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CORSO DI DOTTORATO DI RICERCA IN SCIENZE BIOMEDICHE SPERIMENTALI

CICLO XXX

# The role of Mitok$_{ATP}$ in skeletal muscle

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# TABLE OF CONTENTS

## SUMMARY

Mitochondria ATP-sensitive potassium channels (mitoK$_{ATP}$) were first described in 1991 by direct patch clamp of isolated mitoplasts. Different approaches have been used to characterize the pharmacological profile of the channel. These studies strongly support the idea that cellular protection from damage well correlates with the opening of mitoK$_{ATP}$. Indeed, pharmacological activation of the mitoK$_{ATP}$ can efficiently protect the heart from ischemia/reperfusion injury. However, despite its huge therapeutic potential, the molecular identity of the mitoK$_{ATP}$ is still unknown.

In my laboratory, a novel protein complex that recapitulates the main electrophysiological features and the pharmacological profile of mitoK$_{ATP}$ has been identified. In particular, it has been demonstrated that the mitoK$_{ATP}$ is a heteroligomer composed by a tetramer of mitochondrial $K^+$ channel forming subunits (mitoK) associated with four ATP-sensitive regulatory subunits (mitoSUR) arranged in an octameric complex.

MitoK$_{ATP}$ is believed to act as a sensor of the cellular metabolic state. While in physiological conditions, i.e. when ATP is abundant, it remains mainly closed, a decrease in intracellular ATP levels triggers its opening, an event considered to be protective in a variety of stress conditions. However, although in stress condition the opening of mitoK$_{ATP}$ channel is protective, in basal condition, when intracellular ATP concentration is sufficient, its constitutive activation is not favorable. Experiments performed in cells indicate that the overexpression of mitoK, the potassium permeant subunit of the channel, induces a decrease in mitochondrial $Ca^{2+}$ transients evoked by agonist stimulation, a drastic reduction of mitochondria membrane potential, fragmentation of the mitochondrial network and derangement of the IMM ultrastructure, with the total collapse of the cristae. These severe changes in mitochondrial structure and function are due to the uncontrolled influx of $K^+$ and the subsequent osmotically obligated water into the mitochondrial matrix. Indeed, the overexpression of the potassium channel

forming subunit (mitoK) causes the constitutive opening of the mitoK$_{ATP}$ channel, which is not anymore controlled by the ATP sensitive subunits.

Overall, these data demonstrate that mitoK plays an important role in the control of mitochondria structure and function.

Due to the importance of mitochondrial function in muscle homeostasis, we investigate the pathophysiological role of the mitoK$_{ATP}$ in skeletal muscle. Firstly, we decided to investigate the effect of mitoK overexpression during post-natal development. For this purpose, newborn mice were injected with AAV9 (Adeno Associated Virus Serotype 9) particles expressing mitoK (AAV9mitoK). Four weeks after infection, we observed a great reduction of muscle weight, which was due to decreased myofibers size. Electron microscopy analysis demonstrated alterations in the mitochondrial structure: the internal cristae were totally collapsed and mitochondria were swollen. Inside the organelles, many vacuoles were also present indicating that damaged mitochondria were being degraded. In skeletal muscle mitoK overexpression induces a broad stress response, as indicated by the expression profile of specific genes, and activates cell proteolytic pathways to eliminate damaged organelles and dysfunctional proteins. Mitok overexpressing muscles were also characterized by a great increase in protein polyubiquitination and a block of the late stage of autophagic flux. As compensatory mechanism to counteract muscle wasting, protein synthesis was activated and IGF/AKT pathway, one of the major signalling pathways involved in the control of muscle mass, was induced. Taken together our results indicate that mitoK plays a role on the control of muscle mitochondria function and on muscle trophism.

## RIASSUNTO

I canali mitochondriali del potassio sensibili all'ATP (mitoK$_{ATP}$) sono stati descritti per la prima volta nel 1991 in seguito ad esperimenti di patch clamp su mitoplasti isolati. Diversi approcci sono stati utilizzati per caratterizzare il profilo farmacologico di questi canali. Questi studi sostengono fortemente l'idea che l'apertura di mitoK$_{ATP}$ sia correlata con la protezione cellulare da danni.

In particolare, l'attivazione farmacologica del mitoK$_{ATP}$ può efficientemente proteggere il cuore dal danno da ischemia/riperfusione. Tuttavia, nonostante il loro enorme potenziale terapeutico, la composizione molecolare dei canali mitoK$_{ATP}$ è ancora sconosciuta.

Nel mio laboratorio è stato identificato un nuovo complesso proteico in grado di riassumere le principali caratteristiche elettrofisiologiche e il profilo farmacologico del mitoK$_{ATP}$. In particolare, abbiamo dimostrato che il canale mitocondriale del potassio è un eteroligomero, organizzato in una struttura ottamerica, composto da un tetramero di subunità permeabili al catione potassio e da quattro subunità regolatorie sensibili all'ATP.

Si ritiene che i mitoK$_{ATP}$ siano in grado di agire da sensori dello stato metabolico cellulare. Mentre in condizioni fisiologiche, quando l'ATP disponibile è sufficiente, i canali restano chiusi, una riduzione dei livelli intracellulari di ATP causa la loro apertura, evento considerato protettivo in varie condizioni di stress cellulare.

Tuttavia, nonostante in condizioni di stress cellulare l'attivazione di mitoK$_{ATP}$ risulti vantaggiosa, in condizioni basali, quando la concentrazione intracellulare di ATP è sufficiente, l'apertura non controllata del canale risulta dannosa. Esperimenti svolti in cellule indicano che la sovraespressione di mitoK, la subunità del canale sensibile al potassio, induce una riduzione dell'accumulo mitocondriale dello ione calcio in risposta a stimoli, una drastica riduzione del potenziale di membrana mitocondriale e la frammentazione della morfologia mitocondriale caratterizzata dalla perdita totale delle creste.

Questi drastici cambiamenti della struttura e della funzione cellulare, sono dati dall'incontrollato influsso di potassio e da una conseguente entrata di acqua per

osmosi all'interno della matrice mitocondriale. Infatti, la sovraespressione della subunità del canale permabile al potassio (mitoK) causa una costitutiva apertura del canale, il quale non risulta più controllato dalle subunità regolatorie ATP-sensibili. Nel complesso, i nostri dati indicano che mitoK svolge un ruolo importante nel controllo della funzione e della struttura mitocondriale.

Data l'importanza della corretta funzionalità mitocondriale nel mantenimento dell'omeostasi muscolare, abbiamo indagato il ruolo patofisiologico dei mitoK$_{ATP}$ nel muscolo scheletrico. Come prima cosa abbiamo deciso di analizzare gli effetti della sovraespressione di mitoK durante lo sviluppo post-natale. A questo scopo abbiamo iniettato topi neonati con particelle di AAV9 (Virus Adeno Associato Serotipo 9) esprimenti mitok (AAV9mitoK). Quattro mesi dopo l'infezione abbiamo osservato un fenotipo fortemente atrofico, caratterizzato da una forte riduzione della grandezza delle fibre muscolari. Analisi di microscopia elettronica indicano che la sovraespressione di mitok altera la struttura mitocondriale: le cristae mitocondriali interne sono completamente collassate e i mitocondri appaiono rigonfi. All'interno degli organelli sono inoltre presenti molti vacuoli i quali suggeriscono l'attivazione di sistemi di degradazione dei mitocondri disfunzionali. Nel muscolo scheletrico, la sovraespressione di mitoK induce un'ampia risposta di stress cellulare che porta all'attivazione di sistemi di degradazione degli organelli danneggiati e delle proteine disfunzionali. Nei muscoli sovraesprimenti mitoK abbiamo infatti riscontrato un sostanziale aumento delle proteine ubiquitinate, destinate alla degradazione, ed un blocco delle fasi tardive del flusso autofagico. Per contrastare la perdita eccessiva di materiale citoplasmatico, nelle fibre muscolari viene incrementata la sintesi proteica e attivata una delle principali vie di segnalazioni cellulare implicata nel controllo della massa muscolare, la via IGF/AKT.

Nel complesso i nostri dati indicano che mitoK gioca un ruolo importante nel controllo della funzionalità mitocondriale nel muscolo e nel controllo del trofismo del muscolo scheletrico.

# INTRODUCTION

## MITOCHONDRIA: THE GENERAL FRAMEWORK

Mitochondria are dynamic organelles essential for maintaining cellular energy balance and other essential cellular processes. They are the main site of ATP synthesis achieved via oxidative phosphorylation. Beside the well-known role of the mitochondria in energy metabolism, regulation of both necrotic and apoptotic cell death pathways have been recently proposed as additional features of these organelles. This function in cell death seems to be closely linked to their role as the major intracellular source of reactive oxygen species (ROS). An excessive ROS production can lead to the oxidation of macromolecules and has involved in ageing and cell injury. Given these vital functions, it might be expected that mitochondrial dysfunction is linked with many human diseases, including neurodegenerative diseases (Alzheimer's, Parkinson's, Hungtington's), motoneuron disorders (amyotrhophic lateral sclerosis, type 2A Charcot-Marie-Tooth neurophaty), autosomal dominant optic atrophy, ischemia-reperfusion injury, diabetes, ageing and cancer (Duarte et al., 2014; Gaude and Frezza, 2014).

From a morphological point of view, mitochondria are defined by two structurally and functionally different membranes: the plain outer membrane (OMM), permeable to ions and metabolites up to 5 kDa, and the highly selective inner membrane (IMM), characterized by typical invaginations called *cristae* which enclose the mitochondria matrix. The space between the two membranes is called intermembrane space (IMS). The cristae define internal compartments formed by profound invaginations originating from narrow tubular structures called *cristae junctions* (Mannella, 2006) that limit the diffusion of molecules from the intra-cristae space towards the IMS, thus creating a micro-environment where the mitochondrial Electron Transport Chain (mETC) complexes are hosted. The oxidative phosphorylation system is composed by four different protein complexes: complex I (NADH dehydrogenase), complex II, (Succinate dehydrogenase), complex III (Ubiquinol cytochrome C reductase) and complex IV

(cytochrome c oxidase) plus complex V which is represented by the ATP synthase. Electrons from substrate oxidations are transferred from NADH and $FADH_2$ into the electron transport chain at complex I or II, then subsequently flow to complex III, IV and, at the end, to molecular oxygen. During the electron transport along the respiratory chain, energy is stored as an electrochemical gradient across the inner membrane thus creating a negative mitochondrial membrane potential (around -180mV against the cytosol). Protons return into the matrix mainly through complex V, the ATP synthase, which couples this proton driving force to ADP phosphorylation, according to the chemiosmotic theory.

## MITOCHONDRIAL POTASSIUM CYCLE

Mitochondria play a key role in energy production within the cell and are involved in the regulation of survival and cell death, thus the maintenance of the integrity of internal mitochondrial membranes is essential. Within this context, potassium channels are fundamental for the maintenance of proper mitochondrial structure and in triggering cardio- and neuro-protection (O'Rourke, 2004; Szewczyk et al., 1999). The transport of potassium across mitochondrial membrane is called "$K^+$ cycle" and consists of influx and efflux pathways for $K^+$ , $H^+$ , and anions (Garlid and Paucek, 2001). The chemiosmotic theory, postulated by Peter Mitchell, is the basis for understanding the mitochondrial potassium cycle. The high negative electrical membrane potential across the IMM, generated by protons extrusion through the electron transport system, represent a driving force for $K^+$ influx from the IMS to the matrix. Mitochondrial ATP-sensitive $K^+$ channels (called mito$K_{ATP}$) allows the diffusion of $K^+$ inside the mitochondrial matrix. Net uptake of $K^+$ is accompanied by osmotically obligated water, permeating through mitochondrial aquaporins, resulting in matrix swelling. Excess matrix $K^+$ is then extruded by the electroneutral $K^+/H^+$ antiporter, which uses the energy stored in the proton gradient to export $K^+$ (Figure 1). Thus, dynamic regulation of mitochondrial $K^+$ flux is essential for maintaining the structural and functional mitochondrial integrity necessary for

oxidative phosphorylation. This cycle can potentially modulate the tightness of coupling between respiration and ATP synthesis, thereby maintaining a balance between energy supply and demand in the cell and controlling the magnitude of the Δψ and ΔpH. The mitochondrial potassium cycle is tightly regulated to respond to changes in mitochondrial matrix.

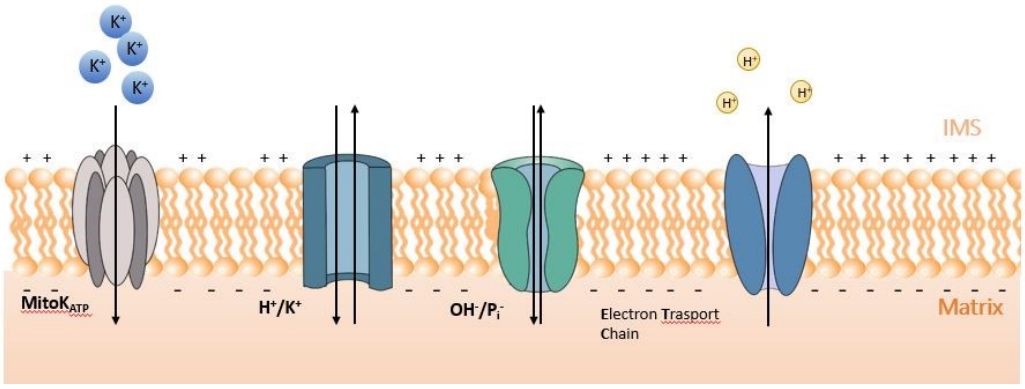


***Figure 1 – Schematic representation of $K^+$ cycle across mitochondrial membrane.***

Under physiological conditions, $K^+$ influx, accompanied by anions and water, is counteracted by the $K^+$ /$H^+$ antiporter, whose activity increases in response to increase in matrix volume. A decrease of matrix volume causes decreased $K^+$ /$H^+$ antiporter, and a new steady state balance is achieved.

Beside the control of matrix volume, potassium transport plays additional roles in mitochondria. It is essential to prevent excess matrix contraction, which can inhibit electron transport and perturbate the structure and function of the intermembrane space. Another important function of potassium cycle is the protection of cells from ischemia-reperfusion injury. This function strictly depends on mitoK$_{ATP}$ channel: the opening of this channel triggers increased mitochondrial production of reactive oxygen species involved in the signalling pathways of cardioprotection.

## MITOCHONDRIAL POTASSIUM CHANNELS

Mitochondria are complex organelles composed by two different membranes: the outer mitochondrial membrane (OMM) and the inner mitochondrial membrane (IMM). The IMM contains the essential components of the electron transport chain, which carry out the oxidative phosphorylation. The passive ion movement across membranes is driven by its electrochemical gradient ($\Delta\mu_{ion}$), which has two component: the electrical one ($\Delta\psi_m$), represented by the voltage difference between the two side of the membrane, and the chemical one ($\Delta p_{ion}$) reported as the concentration of ion on the two sides. The membrane potential difference ($\Delta\psi_m$) created by proton efflux from the matrix is a major component of ($\Delta\mu_{ion}$) across IMM for any ion (Szabo and Zoratti, 2014).

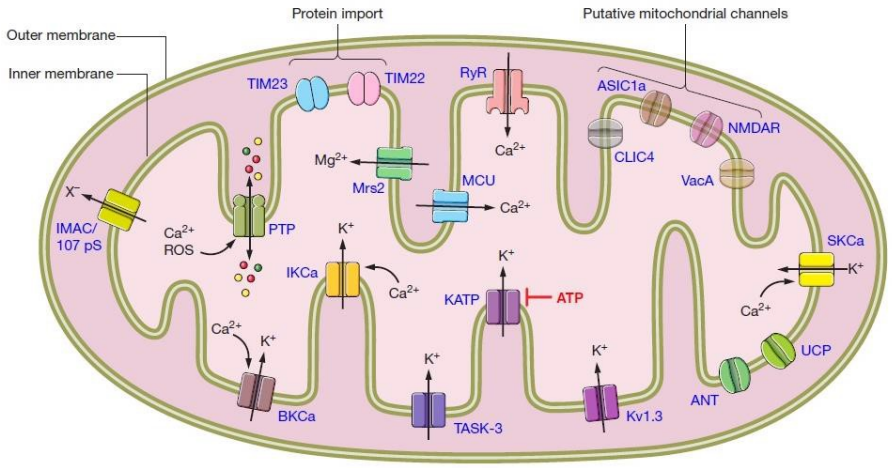


***Figure 2 - Overview of all electrophysiological activities in the IMM.***

Recently the field of mitochondrial ion channel has made great progress and it is now clear that potassium channels are essential for energy supply by mitochondria and have a great impact on the fate of cell (Figure 2).

$K^+$ is the major monovalent cation in the cytoplasm (~150mM), so the electrogenic transport of this ion inside the mitochondrial matrix is strictly ion channel dependent and highly regulated. Up to now, different mitochondrial potassium

channels, present in the inner mitochondrial membrane, have been described. Among them there are: the ATP-regulated potassium channels (*mitoKATP*) (Inoue et al., 1991), the large-conductance $Ca^{2+}$ activated potassium channels (*mitoBKCa*) (Siemen et al., 1999), a voltage-dependent potassium channels (*mitoKv 1.3*) (Szabò et al., 2005) and *twin-pore TASK-3* potassium channels (Rusznák et al., 2008). More recently other two $Ca^{2+}$dependent potassium channel has been discovered: the intermediate-conductance calcium activated potassium channel (*mitoIKCa*) (De Marchi et al., 2010) and a small-conductance calcium-activated potassium channel (*mitoSKCa*) (Stowe et al., 2013). The basic biophysical properties of these channels are similar to some potassium channels present in the plasma membrane of various cell types.

## The mitochondrial ATP sensitive potassium channels

One of the most debated topic in the field of mitochondrial channels is the existence of mitochondrial $K_{ATP}$ channels (mito$K_{ATP}$).

In the 1980s, several groups observed $K^+$ channel activity of proteins purified from mitochondrial membranes and reconstituted into bilayer lipid membranes (BLM). In 1980 and in 1988, the groups of Mironova and Marinov tried to reconstitute a specific $K^+$ uniporter. They isolated a 53-57 kDa protein that conferred a Mito$K_{ATP}$ channel activity in BLM (Mironova et al., 1981). However, they did not provide the exact amino acid sequence. The first evidence of the existence of the mito$K_{ATP}$ channel derived from the study of Inoue and co-workers. They identified the mito$K_{ATP}$ channel in single channel patch-clamp recordings of rat liver mitochondrial inner membrane (Inoue et al., 1991). These authors identified a single $K^+$ selective channel, which was reversibly inactivated by ATP. It is also inhibited by 4-aminopyridine and glibenclamide. They named this channel mitochondrial ATP-sensitive $K^+$ channel (mito$K_{ATP}$). Later, mito$K_{ATP}$ was also described in heart, brain, kidney, skeletal muscle and T lymphocytes. In 1998,

Garlid's group, demonstrate that mito$K_{ATP}$ exhibits many properties similar to those of the plasma membrane $K_{ATP}$ channel.

The plasma membrane $K_{ATP}$ channel (pm$K_{ATP}$) acts as an important sensor of the cellular energetic state. It is closed when energy (ATP) is abundant and opens when ATP decreases. In 1983, pm$K_{ATP}$ channels were described for the first time by patch clamping experiment in cardiac myocytes (Noma, 1983). Subsequently, they have been found in many other cell types like pancreatic ß-cells, where they regulate the glucose-stimulated insulin secretion (Ashcroft and Gribble, 1999), vascular smooth muscle cells, in which they regulate vascular tone, and skeletal muscle fibers, where pm$K_{ATP}$ may be active during fatigue (Davies et al., 1991). In cardiac muscle, they were correlated with ischemic preconditioning and ischemic protection (Grover et al., 1989). From the molecular point of view, $K_{ATP}$ channels are composed by a tetramer of ~40kDa Kir monomers, which represent the potassium channel-forming subunits, associated with four regulatory sulfonylurea receptor (SUR)~160kDa subunits. "Kir" is the acronym of "$K^+$-selective inward rectifier" and the expression "inward rectification" is used to indicate the flow of ions in the inward direction (into the cell) rather than in the outward direction (out of the cell). The phenomenon of inward rectification of $K_{ir}$ channels is the result of high-affinity block by endogenous polyamines, as well as $Mg^{2+}$, that induce an efficient conduction of current only in the inward direction. The $K_{ATP}$ sensitive channels are formed by the members of the Kir6 group, which comprises Kir6.1 and Kir6.2 (mainly expressed in heart and skeletal muscle) and Sulfonylurea receptors (SURs), members of the family of ATP binding cassette (ABC) transporters. ABC transporters have been classified into seven subfamilies. The ones implicated in the formation of $K_{ATP}$ channels belong to the ABCC/MRP subfamily. The SUR subunit is essential for the regulation $K_{ATP}$ channel activity. Each SUR monomer has three transmembrane domains (TMD0-2) and two cytoplasmic nucleotide binding folds (NBFs), each composed of a Walker A and B domain separated by a linker region that create two site of interaction with $Mg_{ATP}$/$Mg_{ADP}$. These interaction sites are involved in the regulation of channel

activity. The major SUR forms, generally considered the interactors of Kir6.x in the formation of pmK$_{ATP}$, are SUR1, with high affinity for sulfonylurea, SUR2A and SUR2B, respectively present in the heart and smooth muscle, with lower affinity for sulfonylureas.

The molecular composition of mammalian mitoK$_{ATP}$ channel is still unknown. It is likely that mitoK$_{ATP}$ may also consist of Kir and SUR subunits. In support of this hypothesis, Garlid's group has purified a mitochondrial fraction containing a 63 kDa sulfonylurea binding protein and a putative pore-forming subunit of 55 kDa. However, the identity of these proteins remains elusive and until now is not clear which are the proteins that compose the mitoK$_{ATP}$ channel.

Since the molecular identity of mitoK$_{ATP}$ is still unclear, the study of the function of this channel is intricate. All data about mitoK$_{ATP}$ function derived from the use of compounds that are able to inhibit or activate mitoK$_{ATP}$ channels (Table 1). However, some of these chemical compounds are not specific for the mitochondrial channel and can also modulate the activity of surface K$_{ATP}$ channels.

| | mitoK$_{ATP}$ | mitoK$_{ATP}$ and surface K$_{ATP}$ | Surface K$_{ATP}$ |
|---|---|---|---|
| Openers | Diazoxide<br>Nicorandil<br>BMS-180448<br>BMS-191095 | Cromakalim<br>Pinacidil<br>P-1060<br>Sildenafil<br>Isoflurane<br>EMD60480<br>Aprikalim | P-1075[1]<br>MCC-134[2] |
| Blockers | 5-HD<br>MCC-134 | Glibenclamide | HMR1098 (1833)<br>Glimepiride[3] |

***Table 1 - Modulators of $K_{ATP}$ channels and their selectivity toward mitochondrial and surface channels.***

MitoK$_{ATP}$ channel is implicated in the protection of the heart from ischemia reperfusion injury, preconditioning and post conditioning. Ischemic

preconditioning (IPC) is a process in which short episodes of hypoxia protect against a subsequent, more prolonged, period of ischemia (Cohen et al., 2000; Kloner et al., 1998; Murry et al., 1986). It consists of a reduction in both infarct size and the incidence of cardiac arrhythmias.

How the opening of mitoK$_{ATP}$ leads to cardioprotection is matter of debate. Three hypotheses have emerged to explain the link between mitoK$_{ATP}$ channel opening and cardioprotection: (1) a decrease in the mitochondrial $Ca^{2+}$ uptake, (2) mitochondrial swelling and changes in ATP synthesis, and (3) changes in the levels of reactive oxygen species (ROS).

Regarding the first point, the hypothesis that mitoK$_{ATP}$ opening attenuates $Ca^{2+}$ accumulation during IPC was proposed for the first time by Liu et.al in 1998 (Liu et al., 1998). This effect on calcium uptake is reduced by diazoxide and reversed by 5-HD (Holmuhamedov et al., 1999). It was proposed that this change in mitochondrial $Ca^{2+}$ uptake is mediated by a partial depolarization of $\Delta\Psi_m$ in response to mitoK$_{ATP}$ opening.

Regarding the second hypothesis, it has been reported that the opening of mitoK$_{ATP}$ causes a 15-20% increase in steady state matrix volume in mitochondria from rat heart, liver and brain and this effect is blocked by 5-HD. The increase in matrix volume in turn activates the respiratory chain providing more ATP to support the recovering myocardium (Grover et al., 2000; Halestrap, 1989). Garlid's group proposed that matrix swelling increases the contact between membrane proteins on the inner and outer mitochondrial membranes, which may cause an increase in ADP transport and ATP synthesis.

About the third hypothesis, is has reported that mitoK$_{ATP}$ opening increases ROS production and this increase, generated during preconditioning period, is thought to be protective (Forbes et al., 2001; Vanden Hoek et al., 1998; Pain et al., 2000). However, the ROS produced during reperfusion causes cell death (Ozcan et al., 2002) so the hypothesis is that mitoK$_{ATP}$ opening results in an increase of the protective ROS produced during preconditioning phase and a decrease in the levels of ROS generated during reperfusion stage (O'Rourke, 2004).

## Calcium-dependent potassium channels

Two different calcium-activated potassium channels have been described in various cell type: mitoBKCa (Big conductance Potassium Channel) and mitoIKCa (Intermediate conductance Potassium Channel).

MitoBKCa channel has been identified in glioma cell line as well as in ventricular cells, rat skeletal muscle and brain (Skalska et al., 2008). It is characterized by a conductance of 100-300ps, is activated by micromolar concentrations of calcium, general drugs like 12,14-dichlorodehydroabietic acid (diCl-DHAA) and more specific inhibitors such as charybdotoxin and paxilline (Heinen et al., 2006). About the molecular structure of mitoBKCa, it seems to be similar to the plasmamembrane channel. MitoBKCa has been reported to be activated under pathophysiological conditions in response to increased mitochondrial $Ca^{2+}$ uptake. As well as mito$K_{ATP}$, mitoBKCa opening protects the heart and other tissue by ischemia-reperfusion injury (O'Rourke, 2007). This protective effect was correlated with the increase in $K^+$ uptake, which prevent $Ca^{2+}$ overload and the opening of permeability transition pore (PTP) (Cheng et al., 2008).

MitoIKCa was first described by Szabo's group by patch clamping mitoplasts isolated from human colon carcinoma cells (De Marchi et al., 2010). Subsequently, it was described also in other cell types such as in HeLa cells and in mouse embryonic fibroblasts (Sassi et al., 2010). MitoIKCa and pmIKCa display the same pharmacological properties and molecular structure (Sassi et al., 2010). The physiological role of MitoIKCa is unknown, however a protective role, similar to that reported for the other mitochondrial potassium channels, was proposed.

## Mitochondrial Kv1.3 potassium channel

Mitochondrial Kv1.3 is an highly selective channel expressed in different cell types like lymphocytes, macrophages, hippocampal neurons and presynaptic neurons. While pmKv1.3 activation is associated with cell proliferation (Cahalan and Chandy, 2009), its mitochondrial counterpart has been found to play a role in cell death. Regarding the mechanism, mitoKv1.3 was identified as a novel target of Bax, a pro-apoptotic cell Bcl-2 family protein, in apoptotic cells. During apoptosis, inhibition of mitoKv1.3 by Bax leads to hyperpolarization, which results in the reduction of respiratory chain components and in enhanced production of ROS (Szabò et al., 2005). ROS is emerging as a key player in promoting cytochrome c release from mitochondria and are indeed able to activate the PTP whose opening leads to depolarization and apoptosis (Kaul et al., 2005).

## Two-pore potassium channel TASK-3

TASK-3 (Twin-related acid-sensitive $K^+$ channel-3;KCNK9) is a two-pore potassium channel recently identified in mitochondria of melanoma and keratinocyte cells by immunochemical and molecular biology methods (Rusznák et al., 2008). In the pm, TASK-3 has a role in apoptosis and tumorigenesis (Patel and Lazdunski, 2004) while the function of mitochondrial TASK-3 is not clear. Notably, a TASK-3 knock down melanoma cell line displays an alteration of mitochondrial function, suggesting that TASK-3 is essential for the survival of these cells (Kosztka et al., 2011).

## IDENTIFICATION OF MITO$K_{ATP}$ CHANNEL

The main features of the mito$K_{ATP}$ channel were described in early the 90s through direct patch clamp of mitoplasts. Since then, mito$K_{ATP}$ has been well described

from the pharmacological point of view. However, until recently, the molecular identity of this channel has remained elusive.

In my laboratory, my colleagues finally identified a novel protein complex localized in the inner mitochondrial membrane (referred to as mitoK$_{ATP}$) that accounts for ATP-sensitive potassium currents (Paggio et al., manuscript under revision). This complex is composed by a channel forming subunit (mitoK) and a regulatory subunit carrying the ATP-binding domain (mitoSUR). Starting from an *in silico* screening of the mitochondrial proteins with unknown function, our laboratory selected a subset of proteins whose overexpression profoundly impaired mitochondrial physiology. Among the positive hits, we focused our attention on the candidate encoded by CCDC51 gene, hereafter named MitoK. This gene encodes a 45kDa protein, which presents one coiled coil domain and two predicted transmembrane domains. The protein is expressed in all human and mouse tissues and it is well conserved in vertebrates. First, the mitochondrial localization of mitoK was validated by immunofluorescence and subcellular fractionation. These experiments indicated a full colocalization of mitoK with mitochondrial markers and an enrichment of mitoK in mitochondria and mitoplasts, suggesting that mitoK is a mitochondrial protein located in the IMM.

In addition, proteinase K protection assay revealed that both the N-terminal and C-terminal region reside inside the organelle matrix.

Next, we investigated the effect of mitoK overexpression on mitochondrial physiology in cultured HeLa cells. In terms of morphology, mitoK overexpression causes an extensive fragmentation of organelle network accompanied by a complete collapse of internal cristae and mitochondrial swelling. From a functional point of view, the overexpression of mitoK caused a drastic decrease of $\Delta\psi_m$ and a decrease of $Ca^{2+}$ accumulation inside the organelles. Taken together, these data indicate that mitoK overexpression causes a drastic alteration of mitochondrial structure and function. To unequivocally demonstrate the channel activity of mitoK, we have exploited a reconstitution approach in heterologous systems, i.e. the planar lipid bilayer. We observed a clear channel activity, characterized by

cooperative gating with a conductance of 75 pS. Interestingly, the activity is rapidly bloked by addition of tetra-ethyl-ammonium (TEA), a general inhibitor of potassium channels. Based on this electrophysiological properties, we pursued the hypothesis that it could be the long-sought, and still highly debated, mitoK$_{ATP}$, although the purified mitoK protein alone showed no inhibition by ATP. However, we reasoned that ATP sensitivity could be conferred by a regulatory SUR-like subunit, similarly to what happens with pmK$_{ATP}$. Indeed, ten different ATP-Binding Cassette (ABC) proteins can be detected in mitochondria, in particular those belonging to the ABCB subfamily. We focused on ABCB8, hereafter named mitoSUR, since i) among all mitochondrial ABC proteins, its tissue expression best correlates with mitoK, and ii) it was already suggested to be part of the mitoK$_{ATP}$ channel. The recombinant mitoK and mitoSUR subunits were co-expressed, purified and reconstituted into liposomes. After the insertion in a planar lipid bilayer the two proteins were able to form a channel inhibited by ATP, activated by diazoxide and blocked by 5-HD and the sulphonylurea glibenclamide. The complex formed by mitoK and mitoSUR recapitulates the main electrophysiological properties and pharmacological profile of mitoK$_{ATP.}$ Based on these findings, we hypotizised that mitoK correspond to the potassium-permeant subunit of the channel, whereas mitoSUR is the ATP-sentitive regulatory subunit which confers the correct gating and closes the channel in normal condition. To validate this hypotesis, the interaction between mitoK and mitoSUR was evaluated *in situ*. Co-immunoprecipitation experiments and blue native electrophoresis demonstrate the physical interaction between the two protein. Accordingly, the concomitant overexpression of mitoK and mitoSUR restored the mitochondrial dysfunction caused by the overexpression of mitoK alone. Indeed, the combined overexpression of the two channel subunits fully rescued $\Delta\psi_m$ and mitochondrial $Ca^{2+}$ dynamics, indicating the restoration of a proper channel gating.

Overall, our data indicate that mitoK and mitoSUR form a complex responsible for the ATP-sensitive mitochondrial transport and strongly support the idea that the two proteins form the long-sought and debated mitoK$_{ATP}$ channel.

## SKELETAL MUSCLE PLASTICITY

Maintenance of skeletal muscle mass is essential for organism's health and survival. Skeletal muscle shows an enormous plasticity to adapt to different stimuli such as contractile activity (endurance exercise, electrical stimulation, denervation), loading conditions (resistance training, microgravity), substrate supply (nutritional interventions) or environmental factors (hypoxia) (Flück and Hoppeler, 2003).

Skeletal muscle mass changes according to physiological and pathological conditions. An increase in muscle mass and fiber size, i.e. muscle hypertrophy, occurs during development and in response to mechanical overload or anabolic hormonal stimulation. A decrease in muscle mass and fiber size, i.e. muscle atrophy, results from aging, starvation, cancer, diabetes, bed rest, loss of neural input (denervation, motor neuron disease) or catabolic hormonal stimulation (Schiaffino et al., 2013). This great plasticity is due to several mechanisms that regulate the rate of muscle growth and muscle loss and essentially reflects the balance between protein turnover and protein degradation within the muscle fibers. Muscle growth and hypertrophy are mainly due to an active protein synthesis. Conversely, a massive protein degradation together with the loss of organelles and cytoplasm are the major causes of muscle atrophy.

However, changes in protein turnover leading to muscle hypertrophy or atrophy do not always proceed according to this simple equation. An increase in protein synthesis together with an increase in protein degradation has been reported in different hypertrophic condition. Goldberg's group demonstrated that during hypertrophy of rat soleus muscle, induced by tenotomy of the gastrocnemius, decreased protein catabolism and increased synthesis of new proteins occur, while during hypertrophy of the soleus induced by growth hormone, there is increased protein synthesis without any change in protein degradation rates (Goldberg, 1969). Just as hypertrophy, so too muscle atrophy is accompanied by increased protein synthesis in some condition. Starvation causes decreased

protein synthesis and increased protein degradation in both fast and slow rat muscles (Li and Goldberg, 1976), however muscle denervation is accompanied by increased protein degradation and increased rather than decreased protein synthesis (Argadine et al., 2009; Quy et al., 2013).

## Signaling pathways that control skeletal muscle mass

Muscle mass and muscle performance is regulated by a broad network of different signalling pathways that coordinate protein degradation and protein synthesis simultaneously (Figure 3).

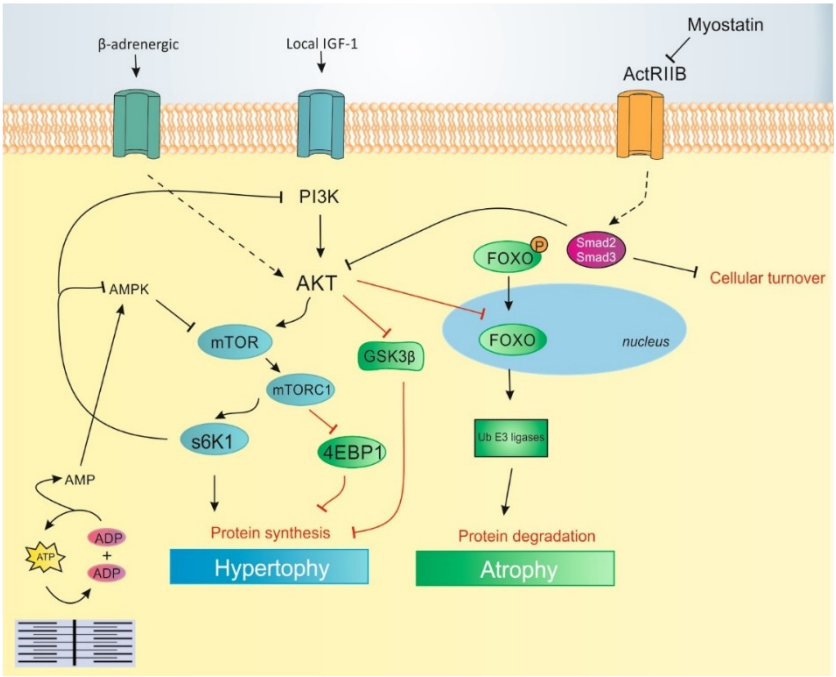


***Figure 3 – Scheme of the major signalling pathways that control muscle mass.***

IGF1 is one of the best characterized muscle growth-promoting factors. In addition to circulating IGF1, local production by skeletal muscle of distinct IGF1 splicing products, has recently described. Muscle-specific overexpression of an IGF1 isoform locally expressed in skeletal muscle results in hypertrophy and increased

strength (Musarò et al., 2001). According to these results it has been proposed for local IGF1 an autocrine/paracrine role for inactivity-dependent muscle plasticity. IGF1/insulin signalling is able to block protein breakdown (Sacheck et al., 2004). Local IGF1 activates Akt through the generation of phosphatidylinositol-3,4,5-triphosphates produced by PI3K. PI3 recruits Akt to the plasmamembrane, where it is phosphorylated by two different kinases: PDK1 and mTOR complex. In skeletal muscle the overexpression of a constitutive form of AKT induces hypertrophy and protects form denervation-induced atrophy (Yang et al., 2004), whereas Akt1 deletion induces muscle atrophy and growth retardation, suggesting that it plays a key role in muscle growth and in the control of protein degradation.

Akt is able to control muscle mass by acting on different downstream targets. The three major downstream branches of the Akt pathway are the mTOR pathway, activated by Akt, glycogen synthase kinase 3 (GSK3) and FoxO pathways, which are blocked by Akt. GSK3 is inhibited by Akt and in turn blocks the eukaryotic initiation factor 2B (eIF2B), which is involved in protein synthesis.

mTOR (mammalian Target Of Rapamycin) is a key regulator of cell growth that controls the status of protein synthesis and other important cell function through the integration of growth signals and energy status (Hay and Sonenberg, 2004). As the name suggests, mTOR is selectively inhibited by rapamycin. This protein is indirectly activated by Akt through the inhibition of tuberous sclerosis 2 (TSC2), a GTPase activating protein, that blocks the small G protein Rheb involved in the activation of mTOR complex. The mTOR complex comprises two multiprotein complexes: mTORC1, which contains Raptor and is required for signalling to S6K and 4EBP1, and mTORC2, which contains Rictor and is important for signalling to Akt-FoxO (Sandri, 2008). mTORC1 phosphorylates the ribosomal protein S6 kinases (S6K1 and 2) and 4E-BP1, a repressor of eIF4E cap-binding protein, thus promoting protein synthesis. S6K1 null mice displayed muscle atrophy and their response to IGF1 and Akt is blunted (Ohanna et al., 2005). Akt is also able to suppress catabolic pathways: it blocks the upregulation of atrophy-related genes, usually activated during atrophy, functioning through negative regulation of FoxO

family of transcription factors (Lee et al., 2004; Sandri et al., 2004; Stitt et al., 2004). Akt-dependent phosphorylation of FoxOs facilitates their export from the nucleus to the cytoplasm, thus inhibiting the upregulation of atrogin-1/MAFbx and MuRF1, two ubiquitin-ligases involved in the atrophy.

Another important factor in the regulation of muscle mass is myostatin. It is a negative modulator of muscle growth: loss of function mutations in its gene leads to increased muscle mass (Lee and McPherron, 2001). The increase in muscle mass is in part due to hyperplasia, an increase in the number of myofibers. Indeed, myostatin pathway inhibits satellite cell activation and differentiation acting on Pax7, MyoD and Myogenin expression (McFarlane et al., 2008). The effects of myostatin overexpression in skeletal muscle are not so clear. In muscle cell culture, myostatin treatment is able to block IGF1/Akt pathway and to activate FoxO1, causing the induction of atrogin-1 (McFarlane et al., 2006). A recent study dissected the myostatin pathway, both in vitro and in vivo, and showed that Smad2 and Smad3 are the transcription factors mediating myostatin effects on muscle mass (Sartori et al., 2009). Myostatin binds to the activin receptor IIB (ActRIIB), a type II TGFβ receptor, which, in turn, activates activin receptor-like kinase 4 (ALK4) or ALK5, both type I serine/threonine kinase receptors (Rebbapragada et al., 2003). However, the downstream targets of the myostatin pathway and their role in the regulation of protein synthesis and protein degradation are still unclear.

Fiber size is also controlled by metabolic programme. One of the key factor in the modulation of skeletal muscle mass is AMPK. It acts as a cellular sensor of energy balance and is activated in stress condition, when AMP levels increased. It is a heterotrimetic complex, composed by a catalytic α-subunit and the regulatory β and γ-subunits, which orchestrate the balance between energy production and energy consumption. AMPK is activated, upon phosphorylation of a conserved threonine residue (Thr172) in the catalytic domain, by upstream kinases LKB1 (a tumor suppressor), CAMKKβ ($Ca^{2+}$/calmodulin- dependent protein kinase kinase β) and TAK1 (transforming growth factor β-activated kinases) (Hardie, 2008).

AMPK activation occurs when the cellular AMP/ATP ratio is high. Its phosphorylation results in the net suppression of protein synthesis and cell growth. In skeletal muscle, the activation of AMPK causes metabolic changes that help myofibers in adapting to energy deprivation through an increase of oxidative capacity (Hardie and Sakamoto, 2006; Palacios et al., 2009).

There is a strictly crosstalk between AMPK and mTORC1. Loss of AMPK is sufficient to activate mTORC1 signalling, leading to increased muscle mass (Lantier et al., 2010). The two catalytic isoforms of AMPK, AMPKα1 and AMPKα2, exert different functions in skeletal muscle and in the crosstalk between mTORC1/AMPK. AMPKα2 is activated in response to energy deprivation by LKB1, whereas, in response to mechanical overload, it is activate by CAMKK. AMPKα1 inhibits mTORC1 signalling by the direct phosphorylation of raptor, mTOR and TSC1/TSC2, triggering a decrease of protein synthesis and muscle cell growth. On the other hand, in response to exercise mTORC1 inhibits AMPK activity by affecting intracellular energy levels and induces protein synthesis promoting muscle cell growth (Lantier et al., 2010).

## PROTEIN BREAKDOWN IN MUSCLE ATROPHY

### Ubiquitin-proteasome system

Atrophy is defined as a decrease in the size mainly caused by the loss of organelles, cytoplasm and proteins. In skeletal muscle, the ubiquitin-proteasome system is one of the most important catabolic pathways that controls protein turnover and it is required to remove damaged sarcomeric proteins during muscle wasting. A decrease in muscle mass is associated with: (1) increased conjugation of ubiquitin to muscle proteins; (2) increased proteasomal ATP-dependent activity; (3) increased protein breakdown; and (4) upregulation of transcripts encoding

ubiquitin, some ubiquitin-conjugating enzymes (E2), ubiquitin-protein ligases (E3) and several proteasome subunits (Lecker et al., 2006) (Figure 4).

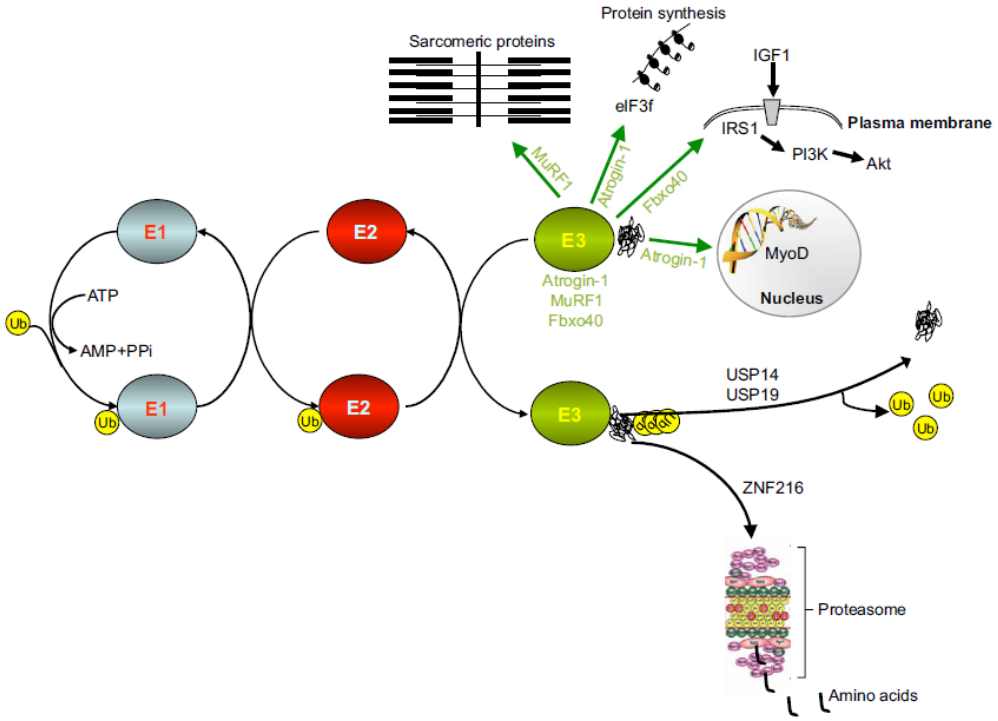


***Figure 4 - Ubiquitin-proteasome systems in skeletal muscle.*** *E1 enzymes activate ubiquitin proteins after the hydrolisis of ATP. The ubiquitin is then moved from E1 to members of the E2 enzyme class. The final steps is mediated by members of the E3 enzyme class, which induces the transfer of ubiquitin from E2 to the substrate. Polyubiquitylated substrate is then delivered to the proteasome for degradation. De-ubiquilylating enzymes (USP) can remove polyubiquitin chains from substrate.* (Bonaldo and Sandri, 2013).

In the human genome, more than 650 ubiquitin ligases are involved in the specific regulation of metabolism, transcription, cell cycle and muscle size (Lee and Goldberg, 2011). In 2001, two independent labs performed genome-wide expression profiling and identified ubiquitin ligases involved in muscle atrophy (Bodine et al., 2001a; Gomes et al., 2001). They compared gene expression in different models of muscle atrophy and drew up a set of genes commonly up or

downregulated in atrophying muscle. They called these genes “atrophy-related genes” or atrogenes”. Among them, the first two identified genes encoded muscle specific ubiquitin ligases: Atrogin1 and MuRF1. Atrogin-1 promotes degradation of MyoD, a key muscle transcription factor, and eIF3f, an activator of protein synthesis (Csibi et al., 2010; Tintignac et al., 2005). MuRF1 is able to interact and control the half-life of important muscle structural proteins, including troponin I (Kedar et al., 2004), myosin heavy chains (Clarke et al., 2007), myosin binding protein C and myosin light chain (Cohen et al., 2009).

Recently, several other E3s, activated during muscle atrophy and responsible for clearance of damaged protein, have been described. Among them Trim32 has been reported as a crucial E3 ligase for the degradation of thin filaments (actin, tropomyosin and troponins), α-actinin and desmin (Cohen et al., 2012). Another ligase involved in muscle atrophy is TRAF6 (Paul et al., 2010), an E3 ubiquitin ligase implicated in the conjugation of Lys63-linked polyubiquitin chains to its target proteins. The deletion of TRAF6 protects mice from muscle loss induced by denervation, cancer or starvation (Kumar et al., 2012; Paul et al., 2010, 2012). This protection is guaranteed by the activation of JNK, AMPK and NFkB, three different regulators of Atrogin-1 and MuRF1, and by the control of several autophagy-related genes (Paul et al., 2012). Mitochondrial ubiquitin ligase 1 (Mul1) is identified by three independent groups (Li et al., 2008; Neuspiel et al., 2008; Zhang et al., 2008). It ubiquitinates the mitochondrial pro-fusion protein mitofusin2, leading to its degradation by proteasome. Although the direct correlation between Mul1 and loss of muscle mass is still controversial, it has been reported that mitochondrial fission and remodelling contributes to muscle atrophy (Romanello et al., 2010). Some muscle specific E3 ligases also showed important regulatory functions in signalling pathways. For example, Fbxo40 ubiquitinates insulin receptor substrate 1 (IRS1) and affects its degradation, thus controlling insulin signalling (Shi et al., 2011). Despite the extensive study of E3 ligases involved in muscle atrophy, little is known about the recognition and delivery of ubiquitinated proteins to the proteasome. ZNF216 has been identified as an important player in

this process. Indeed, it is upregulated by FoxO transcription factors in atrophying muscles, and ZNF216-deficient mice are partially resistant to muscle loss during denervation (Hishiya et al., 2006). Much research in this field is focused on the ubiquitylation process, while information on the role of the de-ubiquitylating system on muscle atrophy is very poor. The major class of de-ubiquitylating enzymes are the ubiquitin-specific processing proteases (USPs). So far, only two (USP14 and USP19) have been found to be upregulated in atrophying muscles (Combaret, 2005).

## AUTOPHAGY IN SKELETAL MUSCLE

Autophagy is a highly conserved homeostatic mechanism essential for the degradation and recycling of bulk cytoplasm, long-lived proteins and organelles (Mizushima and Komatsu, 2011). Several years ago the autophagy-lysosome system was described to be activated in myofibers during catabolic conditions (Bechet et al., 2005; Deval et al., 2001; Tassa et al., 2003). Recently, a great interest has emerged for this process that in skeletal muscle seems to be modulated by a large spectrum of pathophysiological conditions including cancer (Penna et al., 2013), ageing (Wohlgemuth et al., 2010), fasting (Mammucari et al., 2007; Mizushima et al., 2003), disuse (Brocca et al., 2012) and denervation (O'Leary and Hood, 2008; Zhao et al., 2007).

Three different mechanisms have been described in mammals: macroautophagy, chaperone-mediated autophagy (CMA) and microautophagy. However, most data on the role of the autophagy in muscle are related to macroautophagy.

### Molecular mechanism of autophagy in skeletal muscle

Differently from other important metabolic tissues such as liver and pancreas, skeletal muscle shows a persistent and prolonged autophagy induction

(Mizushima et al., 2003). In skeletal muscle, multiple signalling pathways coordinate autophagic flux defined as the entire process of autophagy: from autophagosome formation to lysosomal degradation of autophagic substrates. Autophagic flux is determined by the balance between inhibitors and activators of autophagic machinery (Sandri, 2010) (Figure 5). In skeletal muscles, few autophagy suppressors have been reported, the first of which was Runx1. This is a transcription factors required to preserve the structural integrity of myofibers and it is strongly upregulated in denervated muscles where it induces autophagy (O'Leary and Hood, 2008, 2009; Wang et al., 2005). The mechanism by which Runx1 regulate autophagy is unclear, however recently was shown that Runx1 is able to modulate FoxO3 action (Wildey and Howe, 2009).

Another negative regulator of autophagy is the phosphatase Jumpy. It blocks autophagosome formation by reducing PI3P levels, however the regulation of this phosphatase in normal and atrophic muscles is still unknown (Sandri, 2010). The major autophagy inhibitor in skeletal muscle is Akt. Its activation completely inhibits autophagosome formation and lysosomal-dependent protein degradation during fasting (Mammucari et al., 2007, 2008; Zhao et al., 2007, 2008). This kinase is able to regulate autophagy in two different ways: a rapid mechanism through the regulation of mTOR activity and a slower mechanism, which requires gene transcription via FoxO3. The mTOR kinase is a cellular nutrient sensor essential for cell growth. In skeletal muscle, autophagy is mainly regulated by mTORC1, the complex of mTOR which interact with Raptor. Akt is able to modulate mTORC1 activity directly phosphorylating mTOR or through the tuberous sclerosis complex (TSC). The phosphorylation of TSC2 inhibits TSC complex, thus activating the mTORC1 pathway (Bodine et al., 2001). Akt is also able to regulate autophagy at the level of gene transcription acting on FoxO3. The expression of FoxO3 activates lysosomal-dependent protein degradation both *in vitro* and *in vivo* (Romanello et al., 2010a; Wohlgemuth et al., 2010). Different autophagy genes, such as LC3, GABARAP, Bnip3, Vps34 and Atg12, are under the control of FoxO3. Bnip3, a BH3-only protein, was identified as a central player downstream of FoxO3. Indeed, its

overexpression is able to induce autophagy, and its downregulation mitigates the action of FoxO3 (Grumati et al., 2010; Mammucari et al., 2007; Tracy and Macleod, 2007).

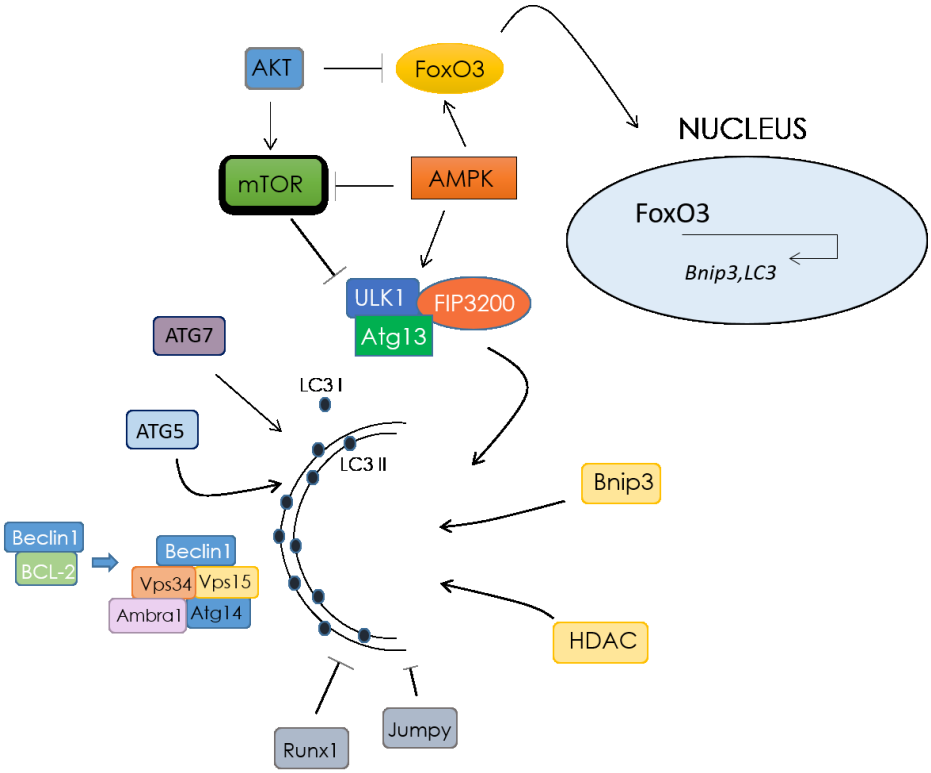


***Figure 5 - Scheme that represents positive and negative regulators of autophagy in skeletal muscle.*** *Modified by Grumati and Bonaldo, 2012.*

Beside repressor of autophagy, there are several factors important for autophagy activation. One potent autophagy activator is AMPK (Hardie, 2007) a kinase activated by AMP when nutrient supply decreases. AMPK regulates cell growth and autophagy through the inhibition of mTORC1 complex by direct phosphorylation of Raptor (Gwinn et al., 2008; Kalender et al., 2010). AMPK can also induces autophagy by phosphorylating, thus activating, ULK1 (Unc-51 Like autophagy activating Kinase 1). AMPK is able to trigger autophagy in two different ways: through a direct activation of ULK1 or by the indirect suppression of

mTORC1. Among the inducer of autophagy, there are Atg5 and Atg7. Muscle-specific inactivation of these two critical autophagy-related genes in mice determines a severe muscle wasting associated with ultrastructural alterations of cytosolic organelles, such as mitochondria, and myofibers degeneration (Masiero et al., 2009; Raben et al., 2008). One of the most important regulatory element on autophagic system is the beclin1/Vps34/Vps15 complex. Vps34 is a Class III phosphatidylinositol 3-kinase (PI3K) enzyme that generates PI3P from PI2P, while Vps15 is a regulatory subunit, required for Vps34 activity. The large complex beclin1/Vps34/Vps15/Atg14L is implicated in the regulation of autophagosome biogenesis and endosome trafficking (Bonaldo and Sandri, 2013).

Histone deacetylases (HDACs) have been described as activators of autophagy in skeletal muscles. HDAC1/2 knockout mice display a progressive myopathy characterized by autophagy dysregulation. It was found that in skeletal muscle HDAC1 and HDAC2 are able to regulate autophagy by inducing the expression of autophagy genes (Moresi et al., 2012).

## Autophagy in Muscle Homeostasis

Autophagic flux was induced upon different catabolic conditions, such as fasting (Mizushima et al., 2004, 2008, 2010), atrophy (Sandri, 2008) and denervation (Schiaffino and Hanzlíková, 1972). Fasting is one of the strongest stimuli that induces autophagy in muscle. Since muscles are the major reserve of aminoacids in the body, during fasting autophagy plays a fundamental role in the maintenance of a proper aminoacid pool by digesting proteins and organelles (Mizushima et al., 2004). Denervation is also able to induce autophagy in skeletal muscle, although at a slower rate than fasting. During atrophy, the activation of autophagy seems to worsen muscle loss. In this context, protein breakdown is mediated by atrogenes, which are under the control of FoxO transcription factors (Sandri et al., 2004) that are able to regulate independently the ubiquitin-proteasome system and the autophagy-lysosome machinery in vivo and in vitro (Mammucari et al.,

2007; Zhao et al., 2007). On the other hand, the impairment of autophagy flux has been correlated with peculiar feature such as aged protein accumulation, abnormal and dysfunctional mitochondria and dilated sarcoplasmic reticulum. Protein aggregates positive for ubiquitin and p62/SQSTM1 have been found inside myofibers of patients affected by sporadic inclusion body myositis (Nogalska et al., 2009). Moreover, accumulation of dysfunctional mitochondria and dilated sarcoplasmic reticulum was described in two inherited muscle diseases: Bethlem myopathy and Ullrich congenital muscular dystrophy (Irwin et al., 2003). The study of Atg5 and Atg7 muscle-specific knockout mice was important for understanding the role of autophagy in muscle physiology. In these knockout models, suppression of autophagy induces muscle weakness and atrophy (Masiero et al., 2009; Raben et al., 2008), with also a significant reduction of the global body weight. During catabolic conditions, like fasting and denervation, increased autophagy is crucial, but on the other side, it is harmful when completely blocked, so these two opposite condition are detrimental for muscle homeostasis (Figure 6). The main difference in these two conditions is the timing. Muscle wasting associated to autophagy inhibition becomes evident only after the accumulation of a number of altered proteins and dysfunctional organelles within the myofiber that can interfere with the normal physiological functions. In this scenario, insufficient induction of autophagy becomes harmful only after some months or even years. On the contrary, excessive induction of autophagy can induce a very rapid loss of muscle mass within days or weeks (Sandri, 2010). Alteration of autophagy-lysosome system also occurs during certain physiological processes, such as aging. Recent findings indicate that the expression of Atg proteins and other proteins required for autophagy induction was reduced in aged tissues and that autophagy diminishes during aging (Rubinsztein et al., 2011). For example in human brain, Atg5, Atg7, and Beclin1 are down-regulated during aging (Lipinski et al., 2010), while in osteoarthritis Ulk1, Beclin1, and LC3 are down-regulated (Caramés et al., 2010). About the involvement of autophagy in age-dependent muscular changes, there are few information; however, several data indicate that

the molecular mechanisms of protein quality control play a central role in the onset of weakness during aging.

Another physiological condition correlated with altered autophagy is physical exercise (Grumati et al., 2011; Jamart et al., 2012). Physical activity represent the most stressful condition for skeletal muscle: during exercise, contractile proteins and organelles are subjected to an intense load of work and this may lead to their exhaustion. In this condition, the autophagic machinery efficiently eliminate dysfunctional organelles (Mizushima and Komatsu, 2011). All these findings indicate that a proper balance of autophagic flux is a key point for muscle health, although the mechanisms that regulate autophagy in skeletal muscles are still to be determined.

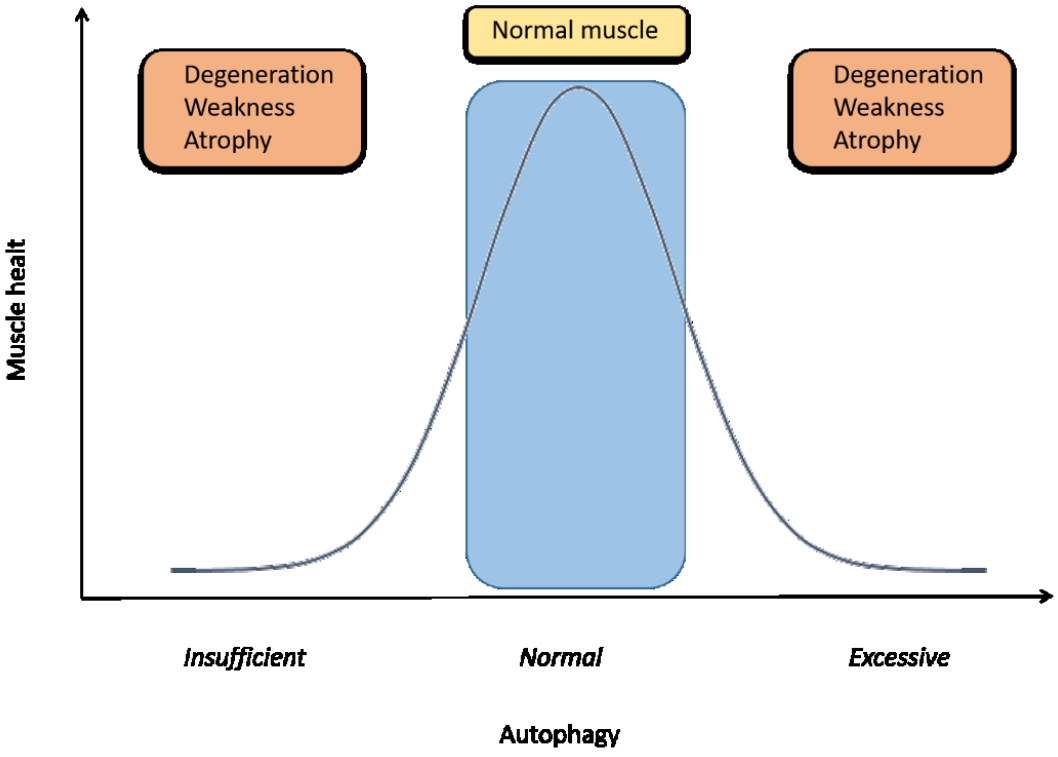


***Figure 6 – Scheme of detrimental effects of insufficient or excessive autophagy on muscle health***

# AIM

The existence of the mitochondrial $K_{ATP}$ channel (mitoK$_{ATP}$) and its physiological function is one of the most controversial topic in the field of mitochondrial channels. The information available on the molecular composition of mitoK$_{ATP}$ is fragmented and contradictory, thus the study of the physiological function of these channels is intricate. According to the available literature, mitoK$_{ATP}$ channels are proposed to play a protective function in ischemia-reperfusion injury. However, the broad conservation profile among all vertebrates suggests that the mitoK$_{ATP}$ should also have a primary housekeeping function.

Recently, my laboratory identified the molecular composition of mitoK$_{ATP}$. It is composed by four channel-forming subunits (mitoK) and a tetramer of ATP-sensitive regulatory domain (mitoSUR) (Paggio et al., unpublished). Our *in vitro* data indicates that mitoK$_{ATP}$ plays a central role in the regulation of mitochondrial function and in the maintenance of a proper mitochondrial structure. The aim of my PhD research was to address the physiological role of mitoK$_{ATP}$ *in vivo*. In particular, we studied the role of mitoK, the potassium permeant subunit of the channel, in skeletal muscle, a tissue that rely on oxidative metabolism and strongly requires proper mitochondrial functionality. Taken together, our findings indicate that mitoK$_{ATP}$ plays a central role in the control of muscle trophism.

# RESULTS

## MitoK overexpression regulates muscle mass and muscle size of developing muscle

Data obtained in our laboratory indicate that mitoK overexpression leads to a dysregulation of multiple mitochondrial parameters, including organelle fragmentation and swelling, collapse of internal cristae, loss of membrane potential and decrease of mitochondrial $Ca^{2+}$ accumulation.

Due to the importance of mitochondria in the energy metabolism of skeletal muscle and in the maintenance of muscle homeostasis, we decided to study the effects of mitoK overexpression on mitochondrial structure and function in this tissue. In order to analyze the effects of mitoK overexpression we used adeno-associated viral vector, serotype 9, which is mainly expressed in the heart and skeletal muscle and ensures a good transduction of the gene of interest with a minimal pathogenicity.

We injected hindlimb muscles of newborn mice with adeno-associated virus overexpressing mitoK linked to a flag tag (AAV9mitoK) and analyzed muscles after the infection. We confirmed the expression of AAV9mitoK by western blot and immunofluorescence analysis (Figure 7) that revealed about 70% of infected myofibers.

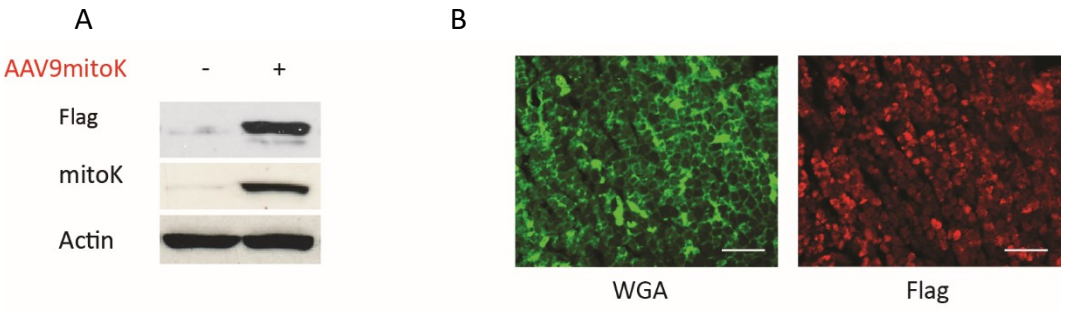


***Figure 7. MitoK overexpression in skeletal muscle.*** *Hindlimb muscles of newborn mice (4–6 days old) were injected with AAV9mitoK. One month later muscles were isolated and processed for further analysis. (A) Immunoblotting of total protein lysates of TA muscle. Anti-Flag and anti – mitoK antibodies were used to detect AAV9mitoK. Actin was used as protein loading*

*control. (B) TA muscle cryosections were immunostained with anti-Flag antibodies. Wheat germ agglutinin (WGA) was used to label the sarcolemma. Scale bar 100 µm.*

In order to investigate the phato-physiological role of mitoK during postnatal development, hindlimbs of newborn mice were infected with AAV9mitoK, collected and analyzed either 4 or 8 weeks later (Figure 8A).

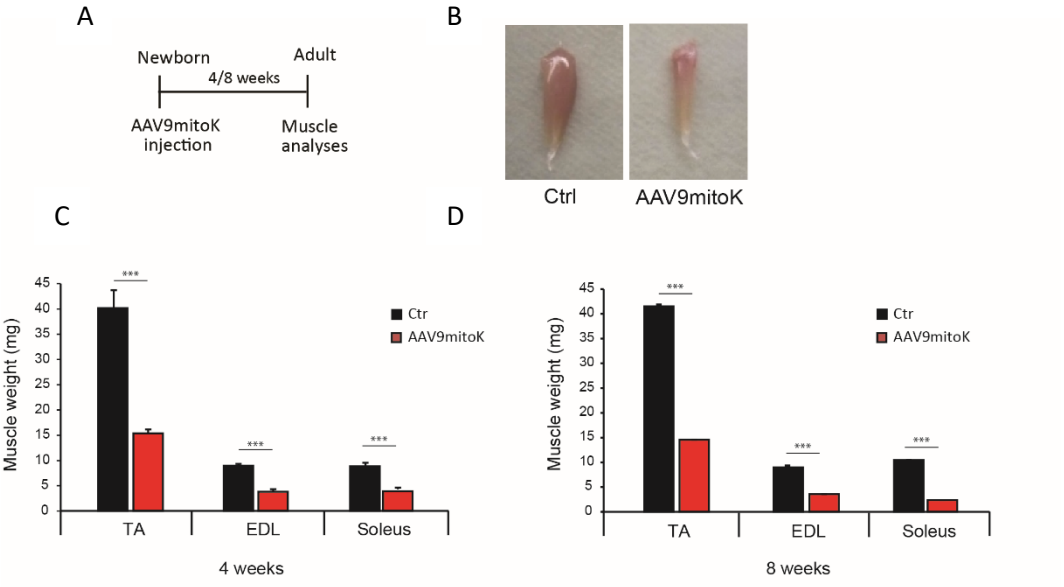


***Figure 8. MitoK overexpression strongly decreases muscle mass.***

*(A) Representative scheme of experimental procedure. (B) Representative pictures of TA muscle of newborn mice injected with AAV9mitoK, 4 weeks after infection, and contralateral, not injected, control muscle. (C) Weight of TA, EDL and Soleus muscle of newborn mice 4 weeks after AAV9mitoK injection. (D) Weight of TA, EDL and Soleus muscle of newborn mice 8 weeks after AAV9mitoK injection. n=8, ***p<0.005, t test (two-tailed, unpaired). Data are presented as mean ±SD.*

The analysis indicates that mitoK strongly influence muscle mass and muscle size during post-natal growth. Indeed, mitoK injected mice shows a dramatic reduction of muscle dimension compared to control (Figure 8B). The reduction of muscle weight in AAV9mitoK injected muscles was more than 50% for tibialis anterior (TA), Extensor digitorum lungus (EDL) and Soleus muscle, 4 weeks after the infection (Figure 8C). This percentage grows up to 60-70% for TA and EDL muscle

8 weeks post-infection (Figure 8D). The reduction of muscle mass in mitoK overexpressing muscles was accompanied by a strong decrease in fiber size. We found a decrease of 50% and 60% of average fiber area respectively in EDL and Soleus muscles compared to controls (Figure 9A). A major decrease of fiber size of EDL muscle was found 8 weeks post-infection, when we observed a 70% reduction (Figure 9B).

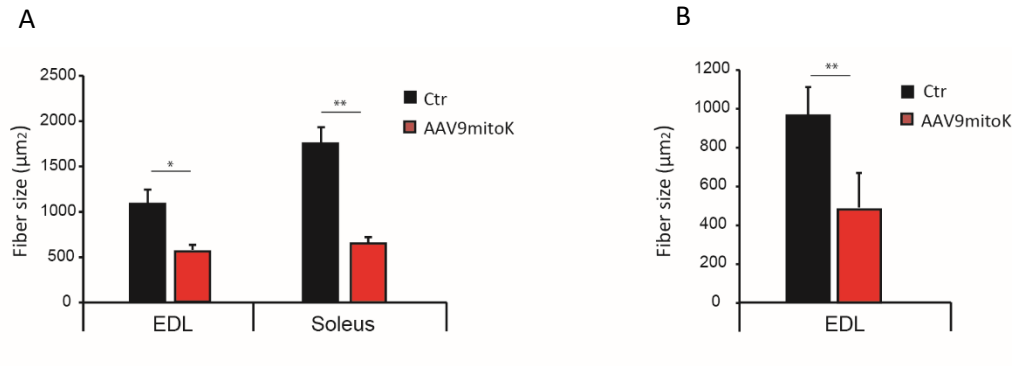


***Figure 9. MitoK overexpression triggers muscle atrophy.***

*(A) Average fiber area of EDL and Soleus muscle 4 weeks after AAV9mitoK injection. (B) Average fiber area of EDL muscles 8 weeks after AAV9mitoK injection. *p<0,05; **p<0,01; t test (two-tailed, unpaired). n=6. Data are presented as mean±SD.*

Then, in order to understand whether mitoK overexpression affects not only muscle dimension but also muscle structure we performed morphological analysis (Figure 10). Hematoxylin-eosin (H&E) staining revealed the presence of abnormal myonuclei with an accumulation of Hematoxylin-positive inclusions and central-nucleated myofibers in mitoK overexpressing TA muscles both 4 and 8 weeks after AAV9mitoK injection. These data suggest that mitoK overexpression alters myofibers structures and triggers muscle damage, expecially 8 weeks post infection. Then we wondered whether mitoK overexpression induces fibrosis, a process that generally occurs after muscle damage.

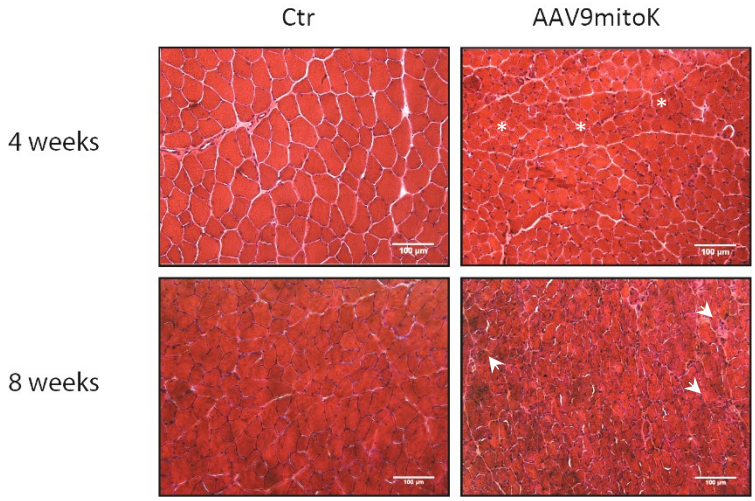


***Figure 10. MitoK overexpression alters muscle structure.***

*H&E staining performed on TA muscles overexpressing mitoK and control muscle, 4 and 8 weeks after the infection. In mitoK overexpressing TA muscle central-nuclated myofibers (white arrowhead) and abnormal myonuclei (white asterix) are present. Scale bar 100 µm.*

We took advantage of Sirius Red staining that allows the visualization of collagen I and II depositions, markers of fibrotic tissue (Figure 11). As reported in the figures we found a significant accumulation of collagen in mitoK overexpressing muscle.

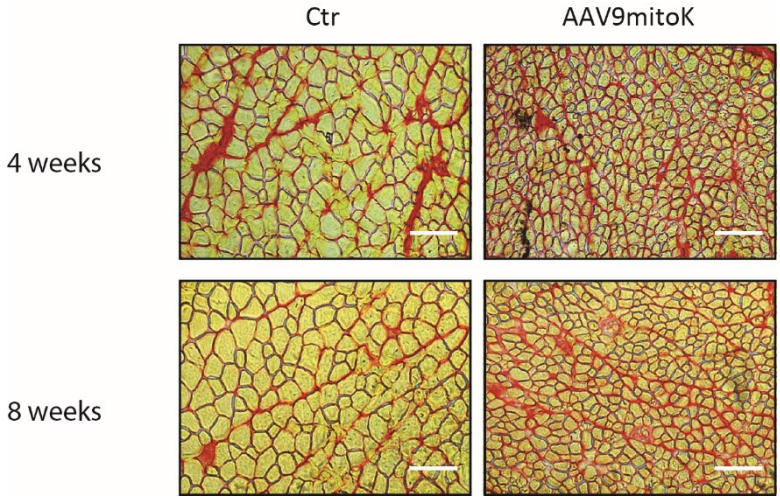


***Figure 11. MitoK overexpression induces muscles fibrosis.*** *Sirius red staining performed on EDL muscle of AAV9mitoK injected and control mice, 4 and 8 weeks post-infection. Scale bar 100 micron.*

## MITOK OVEREXPRESSION ALTERS MITOCHONDRIAL STRUCTURE AND FUNCTION

As previously described in HeLa cells, mitoK overexpression induces a great mitochondrial remodeling with a strong alteration of mitochondrial ultrastructure and mitochondrial swelling (Paggio A., unpublished). For this reason, we investigate mitochondrial morphology and functionality after mitoK overexpression *in vivo,* in skeletal muscle. We analysed mitochondrial structure in mitoK overexpressing muscles, by electron microscopy (EM). For these analyses, we isolated EDL muscle from control and AAV9mitoK overexpressing mice, fixed muscles and analysed mitochondrial distribution and morphology on longitudinal sections (Figure 12).

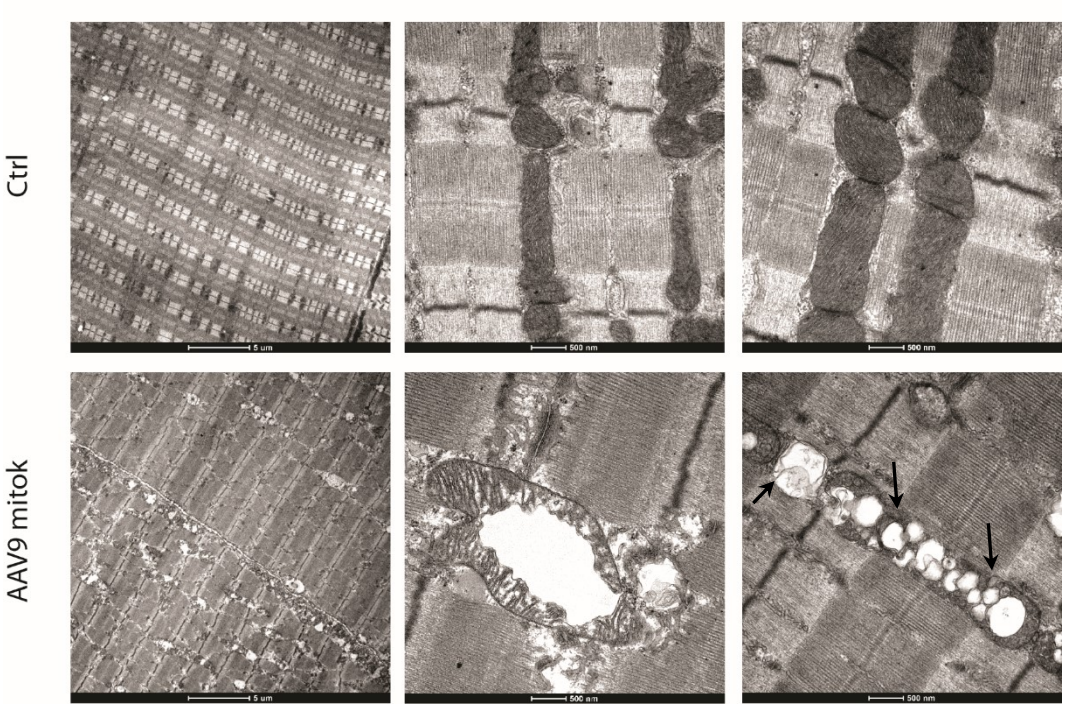


***Figure 12. MitoK overexpression induces mitochondrial swelling.*** *TEM analysis performed on longitudinal section of EDL muscles overexpressing mitoK and control muscles. These pictures show the presence of swollen mitochondria deprived of internal cristae that are being degraded (black arrows).*

TEM pictures indicates that mitoK overexpression strongly affects mitochondrial morphology. The internal cristae are totally collapsed and mitochondria are empty and swollen. Inside the organelle, lamellar structures and vacuoles are also present, indicating that damaged mitochondria are being eliminated.

The alteration of mitochondrial morphology was also supported by immunoblot analysis. This analysis indicates that mitoK overexpression modulates the expression of different mitochondrial proteins depending on their localization inside the mitochondria. In particular, we assessed the expression levels of three different proteins (IMMT, Timm44, COX IV) located in the inner mitochondrial membrane and a protein mainly present inside the matrix (GRP75) (Figure 13).

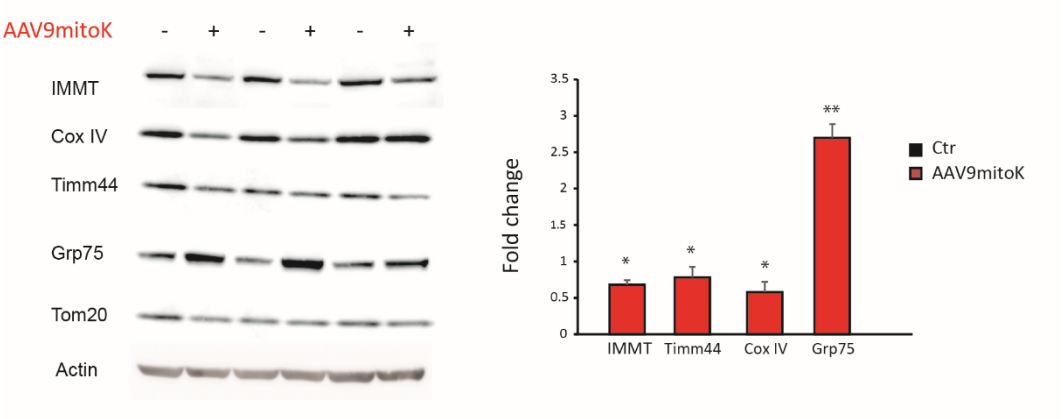


***Figure 13. MitoK overexpression induces a mitochondrial remodeling.***

*Proteins were extracted from TA muscle overexpressing mitoK and control muscles and probed with IMMT, COX IV, TIMM44, GRP75 antibodies. On the left: Immunoblotting of AAV9mitoK infected TA muscles. Actin and Tom20 were used as protein-loading control. On the right: quantification by densitometry. Data are reported as fold increase of each proteins, normalized for the relative actin, compared to control. *p<0,05,**p<0,01, t test (two-tailed, unpaired). n=6. Data are presented as mean ±SD.*

In mitoK overexpressing muscles we found a decrease in the expression of proteins located in the internal membrane and an increase in the protein confined within the matrix. This pattern is in line with an alteration of mitochondrial morphology, characterized by an increase of matrix volume, as suggested by the increase of GRP75 protein level, and a collapse of internal cristae, as indicated by the decrease

in COX IV expression level. This findings allowed us to speculate that mitoK overexpression in skeletal muscle, as well as in Hela cells, causes a constitutive opening of mitoK$_{ATP}$ channel, that induce a net influx of potassium cations leading to water entry and subsequent mitochondrial swelling.

At this point, in order to test if the alteration of mitochondrial structures was accompanied by a dysregulation of respiratory chain complexes, we analysed the overall expression levels of ETC components through western blot analysis (Figure 14).

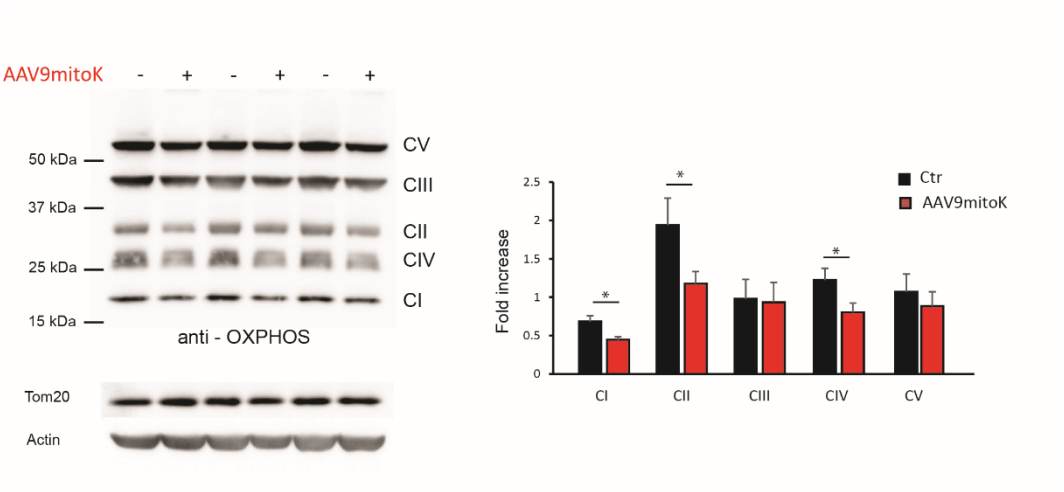


***Figure 14. MitoK overexpression affects protein levels of ETC complexes.***

*On the left immunoblotting analysis of ETC components in TA muscle overexpressing mitoK and control muscles. ETC complex components were probed with anti-Oxphos antibody, while Actin and Tom20 were used as protein-loading control. The graph on the right shows the quantification by densitometry. Data are reported as fold change of each ETC components, normalized for the relative housekeeping, compared to control. *p<0,05, t test (two-tailed, unpaired). n=6. Data are presented as mean ±SD.*

This analysis revealed that mitoK overexpression significantly decreases the expression of the subunit B8 of NADH Dehydrogenase (complex I), the SDHB subunit of complex II and the subunit I of complex IV. This alteration can be correlated with an impairment of mitochondrial functionality.

Then, we decided to understand whether mitochondrial functionality was also compromised after mitoK overexpression. To assess mitochondrial activity we performed SDH staining on TA, EDL and Soleus muscles cryosections (Figure 15).

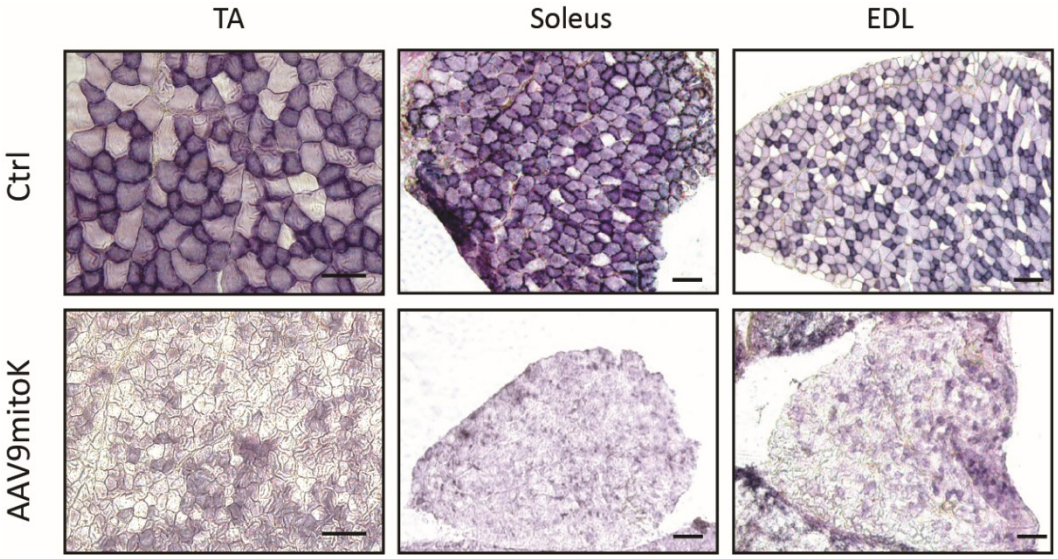


***Figure 15. MitoK overexpression dramatically compromises mitochondrial functionality.*** *SDH staining was performed on TA (Tibialis Anterior), Soleus, EDL (Extensor Digitorum Lungus) muscles 4 weeks after AAV9mitoK injection. Scale bar 100 micron.*

This staining consists in an enzymatic reaction that allows the determination of succinate dehydrogenases activity, (complex II of respiratory chain) in single myofibers. As expected in mitoK overexpressing fibers the number of positive myofibers strongly decreases compared to control muscles, suggesting that mitochondrial function was strongly impaired. Overall, these data indicates that mitoK overexpression induces a strong alteration of mitochondrial ultrastructure and a decrease of mitochondrial function that could be responsible for the decrease in muscle mass.

## MITOK OVEREXPRESSION INDUCES MUSCLE ATROPHY

We found that mitoK overexpression in skeletal muscle induces atrophy, a process defined as a decrease in the size of a tissue or organ mainly caused by loss of organelles, cytoplasm, and protein (Sandri M.,2008). This decrease results from imbalance between protein synthesis and protein degradation that control muscle mass and muscle size. Atrophy is generally correlated with an increase of protein degradation and/or a decrease of protein synthesis.

In order to understand what are the signalling pathways accounting for the phenotype of mitoK overexpressing muscles, we started to study one of the most important cell degradative pathways generally dysregulated in atrophying muscles: the ubiquitin-proteasome system. Immunoblot analysis indicates that mitoK overexpression induces a 60% increase of protein ubiquitination (Figure 16).

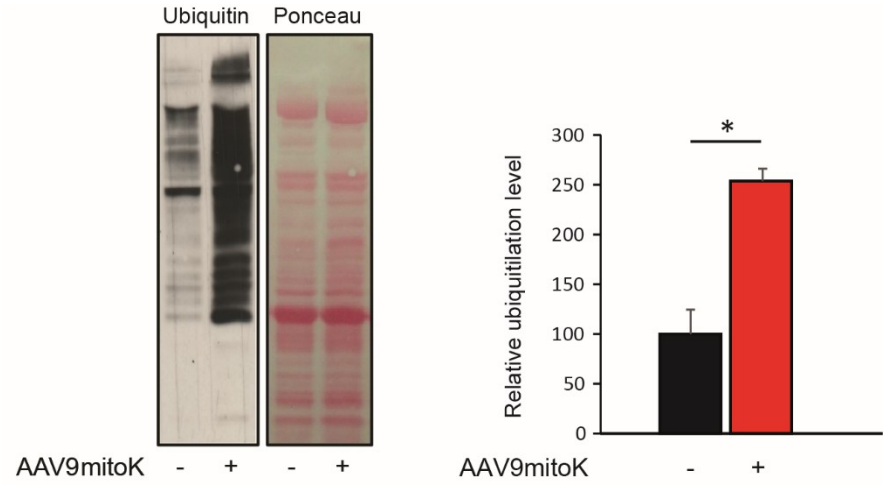


***Figure 16. MitoK overexpression increases protein ubiquitination.***

*On the left hand side western blotting analysis of poliubiquitinated proteins level in TA (Tibialis anterior) muscles injected with AAV9mitoK, 4 weeks after the infection and control mice. Ponceau S staining was used as loading control. The graph on the right shows the quantification by densitometry. *p<0,05, t test (two-tailed, unpaired). n=4. Data are presented as mean ±SD.*

This generally correlates with an increase of Ubiquitin E3 ligases, in line with the activation of muscle atrophy.

Atrophy is an active process that requires the activation of a specific transcriptional program and the induction of a precise set of genes. The commonly up or down regulated genes in atrophying muscles are called “atrogenes” or “atrophy-related-genes”. They are coordinately induced or suppressed in muscle atrophying from different causes like starvation, cancer cachexia and diabetes mellitus (Lecker et al., 2004). Among the strongly induced genes, many are involved in protein degradation, including E3 ubiquitin ligases, E2 ubiquitin-conjugating enzyme, the subunits of the 20S proteasome and specific transcription factors.

In order to dissect the pathway responsible for muscle atrophy in mitoK overexpressing mice we performed qPCR analysis on TA muscle injected with AAV9mitoK 4 weeks after the infection (Figure 17). We analysed the expression of different E3 ubiquitin ligases like Atrogin1, Murf1, Mul1, Traf6, upregulated in atrophying muscles, but we did not find any difference in mitoK overexpressing muscles compared to control, indicating that in our condition other E3 ubiquitin ligases, responsible for the increase in protein degradation, are activated (Figure 17A). Then we analyzed the expression of three transcription factors positively regulated during muscle atrophy: ATF4, TGIF and XBP1 (Figure 17B). In skeletal muscle fibers, ATF4 is a critical component of a complex signaling network that causes muscle atrophy during aging, fasting, and immobilization (Adams et al., 2017). Another transcription factor implicated in atrophy induction is transforming growth-induced factor (TGIF). It is an active transcriptional co-repressor of SMAD2 that is recruited by TGFβ – activated Smad complex (Wotton et al., 1999). X-box-binding protein 1 (XBP1) is generally activated upon accumulation of unfolded proteins in the endoplasmic reticulum. Xbp1 mRNA is processed to an active form by an unconventional splicing mechanism that forms a functionally active isoform. This protein is a marker of ER-stress and is correlated with increased muscle wasting.

As reported in the graph, among these genes, only TGIF was significantly upregulated in AAV9mitoK injected muscle, suggesting an involvement of TGFβ signalling pathway on muscle atrophy.

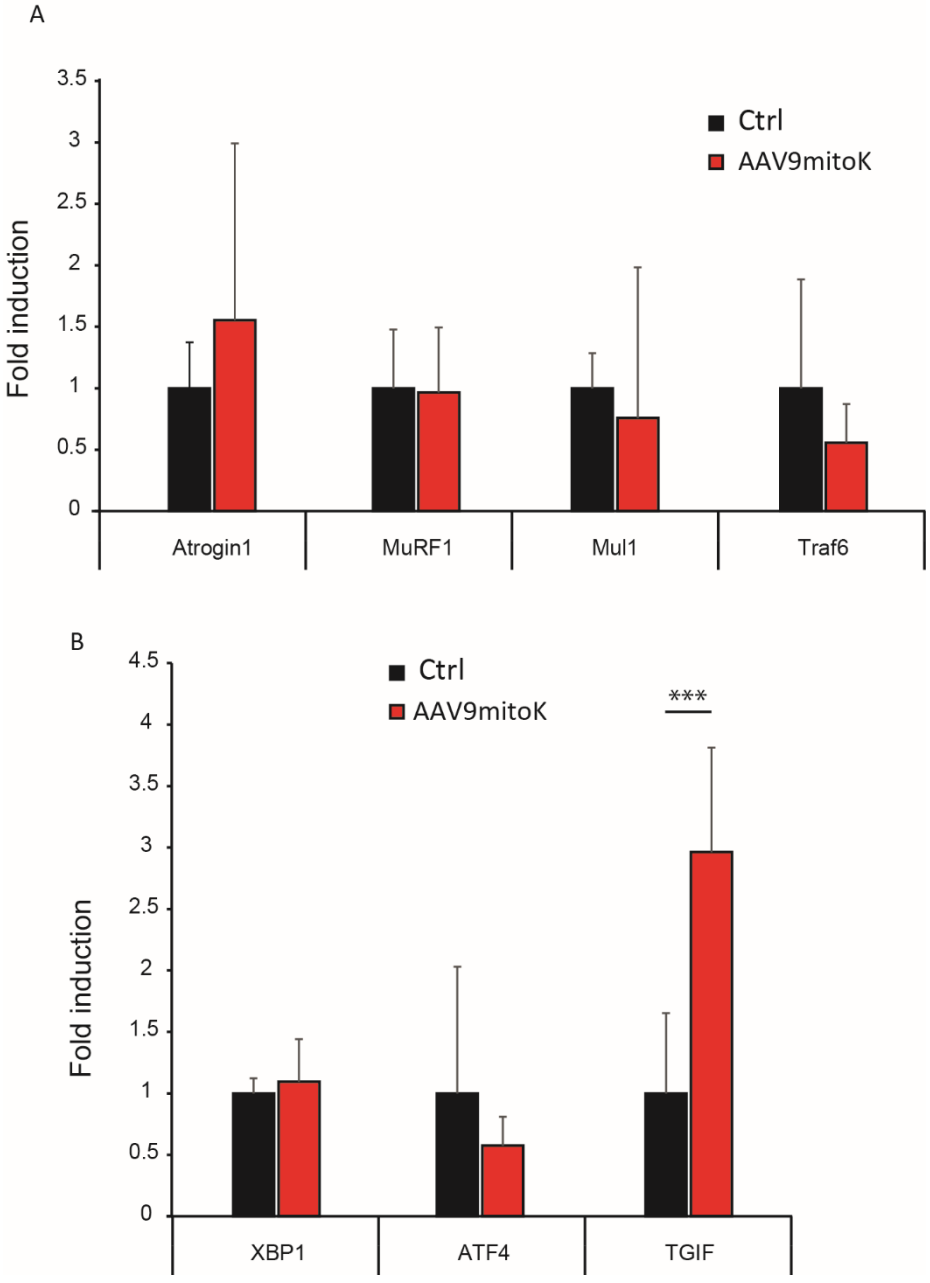


***Figure 17. MitoK overexpression does not modulate the expression of atrophy-related genes.***

*(A) Atrogin1, MuRF1, Mul1, Traf6, relative mRNA expression levels in mitoK overexpressing and control TA muscles measured by Real-time PCR. (B) Xbp1, Atf4, TGIF relative mRNA expression*

*levels in mitoK overexpressing and control TA muscles measured by Real-time PCR. Expression levels are normalized for PolR2F and quantification data are expressed with ΔCt method. ***p < 0.005, t-test (two-tailed, paired) of 8 muscles per group. Data are presented as mean ±SD.*

Overall, gene expression analysis shows that the most common atrophy-related genes, like MuRF1 and Atrogin1, did not change in mitoK overexpressing muscle. Next, we extended the analysis to other genes activated in response to muscle damage and muscle inactivity (Figure 18).

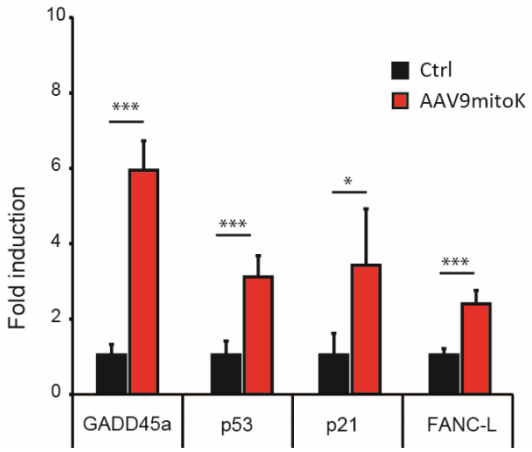


***Figure 18. MitoK overexpression induces different stressor genes.***

*GADD45a, p53, p21, FANC-L relative mRNA expression levels in mitoK overexpressing and control TA muscles measured by Real-time PCR. Expression levels are normalized for PolR2F and quantification data are expressed with ΔCt method. *p<0,05, ***p < 0.005, t-test (two-tailed, paired) of 8 muscles per group. Data are presented as mean ±SD.*

This analysis revealed that in mitoK overexpressing muscle different genes implicated in cellular stress response and DNA repair are induced. We found an increase in FANCL, one of the 15 genes of the Fanconi Anemia (FA) family, required for the activation of the so-called FA pathway. This pathway employs a unique nuclear protein complex that trigger DNA repair together with BRCA protein. Our analysis indicated also an increase in p53 and its downstream target p21. P53 is one of the most studied stress-induced transcription factor, recently associated

with immobilization-induced muscle atrophy (Fox et al., 2014). In skeletal muscle, p53 activates muscle atrophy, while loss of p53 expression partially protect from immobilization-induced atrophy. p21, a well-known cell cycle inhibitor activated by p53, plays an important role in different type of muscle atrophy like denervation, fasting and hindlimb unloading. It has been reported that it promotes atrophy by reducing expression of spermine oxidase, an enzyme important for the maintenance of muscle fiber size under normal conditions (Bongers et al., 2015).

The most interesting finding is the great induction of growth arrest and DNA damage-inducible 45α (GADD45a) in mitoK overexpressing muscles. GADD45 is a small nuclear protein of 18 kDa that has been described as a stress sensor activated by different stimuli and implicated in the regulation of many cellular function including DNA repair, cell cycle arrest and apoptosis. Recently it has been reported that GADD45a is an important molecular mediator of both denervation and fasting-induced muscle atrophy (Bongers et al., 2015; Ebert et al., 2012) so we hypothesize that GADD45a could have a role in the induction of muscle atrophy in AAV9-MitoK injected mice.

Taken together, these data indicates that in skeletal muscle mitoK overexpression induces a wide stress response. Another indicator of a stress-induced response in MitoK overexpressing muscles is the upregulation of heat shock proteins. Heat shock proteins (HSPs) play a fundamental role in the maintenance of muscle homeostasis (Figure 19). They functions as a molecular chaperone maintaining denaturated proteins in a folding-competent state. The family of HSPs is considered an important defense system induced by a variety of environmental stressors such as oxidative stess, inflammation and muscle damage (Moseley, 1998; Welch, 1992). In mitok overexpressing TA muscles we found an induction of HSP27 and HSP90 suggesting that these proteins are required to preserve muscle integrity and to stabilize cytoskeleton.

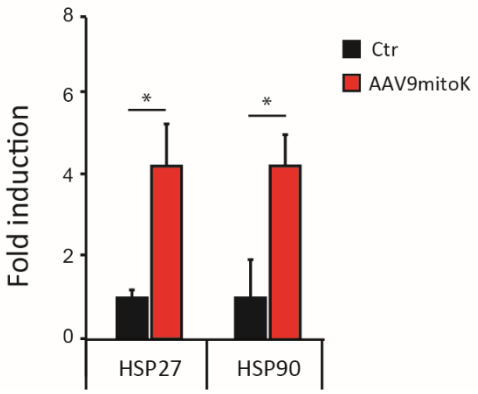


***Figure 19. MitoK overexpression induces heat shock proteins.***

*Hps27 and Hps90 relative mRNA expression levels in mitoK overexpressing and control TA muscles measured by Real-time PCR. Expression levels are normalized for Tioredoxin and quantification data are expressed with ΔCt method. *p < 0.05, t-test (two-tailed, paired) of 8 muscles per group. Data are presented as mean ±SD.*

## MITOK OVEREXPRESSION CAUSES A BLOCK OF AUTOPHAGIC FLUX

Muscle homeostasis is finely modulated by the balance between catabolic and anabolic process. In skeletal muscle the two major catabolic pathways are the ubiquitin-proteasome and the autophagy-lysosome systems. The Ubiquitin-proteasome pathway provides rapid degradation of single proteins or small aggregates (Grumati and Bonaldo, 2012), while autophagy is believed to account for the majority of degradation of long-lived proteins and entire organelles. A proper regulation of the autophagy is essential for skeletal muscle homeostasis in physiological situations and in stress conditions. Indeed, defective or excessive autophagy is detrimental for muscle health (Grumati and Bonaldo, 2012). In several catabolic conditions, such as fasting, atrophy and denervation, aut-ophagy is activated, thus promoting protein degradation. On the other hand, an

impairment of autophagy is correlated with abnormal and dysfunctional mitochondria and protein accumulation.

The study of Atg5 and Atg7 muscle-specific knockout mice has played a fundamental role in understanding the importance of autophagy in skeletal muscle. These knockout models showed that suppression of autophagy is detrimental for muscle homeostasis: both models display muscle weakness and atrophy (Masiero et al., 2009; Raben et al., 2008).

For all these reason, after the analysis of the ubiquitin-proteasome system, we focused on the status of autophagy - lysosome machinery. Autophagy can be easily monitored following LC3 lipidation and p62 degradation. LC3I (Microtubule-associated protein 1A/1B-light chain 3) is lipidated after the recruitment to the autophagic vacuoles (LC3II) and is essential for autophagosomal membrane expansion and fusion. p62 (SQSTM-1) is a ubiquitin-binding scaffold protein that is able to interact with ubiquitinated proteins and binds directly to LC3. The protein is itself degraded by lytic enzymes after the fusion of the autophagosome with the lysosome and may serve to link ubiquitinated proteins to the autophagic machinery to promote their degradation within the lysosome. To monitor autophagy we analysed the protein level of p62 and LC3. Immunofluorescences indicates that p62 accumulates into the myofibers of mitoK overexpressing TA muscles, while western blot indicates an accumulation of both LC3 I and II, respectively the cytosolic form of LC3 and the membrane-bound form of LC3, conjugated to phosphatidylethanolamine (Figure 20). These data suggest an accumulation of autophagosomes as indicated by the increase in LC3 lipidation and increase in p62 expression level. An increase in the number of autophagosomes not always indicate autophagy activation: it may represent either autophagy induction or suppression of autophagosomes maturation or degradation. Indeed, autophagosomes are transient and intermediate structures of a dynamic process. The LC3II levels, that indicate the number of autophagosomes, represent the autophagic activity at that specific time point and does not provide any information about the entire process. The blockade of a

downstream step in autophagy as well as autophagy activation, cause an increase in the number of autophagosomes. For this reason, it is necessary to distinguish whether autophagosome accumulation is caused by autophagy induction or by a block of autophagic process.

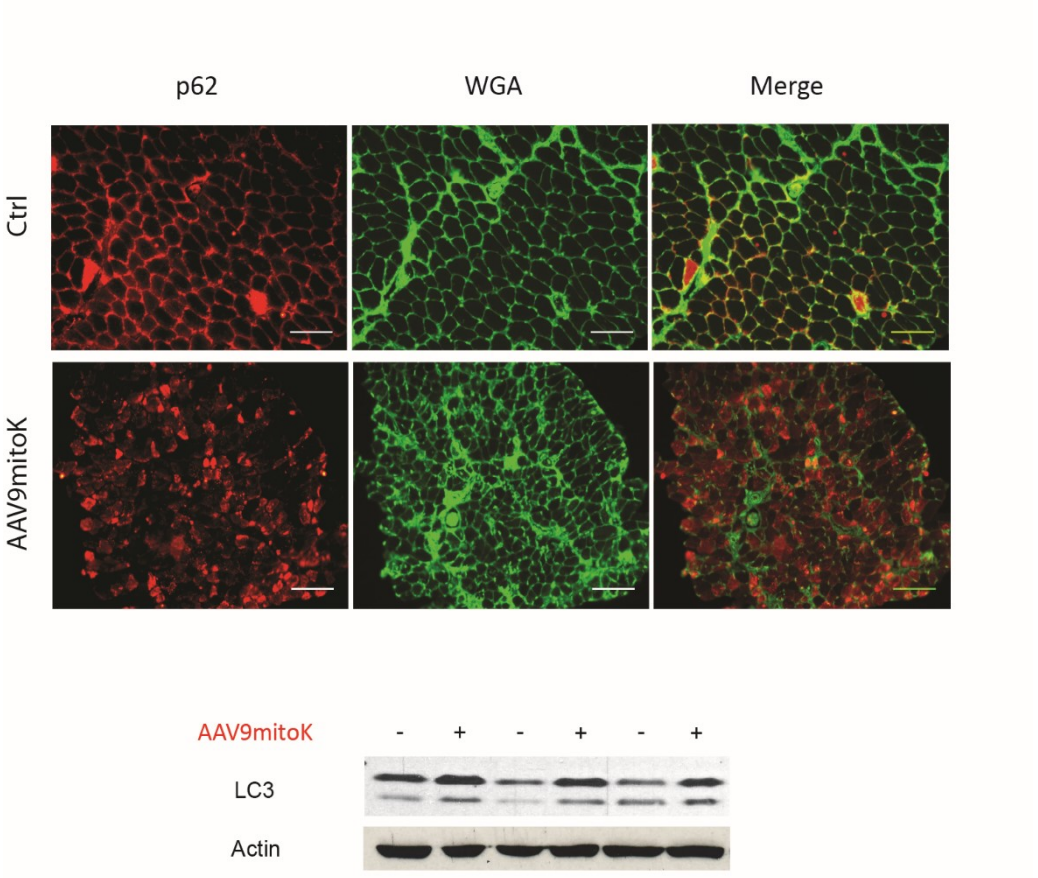


***Figure 20. MitoK overexpression affects the autophagy pathway.***

*The expression of p62 and LC3II was monitored in control and AAV9mitok injected muscles. On the top: immunoflourescence analysis performed on transversal cryosections of Soleus muscle, overexpressing MitoK and control muscle using p62 antibody. Scale bar: 100 micron. On the bottom: Immunoblot analysis of total lysate of TA muscle injected with AAV9mitok.*

To discriminate between these two possibilities we performed an autophagy flux experiment. Autophagy flux is defined as a measure of autophagic degradation activity. It refers to the entire and dynamic process of autophagy: the formation of autophagosomes, the delivery of autophagic substrates to the lysosome and the degradation of autophagic substrates within the lysosome.

One of the major methods for the measurement of autophagic flux is the monitoring of LC3 turnover in the presence or absence of autophagy inhibitors that blocks autophagosomes degradation or maturation resulting in LC3II accumulation. The difference in the amount of LC3-II in the presence or not of autophagy inhibitors represent the amount of LC3 delivered to lysosomes for degradation, that indicates the rate of basal autophagic flux (Klionsky et al., 2016; Mizushima et al., 2010). Together with LC3, p62 is generally used to monitor autophagic flux. It is selectively incorporated inside the autophagosomes and degraded in the last step of the autophagic process, thus the expression level of p62 inversely correlate with autophagic flux.

In order to understand whether autophagic flux was blocked or activated in mitoK overexpressing muscle we analyzed LC3I conversion and p62 accumulation after treatment with colchicine. This is a microtubule depolarizing agents, which inhibits vesicles transport thus blocking the fusion between autophagolysosome and lysosome. In basal condition, if autophagic flux is activated, the treatment with colchicine results in increased LC3II that is due to the accumulation of autophagosomes. Otherwise, if autophagic flux is blocked, LC3 lipidation does not change upon colchicine treatment, indicating that autophagy is already blocked.
As reported in the scheme (Figure 21A), we injected hindlimbs of newborn mice with AAV9mitoK and 4 weeks later we treated mice with colchicine. After 24 hours, we sacrificed mice and isolated muscles for the analysis. In this experiment, we have four groups of mice: two groups of control mice treated or untreated with colchicine, and two other groups of mice injected with AAV9 MitoK, treated or not with colchicine.
In the control group of mice, colchicine increases LC3 lipidation and induces an accumulation of p62 protein, indicating that the treatment increases the number of autophagosomes by blocking their fusion with lysosome.
In the group of mice injected with AAV9mitoK, the treatment with the blocker of autophagy has no effect, since the expression level of LC3II and p62 is comparable

with the control. This suggest that, in mitoK overexpressing muscles, there is a consistent accumulation of autophagic vesiscles also in basal condition, meaning that autophagy is already blocked (Figure 21B).

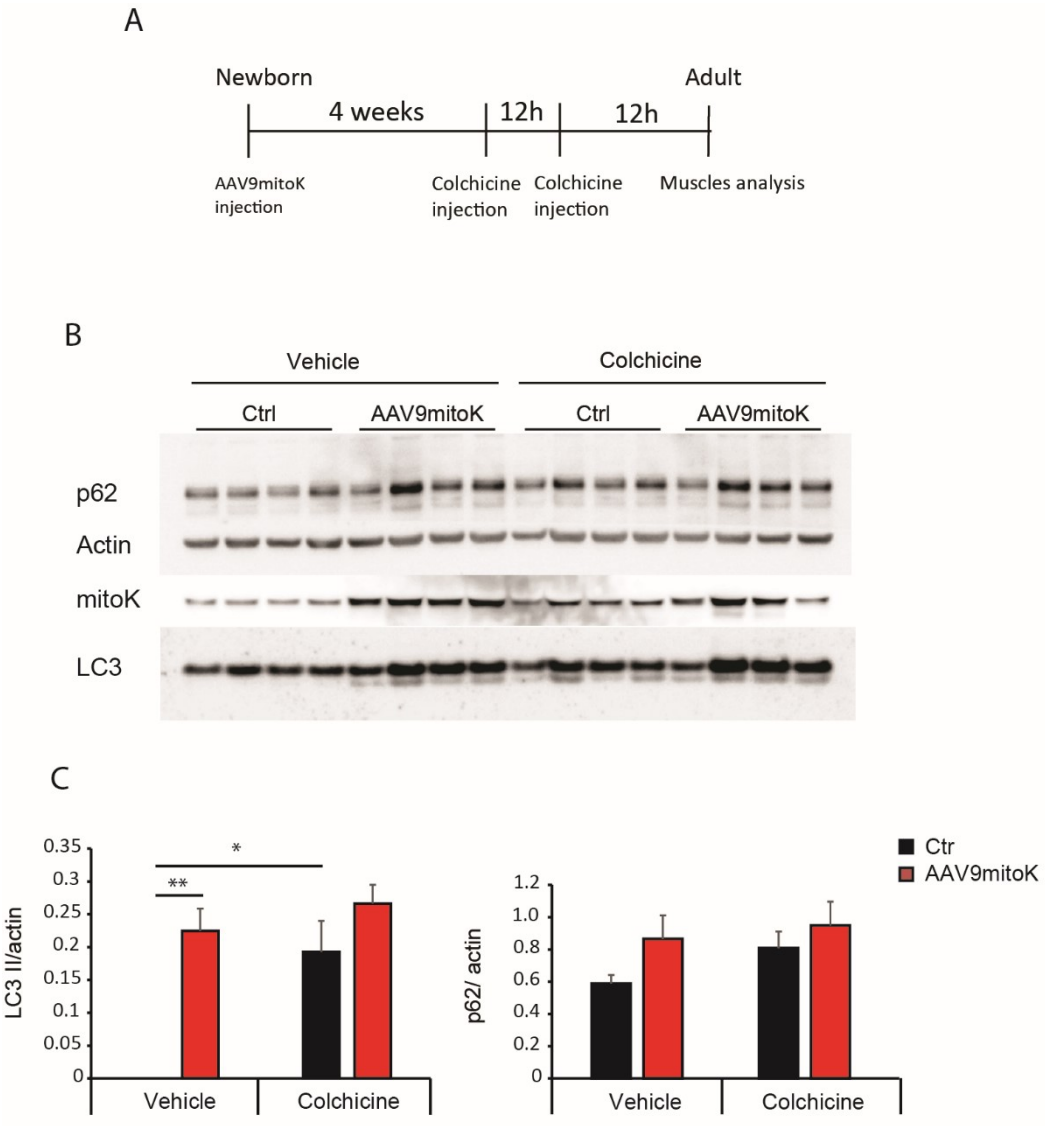


***Figure 21. MitoK overexpression induces a block of the autophagic flux.***

*(A) Representative scheme of experimental protocols: 4 weeks after AAV9mitoK infection mice were intraperitoneally injected with 0.1 mg/Kg colchicine; the treatment was repeated 12h later. The control group of mice received an equal amount of NaCl 0,9%. 24h after the first injection we sacrificed mice and collected muscles.*

*(B) Immunoblot analysis of AAV9 mitoK TA muscles and control muscles treated or not treated with colchicine. Anti-mitoK antibody was used to confirm MitoK overexpression. Actin was used as loading control. (C) The graph on the bottom shows the quantification by densitometry of the ratio between LC3II/actin and p62/Actin. *p<0,05; **p<0,01, t test (two-tailed, unpaired). n=4. Data are presented as mean ±SD.*

In untreated AAV9mitoK injected muscle, LC3 and p62 protein levels are similar with that induced by autophagy inhibitors in control muscles, supporting the idea that mitoK overexpression blocks autophagic flux. With this experiment, we established that in skeletal muscle the overexpression of mitoK induces a block of autophagic flux, thus an accumulation of autophagosomes. A block of autophagy was also confirmed by TEM analysis (Figure 22).

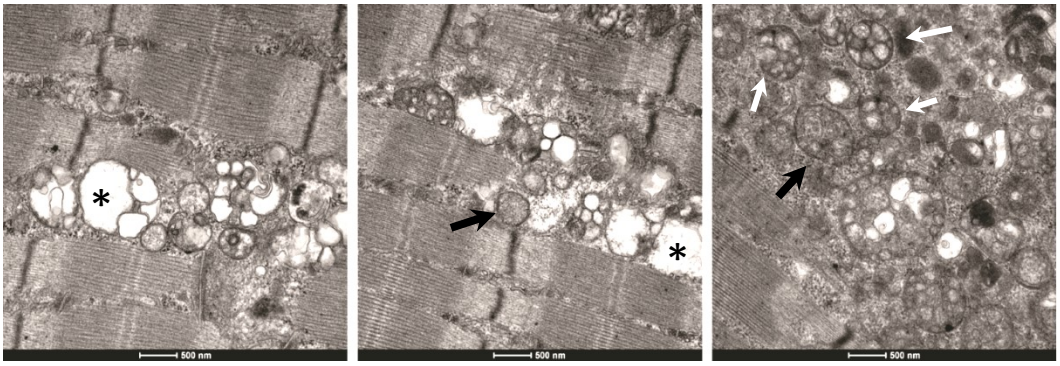


***Figure 22 - MitoK overexpression induces an accumulation of single and double-bounded vesicles.*** *TEM analysis performed on longitudinal sections of EDL muscles overexpressing mitoK. In these muscles, different vesicle types are present: multivesicolar bodies (white arrows), single membrane-bounded vesicles (black asterix) and double membrane-bounded vesiscle (black arrows) that surrounded swollen mitochondria.*

EM pictures revealed the presence of double-membrane autophagosomes dispersed inside the myofibers and at the sarcolemma. In addition, we also observed many different structures of various size and morphology: multivescicolar bodies, lamellar bodies and single-membrane bounded vesicles.

These images indicate that in mitoK overexpressing myofibers there are many damaged organelles, especially mitochondria. Ubiquitin-proteasome system is activated to counteract the accumulation of dysfunctional organelles, however, autophagy- lysosome machinery is blocked at the downstream steps so the two catabolic pathway are not able to cope muscle wasting.

### IN MITOK OVEREXPRESSING MUSCLE PROTEIN SYNTHESIS IS ACTIVATED

Muscle mass is determined by a precise balance between de novo protein synthesis and degradation of pre-existing proteins and that molecular pathways controlling these two processes are tightly regulated and interrelated. For this reason, we decided to investigate the effect of mitoK overexpression on protein synthesis (Figure 23).

We analyzed protein synthesis by ‘‘surface sensing of translation’’ (SUnSET), a method based on the incorporation of puromycin, an aminonucleoside antibiotic, into nascent peptide chains during translation (Goodman et al., 2011). These peptides can be detected by means of an anti-puromycin antibody, thus allowing the precise measurement of the protein synthesis rate. As reported in the scheme (Figure 23A), four weeks after AAV9mitoK injection, we injected puromycin intraperitoneally and 30 minutes later we isolated muscles and performed immunoblot analysis. Based on the strong atrophy displayed by mitoK overexpressing mice, although we would have expected a decrease in protein synthesis, we found a drastic increase in protein synthesis rate.

To better characterize the signalling pathway responsible for the induction of protein synthesis, we first looked at IGF/AKT pathway, one of the major pathways involved in the control of muscle mass.

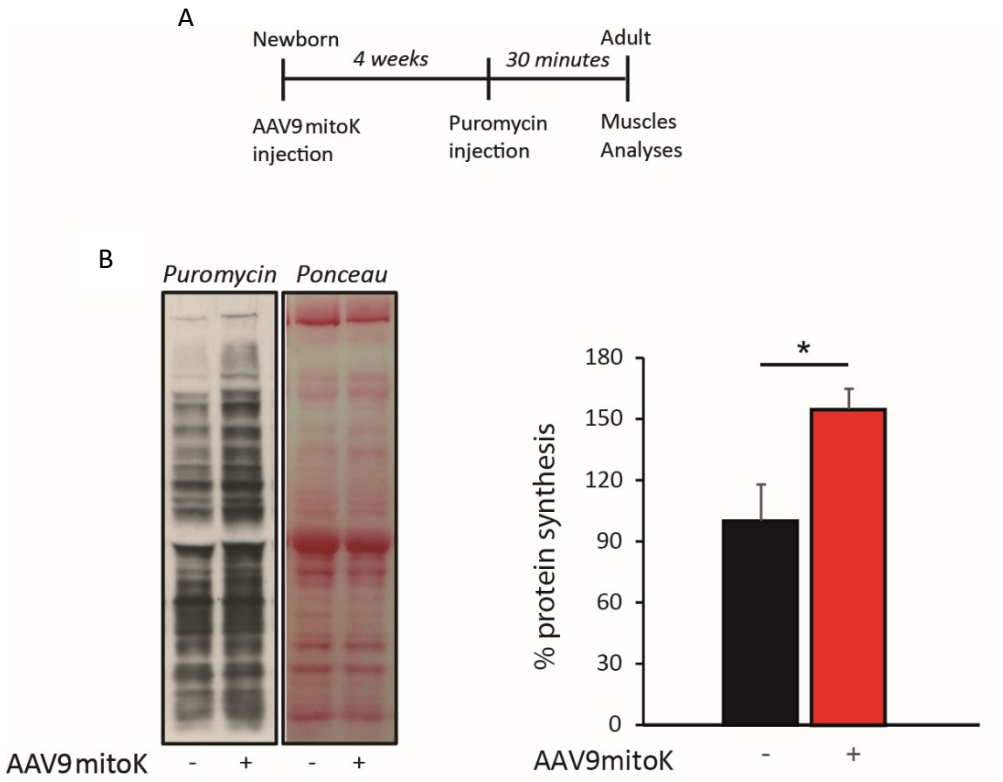


***Figure 23 - Mitok overexpression induces protein synthesis***.

*Analysis of protein synthesis in TA muscles injected with AAV9mitoK. (A) Scheme of experimental procedure: 4 weeks after AAV9mitoK infection mice were intraperitoneally injected with puromycin. 30 minutes after the injection mice were sacrificed and muscles were collected. (B) On the left: Immunoblot analysis of TA muscles injected with AAV9mitoK and control muscles. Protein estracts were probed with anti-puromycin antibody. Ponceau staining was used as protein loading control. The graph on the right shows the quantification by densitometry expressed in percentage of the ratio between Puromycin and Ponceau. *p<0,05; t test (two-tailed, unpaired). n=5. Data are presented as mean±SD.*

We found that in AAV9mitoK injected mice, AKT pathway was induced, showing an increase in the phosphorylation of AKT and its downstream effectors. As reported in the Figure 24, mitoK injected TA muscles showed an increase in the phosphorylation of 4EBP1, an inhibitor of protein translation, inactivated by AKT dependent phosphorylation.

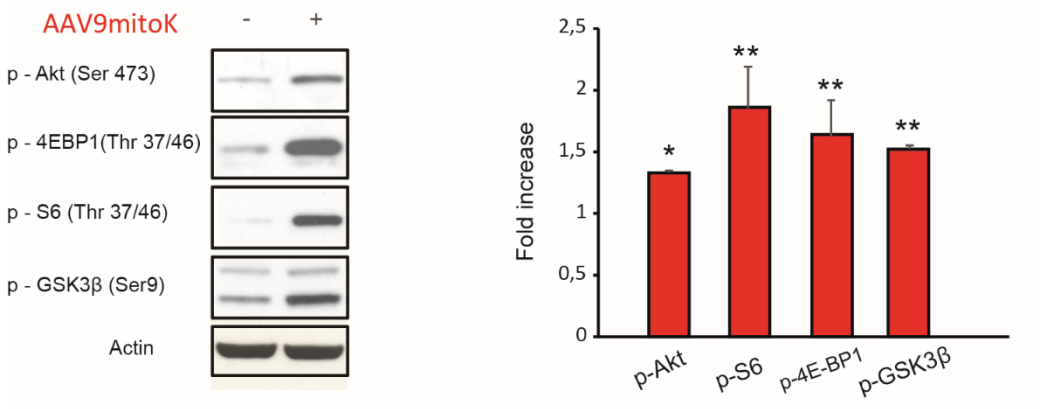


***Figure 24. Mitok overexpression activates AKT pathway.*** *On the left: Immunoblot analysis of AAV9mitoK injected and control mice. Protein extracts from TA muscles were probed with: anti-pAkt (Ser473), anti- p4EBP1(Thr 37/46), anti p-S6 (Ser 240/244) and anti-pGSK3β (Ser9) antibodies. Actin was used as protein loading control. The graph on the right shows the quantification by densitometry expressed in fold increase of each phosphorylated proteins, normalized to actin, compared to control. *p<0,05, **p<0,01; t test (two-tailed, unpaired), n=8. Data are presented as mean ±SD.*

Phosphorylated form of S6, a positive regulator of protein translation, also increased in mitoK overexpressing muscles. Another downstream target of AKT that increases after mitoK overexpression is GSK3beta, a positive regulator of protein synthesis. These results suggest that a compensatory mechanism, counteracting muscle atrophy, is activated in mitoK overexpressing muscles.

GSK3 beta is a serine-threonine kinase that negatively regulates glycogen synthesis. It is inactivated by an AKT-dependent phosphorylation, while glycogen synthase is activated leading to increased rates of glycogen synthesis. In mitoK overexpressing muscles, the phosphorylation of GSK3β significantly increase, so we wondered if this induction results in increased glycogen levels (Figure 25).

To assess the presence of glycogen deposits we performed a PAS staining on TA muscles cryosections. As reported in the figure, we did not find any significant differences between mitoK overexpressing muscles and control. Since this staining is generally used to disclose gross variation in glycogen content, we decided to measured glycogen quantity in TA muscle injected with MitoK, using a more sensitive quantification method. Either in this case we did not find any significant differences in the two groups of mice.

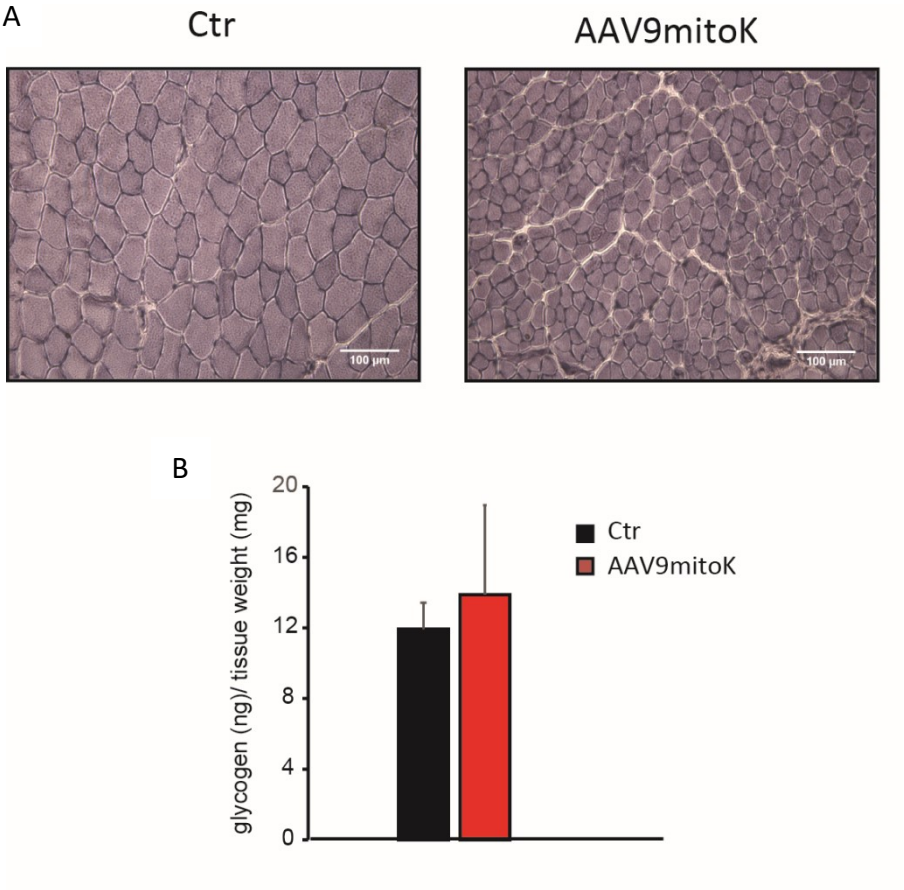


***Figure 25. Mitok overexpression does not alter glycogen content***. (A) *PAS staining performed on TA cryosections of muscles overexpressing mitoK, 4 weeks after the infection. Scale bar 100 µm. (B) Glycogen content measurement performed on TA muscles overexpressing mitoK, 4 weeks post-infection and control muscles.*

## MITOK CONTROLS MUSCLE SIZE IN ADULT MICE

The growth of skeletal muscle mass depends on both protein turnover and cell turnover (Sartorelli and Fulco, 2004). It is well established that cellular turnover plays a major role during muscle development. This means that a decrease of muscle mass and fiber size in the adulthood can be due to a failure in muscle growth and not by the activation of atrophy process.

In order to understand whether in our model there is a defect in muscle growth or the activation of an atrophy program we proceeded with the analysis of adult mice. For this scope, adult EDL muscles were infected with AAV9mitoK and analysed 2 weeks later. In EDL muscle, mitoK overexpression, caused no alteration of muscle structure, as indicated by the WGA and H&E stainings. The SDH staining, an indicator of mitochondrial activity, shows that mitoK overexpression does not affect significantly mitochondrial functionality (Figure 26).

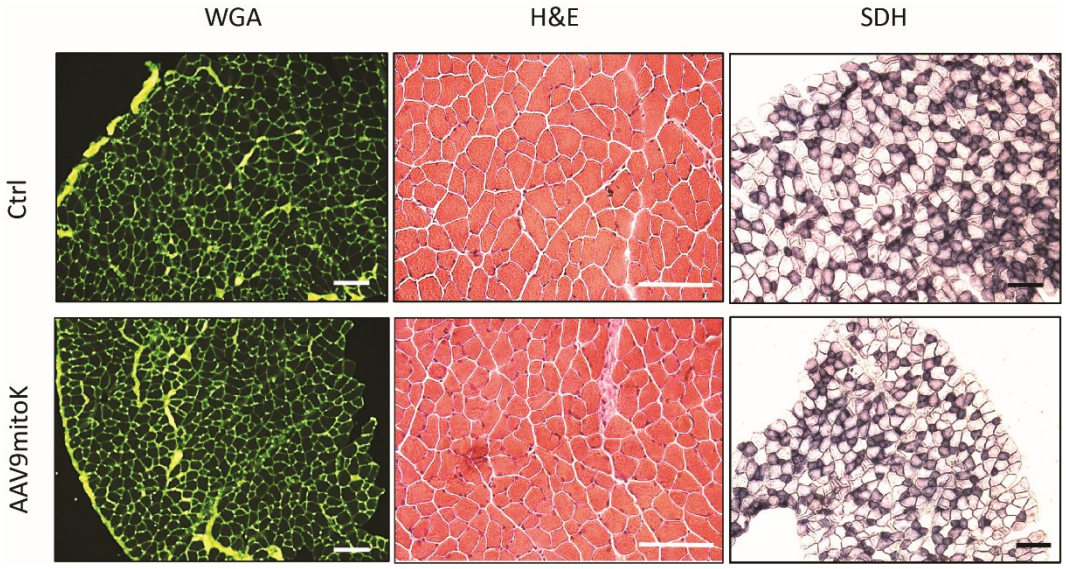


***Figure 26. Mitok overexpression does not significantly alter muscle structure and mitochondrial function in Adult****. From left to right: wheat germ agglutinin (WGA) staining, H&E staining and SDH staining performed on TA cryosection of muscles overexpressing MitoK and control muscles. Scale bar 100 µm.*

Since we found a drastic decrease in muscle mass after mitoK overexpression in newborn mice we measured fiber size of adult EDL muscles (Figure 27B). MitoK overexpression in adult mice triggers a 25% decrease of fiber size, suggesting that the effect on muscle mass induced by mitoK are not growth-dependent.

Then we dissected the signalling pathways activated in this context. Adult mice overexpressing mitoK are characterized by a dysregulation of mitochondrial markers and an increase of p62 and LC3 (Figure 27C).

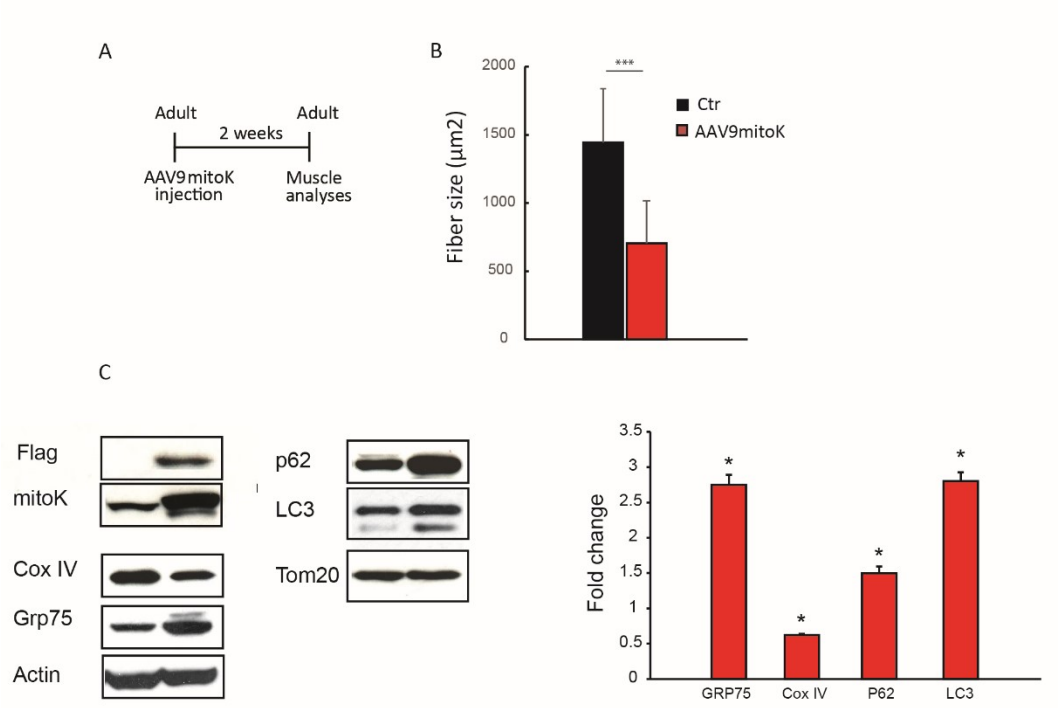


***Figure 27. Mitok overexpression induces muscle atrophy and causes a dysregulation of autophagy in Adult mice.***

*(A) Representative scheme of experimental procedure: EDL muscles of adult mice were injected with AAV9MitoK. 15 days after the infection mice were sacrificed. (B) Average fiber size of EDL muscles. n=4; ***p<0,005, data are expressed as mean±SD. (C) On the Left: Immunoblotting performed on TA muscle overexpressing mitoK 4 weeks after the infection. Protein estracts were probed with anti-Flag, anti mitoK, anti COX IV, anti-GRP75, anti-p62, anti LC3 antibodies. Actin and Tom20 were used as protein-loading control. On the Right: quantification by densitometry. Data are reported as fold increase of each proteins, normalized for the relative housekeeping, compared to control. n=8; *p<0,05, Data are expressed as mean±SD.*

In conclusion, in adult mice overexpressing mitoK we found a decrease in fiber size and a variation in the content of mitochondrial proteins, which suggest a mitochondrial remodelling, together with an increase in LC3 and p62, that indicates a block of autophagic flux and an activation of compensatory hypertrophy.

Taken together our data indicate that in adult as well as in newborn mice mitoK overexpression induces muscle atrophy, accompanied by a dysregulation of autophagy.

# DISCUSSION AND CONCLUSION

Potassium is the most abundant cation inside the cell and plays a key role in maintaining cell function. Different studies indicate that, in addition to the $K^+$ channels in the plasma membrane, mitochondrial $K^+$ channels play important roles in cell survival (Yu, 2003). Six mitochondrial potassium channels have been described until now: *mitoK$_{ATP}$* (Inoue et al., 1991), *mitoBKCa* (Siemen et al., 1999), *mitoKv 1.3* (Szabò et al., 2005), *twin-pore TASK-3* (Rusznák et al., 2008), *mitoIKCa* (De Marchi et al., 2010) *and mitoSKCa* (Stowe et al., 2013). The existence of the mitochondrial $K_{ATP}$ channels (mitoK$_{ATP}$) has been long debated. Even if the discovery of an ATP-dependent mitochondrial potassium channel dates back to 1991, its molecular composition remained unknown for a long time. After the identification of mitoK$_{ATP}$ channels, the vast majority of research was focused on the role of these channels in the heart. These studies indicates that the opening of mitoK$_{ATP}$ correlates with cardioprotection and plays a central role in the process of ischemic preconditioning (IPC) and mitochondrial matrix swelling (Liu et al., 1998). However, the physiological function of mitoK$_{ATP}$ in skeletal muscle is a poorly explored field. Studies performed on L6 skeletal muscle myoblast cell line and isolated rat skeletal muscle, suggested that the pharmacological activation of mitoK$_{ATP}$ channel has a direct effect on mitochondrial respiration, membrane potential and matrix volume (Debska et al., 2002). Other studies indicate that the pharmacological opening of mitoK$_{ATP}$ significantly reduces muscle fatigue (García et al., 2009). Nevertheless, to date, many questions remain unanswered: how does mitoK$_{ATP}$ activation protect cells from death and how does its activation lead to the process of IPC and regulate muscle fatigue?

Recently, the laboratory of Professor Rizzuto at the University of Padova, disclosed the molecular composition of this channel. It has been established that, similarly to the plasma membrane potassium channels ($K_{ATP}$), mitoK$_{ATP}$ is composed by four channel-forming subunits (mitoK) and four ATP-sensitive domains (mitoSUR) arranged in an octameric structure (Paggio A. et al., unpublished). Experiments

performed in HeLa cells indicate that the constitutive opening of mitoK$_{ATP}$ channels causes severe dearrangments of the mitochondrial ultrastructure and a strong decrease of mitochondrial membrane potential (Paggio et al., unpublished). The recent identification of the molecular composition of mitoK$_{ATP}$ channels finally allows the investigation of their structure, function and pathophysiological roles.

Mitochondria play a central role in skeletal muscle metabolism. Indeed, to support the high demand of ATP required during contraction, skeletal muscles rely on oxidative metabolism for energy production, which is sustained by the great mitochondrial density of this tissue. Thus, maintaining skeletal muscle mitochondrial content and function is fundamental for maintaining muscle homeostasis throughout the lifespan (Gouspillou et al., 2016). Recent findings indicate that the alteration of mitochondrial network and function strongly influence skeletal muscle homeostasis. Indeed, the activation of proper mitochondria quality control pathways is particularly important for tissues, like striated muscles, constituted by post-mitotic cells that cannot dilute dysfunctional organelles through cellular division (Gouspillou et al., 2016).

In this work, we studied the effect of mitoK, i.e. the channel forming subunit of the mitoK$_{ATP}$, overexpression in developing muscle and in adult skeletal muscle. To overexpress mitoK during development, we took advantage of AAV9 vectors carrying the coding sequence of mitoK downstream of a CMV promoter (AAV9mitoK) which, topically injected into the hindlimb of 4-6 day-old mice, allow continuous overexpression of mitoK until adulthood (1-2 month-old mice). Within this experimental setup, mitoK overexpression causes severe muscle atrophy, as demonstrated by the reduction of muscle mass and the strong decrease in fibers size. Four weeks after AAV9mitoK injection (thus in 1 month-old mice), the muscle weight was reduced of more than 50% compared to controls, and this reduction went up to 60% in two month-old mice injected with AAV9mitoK when they were pups (Figure 8).

A comparable reduction in muscle mass has been also reported in other mouse model characterized by mitochondrial dysfunction like *Mfn1 and Mfn2 KO* mouse model (Chen et al., 2010) and muscle specific *Opa1* KO mice (Tezze et al., 2017).
Since 1960 numerous studies indicates that mitochondria play an important role triggering signals that contribute to muscle atrophy (Carafoli et al., 1964; Powers et al., 2011a; Romanello and Sandri, 2010). The first published reports indicate that muscle atrophy was accompanied by changes in mitochondrial shape, number and function. Then, many other studies confirmed that muscle atrophy correlates with decreased expression of mitochondrial proteins, mitochondrial respiratory dysfunction and increased mitochondrial ROS production (Abadi et al., 2009; Adhihetty et al., 2007; Muller et al., 2007; Powers et al., 2011b; Singh and Hood, 2011). In particular, Romanello et al. (Romanello et al., 2010b) established a causal link between mitochondrial network and muscle atrophy demonstrating that dysregulation of mitochondrial dynamics affects the signaling pathways that control muscle mass.
In line with these reports, we found significant changes in mitochondrial structure and bioenergetics function of mitoK overexpressing muscle. Electron microscopy studies performed on EDL muscles revealed a strong alteration of mitochondrial ultrastructure upon mitoK overexpression. In mitoK overexpressing muscles, the internal cristae are totally collapsed and mitochondria are swollen and fragmented. This peculiar profile is very similar to the one induced by the treatment with valinomycin, which acts as a potassium ionophore thus increasing potassium permeability of the inner mitochondrial membrane, leading to mitochondrial swelling (Ogata and Rasmussen, 1966). These data support the hypothesis that the overexpression of mitoK alone, without co-expression of the regulatory mitoSUR subunits, induces the constitutive opening of the channel with consequent increase of $K^+$ influx into the mitochondrial matrix. The net $K^+$ influx is followed by water entry, resulting in increased mitochondrial matrix volume and in the disruption of the internal membrane. EM pictures also revealed the presence of many vacuoles inside the mitochondria, in agreement with damaged

mitochondria being degraded. Immunoblot analyses confirm the alteration of mitochondrial structure triggered by mitoK overexpression. As reported in Figure 13, the overexpression of mitoK modulates the expression levels of different mitochondrial proteins according to their specific localization. The levels of proteins confined within the matrix, like GRP75, increased, while the expression of protein located in the inner membrane, such as COX IV, Timm44 and IMMT, decreased. This particular expression profile suggests a remodeling of the mitochondrial structure, with expansion of the mitochondrial matrix and disruption of the inner membrane. Since the alteration of mitochondrial morphology generally correlates with a dysregulation of organelle function, we analyzed the overall expression level of electron transport chain components. We found that the overexpression of mitoK induces a significant reduction in the expression of Complex I, II and IV (Figure 14). In different types of atrophic conditions, a comparable decrease in the expression of mitochondrial proteins, implicated in the electron transport and in ATP production, was reported (Lecker et al., 2004) confirming that the mitochondrial functionality is essential for muscle homeostasis. The decrease in the expression of complex II, the succinate dehydrogenase (SDH), was also accompanied by a decreased activity both in soleus and in EDL muscles overexpressing mitoK (Figure 15). Overall, our data suggest that the changes of mitochondrial network are the primary cause of muscle wasting and the subsequent atrophy.

Muscle atrophy is a process determined by a decrease in muscle size, which is controlled by a tight balance between protein degradation and protein synthesis. During muscle atrophy protein degradation overcomes protein synthesis, thus inducing a decrease in muscle mass. We analyzed the activity of the ubiquitin-proteasome system, one of the major catabolic pathway in skeletal muscles, in mitoK overexpressing muscles. As reported in Figure 16, we observed a 60% increase of protein ubiquitination after mitoK overexpression compared to

controls, indicating that protein degradation is increased, promoting the shrinkage of muscle fibers.

Muscle atrophy is a complex process resulted from the activation of a precise set of genes, commonly defined “atrogenes”. Among these genes, some are implicated in protein degradation, others in cellular metabolism, and others in the regulation of protein translation and gene transcription (Lecker et al., 2004). The most induced genes in atrophic condition are atrogin-1/MAFbx and MuRF1, two ubiquitin ligases. Atrogin-1 and MuRF1 are relatively low expressed in resting skeletal muscle, while increase significantly within 24 h of inactivity. QPCR analyses performed on TA muscle indicate that the expression of MuRF1 and atrogin-1 is not affected by mitoK overexpression (Figure 17). However, in rodent models, the mRNA expression profile of MuRF1 and atrogin-1 is characterized by a rapid increase, within the first 48h after the injury or inactivity, and then a decrease to baseline after 14 days (Bodine and Baehr, 2014). Thus, it is possible that this type of prolonged treatment, that mimics a chronic condition, does not allow the detection of any variation in atrogin-1 and MuRF1 mRNA expression.

In the context of muscle atrophy, an important role is played by transcription factors that mediate the suppression of muscle growth and the activation of genes involved in protein degradation. The analysis performed on mitoK overexpressing muscles indicates an increase in TGIF expression, an homeobox gene codifying for an active transcriptional co-repressor of TGF-beta/SMAD (Derynck and Zhang, 2003). The upregulation of TGIF suggests that TGFβ pathway is activated, so it may play a role on muscle atrophy. As reported in the Figure 18, other genes activated in stress conditions and involved in muscle damage response, are induced in mitoK overexpressing muscles. Some of these genes are implicated in DNA repair and in the control of cell cycle. We found a 3.5 fold induction of FANCL, a member of Fanconi Anemia family of genes, upon mitoK overexpression. The well characterized function of FA genes is the repair of DNA interstrand crosslinks formed in the presence of ROS or aldehyde substrate (Garaycoechea and Patel, 2014). After DNA damage, FA proteins form a FA core complex which contains the

E3 ubiquitin ligase FANCL, that mono-ubiquitinates FANCD2 and FANCI orchestrating downstream DNA repair (Garaycoechea and Patel, 2014). Recently, FANCL and other members of FA pathway were reported as a previously undescribed selective autophagy factors. They are implicated in mitochondrial quality control, the maintenance of mitochondrial respiratory function, suppression of intracellular ROS levels (Pagano et al., 2015) and reduction of inflammasome activity (Garbati et al., 2013).

These findings indicate the existence of a strict crosstalk between nucleus and mitochondria and elucidate how, in mitoK overexpressing mice, mitochondrial dysregulation results in the activation of nuclear pathways and the reprogramming of gene expression.

Starting from these data, we analyse the mRNA levels of other important genes activated in stress conditions like p53 and p21. Both these proteins, important regulators of cell cycle which are activated in response to stress conditions, have been linked with the activation of muscle atrophy upon immobilization (Fox et al., 2014). p53 and p21 are upregulated by mitoK overexpression in TA muscles, suggesting a possible role, either direct or indirect, of the two proteins in the induction of atrophy (Figure 18). Another stress sensor, strongly activated by mitoK overexpression, is GADD45a. This protein induces a great myonuclear remodelling and coordinates the reprogramming of gene expression during muscle atrophy. It is able to induce pro-atrophy genes through the repression of genes involved in anabolic signalling and energy production (Ebert et al., 2012). These results suggest that GADD45a could reprogram muscle gene expression to induce atrophy in response to stress-induced pathways in AAV9mitoK injected mice. Finally, we found a significant increase in HSP90 and HSP27 expression, two important chaperones generally required to support protein re-folding in stress condition (Figure 19).

Muscle atrophy results from excessive protein degradation, mainly due to the activation or dysregulation of the two major catabolic pathways in skeletal muscle: the ubiquitin proteasome system and autophagy-lysosome system. As reported in

Figure 16, in mitoK overexpressing muscles we found an increase in protein ubiquitination. Starting from these data, we decided to explore the activity of the autophagy/lysosome pathway, essential for the degradation of dysfunctional organelles and large portions of cytoplasm. As described in the introduction, a proper regulation of autophagy is fundamental for muscle homeostasis (Grumati and Bonaldo, 2012). However, the role of autophagy in the maintenance of muscle health is not completely understood. Indeed, in some atrophic conditions, the activation of autophagy promotes muscle wasting, while, in other catabolic settings, autophagy is dysregulated. Immunofluorescence and immunoblot analyses indicate that mitoK overexpression increases LC3 II levels and induces p62 accumulation in the myofibers of TA muscles, suggesting an involvement of this degradative pathway in the activation of muscle wasting (Figure 20). Since an increase in the markers of autophagy, p62 and LC3II, indicate only an increase in the total number of autophagosomes, without giving information about the dynamics of the process, we performed an autophagic flux experiment. This analysis indicated that the overexpression of mitoK causes a block of the autophagic flux, with a subsequent accumulation of autophagosomes (Figure 21). The increase of autophagic vesicles was also supported by TEM analysis that revealed the presence of vacuoles and vesicles of various morphology surrounding mitochondria. Taken together our data shows that, in mitoK overexpressing muscles, the ubiquitin-proteasome system is activated to eliminate dysfunctional protein, while the autophagy-lysosome machinery is blocked. We suppose that the autophagy pathway is functional: the early stages of the process, until the formation of autophagic vesicles, are not compromised. However, the block of the last step of the autophagic flux causes a great increase in the amount of autophagic vesicles that are not properly degraded by lysosome. This defect in the downstream steps of the autophagic pathway can be responsible for the accumulation of poliubiquitinated proteins, thus it can exacerbate muscle damage. Nevertheless, we have to better characterize the role of autophagy and the activity of proteasome in mitoK overexpressing mice.

Since muscle mass is determined by the balance between catabolic and anabolic pathways, after the analysis of proteolytic system, we moved to the analysis of protein synthesis using the ‘‘surface sensing of translation’’ (SUnSET) method, which allow the measurement of protein translation rate. MitoK overexpression resulted in increased protein synthesis, which is accompanied by the activation of the IGF/AKT pathway, one of the major signalling pathways involved in the control of muscle mass (Figure 23 and Figure 24). These results could be counterintuitive; indeed in atrophying muscle a decrease in protein synthesis is expected. However, several studies indicate that active protein synthesis accompanies protein degradation in atrophic conditions (Argadine et al., 2009; Goldspink, 1976). Recent data suggest that the increased protein translation during atrophy can be due to a direct activation of mammalian target of rapamycin complex 1 (mTORC1) which is mediated by free amminoacids generated from proteasome-mediated degradation (Quy et al., 2013). A possible mechanism of action was also proposed: mTORC1 activation probably occurs at the lysosomes and involves an aminoacid sensing cascade that requires the activation of RAG GTPases, Ragulator and vacuolar v-ATPase (Jewell et al., 2013). Thus, mTORC1 activation may play a key anabolic role during muscle atrophy. We suppose that the paradoxical activation of protein translation and mTORC1 pathway, in mitoK overexpressing muscles, could be an adaptive response required to counteract muscle atrophy. This hypothesis is supported by the increased expression of two Heat shock proteins, HSP27 and HSP90, in mitoK overexpressing muscles. HSPs have a role in the activation of compensatory hypertrophy after functional overload (FO) (Huey et al., 2004, 2010), demonstrating that HSPs induction well correlates with a compensatory mechanism, activated in response to muscle atrophy. HSP90 is also involved in the maintainance of Akt kinase activity by preventing PP2A-mediated dephosphorylation (Sato et al., 2000).

In the first part of our study, in order to understand whether mitoK influence muscle growth, we analyse the phenotype of newborn-treated mice, 1 month

after AAV9mitoK infection. One of the most controversial issue of the data presented so far is that muscle atrophy, triggered in the adult by overexpressing mitoK since infancy, could be linked with defects in fibers growth and muscle development rather than with the activation of a specific atrophy program. Indeed, during muscle development, muscle growth is the major process that controls muscle mass, while in adulthood, muscle mass and muscle size are mainly defined by protein turnover (Moss and Leblond, 1971; Sartorelli and Fulco, 2004). To discriminate whether the severe reduction of muscle weight and fiber size is or not dependent on the impairment of muscle growth, we performed an additional protocol of mitoK overexpression. In this case, we injected the EDL muscles of 2-month old mice with AAV9mitoK and performed subsequent analyses 15 days later. These analyses revealed a 25% decrease of the average fiber area, compared to controls. However, no major alterations were observed in the myofiber morphology and in mitochondrial activity, as indicated by H&E staining and the SDH activity assay (Figure 26). As newborn-treated mice, also mice treated during adulthood displayed a similar variation in mitochondrial proteins expression upon mitoK overexpression, suggesting a remodelling of mitochondrial network. The autophagic process was also dysregulated: the increase in LC3 II and p62 (Figure 27) suggests a block of the autophagic flux.

To summarize, adult-treated mice displayed an atrophic phenotype, as indicated by the reduction of muscle mass, although in a lesser extent compared to newborn-treated mice. These data demonstrate that the molecular mechanism of atrophy is conserved in the two experimental models. Nonetheless, proper muscle structure is maintained in the adult-treated animals, indicating a milder effect, which does not evolve toward cell death and muscle degeneration.

Overall, our results suggest that mitoK plays a role in the control of mitochondrial function and muscle trophism. MitoK overexpression causes an excessive accumulation of potassium inside the mitochondrial matrix, causing organelle swelling and energy stress (Figure 28). These drastic alterations of mitochondrial

structure and function, in turn trigger the activation of stress-response pathways affecting muscle homeostasis and promoting the activation of degradative systems, thus causing muscle atrophy. The accumulation of dysfunctional proteins and damaged organelles is accompanied by a significant accumulation of autophagomes that are not properly eliminated by lysosomes, causing a block of the last steps of this degradative pathway. To counteract muscle wasting, protein synthesis and IGF/AKT pathway are activated as compensatory responses. Based on the severe effects of mitoK overexpression on mitochondria, we suppose that muscle atrophy is a consequence of mitochondrial dysfunction. However, the molecular mechanisms induced by mitoK overexpression remain to be defined.

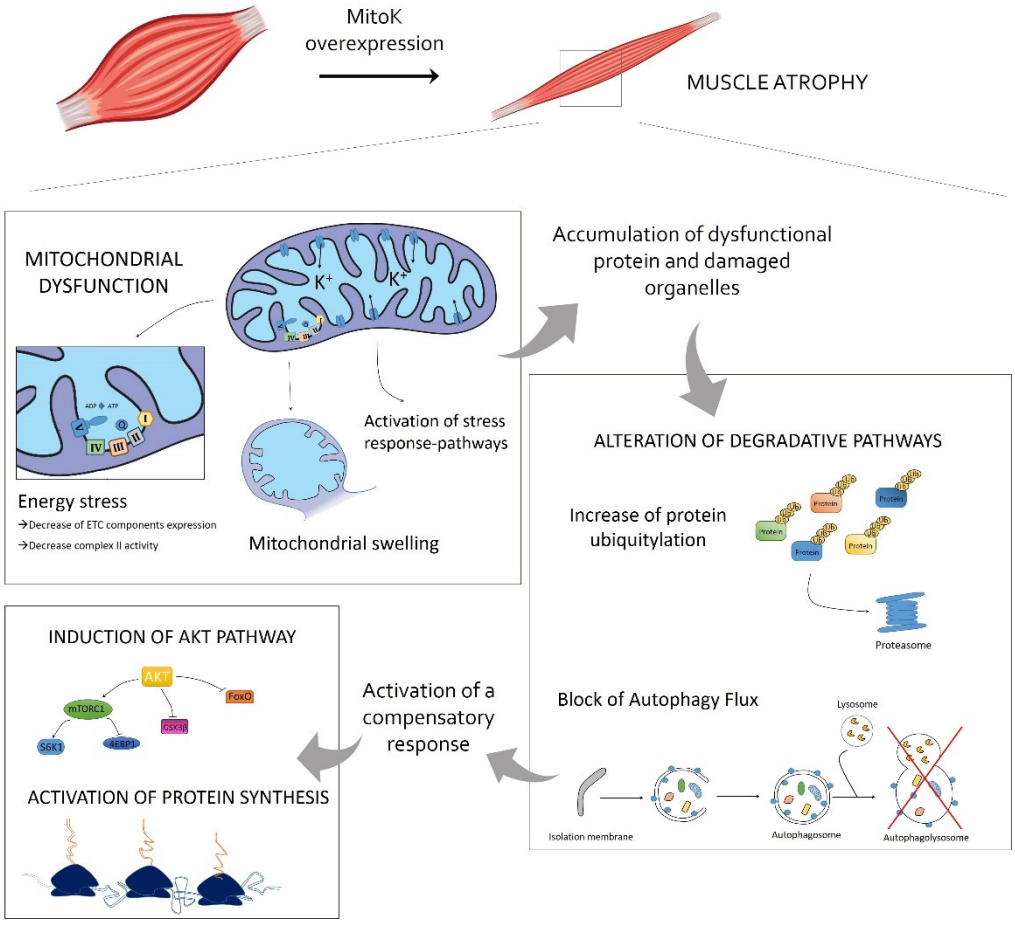


***Figure 28. Representative scheme of mechanisms activated upon mitoK overexpression.***

# MATHERIALS AND METHODS

## ANIMALS

CD1 mice was provided by Charles River. In vivo experiments were performed in accordance with the Italian law D. L.vo n°26/2014.

## AAV PRODUCTION

AAV9MitoK used for experiments was provided by Vector Biolabs. (www.vectorbiolabs.com).

## AAV9 INFECTION

For experiments in the newborn, $10^{10}$ vg were injected into the hindlimb of 4- to 6-day-old male CD1 mice. Muscles were subsequently analyzed 1 or 2 months post-injection as reported in results. An average of 70% of fibers were positive for the AAV infections. For experiments in the adult, male CD1 mice were used. EDL muscles were isolated through a small hindlimb incision, and $10^{10}$ vg were injected along the muscle length. Muscles were analyzed 15 days post-injection.

## CONSTRUCTS

Mouse CCDC51 was amplified from mouse skeletal muscle cDNA library by PCR using the following primers:

For the cloning of CCDC51-Flag into pzac 2.1:

fw, 5'AAGCTTATGACAGGGTGCAGCCCCGT-3';

rv, 5'-CTCGAGTTACTTATCGTCGTCATCCTTGTAATCACTGGTCTTGAACAGCATGT-3'.

The PCR fragment was cloned into HindIII and XhoI sites in pzac 2.1

## AUTOPHAGY FLUX ANALYSIS

Autophagic flux analysis were performed as previously described (Ju et al., 2010) with some modification. Hindlimbs muscle of newborn mice were injected with AAV9MitoK. Colchicine was dissolved in water and stored at -20°C, as a stock solution, at a concentration 4 mg/ml. On day of treatment, colchicine was diluted

to 0.1 mg/ml in water and 0.1 mg/Kg colchicine was i.p injected. Control mice received an equal volume of NaCl 0.9%. The treatment was repeated 12 hours after the first injection. Mice were sacrificed 24 hours after the first injection, TA muscles were harvested and frozen in liquid nitrogen-cooled isopentane.

## RNA EXTRACTION, REVERSE TRANSCRIPTION, AND QUANTITATIVE REAL TIME PCR

Total RNA was extract from TA muscles using SV Total RNA isolation kit (Promega). Following the manufacturer's instructions. The RNA was quantified with Nanodrop (Thermo fisher scientific). From 400 nmol of total RNA of each sample, complementary DNA was generated with a cDNA synthesis kit (SuperScript II, Invitrogen) and analysed by real-time PCR using IQ5 thermocycler and SYBR green chemistry (Bio-Rad). The primers were designed and analysed with Primer3 (Rozen and Skaletsky, 2000). Real-time PCR standard curves were constructed by using serial dilution of cDNAs of the analysed samples, using at least four dilution points and the efficiency of all primer sets was between 85 and 102%. The housekeeping genes Tioredoxin and PolRIIf were used as an internal control for cDNA quantification and normalization of the amplified products. Real-time PCR primer sequences were as follows:

**Atrogin-1**

FW: GCAAACACTGCCACATTCTCTC

RV: CTTGAGGGGAAAGTGAGACG

**MuRF-1**

FW: CCTTCCTCTCAAGTGCCAAG

RV: CCTCAAGGCCTCTGCTATGT

**Mul-1**

FW: AGGGCATTCTTTCAGAAGCA

RV: GGGGTGGAACTTCTCGTACA

**Traf6**

FW: GCAGTGAAAGATGACAGCGTGA

REV: TCCCGTAAAGCCATCAAGCA

**Fanc-L**

FW: GCACGCAGGATTGCATTAGG

RV: GCTACCACTCAGCTTCATTCC

**p21**

FW: CGGTGTCAGAGTCTAGGGGAA

RV: GAACAGGTCGGACATCACCA

**P53**

FW: GGCGTAAACGCTTCGAGATG

RV: TCAGGTAGCTGGAGTGAGCC

**GADD45α**

FW: GAAAGTCGCTACATGGATCAGT

RV: AAACTTCAGTGCAATTTGGTTC

**Tioredoxin**

FW: GGCTTCAAGCTTTTCCTTGTT

RV: TCCAATGTGGTGTTCCTTGA

**PolR2Ff**

FW: CGACGACTTTGATGACGTTG

RV: GCTCACCAGATGGGAGAATC

**HSP27**

FW: TGACCCAGGCTGGAGTAGAA

RV: TGGCTCGGGACAACAACATT

**HSP90**

FW: GGAGATTTTCCTCCGCGAGT

RV: GTCATGCCAATGCCTGTGTC

## WESTERN BLOTTING AND ANTIBODIES

To monitor protein level, frozen muscles were dissolved by means of Quiagen Tissue Lyser and protein estracts were prepared in a buffer containing: 50mM Tris ph 7.50, 150 mM NaCl, 5 mM MgCl2, 1mM DTT, 10% glycerol, 2% SDS, 1% Triton X-100, Roche Complete Protease Inhibitor Cocktail, 1 mM PMSF, 1mM NAV=3, 5mM NaF and 3mM b-glycerophosphate. 40-60 mg of total proteins were loaded, according to BCA quantification. Proteins were separetad by SDS-Page electrophoresis, in commercial 4-12% acrylamide gels (Thermo Fisher Scientific) and transferred into nitrocellulose membranes (Thermo Fisher Scientific) by wet electronphoretic transfer. Blot were blocked 1 hour at RT with 5% non-fat dry milk (Bio-Rad) in TBS-tween (0.5M Tris, 1.5 M NaCl, 0.01% Tween) solution and incubated o.n. at 4°C with primary antibodies. Secondary antibodies were

incubated 1h at RT. Washes after antibody incubations were done on an orbital shaker, three times for 10'each, with TBS-0,01% tween. The following antibodies are used:

- anti-pAKT (1:100 Cell signalling)
- anti-Actin (1:10000 Santa Crus)
- anti LC3B (1:500 Cell Signalling)
- anti p62 (1:3000, Sigma)
- anti-TOM20 (1:20000 Santa Cruz)
- anti-CCDC51Nterm (1:1000 Sigma)
- anti-GRP75 (1:1000, Santa Cruz)
- anti-Tim44 (1:1000, Sigma)
- anti IMMT (1:1000, Sigma)
- anti pS6 (Ser 240/244; 1:1000, Cell Signalling)
- anti-pGSK3β (Ser9, 1:1000, Cell Signalling)
- anti-p4EBP1 (Thr 37/46, 1:1000, Cell Signalling),
- anti-Flag (1:1000 Cell Signalling)
- anti-Puromycin (1:500, Millipore).

Secondary HRP-conjugated antibodies were purchased from Bio-Rad and used at 1:5000 dilution.

## IMMUNOFLUORESCENCE

For immunohistochemistry analysis AAV9MitoK injected muscles were transversally cut in serial slices 20 µm thickness. Muscle slices were fixed with PFA 4% for 20 minutes, incubated with saline solution of NH4Cl 50mM for 20 minutes and blocked with a saline solution 0.5% BSA and 10% goat serum. The primary antibody was diluted in a saline solution 0.5% BSA. Muscle slices were incubated with primary antibody for 1h at 37°C. The following antibodies were used:

- Anti - flag (1:100, Cell Signaling)
- Anti - p62 (1:100, Sigma)

Secondary antibodies were diluted in a solution 2% goat serum, 0.5% BSA and slices were incubated for 1h at RT. For the detection of sarcolemma WGA antibody (1:1000) was used, while Hoechst was used to mark nuclei.

## HEMATOXYLIN AND EOSIN STAINING

Hematoxylin and Eosin (H&E) staining was performed on 20-µm-thick cryosections of TA and EDL muscles. The staining was carried out using the Rapid Frozen Sections H&E staining Kit (Bio-Optica).

## SIRIUS RED STAINING

Sirius Red staining was performed on cryosections 20µm-thick of EDL muscles. Sections were incubated o.n. at RT in a Bouin Solution (saturated picric acid, 37% formaldehyde, 5% acetic acid). After washing in running water, slides were incubated in a solution of 0.1% direct red in saturated picric acid for 1h at RT. After washing with 2% acetic acid slides were dehydrated with 40-50% ethanol, 70% ethanol, 96% ethanol, 100% ethanol, 1:1 ethanol-xylen (100%), 100% xylen. Slides were mounted with Eukitt.

## SDH STAINING

Cryosections 20µm-thick of TA, EDL and Soleus muscles were incubated with SDH incubating solution composed by SDH stock solution (0.2M Na succinate, 0.2M Phosphate buffer ph 7.4) and NBT (nitroblue tetrazolium) 1mg/mL, for 20 minutes at 37°C. Section were rinsed three times in water and assembled with Mowiol.

## GLYCOGEN DETECTION

Glycogen was detected using Periodic Acid-Shiff Staining Kit (SLBC4696, Sigma) and Glycogen Assay Kit II (ab169558, Abcam).

## ELECTRON MICROSCOPY

Electron Microscopy EDL muscles were fixed with fixative solution (3.5% glutaraldehyde in 0.1 M NaCaCo buffer, pH 7.2) at RT. Small bundles of fibers were post-fixed in 2% OsO4 in the same buffer for 2 h and then block-stained in aqueous saturated uranyl acetate. After dehydration, specimens were embedded in an epoxy resin (Epon 812). Ultrathin sections (approximately 30-40 nm) were stained in 4% uranyl acetate and lead citrate.

## STATISTICAL ANALYSIS OF DATA

Statistical data are expressed as mean ± SD unless otherwise specified. For comparison between two independent groups, unpaired t tests were used. Adjusted *$p < 0.05$, **$p < 0.01$, and ***$p < 0.005$.

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