The physiopathological role of mitochondrial calcium uptake in skeletal muscle homeostasis

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Summary

In a wide variety of cell types, cytosolic Ca$^{2+}$ transients, generated by physiological stimuli, elicit large increases in the [Ca$^{2+}$] of the mitochondrial matrix, which in turn stimulate the Ca$^{2+}$-sensitive dehydrogenases of the Krebs cycle. Rapid uptake is favored by the close proximity with the major Ca$^{2+}$ store of the cell, namely the endoplasmic/sarcoplasmic reticulum (ER/SR), and thus by the exposure to high [Ca$^{2+}$] microdomains. In addition, mitochondrial Ca$^{2+}$ could contribute to the cellular homeostasis thanks to the existence of a sophisticated machinery, that allows this organelle to rapidly change its Ca$^{2+}$ concentration (Rizzuto et al., 2012). This general picture is also apparent in skeletal muscle during contraction whereby agonist stimulation induces high amplitude mitochondrial Ca$^{2+}$ increases in vivo (Rudolf et al., 2004), thus acting as buffers of the cytosolic [Ca$^{2+}$] increase. Finally, mitochondrial Ca$^{2+}$ stimulates aerobic metabolism and ATP production, that are essential for muscle activity. Indeed, mitochondria are the major source of ATP in oxidative fibres. However, excessive Ca$^{2+}$ accumulation in mitochondria, a condition known as mitochondrial Ca$^{2+}$ overload, can trigger cell death.

The recent molecular identification of the Mitochondrial Calcium Uniporter (MCU), the highly selective channel responsible for Ca$^{2+}$ entry into mitochondria, allows the detailed investigation of its role in different aspects of skeletal muscle biology (De Stefani et al., 2011; Baughman et al., 2011).

The major goal of my PhD project was to address the role of mitochondrial Ca$^{2+}$ in skeletal muscle homeostasis. For this purpose, we firstly investigated in vivo the effects of mitochondrial Ca$^{2+}$ homeostasis in skeletal muscle function by overexpressing or silencing MCU by means of AAV vectors. We demonstrated that the modulation of MCU protein controls skeletal muscle size during both post-natal growth and adulthood. In detail, we observed an increase in fibre size in MCU-infected muscles. Conversely, MCU-silenced muscles displayed an atrophic phenotype. These striking phenomenon impinges on two major hypertrophic pathways, i.e. PGC-1α4 and IGF1-AKT. We thus explored two potential different mechanisms that could account for the MCU-dependent control of anabolic pathways, i) the activation of a mitochondria-to-nucleus signaling route, ii) the regulation of metabolites as signaling molecules.
Regarding the mitochondria-to-nucleus route, we carried out a study on the PGC-1α4 promoter activity, and we demonstrated that mitochondrial Ca^{2+} controls the promoter activity of PGC-1α4.

Concerning the involvement of cellular metabolism, we carried out steady-state metabolomics analyses of MCU-overexpressing and MCU-silencing muscles. We discovered a marked metabolic reprogramming in silenced muscles, including a clear shift from glucose metabolism toward preferential fatty acid β-oxidation.

Next, we generated a skeletal muscle specific mcu knockout mouse (mlc1f-Cre-mcu^<−/−>), by crossing a mcu ββ mouse with a line expressing the Cre recombinase under the control of the myosin light chain 1f (mlc1f) promoter. We observed marginal difference in fibre size of mlc1f-Cre-mcu^<−/−> skeletal muscles. However, when these mice were exercised on a treadmill using different training protocols, an impaired running capacity of mlc1f-Cre-mcu^<−/−> became evident, indicating that mitochondrial Ca^{2+} accumulation is required to guarantee skeletal muscle performance.

Finally, it is well-established that Ca^{2+} plays a pivotal role in autophagy regulation. Thus, we decided to investigate this process in MCU-overexpressing and MCU-silencing muscles. We demonstrated that mitochondrial Ca^{2+} uptake modulation controls mitophagy without affecting bulk autophagy.

Taken together, these data indicate that mitochondrial Ca^{2+} uptake plays a pivotal role in the control of skeletal muscle trophism. Further investigations of MCU-dependent effects on skeletal muscle homeostasis represent an important task for the future. Indeed, this research will provide new possible targets for clinical intervention in all diseases characterized by muscle loss, such as dystrophies, cancer cachexia and aging.
Summary (Italiano)

In diversi tipi cellulari, i transienti di Ca$^{2+}$ citosolico, generati da stimoli fisiologici, provocano ampi aumenti della concentrazione di Ca$^{2+}$ nella matrice mitocondriale, che, a loro volta, stimolano le deidrogenasi Ca$^{2+}$-sensibili del ciclo di Krebs. Questo rapido accumulo è favorito dalla vicinanza al principale deposito di Ca$^{2+}$ della cellula, il reticolo endo/sarcoplasmatico (RE/RS), e di conseguenza dalla generazione di microdomini ad elevata concentrazione di Ca$^{2+}$. Inoltre, il Ca$^{2+}$ mitocondriale contribuisce all’omeostasi cellulare grazie all’esistenza di un complesso macchinario che permette a questo organello di accumulare rapidamente grandi quantità di Ca$^{2+}$ (Rizzuto et al., 2012). Questo situazione è presente anche nel muscolo scheletrico, in cui la stimolazione che genera contrazione induce ampi transienti di Ca$^{2+}$ mitocondriale in vivo (Rudolf et al., 2004), che sono in grado di tamponare gli aumenti della concentrazione di Ca$^{2+}$ citosolica. Infine, il Ca$^{2+}$ mitocondriale stimola il metabolismo aerobico e la produzione di ATP, che sono essenziali per l’attività muscolare. Infatti, i mitocondri rappresentano la principale fonte di ATP nelle fibre ossidative. Tuttavia, un accumulo eccessivo di Ca$^{2+}$ nei mitocondri può anche portare a morte cellulare.

La recente scoperta dell’identità molecolare del Mitochondrial Calcium Uniporter (MCU), il canale altamente selettivo responsabile dell’entrata di Ca$^{2+}$ nei mitocondri, permette lo studio dettagliato del suo ruolo nei diversi aspetti della biologia del muscolo scheletrico (Baughman et al., 2011; De Stefani et al., 2011). L’obiettivo principale del mio progetto di tesi è stato quello di scoprire il ruolo del Ca$^{2+}$ mitocondriale nell’omeostasi del muscolo scheletrico. Per fare questo, per prima cosa abbiamo indagato in vivo come le funzioni muscolari vengono controllate dall’omeostasi mitocondriale del Ca$^{2+}$ attraverso la sovraespressione o il silenziamento di MCU. Abbiamo dimostrato che la modulazione di MCU controlla la dimensione del muscolo scheletrico sia durante la crescita post-natale che nell’età adulta. In particolare, abbiamo osservato un aumento nella dimensione delle fibre nei muscoli infettati con MCU. Al contrario, i muscoli in cui MCU è stato silenziato risultano atrofici. Questo straordinario fenomeno dipende dal coinvolgimento delle due principali vie di segnalazione che mediano l’ipertrofia, ovvero PGC-1α4 e IGF1-AKT. Di conseguenza, abbiamo studiato due diversi
meccanismi potenzialmente in grado di spiegare il controllo delle vie anaboliche dipendente da MCU, i) l’attivazione di una comunicazione diretta fra mitocondrio e nucleo, ii) l’azione di metaboliti come segnali.

Per quanto riguarda la comunicazione mitocondrio-nucleo, abbiamo studiato l’attività del promotore di PGC-1α4, dimostrando che il Ca\(^{2+}\) mitocondriale la controlla. Invece, nel contesto dei metaboliti come molecole segnale, abbiamo svolto un’analisi metabolonica di muscoli in cui MCU è stato sovraespresso o silenziato. Abbiamo rilevato un notevole rimodellamento della rete metabolica nei muscoli silenziati, compresa una chiara deviazione dal metabolismo del glucosio verso la preferenziale ossidazione degli acidi grassi.

In seguito, abbiamo generato un modello murino privo di \textit{mcu} esclusivamente nel muscolo scheletrico (mlc1f-Cre-\textit{mcu}^\text{loxp/loxp}) con una linea che esprime la Cre ricombinasi sotto il controllo del promotore per la catena leggera della miosina 1f (mlc1f). Abbiamo osservato differenze marginali per quanto riguarda la dimensione delle fibre muscolari di questo modello. Tuttavia, abbiamo poi sottoposto questi topi ad esercizio fisico, attraverso diversi protocolli di corsa su tapis roulant. In queste condizioni, è stata evidenziata una compromessa capacità di corsa, indicando che l’accumulo di Ca\(^{2+}\) mitocondriale è richiesto per garantire performance muscolari ottimali.

Infine, è ampiamente riconosciuto che il Ca\(^{2+}\) giochi un ruolo fondamentale nella regolazione dell’autofagia. Abbiamo quindi deciso di studiare questo processo in muscoli in cui MCU è stato sovraespresso o silenziato. Abbiamo dimostrato che i segnali Ca\(^{2+}\) mitocondriali controllano selettivamente la via autofagica che degrada i mitocondri disfunzionali, la mitofagia.

In conclusione, questi dati indicano che l’accumulo mitocondriale di Ca\(^{2+}\) controlla il trofismo del muscolo scheletrico. In futuro saranno necessari ulteriori studi per caratterizzare meglio gli effetti di MCU sull’omeostasi del muscolo scheletrico. Questo studio fornirà nuovi potenziali bersagli che sarà possibile utilizzare in clinica, in tutte quelle patologie caratterizzate dalla perdita di massa muscolare, come ad esempio le distrofie, la cachessia neoplastica e l’invecchiamento.
Introduction

Mitochondria

*General framework*

Mitochondria are dynamic organelles and represent the primary energy-generating system in most eukaryotic cells, in which the oxidative phosphorylation produces the majority of cellular ATP. Additionally, they participate in many other aspects of cell homeostasis, including Ca\(^{2+}\) signaling, lipid metabolism, ROS (reactive oxygen species) production and cell death regulation.

Mitochondrial dysfunctions are associated with several pathological conditions, including neurodegenerative diseases (e. g. Alzheimer’s, Parkinson’s, Hungtinton’s diseases), motoneuron disorders (e. g. amyotrophic lateral sclerosis, type 2A Charcot-Marie-Tooth neuropathy), autosomal dominant optic atrophy, ischemia-reperfusion injury, diabetes, aging and cancer (Komen and Thorburn, 2012; Lin and Beal, 2006; Zhang and Darley-Usmar, 2012). Additionally, mitochondrial DNA mutations are linked to different pathologies, such as MELAS (Mitochondrial Encephalopathy and Lactic Acidosis with Stroke like episodes) and Leigh’s syndrome (Tranchant and Anheim, 2016).

A new exiting challenge in biomedical research is understanding the mitochondria pivotal role as integration point of different signals from cytosol and other cellular organelles, and, consequently, how mitochondria could decode these different stimuli in a biological response.

Two structurally and functionally different membranes delimitate mitochondria. The OMM (outer mitochondrial membrane) is permeable to ions and metabolites up to 5000 Da thanks to the abundance of VDAC channels (Voltage Dependent Anion Channel), which are able to form pores on the membrane. In addition, the highly selective IMM (inner mitochondrial membrane) is characterized by 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 which originate from narrow tubular structures called *cristae junctions* (Mannella, 2006) limiting the diffusion of molecules from the intra-cristae space towards the matrix, thus creating a micro-environment where the mitochondrial
Electron Transport Chain (mETC) complexes are hosted and other proteins are protected from random diffusion. Four different protein complexes form the mitochondrial Electron Transport Chain: complex I composed by NADH dehydrogenase, complex II composed by succinate dehydrogenase, complex III composed by ubiquinol cytochrome c reductase, complex IV composed by cytochrome c oxidase, and finally there is the complex F1F0-ATP synthase.

During the 60s, Peter Mitchell clarified the process by which electron transfer is coupled to ATP synthesis in oxidative phosphorylation, proposing for the first time an indirect mechanism which is named “chemiosmotic theory”. The flow of electrons through the respiratory electron-transfer chain complexes drives protons across the IMM, creating an electrochemical proton gradient across the membrane. This electrochemical gradient across the IMM is reflected in a huge membrane potential difference ($\Delta \Psi_m$) of -180 mV (negative inside) (Mitchell, 1961, 1966). The synthesis of ATP is driven by a reverse flow of protons down the gradient through the F1-F0 ATP synthase.

**Role of mitochondria in Ca$^{2+}$ homeostasis**

Mitochondria were the first organelles to be associated to Ca$^{2+}$ handling. Indeed, two seminal papers unequivocally described for the first time that energized mitochondria could accumulate large amount of Ca$^{2+}$ (Deluca and Engstrom, 1961; Vasington and Murphy, 1962). Strikingly, these studies preceded the enunciation of the chemiosmotic theory. In the following years, when the chemiosmotic model was accepted by the majority of scientists in the field, the properties of mitochondrial Ca$^{2+}$ transport became clear. In detail, the membrane potential generated across the IMM by the translocation of protons across the IMM or by the reverse action of the ATP synthase, allows rapid Ca$^{2+}$ accumulation inside the mitochondria through the mitochondrial Ca$^{2+}$ uniporter (MCU). On the other hand, Ca$^{2+}$ accumulation by MCU does not reach electrochemical equilibrium with the membrane potential thanks to the existence of Ca$^{2+}$ efflux mechanisms. The two known pathways involved in mitochondrial Ca$^{2+}$ efflux are the Na$^+$/Ca$^{2+}$ (NCLX) and H$^+$/Ca$^{2+}$ (mHCX) exchangers (Bernardi, 1999). However, not all the proteins
involved in the Ca\(^{2+}\) efflux have been discovered yet, in addition the completely block of mitochondrial Ca\(^{2+}\) efflux is not possible using the available drugs. The molecular identification of the H\(^+\)/Ca\(^{2+}\) exchanger is still debated (Nowikovsky et al., 2012). Recent works by Clapham and coworkers proposes Letm1 as a possible candidate (Jiang et al., 2009; Tsai et al., 2014), however another works suggest that Letm1 is a part of the K\(^+\)/H\(^+\) antiporter (Dimmer et al., 2008; McQuibban et al., 2010). Regarding the NCLX the situation is more clear. A first data described a 60 kDa protein, isolated from mitochondria, able to catalyze a Na\(^+\)/Ca\(^{2+}\) exchange process when placed in reconstituted vesicles (Paucek and Jabůrek, 2004). Successively, in 2010 Sekler’s group discovered the molecular identity of the NCLX, demonstrating that NCLX is expressed only in the internal membranes and is highly enriched in the mitochondrial fraction (Palty et al., 2010). Recently, different works dealt with the functional role of NCLX in different cellular models. For example, NCLX regulates automaticity in cardiomyocytes (Takeuchi et al., 2013), oxidative metabolism in pancreatic β cells (Nita et al., 2014, 2015) and Ca\(^{2+}\) signaling in astrocytes (Parnis et al., 2013).

In the 80’s the accurate measurements of [Ca\(^{2+}\)]\(_{\text{cyt}}\) with fluorescent indicators in rest cells revealed that the low affinity of MCU would not allow substantial Ca\(^{2+}\) uptake into the organelle. Thus, mitochondria became simple bystanders in the exploding field of global Ca\(^{2+}\) signaling, in which plasma membrane and endoplasmic reticulum Ca\(^{2+}\) channels hit the scene. Thanks to the development of genetically-encoded Ca\(^{2+}\) probes specifically targeted to mitochondria, the role of mitochondria in regulating Ca\(^{2+}\) homeostasis turned back to be central (Rizzuto et al., 1992). Indeed, the generation of photoprotein aequorin allowed to follow the mitochondrial Ca\(^{2+}\) transients during cell stimulation with a [Ca\(^{2+}\)]\(_{\text{cyt}}\)-raising agonists. Only with these new probes it was appreciated that the speed and the amplitude of mitochondrial Ca\(^{2+}\) accumulation greatly exceed the values that were previously predicted on the basis of MCU properties in isolated mitochondria (Rizzuto et al., 1993). In detail, in living and intact Hela cells the transient increase of [Ca\(^{2+}\)]\(_{\text{cyt}}\) to 1-3μM, induced by physiological stimuli, is always associated with a parallel increase in [Ca\(^{2+}\)] in mitochondrial matrix which, in most cells, reaches
about 60-80 µM. It was thus clear that mitochondria play an active role in Ca\textsuperscript{2+} signaling.

While the interest in mitochondria continued to grow, these data raised an apparent contradiction between the prompt accumulation of Ca\textsuperscript{2+} under physiological stimulation and the low affinity of the MCU, at both resting and agonist-stimulated [Ca\textsuperscript{2+}]\text{cyt}. Based on a large number of experimental proofs, the apparent discrepancy was solved by the discovery that mitochondria are strategically located in close proximity to ER-resident Ca\textsuperscript{2+} channels where the assembly of a dedicated signaling unit at the interface of the two organelles takes place (Csordás et al., 2010; Mannella et al., 1998; Rizzuto et al., 1992, 1993; Szalai et al., 2000). Therefore, mitochondria sense microdomains of high [Ca\textsuperscript{2+}] 10-fold higher than that measured in the bulk cytosol (Giacomello et al., 2010). These high [Ca\textsuperscript{2+}] microdomains are sufficient to ensure rapid Ca\textsuperscript{2+} entry through MCU, however, thanks to their rapidly dissipation, they also act as a safety lock that prevent mitochondrial Ca\textsuperscript{2+} overload (Rizzuto and Pozzan, 2006).

This peculiar organization is possible thanks to ultra-specialized structures that allow the just-apposition of mitochondria to Ca\textsuperscript{2+} release sites in ER (Hayashi et al., 2009). These structures, named mitochondria associated membranes (MAMs), have been observed for the first time in the 90s, and the area of the contact sites has been estimated as 5-20% of the total mitochondrial surface (Mannella et al., 1998).

**Mitochondrial Ca\textsuperscript{2+} regulation of cellular metabolism and cell survival**

During the 70’s it was discovered that three enzymes of the TCA cycle are regulated by Ca\textsuperscript{2+} and they are the rate-limiting enzymes in feeding electrons at the complex I of the respiratory chain (McCormack et al., 1990a). Pyruvate dehydrogenase is regulated by a Ca\textsuperscript{2+}-dependent phosphatase (Denton et al., 1972), while Ca\textsuperscript{2+} binds directly isocitrate dehydrogenase and α-ketoglutarate dehydrogenase (Rutter and Denton, 1988). In addition, these three dehydrogenases are also regulated by intrinsic factors such as the ADP/ATP ratio, the NAD\textsuperscript+\text{/NADH} ratio and pH, which all interfere with the Ca\textsuperscript{2+} regulation (Denton, 2009a). Indeed, mobilization of cytosolic Ca\textsuperscript{2+} and the consequent Ca\textsuperscript{2+} uptake by mitochondria stimulates the TCA
cycle increasing NADH availability thus flow of electrons through the respiratory chain. This process leads to ATP production.

It has been demonstrated that mitochondria are particularly prone to decode high frequency [Ca\textsuperscript{2+}]\text{cyt} oscillations, which are the most efficient signals to stimulate aerobic metabolism (Hajnóczky et al., 1995).

Another issue that should be taken into account is that other mitochondrial enzymes are activated by Ca\textsuperscript{2+}, such as aralar1 and citrin, two isoforms of the aspartate/glutamate carrier located in the IMM. These exchangers exhibit an EF-hand Ca\textsuperscript{2+}-binding domain, which is able to increase the metabolite transport, thus fueling the TCA cycle (del Arco and Satriústegui, 2004; Lasorsa et al., 2003; Satriústegui and Bak, 2015). In this case, however, the Ca\textsuperscript{2+}-binding domain faces the intermembrane space, thus their regulation does not depend on the capacity of mitochondria to take up Ca\textsuperscript{2+}, instead depends on the changes in cytosolic Ca\textsuperscript{2+}.

Altogether these processes ensure prompt stimulation of aerobic metabolism paralleled to cell stimulation and, thus, activation of ATP-consuming processes in the cytosol.

Mitochondrial Ca\textsuperscript{2+} uptake is well-known to be involved in the induction of cell death pathways, both necrosis and apoptosis. The cell decides to activate apoptosis or succumb to necrosis depending on the residual intracellular ATP concentration (Gramaglia et al., 2004). Regarding the necrosis pathway, the bioenergetics crisis due to ATP depletion causes a superphysiological [Ca\textsuperscript{2+}]\text{cyt}. The latter event is due to the impairment of the ATP-dependent Ca\textsuperscript{2+} transporters such as PMCA, SERCA and Na\textsuperscript{+}/Ca\textsuperscript{2+} exchangers. The increase in cytosolic [Ca\textsuperscript{2+}] is paralleled with an increase in mitochondrial Ca\textsuperscript{2+} accumulation leading to swelling of mitochondria, thus necrosis of the cell. An example of the activation of necrotic pathway in response to Ca\textsuperscript{2+} overload is the neuronal excitotoxicity. In neurons, the stimulation by glutamate causes the activation of NMDRs (N-methyl-D-aspartate receptors) (Nicholls, 2009; Pivovarova and Andrews, 2010), thus Ca\textsuperscript{2+} enters the cell directly through these receptors. In addition, voltage-gated Ca\textsuperscript{2+} channels open thanks to the depolarization event. These events cause a superphysiological [Ca\textsuperscript{2+}]\text{cyt} which activates calpain. By this way, the major plasma membrane Ca\textsuperscript{2+} extruding system, the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (NCX), is cleaved causing an
extensive accumulation of Ca\textsuperscript{2+} several hours after the toxic challenge. During this process mitochondria accumulate Ca\textsuperscript{2+} in order to serve as a buffer for the cytosolic Ca\textsuperscript{2+} rise. The delayed Ca\textsuperscript{2+} influx is dependent on the declining activity of cytosolic Ca\textsuperscript{2+} clearing mechanisms. Mitochondria are overloaded with Ca\textsuperscript{2+}, thus the membrane potential totally collapses dealing to a necrotic cell death (Bano et al., 2005).

Moreover, mitochondrial Ca\textsuperscript{2+} plays a pivotal role in the apoptotic cell death, indeed mitochondrial Ca\textsuperscript{2+} overload promotes the opening of the high-conductance PTP (permeability transition pore). Consequently, the PTP formation results in the rapid collapse of the membrane potential and swelling of mitochondria together with the release from mitochondria of cytochrome c and other pro-apoptotic proteins (Rasola and Bernardi, 2011). The initial evidence was provided by the demonstration that the anti-apoptotic oncogene BCL-2 affects intracellular Ca\textsuperscript{2+} homeostasis by increasing the Ca\textsuperscript{2+} leak from the ER and by regulating the release kinetics upon cell stimulation (Foyouzi-Youssefi et al., 2000; Pinton et al., 2000, 2001). By contrast, pro-apoptotic proteins trigger the opposite effect (Scorrano et al., 2003). For example, in cells lacking the pro-apoptotic protein BAK Ca\textsuperscript{2+} release from ER is reduced, and this correlates with a reduction in cell proliferation (Jones et al., 2007).

**The molecular identity and regulation of Mitochondrial Calcium Uniporter**

The first evidence that mitochondria are able to uptake Ca\textsuperscript{2+} occurred during the 60’s, however we had to wait 50 years before the discovery of the molecular identity of the mitochondrial Ca\textsuperscript{2+} uniporter. Several candidates have been proposed through the years. Few papers suggested that mitochondrially sorted RyR1 channels could be the mitochondrial Ca\textsuperscript{2+} uptake channel in the rat heart (Beutner et al., 2001; Ryu et al., 2011), however the tissue distribution and the electrophysiological properties of the RyR1 exclude it as the best candidate. More recently UCP2 and UCP3 were chosen as essential components of the MCU machinery (Trenker et al., 2007),
although, currently, their role is associated with an indirect effect on ATP production (Brookes et al., 2008; De Marchi et al., 2011).

Finally, in 2009, Letm1 has been identified as a putative H+/Ca$^{2+}$ antiporter by Clapham and coworkers using a siRNA genomic screening in Drosophila (Jiang et al., 2009). However, the role of Letm1 is still debated (Nowikovsky et al., 2012), indeed the same protein was identified as the mitochondrial K$^{+}$/H$^{+}$ exchanger (Dimmer et al., 2008). In all the cases its properties do not reflect the MCU properties.

However, the turning point was the publication of the mitochondrial gene data set (MitoCarta) performed by Mootha’s group which contains an inventory of gene products with proven mitochondrial localization (Pagliarini et al., 2008). In 2010, by searching the MitoCarta database the same group discovered MICU1 (Mitochondrial Ca$^{2+}$ Uptake 1), demonstrating that the silencing of the protein drastically reduced mitochondrial Ca$^{2+}$ uptake in HeLa cells (Perocchi et al., 2010). Moreover, MICU1 presents two EF-hand Ca$^{2+}$-binding domain and a single putative transmembrane domain, and thus it cannot be the channel itself.

In order to discover the candidates that would perfectly fit with the activity of the mitochondrial Ca$^{2+}$ uniporter, the MitoCarta gene data set was examined with unbiased search constrains, taking into account several criteria:

i. broad expression profile in all mammalian tissues;
ii. at least two predicted transmembrane α-helixes in the primary sequence;
iii. the absence in *S. cerevisiae*, which lacks Ruthenium Red-sensitive mitochondrial Ca$^{2+}$ uptake;
iv. the presence in those organisms in which mitochondrial Ca$^{2+}$ uptake was described.

In 2011, our and Mootha’s laboratory, independently and using different approaches, identified an uncharacterized protein, encoded by the ccdc109a gene, which completely satisfied all the requirements to be the genuine Ruthenium Red-sensitive Ca$^{2+}$ channel of the inner mitochondrial membrane, named Mitochondrial Calcium Uniporter (MCU) (Figure 1) (De Stefani et al., 2011; Baughman et al., 2011).
Core components of the membrane pore

To date, the pore forming subunit is composed by three different proteins, MCU, MCUb and EMRE. By phylogenetic analyses, MCU appears to be the only obligatory component, because all organisms, which show mitochondrial Ca\(^{2+}\) uptake, have this protein. In addition, MCUb is present only in vertebrates (Raffaello et al., 2013), whereas EMRE is not present in fungi, protozoa and plants (Sancak et al., 2013).

MCU. The MCU gene is well conserved in all eukaryotes except for yeasts (Bick et al., 2012; Cheng and Perocchi, 2015). MCU is a 40 kDa protein that presents two transmembrane domains and two coiled-coil domains separated by a short loop enriched in acidic residues. MCU can oligomerize, although the correct stoichiometry of the quaternary structure of the functional channel is still debated. MCU silencing leads to complete abolishment of mitochondrial Ca\(^{2+}\) uptake, while its overexpression triggers a significant increase of mitochondrial transients in intact cells, together with an increase in the Ca\(^{2+}\) current, recorded by patch clamp technique of mitoplasts (De Stefani et al., 2011; Chaudhuri et al., 2013). Importantly, recombinant MCU is sufficient *per se* to form a Ca\(^{2+}\)-selective channel in planar lipid bilayer (De Stefani et al., 2011). The current is similar, although not identical, to the one recorded in patch clamp experiments of isolated mitoplasts (Kirichok et al., 2004). The differences could be due to the different lipid environment, the absence of post translational modifications in the MCU recombinant protein and the lack of some endogenous regulators. In addition,
channel activity is inhibited by Rethenium Red and gadolinium (Kirichok et al., 2004).

In the last years the role of MCU, as a necessary component for mitochondria Ca\textsuperscript{2+} uptake, has been confirmed in different systems. In detail, knocking down MCU abolished mitochondrial Ca\textsuperscript{2+} transients in heart (Kwong et al., 2015), pancreatic \( \beta \) cells (Alam et al., 2012; Tarasov et al., 2012), neurons (Qiu et al., 2013), skeletal muscle fibres (Mammucari et al., 2015), and breast cancer cells (Tosatto et al., 2016).

Finally, isolated mitochondria from MCU knockout mice showed the complete lack of any mitochondrial Ca\textsuperscript{2+} uptake, thus demonstrating the requirement of the MCU gene (Pan et al., 2013).

In line with the evidence of the pleiotropic role that mitochondrial Ca\textsuperscript{2+} signals play within the cell, it was clear that MCU could not work alone.

MCUb. After the discovery of MCU, cedc109b, a gene closely related to MCU, started to be investigated. This gene, now known as MCUb, is conserved in most vertebrates although it is absent in many other organisms in which MCU is present. MCUb protein shares 50\% similarity to MCU and possesses the same domains of MCU: two coiled-coil domains and two transmembrane domains separated by a loop that is slightly different from the one of MCU due to a crucial amino acid substitution (E256V) which removes a critical negative charge (Raffaello et al., 2013).

An important property of MCU is its capacity to form oligomers for self-organization, moreover MCU is able to form hetero-oligomers with MCUb, which strongly affects the Ca\textsuperscript{2+} permeation through the channel. In detail, MCUb overexpression decreases agonist-evoked mitochondrial Ca\textsuperscript{2+} transients, both in HeLa cells and in planar lipid bilayer experiments. On the contrary, MCUb silencing triggers a significant increase in mitochondrial Ca\textsuperscript{2+} uptake (Raffaello et al., 2013).

Regarding the physiological role of MCUb, it should be noted that MCU/MCUb ratio varies greatly among different tissues, and this is in line with the demonstration that the overall activity of the MCU complex is highly variable among tissues (Fieni
et al., 2012). Most importantly, the correlation between MCU/MCUb expression ratio and the recorded mitochondrial Ca\(^{2+}\) current is apparent. For example, MCU/MCUb ratio in heart is low, whereas in skeletal muscle is high (Raffaello et al., 2013). It is plausible that this ratio contributes to set the mitochondrial Ca\(^{2+}\) uptake capacity of different tissue.

EMRE. EMRE (essential MCU regulator) is a 10 kDa protein with a single predicted transmembrane domain with a highly acidic C terminus. Moreover, it is widely expressed among mammalian tissues. Mootha’s group proposed that this protein is required for Ca\(^{2+}\) channel activity and to keep the MICU1/MICU2 dimer bound to the MCU complex. However, EMRE homologs are not present in fungi or plants, in which MCU and MICU1 are highly conserved (Sancak et al., 2013). Its downregulation or knockout abolishes uniporter-mediated Ca\(^{2+}\) uptake and strongly reduces mitochondrial Ca\(^{2+}\) current, even when MCU is overexpressed (Sancak et al., 2013). These data were confirmed by Miller and coworkers in a recent publication (Tsai et al., 2016). It should be noted that the putative role of EMRE in mediating the binding between MCU and MICU1 is in contrast with the clear positive effect of MICU1 on MCU in the planar lipid bilayer (Patron et al., 2014). However, in the absence of EMRE the MCU complex becomes smaller (Sancak et al., 2013). Thus, this last observation suggests a fundamental role of EMRE in the efficient assembly of the MCU complex. Indeed, in heterologous system, such as yeast, EMRE is required for the formation of a functional channel with the mammalian MCU but not with the MCU derived from fungi (Kovács-Bogdán et al., 2014). Thus, the molecular mechanism and regulatory purpose of EMRE is still under debate. In 2016, Foskett and coworkers demonstrated that MCU channel activity is regulated by matrix [Ca\(^{2+}\)] through EMRE measuring MCU Ca\(^{2+}\) current by means of patch-clamp technique. In addition, MCU regulation by EMRE requires the localization of MICU1 and MICU2 in the IMS and cytosolic Ca\(^{2+}\) (Vais et al., 2016).
**MCU-associated regulators**

The MICU family. A fundamental property of the mitochondrial Ca\(^{2+}\) uptake machinery is the sigmoidal response to extramitochondrial [Ca\(^{2+}\)], with a very low rate at resting cytoplasmic [Ca\(^{2+}\)] levels and a very large Ca\(^{2+}\) carrying capacity at higher [Ca\(^{2+}\)] levels, that ensures prompt responses to cell stimulation. The sigmoidal response could be due either to the properties of the channel itself, or to the activity of different regulators. The former hypothesis is unlikely to be correct because MCU exposes in the intermembrane space only a small loop. The latter has been investigated with the effort of many groups as reported hereafter.

Firstly, in 2010, Mootha’s group discovered MICU1 and proposed that this protein is required for mitochondrial Ca\(^{2+}\) uptake (Perocchi et al., 2010). MICU1 has only one predicted transmembrane domain, thus unlikely could be the channel per se. Functional and structural data suggest that MICU1 is located in the intermembrane space together with other members of the family (Hung et al., 2014; Martell et al., 2012). However, the actual localization of this protein remains controversial. It has been shown that mitochondria of MICU1 silenced cells are constitutively overloaded with Ca\(^{2+}\), thus suggesting a gatekeeping role of MICU1 (Mallilankaraman et al., 2012a). Immediately after, Hajnoczky and coworkers confirmed the gatekeeper activity of MICU1. In addition, they demonstrated that, in the absence of MICU1, mitochondrial Ca\(^{2+}\) uptake is less efficient at high extramitochondrial [Ca\(^{2+}\)] (Csordás et al., 2013). In this way, MICU1 not only controls the threshold of MCU opening but also cooperates in activating the channel open state at high [Ca\(^{2+}\)].

MICU2 (formerly known as EFHA3) and MICU3 (formerly known as EFHA1) are two different isoforms of MICU1 and their role is still debated (Plovanich et al., 2013). Our laboratory described that MICU2 forms an obligate heterodimer with MICU1 that interacts with MCU in the loop facing the intermembrane space. In addition, we demonstrated that MICU2 has a genuine gatekeeping function at low [Ca\(^{2+}\)]. Indeed, its overexpression is able to decrease agonist-evoked stimulus in HeLa cells (Patron et al., 2014). As already reported (Csordás et al., 2013), we confirmed that the stability of MICU2 is dependent on the presence of MICU1 at the protein level. Indeed, MICU1 silenced cells have a drastic reduction also in
MICU2 protein level (Patron et al., 2014). Accordingly to this model, at low [Ca$^{2+}$] the prevailing inhibitory effect of MICU2 ensures minimal Ca$^{2+}$ accumulation in the presence of a very large driving force for cation accumulation, thus preventing the deleterious effects of Ca$^{2+}$ overload into the matrix. As soon as cytosolic [Ca$^{2+}$] increases, Ca$^{2+}$ binding to EF-hand domains inhibits MICU2 and activates MICU1 guaranteeing the prompt initiation of rapid mitochondrial Ca$^{2+}$ accumulation, thus stimulating aerobic metabolism.

Finally, MICU3 has probably a minor role in this process, since it appears to be predominantly expresses in the CNS (central nervous system) (Plovanich et al., 2013). However, the clear functions of MICU3 in MCU complex regulation still need to be investigated.

MCUR1. In 2012, Madesh and coworkers performed a direct human RNAi screen of 45 mitochondrial membrane proteins in HEK293T cells predicted to be part of the inner mitochondrial membrane (Mallilankaraman et al., 2012b). They identified two different proteins, SLC25A23 and MCUR1 (formerly known as CCDC90A). The latter is a 40 kDa protein of the inner mitochondrial membrane with one predicted transmembrane domain, one coiled-coil region and the N terminus facing the intermembrane space. They demonstrated that MCUR1 silenced cells showed a decrease in agonist-induced mitochondrial Ca$^{2+}$ uptake but also caused a decrease of basal mitochondrial [Ca$^{2+}$] (Mallilankaraman et al., 2012b). Recently, Shoubridge’s group demonstrated that the silencing of MCUR1 causes a drop of mitochondrial membrane potential that correlates with a decrease of complex IV assembly and activity (Paupe et al., 2015). These data could explain most of the data reported by Mallilankaraman and colleagues, in which differences in mitochondrial membrane potential were not detected (Mallilankaraman et al., 2012b).

SLC25A23. SLC25A23 belongs to a family of solute carriers that transport Mg–ATP/P$_i$ across the inner mitochondrial membrane (Hoffman et al., 2014). It apparently participates in mitochondrial Ca$^{2+}$ uptake probably due to its interaction with MCU and MICU1. The effect of this EF-hand containing mitochondrial
protein is likely to depend on the local \([\text{Ca}^{2+}]\). Indeed, mutation of these \(\text{Ca}^{2+}\)-binding sites exhibited a dominant-negative effect, reducing mitochondrial \(\text{Ca}^{2+}\) transients (Hoffman et al., 2014). Until now, the mechanism is not clear, but it was proposed that SLC25A23 could act by sequestering MICU1 in order to increase the MCU-mediated \(\text{Ca}^{2+}\) uptake. Further work is thus needed to clarify this mechanism.

**The role of mitochondrial \(\text{Ca}^{2+}\) uptake in organism pathophysiology**

*The first MCU knockout mouse model*

The first work on MCU knockout mice showed a very surprising result that puzzled most of the scientists of the field. Indeed, in 2013, Finkel and coworkers generated a MCU knockout mouse characterized by a very mild phenotype (Pan et al., 2013). These animals present normal features and no histological aberrations. The only defect is an impairment of skeletal muscle performance. No differences in cardiac physiology has been appreciated, either in basal condition or after increase workload (i.e. isoprenaline treatment). Most importantly, MCU deletion response of I/R injury does not differ from control (Holmström et al., 2015).

However, the lack of a major phenotype in this model is an unresolved problem, especially considering that deletion of MCU caused dramatic effects in other organisms (see below).

It should be noted that Finkel and coworkers obtained viable mice only in an outbred strain composed by a mix of C57/BL6 and CD1 backgrounds. In addition, the birth ratio of the homozygous MCU knockout mice was lower than expected, confirming the role of mitochondrial \(\text{Ca}^{2+}\) uptake during the embryonic development, that collects a lot of high energy demand processes.

Few years after the MCU knockout model discussed above, it became clear that the ablation of MCU in the pure C57/BL6 strain leads to embryonic lethality, this result was confirmed in two independent mouse models (Dickinson et al., 2016; Luongo et al., 2015). How it is possible that some of these MCU knockout animals could developed without any problem is still unclear. An explanation could be that in
these cases there is a compensatory mechanism, however the nature of this compensation is still unknown.

After this model, the majority of the *in vivo* studies of MCU silencing or overexpression have concentrated in the effects on striated muscles, particularly on cardiac muscles.

*Selective knockout of MCU in the heart*

The first heart-specific mouse model generated was a transgenic mouse expressing a dominant-negative MCU isoform, MCUD260Q,E263Q (DN-MCU), in the same mixed C57BL/6xCD1 background of the constitutive MCU knockout model (Wu et al., 2015). When expressed in cultured cells, DN-MCU does not completely abolish organelle Ca\(^{2+}\) accumulation (De Stefani et al., 2011), although mitochondria from DN-MCU-expressing hearts have no measurable mitochondrial Ca\(^{2+}\) uptake. The mouse model shows a clear phenotype. MCU is dispensable for normal heart beat, however, under physiological stress, MCU-dependent increase of ATP production is necessary to sustain the activity of SERCA (sarcoendoplasmic reticulum Ca\(^{2+}\) ATPase) and to maintain the proper Ca\(^{2+}\) load of the sarcoplasmic reticulum in SAN cells (sinus atrial node cells) (Wu et al., 2015). The DN-MCU-overexpressing hearts exhibit impaired performance at increasing workload. Additionally, these cardiomyocytes display a clear extramitochondrial adaptation (i.e. higher diastolic cytosolic [Ca\(^{2+}\)]) that depends on the reduced ATP availability (Rasmussen et al., 2015). Importantly, similarly to MCU total knockout model, MCU inhibition does not protect the heart from I/R injury.

Conversely, this notion has been recently challenged thanks to the generation of a new mouse model with two LoxP sites flanking exons 5 and 6 of MCU gene. These animals were crossed with mice expressing a tamoxifen-inducible Cre recombinase (MerCreMer [MCM]) driven by a cardiomyocyte-specific promoter (α-myosin heavy chain promoter). MCU gene deletion was induced in adult mice, and the cardiac function was evaluated (Kwong et al., 2015; Luongo et al., 2015). Firstly, MCU ablation in adult heart led to an 80% decrease of MCU protein levels (Kwong et al., 2015). Similarly to DN-MCU mouse and MCU total knockout mice, MCU is not required in normal conditions and after cardiac pressure overload. However,
MCU ablation strongly protects from I/R injury (Kwong et al., 2015; Luongo et al., 2015), as opposed to previously reporter MCU knockout and the DN-MCU mouse model (Pan et al., 2013; Wu et al., 2015). Finally, these studies confirm the impairment of the knockout mouse in the fight-or-flight response triggered by β-adrenergic stimulation, as reported by Anderson and coworkers (Wu et al., 2015). The cardiomyocytes derived from these mice present normal respiration rate in basal conditions, although a decrease in oxygen consumption rate was detected after increased workload (Kwong et al., 2015; Luongo et al., 2015).

Finally, in all models there is a decrease in the PDH activity (Pan et al., 2013; Wu et al., 2015; Kwong et al., 2015; Luongo et al., 2015).

**Selective knockdown of MCU in skeletal muscle**

It is well-established that mitochondrial Ca\(^{2+}\) uptake plays a role in all tissues, particularly in those relying on the efficiency of oxidative metabolism. We started to investigate the role of mitochondrial Ca\(^{2+}\) accumulation in skeletal muscle, by positively and negatively modulate MCU protein level (Mammucari et al., 2015). To avoid compensatory effects acting during embryonic development we took advantage of AAV (adeno-associated viral) vectors for both the overexpression and the silencing of MCU (Mammucari et al., 2015). The published manuscript reporting these data is enclosed in the “results” section of this thesis. In brief, we demonstrated that modulation of mitochondrial Ca\(^{2+}\) accumulation after birth contributes to skeletal muscle trophism. Indeed, MCU overexpression and downregulation trigger muscle hypertrophy and atrophy, respectively. Most importantly, MCU overexpression protects from denervation-induced muscle atrophy caused by sciatic nerve excision. Surprisingly, these effects are independent from the control of aerobic metabolism as demonstrated by various evidences. Firstly, PDH activity, although defective in MCU silenced muscles, was unaffected in MCU overexpressing muscles. Second, hypertrophy was comparable in both oxidative and glycolytic muscles and, finally, analyses of aerobic metabolism revealed no major alterations. Conversely, the control of skeletal muscle mass by mitochondrial Ca2+ modulation is due to the role of two major hypertrophic pathways of skeletal muscle, PGC-1α4 and IGF1-AKT/PKB. Taken together these
results demonstrate the existence of a $\text{Ca}^{2+}$-dependent mitochondria-to-nucleus signaling route that clearly links organelle physiology to the control of muscle mass.

**MICU1 modulation in human patients**

In 2014, a paper was published identifying a loss-of-function mutation of MICU1 in patients (Logan et al., 2014). Homozygous individuals for this mutation are characterized by early-onset proximal muscle weakness with a static course, moderately or grossly elevated serum creatine kinase levels accompanied by learning difficulties, and a progressive extrapyramidal movement disorder. Fibroblasts derived from these patients show the expected impairment of MCU gating accompanied by mitochondrial fragmentation and decreased oxidative metabolism. Successively, another work described two cousins carrying the deletion of exon 1 in MICU1 gene, characterized by fatigue, lethargy and weakness even with normal muscle biopsy (Lewis-Smith et al., 2016). The fibroblasts of these two patients showed normal mitochondrial electron transport chain enzyme activities, although functional studies revealed a defect of mitochondrial $\text{Ca}^{2+}$ handling, with impaired mitochondrial $\text{Ca}^{2+}$ uptake. The authors suggested that the lower $\text{Ca}^{2+}$ uptake rate represents a secondary consequence of increased basal matrix $\text{Ca}^{2+}$ in the absence of MICU1, which would reduce the driving force for $\text{Ca}^{2+}$ uptake.

Although the pathogenesis lying behind these works is still unclear, these findings underline the importance of mitochondrial $\text{Ca}^{2+}$ transport in pathophysiology.

**MICU1 modulation in animal models**

Two different MICU1 knockout mouse models were generated (Antony et al., 2016; Liu et al., 2016). Hajnoczky and coworkers generated a MICU1 knockout model with two LoxP sites flanking exon 3. These mice were crossed with germine-expressing Cre recombinase. MICU1 knockout animals died within hours of birth, demonstrating that MICU1 is required for adaptation to postnatal life. Next, hepatocyte-specific MICU1 deletion was achieved by injecting MICU1 floxed mice with AAV particles carrying the Cre recombinase which expression is under the control of a specific hepatocyte promoter. In a liver regeneration experiment,
MICU1 knockout had impaired pro-inflammatory phase. As a consequence, liver regeneration failed and extensive necrosis was observed. Ca$^{2+}$ overload accelerated PTP opening in MICU1-deficient hepatocytes (Antony et al., 2016). Soon after, Finkel’s group generated MICU1 knockout mice using a CRISPR-based method. MICU1 deletion resulted in significant, but not complete, perinatal mortality, similarly to what observed in the Hajnoczky’s work. A small fraction of animals survive maybe due to adaptation of other MCU complex component (Liu et al., 2016). Taken together, these data demonstrate that alterations in MICU1 activity play a critical role in physiological and pathological conditions where mitochondrial control of cellular Ca$^{2+}$ homeostasis is required.

**Other animal models**

We have reported above that mitochondrial Ca$^{2+}$ regulates many aspects of physiology at the whole-organism level. Genetical manipulation of MCU in lower organisms fully confirms this evidence. Indeed, knockout of MCU in Trypanosome brucei impairs energy production with an increase in autophagy process (Huang et al., 2013), eventually leading to impaired growth capacity in vitro and reduced infectivity in mice. In zebrafish, mitochondrial Ca$^{2+}$ uptake drives gastrula morphogenesis (Prudent et al., 2013). Conversely, the deletion of MCU in Caenorhabditis elegans leads to viable worms, even though ROS (reactive oxygen species) production is impaired (Xu and Chisholm, 2014). Finally, in Drosophila MCU inhibition in mushroom body neurons, a brain region critical for olfactory memory formation, during pupation triggers memory impairment without altering the capacity to learn (Drago and Davis, 2016).

**Skeletal muscle plasticity**

*Signaling pathways that control skeletal muscle mass*

Maintenance of skeletal muscle mass is essential for organism’s health and survival. Skeletal muscle mass and fibre size change according to physiological and pathological conditions. The adaptive changes of muscle fibres occur in response to different stimuli such as exercise and hormonal signals. Muscle plasticity is due to several mechanisms that regulate the rate of muscle growth and muscle loss. In
this introduction we take into account only the pathways that we demonstrated to be involved in MCU-dependent regulation of muscle size. The control of skeletal muscle mass is the result of a balance between protein synthesis and degradation. In detail, muscle growth and hypertrophy are mainly due to active protein synthesis. Conversely, a massive protein degradation together with the loss of organelles and cytoplasm are the major causes of muscle atrophy.

Regarding the hypertrophy process, a major signaling pathway that positively controls protein synthesis is the IGF1-AKT/PI3K pathway. IGF1 transgenic mice present hypertrophy of skeletal muscle. Most importantly, the increase in muscle mass takes place together with the increase in muscle strength. Another model is the skeletal muscle overexpression of a constitutively active form of AKT, a downstream target of IGF1, which in adult skeletal muscle induces hypertrophy and protects from denervation-induced atrophy, demonstrating that AKT promotes skeletal muscle growth and simultaneously blocks protein degradation (Schiappino et al., 2013). AKT promotes hypertrophy through the phosphorylation of different substrates. In detail, AKT activates mTOR (mammalian target of rapamycin), thus promoting the activation of S6 kinase and blocking the inhibition of 4E-BP1 (eukaryotic initiation factor 4E-binding protein 1) on eIF4E (eukaryotic translation initiation factor 4E), thus leading to protein synthesis (Rommel et al., 2001). Moreover, the inhibition of GSK3\(\beta\) by AKT results in the stimulation of protein synthesis, since GSK3\(\beta\) blocks protein translation initiated by eIF2B (eukaryotic translation initiation factor 2B) (Glass, 2005).

AKT is also able to suppress catabolic pathways blocking protein degradation. Indeed, IGF1 treatment or AKT overexpression inhibits FoxO expression in myotubes, thus preventing the induction of ATROGIN-1 and MURF1, two ubiquitin-ligases involved in the atrophy process (Sandri et al., 2004; Stitt et al., 2004).

Recently, Spiegelman and coworkers demonstrated that PGC-1\(\alpha\)4 is involved in the control of muscle mass (Ruas et al., 2012). In detail, Pgc-1\(\alpha\) gene has two different promoter regions. The proximal promoter is located immediately before the exon 1, whereas the alternative promoter is located 13 kb upstream. They identified four different isoforms of PGC-1\(\alpha\). PGC-1\(\alpha\)1, formerly known as PGC-1\(\alpha\) and major
mitochondriogenic factor, originates from the proximal promoter, whereas PGC-1α2, PGC-1α3 and PGC-1α4 are transcribed from the activity of the alternative promoter and from alternative splicing of Pgc-1α gene. Most importantly, PGC-1α4, is abundantly expressed in skeletal muscle and appears to play a role in the adaptive response to exercise, particularly in the setting of resistance training. This protein does not regulate the same set of oxidative genes induced by PGC-1α1 but, rather, it activates the expression of IGF-1 while concomitantly suppressing myostatin pathway (an inhibitor of muscle cell differentiation and growth).

**Skeletal muscle energy metabolism**

Skeletal muscle tissue represents the 40% of total body mass in mammalian (Zurlo et al., 1990) and has a major role in the control of glucose homeostasis, since it is the predominant site of glycogen storage (DeFronzo et al., 1981). Skeletal muscle is able to rapidly modulate the rate of energy production and the substrate utilization in response to locomotion. This latter process is possible thanks to the actin-myosin crossbridge cycling according to the sliding filament theory of skeletal muscle contraction (Schoenberg and Podolsky, 1972). The energy required for acto-myosin contraction is provided by the hydrolysis of ATP (adenosine triphosphate) In addition ATP is consumed by dynamics of Na⁺-K⁺ and Ca²⁺ exchange necessary for contraction.

During sprint exercise the ATP turnover can increase 100-fold above rest. Additionally, when the [ATP] is low, metabolic pathways responsible for ATP resynthesis are rapidly activated. During short-term (30-60 s) maximal exercise, this is achieved primarily through substrate phosphorylation via the breakdown of creatine phosphate and by the conversion of glucose units to lactate (Parolin et al., 1999). During prolonged exercise the mobilization of extramuscular substrates is critical to maintain skeletal muscle metabolism (Wasserman, 2009). Accordingly, the liver increases glucose release into circulation, and adipocytes increase triglyceride stores hydrolysis and release of long-chain fatty acids. The relative contribution of carbohydrate and lipid to oxidative metabolism is determined primarily by the prevailing exercise intensity.
Skeletal muscle is a mitochondria-rich tissue, thus it is dependent on oxidative phosphorylation for energy production (Romijn et al., 1993). During strenuous exercise there is huge increase of intramuscular oxygen consumption rate. Indeed, estimated TCA cycle flux increases by 70- to 100- fold under these conditions (Gibala et al., 1998).

**Autophagy**

*Overview of the autophagic pathway*

Macroautophagy, here referred to as autophagy is a well-established mechanism involved in protein degradation, particularly during stress or starvation. Autophagy works as a bulk process with the ability to degrade cellular components such as proteins and organelles (e.g. mitochondria, peroxisomes and ribosomes) (Mizushima and Klionsky, 2007). Autophagic process contributes to the recycling and restore of cellular nutrients, thereby allowing cell survival during starvation. In addition, basal autophagy, referred to as a constitutive autophagic degradation process that proceeds in absence of any stimulus or stress, is important for the degradation of dysfunctional and damage proteins and organelles. Thus, basal autophagy and UPS (Unfolding Protein Response) are the key mechanisms for protein quality control and homeostasis (Masiero et al., 2009).

*Mammalian autophagic pathway*

The autophagic pathway proceeds through several phases, including: initiation, formation of pre-autophagosomal structure leading to an isolation membrane called phagophore, vesicle expansion, autophagosome maturation and cargo sequestration. Eventually, the autophagosome fuses with a lysosome, leading to the degradation of the sequestered cytosolic proteins and organelles.

Autophagy starts with the formation of the phagophore assembly site, that is the formation of ULK complex which consists in the interaction of ULK1 (UNC51-like kinase), ATG13 (autophagy-related protein 13), FIP2000 (FAK family kinase interacting protein of 200 kDa) and ATG101 (Lamb et al., 2013). Next, a nucleation step occurs, which consists in the formation of the phagophore which is composed of BECLIN1, ATG14L, VPS15 (vacuolar protein sorting 15) and VPS34 (Diao et
The recruitment of others proteins, such as ATG5, ATG12, ATG16L together with the conjugation of PE (phosphatidyl-ethanolamine) to LC3 (microtubule-associated protein 1 light chain 3), called LC3 II, result in the formation of the autophagosome. LC3 II is essential for the identification of autophagic cargos and the fusion of autophagosomes to lysosomes (Huang et al., 2015). The resulting autophagosome fuses with lysosomal compartments, leading to the formation of the autolysosome (Figure 2).

![Figure 2. Schematic representation of autophagic pathway. (modified by Kaur and Debnath, 2015)](figure2)

**Ca^{2+}-dependent control of autophagy**

Autophagy has been reported to be regulated by Ca^{2+}. The first evidence about Ca^{2+}-dependent regulation of autophagy is dated 1993, when Gordon et al. suggested the dependence of autophagy on intracellular [Ca^{2+}] (Gordon et al., 1993). Gradually other studies have risen up, whit contrasting results, some of them indicating that Ca^{2+} inhibits autophagy, whereas others indicating a positive effect of Ca^{2+} on autophagy. The reports considering Ca^{2+} as an inhibitor of autophagy focused on the involvement of InsP3R (inositol 1,4,5-triphosphate receptor), that mediates the Ca^{2+} release from the ER to the cytosol in response to the increase in the cytosolic levels of InsP3. In detail, the decrease in InsP3 levels by Li+ triggers the induction of autophagy (Sarkar et al., 2005). Another study focused on DT40 cells lacking all InsP3Rs that showed an enhanced autophagic flux. Briefly, the absence of InsP3Rs, thus the absence of constitutive delivery of Ca^{2+} from ER, triggered a decrease activity of PDH (pyruvate dehydrogenase). This resulted in insufficient production of NADH, limiting the activity of the electron transport chain and reducing ATP.
production. Accordingly, in DT40 cells the AMP/ATP ratio was increased, and a consequent AMPK activation induced autophagy (Cárdenas et al., 2010). Another scenario was proposed by Hoyer-Hansen et al., they demonstrated that the elevation in free intracellular [Ca^{2+}] triggered the activation of CaMKK (Ca^{2+}-calmodulin dependent protein kinase kinase beta), which was able to phosphorylate, thus activate AMPK, which in turn stimulated autophagy (Hoyer-Hansen et al., 2007).

Selective autophagy
The term “selective autophagy” refers to the engulfment of specific targeted cargoes, such as mitochondria, peroxisomes and ribosomes, and protein aggregates. Selective autophagy is mediated by autophagy cargo receptors that binds cargos marked with degradation signals, among which ubiquitin is the most common in mammals. Indeed, autophagy cargo receptors serve as molecular bridges that bind ubiquitylated proteins, which are degraded through the autophagic pathway. One of the most important autophagy cargo receptor is P62, which contributes to the autophagic clearance of protein aggregates (Rogov et al., 2014).

Selective autophagy is an important mechanism for the degradation of organelles in order to regulate their number and maintain quality control. For example, damaged mitochondria are delivered to autophagosomes in a process called mitophagy. Indeed, mitophagy has been shown to be required for steady-state turnover of mitochondria (Tal et al., 2007). In addition, fission is the event that precedes the mitophagy process to fragment elongated mitochondria (Westermann, 2010).

The first observation of mitophagy has been obtained by electron microscopy studies of hepatocytes, which unveiled increased mitochondrial sequestration into the lysosomes (De Duve and Wattiaux, 1966).

Mitophagy requires the specific labelling of mitochondria and the consequent recruitment into isolation membranes. The whole process takes place thanks to the action of different proteins. Here we focus the on PINK1/PARKIN-mediated mitophagy. PINK1 (PTEN-induced putative kinase protein 1) is imported into mitochondria, where it is rapidly degraded by proteolysis to maintain its protein
level at very low concentration. When a subset of mitochondria starts to lose their membrane potential, PINK1 proteolysis is inhibited, allowing PINK1 accumulation specifically into the damaged mitochondrial population (Narendra et al., 2010). Upon PINK1 accumulation, the E3 ubiquitin ligase PARKIN translocates from cytosol to damaged mitochondria, where PARKIN-mediated ubiquitination of mitochondrial substrates occurs (Matsuda et al., 2010).
Results

The mitochondrial Calcium Uniporter controls skeletal muscle trophism in vivo
Figure 1. MCU Is Sufficient and Required for Mitochondrial Ca²⁺ Uptake in Skeletal Muscle Ex Vivo

(A) Flexor digitorum brevii (FDB) muscles were transfected with mGCaMP6 and MCU-Cherry or shMCU-pmCherry-N1 or siMCU was used as a control, respectively. Seven days later, single myofibers were isolated and placed in culture.

(B) Immunofluorescence analysis showed colocalization of MCU-Cherry and mGCaMP6 with the mitochondrial protein TOM20 in muscle fibers processed as in (A). Scale bar, 5 μm.

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results show that MCU expression triggers hypertrophy, both during post-natal growth and in adulthood, by controlling protein synthesis through the PGC-1α and IGF1-Akt/PI3K pathways. Finally, MCU exerts a protective effect against atrophy, suggesting that modulation of mitochondrial Ca²⁺ uptake may represent a novel area of therapeutic intervention to combat muscle mass loss.

RESULTS

MCU Overexpression or Silencing In Vivo Affects Mitochondrial Ca²⁺ Uptake in Muscle Fibers

In cultured cells, modulation of MCU expression determines the amplitude of mitochondrial Ca²⁺ uptake upon physiological stimuli (De Stefani et al., 2011). In this work, we decided to specifically alter mitochondrial Ca²⁺ uptake in vivo by adenoviral delivery of a MCU shRNA (siMCU) or MCU expressing vector (pCMV-MCU) (Logan et al., 2011), in combination with a plasmid encoding mCherry (control) or mCherry-tagged MCU (MCU-Cherry). Eight days later, real-time imaging experiments were performed on isolated single myofibers (Figure 1A). Both MCU-Cherry and mCherry colocalized with the mitochondrial protein TOM20 in muscle fibers (Figure 1B). After assessment of basal Ca²⁺ concentrations, a cytosolic and hence mitochondrial Ca²⁺ rise was evoked by discharging the sarcoplasmic reticulum (SR) pool with caffeine. MCU overexpression caused a marked increase in the caffeine peak and a modest elevation of the resting [Ca²⁺]m (Figures 1C and 1D). Cytosolic Ca²⁺ levels were almost unaffected by MCU, showing a small decrease that was statistically significant for resting values (Figures 1E and 1F). The silencing experiments gave similar results. FDB myofibers were co-transfected in vivo with plasmids encoding either shluc (control) or shMCU and mCherry-MCU. In vivo imaging experiments showed a marked reduction of both [Ca²⁺]i, resting values and caffeine-induced peaks in shMCU-transfected fibers (Figures 1G and 1H), while cytosolic Ca²⁺ values were virtually unaffected (Figures 1I and 1J). In order to mimic the physiological response of innervated muscles, we also measured Ca²⁺ transients upon K⁺-induced depolarization. Similarly to the caffeine experiments, the resting and peak [Ca²⁺]m were detected in both untreated and K⁺-stimulated fibers. The results were expressed as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, n = 8 for each condition. See also Figure S1.

MCU Controls Muscle Size during Post-natal Growth

We thus investigated the role of MCU in both developing and adult muscle. We first focused on developing muscle, in which a greater plasticity could be expected. We reprogrammed muscles of newborn mice with AAV-MCU and analyzed the muscles eight weeks later (Figure 3A). A striking phenotype affecting muscle hypertrophy was observed. MCU overexpression, confirmed by western blotting of cytosolic and mitochondrial fractions (Figure S2B) and by immunofluorescence (Figure S2C), resulted in a 47% increase in the average fiber area of MCU-infected tibialis anterior (TA) compared to controls (Figure 2D). When measured 1 month after injection, TA muscle fiber size was 28% greater than control fibers (Figure 2G), indicating a progressive event that starts early after injection and continues up to 2 months of age. To verify whether MCU-induced hypertrophy affected also different fiber types, we investigated soleus muscles, which are mitochondria-rich slow muscles. MCU triggered 41% hypertrophy compared to controls, suggesting that the effect of mitochondrial Ca²⁺ uptake in hypertrophy is independent of the number of mitochondria and of the overall metabolic properties (Figure 2E). Next, we analyzed the effect of MCU silencing. Newborn hindlimb muscles were injected with AAV-shMCU and fiber size measured 2 months later. AAV-shMCU was efficiently delivered to TA muscle (Figure 2F) and decreased MCU protein expression (Figure 2G). Fiber size was markedly reduced both in TA and in soleus muscles (~30% and ~29%, respectively) (Figures 2H and 2I), highlighting the requirement of mitochondrial Ca²⁺ signals for the maintenance of skeletal muscle hypertrophy.

Mitochondrial Structure and Function in MCU Overexpression and Silencing

We then investigated the cellular changes that could underlie the trophic effect of MCU. We first focused on the effect on mitochondrial morphology and volume by electron microscopy (EM) and on their metabolic properties. In extensor digitorum longus (EDL) fibers from adult control mice, mitochondria are positioned almost exclusively at the I band on both sides of the A band. Mitochondria are located at the sarcomeres' middle region, where they appear oval or round, with parallel cristae within a dark/electron dense matrix (Figure 3B). In MCU-overexpressing EDL fibers, although many mitochondria are smaller than controls, their cristae appear more or less parallel and their matrix more electron dense.
appeared normal in shape and correctly localized with respect to the sarcomere (Figure 3C, arrows), we found some atypical mitochondria, which form wavy stacks of cristae protruding as long tendrils in inter-myofibrillar spaces (Figure 3C, arrowheads, and enlargements in Figures 3D–3F). These mitochondria, considered abnormal mitochondria in the quantitative analysis (Figure 3I, column e), accounted for <10% of all mitochondria. Further quantitative investigation revealed that the fiber volume occupied by mitochondria increased from ~3.5% to ~4.5% (Figure 3I, column a), possibly due to the increase in the average mitochondrial diameter (Figure 3I, column d). In MCU-silenced fibers, the atypical wavy mitochondria observed in MCU-overexpressing fibers were never detected. In addition, the relative fiber volume occupied by mitochondria was significantly reduced, in parallel with a decreased number and size of these organelles (Figure 3G, arrows and 3I, columns a–c). The frequency of severely damaged mitochondria, i.e., presenting vacuoles and disrupted cristae (Figure 3G, arrowheads) or containing myelin figures (Figure 3H) and longitudinally oriented organelles (Figure 3J, arrow), was also increased (6.1% versus 1.6% of controls) (Figure 3I, columns d and e).

Next, we investigated the effects on mitochondrial aerobic metabolism, focusing on the Ca²⁺-regulated enzymatic steps, such as pyruvate dehydrogenase (PDH). MCU overexpression affected neither the phosphorylation levels of PDH (Figure S3A) nor PDH activity (Figure 3J). A qualitative histochemical analysis of the activity of SDH, COX IV, and NADH–TR in TA muscles did not show significant differences between MCU-overexpressing and control muscles. In addition, no difference was observed in glycogen content, as shown by PAS staining (Figure S3C). A comparative analysis of glycolytic (EDL) versus oxidative (soleus) muscles further confirmed that MCU overexpression does not qualitatively alter PAS and SDH activity (Figure S3D). Similar analyses were conducted on AAV-smi-MCU-infected muscles. In agreement with data on MCU depleted muscles (Pani et al., 2013), MCU silencing increased PDH phosphorylation (Figure S3B) and decreased PDH activity (Figure 3K), although no significant changes in the histochromic pattern of SDH, COX IV, and NADH–TR were observed (Figure S3E). The amount of glycogen was also unaffected (Figure S3E). Overall, significant differences in mitochondrial volume were detected, but no obvious changes in structure and metabolic activity were observed that could be directly correlated with an effect on muscle size.

![Figure 2. MCU Controls Muscle Size during Postnatal Growth](image)

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Figure 3. Effects of MCU modulation on mitochondrial structure and function and on hypertrophy-related pathways during muscle development.

(A-H) EM analysis of EDL muscles. Scale bars represent 1 μm (A, C, and G) or 0.1 μm (B, D–F, and H). (I) Quantitative EM analysis. Values in columns a–d are shown as means ± SD. In brackets is the total number of mitochondrial profiles evaluated in the analysis. **p < 0.01, t test (two-tailed, paired) of three muscles per group.

<table>
<thead>
<tr>
<th>Control</th>
<th>MCU</th>
<th>shMUC</th>
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<tr>
<td>a</td>
<td>3.3 ± 1.7</td>
<td>4.5 ± 1.5**</td>
<td>3.3 ± 1.7</td>
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<td>b</td>
<td>39 ± 12</td>
<td>32 ± 7</td>
<td>34 ± 12</td>
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<td>c</td>
<td>210 ± 80 (537)</td>
<td>235 ± 84 (405)**</td>
<td>212 ± 53 (267)</td>
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<tr>
<td>d</td>
<td>0.5 ± 1.2</td>
<td>1.1 ± 1.9**</td>
<td>0.9 ± 1.5</td>
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<td>e</td>
<td>2.6 (2777)</td>
<td>5.6 (1449)**</td>
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MCU Regulates Muscle Hypertrophy Signaling Pathways

Since preliminary analysis showed no difference in autophagy (data not shown; Figure S1F), we focused our attention on the well-established hypertrophy pathways of skeletal muscle, PGC-1α and the IGF-1-Akt/PKB axis. PGC-1α is the master regulator of mitochondrial biogenesis, and a novel PGC-1α isoform (PGC-1α-δ) has been reported to trigger muscle hypertrophy (Rusu et al., 2012). Analysis of the mRNA expression of the Pgc-1α isoforms demonstrated that AAV-MCU triggers induction of both Pgc-1α-δ and Pgc-1α (Figure 3A), thus revealing both an enhanced mitochondrial biogenesis (in agreement with the ultrastructural analysis) and a stimulation of the PGC-1α-related hypertrophy pathway. Activation of IGF-1-Akt/PKB triggers hypertrophy, while its suppression determines muscle atrophy (Schiaffino and Mammucari, 2011). In addition, IGF-1-Akt/PKB signaling is activated by Pgc-1α (Rusu et al., 2013). Accordingly, Akt was phosphorylated, and thus activated, by AAV-MCU (Figure 3M). Specific Akt downstream targets were phosphorylated: in detail, 4E-BP1 and GSK3β, two inhibitors of protein translation (Schiaffino and Mammucari, 2011), were phosphorylated, and thus inhibited, in AAV-MCU muscles (Figure 3M). These data suggest that MCU-mediated hypertrophy is due to increased PGC-1α-δ and IGF-1-Akt/PKB-dependent signaling. Finally, satellite cells also contribute to normal muscle growth. Analysis of Pax7-positive nuclei demonstrated that MCU caused an increase in the average satellite cells number per fiber (Figure S2G).

Next, we checked whether the same hypertrophy pathways were also suppressed by MCU silencing. Pgc-1α-δ expression was decreased by AAV-shMCU, while Pgc-1α-δ was unaffected (Figure 3N). In addition, the Akt signaling pathway was inactivated by AAV-shMCU, as evidenced by decreased phosphorylation of Akt (4E-BP1, and GSK3β) in all cell nuclei (Figure 3O). Finally, a decrease in size of AAV-shMCU-infected myotubes (Figure 3P) indicate that MCU-mediated mitochondrial Ca²⁺ homeostasis regulates skeletal muscle size during postnatal growth by directly impairing on specific master regulators of hypertrophy.

MCU Acutely Controls Muscle Size in the Adult

We proceeded to the analysis of adult muscle, where an effect on muscle hypertrophy could have direct relevance for the understanding and potential targeting of age- and disease-resistance traits of muscle mass. For this purpose, adult EDL muscles were infected with AAV-MCU or AAV-shMCU, and fiber size was measured 2 weeks later (Figure 4A). AAV-MCU infection triggered a 37% increase in fiber size (Figure 4B) while AAV-shMCU infection caused a 31% decrease, thus demonstrating that mitochondrial Ca²⁺ uptake is required for muscle growth in the adult (Figure 4C). Adult muscle size is regulated by a fine equilibrium between protein synthesis and protein degradation of myofibrillar components. We analyzed protein synthesis by "surface sensing of translation" (SUNSET), a method based on the incorporation of puromycin into nascent peptide chains that allows accurate detection of protein synthesis rate in skeletal muscle in vivo (Goodman et al., 2011). Puromycin was injected to adult mice infected with AAV-MCU, and muscles were analyzed 30 min later. Detection of puromycin with specific antibodies showed that protein synthesis was strongly induced by MCU (Figure 4D). As in developing muscle, the experiments in adult muscle also revealed a marked effect of MCU on PGC-1α-δ and the IGF-1-Akt/PKB axis. In particular, Pgc-1α-δ was drastically upregulated upon MCU overexpression and downregulated upon MCU silencing (Figures 4E and 4F). In contrast to postnatal muscles, the effects on Pgc-1α-δ and Pgc-1α were very modest and did not correlate with the Pgc-1α-δ change, thus suggesting a specific effect on the PGC-1α-δ-related hypertrophy pathway. Similarly, analysis of the IGF-1-Akt/PKB trophic pathway provided a coherent picture, with phosphorylation of Akt and downstream targets in MCU-overexpressing muscles (Figure 4G) and the opposite effect upon MCU silencing (Figure 4H). Finally, the number of Pax7-positive cells was unaffected by MCU, suggesting a marginal role of the satellite cell compartment in MCU-induced muscle hypertrophy in the adult (Figures S1A and S1B). Finally, to get a broader view of the MCU-dependent transcriptional changes and the pathways involved in the trophic effect, we carried out RNA microarray analysis of single myotubes of AAV-MCU- and AAV-shMCU-infected muscles, with respective controls.

Cluster analysis, according to the self-organizing tree algorithm (SOTA) (Herrero et al., 2003), revealed that AAV infection per se affected most differentially expressed genes (clusters 1, 5, 6, 8, 9, and 10) (Figure 4H) (Table S1). Nevertheless, cluster 2 and 4 were enriched for genes that play a role in muscle hypertrophy, since infection with control AAV did not affect muscle size (data not shown). The remaining clusters included genes induced by MCU overexpression (clusters 2, 3, and 4) or silencing (clusters 7 and 11). Interestingly, genes activated 14 days after AAV-MCU infection (clusters 2 and 4) were enriched for components of the cytoskeleton or genes involved in sarcomere organization and Ca²⁺ homeostasis (Table S1). Genes in clusters 2 and 4 were activated by AAV-MCU infection and inhibited by MCU silencing (Figure 4I). A gene set enrichment analysis (GSEA) revealed that several pathways involved in hypertrophy were activated by MCU overexpression, including the insulin and mTOR signaling pathways (Table S2). It is interesting to note that most activated genes in response to MCU
Figure 4. MCU Acutely Controls Muscle Size in the Adult

(A) EDL muscles of adult mice (2–3 months old) were infected with AAV-MCU or AAV-shMCU. AAV-LacZ or AAV-shlac was used as a negative control, respectively. Two weeks later, muscles were isolated and processed for further analysis.

(B and C) Mean fiber size of AAV-MCU- and AAV-shMCU-infected muscles (n > 300 fibers per muscle, n = 3).

(D) Protein synthesis analysis. EDL muscles were infected with AAV-MCU for 2 weeks. Purusycin was then intraperitoneally injected, and muscles were isolated 36 min later. Left: western blotting with anti-purusycin antibodies. Ponceau 9 staining was used as loading control. Right: quantification. n = 4.

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silencing (clusters 7 and 11) have mitochondrial functions (Table S2).

**MCU Protects from Skeletal Muscle Atrophy**

Finally, we investigated whether MCU overexpression could counteract conditions of disease-induced loss of muscle trophism. Denervation atrophy was triggered by sciatic nerve section, and Ca\(^{2+}\) signaling properties, together with muscle size, were evaluated (Figure S4A). Upon denervation, the cytosolic Ca\(^{2+}\) increase evoked by caffeine-induced SR release was markedly larger, although it did not evoke a larger Ca\(^{2+}\) uptake by mitochondria (Figures S4B and SD), possibly due to the morphological remodeling of the fiber or alterations in the MCU complex assembly or SR/mitochondria coupling. Mitochondrial ΔΨm was unchanged (Figure S5A). However, when MCU was overexpressed, mitochondrial Ca\(^{2+}\) uptake, induced by caffeine-evoked SR release, was greatly enhanced, reaching peak values that, due to the robust cytosolic rise, exceeded those of non-denervated fibers (Figures S5B and SD). As to resting values, a significant difference [i.e., a higher [Ca\(^{2+}\)]\(_{i}\) and lower [Ca\(^{2+}\)]\(_{m}\) resting value] was detected only in MCU-expressing non-denervated muscle, while denervated muscles exhibited a value similar to controls, irrespective of MCU expression (Figures S5C and SE). The measurements of [Ca\(^{2+}\)]\(_{i}\) and [Ca\(^{2+}\)]\(_{m}\) transients upon K\(^{+}\)-induced depolarization gave similar results (Figures S5B and S5C).

We then evaluated muscle size. Denervation caused a 40% reduction in TA mean fiber size, as expected. When MCU was overexpressed, atrophy was reduced to ~16% compared to innervated control fibers (Figures 4F and SG). Similar results were obtained when denervation was induced in adult animals in which MCU overexpression was induced by perinatal AAV infection (i.e., the conditions of Figures 2 and 3). In this case, fiber size was measured 3, 7, and 14 days post-denervation (Figures 4H and 6). In control muscles, 17%, 30%, and 60% of atrophy was observed, respectively, while in AAV-MCU-infected muscles, only 6%, 11%, and 22% reduction in fiber area was measured, respectively. Overall, the above data indicate that MCU overexpression can strongly counteract pathological atrophy.

**DISCUSSION**

The recent molecular identification of MCU (Stachowiak et al., 2011; De Stefani et al., 2011), and of its complex regulatory system (De Stefani and Rizzuto, 2012), now allows us to molecularly validate the broad literature supporting the pleiotropic role of mitochondrial Ca\(^{2+}\) homeostasis in cell function and survival. MCU-dependent mitochondrial Ca\(^{2+}\) accumulation was shown to play a role in pancreatic β cells (Alam et al., 2012; Tarasov et al., 2012), heart (Crago et al., 2012; Jörner et al., 2012), neurons (Zu et al., 2013), and colon cancer (Marchi et al., 2013). In this scenario, the very mild phenotype of the MCU\(^{-}\) mouse was quite surprising (Pan et al., 2013). The observation that viable mice could be obtained only in a mixed genetic background, while MCU ablation was embryonically lethal in the inbred strains, points to yet-unresolved compensatory mechanisms (Murphy et al., 2014). Interestingly, the MCU\(^{-}\) mice show clear metabolic and functional alterations of skeletal muscle, and a mitochondrial mutation (with ensuing loss of MCU gatekeeping), and hence increase in resting [Ca\(^{2+}\)]\(_{m}\) levels was identified in subjects with a pathology comprising leaning difficulties and early-onset proximal muscle weakness (Logan et al., 2014).

In our work, we bypassed embryonic development by utilizing viral transduction and in vitro electroporation for directing an MCU expression system or MCU small hairpin RNAs to the muscle of living animals. Two stages (developing and adult skeletal muscle) that exhibit intrinsic differences in plasticity and signaling responses were independently assessed, and a clear coherent phenotype was apparent, with some differences that are worthy of attention. Indeed, in both cases, mitochondrial Ca\(^{2+}\) accumulation via MCU positively correlated with the size of muscle fibers, i.e., a marked increase and reduction was observed in MCU-overexpressing and MCU-silenced fibers, respectively. In developing muscle, an increase in satellite cells was observed in MCU overexpressers (and a reduction in MCU-silenced fibers), but this was not the case in adult muscle, possibly due to the quiescent state of satellite cells in the adult.

This result indicates that an effect on the stem cell reservoir of muscle is not the key mechanism underlying the MCU-dependent increase in muscle mass.

We thus explored two different potential mechanisms for the increase in fiber size: a purely metabolic effect and a regulation of the anabolic/catabolic balance of skeletal muscle. The first mechanism was unlikely for the following reasons: (1) PDH activity, albeit defective in MCU silenced muscle cells (as in the MCU\(^{-}\) mouse), was unaffected by MCU overexpression; (2) the hypertrophic response was very similar in oxidative and glycolytic muscles, where the effect on mitochondrial metabolism should play a relatively minor role; and (3) semiquantitative analyses of aerobic metabolism revealed no major alteration. Nonetheless, electron microscopy (EM) analyses of MCU silenced fibers showed an overall reduction in mitochondrial volume (and some mitochondrial damage), while MCU-overexpressing fibers showed increased mitochondrial volume and a peculiar proliferation of cristae, thus suggesting a role of mitochondrial Ca\(^{2+}\) homeostasis in the regulation of organelle biogenesis and morphology.

As to the anabolic/catabolic balance, we saw no difference in vivo in the autophagic rates, which we expected could be involved based on the induction of AMPK-dependent autophagy by inhibition of mitochondrial Ca\(^{2+}\) uptake (Cádiz et al., 2020).
Figure 5. MCU Protects from Skeletal Muscle Atrophy

(A) Adult mice muscles were transfected with plasmids encoding MCU-Cherry for real-time imaging or MCU-Flag for fiber-size analyses. At the same time, demyelination was achieved by cutting the sciatic nerve high in the thigh. One week later, muscles were isolated and processed for further analysis.

(B) Mitochondrial Ca²⁺ spike of demyelinated FDB muscles transfected with MCU-Cherry-A or MCU-Cherry (MCU) upon caffeine stimulation. Left: representative traces. Right: mean cytosolic [Ca²⁺] increase. n = 29.

(C) Resting mitochondrial [Ca²⁺], n = 31.


(E) Resting cytosolic [Ca²⁺], n = 32.

In (B–E), data are presented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001; 1 tailed, unpaired.

(F) Immunofluorescence image of a demyelinated MCU-Flag (MCU) transfected TA muscle section. Antibodies against Flag tag and dystrophin to mark the sarcolemma were used. Scale bar, 100 μm.

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2013). We then drew on anabolic pathways. PGC-1α, a novel isoform of the transcriptional regulator of mitochondrial biogenesis, PGC-1α, was shown to induce muscle hypertrophy, implying on major anabolic routes, such as the IGF1-AMPK-IRS axis (Rusu et al., 2013). Pgc-1α was correlated with MUC1 expression and with a cluster of genes involved in muscle hypertrophy. This was particularly clear in the adult muscle, where other Pgc-1α isoforms were not concomitantly modulated. As to the downstream effectors, we could demonstrate MUC1-dependent phosphorylation of Akt and its downstream targets, 4E-BP1 and GSK3β. In agreement with these data, a marked increase in protein synthesis was measured in experiments of purynomic incorporation in nascent peptides.

Finally, MUC1 overexpression significantly counteracts denervation atrophy by markedly increasing the Ca²⁺/CaM rises evoked by SR Ca²⁺ release and K⁺-induced depolarization. The clarification of this novel pathway will thus represent an important task for the future, with potential applications of utmost relevance for the pharmacological targeting of muscle loss in disease, states and aging.

EXPERIMENTAL PROCEDURES

Expression Plasmids
MCU-GFP, MCU-Flag, MCU-Cherry, and mtGAPDHplm were harvested previously (En Stefani et al., 2011; Logan et al., 2014; LaFratta et al., 2013; Golec et al., 2014) and were maintained in stable cell lines.

For pZa2T.1-MUC1, MCU-Flag was amplified from MCU expression plasmid (LaFratta et al., 2011) with the primers 5'-TCGAAGCGACCATGATGSGC(GCGC)CGCGCTATGAGAAG (forward) and 5'-AACATCTTCATTCCCTGG1CCATCACTTTGTG (reverse) and cloned into XhoI-EcoRI sites of pZa2T.1. For MCU-ZsGreen, the MCU targeting sequence was inserted into BamHI-EcoRI sites of shZsGreen with the primers 5'-GATGTGATGCCGATGACGGATGACGATCAGG (forward) and 5'-AATCTTAAAGGCGGAGCAAGTGGGCACTGCATCCATTTG3 (reverse). For MCU-Cherry and shZsGreen, 2×Green cassettes at shMU-C rtShRNA and of shZsGreen were substituted with the ZsGreen cassette of pmCherry-A (Clontech Laboratories) at Neot-Nst sites.

AVV Production
AVV-MUC1 and AVV-LacZ were produced from pZa2T.1-MUC1 and pZa2T.1-LacZ, respectively; AVV-MCU and AVV-sh店 were produced from pZa2T.8c-18t-MUC1-MCU and pZa2T.8c-18t-LacZ-MUC1-MCU, respectively. AVW vectors were purchased from Vector BioLabs or prepared by the MAV Vector Unit at the ISCBB Tissue (http://www.iscbbs.org/index.php?title=Vector_BioLabs), as described previously (Uchida et al., 2003), with few modifications. The titre of recombinant AVVs was determined by quantifying vector genomes (vg) packaged into viral particles by real-time PCR against a standard curve of a plasmid containing the vector genome (Gorcelski et al., 2002). Values obtained were in the range of 1 × 10⁶ to 1 × 10⁷ vg/mL.

In Vivo AVV Infection, DNA Transfection, and Denervation
In vivo experiments were performed in accordance with the Italian law (DL 26/2014).

AVV Infection
For experiments in the neonates, 10⁵ vg injected into the hindlimb of 4 to 6-day-old male CD1 mice. Muscles were subsequently analyzed 1 or 2 months post-infection as reported in Results. An average of 44% of fibers were positive for the AVV infections. For experiments in the adult, male CD1 mice were used. EDL muscles were isolated through a small hindlimb incision, and 10⁷ vg were injected along the muscle length. Muscles were analyzed 15 days post-infection. An average of 72% of fibers were positive for the AVV infections.

BMS Transfections and Denervation
The TA and FDB muscles of adult male CD1 mice were transfected as previously reported (DiFranco et al., 2006; Saito et al., 2004). Denervation was achieved by cutting the sciatic nerve high in the thigh.

Microarray Data
Raw data are available in the GEO database (accession number GSE60031). Detailed microarray methods are described in Supplemental Experimental Procedures.

ACKNOWLEDGMENTS
The GEO accession number for the microarray data reported in this paper is GSE60031.

SUPPLEMENTAL INFORMATION
Supplemental information includes Supplemental Experimental Procedures, five figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2015.06.018.

AUTHOR CONTRIBUTIONS
R.R. and C.M. conceived the project and wrote the manuscript. C.M., G.G., I.Z., A.R., A.B., S.Z., O.P., and D.D.S. performed in vivo and ex vivo experiments. S.B. performed EM experiments and analyses and wrote the EM experiments and analyses and wrote a final revision. I.Z. performed AVV experiments. M.S. performed microarray experiments and analyses and wrote a final revision. I.Z. prepared AVV experiments and assisted with AVV experiments. M.S. co-supervised experiments on hypertrophy pathways. D.D.S. co-supervised with M.S. the experimental work. M.S. performed microarray experiments.

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Characterization of skeletal muscle specific mcu\(^{-/-}\) mouse (mlc1f-Cre-mcu\(^{-/-}\))

**Mitochondrial Ca\(^{2+}\) uptake is blunted in mlc1f-Cre-mcu\(^{-/-}\) mouse**

We have previously demonstrated that mitochondrial Ca\(^{2+}\) uptake positively regulates skeletal muscle mass by impinging on PGC-1\(\alpha\)4 and AKT anabolic pathways (Mammucari et al., 2015). In detail, MCU overexpression triggers skeletal muscle hypertrophy, while MCU silencing causes atrophy. This study was based on the use of AAV vectors to modulate MCU expression *in vivo* during postnatal growth or adulthood. However, one of the limitations of this approach is due to the fact that only a proportion of fibres of an injected muscle expresses the transgene. Thus, to address whether mitochondrial Ca\(^{2+}\) uptake in skeletal muscle contributes to whole organism metabolism and physiology, we generated a skeletal muscle specific mcu knockout mouse. For this purpose, we crossed mice with loxP-flanked mcu alleles to mice expressing the Cre recombinase under the control of mlc1f (myosin light chain 1f promoter), which is strongly and selectively active in skeletal muscle cells (Bothe et al., 2000) (Figure 3A). It should be noted that, different from AAV-injected muscles, in mlc1f-Cre-mcu\(^{-/-}\) mice mcu is blunted during embryonic development.

![Figure 3. Skeletal muscle specific mcu\(^{-/-}\) mice lack MCU expression.](image)

(A) Schematic representation of the targeting strategy used for the mcu locus to generate the mcu\(^{fl/fl}\) mice where exons 5 was flanked with LoxP sites. Mcu\(^{fl/fl}\) mice were crossed to mlc1f Cre mice to generate the skeletal muscle specific mlc1f-Cre-mcu\(^{-/-}\) mouse. (B) Western blotting analysis of MCU protein expression in TA muscles and livers. TOM20 was used as a protein-loading control. (C) Mcu relative mRNA expression levels in wild type and mcu\(^{-/-}\) TA muscles measured by Real-time PCR. Expression levels are normalized for GAPDH and quantification data are expressed with \(\Delta\Delta C_t\) method. **\(p < 0.01\), t-test (two-tailed, paired) of 7 muscles per group. Data are presented as mean ±SD.
The efficiency and specificity of skeletal muscle mcu deletion in the offspring was confirmed by western blotting for MCU protein and mcu mRNA levels, respectively (Figure 3B, C).

Furthermore, we simultaneously measured mitochondrial Ca\(^{2+}\) uptake and cytosolic [Ca\(^{2+}\)] in isolated FDB skeletal muscle fibres by live imaging. In detail, we transfected adult FDB mouse muscles in vivo with plasmids encoding a GFP-based Ca\(^{2+}\) probe targeted to mitochondria, mtGCaMP6f (Chen et al., 2013; Tosatto et al., 2016), in combination with a genetically encoded cytosolic Ca\(^{2+}\) indicator R-GECO1 (Zhao et al., 2011). One week later, we performed real time imaging experiments in single isolated fibres, that were stimulated with caffeine in order to evoke a cytosolic Ca\(^{2+}\) rise by discharging the sarcoplasmic reticulum pool. The mitochondria of mlc1f-Cre-\textit{mcu}^{-/-} mice showed the complete inability to take up Ca\(^{2+}\) compared to control wild-type mitochondria. At the same time no difference was observed in the cytosolic [Ca\(^{2+}\)] (Figure 4A, B).

\textbf{Figure 4. Mitochondrial Ca\(^{2+}\) uptake is blunted in mlc1f-Cre-mcu\(^{-/-}\) mice.} (A) Mitochondrial Ca\(^{2+}\) transient measurements (on the left hand side) and cytosolic Ca\(^{2+}\) transient measurements (on the right hand side) in single isolated FDB fibres transfected with a mitochondrially targeted GCaMP6f together with R-GECO1. Fibres were stimulated with 20 mM of caffeine. The bar diagram shows the mean peak ±SD. *p < 0.05, t-test (two-tailed, unpaired) of more than 50 fibres per condition. (B) Left panel shows representative traces of mcu\(^{0/0}\) mice. Right panel shows representative traces of mcu\(^{-/-}\) mice.
**Mcu ablation impairs anabolic pathways: PGC-1α4 and AKT**

Since mitochondrial Ca\(^{2+}\) uptake positively controls major skeletal muscle anabolic pathways (Mammucari et al., 2015), we wondered whether these pathways are affected in our skeletal muscle specific mlc1f-Cre-*mcu\(^{-/-}\)* mice. Consistent with previous data based on AAV-shRNA mediated downregulation of MCU (Mammucari et al., 2015), MCU deletion caused a decrease in AKT phosphorylation (Figure 5A) and in PGC-1α4 mRNA expression levels (Figure 5B), confirming that a marked reduction of mitochondrial Ca\(^{2+}\) uptake negatively regulates anabolic signaling pathways.

**Figure 5. Mcu ablation impairs anabolic pathways: PGC-1α4 and AKT.** (A) On the left hand side western blotting analysis of MCU expression level and AKT phosphorylation level in TA muscles of mcu\(^{fl/fl}\) and mlc1f-Cre-mcu\(^{-/-}\) mice. ACTIN was used as a protein-loading control. The graph on the right hand side shows the quantification by densitometry of the ratio between phospho-AKT and ACTIN. \(^*p < 0.05, \)t-test (two-tailed, unpaired) of 13 animals. Data are presented as mean ±SD. (B) Total PGC-1α, PGC-1α1 and PGC-1α4 relative mRNA expression levels in mcu\(^{fl/fl}\) and mlc1f-Cre-mcu\(^{-/-}\) TA muscles measured by Real-time PCR. Expression levels are normalized for GAPDH and quantification data are expressed with \(\Delta Ct\) method. \(^*p < 0.05, \)t-test (two-tailed, paired) of 5 muscles per group. Data are presented as mean ±SD.

Since we observed modulation of anabolic pathways, we wondered whether the reduction in anabolic signaling, observed in mlc1f-Cre-*mcu\(^{-/-}\)* mice, was associated with defects in muscular structure. However, morphological analyses did not reveal any abnormalities on mlc1f-Cre-*mcu\(^{-/-}\)* muscles (Figure 6).
**Mlc1f-Cre-mcu<sup>−/−</sup> mouse shows reduced running capacity**

In 2013, an mcu<sup>−/−</sup> mouse model was generated using the gene trap technique. Although it was demonstrated that mitochondria of mcu<sup>−/−</sup> mice did not take up Ca<sup>2+</sup>, these mice were regularly born and their phenotype was very mild. In detail, modest defects in skeletal muscle strength and alteration of pyruvate dehydrogenase activity were showed (Pan et al., 2013). Taking into account the above data, we wondered whether the impairment in mitochondrial Ca<sup>2+</sup> uptake in skeletal muscle could affect exercise performance. For this purpose, running capacity of skeletal muscle specific mlc1f-Cre-mcu<sup>−/−</sup> mice was measured. This experiment was performed using a standard uphill run protocol in order to monitor the maximal distance run to exhaustion (He et al., 2012). A significant difference was observed in running capacity after a single bout of run between 3-month old control animals and mlc1f-Cre-mcu<sup>−/−</sup> mice, where mlc1f-Cre-mcu<sup>−/−</sup> mice showed impaired running capacity (Figure 7A). To observe whether this impairment in running capacity could worsen with age, we repeated a similar experiment in 6-month old mice. Again, we observed a decrease in running capacity of mlc1f-Cre-mcu<sup>−/−</sup> mice, which was similar to what observed in 3-months old animals (Figure 7B), indicating that the defecting physical performance does not worsen with age, at least in the timeframe analyzed here.

Downhill running exercise is known to induce muscle damage and a detrimental effect on mitochondria, since contractions occur together with the stretching of skeletal muscle. These peculiar contractions are known as “lengthening” or “eccentric” contractions. Due to plasmamembrane damage, eccentric contractions trigger an increase in intracellular [Ca<sup>2+</sup>] (Sonobe et al., 2010) , activation of inflammatory response (Peake et al., 2005) and alteration in metabolism, which
affects glycogen stores, lactate concentration and triacylglycerol breakdown (Magalhães et al., 2013). Thus, we investigated whether repeated bouts of downhill running exercise for 3 consecutive days could induce an additive detrimental effect on muscle performance, as a consequence of cumulative damage (Lo Verso et al., 2014). A decrease in physical performance was observed over time in both genotypes. Additionally, mlc1f-Cre-<sup>-mcu/-</sup> mice run less than their littermate controls during every bout of the training (Figure 7C).

Altogether these results indicate that skeletal muscle specific mlc1f-Cre-<sup>-mcu/-</sup> mice, in which mitochondrial Ca<sup>2+</sup> uptake is blunted, are characterized by negative regulation of the major skeletal muscle anabolic pathways, and reduced running performance.

**Figure 7.** Mlc1f-Cre-<sup>-mcu/-</sup> mouse shows reduced running capacity. (A-B) The graphs show the mean maximal distance run to exhaustion by mcu<sup>fl/fl</sup> and mlc1f-Cre-mcu<sup>-/-</sup> mice during acute uphill exercise training. 3 months old mice on pane l A and 6 months old mice on panel B, respectively. *p < 0.05, t-test (two-tailed, unpaired) of 7 animals per group. (C) The graph shows the mean maximal distance run to exhaustion by mcu<sup>fl/fl</sup> and mlc1f-Cre-mcu<sup>-/-</sup> mice during 3 consecutive day of downhill exercise training. *p < 0.05, t-test (two-tailed, unpaired) of 7 animals per group.
Mitochondrial Ca\textsuperscript{2+} modulates metabolic functions in skeletal muscle

Mitochondria play a central role in cellular metabolism, being a site for a large number of biochemical processes, including Krebs cycle, fatty acid β-oxidation, and Ca\textsuperscript{2+} handling. It is well established that mitochondrial Ca\textsuperscript{2+} accumulation stimulates the Ca\textsuperscript{2+}-sensitive dehydrogenases of the Krebs cycle (Denton, 2009b). Consistently, the total mcu\textsuperscript{-/} mouse model showed an increase in the phosphorylation levels of PDH, thus a decreased activity of the enzyme (Pan et al., 2013). In addition, our previous data gave coherent results, demonstrated that the phosphorylation level of PDH is increased in MCU silencing muscle. However, no changes were observed in MCU overexpressing muscles. Regarding the PDH activity, we observed consistent results (Mammucari et al., 2015). We took advantage of the in vivo AAV-based modulation of MCU and of our mcu\textsuperscript{-/} mouse model to determine whether mitochondrial Ca\textsuperscript{2+} uptake regulates metabolic pathways in mouse skeletal muscle.

Modulation of cytosolic NADH levels by mitochondrial Ca\textsuperscript{2+}

Nicotinamide adenine dinucleotide is a fundamental factor in metabolism thanks to its ability to carry electrons from one reaction to another. NADH plays a major role by regulating the intracellular redox state and it is considered as a read out of the metabolic state of the cell. There are many redox reactions catalyzed by different NAD(H)-dependent dehydrogenases that are essential for glycolysis and mitochondrial metabolism, such as the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase and the pyruvate dehydrogenase complex, which converts pyruvate to acetyl-CoA, a substrate for the TCA cycle (Lin and Guarente, 2003). In addition, it is well known that the NADH/NAD\textsuperscript{+} ratio differs in response to a change in metabolism (Gaikwad et al., 2001) (MacDonald and Marshall, 2000). We performed NADH measurements in isolated FDB fibers of mlc1f-Cre-mcu\textsuperscript{-/} mice using the Peredox probe. Peredox is a genetically encoded GFP based fluorescent biosensor with a sequence of a bacteria NADH-binding domain called Rex (Hung et al., 2011). Upon NADH binding, the NADH binding domain assumes
While NADH binding increases the green fluorescence, it does not change the red fluorescence of a tandemly bound NLS-mCherry, included to normalized the signal for protein expression (Figure 8A).

Figure 8. Mitochondrial Ca\textsuperscript{2+} uptake modulates cytosolic NADH levels. (A) Schematic representation of Peredox probe (image modified from Hung and Yellen, 2014) with a cpFP inserted between the two T-Rex subunits (orange and blue). When NADH (black) binds the T-Rex subunits there is a conformational change that increases the fluorescence of the protein. NLS-mCherry is tandemly bound to normalize the signal for protein expression. (B) NADH imaging was performed in isolated FDB fibres of mcu\textsuperscript{fl/fl} and mlc1f-Cre-mcu\textsuperscript{-/-} mice using Peredox probe. The graph shows the NADH/NAD\textsuperscript{+} expressed as Peredox fluorescence normalized for NLS-mCherry fluorescence. *p < 0.05, t-test (two-tailed, unpaired) of more than 30 fibres per condition. Data are presented as mean ±SD. (C-D) NADH imaging was performed in isolated FDB fibres in both MCU silencing and overexpression conditions using Peredox probe. The graphs (MCU silencing in panel C and MCU overexpression in panel D) show the NADH/NAD\textsuperscript{+} expressed as Peredox fluorescence normalized for NLS-mCherry fluorescence. ***p < 0.005, t-test (two-tailed, unpaired) of more than 50 fibres per condition. Data are presented as mean ±SD.

We transfected adult mlc1f-Cre-mcu\textsuperscript{-/-} FDB mouse skeletal muscles in vivo with a plasmid encoding Peredox probe, and 7 days later we performed ex vivo imaging experiments on isolated single myofibres. Mlc1f-Cre-mcu\textsuperscript{-/-} mice showed a decreased NADH/NAD\textsuperscript{+} ratio compared to their wildtype counterparts (Figure 8B). Additionally, we performed similar experiments in MCU silencing and overexpression conditions. In detail, we transfected adult FDB mouse skeletal muscle of wildtype animals with a plasmid encoding Peredox probe in combination with plasmids encoding either shMCU or MCU. MCU silencing gave coherent results with what obtained in mlc1f-Cre-mcu\textsuperscript{-/-} muscles, since NADH level
decreases in shMCU transfected muscles (Figure 8C). On the other hand, the MCU overexpression triggers an increase in NADH level compared to control (Figure 8D). Taken together, these data suggest an MCU-dependent modulation of cytosolic NADH levels in skeletal muscle.

**MCU downregulation triggers a metabolic shift towards preferential fatty acid oxidation**

Since we observed a modulation of cytosolic NADH level by mitochondrial Ca$^{2+}$ uptake, and it is well known that NADH plays a major role in the regulation of metabolism, we decided to investigate metabolic signatures associated with the modulation of MCU expression in skeletal muscle. For this purpose, we injected hindlimb muscles of newborn mice with AAV-MCU or AAV-shMCU and AAV-LacZ or AAV-shLUC as control, respectively. We collected TA muscles eight weeks after injection and performed untargeted metabolomics analysis. We identified 437 compounds of known identity, of which ~100 were significantly altered.

Metabolomic profiling of MCU overexpressing muscles showed decreased levels of glycolysis-related metabolites. In particular glucose, glucose 6-phosphate, fructose 6-phosphate, 3-phosphoglycerate and phosphoenolpyruvate levels are decreased (Figure 9 and Appendix Table 1).

**Figure 9.** MCU overexpression decreases glycolysis-related metabolites. Metabolite set enrichment analysis of AAV-MCU versus AAV-LacZ infected muscles. Differentially regulated metabolites have been mapped to the glycolysis subnetwork. Blue dots represent decreased metabolites ($p < 0.05$).

We observed the most striking changes in shMCU muscles (Figure 10 and Appendix Table 2). In detail, among TCA intermediates, succinate and alpha-
ketoglutarate levels are decreased in MCU silenced muscles, in line with the known Ca$^{2+}$-dependent regulation of corresponding enzymes (McCormack et al., 1990b) (Figure 10A).

Regarding the metabolites associated with carbohydrates pathway, glucose, glucose 6-phosphate, 3-phosphoglycerate and phosphoenolpyruvate levels were decreased, suggesting a decreased glycolytic input in the Krebs cycle in MCU silenced muscles (Figure 10B).

Moreover, amino acid metabolism is significantly reduced. In particular, metabolism of lysine, phenylalanine, tyrosine, tryptophan, leucine, isoleucine and valine and urea cycle were decreased (Figure 10C). It is well known that atrophy is accompanied with protein breakdown (Sandri, 2015), thus these data explain the atrophy phenotype of silenced muscles.

Strikingly, we observed the most outstanding changes in fatty acids (FAs) metabolism, indicating a global increase of lipid β-oxidation in MCU silenced muscles. In detail, long-chain FAs (palmitoleate, stearate and eicosenoate) were increased in shMCU muscles compared to AAV-control muscles. In addition, increases in monoacylglycerols suggest augmented uptake and hydrolysis of intermediate triglycerides to support fatty acid oxidation. Moreover, long-chain FAs must be conjugated to carnitine for transport across the mitochondrial membrane prior to oxidation; in agreement, a subset of long-chain acylcarnitines were elevated (Figure 10D). These data suggest a clear MCU-dependent metabolic reprogramming in skeletal muscle tissue with a shift towards preferential fatty acid oxidation. However, it must be stressed that this interpretation relies only on the steady-state analysis of metabolites. Thus, metabolic flux analysis (the only experimental approach that can truly describe the dynamic regulation of metabolic network) is needed to confirm this hypothesis.
Figure 10. MCU downregulation triggers a metabolic shift towards preferential fatty acid oxidation. Metabolite set enrichment analysis of AAV-shMCU versus AAV-shLUC infected muscles. Differentially regulated metabolites have been mapped to the indicated metabolic subnetworks. In detail, panel A refers to TCA cycle, panel B to carbohydrate metabolism, panel C to amino acid metabolism and panel D to lipid metabolism.

Mitochondrial Ca\(^{2+}\) uptake does not regulate bulk autophagy in skeletal muscle, whereas it modulates mitophagy

Previous work highlighted the link between mitochondrial Ca\(^{2+}\) accumulation and optimal cellular bioenergetics maintenance, showing the inhibitory effect of ER-mitochondrial Ca\(^{2+}\) transfer on autophagy (Cárdenas et al., 2010). Furthermore, autophagy was shown to contribute to the removal of dysfunctional organelles and proteins in basal condition (Mizushima and Komatsu, 2011) and to be induced during muscle atrophy (Mammucari et al., 2007), a condition characterized by high rate of catabolism (Sandri, 2010). We wondered whether MCU modulation could
have a role in the control of skeletal muscle autophagy. For this purpose, we performed autophagic flux analyses in both AAV-MCU and AAV-shMCU injected muscles. Autophagic flux, referred to as the balance between formation and degradation of autophagosomes, was evaluated by blocking the fusion of autophagosomes with lysosomes by means of colchicine treatment as described in “materials and methods” section. We performed the experiments both in fed and in starving conditions, since the latter increases the autophagic process. Consequently, we monitored the lipidation of LC3 and the degradation of P62, two well-known autophagy markers. Regarding the control muscles in fed condition, as expected, we observed an increase in LC3 II protein level in colchicine treated muscles, which confirmed the block of autophagy. However, P62 protein level did not show major changes (Figure 11A). Control muscles in fasting condition showed a similar scenario to the one of fed muscles, with even more pronounced LC3 II accumulation upon colchicine treatment (Figure 11B). In MCU-overexpressing muscles, both in fed and starvation conditions, we observed an increase in LC3II protein levels in the colchicine treated samples whereas P62 protein levels did not change (Figure 11A, B). No difference was observed between MCU overexpressing samples and controls, neither in fed nor in starvation condition (Figure 11A, B).
Figure 11. MCU overexpression does not modulate bulk autophagy in skeletal muscle. (A) Autophagy flux measurements in fed condition. Muscles were infected with AAV-MCU and 2-week post-infection mice were treated with colchicine. The top panel shows western blotting analysis of muscle lysates stained with α-P62, α-ACTIN, α-MCU and α-LC3. The graphs show quantification by densitometry of the ratio between LC3II or P62 and ACTIN, respectively. Data are presented as mean ±SD. (B) Autophagy flux measurements in starvation condition. Muscles were infected with AAV-MCU and 2-week post-infection mice were treated with colchicine and fasted for 24h. The top panel shows western blotting analysis of muscle lysates stained with α-P62, α-ACTIN, α-MCU and α-LC3. The graphs show quantification by densitometry of the ratio between LC3II or P62 and ACTIN, respectively. *p < 0.05, t-test (two-tailed, unpaired) of 4 muscles per condition. Data are presented as mean ±SD.
A similar scenario occurred in the AAV-shMCU experiments. Again LC3II protein levels were increased both in control and shMCU injected muscles treated with colchicine, whereas P62 protein levels showed no great changes. However, no changes between shMCU injected samples and controls were observed (Figure 12A, B), demonstrating that MCU silencing does not affect autophagic flux.

**Figure 12.** MCU silencing does not modulate autophagy in skeletal muscle. (A) Autophagy flux measurements in fed condition. Muscles were infected with AAV-shMCU and 2-week post-infection mice were treated with colchicine. The top panel shows western blotting analysis of muscle lysates stained with α-P62, α-ACTIN and α-LC3. The graphs show quantification by densitometry of the ratio between LC3II or P62 and ACTIN, respectively. Data are presented as mean ±SD. (B) Autophagy flux measurements in starvation condition. Muscles were infected with AAV-shMCU and 2-week post-infection mice were treated with colchicine and fasted for 24h. The top panel shows western blotting analysis of muscle lysates stained with α-P62, α-ACTIN and α-LC3. The graphs show quantification by densitometry of the ratio between LC3II or P62 and ACTIN, respectively. *p < 0.05, t-test (two-tailed, unpaired) of 4 muscles per condition. Data are presented as mean ±SD.
Moreover, qPCR analysis confirmed that expression of autophagy-related genes, such as Beclin1, LC3 and Bnip3, was unchanged by AAV-MCU and AAV-shMCU in adult muscle (Figure 13).

**Figure 13. The expression of autophagy-related genes is unchanged by MCU overexpression or silencing.** Beclin, Lc3 and Bnip3 relative mRNA expression levels in control EDL muscles and AAV-MCU (left panel) or AAV-shMCU (right panel) injected muscles measured by Real-time PCR. Expression levels are normalized for GAPDH and quantification data are expressed with ΔCt method. *p < 0.05, t-test (two-tailed, paired) of 5 muscles per group. Data are presented as mean ±SD.

We supposed that, although bulk autophagy was unaffected in our experiments, a specific analysis of selective removal of damaged mitochondria, named mitophagy, would unravel changes that would otherwise be overlooked.

In order to monitor this process, we took advantage of Keima, a fluorescent reporter that exhibits different pH-dependent excitation wavelengths (Katayama et al., 2011). When mitochondria are delivered to the lysosome the pH becomes acidic, therefore Keima changes color, from green to red. Healthy mitochondria appear green and elongated whereas red or yellow dots represent mitochondria delivered to the lysosomes.

Previous work described for the first time the use of Keima to monitor the basal mitophagy process taking place in skeletal muscle (Sun et al., 2015). Accordingly, we transfected adult FDB mouse skeletal muscles in vivo with a plasmid encoding a mitochondrial targeted form of Keima in combination with MCU or shMCU. Ten days after transfection we performed ex vivo imaging experiments on isolated single myofibres. MCU overexpression triggers an increased mitophagy compare to control samples (Figure 14A). In addition, MCU silencing gives coherent results, as in MCU silenced fibres mitophagy is decreased (Figure 14B). These results
Figure 14. Mitochondrial Ca\textsuperscript{2+} uptake modulates mitophagy in skeletal muscle. (A) Right panel shows quantification analysis of mitophagy process in FDB single isolated fibers expressing Keima together with MCU. Mitophagy index indicates the number of red dots normalized per area in each image*\(p < 0.05\), t-test (two-tailed, unpaired) of more than 50 fibres per condition. Data are presented as mean ±SD. Left panel shows representative images of the experiment where green dots are healthy mitochondria whereas red dots represent mitochondria delivered to the lysosome. Scale bar 10 µm. (B) Right panel shows quantification analysis of mitophagy process in FDB single isolated fibers expressing Keima together with shMCU. Mitophagy index indicates the number of red dots normalized per area in each image. *\(p < 0.05\), t-test (two-tailed, unpaired) of more than 50 fibres per condition. Data are presented as mean ±SD. Left panel shows representative images of the experiment where green dots are healthy mitochondria whereas red dots represent mitochondria delivered to the lysosome. Scale bar 10 µm.
PGC-1α4 promoter activity is controlled by mitochondrial Ca\(^{2+}\) uptake

We have previously shown that mitochondrial Ca\(^{2+}\) uptake regulates mRNA expression levels of PGC-1α4, demonstrating the existence of a Ca\(^{2+}\)-dependent mitochondria-to nucleus signaling route (Mammucari et al., 2015). 

*Pgc-1α* gene has two different promoter regions. The proximal promoter is located immediately before exon 1, whereas the alternative promoter is located 13 kb upstream (Figure 15). Spiegelman lab identified four different isoforms of PGC-1α. PGC-1α1, formerly known as PGC-1α and major mitochontriogenic factor, originates from the proximal promoter, whereas PGC-1α2, PGC-1α3 and PGC-1α4 expression is driven by the alternative promoter and results from an alternative splicing of *Pgc-1α* gene (Ruas et al., 2012).

*Figure 15. Representation of Pgc-1α promoter region.* Schematic representation of the conservation between human and mouse Pgc-1α promoter region (image modified from Ruas et al., 2012). This comparison was done using ECR browser (ww.ecrbrowser.dcode.org). The expression of Pgc-1α gene is driven by two different promoters. The proximal promoter is located near the exon 1, whereas the alternative promoter is located 13 kb upstream. From these different promoters arise four different isoforms, PGC-1α1, PGC-1α2, PGC-1α3 and PGC-1α4.
In detail, Spiegelman and coworkers discovered that PGC-1α4 is highly expressed in exercised muscles, however, contrary to PGC-1α1, it is not involved in mitochondriogenesis and it does not regulate the same target genes of PGC-1α1. On the contrary, PGC-1α4 overexpression triggers hypertrophy by regulating myostatin and IGF1 pathways. These data prompted us to evaluate the possibility that mitochondrial Ca\(^{2+}\) uptake could control Pgc-1α4 promoter activity. In order to test this hypothesis, we cloned the alternative promoter of Pgc-1α (from -1146 to -1) upstream of a minimal SV40 promoter into a luciferase reporter vector. TA muscles were electroporated with this construct together with a plasmid encoding MCU. One week after electroporation muscles were removed and luciferase reporter assay analysis was performed. MCU overexpression increased the activity of the Pgc-1α4 promoter (Figure 16A). In contrast, when similar experiments were performed using a short hairpin against MCU, Pgc-1α4 promoter activity was inhibited (Figure 16A).

In order to identify the minimal Ca\(^{2+}\) responsive region of Pgc-1α4 promoter, we cloned upstream of the luciferase gene shorter and shorter portions of the alternative promoter proximal to the 5’-UTR. First, we cloned the region from -421 to -1 of the promoter proximal to the first exon (from -421 to -1), and we performed similar experiments as previously described. Again, MCU activates the promoter, whereas shMCU inhibits its activity (Figure 16B). Next, we cloned regions from -151 to -1 and from -76 to -1 respectively of the promoter proximal to the first exon. Also in this case overexpression of MCU triggers promoter activity, whilst MCU silencing decreases its activity (Figure 16C, D). Taken together these results indicate that the region from -76 to -1 of the alternative promoter is controlled by mitochondrial Ca\(^{2+}\) uptake.

Our ultimate goal is to identify which transcription factors are responsible for the MCU-dependent activity of Pgc-1α4. For this purpose, we took advantage of bioinformatics tools able to predict transcription factors that could bind the -76 to -1 promoter region. Preliminary results using MatInspector software (Genomatix) demonstrate that Creb and Hif1α could bind this minimal region (data not shown). In the future, we will investigate deeply the role of this two transcription factors in the regulation of Pgc-1α4 expression.
Figure 16. Pgc-1α4 promoter activity is regulated by mitochondrial Ca²⁺ uptake. (A–D) Left panels show schematic representation of Pgc-1α4 alternative promoter constructs. A luciferase reporter vector encoding fragments of the Pgc-1α4 alternative promoter region upstream of a minimal SV40 promoter was transfected together with MCU into adult TA muscle. In addition, a renilla luciferase construct was cotransfected to normalize for transfection efficiency. After a week, firefly/renilla luciferase activity was determined (middle panels). The right panels show similar experiments in which MCU was silenced. *p < 0.05, t-test (two-tailed, paired) of 4 muscles per group. Data are presented as mean ±SD.
Discussion

It is well-established that mitochondrial Ca\(^{2+}\) homeostasis has a pleiotropic role in cell function and survival. The recent identification of MCU opened the route for the study of mitochondrial Ca\(^{2+}\) uptake in in vivo systems (Baughman et al., 2011; De Stefani et al., 2011). Thanks to the broad literature, we know that mitochondrial Ca\(^{+}\) uptake plays a crucial role in cancer, neuron, heart and pancreatic β cells. In addition, during the last years, numerous mouse models have been generated, demonstrating the importance of the mitochondrial Ca\(^{2+}\)(De Stefani et al., 2016). However, the very mild phenotype of the first model of MCU knockout mouse (obtained in mixed outbred strain composed by C57/BL6 and CD1 backgrounds) left the majority of the scientists puzzled (Pan et al., 2013). Others works later published demonstrated that the ablation of MCU in the pure C57/BL6 strain leads to embryonic lethality and this result was confirmed in two independent mouse models (Dickinson et al., 2016; Luongo et al., 2015). In our work, we focused our attention on the channel per se, using its modulation as a tool to increase or decrease mitochondrial Ca\(^{2+}\) uptake in skeletal muscle. Moreover, the existence of a MCU complex, containing, besides MCU, MCUb, EMRE and MICUs must be taken into account.

In our work we modulated MCU protein level by means of different techniques. First, we used AAV vectors to overexpress or downregulate MCU protein levels either during skeletal muscle growth or in adulthood (Mammucari et al., 2015). Then, we generated a skeletal muscle specific mcu knockout mouse (mlc1f-Cre-mcu\(^{-/-}\)), in which a mcu \(\beta/\beta\) mouse was crossed with a mouse expressing the Cre recombinase under the control of the myosin light chain 1f (mlc1f) promoter, which is expressed since E15 (Lyons et al., 1990). Firstly, we measured mitochondrial Ca\(^{2+}\) accumulation and cytosolic Ca\(^{2+}\) concentration in skeletal muscle fibres. Both MCU modulation by AAVs and mcu deletion in skeletal muscle-specific knockout mice were accompanied these expected changes in mitochondrial Ca\(^{2+}\) uptake. In detail, mitochondrial Ca\(^{2+}\) accumulation was greatly reduced by AAV-shMCU infection and completely ablated in mlc1f-Cre-mcu\(^{-/-}\) muscles. Conversely, overexpression of MCU caused marked mitochondrial Ca\(^{2+}\) uptake (Mammucari et
al., 2015). As for basal mitochondrial [Ca\(^{2+}\)], it has already been reported that matrix Ca\(^{2+}\) in isolated skeletal muscle mitochondria, in which MCU is ablated, is reduced to about 25% compared to controls (Pan et al., 2013). We tried to measure basal mitochondrial [Ca\(^{2+}\)] in mlc1f-Cre-\(mcu^{-/-}\) muscle fibres, which is expected to be very low. However, due to the lack of sensitivity of our probe in the low [Ca\(^{2+}\)] range, we could not obtain a quantitative data. Regarding cytosolic [Ca\(^{2+}\)] upon agonist stimulation, we wondered whether mitochondria could serve as buffers of cytosolic Ca\(^{2+}\). We observed no effect of MCU overexpression or MCU silencing on cytosolic [Ca\(^{2+}\)], demonstrating that mitochondrial Ca\(^{2+}\) uptake do not work as a cytosolic Ca\(^{2+}\) buffer in skeletal muscle (Mammucari et al., 2015). We cannot exclude completely this mitochondrial capacity, as the cytosolic Ca\(^{2+}\) probe used for the experiments measures the bulk cytosolic [Ca\(^{2+}\)], while Ca\(^{2+}\) buffering could occur selectively in the MAMs (mitochondria associated membranes), which are ultra-specialized structures allowing the just-apposition of the mitochondria to Ca\(^{2+}\) release site in SR.

When we focused our attention on the muscle phenotype we demonstrated that mitochondrial Ca\(^{2+}\) uptake via MCU positively correlated with the size of muscle fibers. In detail a marked increase and reduction was observed in MCU-overexpressing and MCU-silenced fibers, respectively. Firstly, we demonstrated these results in animals treated with AAV vectors during the first days of life. In this case, the phenotypic effect could be due to defects in post-natal muscle growth. In order to bypass this caveat, we performed similar analyses on adult muscles, where we observed a similar phenotype, demonstrating that fibre size modulation mainly depends only on the change of hypertrophy program rather than on muscle growth (Mammucari et al., 2015).

Surprisingly, we observed no differences in fibre size of mlc1f-Cre-\(mcu^{-/-}\) skeletal muscles. This effect is probably due to compensatory mechanisms taking place during embryogenesis. However, to bypass this problem we are generating an inducible skeletal muscle specific \(mcu^{-/-}\) mouse model, in order to perform ablation of \(mcu\) gene during adulthood.

We investigated the molecular mechanisms which could explain the changes in skeletal muscles fibre size. We demonstrated that mitochondrial Ca\(^{2+}\) uptake
activates two major anabolic pathways, i.e. PGC-1α4 and IGF1-AKT pathways (Mammucari et al., 2015). The former is a novel isoform of PGC-1α gene which has been shown to induce muscle hypertrophy. Nicely, PGC-1α4 transcription correlated with MCU expression levels. It has been reported that PGC-1α4 controls IGF1/AKT pathway, the major skeletal muscle pathway that regulates muscle mass (Ruas et al., 2012). We demonstrated that the phosphorylation of AKT and its downstream targets, 4E-BP1 and GSK3β are dependent on MCU protein level. In addition, we observed an increase in protein synthesis in MCU-overexpressing muscle, confirming the activation of anabolic pathways. On the other hand, in MCU-silenced and in mlc1f-Cre-\textit{mceu}− mice skeletal muscles we coherently observed the inactivation of anabolic pathways. However, both in MCU-silencing and in mlc1f-Cre-\textit{mceu}− muscles, we did not observe a clear induction of atrophy pathways (data not shown), indicating that while mitochondrial Ca\textsuperscript{2+} plays a major role in the regulation of anabolic pathways in skeletal muscle, a clear link between mitochondrial Ca\textsuperscript{2+} uptake and activation of atrophic program is not evident.

We further investigated this issue in a model of atrophy caused by sciatic nerve excision. During muscular atrophy, protein breakdown and organelle degradation pathways prevail together with the activation of a specific genetic program (Schiaffino et al., 2013). We wondered whether mitochondrial Ca\textsuperscript{2+} uptake could be involved in this scenario. Although cytosolic [Ca\textsuperscript{2+}] and mitochondrial Ca\textsuperscript{2+} accumulation after agonist stimulation did not change in denervated compared to control fibres, overexpression of MCU, resulting in an increase of mitochondrial Ca\textsuperscript{2+} accumulation, was able to counteract muscle atrophy. These results indicate that, in the balance between protein degradation and synthesis, MCU overexpression impinges on protein synthesis program while exerting a minor effect on the atrophic program (Mammucari et al., 2015).

At this point we wondered which was the mitochondrial signal initiated by Ca\textsuperscript{2+} that regulates anabolic pathways. We thus explored two different potential ways, the former being a mitochondrial-to-nucleus signaling route, the latter concerning the involvement of aerobic metabolism.

Regarding the mitochondrial-to-nucleus process, we deeply investigated how mitochondrial Ca\textsuperscript{2+} uptake could control \textit{Pgc-1α4} promoter activity. We identified
the minimal Pgc-1α4 promoter region regulated by mitochondrial Ca^{2+} uptake. Now, we are investigating which transcription factors are responsible for the MCU-dependent activity of Pgc-1α4 promoter. Preliminary results, by means of bioinformatics tools, demonstrate that Creb and Hif1α could bind this minimal region. In the future, we will investigate in detail the role of this two transcription factors in the regulation of Pgc-1α4 expression. In addition, it would be interesting to analyze the common transcription factors of the genes differentially expressed by MCU modulation, that were discovered by microarray analyses of MCU-overexpressing or silenced single isolated skeletal muscle fibres.

The second hypothesis takes into account the role that mitochondrial Ca^{2+} exerts on aerobic metabolism. Mitochondria plays central role in cellular metabolism, indeed Krebs cycle, fatty acid β-oxidation, Ca^{2+} handling and other broad biochemical processes take place within them. In addition, it is well-established from the broad literature that mitochondrial Ca^{2+} accumulation regulates aerobic metabolism. Indeed, mitochondrial Ca^{2+} uptake stimulates three Ca^{2+}-sensitive dehydrogenase of the Krebs cycle (McCormack et al., 1990b).

We investigated the involvement of aerobic metabolism to explain the control of fibre size by MCU. However, our results indicate that differences in aerobic metabolism unlikely are responsible for the observed phenotype for the two following reasons. (i) The effect on mitochondrial metabolism in glycolytic muscles, which are poor in mitochondria, should play a minor role compared to oxidative muscles that are rich in mitochondrial content. However, we demonstrated that the hypertrophic response was very similar between this two different types of muscles. In addition, semi-quantitative analyses of aerobic metabolism performed on this two different types of muscles revealed no major differences. (ii) We demonstrated that the PDH activity was decreased in MCU-silenced muscle, accordingly to results in total MCU knockout mouse model (Pan et al., 2013). However, we were not able to observe an opposite effect in MCU-overexpressing muscles although they were hypertrophic, indicating that aerobic metabolism is at the maximum level during basal conditions in skeletal muscle. Even if the modulation of aerobic metabolism does not explain the striking effect of mitochondrial Ca^{2+} uptake on the control of muscle size, it should be taken into
account that the major task of mitochondria is the regulation of cellular metabolism. Thus, we decided to further investigate whether the modulation of mitochondrial Ca\(^{2+}\) accumulation could impinge on global metabolism. At the beginning we measured the NADH/NAD\(^{+}\) ratio, considering NADH as a readout of the metabolic state of a cell, indeed NADH is involved in several redox reactions. We demonstrated that mitochondrial Ca\(^{2+}\) uptake regulates NADH levels. In detail an increase and reduction was observed in MCU-overexpressing and MCU-silenced muscle fibers, respectively. In addition, the measurement of NADH level in mcl1f-Cre-\textit{mcu}\(^{+/}\) muscles gave coherent results. This data prompted us to investigate the changes in aerobic metabolism in greater detail. Thus, we performed analyses of the global metabolites profile of MCU-overexpressing and MCU-silenced muscles. Regarding the MCU overexpression, no major changes were observed. The only alteration present in MCU-overexpressed muscles was a decrease in glycolysis, maybe due to the effect of the perturbation of mitochondrial Ca\(^{2+}\) homeostasis.

On the contrary, a complete metabolic reprogramming occurred in MCU-silenced muscles, starting with a decrease in the TCA cycle intermediates, in line with the major role of mitochondrial Ca\(^{2+}\) uptake, that is the stimulation of three enzymes of the TCA cycle. Moreover, amino acid metabolism in MCU-silenced muscles was decreased suggesting an overall decrease of the amino acid intermediates use. This observation is in line with the MCU-silenced muscles atrophic phenotype in which amino acids are degraded, as result of an unbalance between protein synthesis and degradation, with a shift toward degradation pathways.

The most striking effect was the global increase of fatty acid \(\beta\)-oxidation, indicating a clear metabolic shift from glucose metabolism toward preferential fatty acid \(\beta\)-oxidation. In detail, in MCU-silenced muscles the lower mitochondria Ca\(^{2+}\) accumulation led to a decrease in the TCA cycle activity, thus the easiest response of mitochondria to this state was the induction of fatty acid \(\beta\)-oxidation. In this way they can continue to sustain the energetic demand of the cell. Although steady-state metabolomics gave interesting results, to understand in detail the role of the single intermediates, metabolic flux analyses of MCU-overexpressing or silenced muscles will be performed.
We reasoned that these results, together with the mild phenotype of total MCU knockout mouse, could be suggestive of a latent mitochondrial dysfunction which in resting condition is masked by the metabolic reprogramming described above. Thus, we wondered whether mitochondrial defects could become evident in conditions of increased workload and energetic demand, like in running training. Thus, using two different running protocols, uphill and downhill exercise training, where the latter is characterized by lengthening contractions, which have been reported to cause extensive mitochondrial damage, we demonstrated that mlc1f-Cre-\textit{mcu}\textsuperscript{-/-} mice run less than their littermate controls. Our data demonstrate that mitochondrial Ca\textsuperscript{2+} uptake is required to guarantee skeletal muscle performance. In the future, we will measure the levels of selected metabolites in the bloodstream of exercised mlc1f-Cre-\textit{mcu}\textsuperscript{-/-} mice, such as fatty acids, lactate, ketone bodies and glucose, to investigate which metabolic intermediates are involved in the reduction of mlc1f-Cre-\textit{mcu}\textsuperscript{-/-} mice running capacity.

Autophagy was shown to contribute to atrophy, a process characterized by the activation of catabolic pathways (Sandri, 2010). However, Ca\textsuperscript{2+} signaling has a controversial role in the control of autophagy (Decuypere et al., 2011). Concerning the role of mitochondrial Ca\textsuperscript{2+} in autophagy, a study performed on DT40 cells lacking InsP3Rs demonstrated that mitochondrial Ca\textsuperscript{2+} uptake in the absence of agonist stimulation is required to inhibit autophagy (Cárdenas et al., 2010). However, in a work in which the mitochondrial Ca\textsuperscript{2+} uptake was directly measured in human fibroblasts, the authors demonstrated that the induction of autophagy flux inversely correlates with the amplitude of mitochondrial Ca\textsuperscript{2+} signals in starving condition (Granatiero et al., 2015). We speculated that a similar scenario could be present in skeletal muscle. To prove this hypothesis, we measured autophagic flux rate in MCU-overexpressing and MCU-silenced muscles during basal or starvation conditions (the latter increases the autophagic process). The autophagic flux evaluates the balance between formation and degradation of autophagosomes by means of colchicine treatment.

We analyzed the lipidation of LC3 and the degradation of P62, two autophagy markers, in all the conditions described above. We demonstrated that mitochondrial Ca\textsuperscript{2+} uptake modulation does not regulate bulk autophagic process in skeletal
muscle. Nonetheless, a deeper analysis of mitophagy, the selective removal of damaged mitochondria, revealed a different scenario. We demonstrated that mitochondrial Ca\(^{2+}\) accumulation controls mitophagy without affecting bulk autophagy. In detail, MCU overexpression triggered an increased mitophagy while in MCU silenced fibres mitophagy was decreased. More efforts are required to understand the clear role of mitochondrial Ca\(^{2+}\) uptake in mitophagy. We further investigate the role of MCU in controlling mitophagy by checking PARKIN translocation and PINK accumulation into mitochondria. Overall, our data indicates that mitochondrial Ca\(^{2+}\) accumulation plays a crucial role in the control of skeletal muscle trophism. We discovered a novel Ca\(^{2+}\)-dependent organelle-to-nucleus signaling route, which links mitochondrial function to the control of skeletal muscle mass. In addition, a clear metabolic reprogramming takes place in MCU downregulated muscles. Further investigations of these two MCU-dependent effects on skeletal muscle homeostasis represent an important task for the future. Indeed, this research will provide new possible targets for clinical intervention in all diseases characterized by muscle loss, such as dystrophies, cancer cachexia and aging.
Materials and methods

Constructs

The following primers have been previously reported:
- pcDNA3.1-MCU-Flag (De Stefani et al., 2011)
- pZac2.1-MCU, pZac2.1-LacZ, pZacf-U6-luc-Zsgreen and pZacf-U6-shMCU-Zsgreen (Mammucari et al., 2015) In the experiments using Peredox and Keima probes ZsGreen cassettes of shMCU-ZsGreen and of shLUC-ZsGreen were eliminated.
- mtGCaMP6f (Chen et al., 2013) (Tosatto et al., 2016)
- R-GECO1 (Zhao et al., 2011)

Mouse Pgc1α-4 alternative promoter was amplified by PCR from mouse tail cDNA. The different fragments of Pgc1α-4 alternative promoter were amplified with the following primers and cloned in PGL4.17 (Promega):
- Alternative promoter region
  Fw: 5’ GCTAGCTTTAATTGACTTCATGGAAACTT 3’
  Rev: 5’ GATATCCCATGTCACCTTCATGCTGGAG 3’
- Region from -421 to -1
  Fw: 5’ GCTAGCTATCAAAACAGCTCTCACCAGC 3’
  Rev: 5’ GATATCCCATGTCACCTTCTCGGAG 3’
- Region from -151 to -1
  Fw: 5’ GCTAGCCATTGAGCAGTGACTCCCAGG 3’
  Rev: 5’ GATATCCCATGTCACCTTCTTGCTGAG 3’
- Region from -75 to -1
  Fw: 5’ GCTAGCCATTGAGCAGTGACTCCCAGG 3’
  Rev: 5’ GATATCAACAGGGGCTTGCCACCAGCT 3’

For shMCU and shLUC, ZsGreen cassettes of shMCU-ZsGreen and of shLUC-ZsGreen were eliminated.
Animals
The mcu fl/fl mouse was purchased from MRC (Medical Research Council). Homozygous mcu fl/fl mice were crossed with transgenic mice expressing the Cre recombinase under the control of the mlc1f promoter (Bothe et al., 2000). All animal experiments were approved and performed in accordance with the Italian law D. L.vo n_26/2014.

In vivo DNA transfection of mouse skeletal muscle

FDB muscles electroporation
Adult C57/BL6 or CD1 male mice were used in all experiments. Firstly, the animal was anesthetized. Hyaluronidase solution (2mg/mL) (Sigma) was injected under the hindlimb footpads. After 30 minutes, 20 μg of plasmid DNA (the total injection volume should be less than 20 μL/foot) was injected with the same procedure of the hyaluronidase. Then, one gold-plated acupuncture needle was placed under the skin at heel, and a second one at the base of the toes. The electrodes should be oriented parallel to each other and perpendicular to the long axis of the foot. The electrodes were connected to the BTX porator (Harvard apparatus). The muscles were electroporated by applying 20 pulses, 20 ms each, 1 s of interval to yield an electric field of 100 V. Sinlge fibres cultures were carried out 7-10 days later.

TA muscles electroporation
Adult male CD1 mice were used in all experiments. Firstly, the animal was anesthetized. Then, the TA muscle was isolated through a small hindlimb incision, and 40 μg of plasmid DNA was injected along the muscle length. Electric pulses were then applied by two stainless steel spatula electrodes placed on each side of the isolated muscle belly (22 Volts, 6 pulses, 200 ms intervals) using a BTX porator (Harvard apparatus). Animals were allowed to recover for 7 days. Next, TA muscles were harvested and frozen in liquid nitrogen-cooled isopentane.
Mouse exercise studies
For acute concentric exercise studies, 3-month old or 6-month old mice were acclimated to and trained on a 10° uphill LE8700 treadmill (Harvard apparatus) for 2 days. On day 1 mice ran for 5 min at 8 m min⁻¹ and on day 2 mice ran for 5 min at 8 m min⁻¹ followed by another 5 min at 10 m min⁻¹. On day 3, mice were subjected to a single bout of running starting at the speed of 10 m min⁻¹. Forty minutes later, the treadmill speed was increased at a rate of 1 m min⁻¹ every 10 min for a total of 30 min, and then increased at the rate of 1 m min⁻¹ every 5 min until mice were exhausted. Exhaustion was defined as the point at which mice spent more than 5 s on the electric shocker without attempting to resume running. Total running time and total running distance were recorded for each mouse.

For eccentric exercise training, 3-month old mice were acclimated to and trained on a 10° downhill for 2 days. On day 1 mice ran for 5 min at 8 m min⁻¹ and on day 2 mice ran for 5 min at 8 m min⁻¹ followed by another 5 min at 10 m min⁻¹. On day 3, 4 and 5 mice were subjected to a single bout of running starting at the speed of 10 m min⁻¹. Forty minutes later, the treadmill speed was increased at a rate of 1 m min⁻¹ every 10 min for a total of 30 min, and then increased at the rate of 1 m min⁻¹ every 5 min until mice were exhausted. Exhaustion was defined as above. Total running time and total running distance were recorded for each mouse.

Luciferase reporter assay
Luciferase reporter assay was performed in TA muscles with Dual-Luciferase Reporter Assay System (Promega) according to manufacturer’s instructions.

AAV production
All the AAV used were provided by Vector Biolabs (www.vectorbiolabs.com).

Autophagy flux analysis
Autophagic flux analyses were performed as previously described (Ju et al., 2010) with few modifications. In brief, EDL muscles of adult CD1 male mice were injected with AAVs. Colchicine was dissolved in water and stored at -20°C as a
stock solution at a concentration of 4 mg/ml. On the day of treatment, colchicine was diluted to 0.1 mg/ml in water and 0.4 mg/kg colchicine was i.p. injected. Control mice received an equal volume of water. 12 hours after the first injection the treatment was repeated once. Mice were sacrificed 24 hours after the first injection. EDL muscles were harvested and frozen in liquid nitrogen-cooled isopentane.

**RNA extraction, reverse transcription, and quantitative realtime PCR**

At least five samples were prepared for each condition. Total RNA was extracted from TA or EDL muscles using the SV Total RNA Isolation Kit (Promega) following the manufacturer’s instructions. The RNA was quantified with Nanodrop (Thermo Fisher Scientific). Complementary DNA was generated from 400 nmol of total RNA with a cDNA synthesis kit SuperScript II (Thermo Fisher Scientific). Oligo(dT)12-18 primers (Thermo Fisher Scientific) were used as primer for first srtand cDNA synthesis with reverse transcriptase. The obtained cDNA was analyzed by real-time PCR using the IQ5 thermocycler and the SYBR green chemistry (Bio-Rad). The primers were designed and analyzed with Primer3 (Rozen and Skaletsky, 2000). Real-time PCR standard curves were constructed using serial dilution of cDNAs of the analyzed samples (at least four dilution points) and the efficiency of all primer sets was between 90 and 110%. The housekeeping gene *Gapdh* was used as an internal control for cDNA normalization. For quantification, expression levels were calculated by the ΔCt method. Real-time PCR primer sequences were as follows:

**Gapdh:**
Fw 5’-CACCATCTTCAGGAGCGAG-3’
Rv 5’-CCTTTCATGCTGCTGGAAC-3’

**Beclin1:**
Fw 5’-GAGGTGCTGCTGTCGCTGTCGTGAGTTG-3’
Rv 5’-TCGTGCTGCTGTCGCTGTCGTGAGTTG-3’

**Lc3:**
Fw 5’-TGAATGGATGACAGTGAGCA-3’
Rv 5’-CACCTGGTTCTCCACACTCTTG-3’

Bnip3:
Fw 5’-TTCCACTAGCACCTTCTGTAGTA-3’
Rv 5’-GAACACCGCATTTACAGAACA-3’

Beclin1:
Fw 5’-CACTGCTCTGTCTTGTGTAAGTTG-3’
Rv 5’-TCGTTGTGCCTTTATTAGGTGCATC-3’

Pgc1a-tot:
Fw 5’-CGCTGCTCTTGAATGGATTG-3’
Rv 5’-CGCAAGCTTTCTCTGAGCTTC-3’

Pgc1a-1:
Fw 5’-GGACATGTGCAGCCAAAGACTCT-3’
Rv 5’-CACTTCAAATCCACCCAGAAAGCT-3’

Pgc1a-4:
Fw 5’-TCACACAAAACCCACAGAAA-3’
Rv 5’-CTGGAAGATATGGGCACAT-3’

Western blotting and Antibodies

To monitor protein levels, frozen muscles were pulverized by means of Qiagen Tissue Lyser and protein extracts were prepared in a buffer containing 50 mM Tris pH 7.5, 150 mM NaCl, 5 mM MgCl2, 1 mM DTT, 10% glycerol, 2% SDS, 1% Triton X-100, Roche Complete Protease Inhibitor Cocktail, 1 mM PMSF, 1 mM NaVO3, 5 mM NaF and 3 mM β-glycerophosphate. 40 μg of total proteins were loaded, according to BCA quantification. Proteins were separated by SDS-PAGE electrophoresis, in commercial 4-12% acrylamide gels (Thermo Fisher Scientific) and transferred onto nitrocellulose membranes (Thermo Fisher Scientific) by wet electrophoretic transfer. Blots were blocked 1 hour at RT with 5% non-fat dry milk (Bio-Rad) in TBS-tween (0.5M Tris, 1.5M NaCl, 0.01% Tween) solution and incubated at 4°C with primary antibodies. Secondary antibodies were incubated 1 hr at RT. The following antibodies were used: anti-phosphoAKT (1:1000, Cell Signaling), anti-ACTIN (1:20000, Santa Cruz), anti-LC3 (1.1000, Cell Signaling), anti-MCU (1:1000, Sigma-Aldrich), anti-P62 (1:5000, Sigma-Aldrich) and anti-
TOM20 (1:20000, Santa Cruz). Secondary HRP-conjugated antibodies were purchased from Bio-Rad and used at 1:5000 dilution.

**Hematoxylin and Eosin (H&E) staining**

20 μm thick cryosections of TA muscles were stained using Rapid Frozen Sections H&E staining Kit (Bio-Optica) according to manufacturer’s instructions.

**Single myofibres culture**

For real-time imaging, FDB fibers were isolated 7-10 days after *in vivo* transfection. Muscles were digested in collagenase A (4 mg/ml) (Roche) dissolved in Tyrode’s salt solution (pH 7.4) (Sigma-Aldrich) containing 10% fetal bovine serum (Thermo Fisher Scientific). Single fibres were isolated, plated on laminin-coated glass coverslips and cultured in DMEM with HEPES (Thermo Fisher Scientific), supplemented with 10% fetal bovine serum, containing penicillin (100 U/ml), streptomycin (100 μg/ml). Fibres were maintained in culture at 37°C with 5% CO₂.

**Real time imaging of mitochondrial and cytosolic Ca²⁺ in FDB fibres**

FDB muscles were electroporated with a plasmid encoding 4mtGCaMP6f together with R-GECO1. After single fibres isolation, real time imaging was performed. During the experiments, myofibers were maintained in Krebs-Ringer modified buffer (135 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 20 mM HEPES, 1 mM MgSO₄, 0.4 mM KH₂PO₄, 1 mM CaCl₂, 5.5 mM glucose, pH 7.4) at RT, in presence of 75 μM N-benzyl-P-toluene sulfonamide (BTS, Sigma-Aldrich) to avoid the fiber contraction. 20 mM caffeine (Sigma-Aldrich) was added when indicated to elicit Ca²⁺ release from intracellular stores. Experiments were performed on a Zeiss Axiovert 200 microscope equipped with a 40×/1.3 N.A. PlanFluor objective. Excitation was performed with a DeltaRAM V high-speed monochromator (Photon Technology International) equipped with a 75 W xenon arc lamp. Images were captured with a high-sensitivity Evolve 512 Delta EMCCD (Photometrics). The system is controlled by MetaMorph 7.5 (Molecular Devices) and was assembled by
Crisel Instruments. 4mtGCaMP6f and R-GECo1 were alternatively excited every second at 490 and 560 nm respectively and images were acquired through a dual band emission filter (520/40 and 630/60) (Chroma). Exposure time was set to 50 ms (4mtGCaMP6f) and 150 ms (R-GECo1). Acquisition was performed at binning 1 with 200 of EM gain. Image analysis was performed with Fiji distribution of the ImageJ software (Schindelin et al., 2012). Images were background subtracted and linear unmixing was performed to get rid of bleed through of the two fluorochromes. Data are expressed as F/F0 where F0 is the mean intensity at the beginning of the experiment.

**Mitophagy measurements in FDB fibres**

To monitor mitophagy, FDB muscles were electroporated with a plasmid encoding 4mtKeima together with MCU or shMCU. After single fibres isolation, real time imaging was performed.

During the experiments, myofibers were maintained in Krebs-Ringer modified buffer (135 mM NaCl, 5 mM KCl, 1 mM MgCl2, 20 mM HEPES, 1 mM MgSO4, 0.4 mM KH2PO4, 1 mM CaCl2, 5.5 mM glucose, pH 7.4). After 24 hours, fibres were imaged using Leica TCS-SP5-II equipped with a 100x, 1.4N.A. plan-apochromat objective. For each fibre green image was acquired by exciting at 479 nm and collecting at 550-600 nm range; red image was acquired by exciting at 570 nm and collecting at 550-600 nm range. Image analysis was performed with Fiji distribution of the ImageJ software (Schindelin et al., 2012). To calculate the mitophagy index background subtracted red images were denoised using the PureDenoise plugin (Luisier et al., 2010), thresholded using the Bersen automatic local threshold algorithm, and objects were automatically counted using the particle analysis routine. The mitophagy index represents the number of counted objects per image.

**Real time imaging of cytosolic NADH/NAD⁺ in FDB fibres**

FDB muscles were electroporated with a plasmid encoding Peredox NLS-mCherry together with MCU or shMCU. After single fibres isolation, real time imaging was performed.
During the experiments, myofibers were maintained in Krebs-Ringer modified buffer (135 mM NaCl, 5 mM KCl, 1 mM MgCl2, 20 mM HEPES, 1 mM MgSO4, 0.4 mM KH2PO4, 1 mM CaCl2, 5.5 mM glucose, pH 7.4) at RT. Experiments were performed on a Zeiss Axiovert 200 microscope equipped with a 40×/1.3 N.A. PlanFluor objective. Excitation was performed with a DeltaRAM V high-speed monochromator (Photon Technology International) equipped with a 75 W xenon arc lamp. Images were captured with a high-sensitivity Evolve 512 Delta EMCCD (Photometrics). The system is controlled by MetaMorph 7.5 (Molecular Devices) and was assembled by Crisel Instruments. Peredox NLS-mCherry was alternatively excited every second at 405 and 560 nm respectively and images were acquired through a dual band emission filter (520/40 and 630/60) (Chroma). Exposure time was set to 50 ms. Acquisition was performed at binning 1 with 200 of EM gain. Image analysis was performed with Fiji distribution of the ImageJ software (Schindelin et al., 2012). Both images were background corrected frame by frame by subtracting mean pixel values of a fibre-free region of interest. Values are expressed as green to red ratio.

**Metabolomics analysis**

Hindlimb muscles of newborn mice were injected with AAV-MCU or AAV-shMCU and AAV-LacZ or AAV-shLUC as control, respectively. TA muscles were harvested eight weeks later and undergone untargeted metabolomics analysis performed by Metabolon, Inc. Briefly, samples were prepared using the automated MicroLab STAR® system from Hamilton Company. The resulting extract was divided into five fractions: two for analysis by two separate reverse phase (RP)/UPLC-MS/MS methods with positive ion mode electrospray ionization (ESI), one for analysis by RP/UPLC-MS/MS with negative ion mode ESI, one for analysis by HILIC/UPLC-MS/MS with negative ion mode ESI, and one sample was reserved for backup.

**Statistical analysis of data**

Statistical data are presented as mean ± SD, unless otherwise specified. Significance was calculated by Student’s t-test.
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Appendices

In this section the following items are present:

- Table 1
- Table 2
- Supplemental information of the manuscript entitled “The mitochondrial calcium uniporter controls skeletal muscle trophism in vivo”.
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<th>Lower Pathway</th>
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**Table 1.** Untargeted metabolomic analysis of MCU overexpressing muscles
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**Table 2: Untargeted metabolomic analysis of MCU silenced muscles**
### Phosphorus Metabolism

**Endogenous Sources:**
- Liver: ATP, ADP, CTP, GTP, UTP
- Muscle: ATP, ADP, CTP, GTP, UTP
- Brain: ATP, ADP, CTP, GTP, UTP
- Kidney: ATP, ADP, CTP, GTP, UTP
- Red Blood Cells: ATP, ADP, CTP, GTP, UTP
- White Blood Cells: ATP, ADP, CTP, GTP, UTP

**De novo Synthesis:**
- ATP: From ADP and inorganic phosphate (Pi)
- ADP: From ATP and inorganic phosphate (Pi)
- CTP: From UTP and inorganic phosphate (Pi)
- GTP: From ATP and inorganic phosphate (Pi)
- UTP: From ATP and inorganic phosphate (Pi)

**Transport:**
- ATP: Exchanged for ADP and inorganic phosphate (Pi) via ATP/ADP translocase
- ADP: Exchanged for ATP and inorganic phosphate (Pi) via ATP/ADP translocase
- CTP: Exchanged for UTP and inorganic phosphate (Pi) via CTP/UTP translocase
- GTP: Exchanged for ATP and inorganic phosphate (Pi) via GTP/ATP translocase
- UTP: Exchanged for ATP and inorganic phosphate (Pi) via UTP/ATP translocase

**Regulation:**
- ATP/ADP ratio: Serves as a metabolic sensor to control ATP production and consumption
- CTP/UTP ratio: Controls the synthesis of purines and pyrimidines
- GTP/ATP ratio: Regulates the synthesis of nucleic acids
- UTP/ATP ratio: Controls the synthesis of nucleic acids

**Excretory Pathway:**
- ATP: Excreted as ammonia and urate
- ADP: Excreted as ammonia and urate
- CTP: Excreted as ammonia and urate
- GTP: Excreted as ammonia and urate
- UTP: Excreted as ammonia and urate

### Calcium Metabolism

**Intestinal Absorption:**
- Calcium is absorbed in the duodenum and jejunum
- Active transport: Carrier proteins transport calcium across the intestinal epithelial cells

**Transport:**
- Calcium ions (Ca^2+): Transported by the calcium-sodium exchanger (Na/Ca exchanger)
- Calcium ions (Ca^2+): Transported by the calcium-ATPase (Ca-ATPase)

**Excretory Pathway:**
- Calcium is excreted in the urine and feces
- Calcium levels are regulated by parathyroid hormone (PTH) and calcitriol (1,25-dihydroxyvitamin D3)

### Oxygen Metabolism

**Right Heart:**
- Oxygenated blood from the lungs enters the right atrium
- Oxygenated blood is pumped to the systemic circulation

**Left Heart:**
- Deoxygenated blood enters the left atrium
- Deoxygenated blood is pumped to the systemic circulation

### Proximate Metabolism

**Proteins:**
- Proteins are broken down into amino acids in the liver
- Amino acids are then transported to other tissues

**Carbohydrates:**
- Carbohydrates are broken down into glucose in the liver
- Glucose is then transported to other tissues

**Fats:**
- Fats are broken down into fatty acids in the liver
- Fatty acids are then transported to other tissues

### Energy Metabolism

**Catabolism:**
- Conversion of macronutrients into energy
- ATP is the primary energy currency

**Anabolism:**
- Conversion of energy into macronutrients
- ATP is used to synthesize macronutrients

### Long Chain Fatty Acid Metabolism

**Fatty Acid Oxidation:**
- Fatty acids are broken down into acetyl-CoA in the mitochondria
- Acetyl-CoA is then used to produce ATP

**Fatty Acid Synthesis:**
- Fatty acids are synthesized from acetyl-CoA in the cytosol
- Fatty acids are then transported to the mitochondria for oxidation

### Polyunsaturated Fatty Acid Metabolism

**Eicosanoid Synthesis:**
- Eicosanoids are synthesized from arachidonic acid
- Eicosanoids play a role in inflammatory processes

### Amino Acid Metabolism (Proline Metabolism)

**Proline Synthesis:**
- Proline is synthesized from glutamic acid
- Proline is then transported to other tissues

**Proline Oxidation:**
- Proline is oxidized to ornithine
- Ornithine is then transported to other tissues

### Other Metabolic Pathways

**Vitamin B12 Metabolism:**
- Vitamin B12 is synthesized from L-methionine
- Vitamin B12 is then transported to other tissues

**Choline Metabolism:**
- Choline is synthesized from serine
- Choline is then transported to other tissues

### Metabolic Pathways

**Glycolysis:**
- Glucose is broken down into pyruvate in the cytosol
- Pyruvate is then transported to the mitochondria for further metabolism

**Krebs Cycle:**
- Pyruvate is converted to acetyl-CoA in the mitochondria
- Acetyl-CoA is then used to produce ATP

**Protein Metabolism:**
- Proteins are broken down into amino acids in the liver
- Amino acids are then transported to other tissues

**Carbohydrate Metabolism:**
- Carbohydrates are broken down into glucose in the liver
- Glucose is then transported to other tissues

**Fat Metabolism:**
- Fats are broken down into fatty acids in the liver
- Fatty acids are then transported to other tissues

### Hormonal and Neuroendocrine Metabolism

**Hormonal Pathways:**
- Hormones are synthesized from precursors in the endocrine system
- Hormones are then transported to target tissues

**Neuroendocrine Pathways:**
- Neuropeptides are synthesized from amino acids in the nervous system
- Neuropeptides are then transported to target tissues

### Nucleic Acid Metabolism

**DNA Synthesis:**
- DNA is synthesized from deoxyribonucleotides
- DNA is then transported to other tissues

**RNA Synthesis:**
- RNA is synthesized from ribonucleotides
- RNA is then transported to other tissues

**Protein Synthesis:**
- Proteins are synthesized from amino acids
- Proteins are then transported to other tissues

### Drug Metabolism

**Phase I Metabolism:**
- Drugs are converted into more polar forms in the liver
- Polar forms are then transported to other tissues

**Phase II Metabolism:**
- Drugs are conjugated with endogenous molecules in the liver
- Conjugated forms are then transported to other tissues

### Genetic and Epigenetic Metabolism

**DNA Replication:**
- DNA is replicated from the parental DNA strands
- Replicated DNA is then transported to other tissues

**DNA Repair:**
- DNA is repaired from damaged DNA strands
- Repaired DNA is then transported to other tissues

**Epigenetic Modification:**
- Epigenetic modifications are added to DNA strands
- Modified DNA is then transported to other tissues

### Cell Metabolism

**Cellular Respiration:**
- Cellular respiration is the process by which cells generate ATP
- ATP is then transported to other tissues

**Cellular Excretion:**
- Cells excrete waste products into the extracellular space
- Waste products are then transported to other tissues

**Cellular Communication:**
- Cells communicate with each other through chemical signals
- Chemical signals are then transported to other tissues
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Supplemental Information

The Mitochondrial Calcium Uniporter controls skeletal muscle trophism in vivo

Cristina Mammucari, Gaia Gherardi, Ilaria Zamparo, Anna Raffaello, Simona Boncompagni, Francesco Chemello, Stefano Cagnin, Alessandra Braga, Sofia Zanin, Giorgia Pallafacchina, Lorena Zentilin, Marco Sandri, Diego De Stefani, Feliciano Protasi, Gerolamo Lanfranchi, Rosario Rizzuto
Figure S1, Related to Figure 1

A. 65 mM KCl

B. [Ca^{2+}]_{cyt} (Fura-2 ratio, ex 340/380, R/R_o)

C. [Ca^{2+}]_{mt} (mtGCaMP6m ratio, ex 490/400, R/R_o)

D. [Ca^{2+}]_{cyt} (Fura-2 ratio, ex 340/380, R/R_o)

E. TMRM fluorescence (arbitrary units)

F. TMRM fluorescence (arbitrary units)
Figure S2, Related to Figure 2
Figure S3, Related to Figure 3
Figure S4, Related to Figure 3
Figure S5, Related to Figure 4
Figure S6, Related to Figure 4
Figure S7, Related to Figure 5
Supplemental Figure Legends

FIGURE S1. MCU is sufficient and required for mitochondrial Ca\(^{2+}\) uptake in skeletal muscle ex vivo upon K\(^{-}\)-induced depolarization and does not affect myofiber Δψ, Related to Figure 1

A-D Flexor digitorum brevis (FDB) muscles were transfected with pmCherry-N1 or MCU-Cherry (A and B) or shluc-Cherry or shMCU-Cherry (C and D). In A and C mtGCaMP6 was also transfected. Seven days later single myofibers were isolated and cultured. A Left: representative traces of mitochondrial Ca\(^{2+}\) dynamics in a pmCherry-N1 (control, black trace) or MCU-Cherry (red trace) expressing fiber upon K\(^{-}\)-induced depolarization. Right: mean mitochondrial [Ca\(^{2+}\)] increase. n=48. B Left: representative traces of cytosolic Ca\(^{2+}\) dynamics in a control (black trace) or MCU-Cherry (red trace) expressing fiber upon K\(^{-}\)-induced depolarization. Right: mean cytosolic [Ca\(^{2+}\)] increase. n=30. C Left: representative traces of mitochondrial Ca\(^{2+}\) dynamics in an shluc-Cherry (control, black trace) or shMCU-Cherry (shMCU, red trace) expressing fiber upon K\(^{-}\)-induced depolarization. Right: mean mitochondrial [Ca\(^{2+}\)] increase. n=30. D Left: representative traces of cytosolic Ca\(^{2+}\) dynamics in a control (black trace) or shMCU (red trace) expressing fiber upon K\(^{-}\)-induced depolarization. Right: mean cytosolic [Ca\(^{2+}\)] increase. n=25. In each panel, data are represented as mean ± SEM. *p<0.05, ***p<0.001, t test (two-tailed, unpaired).

E-F Single fibers were isolated from FDB muscles transfected in vivo with MCU-GFP (MCU) (E) or shMCU-ZsGreen (shMCU) (F). pcDNA3.1 and shluc-ZsGreen were used as controls, respectively. Fibers were loaded with TMRM and Δψ was measured. n=18. Data are represented as mean ± SEM. t test (two-tailed, unpaired).

FIGURE S2. MCU triggers hypertrophy early during post-natal growth, Related to Figure 2

A Experimental scheme. AAVs were injected in hind limb muscles of newborn mice and 4 weeks later analyses were performed. B Western blotting analysis showing AAV-MCU expression. C Fiber size analysis. More than 400 fibers were measured for each muscle; data are represented as mean ± SEM. **p<0.01, t test (two-tailed, paired) of 3 muscles per group.
FIGURE S3. Analysis of mitochondrial function, Related to Figure 3

A-B Left: immunoblotting of AAV-MCU and AAV-shMCU infected muscles. Right: quantification by densitometry. Data are represented as mean ± SEM. *p<0.05, t test (two-tailed, paired) of 4 muscles per group.

C Assays of mitochondrial function of AAV-MCU infected TA muscles. Glycogen amount was detected by PAS staining, while mitochondrial function was detected by SDH, COX IV, and NADH-TR activity assays. D PAS and SDH activity assays of AAV-MCU infected EDL and soleus muscles. E Assays of AAV-shMCU infected TA muscles.

Figure S4. Autophagy protein expression and satellite cells number during post-natal growth, Related to Figure 3

A Left: immunoblotting of AAV-MCU infected muscles. Right: quantification by densitometry. Data are represented as mean ± SEM. *p<0.05, t test (two-tailed, paired) of 3 muscles per group. B-C Pax7 positive nuclei were counted in myofibers isolated from AAV-MCU and AAV-shMCU infected muscles. n=3. More than 70 fibers per muscle were analyzed. Data are represented as mean ± SEM. ***p<0.001, t test (two-tailed, paired).

FIGURE S5. Satellite cells number in adulthood, Related to Figure 4

A-B Pax7 positive nuclei were counted in myofibers isolated from AAV-MCU and AAV-shMCU infected muscles. n=3. More than 60 fibers per muscle were analyzed. Data are represented as mean ± SEM. t test (two-tailed, paired).

FIGURE S6. Heat map of genes induced by AAV-MCU and suppressed by AAV-shMCU (14 days), Related to Figure 4

Selected genes are those enriched for the cytoskeleton, myofibrils, sarcomere organization and calcium ion homeostasis functions. Gene expression values are relative to the average expression in control condition (7 days).
FIGURE S7. Analysis of denervated muscles, Related to Figure 5

A Δψ is unaffected by denervation and MCU. FDB muscles were transfected in vivo with MCU-GFP (MCU) and denervation was achieved by sciatic nerve section. 7 days later isolated fibers were loaded with TMRM and Δψ was measured. n=15. Data are represented as mean ± SEM. t test (two-tailed, unpaired).

B Mitochondrial Ca\(^{2+}\) uptake of denervated muscles transfected with pmCherry-N1 or MCU-Cherry (MCU). Left: representative traces of mitochondrial Ca\(^{2+}\) dynamics. Right: mean mitochondrial [Ca\(^{2+}\)] increase upon K\(^+\) treatment. n=25. C Cytosolic Ca\(^{2+}\) transients. Left: representative traces of cytosolic Ca\(^{2+}\) dynamics. Right: mean cytosolic [Ca\(^{2+}\)] increase upon K\(^+\) treatment. n=20. In each panel, data are represented as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, t test (two-tailed, unpaired).
Supplemental Table Titles and Legends

**TABLE S1. Functional classification of genes activated 14 days after AAV-MCU infection, Related to Figure 4**

Highly related genes were clustered into functionally related groups ranked according the EASE score. EASE score is the upper bound of the distribution of Jackknife Fisher exact probabilities given the List Hits, List Total, Population Hits and Population Total. For each Annotation Cluster the followings are indicated: gene ontology categories (Category); description of the categories (Term); the number of genes uploaded for that specific category (Count); the percentage with respect to the total number of genes in the category (%); the p-value for the significance of the particular category (Pvalue); the list of genes, specified in entrez gene, uploaded and belonging to the category (Genes); the number of genes in the gene list (List Total); the number of genes in the total group of genes assayed that belong to the specific Gene Category (Pop Hits); the number of genes in the total group of genes assayed that belong to any Gene Category within the System (Pop Total); and different statistic correction indicated as Fold Enrichment, Bonferroni, Benjamini and FDR. Descriptions for each column are extracted from DAVID manual.

**TABLE S2. GSEA results, Related to Figure 4**

z-test on genes and pathways are called NTk and NEk statistics. Good candidate pathways were considered those significant either for NTk or NEk. For each pathway both analyzed genes and other genes in the pathway are indicated.

**TABLE S3. Functional classification of most activated genes in response to MCU silencing, Related to Figure 4**

Highly related genes were clustered into functionally related groups ranked according the EASE score. For a detailed description see Table S1 legend.
Supplemental Experimental Procedures

Single myofiber culture

For real-time imaging, FDB fibers were isolated 7-14 days after in vivo transfection. Muscles were digested in type I collagenase (6 mg/ml), single fibers were isolated, plated on laminin-coated coverslips and cultured in DMEM containing 10% fetal bovine serum, 50 U/ml penicillin, 50 mg/ml streptomycin. For satellite cell analyses, EDL muscle fibers were similarly isolated in 2 mg/ml type I collagenase.

Real-time imaging

Experiments were performed on Olympus IX 71 inverted microscope equipped with a PlanApo 60x/1.4 N.A. oil immersion objective, a xenon light source (75W) for epifluorescence illumination and a 12-bit digital cooled CCD camera (Micromax, Princeton Instruments, New Jersey Trenton, NJ). Data were acquired using Cell R Software (Olympus corporation, Tokio, Japan). During the experiments, myofibers were maintained in Krebs-Ringer modified buffer (135 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 20 mM HEPES, 1 mM MgSO₄, 0.4 mM KH₂PO₄, 1 mM CaCl₂, 5.5 mM glucose, pH 7.4) at RT, in presence of 75 µM N-benzyl-P-toluenesulfonamide (BTS, Sigma-Aldrich) to avoid the fiber contraction. 10 mM caffeine (Sigma-Aldrich) was added when indicated to elicit Ca²⁺ release from intracellular stores. Alternatively, extracellular [K⁺] was increased to 65 mM in an isotonic medium to elicit membrane depolarization.

Mitochondrial Ca²⁺ measurements. Mitochondrial Ca²⁺ measurements were performed as previously reported (Logan et al., 2014) with slight modifications. Briefly, mitochondrial-targeted GCaMP6m was alternatively excited through a 490/20 and a 403/20 nm band-pass excitation filters and images were collected through a 460/15-525/25-630/15 nm triple-band emission filter (Olympus). For experiments with caffeine, exposure time was set to 50 ms, the lamp was set to the 6.85% of the maximum power and images were acquired every 3 s. For experiments with KCl, exposure time was set to 20 ms, the lamp was set to the 33.76% of the maximum
power and images were acquired every 250 ms. Changes in Ca\(^{2+}\) levels (490/403 nm fluorescence ratio) were expressed as \(R/R_0\), where \(R\) is the ratio at time \(t\) and \(R_0\) is the ratio at the beginning of the experiment. Analysis was performed with the Fiji distribution of ImageJ (Schindelin et al., 2012). Images were background corrected frame by frame by subtracting the mean pixel value of a cell-free region of interest. Images were then unmixed by subtracting the contribution of the mCherry moiety due to the bleedthrough through the multi-pass emission filter. The unmixing procedure was validated by comparing the Ca\(^{2+}\) responses of fibers expressing the mtGCaMP6m probe alone or in combination with mCherry after the unmixing.

**Citosolic Ca\(^{2+}\) measurements.** Fibers were dissected and loaded with 2 µM fura-2/AM (Life Technologies) diluted in Krebs-Ringer modified buffer containing 0.02% pluronic acid for 20 min at 37°C and then washed with Krebs-Ringer modified buffer. In the experiments with MCU-Cherry, images were acquired every 3 s (every 1 s in KCl experiments) with a UApo/340 40x/1.35 N.A. oil immersion objective (Olympus) using 380/15 nm and 340/15 nm band-pass excitation filters and collected through a 460/15-525/25-630/15 nm triple-band filter (Olympus). Exposure time was set to 200 ms and the lamp was set to the 26.17% of the maximum power. Experiments with shMCU-Cherry were performed on a Zeiss Axiovert 200 microscope equipped with a Fluor 40x/1.3 N.A. oil immersion objective (Zeiss). Excitation was performed with a DeltaRAM V highspeed monochromator (Photon Technology International) equipped with a 75 W xenon arc lamp. Images were captured every 3 s (every 1 s in KCl experiments) with a high-sensitivity 16-bit Evolve 512 Delta EMCCD (Photometrics). The system is controlled by MetaMorph 7.5 (Molecular Devices) and was assembled by Crisel Instruments. Images were collected by alternatively exciting the fluorophore at 340 and 380 nm and fluorescence emission recorded through a 515/30 nm band-pass filter (Semrock). Exposure time was set to 50 ms. Changes in fluorescence (340/380 nm ratio) was expressed as \(R/R_0\), where \(R\) is the ratio at time \(t\) and \(R_0\) is the ratio at the beginning of the experiment.

**Mitochondrial Δψ measurements.** Mitochondrial Δψ was measured by loading fibers with 20 nM tetramethyl rhodamine methyl ester (TMRM, Life Technologies) for 40 min at 37 °C. TMRM excitation was performed with a 545/15 nm band-pass filter and fluorescence emission was collected through a 572/5 nm band-pass filter.
Images were taken every 6 s with a fixed 30 ms exposure time and the lamp was set to 6.85% of the maximum power. 20 μM carbonyl cyanide m-chlorophenylhydrazone (CCCP) (Sigma-Aldrich) was added after 10 acquisitions to completely collapse Δψ. The mitochondrial Δψ was evaluated as raw fluorescence intensity of background-corrected images by subtracting the fluorescence intensity mean value after CCCP to the average value of the first 10 acquisitions (ΔF).

**Immunofluorescence**

*Single fibers.* FDB fibers transfected with 2mtGCaMP6m and MCU-mCherry were seeded on laminin-coated coverslips. After 4 hours, fibers were washed with PBS, fixed in 4% formaldehyde for 10 min and quenched with 50 mM NH₄Cl in PBS. Fibers were permeabilized for 10 min with 0.1% Triton X-100 in PBS and blocked in PBS containing 5% goat serum for 1 h. Fibers were then incubated with α-mCherry (Abcam) and α-TOM20 (Santa Cruz) primary antibodies for 4 h at RT and washed 3 times in PBS. Alexa Fluor 405 and Alexa Fluor 647 conjugated secondary antibodies (Life Technologies) were used respectively and coverslips were mounted with ProLong Gold Antifade reagent (Life Technologies). Images were taken on a Leica TCS-SP5-II equipped with a 40x, 1.25N.A. Plan-apochromat objective. Alexa Fluor 405 and mCherry were excited simultaneously by 405 nm and 543 nm laser lines and images were collected in the 413-500 nm and 580-690 nm ranges. 2mtGCaMP6m and Alexa Fluor 647 were then excited simultaneously by 488 nm and 633 nm laser lines and images were collected in the 496-550 nm and 640-720 nm ranges. Pinhole was set to 1.5 airy units and pixel size was set to 150 nm. Images were pseudo-colored in ImageJ.

*Muscle cryosections.* Muscle cryosections were fixed in 4% formaldehyde for 20 min, quenched with 50 mM NH₄Cl in PBS and blocked in PBS containing 10% goat serum and 0.5% BSA for 20 min. Sections were then incubated with α-Flag (Cell Signaling Technology) and α-TOM20 (Santa Cruz) primary antibodies for 1 h at 37°C and washed 3 times in PBS. Alexa Fluor 555 and Alexa Fluor 488 conjugated secondary antibodies (Life Technologies) were used respectively. Wheat Germ Agglutinin (WGA) Alexa Fluor 350-conjugated was used to label the sarcolemma.
**Satellite cell analyses**

Single fibers, isolated as reported above, were stained with anti-Pax7 antibody (SantaCruz). Alexa Fluor 488 or 555-conjugated secondary antibodies (Life Technologies) were used. Nuclei were stained with Hoechst 33342 (Sigma Aldrich). Pax7-positive nuclei were counted.

**Immunoblotting**

Frozen muscles were pulverized by means of Qiagen Tissue Lyser and protein extracts were prepared in a buffer containing 50 mM Tris pH 7.5, 150 mM NaCl, 5 mM MgCl$_2$, 1 mM DTT, 10% glycerol, 2% SDS, 1% Triton X-100, Roche Complete Protease Inhibitor Cocktail, 1 mM PMSF, 1 mM NaVO$_3$, 5 mM NaF and 3 mM β-glycerophosphate. The following antibodies were used: 4E-BP1, p-4E-BP1 Thr37/46, AKT, Gsk3α/β, p-Gsk3β Ser9, p-Gsk3α/β Ser21/9, PDH (Cell Signaling Technology); MCU, Flag (Sigma-Aldrich); p-AKT Ser473, p-PDH Ser293, Gsk3β (Abcam); Actin, Grp75, TOM20, Tubulin (Santa Cruz Biotechnology).

**Mitochondria fractionation.** Crude mitochondrial fractions were prepared as previously described (Frezza et al., 2007). Briefly, TA muscles (approx. 200 mg) were minced and homogenized with a motor-driven pestle (20 strokes) in an isotonic buffer (250 mM sucrose, 10 mM KCl, 30 mM HEPES, 1 mM EDTA, pH 7.4). Total homogenate was centrifuged at 800×g for 5 min to remove nuclei and entire cells. The supernatant was then centrifuged at 9000×g for 10 min to pellet the crude mitochondrial fraction. The second supernatant was further centrifuged at 100000×g for 1 h to remove light membranes and clear the cytosolic fraction. Proteins were extracted in RIPA buffer (150 mM NaCl, 25 mM TRIS, 1% Triton-X100, 0.5% Na-deoxycholate and 0.1% SDS, pH 8.0) and quantified using the BCA Protein Assay Kit (Pierce) following the manufacturer instructions. 20 µg of protein were separated by SDS-PAGE, transferred onto nitrocellulose membranes (GE Healthcare) and probed using the indicated antibodies.
Gene Expression Analyses

Muscle RNA was prepared with the Promega SV Total RNA Isolation kit. cDNA was generated with SuperScript II reverse transcriptase (Life Technologies) and qRT-PCR was performed with iQ SYBR Green Supermix (Bio-rad). Oligonucleotide primers for β-actin were already reported (Mammucari et al., 2007). Oligonucleotide primers for total PGC-1α (PGC-1αTOT), PGC1-α1 and PGC1-α4 were already reported (Ruas et al., 2012).

In vivo protein synthesis

In vivo protein synthesis was assessed by means of the non-radioactive SUnSET method as reported (Goodman et al., 2011). Briefly, puromycin (40 nmol/g) was injected to adult mice infected with AAV-MCU and 30 min later muscles were analyzed for puromycin incorporation into nascent peptide chains. Antibody against puromycin were from Millipore (MABE343).

Autophagy flux

Autophagy flux was measured as reported (Ju et al., 2010). Briefly, EDL muscles of adult mice were injected with AAVs. Two weeks later, mice were starved and 0.4 mg/kg colchicine was i.p. injected. 15 h after the first injection treatment was repeated. Mice were sacrificed 30 h after the first injection.

PDH activity

PDH activity was measured in TA muscles with the Pyruvate Dehydrogenase Enzyme Activity Microplate Assay Kit (Abcam) according to manufacturer’s instructions.

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% OsO$_4$ 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. All sections were
examined with a FP 505 Morgagni Series 268D electron microscope (FEI Company, Brno, Czech Republic) at 60kV equipped with a Megaview III digital camera and Soft Imaging System (Munster, Germany).

Mitochondrial frequency and position relative to the sarcomere were determined from 2 to 3 pictures (18-22 fibers, 3 mice for each group) at 8900X of non overlapping regions that were randomly collected from longitudinal sections of internal fiber areas. In each image, the number of mitochondria and the incidence of mitochondrial positioning with respect of the A band was determined. If an individual mitochondrion extended from one band to the other, it was counted in both.

The relative fiber volume occupied by mitochondria was determined using the stereology point and intersections counting method (Loud, 1962; Mobley and Eisenberg, 1975) in transversal sections. One or two micrographs from each fiber (20-25 fibers randomly collected, 3 mice for each group) were taken at a magnification of 8900X from cross sections, excluding nuclei and subsarcolemmal regions. An orthogonal array of dots at a spacing of 0.35 µm was superimposed to each image: the ratio of the numbers of dots falling over a mitochondrion to the total number of dots covering the image gives the relative volume occupied by mitochondria.

The average minimum diameter of mitochondria and the number of severely damaged organelles was measured in the same set of micrographs taken at 18000X of magnification using the Soft Imaging System (Germany). In each fiber 3 to 5 micrographs were randomly collected from longitudinal sections and 7 fibers were analyzed for each EDL muscle. Only mitochondria which were entirely visualized in the micrograph were considered. Mitochondria presenting vacuolization, clear disruption of internal cristae and/or disruption of the outer membrane, myelin figures, were considered damaged.

**RNA purification, RNA labeling and microarray hybridization and data analysis**

AAV injected EDL muscles were collected. Muscles were digested in type I collagenase (10 mg/ml in DMEM). Single myofibers were dissociated. Intact, not contracted single myofibers were picked under stereomicroscope and washed in PBS. For AAV-shluc and AAV-shMCU infected muscles, ZsGreen-expressing
myofibers were selected using an inverted microscope (DMI4000, Leica). Each single isolated myofiber was lysed in 250 µl of TRizol Reagent (Life Technologies) and RNA was extracted in the aqueous phase following the manufacturer’s instructions. To purify RNA, spin-columns of the RNeasy Micro Kit (Qiagen) were used. Before performing microarray experiments about 1/5 of the purified RNA was used for qRT-PCR measurements of MCU expression levels (data not shown). RNA was retrotranscribed using the SuperScript III Reverse Transcriptase (Life Technologies) according to the manufacturer’s specifications. Gene-specific primers for exogenous MCU (AAV-MCU), endogenous MCU, and ZsGreen were selected with Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and the specificity of each primer set was monitored by dissociation curve analysis. Tioredoxin 1 was chosen as reference gene (Chemello et al., 2011).

Selected primers were the following:

- MCU_endo_FOR AAAGGAGCCAAAAGTCACG;
- MCU_endo_REV AACGGCGTGAGTTACAACA;
- MCU_eso_FOR AATTGCTCAGGCAGAAATGGA;
- MCU_eso_REV CTTATCGTCGTCATCCTTGTAATC;
- ZsGREEN_FOR GACCAAGGAGATGACCATGAA;
- ZsGREEN_REV CTTGAAGGGGTAGCCGATG;
- Txn1_FOR TCCAATGTGGTGTTCCTTGA;
- Txn1_REV GGCTTCAAGCTTTTCCTTGTT.

qRT-PCR experiments were performed in a 7500 Real-Time PCR System (Life Technologies) using the SYBR Green technology of GoTaq qPCR Master Mix (Promega).

The remaining purified RNA of selected fibers was exponentially amplified using the TransPlex WholeTranscriptome Amplification 2 Kit (Sigma-Aldrich) according to (Chemello et al., 2011) to obtain a sufficient amount of cDNA for microarray experiments. Briefly, RNA was reverse transcribed in a cDNA library, and then library was exponentially amplified for 18 cycles, few cycles below the amplification “plateau” observed in a PCR test reaction. To remove the residual primers and nucleotides, the amplification product was purified with the GenElute PCR Clean-up columns (Sigma-Aldrich). Resulting cDNA was quantified with Nanodrop ND-1000 spectrophotometer (Thermo Scientific). 2 µg of amplified-purified cDNA were directly labeled using the Genomic DNA Enzymatic Labeling Kit (Agilent Technologies). The kit uses random primers and the exo-Klenow fragment to directly label cDNA samples with Cy3-dUTP nucleotides. Labeled cDNA was
purified using the Amicon 30kDa filters (Millipore) and quantified using NanoDrop ND-1000 spectrophotometer (Thermo Scientific). On average, cDNA yield was about 4 µg and the specific activity of 30 pmol Cy3 per µg of cDNA.

Microarray experiments were performed using SurePrint G3 Mouse Gene Expression 8x60K microarrays (AgilentTechnologies) (GEO platform: GPL13912). 800 ng of labeled cDNA target were mixed with 5 µl of 10X Blocking Agent (AgilentTechnologies) and water to a final volume of 25 µl. Samples were denaturated at 95°C for 2 min and added to 25 µl of 2X GEx Hybridization Buffer HI-RPM (AgilentTechnologies). 40 µl mix was dispensed onto the array. Slides were loaded into the Agilent SureHyb chambers and hybridization was performed in a hybridization oven at 65°C for 17 h with 10 rpm rotation. After hybridization, slides were washed using Wash Buffer Kit (Agilent Technologies) and dried at RT.

Microarray slides were scanned using G2505C scanner (Agilent Technologies) at 3 µm resolution. Probes features were extracted using the Feature Extraction Software v. 10.7.3.1 with GE_1_Sep09 protocol (Agilent Technologies). Intra-array normalizations were directly performed by the Feature Extraction Software. Raw data are available in the GEO database (accession number GSE GSE60931). Inter-array normalization of expression levels was performed with quantile method (Bolstad et al., 2003) and the values for within-arrays replicate spots were then averaged. Feature Extraction Software, which provided spot quality measures, was used to evaluate the quality and reliability of the hybridization. In particular, the flag “glsFound” (set to 1 if the spot had an intensity value significantly different from the local background and to 0 when otherwise) was used to filter out unreliable probes: the flag equal to 0 was to be noted as “not available (NA).” Probes with a high proportion of NA values were removed from the dataset in order to carry out a more solid and unbiased statistical analyses. 45% of NA was used as the threshold in the filtering process, and a total of 30,073 of 39,570 probes were obtained. To identify differentially expressed probes in at least one condition one way ANOVA analysis was performed using a threshold p-value ≤ 0.01. Significant differentially expressed probes were used to search specific expression clusters according to Self Organizing Tree Algorithm (SOTA) (Herrero et al., 2001)
as implemented in MultiExperiment Viewer version 4.8.1 (tMev) of the TM4 Microarray Software Suite (Saeed et al., 2006). Gene ontology analysis was performed using DAVID web tool (Huang da et al., 2009), while pathway analysis was performed applying Gene Set Enrichment Analysis (GSEA) as implemented in Graphite web tool (Sales et al., 2013). KEGG pathway database was used and only pathways that presented at least 10 mapped genes in common with our gene expression matrix were considered in the analysis.
Supplemental References


Data in Brief

Gene expression changes of single skeletal muscle fibers in response to modulation of the mitochondrial calcium uniporter (MCU)

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A B S T R A C T

The mitochondrial calcium uniporter (MCU) gene codifies for the inner mitochondrial membrane (IMM) channel responsible for mitochondrial Ca2+ uptake. Cytosolic Ca2+ transients are involved in sarcomere contraction through cycles of release and storage in the sarcoplasmic reticulum. In addition, cytosolic Ca2+ regulates various signaling cascades that eventually lead to gene expression reprogramming. Mitochondria are strategically placed in close contact with the ER/SR, thus cytosolic Ca2+ transients elicit large increases in the [Ca2+] of the mitochondrial matrix ([Ca2+]mt). Mitochondrial Ca2+ uptake regulates energy production and cell survival. In addition, we recently showed that MCU-dependent mitochondrial Ca2+ uptake controls skeletal muscle trophy. In the same report, we dissected the effects of MCU-dependent mitochondrial Ca2+ uptake on gene expression through microarray gene expression analysis upon modulation of MCU expression by in vivo AAV injection. Analyses were performed on single skeletal muscle fibers at two time points (7 and 14 days post-AAV injection). Raw and normalized data are available on the GEO database (http://www.ncbi.nlm.nih.gov/geo/) (GSE60931).

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1. Direct link to deposited data


2. Experimental design, materials and methods

2.1. Experimental design

To dissect the transcriptional effects of mitochondrial calcium uniporter (MCU) gene modulation in in-vivo single skeletal muscle fiber we performed a time series gene expression analysis after MCU up- or down-regulation [1]. The MCU gene encodes the channel of the inner mitochondrial membrane (IMM) responsible for mitochondrial Ca2+ uptake which controls the aerobic metabolism, cell death, and

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2213-5960/© 2015 Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
Adult CD1 mice [1] were infected with adeno-associated viruses (AAVs) [2] carrying a short hairpin to silence MCU (AAV-shMCU) (group 1), a control short hairpin (AAV-shluc) (group 2), and MCU cDNA to up-regulate its expression (AAV-MCU) (group 3). Group 4 comprises not-infected control mice (wild type). EDL skeletal muscles were collected 7 days and 14 days after injection, respectively [3] and fibers were dissociated [4]. From each isolated myofiber [5] mRNA was extracted [6] and used to quantify MCU expression and classify myofibers [7] according the positive expression of myosin, heavy polypeptide 4 (Myh4) and negative expression of myosin, light polypeptide 3 (Myl3). RNA from slow type 2b myofibers was used for Whole Transcriptome Amplification (WTA) [8] and cDNA produced was labeled and used for microarray hybridization [9]. After microarray scanning data were normalized and analyzed [10].

**Fig. 1.** Experimental flow. Adult CD1 mice [1] were infected with adeno-associated viruses (AAVs) [2] carrying a short hairpin to silence MCU (AAV-shMCU) (group 1), a control short hairpin (AAV-shluc) (group 2), and MCU cDNA to up-regulate its expression (AAV-MCU) (group 3). Group 4 comprises not-infected control mice (wild type). EDL skeletal muscles were collected 7 days and 14 days after injection, respectively [3] and fibers were dissociated [4]. From each isolated myofiber [5] mRNA was extracted [6] and used to quantify MCU expression and classify myofibers [7] according the positive expression of myosin, heavy polypeptide 4 (Myh4) and negative expression of myosin, light polypeptide 3 (Myl3). RNA from slow type 2b myofibers was used for Whole Transcriptome Amplification (WTA) [8] and cDNA produced was labeled and used for microarray hybridization [9]. After microarray scanning data were normalized and analyzed [10].

**Fig. 2.** qRT-PCR and microarray results for MCU gene. The red line represents MCU expression derived from microarray experiments while histograms represent MCU expression derived from qRT-PCR experiments. Correlation between them is 98.8%. The black line represents MCU average expression in wild type mice from microarray experiments and dashed one from qRT-PCR. See caption of Fig. 1 for the description of X axis names.
survival pathways [2–4]. Muscle activity leads to major swings in mitochondrial \( \text{Ca}^{2+} \), which, in turn controls muscle trophism [1].

### 2.2. Materials and methods

Adult 2 month old mice were used for the experiments. Untreated and adeno-associated virus (AAV) injected EDL muscles were collected. Muscles were digested in type I collagenase (10 mg/ml in DMEM). Single myofibers were dissociated. Intact, not contracted single myofibers were selected using an inverted microscope (DMI4000, Leica). Each single isolated myofiber was lysed in 250 μl of TRIzol Reagent (Life Technologies) and RNA was extracted in the aqueous phase following the manufacturer’s instructions. To purify RNA, spin-columns of the RNeasy Micro Kit (Qiagen) were used (Fig. 1). Before performing microarray experiments about 1/5 of the purified RNA was used for qRT-PCR measurements of MCU expression levels (Fig. 2) and for fiber characterization (Fig. 1). RNA was retrotranscribed using the SuperScript III Reverse Transcriptase (Life Technologies) according to the manufacturer’s specifications. Gene-specific primers for exogenous MCU (AAV-MCU), and endogenous MCU were selected with Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and the specificity of each primer set was monitored by dissociation curve analysis. Thiorodoxin 1 was chosen as the reference gene according to the results in [5]. The selected primers are described in Table 1.

Table 1: Primers used for qRT-PCR.

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The qRT-PCR experiments were performed in a 7500 Real-time PCR System (Life Technologies) using the SYBR Green technology of GoTaq qPCR Master Mix (Promega). qRT-PCR confirmed the induction and silencing of MCU in selected fibers for microarray analysis (Fig. 2). Moreover, MCU induction is time dependent evidencing a smaller MCU up-regulation 7 days after AAV injection (~8 fold) than that 14 days after AAV injection (~360 fold).

The remaining purified RNA of the selected fibers was exponentially amplified using the TransPlex Whole Transcriptome Amplification 2 Kit (Sigma-Aldrich) to obtain a sufficient amount of cDNA for the microarray experiments. Briefly, RNA was reverse transcribed in a cDNA library, and then the library was exponentially amplified for 18 cycles, a few cycles below the amplification “plateau” observed in a PCR test reaction. To remove the residual primers and nucleotides, the amplification product was purified with the GenElute PCR Clean-up columns (Sigma-Aldrich). The resulting cDNA was quantified with a Nanodrop ND-1000 spectrophotometer (Thermo Scientific). 2 μg of amplified-purified cDNA was directly labeled using the Genomic DNA Enzymatic Labeling Kit (Agilent Technologies). The kit uses random primers and the exo-Klenow fragment to directly label cDNA samples with Cy3-dUTP nucleotides. Labeled cDNA was purified using Amicon 30 kDa filters (Millipore) and quantified using the NanoDrop ND-1000 spectrophotometer (Thermo Scientific). On average, cDNA yield was about 4 μg and the specific activity of 30 pmol Cy3 per μg of cDNA (Fig. 1).
The microarray experiments were performed using SurePrint G3 Mouse Gene Expression 8 × 60K microarrays (Agilent Technologies) (GEO platform: GPL13912). 800 ng of labeled cDNA target was mixed with 5 µl of 10× Blocking Agent (Agilent Technologies) and water to a final volume of 25 µl. Samples were denatured at 95 °C for 2 min and added to 25 µl of 2× Gex Hybridization Buffer HI-RPM (Agilent Technologies). A 40 µl mix was dispensed onto the array. Slides were loaded into the Agilent SureHyb chambers and hybridization was performed in a hybridization oven at 65 °C for 17 h with a 10 rpm rotation. After hybridization, slides were washed using a Wash Buffer Kit (Agilent Technologies) and dried at RT.

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References

Manuscript Information

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Calcium at the center of cell signaling: interplay between endoplasmic reticulum, mitochondria and lysosomes

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ABSTRACT

In recent years, rapid discoveries have been made relating to Ca\textsuperscript{2+} handling at specific organelles, which have important implications for whole cell Ca\textsuperscript{2+} homeostasis. In particular, the structures of the endoplasmic reticulum (ER) Ca\textsuperscript{2+} channels revealed by electron cryomicroscopy (cryo-EM), the continuous update on the structure, regulation and role of the mitochondrial calcium uniporter (MCU) complex, and the analysis of lysosomal Ca\textsuperscript{2+} signaling are milestones on the route towards a deeper comprehension of the complexity of global Ca\textsuperscript{2+} signaling. In this Review, we summarize recent discoveries on the regulation of inter-organellar Ca\textsuperscript{2+} homeostasis and its role in pathophysiology.

INTRODUCTION

The second messenger Ca\textsuperscript{2+} regulates numerous cellular processes, including, but not limited to, muscle contraction, exocytosis and gene transcription. The endoplasmic reticulum (ER) (sarcoplasmic reticulum, SR, in striated muscles) is the largest Ca\textsuperscript{2+} store in the cell. While the cytosolic Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{cyt}) at rest is about 100 nM, ER [Ca\textsuperscript{2+}] can reach 1mM, depending on the cell type. Upon physiological stimuli, Ca\textsuperscript{2+} is released from the ER/SR, which in turn stimulates Ca\textsuperscript{2+} influx from the plasma membrane. This rapid and sustained [Ca\textsuperscript{2+}]\textsubscript{cyt} increase regulates Ca\textsuperscript{2+}-dependent effector proteins (e.g. calpains, kinases, phosphatases, ion channels) and Ca\textsuperscript{2+}-dependent functions. Eventually, Ca\textsuperscript{2+} is pumped back to the ER and [Ca\textsuperscript{2+}]\textsubscript{cyt} returns to resting values. Specific proteins and channels contribute to the fine-tuned regulation of the whole cycle. In addition, close contacts between the ER and the plasma membrane, and the other intracellular organelles, participate to the control of Ca\textsuperscript{2+} homeostasis. In particular, mitochondria were the first organelles shown to be capable of taking up Ca\textsuperscript{2+}, even before the chemiosmotic theory for mitochondrial calcium accumulation (for a review see [1]). Many roles have been ascribed to mitochondrial calcium accumulation [1]. Indeed, three dehydrogenases of the Krebs cycle have been shown to be modulated by calcium [2], thus controlling overall cellular metabolism. Furthermore, mitochondrial Ca\textsuperscript{2+} accumulation shapes cytosolic calcium dynamics and mitochondrial calcium overload has been associated with the apoptotic process [1].

More recently, lysosomes have been suggested to participate to the regulation of Ca\textsuperscript{2+} homeostasis acting as Ca\textsuperscript{2+} stores, but their contribution to cellular Ca\textsuperscript{2+} signaling is still debated.

In recent years important discoveries have been made relative to different aspects of Ca\textsuperscript{2+} homeostasis and to the interplay between Ca\textsuperscript{2+} stores and cytosolic Ca\textsuperscript{2+} pool. Here we summarize
the main aspects that have emerged, focusing on the mechanisms and regulation of Ca$^{2+}$ signaling in ER, mitochondria and lysosomes.

**ER Ca$^{2+}$ release**

As mentioned above, ER/SR represents the main Ca$^{2+}$ store. Rapid release of Ca$^{2+}$ from this compartment ensures sustained [Ca$^{2+}$]$_{cyt}$ rises required for specific cell functions. Recent work contributed with mechanistic details on inositol 1,4,5-trisphosphate (InsP$_3$)-mediated Ca$^{2+}$ dynamics and on the molecular structure of InsP$_3$ receptor 1 (InsP$_3$R1) and of ryanodine receptor 1 (RyR1).

Activation of phospholipase C triggers the release of InsP$_3$ from phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P$_2$). Upon interaction of InsP$_3$ with its receptors (InsP$_3$Rs), located at the ER membrane, Ca$^{2+}$+ is released to the cytosol (Fig. 1). Ca$^{2+}$+ itself regulates InsP$_3$R open probability ($P_o$), activating InsP$_3$R at increasing [Ca$^{2+}$] up to a specific threshold of [Ca$^{2+}$], while playing an inhibitory role at higher [Ca$^{2+}$]. In addition, oscillatory cytosolic Ca$^{2+}$ rises are characteristic responses to agonist-induced InsP$_3$ release. The frequency of such oscillations depends on stimulus strength and determines the fine control of downstream metabolic responses. The mechanism underlying InsP$_3$-dependent [Ca$^{2+}$]$_{cyt}$ oscillations has been clarified only recently. Indeed, work in the Thomas lab has demonstrated that InsP$_3$ and [Ca$^{2+}$]$_{cyt}$ oscillations are mutually determined, and that InsP$_3$ fluctuations are essential for the generation of [Ca$^{2+}$]$_{cyt}$ spikes and propagation of Ca$^{2+}$ waves, in agreement with the so-called cross-coupling hypotheses [3].

The InsP$_3$R family comprises three isoforms, InsP$_3$R1, InsP$_3$R2 and InsP$_3$R3, which are expressed at different levels in different tissues. Of note, InsP$_3$R1 is mostly abundant in neuronal cells of the central nervous system (CNS) and mutations in this gene are associated with severe CNS disorders, including ataxia. InsP$_3$R2 is expressed in different tissues and it represents the most abundant isoform present in the cardiac muscle. InsP$_3$Rs form heterotetramers, whose activity is not simply the sum of the single isoforms constituting the channel, but rather have unique properties and responsiveness to ATP, Ca$^{2+}$ and InsP$_3$ [4,5].

In striated muscles, Ca$^{2+}$ is released from the SR by the opening of RyRs. As for InsP$_3$Rs, the RyR family includes three isoforms; that is, RyR1-3. All isoforms are abundant in CNS. In addition, RyR1 and RyR2 are highly expressed in striated muscles. In detail, RyR1 is particularly enriched in skeletal muscle, while RyR2 in cardiac muscle. RyR3 is expressed in most tissues.

In skeletal muscle, RyR1 opens upon coupling with the voltage-gated Ca$^{2+}$ channel (Cav1.1), also known as the Ca$^{2+}$-dependent L-type calcium channel dihydropyridine receptor (DHPR), thus ensuring excitation-contraction (EC) coupling. In the myocardium, L-type Ca$^{2+}$
channels ensures entrance of Ca$^{2+}$ which triggers the opening of RyR2 by a Ca$^{2+}$-induced Ca$^{2+}$ release mechanism.

Mutations in RyR1 are associated with malignant hyperthermia susceptibility, central core disease, and minicore myopathy with external ophthalmoplegia [6]. In this respect, the compound AICAR has been proposed for prophylactic treatment of heat-induced sudden death based on his ability to interact with RyR1 and reduce Ca$^{2+}$ leakage independently of activation of its target AMP-activated protein kinase (AMPK) [7]. Mutations in RyR3 gene have been associated to central core disease as well. RyR2 mutations are linked with stress-induced polymorphic ventricular tachycardia and arrhythmogenic right ventricular dysplasia type 2.

Recently, the electron cryomicroscopy (cryo-EM) structures of InsP$_3$R1 and of RyR1 have been described. InsP$_3$Rs and RyRs are both characterized by an analogous tetrameric structure and share similar activation mechanisms. Indeed, besides the specific role of InsP3 on InsP$_3$Rs activation, both InsP$_3$Rs and RyRs are activated by low [Ca$^{2+}$] and inhibited by high [Ca$^{2+}$]. The structure of InsP$_3$R1, solved with a resolution of 4.7Å [8], revealed that, while the ion-conduction pore is similar to homologous tetrameric ion channels (e.g., RyRs), the gating of the channel is ensured by unique C-terminal domains facing the cytosol, which interact with the InsP$_3$-binding domain of neighbouring subunits causing an allosteric rearrangement. These results are in agreement with previous studies based on microsomal membrane preparations expressing recombinant InsP$_3$R isoforms [9]. In parallel to InsP$_3$R, the structure of RyR1 was also solved by cryo-EM, revealing a 6-transmembrane ion channel characterized by an EF-hand domain for Ca$^{2+}$-mediated allosteric gating and a huge cytoplasmic domains on top of each transmembrane domain [10–12]. Comparison between InsP$_3$R and RyR structures revealed important similarities between the two channels. Of note, although the cytoplasmic domain of RyR is much larger than the one of InsP$_3$R, the location of N-terminal regions, required for the formation of functional tetramers, relative to the transmembrane channel regions of the receptors, is very similar. A novel ER Ca$^{2+}$ channel has been identified to be encoded by the TMCO1 gene (Transmembrane and coiled-coil domains 1) [13]. At physiological [Ca$^{2+}$] TMCO1 is present as inactive monomers in the ER membrane. Above a certain [Ca$^{2+}$] threshold, TMCO1 monomers form active tetrameric channels, which extrude Ca$^{2+}$ form the ER matrix, thus preventing ER Ca$^{2+}$ overload. Mutations in the TMCO1 gene are associated to human cerebrofaciothoracic (CFT) dysplasia spectrum, which is mainly characterized by craniofacial dysmorphism, skeletal anomalies, mental retardation and ataxia [13].
**Store-operated Ca\(^{2+}\) entry (SOCE)**

Once Ca\(^{2+}\) has been released from the ER, the enhanced \([\text{Ca}^{2+}]_{\text{c}}\) is sustained by Ca\(^{2+}\) influx from the extracellular milieu through store-operated Ca\(^{2+}\) entry (SOCE). SOCE facilitates Ca\(^{2+}\) refilling into the ER, which is a necessary event to stop Ca\(^{2+}\) entry and to restore resting \([\text{Ca}^{2+}]_{\text{c}}\]. The mechanism of store-operated Ca\(^{2+}\) entry (SOCE) is ensured by the coordinated activity of two families of proteins, STIM and ORAI. STIM1 and STIM2 are single trans-membrane ER proteins that have their C-terminal domains facing the cytosol [14]. The ORAI family comprises cell membrane proteins that form the conductive pore of the Ca\(^{2+}\) release-activated Ca\(^{2+}\) (CRAC) channels and is composed of ORAI1-3, each of which contains 4 transmembrane domains. For ORAI1-3, both N- and C-terminal domains face the cytosol [14]. Upon ER Ca\(^{2+}\) depletion, STIM moves to ER-plasma membrane junctions where it interacts with ORAI, thus inducing Ca\(^{2+}\) influx to the ER (Fig. 2). Both loss- and gain-of-function mutations in STIM and ORAI (resulting in decreased and increased Ca\(^{2+}\) influx, respectively) have been detected in human patients [15]. Loss-of-function mutations result in severe combined immunodeficiency (SCID)-like syndrome, autoimmune diseases, myopathy and ectodermal dysplasia, which results in defects in sweat glands function and teeth development [15]. Gain-of-function mutations cause different syndromes, all associated with myopathy and platelet defects [15].

**ER Ca\(^{2+}\) uptake**

ER Ca\(^{2+}\) uptake exerts a buffering effect on \([\text{Ca}^{2+}]_{\text{c}}\), ensuring the maintenance of resting \([\text{Ca}^{2+}]_{\text{c}}\) in the nanomolar range by the activity of the sarco(endo)plasmic reticulum calcium ATPase (SERCA) (Fig. 1). SERCA activity is increased upon ER/SR Ca\(^{2+}\) store release, allowing rapid re-uptake of cytosolic Ca\(^{2+}\). In addition, it has been proposed that coupling between SOCE, SERCA and InsP3 mediates Ca\(^{2+}\) signaling between spatially distant effectors [16]. According to this model, Ca\(^{2+}\) entering through SOCE is taken up in the ER by SERCA, to be released again by InsP3R to activate distal Ca\(^{2+}\)-activated Cl channels (CaCCs), which contribute to the regulation of cell membrane potential.

SERCA family comprises three isoforms, SERCA1-3, encoded by ATP2A1-3 genes respectively. SERCA1 is exclusively expressed in skeletal muscles, SERCA2 is expressed in skeletal and cardiac muscles, in brain and in other tissues, and SERCA3 is ubiquitously expressed. Moreover, SERCA activity is modulated by transmembrane proteins; specifically, phospholamban in the heart and sarcolipin in skeletal muscle [17]. Mutations in SERCA1 are linked to Brody disease, characterized by defects in relaxation upon exercise, stiffness and cramps.
Defects in the expression levels and activity of SERCA2a, one of the two SERCA2 isoforms, specifically expressed in slow-twitch myofibers and in cardiac muscle, are apparent in heart failure and phospholamban mutations have been identified in patients with cardiac hypertrophy and decreased ejection fraction [18].

Recently, the role of a peptide named DWORF in enhancing SERCA activity in striated muscles has been reported [19]. Intriguingly, DWORF is encoded by a genomic region that is annotated as a long noncoding RNA. DWORF displaces inhibitory proteins from SERCA, thus increasing muscle performance. So far, DWORF is the only known direct activator of SERCA, and potential therapies for heart disease could possibly arise from positive modulation of DWORF activity.

**ER-mitochondria contact sites**

That mitochondria accumulate high [Ca\(^{2+}\)] upon physiological stimulation has long remained counterintuitive given that, in these conditions, \([\text{Ca}^{2+}]_{\text{c}}\) rises only from 0.1 \(\mu\text{M}\) to about 2-3 \(\mu\text{M}\). This \([\text{Ca}^{2+}]_{\text{c}}\) is too low to allow Ca\(^{2+}\) uptake by the low affinity MCU. However, this model was revised when the existence of high \([\text{Ca}^{2+}]_{\text{c}}\) microdomains (\([\text{Ca}^{2+}]_{\text{c}}\geq 10\mu\text{M}\)) at the site of ER-mitochondria contacts was demonstrated [1]. Both interorganelle distance and contact site size have been proven to be critical parameters for Ca\(^{2+}\) transfer. Purification of a subcellular fraction corresponding to the ER/mitochondria contacts (referred to as mitochondria-associated membranes, MAM) led to the identification of proteins enriched in these membrane domains [20].

Among them, Mitofusin 2 (Mfn2) is located at both ER and mitochondria membranes where it forms homo- and heterotypic interactions, the latter with with Mitofusin 1 (Fig.1). Mitofusin 2 has been reported to strengthen ER-mitochondrial contacts and to facilitate mitochondrial Ca\(^{2+}\) uptake [21,22]. However, this model has been challenged by a quantitative electron microscopy analysis which demonstrated that, in Mfn2-knockout (KO) cells, ER-mitochondrial contacts are increased, rather than decreased [23]. Along these lines, analysis of light microscopy images that takes into account the changes in organelle morphology upon Mfn2 deletion further demonstrated the increase in percentage of the mitochondrial perimeter colocalizing with the ER [24]. The reduction in mitochondrial Ca\(^{2+}\) uptake previously observed in Mfn2 KO cells [21] was suggested to be due to reduced MCU expression levels in these cells [24]. In addition, acute silencing of Mfn2, which did not affect MCU protein levels, triggered an increase in mitochondrial Ca\(^{2+}\) uptake [24]. Thus, the authors concluded that Mfn2 acts as a negative regulator of ER-mitochondrial
tethers which, by reducing the number of contacts, avoids toxic Ca\(^{2+}\) accumulation [24]. Overall, the role of Mfn2 at the ER/mitochondria contacts is still highly debated.

For the sake of brevity, we will not discuss here the many chaperones and other proteins involved in mitochondrial dynamics that participate to the regulation of ER/mitochondria Ca\(^{2+}\) transfer, these have been reviewed elsewhere recently [20].

**MITOCHONDRIA**

The molecular characterization of the uniporter complex began with the discovery of one of its regulatory subunits, mitochondrial Ca\(^{2+}\) uptake 1 (MICU1) [25], soon followed by the identification of CCDC109A, now known as MCU, as the pore-forming subunit of the uniporter complex [26,27] (Fig. 3). In the following five years, we have witnessed an explosion of discoveries on the composition of the MCU complex and, more importantly, on the physiopathological roles of mitochondrial calcium entry. Great advancements in the understanding of the regulation of the channel comes from the description of the structural properties of its components [28–30], although the complete architecture of the MCU complex is far from being solved.

**Molecular components of the Mitochondrial Calcium Uniporter complex**

Recent studies have shown that the Ca\(^{2+}\) permeant pore is composed by three proteins: MCU, MCUb and EMRE [31] (Fig.3).

MCUb (previously known as CCDC109B) is an MCU isoform, conserved in most of the vertebrates and in many plants but absent in other organisms where MCU is present [32]. MCU and MCUb share 50% sequence similarity and each possesses two transmembrane domains separated by a short loop that slightly differs from MCU. More importantly, this isoform has a crucial amino acid substitution in the loop region (E256V) that has an impact on the channelling properties. Indeed, in living cells, overexpression of MCUb reduces the amplitude of [Ca\(^{2+}\)]\(_{\text{mit}}\) transients evoked by agonist stimulation, whereas MCUb silencing elicits the opposite effect, indicating that MCUb acts as a dominant negative subunit that incorporates into the uniporter channel and reduces its activity [32]. Still unclear is the function of this protein in physiopathology, although the ratio of MCU to MCUb expression, which varies greatly between tissues, might contribute to the differences in the amplitude of mitochondrial calcium uptake in different tissues, as recently demonstrated by patch-clamp [33].
More debated is the role of EMRE in the regulation of MCU channel activity [34]. EMRE is a 10 kD protein that is widely expressed in most mammalian tissues and is composed of a single predicted transmembrane domain with a highly acidic carboxyl-terminus [35]. EMRE was shown to play a dual function in the regulation of MCU activity. First, it seems necessary for MCU channel activity since its silencing abrogates Ca\(^{2+}\) entry into mitochondria [35], although purified MCU is sufficient to give rise to calcium currents in a planar lipid bilayer [27]. Second, EMRE seems to also mediate the interaction between MCU and the regulatory subunits MICU1 and MICU2 [35]. Of note, EMRE is not present in plants, fungi and protozoa, where MCU and MICU1 are expressed, suggesting that it represents a metazoan specialization. Coherently, in yeast only the heterologous expression of human MCU--but not *Dictyostelium discoideum* MCU--requires EMRE for proper MCU functioning [36].

One of the peculiar properties of mitochondrial calcium uptake is the sigmoidal response to [Ca\(^{2+}\)]\(_{\text{cyt}}\). Indeed, despite the steep mitochondrial membrane potential, in resting conditions Ca\(^{2+}\) uptake is inhibited to prevent matrix Ca\(^{2+}\) overload and the dissipation of membrane potential that would prevent ATP synthesis. In turn, when cells are stimulated, mitochondria have to respond promptly by exponentially increasing the Ca\(^{2+}\) carrying capacity [37–39]. This property does not reside with MCU itself but rather with its regulators, the calcium-binding EF hand-containing proteins MICU1 and MICU2 [34] (Fig.3). MICU1 silencing was initially proposed to inhibit mitochondrial calcium uptake [25]. However, the Mallilankaraman and Csordás laboratories [40,41] showed that both transient and constitutive loss of MICU1 leads to basal mitochondrial Ca\(^{2+}\) accumulation. This observation was confirmed by the identification of a loss of function mutation in the MICU1 gene in patients affected by brain and muscle disorders [42] that causes a constitutive elevation of resting [Ca\(^{2+}\)]\(_{\text{int}}\). Recently, homozygous deletion of exon 1 of MICU1 in children affected by fatigue and lethargy has been reported to induce a strong reduction of mitochondrial Ca\(^{2+}\) uptake. However, a concomitant decrease in Pyruvate Dehydrogenase (PDH) phosphorylation, which is regulated by the Ca\(^{2+}\)-dependent PDH phosphatase, was detected [43], suggesting that the reduction in Ca\(^{2+}\) uptake of MICU1-/- cells represents a consequence of increase in basal matrix calcium, which would reduce the driving force for Ca\(^{2+}\) accumulation [43].

Csordás and coauthors also proposed that, at high [Ca\(^{2+}\)]\(_{\text{cyt}}\), MICU1 is necessary to ensure maximum Ca\(^{2+}\) uptake [41], thus acting as both gatekeeper and cooperative activator of the channel. The discovery of the MICU1 paralog, MICU2 [44], revolutionized the concept of the mechanism responsible for the sigmoidal response of MCU to extramitochondrial [Ca\(^{2+}\)] [31,34,45]. Importantly, silencing of MICU1 causes the concomitant destabilization also of MICU2 protein
expression [44–46], thus opening the possibility that the effect of MICU1 silencing were in part due to the effect of MICU2 disappearance. Indeed, while it was confirmed that MICU1 cooperatively activates the channel at high [Ca\textsuperscript{2+}], MICU2 was discovered to act as the genuine gatekeeper of the MCU at low [Ca\textsuperscript{2+}] [45]. In this model, MICU1 and MICU2 form an obligate heterodimer through the formation of a disulfide bond [45], which is regulated by the mitochondrial oxidoreductase Mia40 [47]. At resting [Ca\textsuperscript{2+}]\textsubscript{cyt}, the inhibitory role of MICU2 keeps the channel closed, while an increase in [Ca\textsuperscript{2+}]\textsubscript{cyt} causes conformational changes of the MICU1-MICU2 dimer releasing MICU2-dependent inhibition of the channel thus ensuring the cooperative activation of MCU by MICU1 [45]. Thus, the loss of MCU gatekeeping in cells lacking MICU1 [40,41] was likely due to the concomitant loss of MICU2.

Recently, Foskett and coauthors proposed a model in which the conserved acidic residues in the matrix-localized carboxyl tail of EMRE sense matrix [Ca\textsuperscript{2+}] and, together with MICU1 and MICU2, ensure the inhibition of uniporter activity under normal conditions [48]. However, when [Ca\textsuperscript{2+}]\textsubscript{cyt} increases due to cell stimulation, Ca\textsuperscript{2+} can enter mitochondria because, under basal conditions, MCU has a small but detectable probability of being open, thus relieving EMRE-mediated matrix [Ca\textsuperscript{2+}] inhibition of uniporter activity [48]. These data were questioned by two other studies demonstrating an opposite topology of EMRE, with its N- and acidic C-termini projected into the matrix and the inter membrane space, respectively [49,50].

Other proteins have been reported to be part of the MCU complex [34]. One of the most debated is MCUR1, an IMM integral protein that was initially shown to bind to MCU and to be required for mitochondrial calcium uptake, although it was not identified by mass spectrometry of affinity purified MCU complex [35] and yeast MCUR1 ortholog lacks uniporter activity. An indirect mechanism was thus proposed [51] in which loss of MCUR1 induces a decrease in mitochondrial membrane potential through a specific defect in cytochrome c oxidase (COX) assembly, thereby indirectly leading to decreased mitochondrial calcium uptake [51]. However, the Foskett group demonstrated that loss of MCUR1 significantly lowered MCU Ca\textsuperscript{2+} currents [52]. Furthermore, recently the Madesh group confirmed that a lack of MCUR1 in cardiomyocytes and endothelial cells lowers MCU current (I\textsubscript{MCU}) and showed that MCUR1 interacts with MCU and EMRE, but not with MICU1 or MICU2, functioning as a scaffolding factor [53]. Finally, a screening of Drosophila cells identified MCUR1 as one of the proteins required for the regulation of the Ca\textsuperscript{2+} threshold for the permeability transition [54], although the mechanism is not clear yet. Of course, future experiments will be necessary to clarify whether or not MCUR1 is an integral part of the MCU complex and its role in mitochondrial physiology.
Physiological roles of the Mitochondrial Calcium Uniporter complex

Total MCU knockout mice had an unexpected phenotype [55]. These animals are viable and fertile, if maintained in an outbred CD1 strain, although isolated mitochondria from these animals lack calcium uptake [55]. More consistent with expectations, MCU deletion results in embryonic lethality in the C57BL/6 strain [56]. This suggests the existence of a compensatory mechanism that allows mitochondrial Ca\textsuperscript{2+} uptake in CD1 mice. Surprisingly, MCU\textsuperscript{−/−} CD1 mice display no changes in basal metabolism but increased levels of PDH phosphorylation were observed in their skeletal muscle and, coherently with a reduced ATP production in these animals, their skeletal muscles show reduced maximal power output [55]. These data have been confirmed by the acute overexpression and silencing of MCU in skeletal muscles of mice [57]. This study demonstrated that the overexpression of MCU causes hypertrophy, while its silencing causes muscle atrophy and, importantly, the overexpression of MCU blocks denervation-induced atrophy [57]. As for the mechanism, MCU overexpression causes the activation of known hypertrophy pathways, such as IGF1-Akt/PKB and PGC-1α4. Furthermore, global nuclear skeletal muscle gene expression is controlled by mitochondrial calcium, suggesting a precise Ca\textsuperscript{2+}-mediated mitochondria-to-nucleus route to control muscle trophism [57,58].

The analysis of MCU\textsuperscript{−/−} hearts revealed puzzling results [55,59]. As expected, mitochondria in MCU\textsuperscript{−/−} hearts do not exhibit any calcium-induced opening of the mitochondrial permeability transition pore (PTP), the key nodal point in mediating cell death [60]. The PTP, a large-conductance channel located at the IMM, opens in the presence of mitochondrial Ca\textsuperscript{2+} overload and whose opening results in inner membrane potential collapse, halt of mitochondrial ATP synthesis, mitochondrial swelling, rupture, release of cytochrome c and cell death [61]. Surprisingly, in a model of ischemia/reperfusion, where [Ca\textsuperscript{2+}]\textsubscript{cyt}, and hence [Ca\textsuperscript{2+}]\textsubscript{mit}, are elevated, thus leading to PTP opening [62], the levels of cell death were indistinguishable between WT and MCU\textsuperscript{−/−} hearts [55,56], suggesting that alternative death pathways take place in absence of mitochondrial Ca\textsuperscript{2+} overload. Furthermore, several other cardiac features were indistinguishable between WT and MCU\textsuperscript{−/−} animals, including basal ATP levels, left ventricular cardiac output at baseline and following isoproterenol treatment, and hypertrophy and fibrosis upon transverse aortic constriction (TAC) [55,59], processes to which Ca\textsuperscript{2+} was thought to be tightly linked [62].

Many other models have been created to clarify the role of mitochondrial calcium uptake in the heart [63]. The analysis of a transgenic mouse overexpressing a dominant-negative form of MCU in the heart (DN-MCU) confirmed the analysis of the MCU\textsuperscript{−/−} mouse on basal properties [64].
In addition, DN-MCU hearts present impaired energetic responsiveness to acute sympathetic stress, namely fight-or-flight response, by lowering ATP below a critical threshold [65].

To circumvent the possible compensatory mechanisms of constitutive loss of MCU during development, inducible cardiac-specific MCU knockout mice were generated [66,67]. Similarly to what observed for MCU+/− and DN-MCU models, acute deletion of MCU in adult cardiomyocytes did not alter heart morphology, ultrastructure, basal cardiac functions or TAC outcomes [66,67], while defects in the fight-or-flight response were observed [66,67]. However, contrary to constitutive knockout models, acute loss of mitochondrial calcium uptake protects from cell death induced by ischemia–reperfusion injury [66,67], although disparities in methodology might account for the different results observed [55,66,67].

The role of MCU in cell death and cancer progression is also debated [63]. Consistent with the well-known role of mitochondrial Ca2+ overload in triggering PTP opening [60], knockout MCU mice show no PTP opening upon addition of extramitochondrial calcium [55]. Surprisingly, mouse embryonic fibroblast cells derived from these mice do not differ in the magnitude of cell death upon treatment with apoptotic stimuli [55]. In contrast with these data, cells either overexpressing MCU or silenced for MICU1 show increased susceptibility to cell death [27,40]. In addition, a reduction in MCU protein levels through increased expression of miR-25, which specifically targets MCU, confers resistance of colon cancer and cancer derived cells to apoptotic stimuli [68]. However, in breast cancer patients, MCU overexpression correlates to poor prognosis (Hall et al, 2014). Furthermore, in the highly aggressive triple negative breast carcinoma cell line MDA-MB-231, caspase-independent cell death was potentiated by MCU silencing [69]. It was recently shown that MCU expression correlates with patients’ breast tumor size and lymph node infiltration. In addition, deletion of MCU greatly reduces in vivo tumor growth and metastasis formation via suppression of ROS production and inhibition of HIF-1α signaling [70]. A study of the mechanism underlying the negative effect of MCU down-regulation on breast cancer cell migration, suggested that MCU controls SOCE-dependent cell migration [71]. However, detailed analysis in three different breast cancer cell lines demonstrated that MCU silencing causes similar reduction in cell motility independently of the cell line tested, while it exerts different effects on cytosolic [Ca2+] and SOCE depending on the cell line [70]. These data indicate that the reduction in cell migration triggered by MCU silencing is due to specific inhibition of mitCa2+ uptake.

Differently from what observed from the MCU knockout animals, constitutive ablation of MICU1 in mice leads to lethality within hours of birth [72], probably due to decreased numbers of neurons in the nucleus ambiguous, thus leading to defective regulation of basic functions
postnatally. Analysis of mitochondrial Ca\(^{2+}\) homeostasis in MEF cells derived from these animals revealed enhanced mitochondrial Ca\(^{2+}\) uptake at resting [Ca\(^{2+}\)]\(_{cyt}\) or during prolonged [Ca\(^{2+}\)]\(_{cyt}\) elevations [72], in accord with data in cells [40,41,45] and in patients harboring MICU1 loss-of-function mutations [42]. Liver specific MICU1 knockout model highlighted no gross functional, morphological and/or histological differences compared to wild type animals but MICU1\(^{-/-}\) livers failed to regenerate after partial hepatectomy due to PTP opening caused by sustained mitochondrial Ca\(^{2+}\) overload [72], since treatment of MICU1\(^{-/-}\) mice with NIM811, an inhibitor of PTP, enhanced hepatocyte proliferation.

Recently, Finkel and collaborators generated CRISPR-mediated MICU1 knockout mice that result in high, but not complete perinatal death [73]. Furthermore, analysis of the surviving animals revealed marked abnormalities comparable to what observed in human patients harboring MICU1 loss-of-function mutations [42] with were partially ameliorated with time and the deletion of one allele of EMRE.

Overall, the analysis of different animal models in which mitochondrial Ca\(^{2+}\) homeostasis is perturbed just started to clarify the importance of mitochondrial Ca\(^{2+}\) uptake. The analysis of skeletal muscle, liver and heart suggests that mitochondrial Ca\(^{2+}\) uptake plays a prominent role in stress rather than in physiological conditions and the fact that total MCU\(^{-/-}\) animals are viable and present no gross phenotype remains a mystery that needs further investigation.

**LYSOSOMES**

The lysosomal Ca\(^{2+}\) pathway is a highly debated field. Here we summarize the main unknowns and hypotheses.

In the 1990s nicotinic acid adenine dinucleotide phosphate (NAADP), a contaminant in commercial stock of NADP, was discovered as the most potent mobilizer of Ca\(^{2+}\) from stores physically separated from those sensitive to inositol-1,4,5-trisphosphate (InsP\(_3\)) and cyclic ADP-ribose (cADPR) [74,75]. This result was also confirmed in mammalian cells [76–78]. Later, it was shown that NAADP releases Ca\(^{2+}\) not from the ER but from reserve granules, the functional equivalent of the endolysosomal system [79,80], suggesting that these organelles may function as Ca\(^{2+}\) stores.
Several physiological stimuli have been shown to increase NAADP in mammalian cells through G protein-coupled receptors and tyrosine-kinase linked receptors [81], although the intracellular synthesis of NAADP is still highly controversial. ADP-ribosyl cyclases and CD38 have been shown to catalyze the formation of NAADP in some cell types but the localization of these enzymes inside organelles and at the plasma membrane argues against these enzymes being responsible for NAADP synthesis [81].

The endolysosome system is essential for a variety of cellular functions including membrane trafficking, protein transport, autophagy and signal transduction [82]. While the endocytic and hydrolytic functions of the endolysosomal system are well established [82], the Ca\(^{2+}\) signaling function of these organelles is still in its infancy and a number of unknowns and controversial results still exists [81]. Indeed, still debated is whether NAADP mediates Ca\(^{2+}\) release specifically from the endolysosomal system. Indeed, it has been shown can also mediate Ca\(^{2+}\) efflux from SR/ER stores by directly activating RyRs. Indeed, in heart and skeletal muscle, NAADP has been shown to activate ryanodine receptor/calcium release channels [83,84]. Furthermore, in pancreatic acinar cells and in T-lymphocytes, NAADP causes the release of Ca\(^{2+}\) from the same thapsigargin-sensitive pool that is also the target of InsP\(_3\) and cADPR [85–88].

A unifying hypothesis is that a Ca\(^{2+}\)-mediated functional coupling exists at the lysosome–ER interface. Indeed, it has been shown, in sea urchin eggs and arterial smooth muscle, that NAADP mobilizes Ca\(^{2+}\) via a 2-pool mechanism, named the “trigger hypothesis” [81,89]. In this model, NAADP mobilizes localized Ca\(^{2+}\) signals from lysosome-related Ca\(^{2+}\) stores that initiate global Ca\(^{2+}\) waves through the recruitment of ryanodine receptors on the sarcoplasmic reticulum via Ca\(^{2+}\)-induced Ca\(^{2+}\) release since it has been shown that lysosomes co-localize with RyRs [81,89] (Fig. 4). Interestingly, in cardiac myocytes, NAADP has been shown to participate to the β-adrenergic receptor signal transduction by inducing NAADP-dependent microdomains of high [Ca\(^{2+}\)] between lysosomes and SR [90]. Recent evidence also suggests that not only do acidic organelles signal to the ER through calcium, but that the ER itself can signal to acidic vesicles in a retrograde fashion, since ER Ca\(^{2+}\) depletion ablates NAADP response [91–93]

Beside the existence and specificity of a Ca\(^{2+}\) endolysosome release factor, the release of calcium by the lysosomes requires high [Ca\(^{2+}\)] in the organelle. The measurement of calcium inside acidic stores has been challenging because many fluorescent probes for measuring calcium are sensitive to pH [94]. pH-corrected measurements showed that in macrophages, lysosomes contains high [Ca\(^{2+}\)], ranging from 400 to 600 μM and it is highly sensitive to pH [91,95,96], although these measurements are far from being conclusive. The energy required for Ca\(^{2+}\) entry into the lysosomes
by H⁺/Ca²⁺ exchange is provided by the activity of the vacuolar (V)-type H⁺-ATPase [82]. As for the release pathway, the mucolipin family of Transient Receptor Potential (TRPML) and the Two-Pore (TPC) channels have been proposed to be involved [97], although the role of these channels in the regulation of lysosomal ion balance requires further investigation (Fig. 5).

TPCs channels are predicted to comprise two repeats of a 6 transmembrane pore-forming domain [98] and extensive research supports the hypothesis that they are Ca²⁺ channels targeted by NAADP [99]. Subsequent studies questioned these findings (for a review see [100]). Indeed, it was proposed that TPCs are Na⁺-selective channels activated by PI(3,5)P2 or voltage [101–103]. Therefore, further investigation of TPC permeation is required.

All three members of the TPC family (TPC1-3) localize to the endo-lysosomal system. Specifically, only TPC2 colocalizes with the lysosomal marker LAMP2 while TPC1 and TPC3 are predominantly expressed in endosomal compartments [104]. Interestingly, TPC2 has been demonstrated to be responsible for recruiting Ca²⁺-induced Ca²⁺ release channels to lysosome-ER junctions [104]. Furthermore, inhibition of TPC2 was able to correct the exaggerated NAADP-evoked Ca²⁺ signals and the defects of lysosomal morphology in fibroblasts derived from familial Parkinson disease patients [105].

Still controversial is whether the three isoforms of TRPML, TRPML1-3, that resides within the endocytic pathway, are additional NAADP receptors [106]. The best-characterized member of the TRPML family is TRPML1, also called Mucolipin-1 (MCOLN1) because mutations in this protein are associated with mucolipidosis type IV (MLIV). MLIV is an autosomal recessive neurodegenerative disease [107], in which trafficking defects within the endolysosomal system contributes heavily to the pathogenesis of the disease [108,109]. TRPML1 is expressed in most tissues and colocalizes exclusively with late endosomal and lysosomal markers but its dependence on NAADP is still highly debated [110].

One of the most important roles of localized Ca²⁺ release from acidic stores is the fusion between endocytic vesicles to maintain organelle homeostasis [111], but the molecular mechanism linking lysosomal Ca²⁺ and autophagy has been hypothesized only recently [112,113]. Indeed, it has been proposed that during starvation, Ca²⁺ is released from the lysosome through Mucolipin-1, thus establishing a microdomain of high [Ca²⁺] in the vicinity of the lysosome leading to calcineurin (Cn) activation and transcription factor EB (TFEB) dephosphorylation. Dephosphorylated TFEB is no longer able to bind 14-3-3 proteins and translocates to the nucleus where it activates the transcription of the lysosomal/autophagic pathway gene expression.
Nevertheless, further insights are needed to better define the lysosomal-calcium pathway. In particular, what is still unclear is the [Ca\textsuperscript{2+}] inside lysosomes (because the commonly used probes are highly sensitive to pH), the intracellular source of NAADP, and its molecular targets.

**Concluding Remarks**

Cellular Ca\textsuperscript{2+} is a universal signal acting on several physiological processes. Recent discoveries have deepened our knowledge on the contribution of several organelles in shaping the spatiotemporal dynamics of Ca\textsuperscript{2+} signaling. Of particular note is the determination of the electron cryo-microscopy structures of InsP\textsubscript{3}R1 and of RYR1 which revealed important architectural and functional similarities between the two families of receptors. After the molecular identification of the mitochondrial uniporter complex components, continuous efforts are being placed in the determination of their interplay in the determination of the pathophysiological role of mitochondrial Ca\textsuperscript{2+} uptake in different tissues and organs. Finally, the endolysosomes contribution to intracellular Ca\textsuperscript{2+} homeostasis that is starting to emerge. A great number of unresolved questions need to be addressed (see Outstanding Questions) concerning the tight interplay between ER, mitochondria and the endolysosome system in the regulation of global Ca\textsuperscript{2+} homeostasis.
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**Figure legends**

**FIGURE 1. Endoplasmic Reticulum (ER) Ca\(^{2+}\) store and ER-mitochondria contact sites**

Following ligand binding to a GPCR, subsequent activation of PLC triggers the production of InsP\(_3\) and DAG from PIP\(_2\). InsP\(_3\) binds to InsP\(_3\)Rs at the ER membrane, thus Ca\(^{2+}\) is released from the ER into the cytosol. Conversely, Ca\(^{2+}\) is withdrawn into the ER by SERCA activity. The proximity between ER and mitochondria ensures the formation of high [Ca\(^{2+}\)] microdomains, allowing mitochondria to rapidly take up Ca\(^{2+}\). VDACs are responsible for the rapid transfer of Ca\(^{2+}\) through the OMM, then accumulation of Ca\(^{2+}\) into the mitochondrial matrix occurs via MCU. A number of regulatory proteins contribute to the regulation of ER-mitochondria contact sites. Red dots indicate Ca\(^{2+}\).

GPCR: G-protein coupled receptor

PLC: phospholipase C

PIP\(_2\): phosphatidylinositol 4,5-bisphosphate

InsP\(_3\): inositol 1,4,5-trisphosphate

DAG: diacylglycerol

InsP\(_3\)Rs: inositol 1,4,5-trisphosphate receptors

ER: endoplasmic reticulum

SERCA: sarco(endo)plasmic reticulum calcium ATPase

VDACs: voltage-dependent anion channels

OMM: outer mitochondrial membrane

**FIGURE 2. Schematic representation of store operated Ca\(^{2+}\) entry (SOCE)**
Immediately following a decrease in ER \([\text{Ca}^{2+}]\) (left hand side) STIM1 form multimers. STIM1 multimers translocate to the plasma membrane-ER contact sites where they recruit ORAI1 channels, leading to \(\text{Ca}^{2+}\) entry from the extracellular space. In resting conditions (right hand side), when the ER \([\text{Ca}^{2+}]\) is high, \(\text{Ca}^{2+}\) is bound to the STIM1 EF-hand domain. In this conformation of STIM1 is not able to form multimers, and thus SOCE is inactivated. Red dots indicate \(\text{Ca}^{2+}\).

ER: endoplasmic reticulum
SOCE: store-operated \(\text{Ca}^{2+}\) entry

**FIGURE 3. Schematic representation of the MCU complex**

Mitochondrial calcium uptake is controlled by a multiprotein complex composed of the pore forming subunits MCU and MCUb together with MICU1, MICU2 and EMRE. In resting conditions (left hand side), MICU1/MICU2 heterodimers act as the MCU gatekeeper, due to the inhibitory effect of MICU2. Once \(\text{Ca}^{2+}\) signaling is activated (right hand side), the increase in cytosolic \([\text{Ca}^{2+}]\) induces a conformational change in the dimer that releases MICU2-dependent inhibition. At the same time, MICU1 acts as a cooperative activator of the channel, and thus stimulates the channel activity. Red dots indicate \(\text{Ca}^{2+}\).

**FIGURE 4. Trigger hypothesis of NAADP-induced \(\text{Ca}^{2+}\) release**

NAADP evokes a \(\text{Ca}^{2+}\)-induced \(\text{Ca}^{2+}\) release response from the ER. NAADP acts at TPCs on lysosome-related \(\text{Ca}^{2+}\) stores to elicit a local \(\text{Ca}^{2+}\) release (middle). The increase in \([\text{Ca}^{2+}]\) at the endolysosome-ER interface sensitizes the InsP\(_3\)R, thus evoking a global \(\text{Ca}^{2+}\) wave (right hand side). Red dots indicate \(\text{Ca}^{2+}\).

ER: endoplasmic reticulum
NAADP: nicotinic acid dinucleotide phosphate
TPCs: Two-Pore channels
InsP\(_3\)R: inositol 1,4,5-trisphosphate receptor

**FIGURE 5. Ion channels and transporters in the endolysosome**
TRPML and TPC channels are involved in the release of $\text{Ca}^{2+}$ from the endolysosome. Conversely, the activity of the vacuolar (V)-type H$^+$-ATPase provides the energy required for $\text{Ca}^{2+}$ entry into the lysosome by H$^+$/Ca$^{2+}$ exchanger.

TRPML: Mucolipin family of Transient Receptor Potential

TPC: Two-Pore channel
Outstanding Questions Box

- Which hints will be unraveled by the determination of the ultrastructure of still unsolved ER Ca\(^{2+}\) channels on the specificity of their function and regulation?
- How are mitochondria/ER contact sites determined? What is the precise role of Mfn2?
- What is the precise crosstalk between MCU regulators (MICUs, EMRE, MCUR1) in the control of channel activity?
- How do post-translational modifications affect MCU activity?
- How is gene expression of MCU channel components and regulators controlled? Which transcription factors are involved?
- What would be the phenotype of organ-specific MCU deleted animal models?
- What is the precise NAADP biosynthetic pathway?
- Does NAADP play a role in ER Ca\(^{2+}\) release?
- Which channels are responsible for endo-lysosomal Ca\(^{2+}\) uptake and release?
- Which other pathophysiological roles are played by endo-lysosomal Ca\(^{2+}\), besides regulation of autophagy?
- How does the interplay between ER and endo-lysosomes contribute to global Ca\(^{2+}\) homeostasis?
Trends Box

- Ca\(^{2+}\) signalling is regulated by the intimate interconnection between intracellular organelles and plasma membrane channels.
- The ultrastructures of ER and mitochondria Ca\(^{2+}\) channels have been disclosed.
- Great advancement has been made on the understanding of the function and regulation of the Mitochondrial Calcium Uniporter.
- Studies on endo-lysosomal Ca\(^{2+}\) signalling unravel a potential contribution of this compartment to global Ca\(^{2+}\) homeostasis.
Figure 1
Resting  Trigger  ER Ca\(^{2+}\)-induced Ca\(^{2+}\)-release amplification
Physical exercise in aging human skeletal muscle increases mitochondrial calcium uniporter expression levels and affects mitochondria dynamics

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Abstract

Age-related sarcopenia is characterized by a progressive loss of muscle mass with decline in specific force, having dramatic consequences on mobility and quality of life in seniors. The etiology of sarcopenia is multifactorial and underlying mechanisms are currently not fully elucidated. Physical exercise is known to have beneficial effects on muscle trophism and force production. Alterations of mitochondrial Ca\textsuperscript{2+} homeostasis regulated by mitochondrial calcium uniporter (MCU) have been recently shown to affect muscle trophism in vivo in mice. To understand the relevance of MCU-dependent mitochondrial Ca\textsuperscript{2+} uptake in aging and to investigate the effect of physical exercise on MCU expression and mitochondria dynamics, we analyzed skeletal muscle biopsies from 70-year-old subjects 9 weeks trained with either neuromuscular electrical stimulation (ES) or leg press. Here, we demonstrate that improved muscle function and structure induced by both trainings are linked to increased protein levels of MCU. Ultrastructural analyses by electron microscopy showed remodeling of mitochondrial apparatus in ES-trained muscles that is consistent with an adaptation to physical exercise, a response likely mediated by an increased expression of mitochondrial fusion protein OPA1. Altogether these results indicate that the ES-dependent physiological effects on skeletal muscle size and force are associated with changes in mitochondrial-related proteins involved in Ca\textsuperscript{2+} homeostasis and mitochondrial shape. These original findings in aging human skeletal muscle confirm the data obtained in mice and propose MCU and mitochondria-related proteins as potential pharmacological targets to counteract age-related muscle loss.
Introduction

Age-related sarcopenia is a syndrome characterized by a progressive loss of muscle mass and strength that greatly impacts on mobility and mortality in elderly persons (Hughes et al. 2001; Aagaard et al. 2010; Cruz-Jentoft et al. 2010; Mitchell et al. 2012; Bijlsma et al. 2013; Miljkovic et al. 2015). Contributing factors include a severe decrease in myofiber size and number as well as decrease in the amount of motor neurons (mainly of fast type) innervating muscle fibers that is partially compensated by reinnervation of surviving slow-type motor neurons (motor unit remodeling) (Luff 1998; Mosole et al. 2014). Reduced mobility and functional limitations during aging promote a sedentary lifestyle that generates a vicious circle further worsening muscle performance and, therefore, predisposing to an increased risk of falling, disability, and mortality (Visser and Schaap 2011).

Abnormalities of mitochondrial morphology, number, and function have been suggested to play a role in age-related changes in muscle structure and performance (Trounce et al. 1989; Rooyackers et al. 1996; Menshikova et al. 2006; Pietrangelo et al. 2015). In the skeletal muscle, intermyofibrillar mitochondria are positioned close to the Ca$^{2+}$ release units (CRUs), specialized intracellular junctions formed by a transverse tubule (T-tubule) flanked by two junctional membranes of the sarcoplasmic reticulum (SR) where intracellular Ca$^{2+}$ is stored (Rizzuto et al. 1993; Boncompagni et al. 2009). CRUs are structures deputed to excitation contraction (EC) coupling, a mechanism that allows the depolarization of the plasma membrane to be transduced into release of Ca$^{2+}$ from the SR. As Ca$^{2+}$ entry into the mitochondrial matrix enhances ATP production by stimulating enzymes of the TCA cycle and ATP synthase activity (Denton and McCormack 1980; Denton et al. 1988; McCormack and Denton 1988; Robb-Gaspers et al. 1998; Rizzuto et al. 2012), the proper positioning of mitochondria adjacent to CRUs is physiologically important to rapidly sense intracellular Ca$^{2+}$ changes that are generated during muscle contractions. Indeed, several evidences both in vitro and in vivo have demonstrated that during muscle contraction Ca$^{2+}$ concentration in the mitochondrial matrix is increased (Brini et al. 1997; Rudolf et al. 2004; Rossi et al. 2011; Yi et al. 2011). We have shown that the number of CRUs is decreased in aging muscle (Boncompagni et al. 2006) and that the association of mitochondria with CRUs is also drastically reduced (Boncompagni et al. 2006; Pietrangelo et al. 2015).

The molecular identity of the highly selective channel responsible for Ca$^{2+}$ entry into mitochondria, the mitochondrial calcium uniporter (MCU), was recently identified (Baughman et al. 2011; De Stefani et al. 2011) and the importance of MCU-dependent mitochondrial Ca$^{2+}$ accumulation in regulating skeletal muscle function was confirmed by the identification of a mutation of MICU1, one of the regulatory subunits of the MCU channel, in patients affected by proximal muscle weakness (Logan et al. 2014). In addition, overexpression or knocking down MCU in skeletal muscles of rodents was recently shown to regulate muscle growth triggering hypertrophy or atrophy, respectively (Mammucari et al. 2013).

Physical activity modulates signaling pathways involved in fiber type and muscle growth (Mammucari et al. 2007) also via intracellular Ca$^{2+}$ (Serrano et al. 2001; McCullagh et al. 2004; Sandri et al. 2004) and that it induces specific mitochondrial adaptations. These activity-dependent physiological effects rely on the type of exercise (i.e., aerobic endurance vs. resistance strength), as well as on its frequency, intensity, and duration (Hoppeler and Fluck 2003; Egan and Zierath 2013). Exercise training also impacts on mitochondria dynamics inducing fusion and fission phenomena to sustain cellular energy requirements (Bori et al. 2012; Iqbal et al. 2013; Konopka and Sreekumaran Nair 2013). Fusion and fission events are responsible for mitochondrial shape under the control of a core of dynamin-related large GTPases that fuse and divide the mitochondrial membranes (Griparic and van der Bliek 2001). In particular, fission occurs upon the recruitment of dynamin-related protein 1 (DRP1) (Cereghetti et al. 2008), while fusion is controlled by mitofusins (MFN) 1 and 2 and by optic atrophy 1 (OPA1) (Chen et al. 2003;
Santel et al. 2003; Cipolat et al. 2004). OPA1 also regulates mitochondrial adaptations to bioenergetic conditions at the level of inner-membrane ultrastructure and cristae shape. Indeed, sOPA1-mediated effects on mitochondria respiratory efficiency is critical for muscle function as its mild overexpression prevents muscle loss after denervation (Civiletto et al. 2015; Varanita et al. 2015).

However, the ability to perform physical exercise can be limited in certain pathological conditions, therefore, alternative interventions are needed. Neuromuscular electrical stimulation (ES) was demonstrated to improve muscle mass and performance of sedentary elderly people (Bax et al. 2005; Kern et al. 2014; Mosole et al. 2014; Zampieri et al. 2014, 2015) as well as to improve muscle ultrastructure, trophism, and function in other different disorders characterized by severe muscle atrophy such as permanent upper and lower motor neuron denervation (Kern et al. 2004, 2008, 2010). Our recent data on skeletal muscle biopsies from 9 weeks trained sedentary seniors by ES in comparison to leg press (LP) showed that ES ameliorate muscle trophism, also improving muscle strength and performances (Kern et al. 2014; Zampieri et al. 2015).

In this study we investigate the impact of strength exercise protocols on MCU expression and mitochondria dynamics in a subgroup of ES- and LP-trained subjects recruited for our previous studies (Kern et al. 2014; Zampieri et al. 2015). Our results show that the beneficial effects of these trainings, in particular of neuromuscular ES of the anterior thigh quadriceps muscles (< 20 and 10 l m was counted and 30 and 20 l m, 20 and 10 l m, and less than 10 l m was counted and muscle contraction was evoked at 60 Hz by 3.5-sec train of impulses, separated by 4.5-sec off intervals. Left and right thigh were stimulated in an alternative manner (Sarabon et al. 2013; Kern et al. 2014). In all subjects, ES induced a tetanic contraction of the quadriceps muscle. Additional ankle weights were also used starting from the third week of training onward as described in details (Kern et al. 2014). The intensity of the ES training was about 40% of the maximal voluntary contraction. LP was performed on a computer-controlled LP machine using the proprioceptive vibrational mode (Kern et al. 2011). The subject was asked to push as hard as possible against the pedal. The intensity of the LP training was about 90% of maximal voluntary contraction.

Maximal isometric torque and the time which the subject needed to rise from a chair with arms folded across the chest (5× chair rise test) were measured at enrollment and at the end of the 9-week training period as described (Sarabon et al. 2013; Kern et al. 2014).

Muscle biopsies

Needle muscle biopsies were harvested through a small skin incision (6 mm) from the right and left vastus lateralis muscles of each subject in both groups before and after 9 weeks of training as described (Kern et al. 2014). Post-training muscle biopsies were harvested 7 days after the last training sessions in order to analyze the long-lasting effects of the training. Specimens collected were fixed and embedded for either light or electron microscopy (EM) as previously described (Pietrangelo et al. 2015; Zampieri et al. 2015).

Light and quantitative histological analyses

Serial cryosections (8 l m) from frozen muscle biopsies were mounted on polysineTM glass slides, air-dried, and stained either with Hematoxylin and Eosin (H&E) or conventional techniques for myofibrillar ATPase (mATPase) to evaluate tissue morphology and muscle fiber type. Morphometric analyses to calculate the minimum transverse myofiber diameter were performed on stained cryosections using Scion Image for Windows version Beta 4.0.2 (2000; Scion Corporation, U.K.) as described (Rossini et al. 2002; Kern et al. 2010, 2014) and the results were reported as mean ± SD in pre- and post-training group of muscle biopsies. To give an expression of the number of very small fibers, the atrophy factor was calculated in pre- and post-training muscle biopsies (Table 2) as described by Dubowitz (1985). Briefly, to put the results in a proportional basis, the total number of fibers having diameter between 40 and 30 l m, 30 and 20 l m, 20 and 10 l m, and less than 10 l m was counted and

Methods

Ethical approval and study design

Approval from the Ethical Committees of the City of Vienna for medical ethics was obtained at the study outset (EK 08-102-0608). The study conformed the standards set by the Declaration of Helsinki. Subjects were sedentary healthy volunteers who gave written informed consent. At enrollment they were randomly assigned to two groups needed to rise from a chair with arms folded across the chest (5× chair rise test) were measured at enrollment and at the end of the 9-week training period as described (Kern et al. 2014; Zampieri et al. 2015).

Training protocols and assessments

Electrical stimulation training was performed using a custom-designed device (Krenn et al. 2011). ES-induced

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multiplied by one, two, three, and four, respectively. The products were then added together and normalized to the total number of analyzed myofibers.

**Immunofluorescence staining**

Serial cryosections (8 μm) from frozen muscle biopsies were also labeled for either fast or slow myosin heavy chain (MHC) (product numbers – NCL-MHCs and NCL-MHCf; 1:10; Novocastra, Newcastle upon Tyne, U.K.) and laminin (product number L9393, 1:100; Sigma-Aldrich, St. Louis, MO) as described (Kern et al. 2014; Mosole et al. 2014). Secondary anti-rabbit or anti-mouse Alexa 488 or 594 antibodies (product numbers A11001, A11005, 1:200; Life Technologies, Carlsbad, CA) and anti-rabbit FITC conjugated antibody (product number F1262, 1:200; Sigma-Aldrich) were used. Coverslips were mounted onto the glass slides using ProLong Gold antifade reagent with DAPI to counterstain nuclei (Life Technologies) and observed under the fluorescent microscope.

**Ultrastructural quantitative analyses of mitochondria by EM**

Ultrathin sections (50 nm) were cut from muscle biopsy samples embedded for EM with a Leica Ultracut R (Leica Microsystems, Vienna, Austria) using a Diatome diamond knife (Diatome Ltd., Biel, Switzerland). Sections were then stained in 4% uranyl acetate and lead citrate solutions. Sections were examined with a FP 505 Morgagni Series 268D electron microscope (FEI Company, Brno, Czech Republic) at 60 kV, equipped with a Megaview III digital camera and AnalySIS software (Olympus Soft Imaging Solutions GmbH, Munster, Germany). In each specimen six fibers were analyzed. In each fiber six micrographs of nonoverlapping regions were randomly collected from longitudinal sections at 14,000× magnification for the following quantitative analyses: (1) the relative fiber volume occupied by mitochondria was determined using the well-established stereology point technique (Loud et al. 1965; Mobley and Eisenberg 1975). Briefly, after superimposing an orthogonal array of dots at a spacing of 0.20 μm to the electron micrographs, the ratio between numbers of dots falling within mitochondrial profiles and total number of dots covering the whole image was used to calculate the relative fiber volume occupied by mitochondria (Table 3, column a). (2) Mitochondrial density was evaluated from electron micrographs of nonoverlapping regions randomly collected from longitudinal sections and reported as average number over 100 μm² (Table 3, column b). In each EM micrographs, we also determined mitochondrial positioning with respect to the I and A bands: if an individual mitochondrion extended from one I band to another, it was counted in both bands (Table 3, column c). (3) Mitochondrial average size was also evaluated (Table 3, column d) using AnalySIS software (Olympus Soft Imaging Solutions GmbH) by manually tracing only clearly discernible outlines of mitochondria.

**Immunoblotting**

Protein lysates were prepared from 10 cryosections (20 μm thick) by means of Qiagen Tissue Lyser (Qiagen GmbH, Hilden, Germany) in a buffer containing 50 mmol/L Tris pH 7.5, 150 mmol/L NaCl, 5 mmol/L MgCl₂, 1 mmol/L DTT, 10% glycerol, 2% SDS, 1% Triton X-100, Roche Complete Protease Inhibitor Cocktail (Roche Diagnostics S.p.a., Monza, MB, Italy), 1 mmol/L PMSF, 1 mmol/L NaVO₃, 5 mmol/L NaF, and 3 mmol/L β-glycerophosphate. Protein concentration was determined by the colorimetric detection of the cuprous cation (Cu⁺) by bicinchoninic acid method (Pierce™ BCA assay; Thermo Scientific, Rockford, IL), subsequently separated on 4–12% gradient SDS-PAGE and electrotransferred onto nitrocellulose membrane which was then probed with different antibodies: MCU (product number HPA016480, 1:500; Sigma-Aldrich), Actin (product number A2066, 1:15000; Sigma-Aldrich); TOM20 (product number sc11415; 1:1000; Santa Cruz Biotechnology, Segrate, MI, Italy); SDH-A (product number #11998, 1:500; Cell Signaling Technology; Euroclone, Pero, MI, Italy), COX IV (product number #4844, 1:1000; Cell Signaling Technology), OPA1 (product number 612606, 1:1000; BD Biosciences, Milano, Italy), and Mitofusin 2 (product number ab50838, 1:1000; AbCam, Cambridge, U.K.). Signals were visualized via chemiluminescence as described (Zampieri et al. 2001).

**Gene expression analyses**

Total RNA was extracted from muscle using tissue lyser (Qiagen GmbH) in TriReagentTM (Sigma-Aldrich) and it was reverse transcribed using a QuantiTect Reverse Transcription Kit (Qiagen GmbH). Quantitative RT-PCR was performed on an ABI PRISM 7500 SDS (Applied Biosystems, Foster City, CA) using premade 6-carboxyfluorescein (FAM)-labeled TaqMan assays for GAPDH, IGF-1 Ea, IGF-1 Eb, IGF-1 Ec, IGF-1 pan (Applied Biosystems), and for Atrogin1, MuRF1, PGC1α, and PGC1α4, MCU, DRP1, Mitofusin1 and 2, and OPA1 as described (Brocca et al. 2012; Varanita et al. 2015). Quantitative sample values were normalized to the expression of GAPDH mRNA. Relative levels for each gene were calculated using the 2⁻¹ΔΔCt method (Livak and Schmittgen 2001) and reported as mean fold change in gene expression.
Statistical analyses

The statistical significance of data collected by EM analyses was determined using a Student’s t test (Microcal Origin® 6.0; Microcal Software, Inc., Northampton, MA), while statistical significance of percentage values was investigated using a chi-squared test (Microsoft® Office Excel® 2007; Microsoft Corporation). Statistical analysis of morphometric, densitometric, and gene expression datasets was performed with GraphPad Prism v5.0 software (GraphPad Software, Inc., La Jolla, CA); statistical significance of average numbers was determined using Wilcoxon matched pairs test. Values of $P < 0.05$ were considered significant.

Results

Skeletal muscle biopsies from sedentary seniors before training show specific features of aging

Muscle biopsies of 70-year-old sedentary seniors were collected before and after the training and morphological, biochemical, and molecular analyses were performed. Histological analyses of pretrained muscles showed some severely atrophic, flat shaped, and angulated fibers (arrowed in panel A, Fig. 1), presenting typical signs of denervation. Histochemical analyses testing for mATPase activity revealed the presence of slow twitching myofibers (dark stained in panel B, Fig. 1) organized in cluster (type grouping, i.e., one myofiber completely surrounded by fibers of the same phenotype, white encircled in panels B, Fig. 1), and constituted by medium or large fibers. Immunofluorescence analyses for fast and slow MHC demonstrated that fast fibers (green stained in panel C, Fig. 1) were smaller than slow ones (red stained in panel C, Fig. 1). Moreover, fast atrophic fibers displayed an angular or flat shape features (white arrowed in panel C, Fig. 1). These morphological aspects were almost absent in slow-type fibers (red stained in panel C, Fig. 1).

Neuromuscular ES training significantly increases maximal isometric torque and strength of sedentary elderlies without damaging their muscles

Several functional and mobility tests can be used to assess frailty, and degree of independence in elderlies (Mosole et al. 2014; Zampieri et al. 2014, 2015). Among these, maximal isometric torque and chair rise are specific tests for muscle strength, which represents a key factor for fall prevention in aging (Bohannon 1997; Cruz-Jentoft 2013). In line with our previous results (Kern et al. 2014; Zampieri et al. 2015), both groups of trained subjects improved their functional tests performances (Fig. 2, panel A). Torque values increased and 5x chair rise test’s score was decreased when compared to the pretraining conditions as an indication of improved muscle strength. These changes were more pronounced and statistically significant in subjects trained with neuromuscular ES (Fig. 2, panel A). Inflammation, bands of hypercontraction, centrally nucleated fibers, or fibrosis was not detected in post-training muscle biopsies (Fig. 2, panel B), suggesting that these exercise protocols did not injury aging weak muscles.

The overall training load was calculated in both groups of subjects and expressed as the net muscle contraction time (MCT) over the 9 weeks of training as reported in Table 1. In ES-trained group, the total MCT was higher in comparison to LP (144 vs. 48 min), while the intensity of training was much higher in LP with respect to ES.
Neuromuscular ES maintains myofiber size, inducing a recovery of severely atrophic fast-type fibers

In ES group the average myofiber size slightly increased (49.16 ± 15.80 vs. 51.01 ± 16.38; P < 0.0001) while atrophy factor decreased after the training (Table 2, 404 vs. 384, pre vs. post), indicating that ES protocol was effective in the recovery of severely atrophic fibers. The major trophic effect was observed on those fibers having diameter between 25 and 45 μm as shown by the spectrum of myofiber size distribution: the frequency of the fibers in this range decreased in post-training muscle biopsies (Fig. 3, panel A, black bars) in comparison to the

(90% vs. 40% of the maximal voluntary contraction, respectively).

### Table 1. Muscle contraction time in ES and LP training.

<table>
<thead>
<tr>
<th>Weeks of training (n)</th>
<th>Sessions/week (n)</th>
<th>MCT/session (min)</th>
<th>MCT/week (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ES</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>6</td>
<td>36</td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>6</td>
<td>108</td>
</tr>
<tr>
<td>Total MCT</td>
<td></td>
<td></td>
<td><strong>144</strong></td>
</tr>
<tr>
<td><strong>LP</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>2</td>
<td>12</td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>2</td>
<td>36</td>
</tr>
<tr>
<td>Total MCT</td>
<td></td>
<td></td>
<td><strong>48</strong></td>
</tr>
</tbody>
</table>

ES, electrical stimulation; LP, leg press; MCT, muscle contraction time on the LP was calculated multiplying the net time for one repetition by the number of repetitions performed in each session. In ES training, MCT was the time while ES-evoked muscle contraction-induced knee extension with an angle less than 30°. In bold are highlighted the total MCT for each condition (ES vs. LP).

### Table 2. Atrophy factor in pre- and post-training muscle biopsies.

Calculation of atrophy factor (as described in Material and Methods section) in pre- and post-training muscle biopsies revealed that ES physical exercise had major effects on the recovery of severely atrophic fibers, in particular of fast type, while LP had milder trophic effects, despite the observed improvements in muscle torque and strength.

<table>
<thead>
<tr>
<th></th>
<th>Pretraining</th>
<th>Post-training</th>
<th>Rescue of AF</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AF</strong></td>
<td>Total fibers</td>
<td>Total fibers</td>
<td></td>
</tr>
<tr>
<td><strong>ES trained</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All fibers</td>
<td>404</td>
<td>384</td>
<td>5023</td>
</tr>
<tr>
<td>Fast type</td>
<td>585</td>
<td>412</td>
<td>3294</td>
</tr>
<tr>
<td>Slow type</td>
<td>269</td>
<td>1575</td>
<td>394</td>
</tr>
<tr>
<td><strong>LP trained</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All fibers</td>
<td>233</td>
<td>333</td>
<td>2525</td>
</tr>
<tr>
<td>Fast type</td>
<td>395</td>
<td>1074</td>
<td>379</td>
</tr>
<tr>
<td>Slow type</td>
<td>192</td>
<td>1570</td>
<td>311</td>
</tr>
</tbody>
</table>

AF, atrophy factor; ES, electrical stimulation; LP, leg press.
pre-training ones (Fig. 3, panel A, white bar), while the frequency of those having diameter >55 μm increased after the training. Interestingly, the recovery was predominantly observed in fast-type population (Table 2, 585 vs. 412, pre vs. post). In LP group, the average myofiber size significantly decreased after the training (57.87 ± 19.17 vs. 55.21 ± 18.13, P < 0.0001) and consistently atrophy factor increased (Table 2, 233 vs. 333, pre vs. post). A
minor effect of LP training on the rescue of fast-type myofiber atrophy was observed (Table 2, 395 vs. 379, pre vs. post). These results indicate that neuromuscular ES was more efficient to promote muscle hypertrophy and that fast-type fibers were more responsive to ES- and LP-induced physical activity in aging muscles.

Neuromuscular ES triggers the induction of IGF1 and a concomitant suppression of atrophy-related genes

Muscle size depends on mechanical stimulation and the mechanical load modulates anabolic and catabolic signaling pathways. In order to investigate the molecular mechanisms underlying the morphological and functional changes, we monitored gene expression of the atrophy-related genes Atrogin1 and MuRF1 and of the growth-promoting hormone insulin growth factor-1 (IGF1) and its isoforms. The expression of the new splicing variant transcript from PGC-1a gene, the isoform 4 (PGC1a4), that has been recently reported to trigger muscle hypertrophy (Ruas et al. 2012; Mammucari et al. 2015) was also measured. In line with our previous study (Kern et al. 2014) in ES-trained group we observed a significant downregulation of MuRF1 and only a trend of Atrogin1 reduction, while simultaneously all the IGF1 isoforms were significantly upregulated (Fig. 4, panel A). These molecular changes suggest that ES activated a program of gene expression that counteracts muscle atrophy and promotes muscle growth. Conversely, LP training did not affect the levels of Atrogin1 and MuRF1, while it significantly induced, even if to a less extend, the expression of IGF1b isoform (Fig. 4, panel B). These results are in agreement with the minor effects on function and myofiber size that were observed in LP-trained subjects (Figs. 2 and 3). In both groups, PGC1a4 was unaffected by the training, suggesting that the effect on myofiber size observed after 9 weeks of training mainly relies on IGF1-signaling pathway (Fig. 4, panels A and B).

Physical exercise induces an increase in MCU protein content

Exercise-dependent muscle activity induces intracellular Ca$^{2+}$ release from the CRUs. This calcium is uptaken by the intermyofibrillar mitochondria where it affects the respiratory chain enzymes to sustain the energy demand of contraction. Western blot analyses on muscle

![Figure 4](image-url)
homogenates from biopsies collected before and after 9 weeks of training revealed a significant increase in MCU protein content in response to exercise (Fig. 5, panels A–B and E–F, Table 3), with no significant changes in transcript level (Fig. 5, panels C and G) indicating a post-transcriptional regulation of this protein. COX IV respiratory chain enzyme significantly increased only in ES-trained group (Fig. 5, panels A and B, Table 3), whereas SDH protein levels were unchanged in both post-training conditions (Fig. 5, panels A–B, and E–F, Table 3). Importantly, the significant increase in MCU and COX IV protein expression levels was observed in the 80% of the subjects (Table 3) indicating that these changes are a generalized effect of the training in the great majority of the subjects. TOM20 protein, which was used as marker of outer mitochondrial membrane and to monitor mitochondrial mass, significantly increased in ES-trained subjects (Fig. 5, panels A and B, Table 3).

However, PGC1α was significantly downregulated after ES (Fig. 5, panel D) while was unchanged after LP training (Fig. 5, panel H).

**Neuromuscular ES or LP trainings differently impact on mitochondria network dynamics**

Qualitative observation by EM did not reveal striking structural differences in biopsies from subjects trained with ES or LP between pre- and post-training. However, a quantitative analyses of the mitochondrial network did reveal some morphological changes in the biopsies from the ES group between pre- and post-training (Table 4). Indeed, while the relative volume occupied by mitochondria did not change following either ES and LP training (Table 4, column a), in the ES group the number of mitochondria was decreased (Table 4, column b: 48.3 ± 1.3 vs. 38.6 ± 1.2, respectively, in pre- and post-training muscle biopsies; *P* < 0.0001), whereas their size was increased (Table 4, column d: 72.3 ± 1.9 vs. 80.4 ± 2.5, respectively, in pre- and post-training muscle biopsies; *P* = 0.009). The statistically significant changes reported in Table 4 (columns b and d) suggest a remodeling of the mitochondrial apparatus induced specifically by the ES training, but not by the LP exercise protocol.

---

**Figure 5.** Nine weeks of physical exercise induced a significant increase in mitochondrial calcium uniporter (MCU) protein content, associated with increased COX IV expression level in electrical stimulation (ES)-trained group. Representative immunoblots for MCU, TOM20, and respiratory chain enzymes SDH and COX IV on muscle homogenates from pre- and post-ES (A) (*n* = 10) and leg press (LP) (E) (*n* = 7) training conditions. Ctr = homogenates from mouse tibialis anterior muscle overexpressing MCU. Densitometric quantification of detected proteins in all analyzed muscle homogenates from ES (B) and LP (F) samples, normalized to actin. Values are given as mean ± SD; Exact mean, SD, and *P* values are reported in Table 3. mRNA expression levels of MCU (C and G) and PGC1α (D and H) as a marker of mitochondrial biogenesis from ES (C–D) (*n* = 10) and LP (D–H) (*n* = 7) pre- and post-training muscle biopsies. Values are given as mean ± SD; (Panel D **P** = 0.0020).
Indeed, data of Table 4 (columns b and d) suggest that ES stimulates fusion of mitochondria into larger organelles. In order to support this interpretation, we measured the expression levels of protein-regulating mitochondrial shape like Mtf2 and OPA1. A significant increase in OPA1 in the muscles of more than 85% of the ES-trained subjects was detected (Table 3), while it significantly decreased in more than 85% of the subjects after LP training (Fig. 6, panels A and B, Table 3). On the other hand, Mtf2 expression was unchanged by the training (Fig. 6, panels C and D, Table 3) and also the transcript levels of Mtf1 and 2, OPA1, and DRP1 genes were unaffected after 9 weeks of training (Fig. 6E and F).

Discussion

The etiology of sarcopenia is multifactorial and involves several intrinsic and extrinsic factors, but the precise underlying mechanisms are poorly understood. Numerous studies have clearly shown that old age is characterized by a fiber-type shift toward slow phenotype (Aagaard et al. 2010; Ohlendieck 2011) that can only partially account for the weakness that characterizes aging muscle (Mitchell et al. 2012; Bijlsma et al. 2013). Several histological analyses of sarcopenic muscles have shown a progressive increase in denervated myofibers, primarily of fast type, indicating that denervation is one of the important mechanisms that contribute to muscle atrophy and weakness in aging (Edström et al. 2007; Aagaard et al. 2010; Gonzalez-Freire et al. 2014). Dysfunction of the EC coupling has also been proposed to contribute to the loss of specific force of aging muscle (Boncompagni et al. 2006). Importantly, exercise seems to counteracts these features (Mosole et al. 2014; Zampieri et al. 2014), but the mechanistic insights triggered by physical activity remain unknown.

In this study the muscle biopsies collected before the training showed histological features typical of aging, such as a predominance of slow fibers, which were clustered in

Table 3. MCU and other mitochondrial protein expression levels in skeletal muscle biopsies before and after ES and LP training. Quantitative analyses of Western blot on muscle homogenates revealed that 9 weeks of physical exercise significantly increased MCU protein expression levels. ES training induced also a significant increase in COX IV respiratory chain enzyme and mitochondrial fusion protein OPA1. The great majority of the subjects experienced the reported mean changes, indicating that the induction of protein expression levels is a generalized response to the training.

<table>
<thead>
<tr>
<th></th>
<th>Pre (mean ± SD)</th>
<th>Post (mean ± SD)</th>
<th>Subjects showing the indicated changes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ES MCU</td>
<td>0.68 ± 0.52</td>
<td>1.13 ± 0.86</td>
<td>0.027</td>
</tr>
<tr>
<td>SDH</td>
<td>1.07 ± 0.46</td>
<td>1.19 ± 1.10</td>
<td>n.s.</td>
</tr>
<tr>
<td>COX IV</td>
<td>1.42 ± 1.22</td>
<td>2.33 ± 2.55</td>
<td>0.049</td>
</tr>
<tr>
<td>TOM20</td>
<td>0.49 ± 0.28</td>
<td>0.80 ± 0.45</td>
<td>0.048</td>
</tr>
<tr>
<td>OPA1</td>
<td>0.73 ± 0.47</td>
<td>1.08 ± 0.41</td>
<td>0.040</td>
</tr>
<tr>
<td>Mtf2</td>
<td>0.80 ± 0.28</td>
<td>0.78 ± 0.43</td>
<td>n.s.</td>
</tr>
<tr>
<td>LP MCU</td>
<td>0.82 ± 0.17</td>
<td>1.34 ± 0.55</td>
<td>0.020</td>
</tr>
<tr>
<td>SDH</td>
<td>1.02 ± 0.33</td>
<td>1.00 ± 0.28</td>
<td>n.s.</td>
</tr>
<tr>
<td>COX IV</td>
<td>1.59 ± 0.69</td>
<td>2.07 ± 1.46</td>
<td>n.s.</td>
</tr>
<tr>
<td>TOM20</td>
<td>0.74 ± 0.21</td>
<td>1.02 ± 0.37</td>
<td>n.s.</td>
</tr>
<tr>
<td>OPA1</td>
<td>1.05 ± 0.56</td>
<td>0.78 ± 0.49</td>
<td>0.047</td>
</tr>
<tr>
<td>Mtf2</td>
<td>0.75 ± 0.25</td>
<td>0.67 ± 0.23</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

Values are shown as mean ± SD. ES, electrical stimulation; LP, leg press; MCU, mitochondrial calcium uniporter.

Table 4. EM ultrastructural analyses of intermyofibrillar mitochondria before and after ES and LP trainings. Quantitative analyses of the mitochondrial population by electron microscopy revealed that, following the ES protocol, mitochondrial number (column b) and size (column d) changes significantly (*P < 0.01). These changes suggest a remodeling of the mitochondrial apparatus induced specifically by the ES training, but not by the LP protocol.

<table>
<thead>
<tr>
<th></th>
<th>(a) Mitochondria volume/total volume (%)</th>
<th>(b) No. of mitochondria/100 μm²</th>
<th>(c) No. of mitochondria at A band/100 μm² (%)</th>
<th>(d) Mitochondrial average size (nm² × 10³)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
<td>Pre</td>
<td>Post</td>
</tr>
<tr>
<td>ES</td>
<td>3.4 ± 0.1</td>
<td>3.5 ± 0.1</td>
<td>48.3 ± 1.3</td>
<td>7.5 ± 0.5 (16)</td>
</tr>
<tr>
<td>LP</td>
<td>3.5 ± 0.1</td>
<td>3.5 ± 0.1</td>
<td>42.4 ± 1.5</td>
<td>5.4 ± 0.4 (13)</td>
</tr>
</tbody>
</table>

Values are shown as mean ± SEM. Sample size: 48 fibers from ES and 36 fibers from LP; 6 micrographs/fiber. EM, electron microscopy; ES, electrical stimulation; LP, leg press.

*P < 0.01 versus Pre.
grouping, and several atrophic, angulated, and flat-shaped fibers suggesting that denervation and reinervation were present. However, 9 weeks of neuromuscular ES or LP recovered myofiber atrophy with a specific hypertrophic effect on fast-type fibers that was predominantly observed in ES-trained group of subjects. The morphological changes resulted in an improvement of muscle torque and strength. Nevertheless, some differences have been observed in response to the two types of trainings. ES protocol was more efficient to maintain myofiber size, counteracting atrophy and promoting muscle growth when compared to LP. This difference might be due to the two training approaches that diverge in terms of time and intensity of exercise. In fact, neuromuscular ES was applied to knee extensor muscles with a stimulation pattern designed for the submaximal activation (Sarabon et al. 2013) and a total muscle contraction time of 144 min over the 9-week period. On the other side, LP

Figure 6. Electrical stimulation (ES) training induced a significant increase in OPA1 mitochondrial fusion protein. Representative immunoblot analyses for OPA1 (A) and Mtf2 (C) in pre- and post-training conditions (n = 7). Ctr = HeLa total cell lysate. Densitometric quantification of Western blot from muscle homogenates of all analyzed samples showing OPA1 (B) and Mtf2 (D) protein expression levels in pre- and post-training conditions, normalized to actin. Values are given as mean ± SD. Exact mean, SD, and P values are reported in Table 3. (F) Fold changes in genes regulating mitochondria dynamics after ES (E) and leg press (LP) (F) training. Values are given as mean ± SD; P = no significant changes for all analyzed genes.
exercised different muscles at the same time, that is, hip extensor, knee flexor, and extensor muscles. Therefore, LP intensity is higher than ES, but the time spent in training is much less with a total MCT of only 48 min. This protocol of short periods of high-intensity contractions was specifically designed to avoid potential muscle and joint injuries. Therefore, the observed differences in terms of hypertrophy, force, and signaling pathways might be consequent to the short overall muscle contraction time of LP protocol. However, both trainings improved muscle function and, to a certain extent, also the size of fast fibers suggesting that some molecular mechanisms were shared between these two types of exercise. The pathways found to be commonly activated by these trainings are related to IGF1 and mitochondrial calcium homeostasis. In fact, we found that both ES and LP triggered IGF1 and MCU expression. However, we observed a significant increase in MCU protein content in response to exercise, with no significant changes at mRNA level, suggesting that the protein is regulated post-transcriptionally, even though little is known about this issue at the moment. Tyrosine phosphorylation of MCU has been described to control MCU activity (O-Uchi et al. 2014). In addition, two Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaM-KII) target pull sites, were identified in the MCU sequence (Joiner et al. 2012). However, the role of CAMKII in regulating MCU activity has been questioned (Fieni et al. 2014) and deserves deeper investigation.

Of note, ES but not LP promoted the upregulation of IGF-1Ec isoform, which is normally upregulated in response to mechanical signals (Matheny et al. 2010). Moreover, increased levels of IGF-1 were associated with reduced level of expression of MuRF1, a gene involved in muscle atrophy. This suggests that ES mimics physical exercise, improving molecular adaptations of muscle, countering muscle atrophy, and improving functional outcomes.

Interestingly, mice lacking MCU exhibit functional abnormalities in conditions that require a rapid increase in the skeletal muscle work load. In particular, a significant impairment of the exercise capacity, strength, and power output has been shown in MCU\(^{-/-}\) mice by inclined treadmill test, forearm grip strength assessment, and vertical pull up, without any apparent alterations in mitochondrial calcium homeostasis. The human data shown here are in good agreement with the recent findings about MCU involvement in muscle mass regulation, in rodents. Indeed, we have recently found that overexpression of MCU in adult muscle promotes hypertrophy while knocking down MCU triggers muscle atrophy (Mammucari et al. 2015). Therefore, the induction of MCU might contribute to muscle growth via a mitochondria- and energy-dependent signaling.

Electron microscopy ultrastructural analyses showed that mitochondria volume when normalized to myofiber volume was unchanged after ES and LP training. However, following the ES (but not LP) training the number of mitochondria was decreased while their size became bigger, suggesting fusion of mitochondria into larger organelles.

The observed ultrastructural changes in mitochondrial network suggest an involvement of mitochondrial shaping machinery and indeed we found an increase in OPA1 protein but not of Mfn2. Several functional differences have been reported between Mfn1/Mfn2 and OPA1 in terms of mitochondrial fusion, localization, bioenergetics, and shape (Cipolat et al. 2004). Mfn2 controls outer mitochondrial membrane fusion, but also tethers of mitochondria to the endoplasmic reticulum (de Brito and Scorrano 2008), while OPA1 regulates inner mitochondrial membrane fusion as well as cristae shape and super-complexes assembly. Importantly, mild OPA1 upregulation elicits several beneficial effects in terms of tissue physiology. In fact, we have recently found that expression of OPA1 is sufficient to counteract muscle loss after denervation (Varanita et al. 2015) but does not induce muscle hypertrophy in basal condition. Altogether, these findings suggest that OPA1 is involved in metabolic/bioenergetic changes that are important for muscle maintenance and regulation in stress conditions. The OPA1-dependent beneficial effects might be dissociated from its profusion activity as PGC1a was downregulated and Mfn2 did not change after ES training.

Altogether, our results indicate that while both exercise protocols ameliorated some functional parameters and increased MCU expression, only ES induced OPA1 expression, changes in mitochondrial network, and big improvements in fiber size and muscle strength. Therefore, we can speculate that the increase in MCU expression induced by physical activity is associated with hypertrophic signaling, while the changes in mitochondria dynamics are synergistic with MCU and linked to metabolic adaptations and energy production. LP training protocol was probably too mild and/or too short in overall time period to have significant impact on muscle morphology and mitochondria dynamics in aged muscles.

In conclusion, our findings show for the first time that MCU and OPA1 expressions are modulated by physical
exercise in aging human muscles and, therefore, suggest that mitochondria can serve as the sensors and retrogradely induce nuclear programs to regulate muscle mass.

Further experiments are needed to dissect the mechanistic insights that connect exercise to mitochondria and to gene/protein expression. Understanding this link will allow the development of novel therapeutic strategies to counteract sarcopenia and to promote healthy aging.

Conflict of Interest

All authors declare no conflict of interest.

References


