in the adult and 2% in the senile (29–30 mo) mice[12].

SC distribution between muscle groups depends on their heterogeneous contentin different muscle type. An increase in satellite cell density has been demonstrated in association with the proximity of capillaries, myonuclei, and motoneuron junctions, in fact oxidative fibers, characterized by increased capillary and motoneuron density compared with glycolytic myofibers, demonstrate a five to six times greater satellite cell content[13].

Instead the decrease in the percentage of satellite cells with aging is the result of a decrease in total number of satellite cells and although the functional consequences of SC number decline are unclear, it is becoming clear that SC function changes profoundly with aging[14].

Morphologically satellite cells populationare characterized by a relatively high nuclear-to-cytoplasmic ratio with few organelles, a smaller nuclear size compared with the adjacent nucleus of the myotube, and an increasedamount of nuclear heterochromatin compared with that of the myonucleus These morphological features are consistent with the finding that satellite cells are relatively quiescent and transcriptionally less active. These features are lossfollowing activation or proliferation of the satellite cells in response to growth, remodeling, or muscle injury. After activation, the satellite cells are more easily identified, they are characterized bycytoplasmic processes that extend from one or both poles of the cell, by reduction of heterochromatin due to increased mitotic activity, by increased cytoplasmic-to-nuclear ratio, and an increase in the number of intracellular organelles[15].

### 1.2.2 Myogenic factors

Many similarities between the activation of satellite cells and somite myogenesis have supported the idea that adult muscle regeneration to a certain extent recapitulates embryonic development through analogous, but not identical, mechanisms[16-17].

Consistently with the establishment of embryonic progenitors in the myotome , the activation, proliferation and differentiation of satellite cells are processes regulated by a family of transcription factors known as myogenic regulation factors (MRFs) and among them, the basic helix–loop–helix (bHLH) transcription factors myogenic factor 5 (MYF5) and myoblast determination protein (MYOD) together with muscle-specific regulatory factor 4 (MRF4; also known as MYF6) and Myogenin are upregulated during myoblast differentiation. Together these myogenic regulatory factors transcriptionally and epigenetically determine the myogenic capacity of muscle progenitors (figure 3).

Mice lacking MyoD have quite normal muscle but express about fourfold higher levels of MYF5. In agreement also MYF5 deficient mice have normal muscle. However, mice lacking both Myf5 and MyoD are totally devoid of myoblasts and myofibres, indicating that these genes are required for the determination of myogenic precursors. [18-19]. As a downstream target of MYOD, myogenin regulates the transition from myoblasts into myocytes and myotubes and, although myogenin-knockout mice show proper compartmentalization of muscle groups, they almost completely lack myofibres and accumulate undifferentiated myoblasts[20]. Thus, MYF5 and MYOD are required for the determination of myogenic precursors and act upstream of myogenin and MRF4, which are required for terminal differentiation.

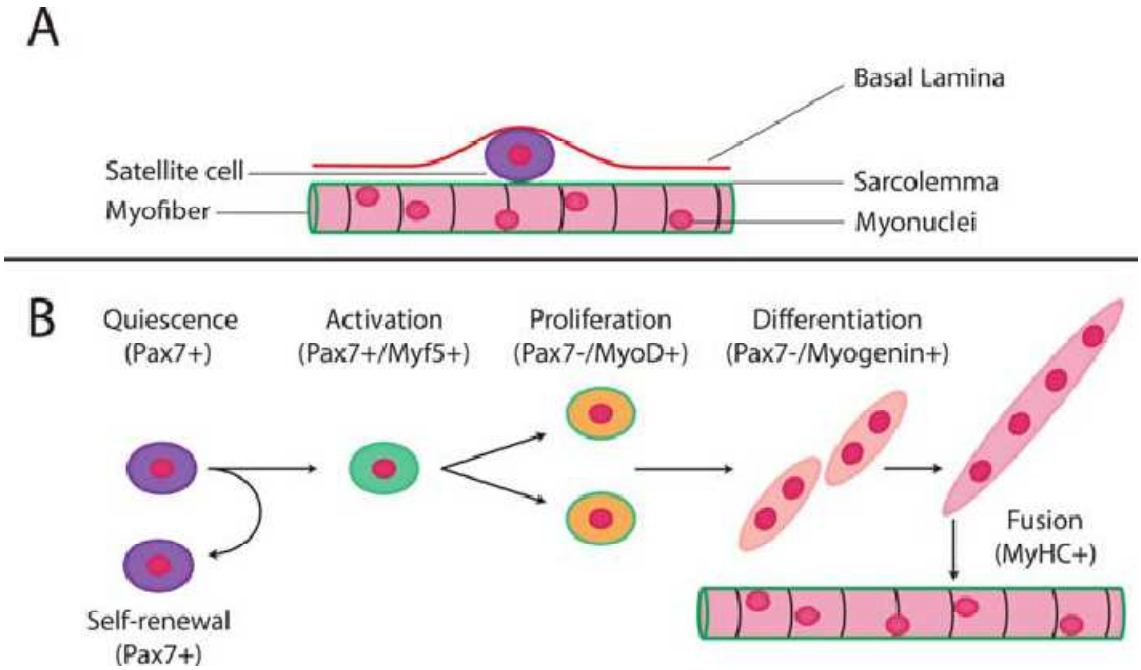



***Fig 3 Satellite cell (SC) location and function****. A) The SC is anatomically located between the myofiber basal lamina and sarcolemma. B) The relationship between gene expression and SC activation, self-renewal, proliferation, differentiation and fusion with existing myofiber.*

Somitic myogenic progenitors, that eventually give rise to satellite cells, express PAX3 and/ or PAX7 two closely related paired domain homeobox transcription factors and do not express MRFs. These progenitor $PAX3^{+}PAX7^{+}$cells upregulate MYF5 and MYOD when they enter into the myogenic differentiation programme, or remain as satellite cells without upregulating MRFs. In particular PAX3 and PAX7 play key rolein maintaining the proliferation of progenitors and preventing early differentiation.

Belonging to the PAX family of transcription factors, PAX3 and PAX7 are paralogues with conserved amino acid sequences and have almost identical sequence-specific DNA-

binding motifs. Despite this homology, PAX3 and PAX7 have overlapping but non-redundant roles in the myogenic programme.

Lineage-tracing studies suggest that $PAX3^{+}$cells contribute to embryonic myoblasts and endothelial lineage, whereas $PAX7^{+}$cells contribute to fetal myoblasts, supporting the notion that these cells represent distinct myogenic lineages[21].

PAX3 expression is abundant in the embryo then after birth it is downregulated, suggesting that it has little function in satellite cells indeed prolonged expression of PAX3, by preventing its proteosomal degradation, results in the inhibition of terminal differentiation[22]. Conversely PAX7 is expressed by all satellite cells and proliferating myoblasts but is quickly downregulated before differentiation[23].

Pax7 is involved in the specification of the myogenic identity and the maintenance of the undifferentiated state. Ectopic overexpression of PAX7 in C2C12 myoblasts and primary myoblasts suggests that PAX7 drives the expression of Myf5 but not MyoD[24].

Moreover, PAX7 has been shown to directly activate Myf5 by promoting the recruitment of the ASH2-like (ASH2L)–MLL2 histone methyltransferase complex at a regulatory element of the Myf5 coding region, indicating that PAX7 can epigenetically specify the myogenic identity of satellite cells. PAX7 expression correlates with an undifferentiated but committed myogenic state, indicating that PAX7 in the early postnatal period probably worksto prevent the transition into terminal differentiation maintaining the myogenic identity by promoting MYF5 expression. Both functions allow PAX7 to specify the satellite cell lineage. Therefore, satellite stem cells (PAX7+MYF5) in adult muscle may represent a lineage of the embryonic PAX3+PAX7+MRF– progenitors[25].

### 1.2.3 Muscle regeneration: quiescence, activation and self renewal

Muscle regeneration is characterized by different myogenic stages, namely: activation, proliferation, differentiation, and self-renewal/return to quiescence. Careful regulation of the cell cycle is essential to ensure appropriate progression through these various overlapping states. In resting adult muscles, satellite cells exist in a dormant state known as quiescence or the reversible G0 state. The ability of satellite cells to maintain quiescence is essential for the long-term conservation of the satellite cell pool [26-27]. This quiescent state is distinct from the cell cycle exit observed prior to differentiation, which allows cells to return to a proliferative state in response to injury. The quiescent state is highly regulated and represents a ‘ready’ state that is primed for activation. Microarray analyses revealed that more than 500 genes are highly upregulated in

quiescent satellite cells compared with cycling myoblasts [28-29]. These quiescence genes act in concert to prevent the early activation of quiescent satellite cells because impairments in the ability of satellite cells to maintain quiescence reduce self-renewal capacity and muscle regeneration.

Mitogenic factors liberated following injury drive quiescent satellite cells to re-enter the cell cycle[30] (Figure 4).

Recent analyses of systemic effects of muscle injury reveal an intermediate state between the G0 quiescent and activated cell states, termed G alert. It has beenshown that, following muscle injury, satellite cells from the controlateral uninjured leg are phenotypically different from classical quiescent cells. They are larger, have more intracellular ATP and higher metabolic activity than quiescent satellite cells and express a number of cell cycle genes similar to those expressed in activated satellite cells, but they do not proliferate or enter in the cell cycle.

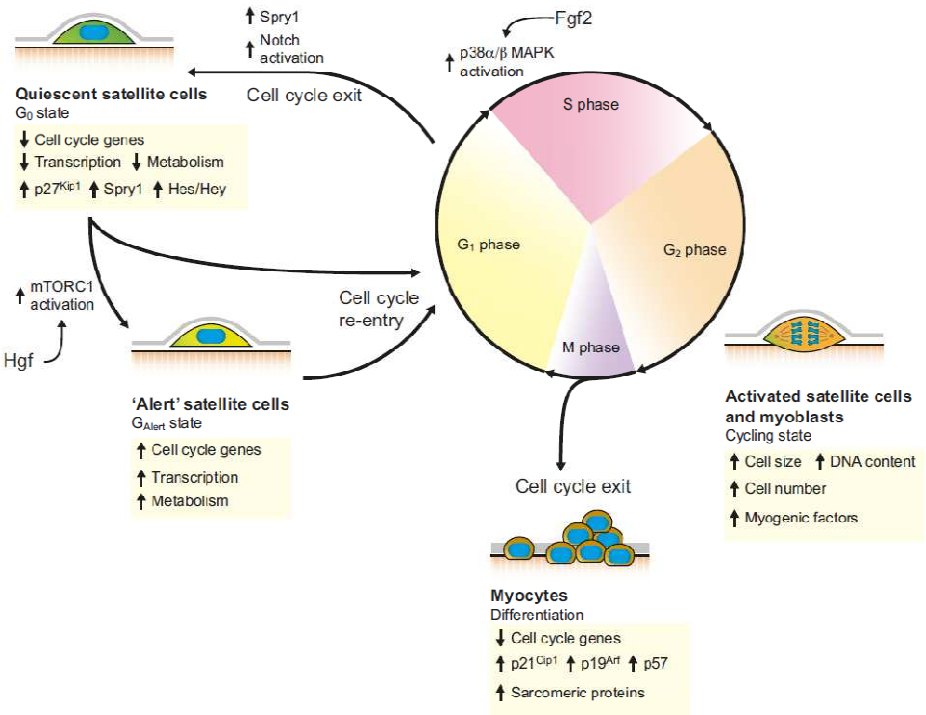



***Figure 4 Regulation of the cell cycle in satellite cells.***

This intermediate GAlert state allows satellite cells to perform their first division faster than satellite cells in the G0 state, finally satellite cells in the GAlert state have greater regenerative potential[31]. This supportsthe existence of a system-wide response mechanism that inducessatellite cells to become activated in a regenerative environment.

Damaged muscles release various growth factors that activate signaling pathways involved in satellite cell cycle entry such as insulin-like growth factor (Igf1) [32-33] and Fgf2[34].

Once activated, satellite cells can undergo differentiation (into myocytes) or return to quiescence, two processes that involve cell cycle exit.

In addition to re-entering the cell cycle, activated satellite cells canalso determine the cell fate of their daughter cells, in particular whether they self-renew or generate myogenic progenitors.

The analysis of MRF expression in proliferating satellite cells shows an asymmetric expression of determination factors, including Myf5, MyoD and Myog, in the daughter cells after satellite cell divisions. Indeed stem cell subpopulations are able to undergo both symmetric and asymmetric types of self-renewal to maintain their numbers. Asymmetric divisions generatestwo identical daughter cells to increase the satellite stem cell pool. Alternatively, satellite stem cells can perform asymmetric divisions, which generate only one stem cell and one committed daughter cell that will go through the myogenic lineage (Figure 5).

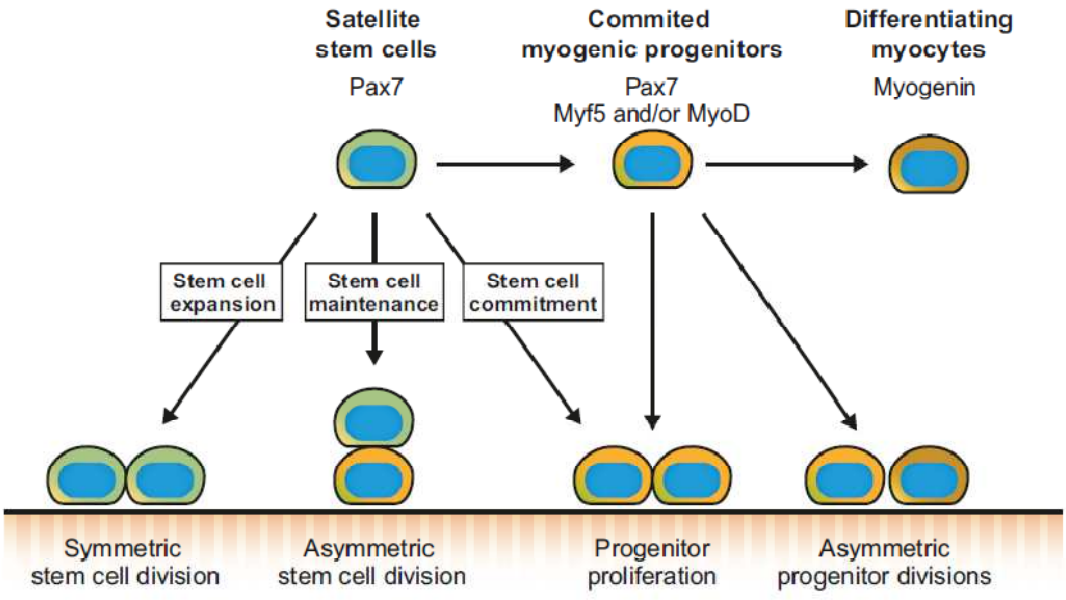



***Figure 5 Satellite cell fate decisions.***

Symmetric division is induced by activation of the planar cell polarity (PCP) pathway, leading to the symmetric distribution of effectors in daughter cells, while during asymmetric division segregation of different cell fate determinants is observed[35].

Satellite cells are able toselectthe type of division to performallowing them to coordinate their activity with the needs of the regenerating muscle. For example, an increased symmetric divisions would be useful for the expansion of the satellite stem cell pool, whereas increased asymmetric divisions would enhanceboth the generation of myogenic progenitors and maintenance of the stem cell pool.

# Chapter 2 Nitric Oxide

In mammals, including humans, Nitric Oxide(NO) is a ubiquitous, diffusible, gaseous messenger and an important regulator involved in many physiological and pathological processes. For instance, in the neuronal, cardiovascular, gastrointestinal and other systems NO mediates its biological effects through hemeproteinguanylatecyclase, which activates the enzyme and thereby raises cGMPlevels.However, other important effects of NO in the vasculature such as cytoprotection and anti-adhesion appear to be mediated through cGMP independent signaling. Likewise, NO regulation of inflammation has frequently been associated withsignal transduction events that do not involve cGMP.

NO has been documented to produce a variety of effects changing from its centralneurotransmitterto peripheral actions which propose it to be a therapeutic target in aging, apoptosis, diabetes, inflammation, ischemic preconditioning .

NO is synthesized from L-arginine and oxygen (figure 6) by NO synthases (NOS) which are the products of three different genes and named as neuronal nNOS (NOS1), inducibleiNOS (NOS2), and endothelial eNOS (NOS3).

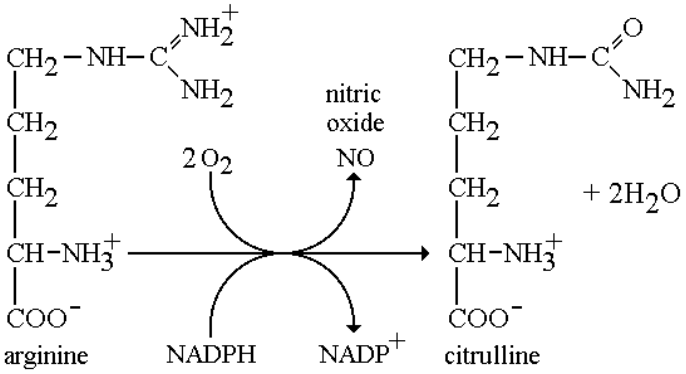



***Figure 6** Nitric oxide synthases catalyze the production of NO and L-citrulline from L-arginine, $O_2$, and NADPH-derived electrons*

All NOS isoforms share between 50 and 60% sequence homology and require same co-substrates (molecular oxygen, NADPH) and co-factors (FMN, FAD, tetrahydrobiopterinand $Zn^{++}$ions). FAD, FMN and heme are involved in redox reactions leading to synthesis of NO.

The three NOS isoforms differ in their mechanisms of activation, regulation and catalytic activity. Neuronal and endothelial NOS are constitutively expressed and require $Ca^{2+}$/calmodulin complex for their activation. Both nNOS and eNOSproduce NO at low, physiological levels (nanomolar range) for short periods. Conversely iNOS is expressed

in skeletal muscle primarily under severe inflammatory conditions, such as in the course of autoimmune inflammatory myophaties[36], and after crash injury [37]. The activity of iNOS is independent of the $Ca^{2+}$/calmodulin complex, and generates NO at high concentrations (micromolar range) for prolonged periods of time.

Studies have recently identified a putative fourth NOS isoform, named mitochondrial NOS (mtNOS), whose nature is still highly debated; it is a constitutive protein sitting on the mitochondrial inner membrane at the matrix side, that generates NO in a $Ca^{2+}$-dependent reaction [38-39]. Most evidence support the notion that mtNOS is mostly a variant of nNOS α.

The different NOS isoforms display tissue and cell type distribution and activity. eNOS is anchored to the outer mitochondrial membrane in neurons and endothelial cells[40], which indicates that mitochondria might regulate NOS activity and, conversely, that eNOS might regulate mitochondrial function. nNOS is expressed in peripheral and central nervous system, where NO is a signal molecule for cell-to-cell communication and neuroplasticity [41]. Finally mtNOS plays a role in the regulation of bioenergetics and $Ca^{2+}$ buffering[38, 42].

NO affects mitochondrial function at different levels: it regulates blood flow to tissues, therefore it supplies respiratory substrates to the organelles and redistributes heat generated by respiring mitochondria. NO also regulates mitochondrial function by reversible binding to cytochrome C oxidase in competition with oxygen to negatively regulate the respiratory complex, inhibiting oxidative phosphorylation at low oxygen concentration. This NO-dependent inhibition of cell respiration is important as part of the adaptive response to stress,*e.g.* in thedefence from alcohol toxicity [43] and in response to cardiac failure [44].

NO also binds to soluble guanylylcyclase in allosteric way and it increases cyclic guanosine monophosphate concentration leading to several cyclic GMP dependent responses. The cGMP-dependent action of NO are widespread and cGMP accounts for most of the physiological action of NO [45].

In addition to its physiological roles NO also has pathophysiological actions; NO reacts with different oxygen species to form highly reactive molecules that can damage cellular components. First NO interacts with superoxide anions ($O_2^-$), which is usually generated during mitochondrial OXPHOS activity to generate peroxynitrate ($ONOO^-$) which can in turn lead to cytochrome C release and blockade of mitochondrial complexes I and IV [46-47]. $ONOO^-$can also interact with cysteine residues to form S-nitrosothiols; this

modification is important for protein regulation but it can also cause protein dysfunction [48].

Finally NO plays a role in glutamate excitotoxicity, under certain condition NO can block NMDA receptor by S-nitrosylation and protects from cell death [49], but in different situation it can enhance neuronal injury, increasing excitotoxicity effects [50]

## 2.1 THE NITRIC OXIDE SYSTEM IN SKELETAL MUSCLE

The role of NO in the regulation of skeletal muscle physiological activity, including excitation–contraction coupling, autoregulation of blood flow, calcium homeostasis and bioenergetics has been investigated extensively. All three NOS isoforms are expressed in skeletal muscle: nNOSis abundant at the surface of type II fibres (fast twitch), whereas it is less represented intype I (slow twitch) fibres[51], eNOS has been described in several muscles as a peripheral membrane protein, which is targeted to endothelial plasmalemmal caveolae through specific interaction with caveolin-1 and caveolin-3.Changes in the expression of these enzymes may occur in skeletal muscle: expression of nNOS is increased by crush injury, muscle activity and ageing [37];while nNOS is enhanced by chronic exercise and shear stress [52]. Conversely iNOS is absent or present at very low levels in skeletal muscles under physiological conditions, but its levels are upregulate when skeletal muscle is subjected to severe inflammatory conditions, such as in the course of autoimmune inflammatory myopathies and after crash injury[36].

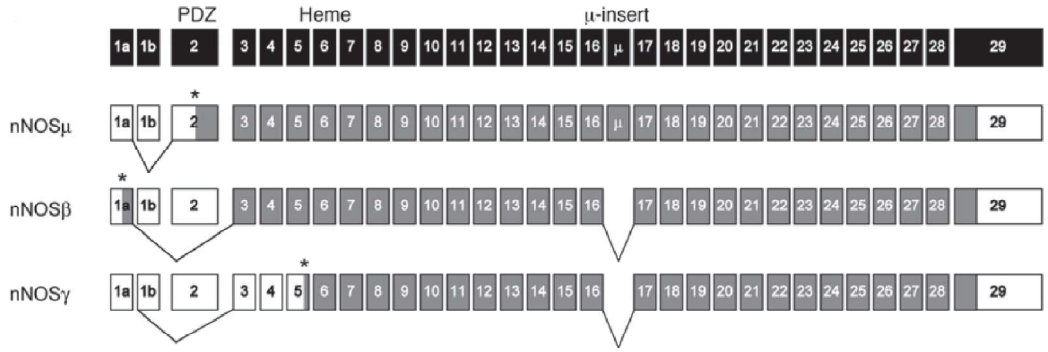



***Fig. 7 Exon structure of the murine Nos1 gene. The coding sequence is shown in gray, and asterisks mark translation initiation sites. The 5′- and 3′-untranslated sequences are shown as white boxes.***

Globally, skeletal muscle expresses three different alternative spliced isoform of nNOS:sarcolemmal nNOSμ, nNOSβ of Golgi apparatus and cytoplasmic nNOSγ[53]. In

mature skeletal and cardiac myocytes the majority of NOS activity is identified with nNOSμ, that contains a 34-aminoacid insert as a result of the alternative splicing of NOS1pre-RNA between exons 16 and 17 (figure 7). Evidences suggest that different nNOS splice variants creates functionally distinct NO signaling microdomains, with local action of NO. In fact the nNOS splice variant, localized to the Golgi complex, regulates microtubule cytoskeleton integrity and is a critical regulator of skeletal muscle fatigue and post exercise force output, sarcolemmal nNOSμ is involved in maintaining blood delivery during exercise, in modulating glucose homeostasis, controlling muscle mass and regulating fatigue resistance while function of cytoplasmic nNOSγ remains to be determined.

Contraction-induced production of NO by sarcolemmal nNOSμ attenuates α-adrenergic receptor mediated vasoconstriction, thus maintaining appropriate blood and oxygen delivery to active muscles[54-56].

The localization of nNOSμ to the dystrophinglycoprotein complex at sarcolemma, is a key aspect that explains the coupling of NO generation with muscle contractile activity (figure 8).

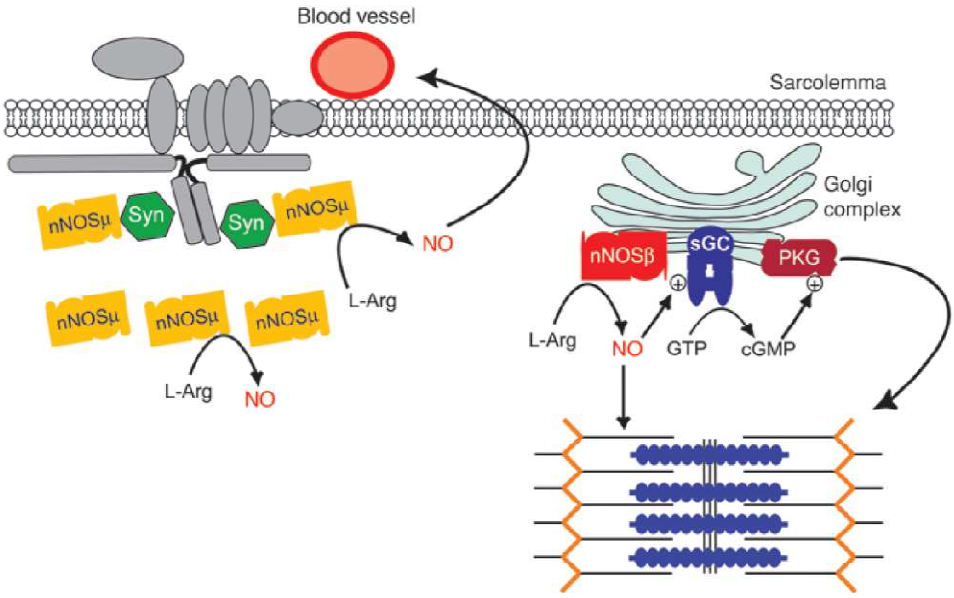



***Fig 8 Model for nNOS splice variant microdomain signaling in skeletal muscle.***

NO exerts its effects in skeletal muscle by activation of NO-dependent guanylylcyclase with formation of cyclic GMP, inhibition of cytochrome c oxidase in the mitochondrial respiratory chain or S-nitrosylation, the covalent attachment of a nitrogen monoxide

group to the thiol side chain of cysteine. Cyclic GMP-dependent signalling plays a major role in NO-dependent vasodilation and vascular responses, and presides over complex intracellular cross-talk events involving calcium and sphingolipids, which are key players in muscle homeostasis [57-58]. In addition, cyclic GMP mediates the NO-dependent biogenesis of mitochondria, that occurs via activation of a pathway involving sirtuin 1, the peroxisome proliferator-activated receptor-γ coactivator 1-α (PGC-1α), the nuclear respiratory factor 1, and the mitochondrial transcription factor A [59]. Inhibition of cytochrome c oxidase in mitochondria that occurs at physiological concentrations of NO is also particularly relevant in skeletal muscle. The third main mechanism of NO action, S-nitrosylation, regulates the activity of several enzymes that are important in skeletal muscle physiology among which are phosphatases, caspases and oxidoreductases[60-64]as well as of several transcription factors, such as p53 and NF-kB[65-66].

A significant contribution to regulation of NO concentrations in muscle, with consequence also on its bioenergeticsrole, comes from myoglobin that acts as a NO scavenger in skeletal muscle, thus regulating its delivery [67]. Myoglobinexpression is developmentally regulated in skeletal muscle, and its expression is higher in oxidative, fatigue-resistantfibres[68]. In myoglobin-deficient mice there is an increased compensatory stimulation of skeletal muscle NO generation, associated with other changes relevant to metabolism such as fibre type transition (type I to type II in the soleus muscle), increased expression of the hypoxiainducible factor 1 α, stress proteins such as heat shock protein 27, and the vascular endothelial growth factor that stimulates angiogenesis.

## 2.2 NITRIC OXIDE IN MYOGENESIS AND SKELETAL MUSCLE

Several studies in the last decade have shown that NO regulates the myogenic precursor cells through a variety of actions, mediated by different signal transduction pathways and downstream effectors. These various actions of NO converge in stimulating, on the one hand, the process of proliferation, activation and differentiation of these cells; on the other hand, in maintaining their reserve pool so that it does not become exhausted during physiological muscle damage. Such mechanisms involvingNOalso explain, at least in part, why NO may have therapeutic efficacy in pathologies, such as muscular dystrophies, where the muscle is subject to repetitive rounds of damage.

NO is implicatedin activation of SC immediately upon damage regulating release of HGF, during skeletal muscle injury or stretchingand HGFis responsible for

triggeringactivation of quiescent satellite cells in vivo[69]. Reduced NOS activity prevents the increase in myogenic cells yield, when they areisolated from the injured muscle, and delays the activation of SC after isolation[70].Further, a single administration of NOS inhibitors in rats subjected to muscle trauma preventsthe increase of SC number[71] andshowsincreased levels of collagen, suggesting that blocking NO signal in the early phase of injury impairs muscle repair, favoring fibrotic scar tissue production.

NO has beenfound to be crucial for the fusion of SC. Of importance, this effect is observed not only on adult SC but also on the presomitic mesoderm, further indicating that NO has an effect at critical stages of pre-postnatal muscle developmental life. In fact,differentiation of embryonic muscle cells involves fusion of mononucleated myoblasts into multinuclear myotubes. These myoblasts have NOS activity as measured by their ability to convert arginine to citrulline and NOS activity increases dramatically in cells competent for fusion but not in proliferating myoblasts (or in myotubes indicating that NOS activity in cultured myoblasts is critically dependent on time of differentiation[72]influencing myoblasts fusion.Moreover, the effect of NO on adult muscle appears of importance as it prevents the exhaustion of the SC pool in case of severe muscle damage, such as in muscular dystrophy. In this conditions, genetic ablation of nNOS in fibres or treatment with NOS inhibitors is sufficient to induce progressive reduction of the muscle regenerative capacity, confirming the obligatory role of NO in maintaining the myogenic precursor pool in vivo[73]. Finally, recently it has been described a role of NO in mitochondrial respiration biogenesis and dynamics, processes that have a role in myogenesis, in particular in differentiating myoblast prior to their fusion, thus NO exerts a quality control check on differentiation by regulating mitochondrial morphology and function.

## 2.3 NO AND MITOCHONDRIA

### 2.3.1 Mitochondrial structure and Dynamics

Mitochondria are intracellular organelles (with a 0.5-1µm diameter) associated with the microtubules which define their distribution and localization in the different cell types, taking up variable portion of the cytoplasm. Mitochondria are organized into two sections, the matrix and the inter-membrane space, by means of two highly specialized membranes playing different functions: the Outer Mitochondrial Membrane, OMM and the Inner Mitochondrial Membrane (IMM). The OMM displays many copies of a carrier

protein called porin, which forms wide aqueous channels through the double phospholipidic layer. Ions and tiny molecules (up to a molecular weight of 5000 daltons) can easily enter into the mitochondria through these channels, thus reaching the inter-membrane space where they are stored up. The inter-membrane space shows a chemical composition very similar to the cytosol, indeedthe matrix contains a well selected portion of molecules, because of the selective permeability of IMM. IMM and matrix are the functional areas of the mitochondria. IMM is a double phospholipidic layer composed by high amount of cardiolipin and no cholesterol. A further relevant feature is the abundance of proteins, correspondent to more than 80% of the IMM's total composition. These proteins are involved in the energy conversion and in the synthesis of ATP, but also in the metabolite exchange through IMM.IMM is bent to build a number of folds, called *cristae,* which remarkably increase IMM surface and the efficiency in the ATP production. The number of *cristae* changes according to the cell type (figure 9).

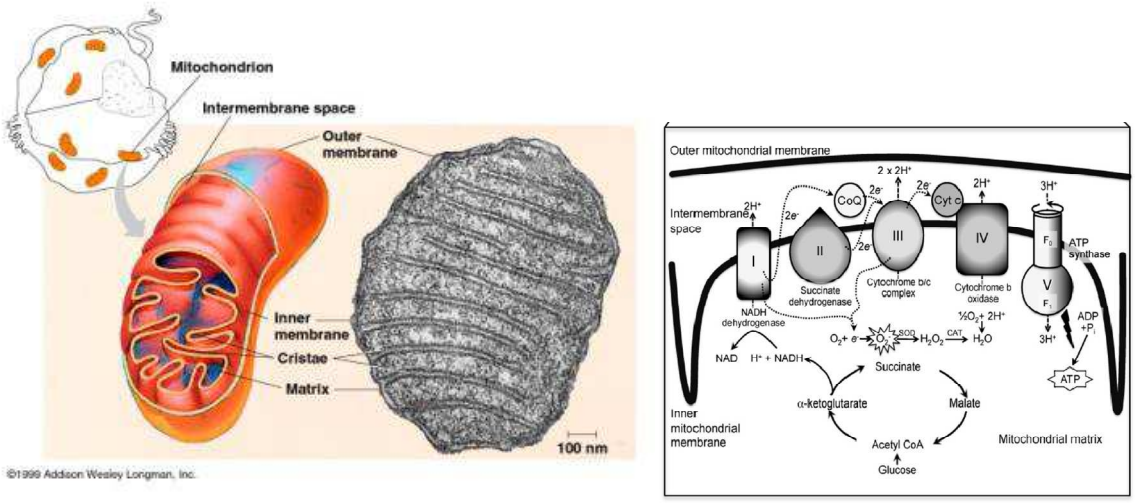



***Figure 9 Mitochondria representation***

The mitochondrial matrix contains all the enzymes meant for the catabolism of the pyruvate and the fatty acids and all the enzymes of the Kreb's cycle that oxidize the acetil CoA to generate reducing equivalents for the respiratory chain. Scattered inside are also ribosomes, tRNA and several copies (from 2 to 10) of mitochondrial DNA (mtDNA). Mitochondria have their own genoma and all that is required for transcription and translation processes. mtDNA codifies 37 genes, 13 of which correspond to complexes I, III, IV and V of the respiratory chain, while the others correspond to tRNA and rRNA.

All the biochemical reactions of the mitochondria take place in the matrix and IMM. They generate $CO_2$, $H_2O$ and ATP from the oxidation of the nutrients. Besides the

important role played in the oxidation of the substrates and in the ATP synthesis, mitochondria are involved in many other functions. In particular they control the intracellular homeostasis of $Ca^{2+}$,the synthesis of a number of compounds ,apoptosis, cell cycle regulation, regulation of cellular redox state, heme and cholesterol synthesis and heat production.

In order to suit the cells' needs, mitochondria have the possibility to change their shape and function very quickly and these morphological changes are indicative of their function and their capacity of playing several roles.Mitochondrial morphology and number depend on the balance between two opposing processes, fusion and fission[74](Figure 10).

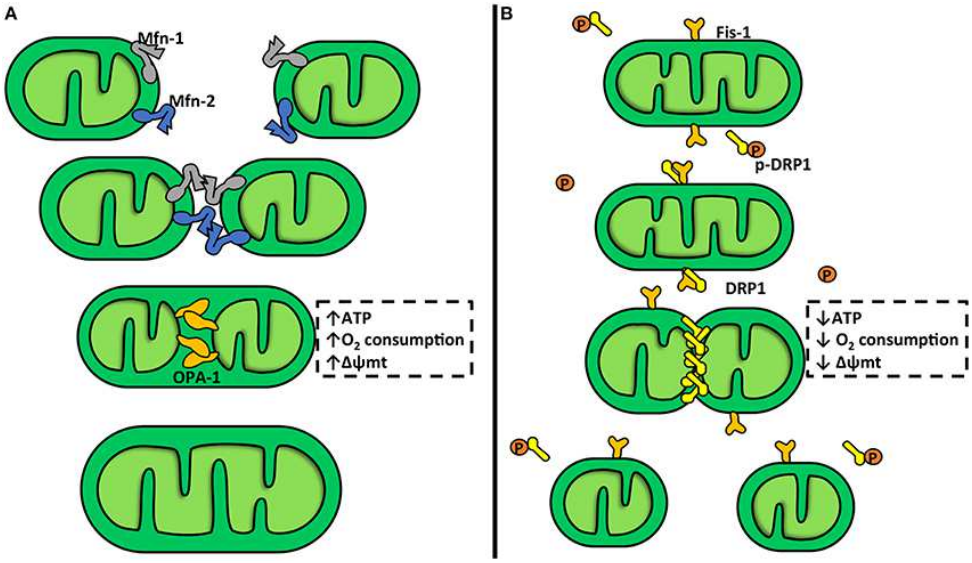



***Fig 10 Mitochondrial dynamics.***

Fusion of isolated mitochondria induces the formation of an extended interconnected mitochondrial network. This change enables mitochondria to mix their contents within the network resulting in the redistribution of metabolites, proteins and mtDNA. Moreover, fusion into a network is a selective process occurring preferentially between mitochondria with high mitochondrial membrane potential and however it generates a population of non-fusing mitochondria.. Conversely, mitochondrial fission or fragmentation is a mechanism that segregates components of the mitochondrial network producing depolarized mitochondria, with reduced expression levels of OPA1 and reduced size, moreover these mitochondria show less fusion capacity and they are removed by autophagy. Both mechanisms are evolutionarily conserved from yeasts to human. In mammals, mitofusin 1 (Mfn 1), mitofusin 2 (Mfn 2) and optic atrophy protein

1 (OPA 1) are required for fusion of both the outer and inner mitochondrial membrane. Both Mfn1 and Mfn 2 are integrated into the OMM and form homo- or hetero- oligomers which promote tethering and fusion of the OMMs from two different mitochondria. OPA 1 is anchored to the IMM by a transmembrane domain at the N terminus but most of the protein is exposed to the intermembrane space. OPA 1 requires Mfn 1 to regulate mitochondrial fusion[75]and, oligomerization of OPA 1 regulates apoptosis by controlling cristae remodeling and cytochrome C release[76].

Mitochondrial fission depends on the GTPase cytosolic dynamin-related protein 1 (Drp1), which shows a cytosolic localization that after recruitment to the OMM and oligomerization forms sub-mitochondrial foci resulting inactive GTP-dependent mitochondrial fission sites[77].

Mitochondrial fusion, fission and mitochondrial turnover (the balance between mitochondrial biogenesis and degradation) are interconnected processes essential for mitochondrial quality control. Mitochondrial biogenesis is controlled by a family of coactivators including PGC-1α and PGC-1 β (peroxisome proliferator-activated receptor-γ coactivator-1α and β). PGC1 family members are preferentially expressed in tissues with high- mitochondrial function like heart, adipose tissue and slow-twitch skeletal muscle. PGC-1 α coordinately increases mitochondrial biogenesis as well as the uptake and utilization of substrates for energy production, being crucial in the maintenance of energy homeostasis. PGC-1α is a powerful coactivator of NRF-1and NRF-2 enhancing the expression of mitochondrial transcription factor A (Tfam), mitofusins and of different nuclear genes encoding mitochondrial proteins. Both Tfam and nuclear gene products are imported into the mitochondria where they regulate the expression of mitochondrial proteins required for ATP synthesis[78].

### 2.3.2 Mitochondria in skeletal muscle

Skeletal muscles are composed of myofibers which differ in mitochondrial content, physiological properties and myosin composition. The relative distribution of the different myofiber types accounts for the functional properties of muscles (figure 11).

Muscles that rapidly generate a great force for a short time are mainly composed of glycolytic fibers, while muscles that produce discrete (lesser) force for prolonged periods are constituted mainly of oxidative fibers (rich in mitochondria). Depending on the frequency, intensity, duration and the type of exercise (resistance or endurance), different signaling pathways modulate changes in mitochondrial content, protein synthesis and

shifts in fiber type. For instance, endurance training induces mitochondrial biogenesis to improve fatigue resistance, resulting in a fast-to-slow fiber-type switch. Conversely, disuse or denervation causes the suppression of β-oxidative program and the conversion from slow oxidative fibers into fast ones[79-80].

During contraction, cytoplasmic calcium concentration transiently increases, as well as ROS production and ATP consumption. The alteration in calcium homeostasis activates calcium-sensitive signaling such as calcium/calmodulin-dependent protein kinases (CaMK) and calcineurin/NFAT pathways.

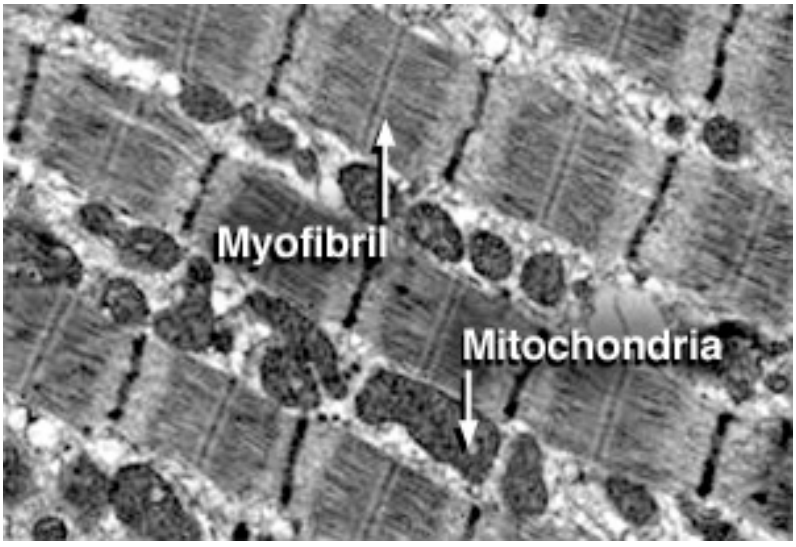



***Figure 11 An electron microscope image of skeletal muscle (25,000x) showing mitochondria (the organelle in a cell responsible for energy metabolism) within the myofibrillar area (responsible for muscle contractile function***

On the other side ATP depletion modifies the AMP/ATP ratio, activating the energy sensor AMP-activated protein kinase (AMPK). Both the calcium-dependent pathways and AMPK modulate the activity and the expression of PGC-1α[81].

In proliferating myoblasts, approximately 30% of the ATP used by the cells is provided by OXPHOS, whereas terminally differentiated myotubes rely on mitochondrial respiration as their major source of metabolic energy (approximately 60%). Intriguingly, the total metabolic rate remains constant throughout the culture period, but there is a steady shift toward a greater reliance on mitochondrial pathways[82]. Taken together, these findings suggest that a metabolic shift from glycolysis to oxidative phosphorylation as the major energy source takes place during myogenesis.

Recent studies have extended our knowledge of the potential role of mitochondrial biogenesis in muscle regeneration . It has been reported that muscle regeneration is impaired when mitochondrial protein synthesis is inhibited with chloramphenicol[83]. Chloramphenicol inhibits protein synthesis in mitochondria but not in mammalian cytoplasmic ribosomal systems.

Chloramphenicol inhibits myogenic differentiation by downregulating Myogenin but not MyoD and Myf5[84]. Interestingly, this downregulation is commonly observed also with FCCP, myxothiazol, rotenone, and oligomycin[85], which affect mitochondria at different levels. These findings suggest that Myogenin could be an important target of mitochondrial activity. Chloramphenicol has no effect on Myogenin mRNA stability, suggesting that mitochondrial activity could regulate Myogenin expression at the transcriptional level [86].

Moreover study show that the muscle specimens analyzed at day 10 after initial freeze injury and injection of chloramphenicol at days 3, 5, and 7, have impairment of mitochondrial activity that results in poor muscle regeneration with small myofibers and increased connective tissues. Overall, this supports *in vitro* data showing that chloramphenicol blocks myogenic differentiation[87].

Understanding how mitochondria are involved in myogenesis will provide a valuable insight into the underlying mechanisms that regulate the maintenance of cellular homeostasis. Recently, it has been reported that the transgenic mice with skeletal musclesspecific expression of PGC-1$\alpha$preserve mitochondrial function as well as neuromuscular junctions and muscle integrity during ageing[88] and mitochondrial gene therapy may be effective in the treatment of muscle injury [89]. These efforts may facilitate to understand the molecular mechanisms of mitochondrial disorders in muscle.

### 2.3.3 NO, mitochondria and muscle

Mitochondria possess several hemoproteins (e.g. cytochrome *c* oxidase), thiols (e.g. glutathione) and cysteine-containing proteins, and they are major cellular sources of superoxide anion. Consequently, mitochondria are important targets of NO and contribute to several of the biological functions of NO.

Some studies have revealed the presence of an NOS isoform on the mitochondrial inner membrane (mtNOS). Whose activity depends, typically, on $Ca^{2+}$. Thus, increased intra mitochondrial $Ca^{2+}$concentration [90] increases the activity of mtNOS, which in turn decreases both the mitochondrial consumption of $O_2$and the mitochondrial

transmembrane potential ($\Delta\psi$).. Conversely, inhibiting the basal activity of mtNOS increases mitochondrial consumption of $O_2$and $\Delta\psi$.

Characteristic property of NO is the ability to compete with $O_2$;[3, 91-92] binding of NO to cytochrome c oxidase triggers intracellular signaling events including ROS generation with potentially hazardous consequences[93]. In particular increased NO concentration results in peroxinitrite production that, in turn, irreversibly inhibits complex I and II of the electron transport chain.

Nitric oxide is a plausible regulator of mitochondrial biogenesis and can directly affect respiration in myocytes. Indeed, long-term exposure to NO triggers mitochondrial biogenesis in mammalian cells and tissues through the activation of guanylatecyclase and generation of cGMP. The NO/cGMP-dependent mitochondrial biogenesis is associated with enhanced coupled respiration and ATP content in skeletal muscle [3].

Nitric oxide interacts with the metabolic sensor enzyme, AMPK (Lira et al., 2007) and together they cooperate to regulate PGC-1a. The AMPKa1 isoform is identified as a mediator of NO-induced effects in skeletal muscle cells and the synergistic interaction with NOS is critical for maintenance of metabolic function in skeletal muscle[94].

Nitric oxide is also important to control mitochondrial shape and function and inhibition of NO synthesis in myogenic precursors enhanced the activity, translocation and docking to mitochondria of Drp-1, leading to inhibition of mitochondrial elongation. Under this condition, differentiating myoblasts displayed a latent mitochondrial dysfunction with impaired bioenergetics activity[5].

Also relevant is the role of NO produced by eNOS. Mice with quantitative reductions in eNOS expression (approximately 40%)display reduced expression of mitochondrial oxidative phosphorylation complexes, impaired ATP level and CaMKII phosphorylation in skeletal muscle. An acute exercise *in vivo* elicits a number of physiological processes that depend on eNOS expression with for instance the exercise-induced AMPK a phosphorylation being reduced in parallel with decrease in eNOS expression. Thus ablation of eNOS results in impaired exercise capacity, hypoglycemia, and increased plasma lactate levels without changing the mitochondrial content. However, effects of NO are rarely unidirectional and ultimately beneficial[95].

# Chapter 3 AUTOPHAGY

Autophagy is an evolutionarily conserved process whose primary task in lower organisms is the maintenance of metabolic homeostasis in the face of changing nutrient availability. In recent years, a growing number of functions have been attributed to autophagy, but almost all of them can be included in one of the following four categories: quality control, cellular source of energy, cell and tissue remodeling and cellular defense. The ability of different autophagic pathways to break down intracellular components (e.g., proteins, lipids, sugars, and nucleic acids) and recycle their constituent elements back to the cytosol makes it an ideal mechanism to supply cells with this elements when nutrients are scarce[96].

Double-membrane vesicles, termed autophagosomes, engulf long-lived proteins, damaged organelles, and even invasive pathogens, and transport these cargos to the lysosomes. There, the outer-membrane of the autophagosome fuses with the lysosomal membrane, and the inner vesicle, together with its cargo, is degraded. The resulting macromolecules can be recycled back to the cytosol for reuse during starvation [97]. This role in recycling is complementary to that of the ubiquitin-proteasome system, which degrades proteins to generate oligopeptides that are subsequently degraded into amino acids.

Cargo recognition and delivery occurs by different mechanisms, depending on the type of cargo and the cellular conditions and giving rise to different modalities of autophagy (Figure 12) [98]. The best characterized in mammalian cells are [99]:

- Chaperone-mediated autophagy (CMA): a targeting motif in the substrate proteins is recognized by a cytosolic chaperone that delivers it to the surface of the lysosome.
- Microautophagy: the lysosomal membrane invaginates to trap regions of the cytosol that are internalized into the lysosomal lumen as single membrane vesicles.
- Macroautophagy: a whole region of the cytosol is sequestered into a double membrane vesicle that fuses with lysosomes for cargo delivery.

These autophagic processes differ not only in terms of mechanistic and morphological characteristics but also in the factors involved. Among these types, macroautophagy

(hereafter autophagy) is the most comprehensively studied and best characterized process.

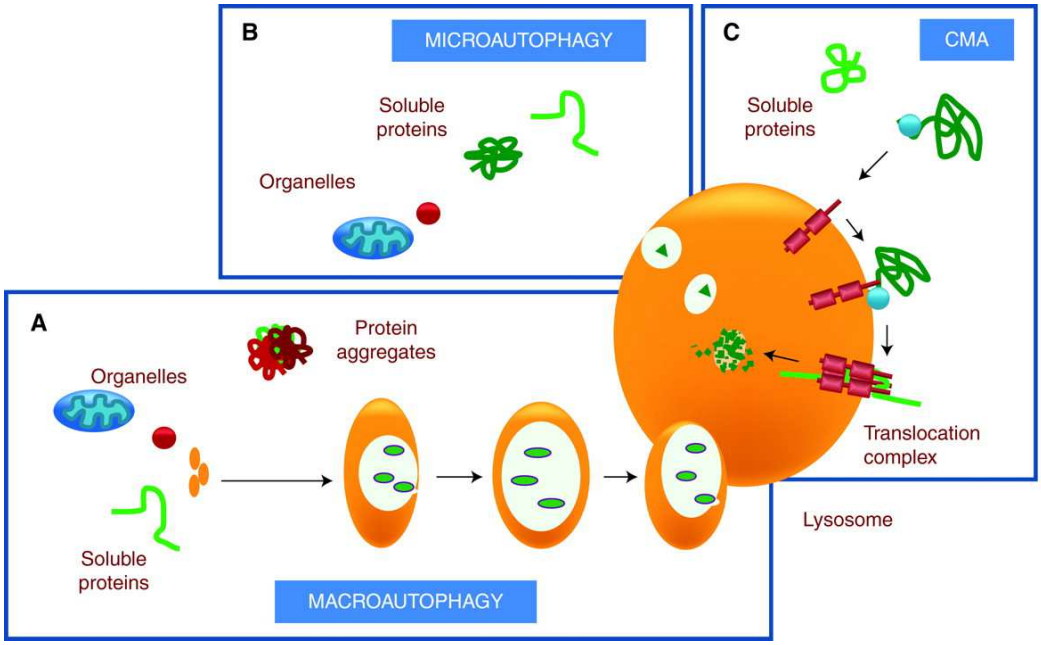



***Figure 12 Autophagic pathways****. Cytosolic proteins can reach the lysosomal lumen for degradation via autophagy through three different mechanism (A) macroautophagy, (B) microautophagy, (C) chaperone-mediated autophagy.*

## 3.1 MACROAUTOPHAGY

Macroautophagy was first described in mammalian cells more than 50 years ago, but only in the past decade that the molecular basis for this process has been illuminated.

The autophagy process is divided into mechanistically distinct steps, including: induction, cargo recognition and selection, vesicle formation, autophagosome-vacuole fusion, and breakdown of the cargo followed by release of the degradation products back into the cytosol (figure 13). These steps require energy and involve different sets of Atg (autophagy-related proteins) proteins [100]. The large majority of these proteins are conserved from yeasts to humans and can be divided into three different functional groups: Atg1-ULK1 kinase complex, required for the induction of autophagy, Atg6 and Atg14 are part of a phosphatidylinositol 3-kinase complex, required for vesicle nucleation and finally, two conjugation systems that are built around the ubiquitin-like proteins Atg8 and Atg12 [101]. Orthologs of most of these yeast Atg proteins exist in complex eukaryotes.

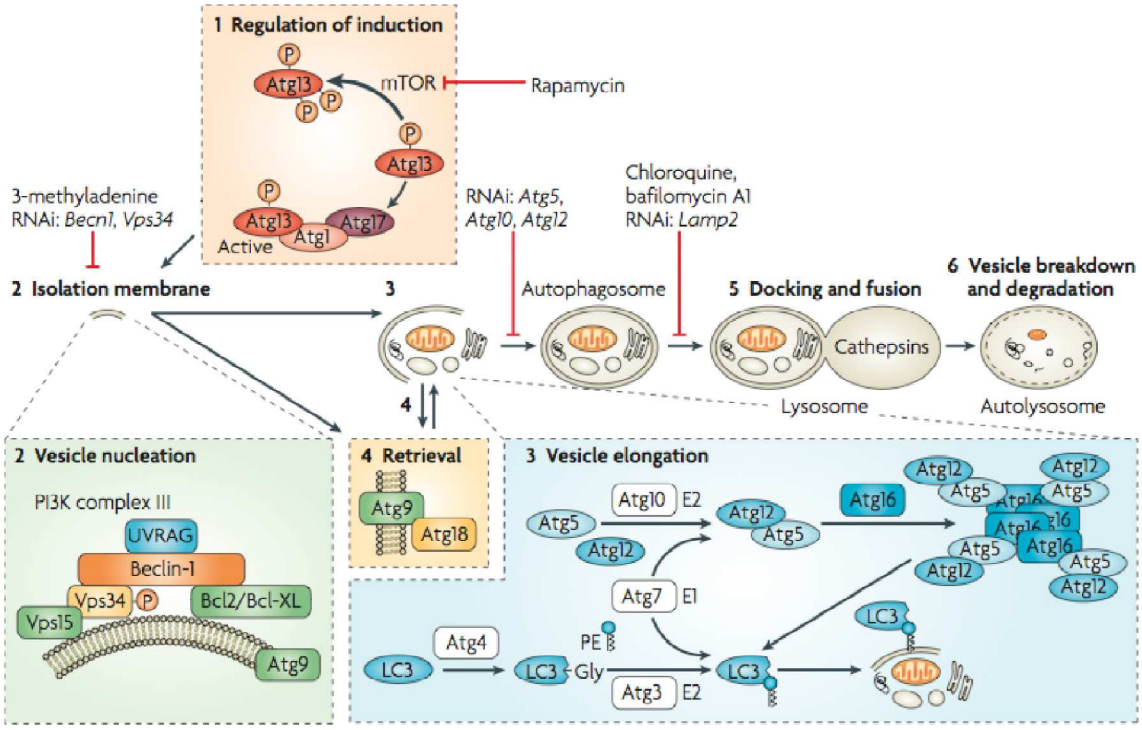



***Figure 13 Process of autophagy.*** *The process of autophagy includes five steps: initiation, elongation, maturation, fusion and degradation.*

## 3.2 MOLECULAR MACHINERY OF AUTOPHAGY

### 3.2.1 Initiation and assembly of the initial phagophore membrane

The membrane source from which autophagosomes arise is still a matter of debate, and there may be multiple sources. It has been hypothesized that autophagosomes can either be generated de novo from preexisting intracellular precursor molecules, or could arise from other intracellular membrane structures like the endoplasmic reticulum (ER) [102] . The formation of new autophagosomes requires the activity of the class III phosphatidylinositol 3-kinase (PI3K) and Vps34. Phosphatidylinositol-3-phosphate (PI-3-P), the product of Vps34 activity, plays an essential role in the early stages of the autophagy pathway. Vps34 is part of the autophagy-regulating macromolecular complex (PI3K complex) consisting of Beclin 1/Atg6, Atg14/barkor, and p150/Vps15 [103]. The activity of Vps34 is enhanced by its interaction with Beclin 1 [104]. The function in autophagy of Beclin 1 binding partners is regulated by Bcl-2 (B-cell lymphoma/leukemia-2), an antiapoptotic protein. In fact, Bcl-2 binding and sequestering

Beclin 1 under nutrient-rich conditions inhibits autophagy and dissociation of Beclin 1 from Bcl-2 is required for autophagy induction.

A second macromolecular complex implicated in the initiation step of autophagosome formation is the [105] FIP200-ULK1/Atg1 complex [106-107]. Atg13 binds ULK1 or its homolog ULK2 and mediates their interaction with FIP200. Under nutrient deprivation conditions, Atg13 and ULK1/2 are dephosphorylated, thereby activating ULK1/2, which phosphorylates FIP200 to induce autophagosome formation.

### 3.2.2 Autophagosome Formation/Elongation

Unlike processes of vesicle formation in most endomembrane trafficking systems, double-membrane autophagosomes appear to be assembled at the pre-autophagosomal structure by addition of new membranes, thus, formation of the sequestering vesicles is likely the most complex step of autophagy. Multiple Atg proteins are recruited to the phagophore to participate in autophagosome formation, and this step requires the highly regulated coordination of all of these proteins. There are two protein conjugation systems necessary for autophagosome formation (figure14), the Atg12-Atg5 and the Atg8-phosphatidyl ethanolamine systems [108]. The mechanism of both conjugation systems closely resemble ubiquitination reaction and the main difference between the two systems is related to the fate of the ubiquitin and ubiquitin-like proteins. While the ubiquitin proteasome pathway recycles ubiquitin molecules, the autophagy-lysosome system progressively loses the ubiquitin-like proteins, forcing the cell to replenish them in order to maintain the autophagic flux.

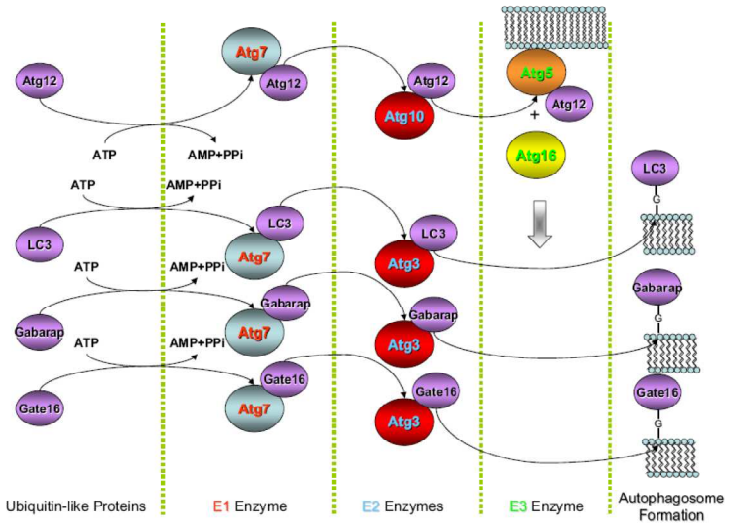



***Figure 11: Schematic representation of the autophagic conjugation system.***

- **Atg12 conjugation system**

Atg12 was the first ubiquitin-like Atg protein to be identified. In the canonical system, ubiquitin is synthesized as a precursor and is processed by a specific protease to expose the carboxy-terminal glycine residue. Activated by an E1 enzyme, ubiquitin is then transferred to an E2 enzyme, forming a thioester bond. An E3 ubiquitin ligase recognizes the target protein and transfers ubiquitin from the E2 to a lysine residue on the target.

Analogous to ubiquitination, there is an E1-like enzyme, Atg7, and Atg12 is activated by forming a thioester bond between the C-terminal Gly 186 of Atg12 and the Cys 507 of Atg7 [109]. The ATP –binding domain of Atg7 is also homologous to the corresponding region in other E1 enzymes and is essential for the formation of the Atg12–Atg5 conjugate. After activation, Atg12 is transferred to Atg10, which is an E2 enzyme, and is eventually conjugated to the target protein Atg5 [101]. Atg5 interacts further with a small coiledcoil protein, Atg16, and Atg12–Atg5-Atg16 forms a multimeric complex through the homo-oligomerization of Atg16 [110]. In addition, no processing enzyme has been identified that cleaves the isopeptide bond between Atg12 and Atg5, suggesting that this conjugation is irreversible.

- **Atg8 conjugation system**

The ubiquitin-like protein Atg8 is attached to phosphatidylethanolamine (PE) and the C-terminal Arg 117 residue of Atg8 is initially proteolytically removed by a cysteine protease, Atg4, to expose Gly 116 [111]. This exposed glycine forms a thioester bond with Cys 507 of Atg7, which is also the site that participates in the Atg12–Atg5 conjugation. Activated Atg8 is then transferred to the E2-like enzyme Atg3, also through a thioester bond. In the final step of Atg8 lipidation, Gly 116 of Atg8 is conjugated to PE through an amide bond[112]; Atg8–PE exists in a tightly membrane-associated form. Unlike Atg12–Atg5 conjugation, lipidation of Atg8 is reversible. Atg8–PE can be cleaved by Atg4 to release free Atg8 and it can still be used for another processes (Kirisako*et al.*, 2000). In mammalian cells, several homologues of yeast Atg8 have been identified: MAP-LC3 (microtubule-associated protein light chain 3), GATE-16 (Golgiassociated ATPase enhancer of 16 kDa), GABARAP (γ-aminobutyric-acid-type- A-receptor-associated protein). All of these undergo a modification process similar to that of their yeast counterpart, which is also catalysed by ATG4, ATG3 and ATG7 (Kabeya*et al.*, 2004; Tanida *et al.*, 2003; Tanida *et al.*, 2006; Tanida *et al.*, 2002; Tanida *et al.*, 2001). Among them, MAP-LC3 (typically abbreviated LC3) has been best characterized as an

autophagosome marker in mammalian cells. LC3 is synthesized as proLC3, and ATG4B processes this precursor into LC3-I with an exposed C-terminal glycine (Kabeya*et al.*, 2004). Catalysed by ATG7 and ATG3, cytosolic LC3-I is transformed to a membrane-bound form, LC3-II, which corresponds to Atg8–PE in yeast. Because LC3-II remains on the inner membrane of autophagosomes until lysosomal enzymes degrade it, increased steady-state levels of LC3-II may be due to induction of autophagosome formation, a blockade in their maturation, or both.

### 3.2.3 Vesicle fusion and autophagosome breakdown.

When autophagosome formation is completed, Atg8 attached to the outer membrane is cleaved from PE by Atg4 and released back to the cytosol. Autophagosome- lysosome fusion is mediated by the same machinery that is involved in homotypic vacuole membrane fusion. In mammalian cells, the fusion event requires the lysosomal membrane protein LAMP-2 and the small GTPase Rab7 [113-114], although the mechanism is less characterized.

After fusion, degradation of the inner vesicle is dependent on a series of lysosomal/vacuolar acid hydrolases, including proteinases A and B and the lipase Atg15 in yeast[115] and cathepsin B, D (a homolog of proteinase A), and L in mammalian cells [116]. The resulting small molecules from the degradation, particularly amino acids, are transported back to the cytosol for protein synthesis and maintenance of cellular functions under starvation conditions.

## 3.3 SIGNALING PATHWAYS REGULATING AUTOPHAGY

The complex molecular machinery of autophagy suggests that its regulation can be extremely complicated and may involve multiple signaling inputs. The classical pathway involves the serine/threonine kinase, mammalian target of rapamycin (mTOR) and, in recent years, are described various pathways and small molecules regulating autophagy via mTOR and mTOR-independent mechanisms.

### 3.3.1 mTORPathway.

One of the key regulators of autophagy is the target of rapamycin, mTOR kinase (figure 15), which is the major inhibitory signal that shuts off autophagy in the presence of growth factors and abundant nutrients [117-118] . The presence of amino acids stimulates Vps34, which leads to mTOR activation and autophagy inhibition. mTOR activity

prevents the formation of Atg complexes including the Atg1-Atg13- Atg17 serine/threonine protein kinase complex and the Vps34-Atg6-Vps15 lipid kinase complex [119]. It also interferes with the two ubiquitin-like conjugation systems of autophagy (Atg12-5 and Atg8). As a result, induction and expansion of the isolation membrane is abrogated. Conversely, TOR is inhibited during nutrient deprivation, inducing the autophagy machinery. Therefore, inhibition of TOR has been suggested to be necessary for the autophagy activation [120]. However it remains to be determined whether TOR inhibition is a universal mechanism for autophagy regulation.

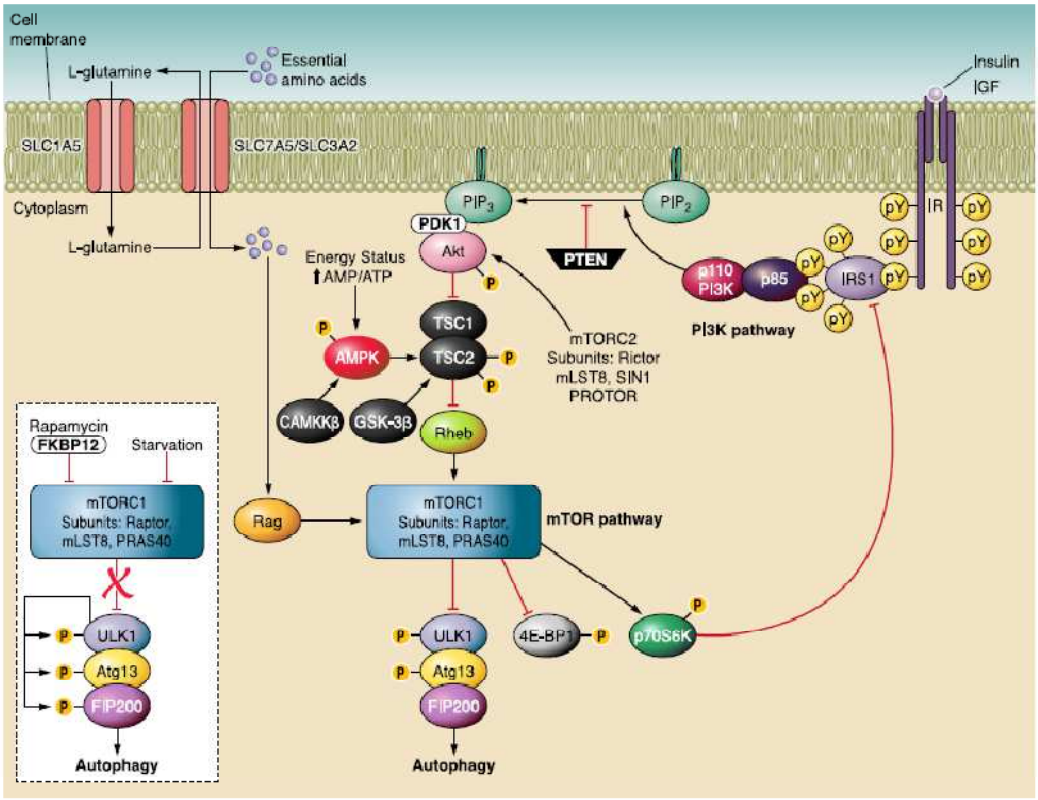



***Figure 15 The phosphatidylinositol 3-kinase (PI3K)-mammalian target of rapamycin (mTOR) pathway regulating autophagy***

### 3.3.2 Insulin/Growth Factor Pathways

When growth factors are withdrawn from the extracellular milieu, in spite of sufficient nutrients, autophagy is induced and is indispensable for maintaining cellular functions and energy production [121]. In higher eukaryotes such as Drosophila and mammalian cells, the pathways through which hormones regulate autophagy are different from those of nutrients, but both converge on TOR (Figure 15). Insulin and insulin-like growth factors regulate mTOR through the class I PtdIns3K. Upon insulin binding,

autophosphorylation of the insulin receptor on tyrosine residues results in the recruitment and phosphorylation of IRS1 and IRS2 (insulin receptor substrate 1 and 2), which creates a docking scaffold that allows binding of adaptor proteins, including subunits of the class I PtdIns3K such as p85. Generation of PIP3 (phosphatidylinositol-trisphosphate) [122], by the class I PtdIns3K, increases membrane recruitment of both protein kinase B (PKB)/Akt and its activator PDK1 (phosphoinositide-dependent protein kinase 1), leading to phosphorylation and activation of PKB/Akt by PDK1 [123]. The 3 -phosphoinositide phosphatase PTEN reverses PIP3 production, decreases the downstream PKB/Akt signaling and thus positively regulates autophagy.

### 3.3.3 Energy Sensing

During periods of intracellular metabolic stress, activation of autophagy is essential for cell viability, and the underlying pathways are understood in considerable detail. In mammalian cells, a reduced cellular energy (ATP) level is sensed by AMPK (5 -AMP-activated protein kinase). AMPK is activated by a decreased ATP/AMP ratio through the upstream LKB1 kinase. Active AMPK leads to phosphorylation and activation of the TSC1/2 complex, which inhibits mTOR activity through Rheb [124]. Autophagy stimulated by mTOR downregulation results in elevated ATP production via recycling of nutrients. In addition, the LKB1-AMPK pathway phosphorylates and activates p27kip1, a cyclin-dependent kinase inhibitor leading to cell cycle arrest, which is essential to prevent cells from undergoing apoptotic death and to induce autophagy for survival in response to bioenergetic stress during growth factor withdrawal and nutrient deprivation [125].

### 3.3.4 Stress Response

Some of the other regulatory molecules that control autophagy include the eukaryotic initiation factor 2α (eIF2α), which responds to nutrient starvation, double-stranded RNA, and Endoplasmic Reticulum (ER) stress (figure16 A) [126].

Furthermore a common intracellular stress that effectively leads to induction of autophagy is the formation of ROS (figure 16 B). Mitochondria are the major source generating ROS, which will in turn damage these organelles. The link between ROS and autophagy induction may be the cysteine protease Atg4, which cleaves Atg8/LC3 from the autophagosome outer-membrane before, or soon after, autophagosome-lysosome fusion. It is not known, however, how ROS levels might be temporally and spatially controlled inside the cell, so that Atg4 can be locally activated to allow delipidation and

recycling of Atg8/LC3, or whether another mechanism is involved. In addition, Atg4 seems to not be the sole molecule that underlies the oxidative regulation of autophagy.

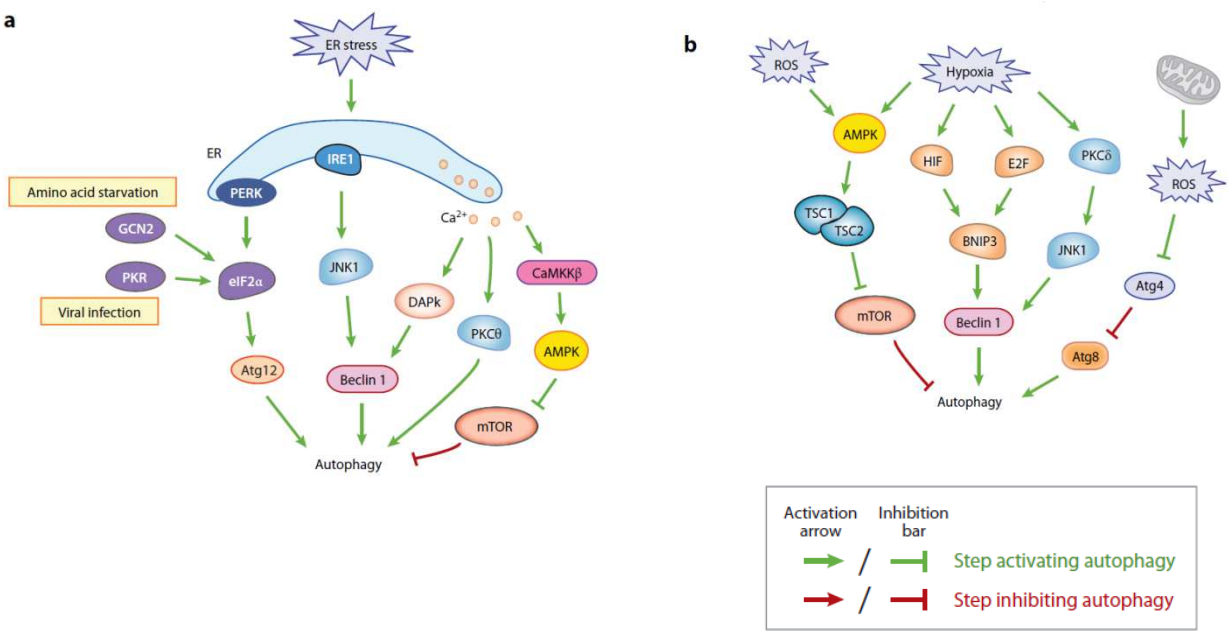



***Figure 16 (A) Autophagy regulation in response to stress, (B) Autophagy inductin by mechanisms sensing hypoxia or oxidative stress .***

### 3.3.5 The Ras/PKA pathway.

The Ras/cAMP dependent protein kinase A (PKA) signaling pathway plays an important role in glucose sensing from yeast to mammals. In nutrient-rich conditions, the small GTPases Ras1 and Ras2 are active and enhance cAMP generation by the adenylyl cyclase and elevated cAMP levels inhibits PKA. Constitutive activation of the Ras/PKA pathway suppresses autophagy induced by mTOR inhibition in yeast [127-128], suggesting that the Ras-PKA pathway downregulates autophagy.

Autophagy inhibition by Ras/PKA may be mediated through regulation of Atg1, which is identified as a phosphorylation substrate of PKA [127-128]. In the presence of nutrients, PKA phosphorylation causes Atg1 to be largely cytosolic and dissociated from the phagophore assembly site, whereas during starvation, Atg1 is dephosphorylated and localized to the assembly site.

## 3.4 MITOPAGY

We use the term mitophagy to refer to mitochondrial degradation by autophagy [129] and is considered to be the central mechanism of mitochondrial quality and quantity control (figure 17).

In mitochondria and sub mitochondrial particles as well as in intact cells, respiration produces reactive oxygen species (ROS) like H2O2 and superoxide anion and as a major source of ROS production, mitochondria are especially prone to ROS damage. Such damage can induce the mitochondrial permeability transition (MPT) caused by opening of non-specific high conductance permeability transition (PT) pores in the mitochondrial inner membrane. ATP depletion from uncoupling of oxidative phosphorylation then promotes necrotic cell death, whereas release of cytochrome c after mitochondrial swelling activates caspases and onset of apoptotic cell death [130].

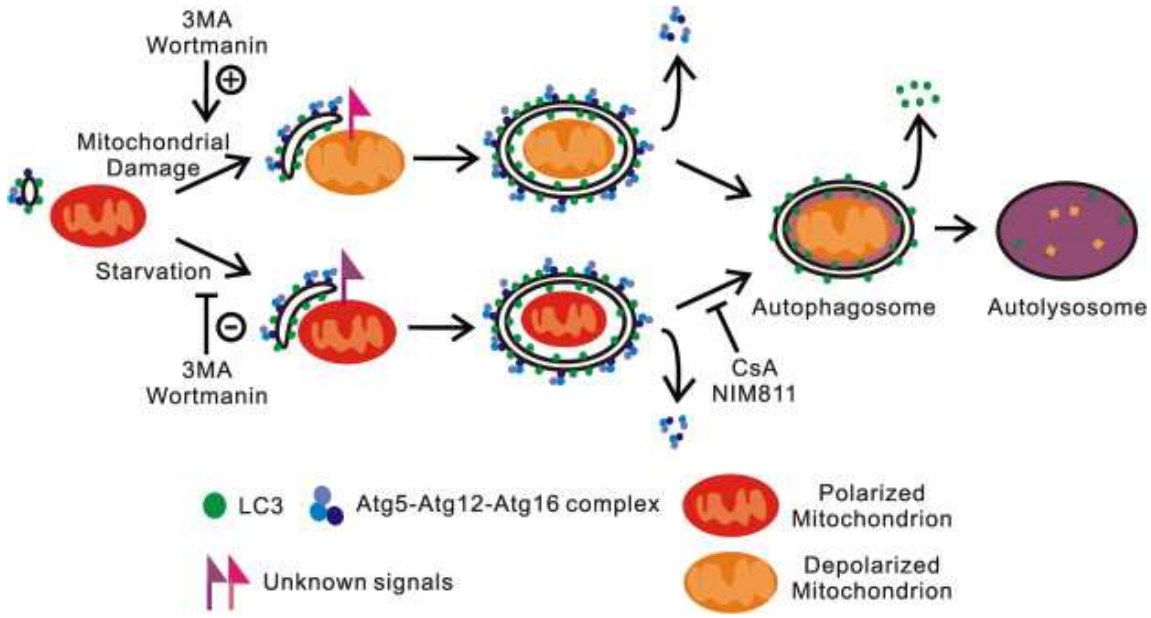



***Figure 17 Scheme of mitophagy.***

Genetic evidence that autophagy is involved in mitochondrial degradation in mammals comes from characterization of the Atg7 conditional knockout mouse model [6]. The defect in autophagy is associated with an accumulation of deformed mitochondria in Atg7-deficient hepatocytes, suggesting that autophagy is involved in turnover of damaged mitochondria in mammalian cells [131].

In mammalian, two critical mediators of mitophagy are PTEN-induced putative kinase 1 (PINK1) and Parkin (figure 18), encoded by genes in which loss of function mutations cause early onset autosomal recessive Parkinson disease.

PINK1 is a mitochondrial kinase that accumulates in damaged mitochondria and initiates the signal for translocation of the Parkin cytosolic E3 ubiquitin ligase specifically to damaged organelles [132]. Parkin-mediated ubiquitination of outer membrane proteins on these damaged mitochondria attracts autophagosomes, thus initiating mitophagy. Relocalization of Parkin from cytosol to mitochondria results in Parkin-mediated ubiquitination of dozens of outer mitochondrial membrane proteins, including mitofusin 1 and mitofusin 2 [133]. Mitochondria engulfed by an autophagosome are transferred to lysosomes and degraded, which minimizes cellular toxicity from ROS that damaged mitochondria often produce and release. Interruption of PINK1-Parkin signaling disrupts mitophagy.

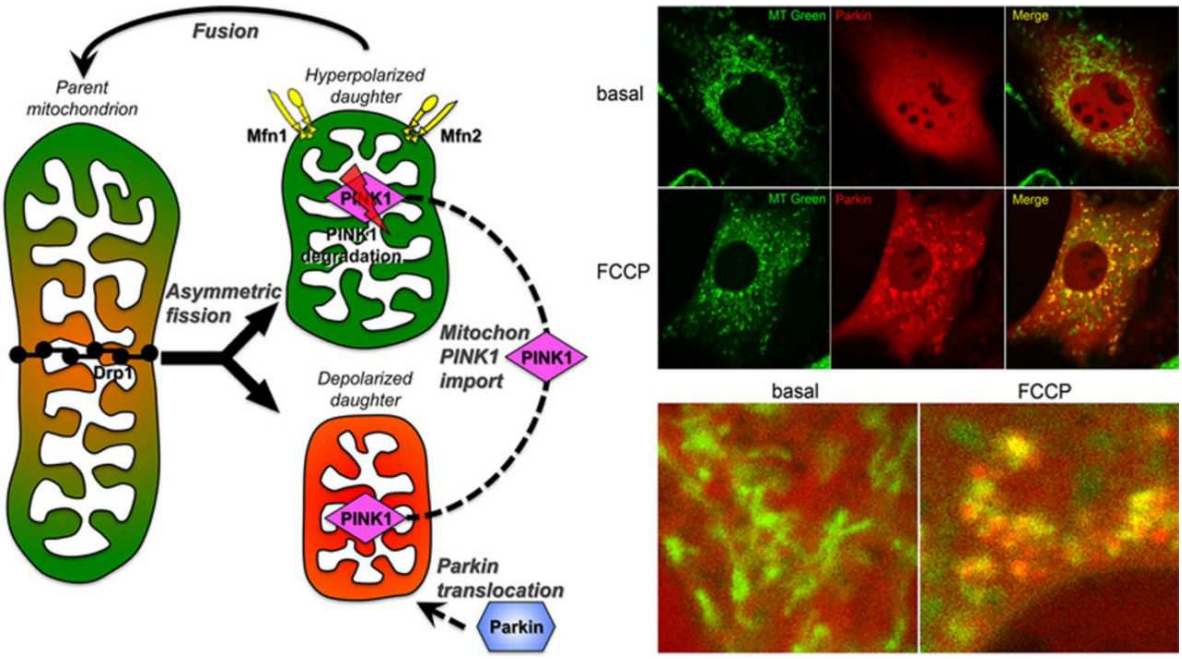



***Figure 18 The (PTEN)-induced putative kinase 1 (PINK1)-Parkin mechanism of mitophagy.***

Timely elimination of aged and dysfunctional mitochondria is essential to protect cells from the harm of disordered mitochondrial metabolism and release of proapoptotic proteins. For example, aging seems to affect mitochondria particularly because of mitochondrial ROS generation, protein damage occurs in mitochondria, and mutations of mtDNA accumulate.

## 3.5 AUTOPHAGY AND MUSCLE

The role of autophagy in skeletal muscle has been investigated extensively [134] since is a peculiar mechanism when compared to other important metabolic tissues such as the liver and pancreas. During fasting, most tissues show a transient activation of autophagy that only lasts a few hours, while skeletal muscle shows a persistent generation of autophagosomes that continues for days [135]. During catabolic conditions, in skeletal muscle occur several changes: proteins are mobilized, mitochondrial and sarcoplasmic networks are remodeled and myonuclei are lost. In addition, the daily contractions can mechanically and metabolically damage/alter muscle proteins and organelles. Therefore, muscle cells require an efficient system for removing and eliminating unfolded and toxic proteins as well as abnormal and dysfunctional organelles (figure 19). Moreover skeletal muscle acts as a large store of protein that can be catabolized during nutrient deprivation or disease to provide free amino acids that can be used for protein synthesis in vital organs (i.e., heart, brain, lungs).

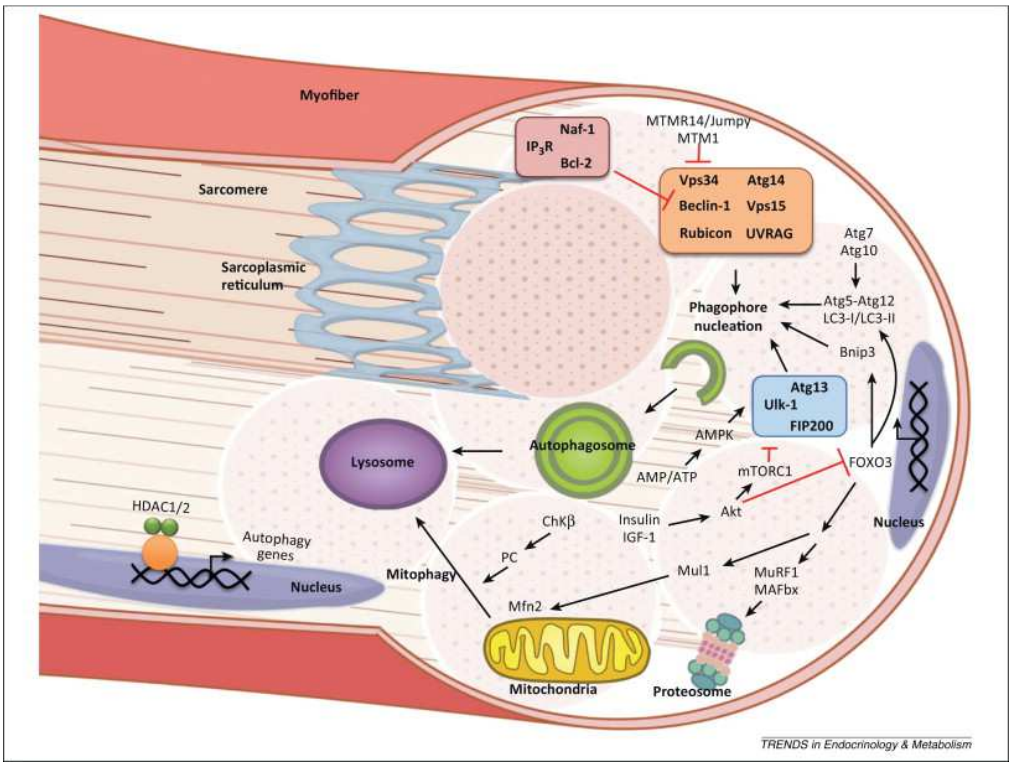



***Figure 19 Overview of signal transduction pathways controlling skeletal muscle autophagy.***

Thus, autophagy is crucial for normal muscle function and may contribute to muscle remodeling. However, aberrant protein degradation in the skeletal muscle is detrimental

for the economy of the body, and may cause myofiber dysfunction driving to muscle wasting and myopathy.

To clarify the role of basal autophagy it was generated many model of knockout mice with muscle-specific inactivation of various genes coding for autophagy- related proteins, such as Atg7 or Atg5. Deletion of this genes causes altered muscle structure, with sarcomere disorganization and myofibers degeneration due to accumulation of protein aggregates, appearance of abnormal mitochondria and of concentric membranous structures that assemble between the myofibrils or just beneath the sarcolemma, induction of oxidative stress and activation of Unfolded Protein Response(UPR) [136] . Altogether, these effects accounted for muscle weakness, atrophy, and other signs of myopathy such as an irregular distribution of fibers' shape as well as fibers with a vacuolated cytosol [137]. The fact that manipulation of animal models to dysregulate autophagy also leads to muscle pathology has prompted studies to investigate whether this process is altered in muscle diseases [138].

Loss of muscle mass occurs in many conditions ranging from denervation, inactivity, microgravity, fasting to a multitude of systemic diseases [139] and in all of these catabolic conditions protein breakdown is enhanced and exceeds protein synthesis resulting in myofiber atrophy [140]. Also autophagosomes have been found in almost every myopathy and dystrophy studied so far and are characteristic of a group of muscle disorders named Autophagic Vacuolar Myopathies (AVM) [141] .

The activation of the major proteolytic systems requires a transcription-dependent program and comparing gene expression in different models of muscle atrophy lead to the identification of a subset of genes that are commonly up- or down-regulated in atrophying muscle [142]. These common genes are thought to regulate the loss of muscle components and were thus designated atrophy-related genes or atrogenes [143]. The atrogenes comprise several proteasome subunits and two muscle specific E3 ligases, atrogin-1/MAFbx (muscle atrophy F box) and muscle RING finger 1 (MuRF1) [142]. The up-regulation of this atrogenes and several autophagy-related genes is normally blocked by Akt through negative regulation of Fork head box O (FoxO) transcription factors [143]. In particular, Akt regulates both the ubiquitin–proteasome system and the autophagy–lysosome pathway, and this action is mediated by FoxO transcription factors. In the presence of growth factors such as insulin or IGF, Akt phosphorylates and inactivates FoxO [144], promoting its export from the nucleus to the cytoplasm. The reduced activity of the Akt pathway observed in various models of muscle atrophy leads

to decreased levels of phosphorylated FoxO in the cytoplasm and a marked increase in nuclear FoxO.

The translocation and transcriptional activity of FoxO members is sufficient to promote atrogin-1 and MuRF1 expression, and muscle atrophy. In addition to activating the transcription of the previously described atrogenes (including MRF1 and MAFbx), FoxO3 can induce expression of a number of autophagy-related genes including LC3, GABARAPl 1, Bnip3, Vps34, Ulk1, Atg12, and Atg4B in mouse muscle in response to fasting and denervation [21, 145]. Moreover, many genetic models have confirmed the role of autophagy during muscle atrophy (figure 20).

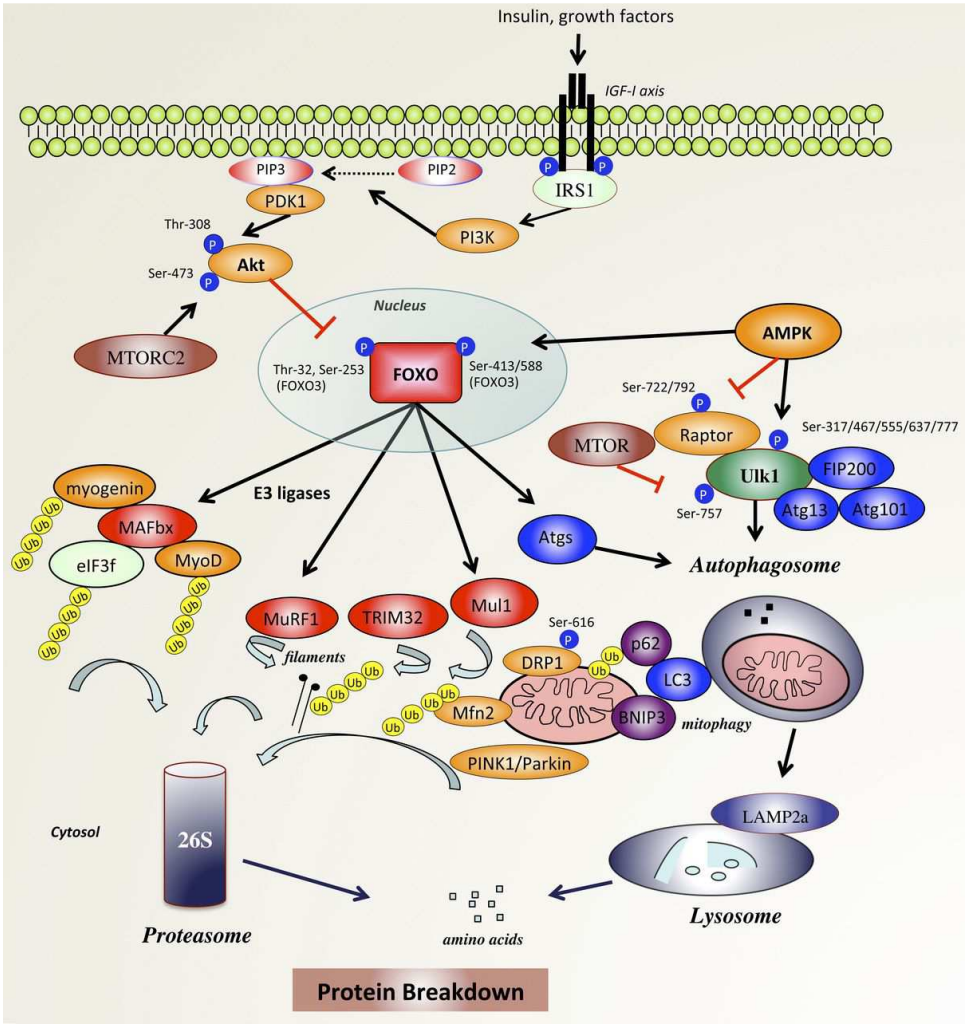
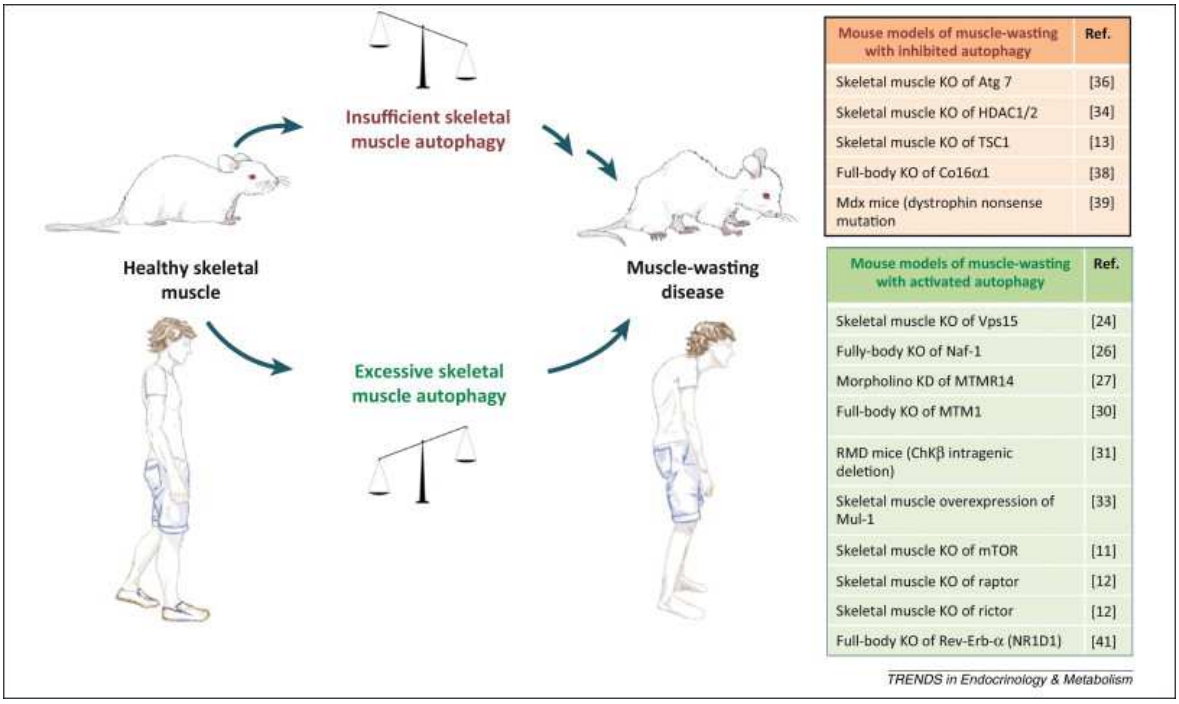



***Figure 20 FOXO-dependent protein breakdown and autophagy in skeletal muscle.***

For example, increased oxidative stress also been reported to occur during denervation and hind limb suspension. During these disuse conditions, neuronal NOS (nNOS) moves from the sarcolemma, where it is bound to the dystrophin-glycoprotein complex, to the

cytosol. Free cytosolic nNOS induces oxidative stress and enhances FoxO3-mediated transcription of the atrophy-related ubiquitin ligases, atrogin 1 and MuRF1, causing muscle loss [146].

Recent data suggest that autophagy may also contribute to sarcopenia, the excessive loss of muscle mass that occurs in the elderly. During ageing there is also a progressive deterioration of mitochondrial function and activation of autophagy. Forced expression of PGC1a, the master gene of mitochondrial biogenesis, in skeletal muscles of old mice ameliorates loss of muscle mass and prevents the age-related increase of autophagy [88]. Thus, autophagy activation has been reported in acute conditions of muscle loss as well as in chronic and long-lasting situations of muscle debilitation and weakness.




| Mouse models of muscle-wasting with inhibited autophagy | Ref. |
|---|---|
| Skeletal muscle KO of Atg 7 | [36] |
| Skeletal muscle KO of HDAC1/2 | [34] |
| Skeletal muscle KO of TSC1 | [13] |
| Full-body KO of Co16α1 | [38] |
| Mdx mice (dystrophin nonsense mutation | [39] |

| Mouse models of muscle-wasting with activated autophagy | Ref. |
|---|---|
| Skeletal muscle KO of Vps15 | [24] |
| Fully-body KO of Naf-1 | [26] |
| Morpholino KD of MTMR14 | [27] |
| Full-body KO of MTM1 | [30] |
| RMD mice (ChKβ intragenic deletion) | [31] |
| Skeletal muscle overexpression of Mul-1 | [33] |
| Skeletal muscle KO of mTOR | [11] |
| Skeletal muscle KO of raptor | [12] |
| Skeletal muscle KO of rictor | [12] |
| Full-body KO of Rev-Erb-α (NR1D1) | [41] |

***Fig A hypothetical diagram of the development of muscle-wasting diseases.*** *The equilibrium of skeletal muscle autophagy is important for maintaining muscle mass and healthy skeletal muscle. An imbalance due to a number of factors such as diet, physical activity, systemic disease, or genetics can cause a shift in the autophagic flux*

Of interest, is the role of detective autophagy in different forms of inherited muscular disorders, including Limb girdle muscular dystrophy type 2B, Ullrich congenital muscular dystrophy, myositis, and Emery–Dreifuss muscular dystrophy has emerged in the past 5 years [147]. Deficient autophagy was also suggested to contribute to Duchenne muscular dystrophy (DMD) pathogenesis. The presence of swollen and damaged mitochondria, protein aggregation, and distension of sarcoplasmic reticulum, which are cyto-pathological hallmarks of DMD, are often observed when autophagy is impaired

[148]. In addition, activation of Akt was significantly higher in muscles from *mdx* mice and dystrophin-deficient primary myotubes, thereby suggesting a defective autophagic process. The causal relationship between DMD pathogenesis and dysfunctional autophagy has been investigated more recently in studies addressing this issue specifically [149].

The beneficial effects of activating autophagy in *mdx* mice have been also confirmed by overexpressing peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1a), a transcriptional coactivator, which is a powerful mediator of muscle plasticity [150] and by pharmacological treatment with an agonist drug against the energy sensor AMPK, i.e., 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) [151].

Therefore unbalanced autophagy might be the pathogenic mechanism (figure 21): at high levels, it can be detrimental and contribute to muscle atrophy; at low levels, it can cause weakness and muscle degeneration, due to the unchecked accumulation of damaged proteins and organelles [134, 147, 152]. Thus, a proper autophagic process is vital for both functional skeletal muscle, which controls the support and movement of the skeleton, and muscle metabolism [138].

# AIM OF THE PROJECT

My phD project is divided in two sections: the formeris focused on the study of the relationship between the NO system, mitochondrial structure/activity and skeletal muscle wasting, paying attention on the role of autophagy and using a mouse model in which NOS1 (nNOSμ) is absent. My data demonstrate that an altered NO signaling leads to mitochondrial dysfunction resulting in enhanced autophagy and reduced muscle growth, however not associated with atrophy induction. Furthermore autophagy is essential to maintain muscle mass, but its role on regenerating population and its impact on muscle growth and development has not been evaluated yet. Starting form conclusion in the second part of my PhD project i focalized my attention on the role of autophagy specifically on satellite cells population, generating a transgenic mice in which autophagy is selectively inhibited in satellite cells and studying their impact on muscle growth. My data suggest that autophagy is involved in the controlling of satellite cells functions. Autophagy loss of function in satellite cells impairs their proliferation rate as well as their capability to fuse and differentiate.

The study of these two different transgenic mice reveals as in muscle, unbalanced autophagy from the early phase of growth affects satellite cells population causing muscle wasting and suggests how during skeletal muscle development is important to have appropriate levels of autophagy

# Materials and Methods

## Animals

NOS1-/- mice homozygous for targeted disruption of the nNOS gene (strain name B6129S4- NOS1tm1Plh/J) were purchased from Jackson Laboratories (Bar Harbor, Maine, USA) (stock no. 002633). In this mouse model, targeted deletion of exon 2 specifically eliminates expression of nNOSμ [37]. NOS1-/- mice were crossed with the wild-type B6129 strain to maintain the original background and to obtain a colony of NOS1-/- mice and wild-type littermate controls, with genotyping performed by tail clippings. Experiments were performed on male mice at postnatal day 10 (P10) and P120. C57BL/6 wildtype mice (strain name C57Bl10SnJ) were purchased from Charles River (Calco, Italy).

To generate mice with specific Atg7 gene deletion in satellite cell population we crossed Atg7-floxed mice (Atg7 $^{f/f}$) (generously provided by Sandri's lab) with a transgenic line expressing Cre recombinase under control of Pax7 gene promoter (generously provided by Pura's lab)[6]. Experiments were performed on male mice at postnatal day 21 (P21). Genotyping of mice Atg7-/- by PCR was performed by tail clippings with the following two primers:

REV:5-TGGCTGCTACTTCTGCAATGATGT-3

FW:5-CAGGACAGAGACCATCAGCTCCAC-3.

Animals were housed in a regulated environment (23 ± 1°C, 50 ± 5% humidity) with a 12-hour light/dark cycle (lights on at 08.00 a.m.), and provided with food and water ad libitum. For specific experiments, mice were killed by cervical dislocation. All studies were conducted in accordance with the Italian law on animal care N° 116/1992 and the European Communities Council Directive EEC/609/86. The experimental protocols were approved by the Ethics Committee of the University of Milano. All efforts were made to reduce both animal suffering and the number of animals used.

## Protein isolation and western blotting

Satellite cells were harvested and homogenised for 10 minutes at 4°C in RIPA lysis buffer, containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA and 0.1% sodium dodecyl sulphate (SDS). Tissue samples from muscles were homogenized in a lysis buffer containing 20 mM Tris-HCl (pH 7.4),

150 mM NaCl, 1% Triton X-100, 10% glycerol, 10 mM EGTA and 2% SDS. Buffers were supplemented with a cocktail of protease and phosphatase inhibitors (cOmplete and PhosSTOP; Roche). Protein concentration was determined using the bicinchoninic acid assay (ThermoFisher Scientific). Using published protocols[156], SDS and β-mercaptoethanol were added to samples before boiling, and equal amounts of proteins (40 μg/lane) were separated by 4% to 20% SDS-polyacrylamide gel electrophoresis (Criterion TGX Stain-free precast gels and Criterion Cell system; Bio-Rad). Proteins were then transferred onto a nitrocellulose membrane using a Bio-Rad Trans-Blot Turbo System. The membranes were probed using the following primary antibodies as indicated in the text: rabbit polyclonal anti-MyoD (C-20) (Santa Cruz Biotechnology, Dallas, TX, USA), rabbit polyclonal anti-Atg7 and rabbit polyclonal anti-LC3B (Sigma-Aldrich, Saint Louis, MO, USA), mouse monoclonal anti-sarcomeric myosin (MF20) (Developmental Studies Hybridoma Bank, Iowa City, IA, USA).

After the incubation with the appropriate horseradish-peroxidase-conjugated secondary antibody (Cell Signaling Technology), bands were visualised using the Bio-Rad Clarity Western ECL substrate with a Bio-Rad ChemiDoc MP imaging system. To monitor for potential artefacts in loading and transfer among samples in different lanes, the blots were routinely treated with the Restore Western Blot Stripping Buffer (ThermoFisher Scientific) and reprobed with rabbit polyclonal anti-calnexin (GeneTex, Irvine, CA, USA), goat polyclonal anti-actin (I-19) or mouse monoclonal anti-vinculin primary antibodies (Sigma-Aldrich, Saint Louis, MO, USA).

**Real-time quantitative PCR**

Satellite cells and muscle tissue samples were homogenised, and RNA was extracted using the TRIzol (Invitrogen-Life Technologies, Monza, Italy). Using published protocols [159], after solubilisation in RNase-free water, first-strand cDNA was generated from 1 μg of total RNA using the ImProm-II Reverse Transcription System (Promega). As show in Table 1, a set of primer pairs amplifying fragments ranging from 85 to 247 bp was designed to hybridise to unique regions of the appropriate gene sequence. Real-time quantitative PCR (qPCR) was performed using the SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) on CFX96 Real Time System. All reactions were run in duplicate. A melt-curve analysis was performed at the end of each experiment to verify that a single product per primer pair was amplified. As a control experiment, gel electrophoresis was performed to verify the specificity and size of the amplified qPCR

products. Samples were analysed using the Bio Rad CFX manager software and the second derivative maximum method. The fold increase or decrease was determined relative to a calibrator after normalising to 36b4 (internal standard) through the use of the formula 2-ΔΔCT [160].

**Table 1 Primer pairs designed for qPCR analysis**

| Name/symbol | Forward primer | Revers primer |
|---|---|---|
| Atrogin-1 (fbxo32) | 5′-GCAAACACTGCCACATTCTCTC-3 | 5′-CTTGAGGGGAAAGTGAGACG-3 |
| MuRF1 (Trim63) | 5′-ACCTGCTGGTGGAAAACATC-3′ | 5′-CTTCGTGTTCCTTGCACATC-3′ |
| Atg4b | 5′-ATTGCTGTGGGGTTTTTCTG-3′ | 5′-AACCCCAGGATTTTCAGAGG-3’ |
| Bnip3 | 5’ -TTCCACTAGCACCTTCTGATGA-3′ | 5′-GAACACGCATTTACAGAACAA-3′ |
| p62 (Sqstm1) | 5′-GAAGCTGCCCTATACCCACA-3′ | 5′-AGAAACCCATGGACAGCATC-3′ |
| MyoD | 5’GACAGGACAGGACAGGGAGGAG | GGAGTGGCGGCGATAGAAGC |
| Myogenin | 5’GACCCTACAGACGCCCACAATC | ACACCCAGCCTGACAGACAATC |

**Primary myogenic cell cultures, immunofluorescience, fusion index and proliferation assay**

Using published protocols [5], myogenic precursor cells (satellite cells) were freshly isolated from muscles derived from the NOS1-/- mice, Atg7-/- mice and wild-type littermate controls.

Briefly, hind limb muscles were digested with 2% collagenase-II and dispase for 10 minutes at 37°C with gentle agitation. Contamination by non-myogenic cells was reduced by pre-plating the collected cells onto plastic dishes where fibroblasts tend to adhere more rapidly. Dispersed cells were then resuspended in Iscove’s modified Dulbecco’s medium supplemented with 20% foetal bovine serum, 3% chick embryo extract (custom made), 10 ng/ml fibroblast growth factor, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μg/ml gentamycin, and plated onto matrigel-coated dishes. Primary myoblasts were cultured in grow media to reach 80% confluence and switched into “differentiation medium” (Iscove’s modified Dulbecco’s medium supplemented with 2% horse serum and the antibiotics) for up to 48 hr.

For immunofluorescence analysis cells were fixed using 4% paraformaldehyde for 10 min at RT, permeabilized (0.1% Triton X-100 PBS) and incubate in blocking solution (PBS, 10% goat serum) (1hr RT). Cells were then incubated with an anti Myosin heavy chain primary antibody (MF20 ibridoma bank) diluted in blocking solution (2hr RT), then

washed in PBS, and incubated in fluorescent secondary antibodies (1 hr RT). Nuclei were counterstained with DAPI solution.

The fusion index is defined as the total number of nuclei in MF20-positive cells divided by the total number of nuclei per field. The number of nuclei per myotube was established counting nuclei within every myotube per microscopic field divided by the number of myotubes in the field, where a myotube is defined as a MF20-positive cell with at least two nuclei.Proliferation was assessed on cells grown on p60 dish using the CytoTrack Cell Proliferation Assay Kits (Bio-Rad) and assayed after 24 and 48h of proliferation counting the percentage of positive cells onto the Integrin $\alpha7^+$ population by flow cytometry.

**Facs analisis**

To determine the percentage of satellite cells resident in muscle, 80 mg of hind limb muscles were minced and digested with 0.2% of Collagenase II (Worthington Biochemical, Lakewood, NJ) following published protocols [161].

For FACS analysis cells were stained with the following antibodies: anti-CD31-phycoerythrin (PE)/Cy7 (PECy7, clone 390), anti-CD45-PECy7 (clone 30-F11) (eBioscience, Inc., San Diego, CA), anti-α7-Integrin-PE (AbLab, UBC Antibody Lab, Vancouver, Canada), anti-LY-6A/E SCA-1-allophycocyanin/Cy7 (APC/Cy7, clone B7, BD Biosciences, Franklin Lakes, NJ). Satellite cells were selected through flow cytometry as Integrin $\alpha7^+/CD34^+$ double positive cells and CD45, CD31, CD11b, Sca1 negative ($Lin^-$) cells (Integrin $\alpha7^+/CD34^+/Lin^-$).

**Single-Fiber Culture Assays.**

Single floating myofibers were prepared from the gastrocnemius of 21 days-old Atg7-/- mice and controls, as described [162].

Individual intact myofibers were cultured in proliferating medium (20% fetal bovine serum [Mediatech, Herndon, VA], 10% HS, 2% CEE in DMEM) for 3 days and then fixed in 4% paraformaldehyde (PFA) for 10 min at room temperature. For immunoflurescence analysis fibres were permeabilized 4 min RT in 0.2% PBS/0.2% Triton X-100 [PBST]), incubated in blocking solution (BSA; 10% goat serum, PBS) for 30 min RT and hybridated with primary antibodies (diluted in blocking solution) overnight at 4C.

The day after, single fibers were washed in PBS, incubated with appropriate flurescent secondary antibodies (diluted in blocking solution) and DAPI 1 hr RT. After several washes in PBS, single fibers were mounted and observed at fluorescence microscopy.
The antibodies used in this study are: anti MyoD antibody (1:50; Dako E3238 or Santa Cruz Biotechnology), anti Pax7 antibody[1:10; Developmental Studies Hybridoma Bank (DSHB)] and Ki67 antibody (1:250; Abcam ) .

**In vivo imaging using two-photon confocal microscopy**

Mitochondrial morphology and autophagosome formation in living animals were monitored in tibialis anterior muscles transfected by electroporation with plasmids encoding pDsRed2-Mito or the LC3 protein fused to the yellow fluorescent protein (YFP-LC3), as described previously [143, 145, 153]. Two-photon confocal microscopy in the live, anaesthetised animals was then performed 12 days later on in situ exposure of transfected muscles. To allow the muscle to recover from the injection-induced swelling, microscopic observation was interrupted for two to five minutes.

**Transmission electron microscopy**

Tibialis anterior muscles were dissected and fixed for one hour in a solution containing 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, immobilised on a Nunc Sylgard coated Petri dish (ThermoFisher Scientific, Waltham, MA, USA) to prevent muscular contraction as previously described [154]. The muscles were rinsed in the same buffer and dissected further into small blocks that were subsequently processed for transmission electron microscopy (TEM) as described elsewhere [155]. Briefly, the samples were postfixed with osmium tetroxide (2% in cacodylate buffer), rinsed, en bloc stained with 1% uranyl acetate in 20% ethanol, dehydrated and embedded in epoxy resin (Epon 812; Electron Microscopy Science, Hatfield, PA, USA) that was baked for 48 hours at 67°C. Thin sections were obtained with a Leica ultramicrotome (Reichert Ultracut E and UC7; Leica Microsystems, Wetzlar, Germany) stained with uranyl acetate and lead citrate, and finally examined with a Philips CM10 TEM (Philips, Eindhoven, The Netherlands). Morphometric analysis of mitochondrial cristae complexity was evaluated with a stereological method. Briefly, a regular grid has been superimposed over 10500X TEM micrographs and the number of intersections between the grid and mitochondrial cristae was recorded. The same grid was used for all the different analysis.

**Confocal microscopy of myogenic precursor cells**

Cells were plated in eight-well Nunc LabTeck Chamber slides (ThermoFisher Scientific). When indicated cells were transfected with YFP-LC3 plasmid. Transfections were performed with the Lipofectamine LTX with Plus reagent (Invitrogen-Life Technologies) according to the manufacturer's instructions. The cells were used 24 hours after transfection in the various experimental settings described. For confocal imaging, the cells were fixed in paraformaldehyde and washed in phosphate-buffered saline [157]. To prevent nonspecific background, cells were incubated in 10% goat serum/phosphate-buffered saline followed by probing with the primary antibody mouse monoclonal anti-cyclophillin D (Abcam). Cells were then incubated with the secondary antibody, Alexa Fluor 546 dye-conjugated anti-mouse IgG (Molecular Probes-Life Technologies, Monza, Italy). Slides were placed on the stage of a TCS SP2 Laser-Scanning Confocal microscope (Leica Microsystems) equipped with an electronically controlled and freely definable Acousto-Optical Beam Splitter. Images were acquired with x63 magnification oil-immersion lenses. Analyses were performed using Imagetool software (Health Science Center, University of Texas, San Antonio, TX, USA). Images of cells expressing YFP-LC3 were thresholded by using the automatic threshold function.

**Muscle immunohistochemistry and histology**

The tibialis anterior (TA) of nNos  mice and wild-type littermate controls at P10, P30 and P120 and tibialis anterior of Atg7-/- mice and wild-type littermate controls at P21 were removed, the proximal extremity fixed on a piece of cork with tragacanth gum and frozen in isopentane

Serial 7 μm cryosections were cut with a cryostat and samples were stored at -80°C.

For histological analyses, serial muscle sections were obtained and stained with Haematoxylin and Eosin (H & E), performed as previously described [149, 158]. The number of fibres was counted and analysed using the ImageJ software

To measure the cross sectional area (CSA) of myofibres, muscle sections were stained in immunoflurescence with an anti-laminin A (a cell-adhesion molecule strongly expressed in the basement membrane of skeletal muscles) antibody (L1293; Sigma-Aldrich diluizione?)and an appropriate fluorescent secondary antibody. Morphometric analyses were performed on sections collected from similar regions of each muscle, using a Leica DMI4000 B automated inverted microscope equipped with a DCF310 digital camera.

Image acquisition was controlled by the Leica LAS AF software. The ImageJ software was used to determine the CSA of 1,000 to 3,000 individual fibres from at least two different fields for each muscle section. Four to nine sections from each muscle were analyzed.

# RESULTS

### nNOSμ deficiency affects mitochondrial network remodelling, and autophagy

NO is an important factor for muscle homeostasis; in vitro indications on cell lines and primary cells show that NO is able to modulate autophagy [147] and mitochondria morphology [2] both processes involved in the maintenance of muscle mass preventing muscle wasting[134, 147].The major source of NO in muscle is the neuronal NOS (NOS1)localized in the sarcolemma and associated with the dystrophin glycoprotein complex (DGC), thus to assess in details the role of NO in vivo on muscle, we studied the NOS1 knockout mice (NOS1-/-). In order to identify the changes in mitochondrial network morphology, tibialis anterior muscles of P120 NOS1-/- and wild-type control mice were imaged using pDsRed2-Mito, a mitochondrially targeted red fluorescent protein, by in situ two-photon confocal microscopy[143, 145, 153]. NOS1-/- mice showed a disorganized mitochondrial network (Fig. 1A). Accordingly, ultrastructural analyses by TEM revealed changes in the subsarcolemmal mitochondria of tibialis anterior muscles of NOS1-/- mice that exhibited, in thin sections, a significant increase in mitochondrial surface area (Fig 1B) and a significant decrease in the density of the cristae (Fig. 1C), as compared with the controls. The analysis of intermyofibrillar mitochondria showed a pattern of enlarged mitochondria indicating that the presence of these mitochondrial alterations in NOS1-/- mice muscle is not restricted to the sarcolemma but is a more general phenomenon.We then analysed autophagy a downstream process linked to mitochondrial stress: the two-photon confocal microscopy of the YFP-LC3 revealed the presence of LC3- positive vesicles, an established marker of autophagosome formation[163] in tibialis anterior muscles of P120 NOS1-/- mice (Fig. 1D). Furthermore, TEM analysis showed the presence of autophagic vacuoles and multivesicular bodies, indicative of an active autophagic pathway [164] (Fig 1B). The enhanced autophagy in the absence of NOS1 in skeletal muscle was confirmed by Western blot analysis. The appearance of a faster migrating band of LC3 protein due to its lipidation and cleavage is a common marker of autophagy induction [163]. As shown in figure 1E, tibialis anterior muscles of P120 NOS1-/- mice exhibited increased lipidated LC3 levels when compared to control mice.

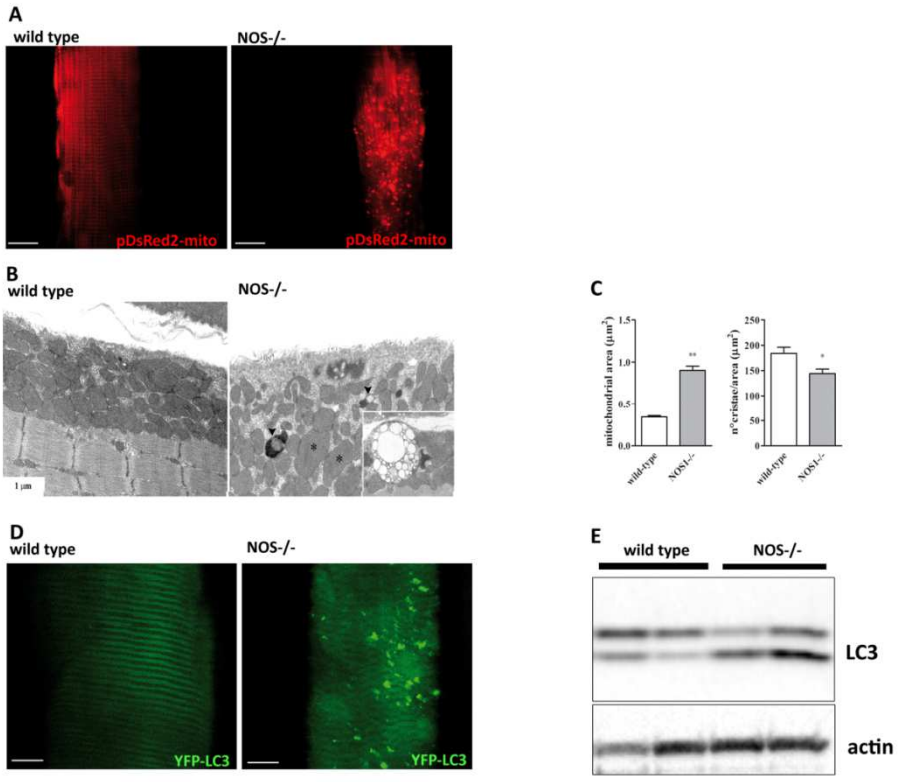



***Figure 1 Mitochondrial morphology and autophagy in skeletal muscles of NOS1-/- mice****Tibialis anterior muscles were isolated from wild-type and NOS1-/- mice at P120. (A) In vivo imaging of the mitochondrial network by two-photon confocal microscopy. Scale bar: 10 µm. (B) TEM images detecting the presence of abnormal, enlarged subsarcolemmal mitochondria (asterisks) or autophagic vacuoles (arrowheads) in NOS1-/- muscles. The inset depicts a multivesicular body in NOS1-/- fibres taken at higher magnification. (C) Subsarcolemmal mitochondrial ultrastructure analysis by TEM (D) In vivo imaging of autophagosome formation by two-photon confocal microscopy. Scale bar: 10 µm. (E) Western blot analysis of LC3 lipidation.*

## nNOSµ deficiency affects muscle growth

We evaluated the effects of the absence of neuronal NOS on skeletal muscle phenotype. Since the body weight and the visceral adipose tissue of NOS1-/- male (figure 2A and 2B) mice were significantly lower than wild-type control [54] we calculated the muscle size relative to body weight..As shown in Figure 2C, the relative mass of the muscles for the P120 NOS1-/- mice was significantly lower than the relative mass of the muscles for the control mice. This excludes the possibility that the changes in muscle mass are simply due to an overall change in the size of the mice. The overall morphology of the tibialis anterior muscle in P120 NOS1-/- mice was normal, without pathological features of necrosis, macrophage infiltration and centronucleatedfibres(Figure 2D). In addition, the number of fibres in tibialis anterior muscles was comparable in both NOS1-/- and control mice (Figure 2E).

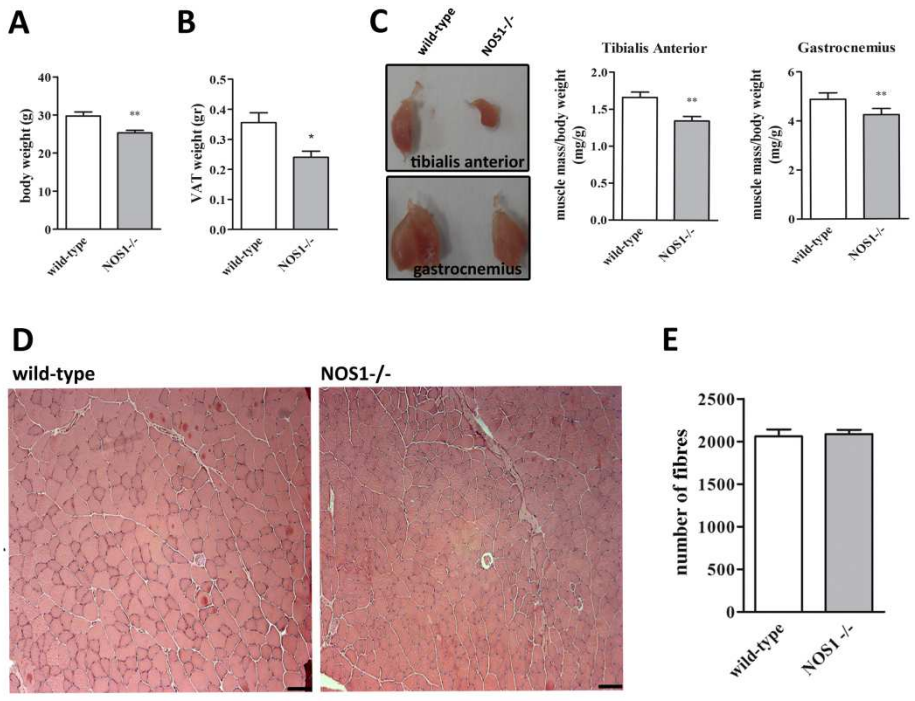



***Figure 2 Skeletal muscle phenotype of wild-type and NOS1-/- mice at P120****. (A)Body weight of mice (B) Weight of visceral adipose tissue (C) Weight of tibialis anterior and gastrocnemius muscles. The muscle size is relative to body weight. (D) Histological sections of* tibialis anterior*stained with H & E. Hematoxylin and eosin staining (E) The number of myofibres in tibialis anterior.*

By contrast, laminin staining of tibialis anterior, used to identify individual muscle fibers, revealed a significant decrease in the mean CSA of tibialis anterior sections in P120 NOS1-/- mice when compared with control (Figure 3A, B and C).

The examination of multiple time points was then carried out in order to establish a possible link between the changes in mitochondrial homeostasis and the reduction in muscle size. The CSA of hind limb muscle fibers were also significantly decreased at P10 (figure 3D-F), P30 in NOS1-/- mice (figure 3G-I) when compared with the respective control.Using NOS1-/- mice it has been previously shown that neuronal NOS modulates the mechanism of disuse-induced atrophy via FoxO transcription factors [146].

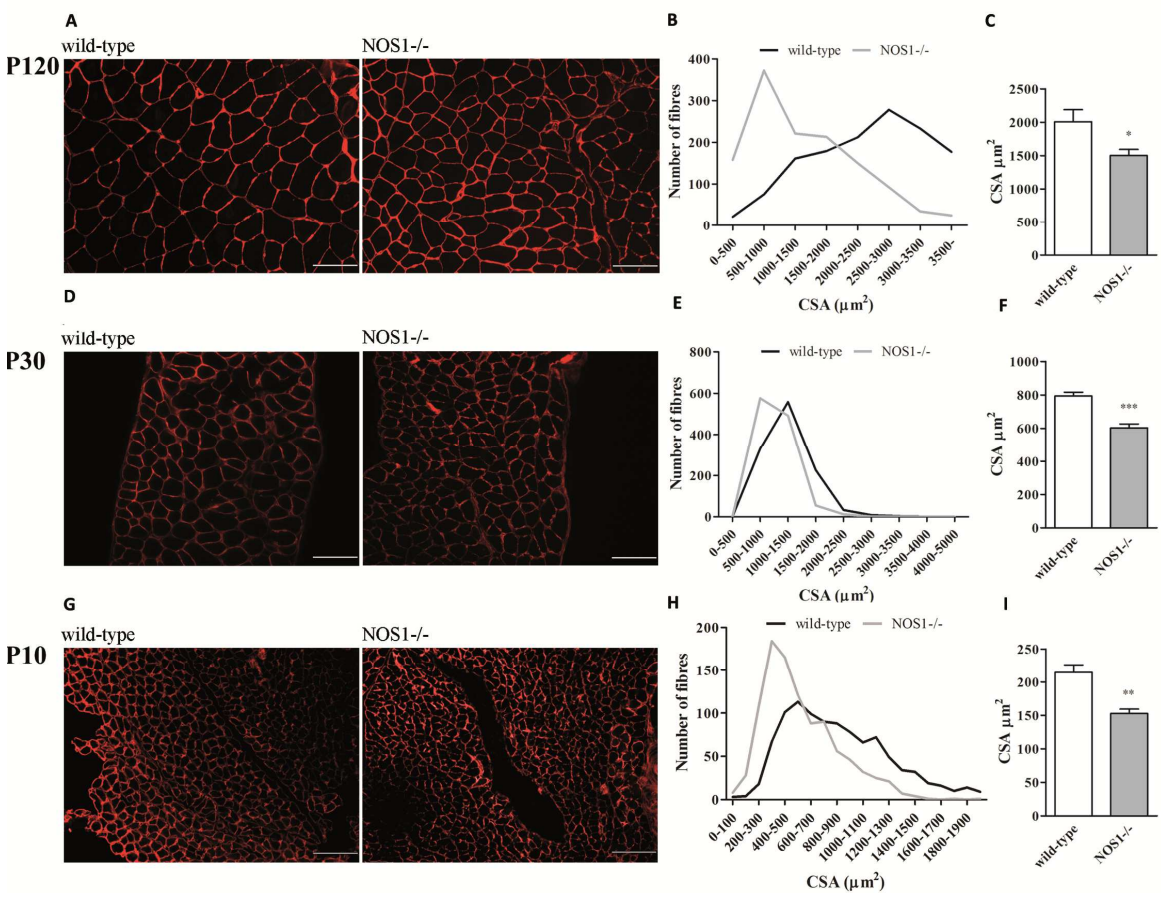



***Figure 3 Muscle size during development****(A-C-D) Laminin staining in tibialis anterior of wild-type and NOS1-/- mice at P120, P30, P10 respectively. (B-D-F) Representative distribution of CSA values and (C-F-I) quantification of CSA in tibialis anterior of wild-type and NOS1-/- mice at P120P30, P10 respectively.*

However, the observation that CSA was always reduced at any time points considered, together with the observation that at different time points NOS1-/- and control mice expressed similar levels of transcripts encoding the classical atrogenesAtrogin-1 and MuRF1 (figure 4A-C)[165], indicates that the atrophy pathways do not play a key role in the development of NOS1-/- muscles. Muscle growth during post-natal development (P0 to P21), but not at later stages, is accompanied by a continuous increase in the number of myonuclei resulting from satellite cell fusion[166].

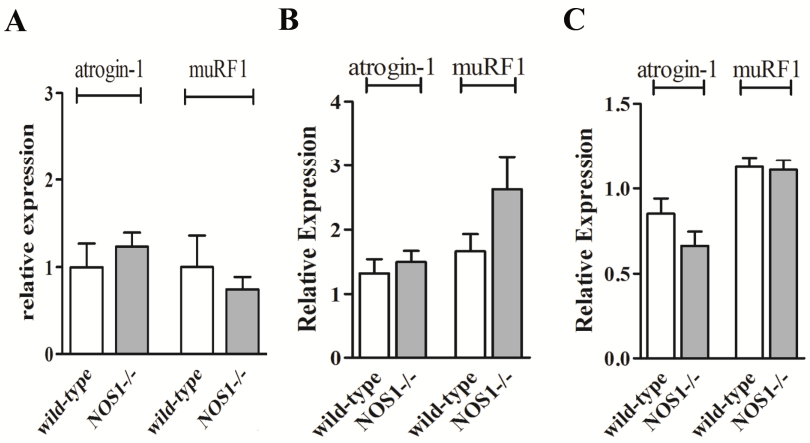



***Figure 4 Analysis of atrophic pathways during muscle development in NOS1-/- mice.****qPCR analysis of mRNA levels for atrogin-1and muRF1 in hind limb muscles of wild-type and NOS1-/- mice at P120(A) P30(B) and P10(C). Values are expressed as the fold change over wild-type.*

As shown in Figure 5A, NOS1-/- cells exhibited lower number of myonuclei inside the fibers at p10 suggesting defects in the proliferation and fusion of satellite cells. In agreement with this, levels of myosin and MyoD, which are markers of myogenic differentiation, were reduced in NOS1-/- satellite cells as compared to control cells (Figure 5B). Interestingly, CycloD staining of differentiating myogenic precursor cells confirmed that the absence of NOS1 induces diffuse mitochondrial fragmentation (Figure 5C) as previously described [5]2010.

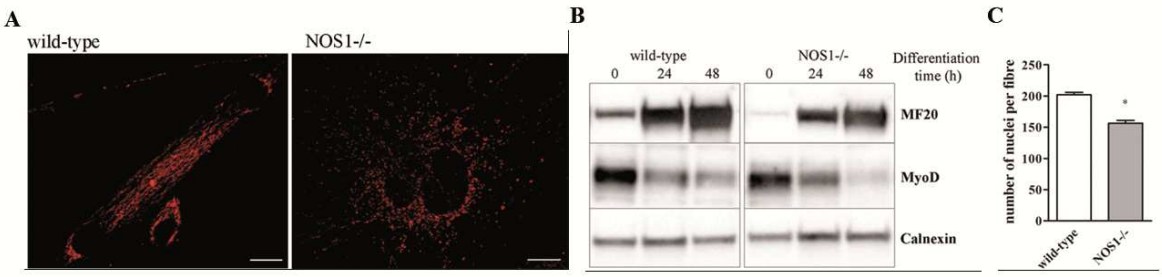



***Figure 5 Satellite cells*** *defects in NOS1 -/- mice. (A) Confocal microscopy imaging of satellite cells isolated from wild-type and NOS1-/- mice and differentiated for 48 hours. Mitochondrial morphology was detected by mitochondrial matrix-specific protein cyclophillin D staining. Scale Bar: 10 μm. (B) Western blot analysis of myosin (MF20) and MyoD expression in satellite cells isolated from wild-type and NOS1-/- mice and differentiated for increasing times (C) Number of myonuclei per fibre in hind limb muscles.*

Taken together, our data argue that the absence of NOS1in muscle form the beginning induces mitochondrial fragmentation, activates autophagy and results in a deficit in satellite cell fusion/differentiation, thus impairing fibers growth . Furthermore NOS1-/- mice could be considered a model of skeletal muscle wasting due to high autophagy induction and affecting satellite cells pool.

**NO signaling regulates autophagy machinery**

Activation of the NO-dependent enzyme guanylatecyclase, with formation of cyclic guanosine monophosphate (cGMP) and activation of a variety of downstream signaling cascades, including cGMP-dependent-protein kinases (PKG), contributes significantly to mediate the physiological effects of NO in muscle [154].

We analyzed the expression of relevant markers of the autophagic signaling pathway, namely LC3, by western blotting and p62, Bnip3 and Atg4 by qPCR analysis L-NAME, ODQ and KT5823 treatments increased lipidated LC3 conversion in differentiated satellite cells and LC3 lipidation induced by L-NAME and ODQ was blocked by DETA-NO or 8Br-cGMP, respectively (Figure 6A). In addition, cells treated with L-NAME, ODQ and KT5823 expressed higher levels of transcripts encoding p62, Bnip3 and Atg4 (Figure 6B).

As shown by confocal microscopy fluorescence analysis of LC3 and the mitochondrial matrix-specific protein cyclophillinD (Figure 6C), in control cells LC3 staining was diffuse and the majority of mitochondria were in the elongated form, indicating myogenic differentiation and a low rate of autophagy. L-NAME, ODQ, and KT5823 treatment, while inducing mitochondrial fragmentation, resulted in LC3 localization into dot cytoplasmic structures, as compared to the diffuse cytoplasmic distribution observed in control cells. The effects of L-NAME and ODQ were prevented by DETA-NO and 8Br-cGMP,respectively. Taken together these experiments recapitulate the results obtained with NOS1-/- satellite cells confirming the induction of autophagy associated with mitochondria fragmentation with a cGMP dependent pathway.

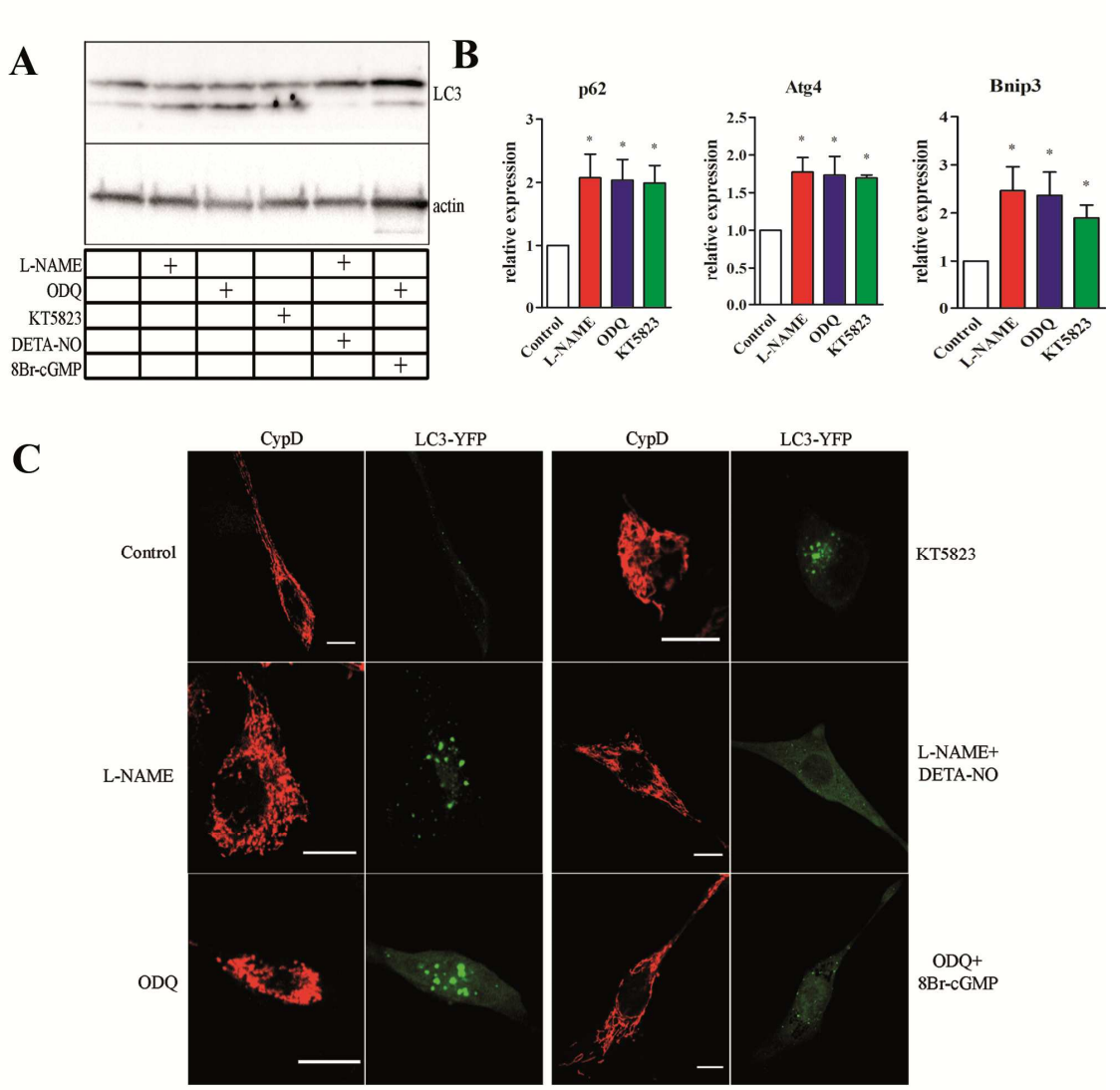



***Figure 6 NO signalling and autophagic pathway in satellite cells.*** *(A) Western blot analysis of LC3 lipidation in cells differentiated for six hours in the absence or in the presence of L-NAME (6 mM), ODQ (10 μM), KT5823 (1 μM), L-NAME + DETA-NO (80 μM), and ODQ +8 Br-cGMP (2.5 mM). (B) qPCR analysis of mRNA levels for p62, Bnip3 and Atg4 in cells differentiated for six hours in the absence (control) or in the presence of L-NAME ODQ, and KT5823. (C) Confocal microscopy imaging of cells transfected with YFP-LC3. Mitochondrial morphology was detected by mitochondrial matrix-specific protein cyclophillin D (CypD) staining. Scale Bar: 10 μm.*

So far these data demonstrate that an altered NO signaling in myogenic precursor cells leads to mitochondrial network fragmentation and increased autophagy induction resulting in significant defects in myogenesis process both in vivo and in vitro, decreasing muscle development and leading to muscle wasting.

Autophagy is essential to maintain muscle mass[136] but its role on regenerating population and its impacton muscle growth and development has not been evaluated yet. We have evidences from the NOS1-/- mice, described above, that high autophagy

induction impaired satellite cells population, however a better model to study this aspect is required.

**Generation of Muscle-Specific Atg7 Knockout Mice**

To investigate the physiological roles of autophagy in satellite cells population and its impact in muscle growth, we crossed Atg7-floxed mice (Atg7 $^{f/f}$) [6] with a transgenic line, expressing Crerecombinase, under the control of Pax7 promoter the best characterized marker of satellite cells, in order to generate mice with specific Atg7 deletion in the satellite cell population , which are hereafter referred to as Atg7-/-.

In figure 7A was shown the schematic representation of the generation of Atg7$^{f/f}$mice while the results of PCR genotyping were shown in figure 7B .

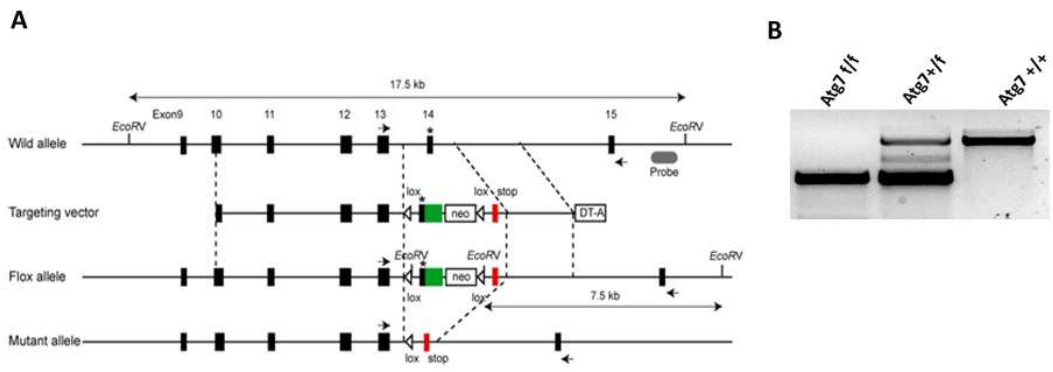



***Figure 7: Generation of Atg7f/f mice. (A)****Schematic representation of the targeting vector and the targeted allele of Atg7 gene. (B) Genotyping from genomic DNA of the Atg7f/f mice.*

Efficient and specific inhibition of autophagy in gastrocnemius skeletal muscles(Figure 8A) and satellite cells (Figure 8B) was confirmed by deletion of ATG7 and suppression of LC3 lipidation and in whole protein extracts.LC3 exists in two forms and the absence of LC3II band confirmed that the reaction of LC3 conjugation to phospholipids was completely blocked, inhibiting the formation of autophagosomes.

Conversely, Atg7 expression was unaffected in other tissues, such as brain liver lung and kidney (Figure 8C). Altogether, these findings validate our genetic mouse model of specific autophagy inhibition in muscle and satellite cells

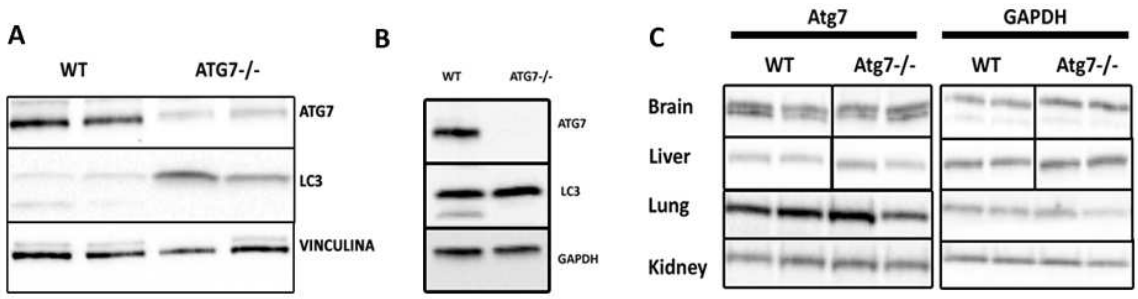



***Figure 8****(A)ATG7 expression and impaired LC3 lipidation in Atg7-/- gastrocnemius muscles and (B) in Atg7-/- satellite cells.(C) Immunoblot analysis of Atg7 and LC3 in homogenates from different tissues.*

## Autophagy inhibition in satellite cells induces muscle growth defects

Atg7-/- mice showed a marked phenotype compared to control mice,characterized by very significant differences in body weight between control and Atg7-/- mice, with the last one very smaller than control littermates (figure 9A).

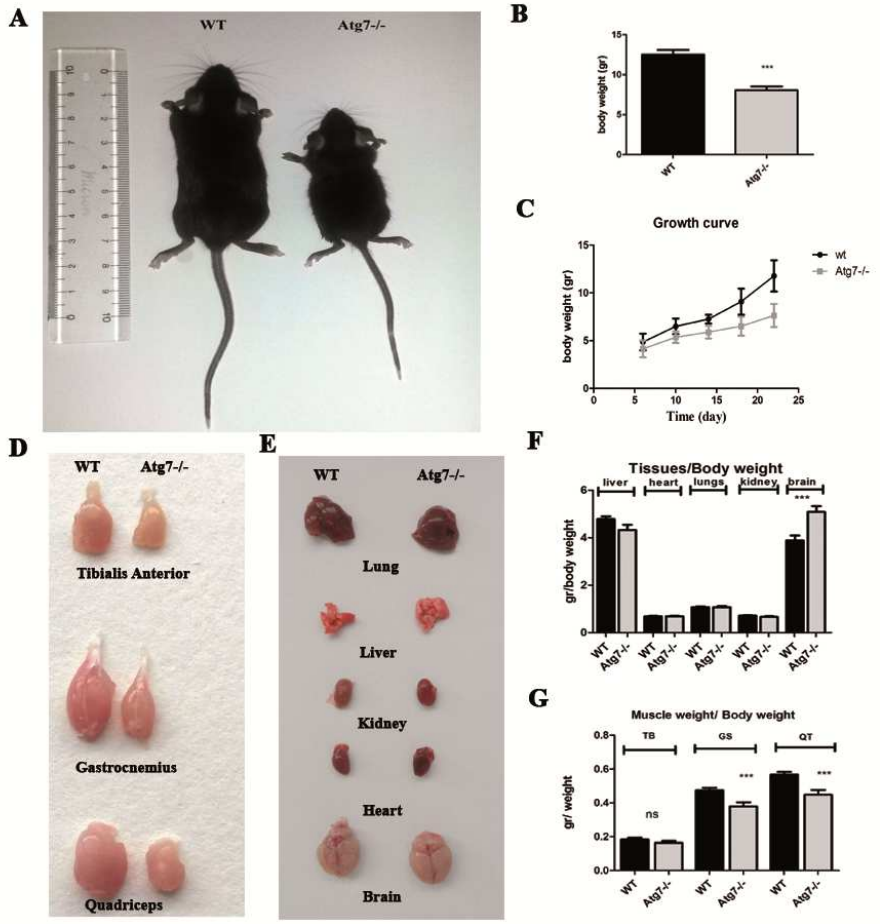



***Figure 9 Atg7 phenotype****(A) Comparison of Atg7-/- and wild-type (WT) bodyweight at 21 days of age. (B) Growth curve(C) Side-by-side comparison of various tissues including skeletal muscle from Atg7-/- and WT mice. (D) The weights of muscles and of various tissues were normalized to body weight.*

In particular, the growth curve showed a severe reduction in body weight of Atg7-/- mice, that was significantly impaired between day 14 and 21 (figure 9B). The first 3 weeks of postnatal life in mouse is a period of intense growth, with body weight increasing 7-8 fold, half of which is accounted for by the increasing in skeletal muscle [167] and in this phase satellite cells are essential to muscle growth. Normalizing all the organs weight to the body weight we demonstrated that only the quadriceps and gastrocnemius muscleswere smaller out-of-proportion to the body weight in Atg7-/- mice, suggesting that a defective autophagy in satellite cells population causes a muscular phenotype more serious, but with some similarities with NOS1 -/- mice, in spite of opposing autophagy conditions (figure 9C and 9D).

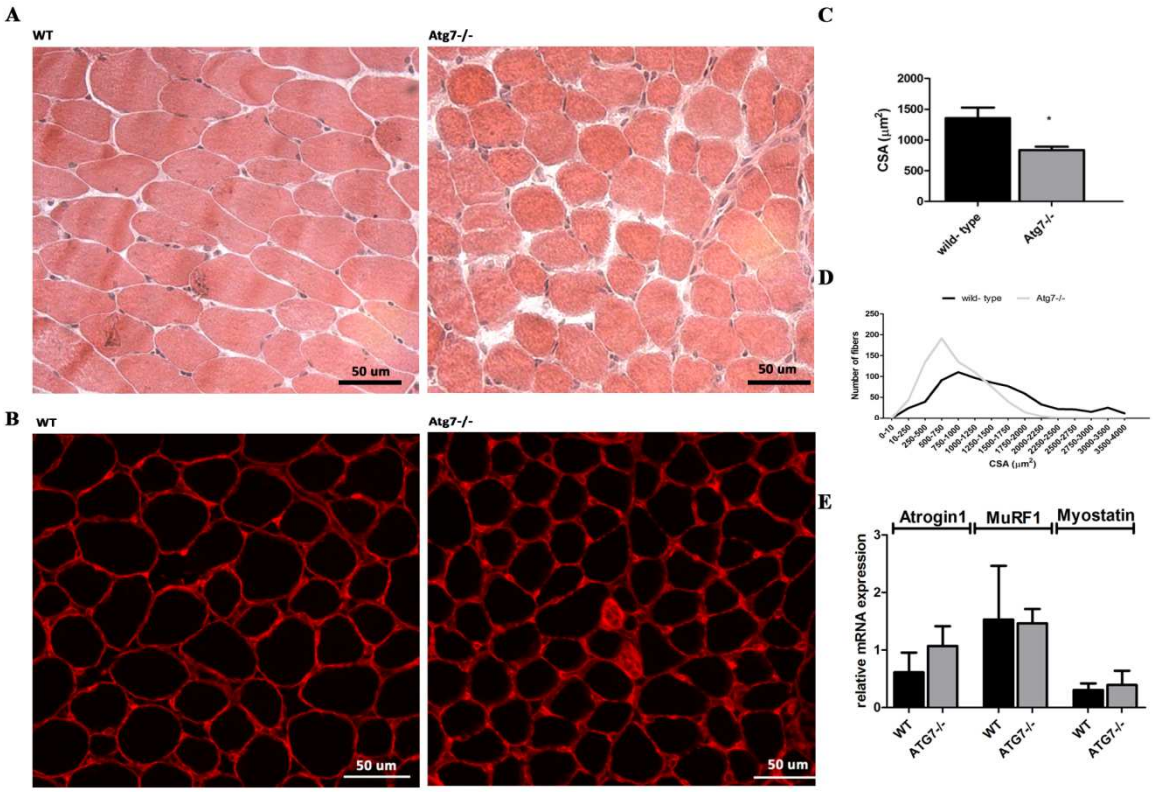



***Figure 10*** *(A)Hematoxylin and eosin staining in tibialis anterior of wild-type and Atg7-/- mice at P21 (B) Immunofluorescence Laminin on tibialis anterior of Atg7-/- and WTmice. (C) Quantification of CSA in tibialis anterior and (D) representative distribution of CSA values and of wild-type and Atg7-/- mice.(E) qPCR analysis of mRNA levels for atrogin-1,muRF1 and Myostatin in gastrocnemius of wild-type and Atg7-/- mice.*

To better understand the nature of the hypotrophic skeletal muscle phenotype observed in Atg7 -/- mice, tibialis anterior muscle sections from male Atg7-/- and control mice at p21 days after birth were stained by hematoxylin and eosin (H&E) (figure 9E) or immunostained with an anti-Laminin antibody (figure 9F). The overall morphology of the

tibialis anterior of Atg7-/- mice was normal, without pathological features such as necrosis, macrophage infiltration and centronucleated fibers, conversely laminin staining revealed a significant decrease in the mean CSA in Atg7-/- mice when compared with control littermates (figure 9G and 9H).

The decrease of muscle mass can be due to activation of atrophic pathway or alteration of pathways involved in the regulation of skeletal muscle growth such as the Myostatin pathway. We examined the mRNA levels of Myostatin[168], MuRF1 and Atrogin[142] for atrophic pathway and none of them were significantly altered, demonstrating that loss of autophagy does not affect these pathways howeveris crucial for early postnatal skeletal muscle growth mostly dependent on satellite cells population (figure 9I).

**Loss of autophagy affects myoblast proliferation and cell fate decision**

Muscle growth during post-natal development (P0 to P21), but not at later stages, is accompanied by a continuous increase in the number of myonuclei resulting from satellite cell proliferation and fusion [165-166]. In Atg7 -/- mice at p21 the number of myonuclei of gastrocnemius muscle fibres were significantly decreased (figure 10C), when compared with the respective control suggesting a defect in the balance between the proliferation of satellite cells, and their differentiation into muscle fibers.

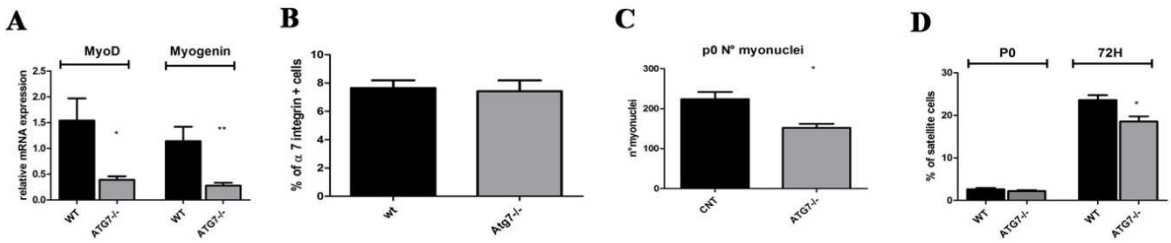



***Fig 11*** *(A) qPCR analysis of mRNA levels for atrogin-1,muRF1 and Myostatin in gastrocnemius muscle of wild-type and Atg7-/- mice.(B) Number of α7 integrin positive cells in skeletal muscle of wild-type and Atg7-/- mice. (C) Number of Myonuclei of isolated Myofibers obtained from wt and Atg7 -/- at p21 (D) Number of satellite cells on isolated myofibersobtained from wt and Atg7 -/- at p21 at two time points: after isolation (p0) and at 72h of proliferation*

The myogenic regulatory factors MyoD and Myogenin are the major components controlling satellite cell activity. The expression patterns of these two proteins provide some of the signals crucial for satellite cell progeny proliferation, differentiation into myocytes, or returning to quiescence [34, 169]. Thus, as surrogate markers of satellite cells activity in muscle; we evaluated the expression of MyoD and Myogenin and as expected Atg7-/- muscle showed significant reduction of both markers compared to control (figure 10A), confirming a defect of satellite cells population in muscle of Atg7-/-

mice. Since an impairment of satellite cells function can be related to reduction in muscle stem cell number, we determined the percentage of satellite cells resident in muscle after labeling withα7 integrin antibody and analysis through flow cytometry (figure 10B) excluding cells positive for CD45, CD31, SCA. Our results showed no significant differences in the average number of α7 integrin positive cells in tibialis anterior of Atg7-/- and control mice. Accordinglyon freshly isolated single myofibers, the average number of satellite cells labeled with Pax7 and MyoD antibodies was similar in Atg7 -/- and controls mice (figure 10D).Conversely after 72 h of proliferation in growth medium condition, when satellite cells undergo their first divisions, Atg7-/- fibers displayed significantly fewer satellite cells than controls (Figure 10D). This last finding suggests that the loss of autophagy in satellite cells population can affect the proliferation rate of these cells.

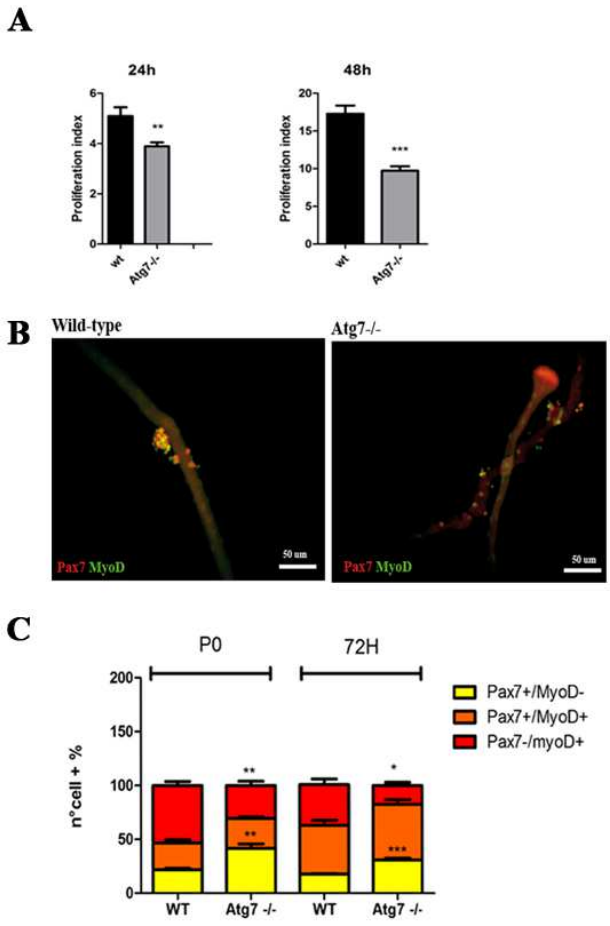



***Figure12* Loss of autophagy affects myoblast proliferation**. *(E) CytoTrack Cell Proliferation Assay analysis on isolated satellite cells (F) Number of proliferating Ki67+ satellite cells on isolated myofibers obtained from wt and Atg7 -/- at p21 at two time points: after isolation(p0) and at 72h of proliferation immunofluorescence for Pax7 and MyoD on single myofibres obtained from wt and Atg7 / mice at p21 (G) and quantification of satellite cells population on isolated myofibers(H) at two time points: after isolation and at 72h of proliferation*

To deepen this aspect, satellite cells isolated from Atg7-/- and control muscles, were labeled with CytoTrack Cell Proliferation Assay Kits and then monitored after 24 and 48h of proliferation by flow cytometry. In agreement with the results obtained on single muscle fibers the analysis of CytoTrack Cell Proliferation Assay dye dilution showed a lower proliferation rate of Atg7-/- satellite cells compared to control satellite cells (Figure 12A). To gain further insight into the role of autophagy on satellite cells we evaluated the myogenic cell determination on isolated myofibers after isolation and at 72h of proliferation in growth condition. Analyzing immunofluorescence for Pax7 and MyoD we observed that the number of MyoD+/Pax7-(cells committed to differentiate) decreased in Atg7-/- fibers (figure 12B). There was no significant differences in the number of MyoD+/Pax7+ cells (activated cells), whereas there was an increased number of $Pax7^{+}$/MyoD-(quiescent cells) cells in Atg7-/- fibers, suggesting a delay of satellite cells to progress into myogenic lineage and the presence of more cells in quiescent state (figure 12C).

Finally, to investigate whether loss of autophagy also regulates the ability of satellite cells to fuse and differentiate, myoblasts isolated from Atg7-/- and control mice were grown and differentiated in vitro. The cells were seeded and allowed to proliferate for 2 days before serum with drawalfor 48h. After this time myotubes were stained with MF20 and myonuclei were counted determining a fusion index (figure 13A)- This experiment demonstrated a reduced ability for Atg7 -/- satellite cells to fuse (figure 13B) into myotubesand moreover the myotubes in Atg7 -/- condition were smaller than control as shown by thesharp (figure 13C) decrease in myotubes diameter.

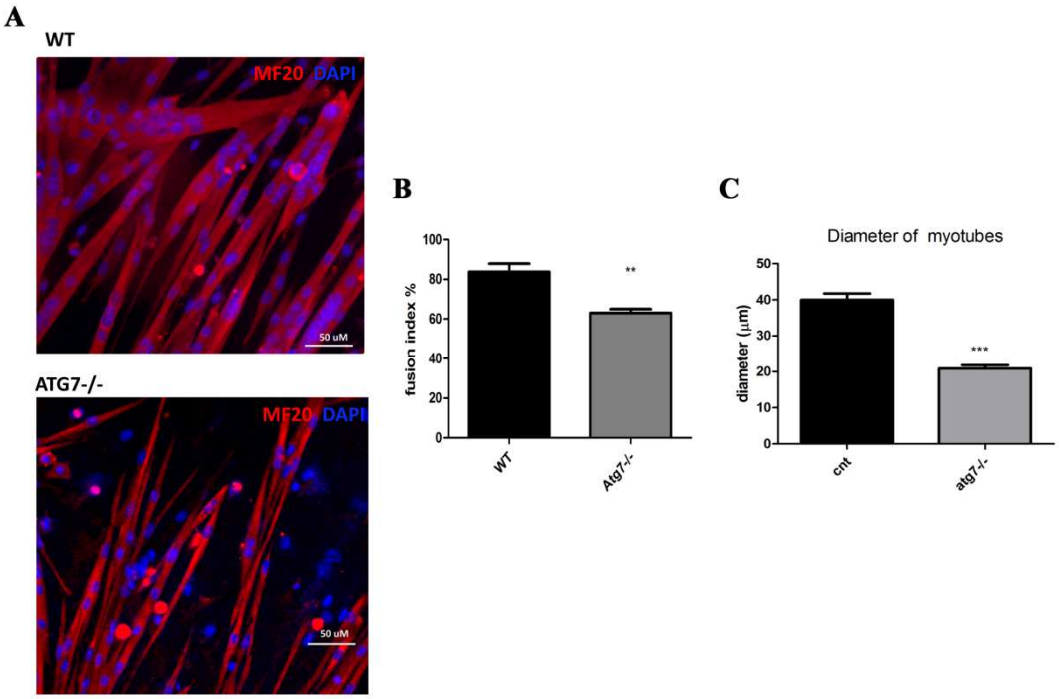



***Figure 13 Fusion index analysis in wt and Atg7 -/- mie****(A) immunofluorescence analysis of myotubes after 48h of differentiation of cultured satellite cells isolated from oAtg7-/- and control muscle and stained with the monoclonal antibody against MyHC (B) Fusion index quantification(C) measurement of myotubes diameter.*

Taken together, our data suggest that autophagy is involved in the control of satellite cells functions. Autophagy loss of function in satellite cells impairs their proliferation rate as well as their capability to fuse and differentiate and these results explain, at least in part, because the muscles of the Atg7-/- mice are smaller than the muscles of control mice. Surprisingly this condition is more severe but similar to another model of muscle wasting, NOS1 -/- mice, in which autophagy is enhanced due to mitochondrial defects, suggesting that unbalanced autophagic process during muscle development can affect in different ways regenerating population impairing their proliferation/differentiation capacity and compromising muscle formation in the early stage of development.

# DISCUSSIONS

This PhD thesis is divided in two sections: the formeris focused on the study of the relationship among the NO pathway, the mitochondrial structure/activity and skeletal muscle wasting, paying particular attention on the role of autophagy and using a mouse model in which NOS1 (nNOSμ) is absent. The latter has examined the role of autophagy in myogenic precursor cells(satellite cells) and muscle development utilizing a model of skeletal muscle specific autophagy-deficient mice (Atg7-/- mice).

The first important observation of this project is that nNOSμ deficiency is *per se* sufficient to induce profound defects in mitochondria, with alterations in mitochondrial distribution, shape, morphology and size accompanied by a latent mitochondrial dysfunction. Nitric oxide has several key functions in mitochondria: it inhibits mitochondrial fission, induces mitochondrial biogenesis and controls mitochondrial respiratory rate by reversible inhibition of complex IV in the mitochondrial respiratory chain [3, 5, 170]. Furthermore, it controls the expression of several enzymes in the Krebs cycle [171]. Derangement of these mitochondrial functions is most likely at the basis of the multiple mitochondrial deficits we observed in NOS1-/- mice. The correlation between mitochondrial defects and muscle impairment is an important aspect we highlight in this project. Alterations in the content, shape or function of the mitochondria appear to occur in damaged muscle and inhibition of mitochondrial fission protects from muscle loss during fasting [153]. Recent findings have also underlined the crucial role of autophagy in the control of muscle mass and functions [134, 152, 165]. Autophagy derangement is involved in a number of inherited muscle diseases [172] end of interest, mitochondria are involved in regulating autophagy[173]. We found an increase of autophagy shown by the increase in expression of molecules relevant to autophagic signalling, namely p62, Bnip3 and Atg4 in nNOSμ muscle deficient. This suggests that nNOSμ deficiency leads to a sufficiently severe mitochondrial deficit but also a enhanced autophagic response that were normalised when the cGMP-dependent signaling was activated, indicating that these events are controlled by NO via its physiological second messenger cGMP.

The second relevant information is that an altered NO system leads to impairment of muscle growth in postnatal phase. We found that skeletal muscles in the absence of nNOSμ are smaller relative to the rest of the body, thus indicating that muscle mass decrease was not simply attributable to a generalised decreased body mass tissues (including adipose tissue) and likely due to a specific reduction in the size of the muscle

fibres themselves. In agreement with this, NOS1-/- mice muscles displayed smaller myofibre CSA when compared to littermate controls, although they did not show any pathological features reminiscent of muscle damage, such as inflammation, necrosis or fibrosis. That the decrease in muscle mass is due to mechanisms other than the decrease in body mass was recently suggested using NOS1-/- mdx mice [174]. The deficiency of nNOSμ is also accompanied by muscle ageing [175] and fibre growth was prevented in the NOS1-/- mice model of skeletal muscle hypertrophy [176] and NOS1-/- mdx mice. Furthermore, the observation that at different time point NOS1-/- and control mice expressed similar levels of transcripts encoding the classical atrophygenes atrogin-1 and MuRF1, indicates that the atrophy pathways do not play a key role in the development of NOS1-/- muscles.

Moreover, in this model the absence of NOS1 altered mitochondrial homeostasis and autophagic pathway in myogenic precursor cells with a decrease in the number of myonuclei per fibres and impaired muscle development at early stages of growth. This suggests that fusion of myogenic precursor cells during perinatal myogenesis is impaired. Accordingly, NO has been shown to stimulate the ability of myogenic precursor cells to become activated and fuse to each other [70, 73, 154]. There is a general agreement that mitochondria change when the myoblasts differentiate into myotubes. Also, NO maintains functional mitochondria and this permits differentiation of myogenic precursor cells in vitro. An altered NO signaling in myogenic precursor cells led to mitochondrial network fragmentation and increased autophagy induction resulting in significant defects in myogenesis process both in vivo and in vitro, decreasing muscle development.

In conclusion, the first part of this work suggests that NO regulates key homeostatic mechanisms in skeletal muscle, namely mitochondrial bioenergetics and autophagy and influences the fate of satellite cells population and their ability to stimulate muscle development.

Mitochondrial dysregulation and autophagy induction alter satellite cells behaviorand differentiation capacity leading to muscle wasting. Of notice, autophagy is essential to maintain muscle mass and impaired autophagy is known to induce skeletal myofiber degeneration and muscle weakness [136-137], but its role on regenerating satellite cells and its impact on muscle growth and development has not been evaluated yet.

Toward this goal, in the second part of my PhD project we generate a transgenic mice in which autophagy is selectively inhibited in satellite cells, crossing Atg7-floxed mice with a transgenic line expressing Cre recombinase under the control of the Pax7 promoter.The

paired-box gene Pax7 has been chosen since it has been reported that homozygous Pax7 mutant mice completely lack muscle satellite cells, thus suggesting that Pax7 is at the top of the molecular hierarchy controlling satellite cell specification and it may be considered as a major regulator of satellite cell renewal and propagation [177].

Measuring body weight after birth we detected very significant differences between control and Atg7 -/- mice with the last one smaller than control littermates. Normalizing all the organs weight to the body weight we demonstrated that only the skeletal muscle tissue was smaller out-of-proportion to the body weight in Atg7-/- mice. KO mice presented with reduced myofiber cross sectional area, absent centralized myonuclei and any difference in the expression of atrophy markers, MuRF-1 and Atrogin-1 or Myostatin, indicating absence of skeletal muscle degeneration (18) and suggesting that a defective autophagy in satellite cells population causes a muscular phenotype more serious, but with some similarities with NOS1 -/- mice, in spite of opposing autophagy conditions.

Reduced myofiber size, probably resulted from defective satellite cells function in muscle, indicated that the loss of Atg7 in Pax7 progenitors affected myocyte size, supporting the contribution of autophagy to skeletal muscle development. Thus, this study identifies autophagy as an important new regulator of postnatal myogenesis that is required for normal muscle growth, maintenance, and regeneration. Skeletal muscle postnatal growth is dependent on the expansion of satellite cells and on the balance between their proliferation and differentiation into muscle fibers. Indeed embryonic muscle development in*Pax7* germ line null mice is grossly normal[178]; however, postnatal muscle growth and regeneration in these mice is severely impaired [23, 179] proving that Pax7 cells contribute to adult myonuclei.

Many diseases involving muscle wasting such as congenital myotonic dystrophy are suggested to involve impaired satellite cell function [180] and several studies have demonstrated the importance of autophagy in the maintenance and function of stem cells [181]. One of these studies, in which atg7 is genetically knocked out in Myf5+ progenitor cells that give rise to muscle and brown adipose tissue, demonstrated the requirement for autophagy in proper brown fat and skeletal muscle development [182]. Another recent study using a hematopoietic stem cell(HSC) specific knockout of atg7 suggests that inhibition of autophagy may lead to a loss of quiescence [183]. Other studies perturbing metabolic pathways that may regulate autophagy show defects in the maintenance of quiescence. Here, we show that autophagy loss of function in satellite cells impairs their

proliferation rate as well as their capability to fuse and differentiate. Furthermore, analyzing immunofluorescence for Pax7 and MyoD on isolated myofibers we observed that the number of cells committed to differentiate decreased in Atg7-/- fibers, whereas the number of quiescent cells increased, suggesting a delay of satellite cells to progress into myogenic lineage and the presence of more cells in quiescent state. Therefore, it is likely that the delayed muscle growth in*Atg7-/- mice* is due to satellite cell/myoblast dysfunction.

Regarding the precise nature of this dysfunction, the exit of a stem cell out of quiescence into an activated state to differentiate is characterized by major metabolic changes associated with increased biosynthesis of proteins and macromolecules. The regulation of this transition is poorly understood. Autophagy, which catabolizes intracellular contents to maintain proteostasis and to produce energy during nutrient deprivation, was induced and its inhibition can suppress the increase in ATP levels and delayed satellite cell activation and differentiation [184].

Stem cells have unique metabolic hallmarks that characterize their quiescent, proliferative, and differentiated states [185]. For instance, quiescent HSCs, which have low mitochondrial content and oxidative activity, primarily generate ATP from glycolysis rather than oxidative phosphorylation [186-187]. Proliferating cells, on the other hand, have an increased demand for biogenesis and primarily use oxidative phosphorylation to generate ATP [188]. As proliferating cells cycle, aerobic ATP production and oxidative phosphorylation fluctuate, increasing in G1 phase [189]. In fact, without sufficient energy and metabolites, proliferating cells will arrest in G1 phase [190].

Furthermore, a recent study of Rando et coll., show that the inhibition of autophagy, obtained by using pharmacological and siRNA approaches to inhibit it acutely,does not lead to a loss of satellite cells quiescence but rather delays the activation of these cells out of the quiescent state. This study demonstrating that autophagy contributes to the metabolic adaptation that satellite cells undergo during activation add to the understanding of the importance of metabolic flexibility in stem cell fate determination. Stem cells adapt their energy usage and production via the manipulation of different metabolic pathways to support the changing bioenergetic needs during quiescence, proliferation, differentiation, or self-renewal [185](36). All these studies suggest that the activation out of quiescence is an example of a stem cell function that requires rapid and dramatic changes in the metabolic activity and that the induction of autophagic activity may be a critical component of those metabolic shifts.

In summary, the data presented here are of major importance to understand the mechanisms and pathways that govern muscle growth and repair processes.In two different models of muscle wasting characterized by two opposite autophagic conditions we detect similar defects in the myogenic precursor population, but with a different extent. In NOS1-/- the upregulation of autophagy induces a mild muscle phenotype associated with slower skeletal muscle development, but not with atrophic program. This defects is due to satellite cells impairments causing reduced proliferation and fusion during perinatal myogenesis. Similarly, in absence of autophagy specifically in satellite cells, their proliferation and fusion is marked impaired resulting in reduced skeletal muscle growth and development with the onset of a severe phenotype.

The study of these two different transgenic mice reveals as in muscle unbalanced autophagy duringthe early phase of growth affects satellite cells population causing muscle wasting and suggests how during skeletal muscle development is important to have appropriate levels of autophagy.

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