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CICLO XXX

Physiopathological characterization of the role of MCUb in skeletal muscle regeneration

Coordinatore: Ch.mo Prof. Paolo Bernardi

Supervisore: Ch.mo Prof. Rosario Rizzuto

Co-Supervisore: Dott.ssa Anna Raffaello

Dottorando: Simona Santina Feno

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1. Summary

Calcium ions play key roles in different intracellular mechanisms, ranging from biological processes such as proliferation, gene transcription, protein post-translational modifications and aerobic metabolism to physiological mechanisms such as muscle contraction, exocytosis, energy metabolism, chemotaxis and synaptic plasticity during learning and memory [1,2]. Key players in the spatio-temporal regulation of cytosolic calcium concentrations ($[Ca^{2+}]_{cyt}$) are mitochondria, intracellular organelles that can accumulate Ca^{2+} in the very rapid time-scale of hundreds of milliseconds [3] and reach values up to 100 μM in some cell types [4].

Mitochondrial Ca^{2+} overload, caused by either abnormal release from internal stores, physical damage of mitochondria or malfunction of receptors and channels present in their membrane, has been long known to be a critical event in the bioenergetic crisis associated with cell death by necrosis [2]. Furthermore, one of the most important roles that highlights the importance of mitochondrial Ca^{2+} in cell metabolism and survival is the mitochondrial Ca^{2+}-dependent control of mitochondrial adenosine triphosphate (ATP) production, the main fuel for sustaining diverse cell functions [5,6].

Thus, the understanding of the role of mitochondria in regulating cellular Ca^{2+} homeostasis has become crucial for the understanding of a series of cell functions. Nevertheless, this analysis was severely limited due to the lack of molecular information on the identity of the protein responsible of mitochondrial Ca^{2+} uptake.

The situation completely reversed in 2011 when Mootha's and our laboratory identified the molecular identity of the mitochondrial Ca^{2+} uniporter (MCU) [7,8]. From that moment, we have witnessed an explosion of studies aimed to characterize the composition and regulation of the complex. It is now clear that MCU exists in a large protein complex that includes pore-forming and regulatory subunits [9]. The pore forming subunit includes MCU, the MCU dominant-negative subunit, MCUb, and the essential MCU regulator, EMRE, whereas the regulatory

subunit includes the mitochondrial calcium uptake protein 1 and 2, MICU1, MICU2 [7,10–13].

One of the most peculiar subunits of the MCU complex is MCUb that was identified in our laboratory in 2013 as one of the components of the pore region of the MCU complex [10]. We have demonstrated that MCUb inhibits mitochondrial Ca^{2+} uptake by acting as a dominant-negative subunit of MCU [10]. Intriguingly, the ratio of expression between MCU and MCUb varies greatly between tissues (e.g.: ~3:1 in heart or lung and ~40:1 in skeletal muscle) [10], and this might contribute to the spatiotemporal regulation of mitochondrial Ca^{2+} uptake. Indeed, the MCU/MCUb ratio correlates with patch clump recording data of mitochondrial Ca^{2+} uptake of isolated mitochondria from different tissues [14].

Our Real Time-PCR experiments demonstrated that MCUb expression levels dramatically increase during skeletal muscle regeneration 3 days after cardiotoxin (CTX)-induced injury. This induction is specific, since the expression of the other components of the MCU complex is unchanged in this condition. Therefore, we hypothesized that MCUb might play a role in the progression of skeletal muscle regeneration after damage. In addition, high MCUb expression levels have been detected in anti-inflammatory macrophages (M2), one of the most important effectors of the later stages of tissue repair [15–17].

Importantly, our *in vitro* results demonstrated that MCUb silencing affects macrophages polarization towards an M2 pro-regenerative phenotype and that, during the progression of skeletal muscle regeneration *in vivo*, MCUb is induced selectively in macrophages.

We therefore asked whether MCUb overexpression in M2 macrophages, occurring during skeletal muscle regeneration, could be crucial for their differentiation and thus for tissue repair. To answer to this question, we performed skeletal muscle regeneration experiments on a total MCUb knockout (KO) mouse model. We observed that MCUb ablation *in vivo* affects macrophage skewing from a pro-inflammatory (M1) to an M2 phenotype.

It is widely accepted that M1 and M2 macrophages actively participate to skeletal muscle regeneration process by releasing cytokines and growth factors that promote skeletal muscle regeneration [15]. Specifically, M1 macrophages promote

the activation and proliferation of satellite cells, while M2 macrophages are involved in the last stages of skeletal muscle repair by promoting the differentiation and fusion of myogenic precursor cells (MPCs) [15]. We hypothesized that an impairment in M1 to M2 transition might also influence skeletal muscle repair capacity by affecting the expression levels of myogenic regulatory factors. Strikingly, our results demonstrated that the lack of MCUb, by influencing macrophage plasticity and function, might negatively influence the expression of early myogenic regulatory genes *Pax7* and *Myod*, crucial regulators of proliferation and differentiation of satellite cells [18,19], causing an impairment in skeletal muscle regeneration process.

Our results strongly support the hypothesis that this altered muscular phenotype is caused by an impairment in macrophages skewing from the M1 to the M2 phenotype, since M2 macrophages are endowed with pro-regenerative capacity [15–17]. This is supported by our latest data showing that macrophages from MCUb KO animals present lower phagocytic capacity compared to wild type animals. These results are in line with published data, demonstrating that the phagocytic activity is fundamental for M2 polarization [20].

Intriguingly, we observed a significant reduction in the number of regenerating myofibers in regenerating muscles of MCUb KO mice compared with WT animals, parameter related to the efficiency of skeletal muscle regenerative capacity.

Furthermore, it is well established that, during skeletal muscle regeneration, satellite cells that are not initiated to differentiation, return to quiescence to replenish the reserve population of satellite cells that will become activated during further rounds of muscle injures [21]. In order to evaluate whether MCUb KO mice show an alteration in the reconstitution of the satellite cells pool, we performed a triple skeletal muscle regeneration experiment, as already performed [22]. Intriguingly, we found a dramatic decrease in the cross-sectional area of regenerating muscle fibers in MCUb KO mice compared with WT animals. This result suggests that a possible exhaustion of the pool of satellite cells might occur in the MCUb KO animals by affecting the myogenesis process after damage. We also observed that MCUb KO mice show a decrease in collagen content suggesting

that MCUb ablation, by affecting M2 polarization, and thus function, alters the reconstitution of muscle structure. These results are in line with data in literature that demonstrate the role of M2 macrophages in collagen production [16].

Finally, we analysed in detail the mechanism responsible for MCUb induction in M2 macrophages. We found that MCUb promoter, and thus its transcription in macrophages, is activated by IL-4. We thus hypothesized that the IL-4-STAT6 pathway, that is known to be involved in M2 macrophages polarization [23], might also regulate MCUb transcription.

In the future, we will analyse also the 5' AMP-activated protein kinase (AMPK) phosphorylation state, the cellular metabolic sensor, that it is known to mediate macrophage skewing from an M1 to M2 phenotype [20]. We believe that MCUb, by regulating the entry of Ca^{2+} into mitochondria, and thus the rate of ATP production, might induce the activation of this pathway.

It is known that perturbations of macrophage function and/or activation may result in impaired regeneration and fibrosis deposition, as described in several pathological disease [15], and that skeletal muscle repair system is compromised during ageing and in patients affected by muscular dystrophies [24]. Therefore, our research might be fundamental for the understanding of the molecular basis of chronic muscular diseases, such as the Duchenne Muscular Dystrophy (DMD).

2. Sommario

Il Ca^{2+} riveste un ruolo fondamentale nella regolazione di un vasto spettro di processi biologici e fisiologici come la proliferazione cellulare, la trascrizione genica, la stimolazione del metabolismo aerobico, la regolazione della contrazione muscolare, l'esocitosi e la plasticità sinaptica [1,2]. I principali organelli intracellulari predisposti alla regolazione della concentrazione di Ca^{2+} citosolica ($[Ca^{2+}]_{cit}$) sono i mitocondri. Quest'ultimi hanno la capacità di accumulare molto velocemente elevate $[Ca^{2+}]$[3] che in alcuni tessuti possono raggiungere livelli superiori ai 100 µM [4].

L'alterato rilascio di Ca^{2+} dalle riserve intracellulari, il danno mitocondriale o il malfunzionamento dei recettori e canali presenti nella loro membrana possono portare ad un accumulo eccessivo di Ca^{2+} all'interno del mitocondrio, fenomeno che è stato dimostrato condurre ad una crisi energetica con conseguente induzione di morte cellulare [2]. In condizioni fisiologiche, il Ca^{2+} mitocondriale riveste un ruolo fondamentale nella sopravvivenza e metabolismo cellulare sostenendo la produzione dell'adenosina trifosfato (ATP), la principale molecola energetica della cellula, necessaria per lo svolgimento di diverse funzioni cellulari [5,6].

Di conseguenza, lo studio del ruolo dei mitocondri nella regolazione dell'omeostasi del Ca^{2+} è diventato cruciale per la comprensione di una elevata serie di funzioni cellulari. Sfortunatamente, l'analisi di questo aspetto è stata fortemente limitata dalla mancata caratterizzazione molecolare della proteina responsabile dell'accumulo di Ca^{2+} mitocondriale.

Nel 2011, nel nostro laboratorio e in quello del Prof. Mootha è stata scoperta l'identità molecolare dell'uniporto del Ca^{2+} mitocondriale (MCU), proteina necessaria e sufficiente a permettere l'ingresso di Ca^{2+} nel mitocondrio [7,8]. Questa scoperta ha permesso l'identificazione di vari componenti dell'uniporto e dei meccanismi che regolano la sua funzione [9]. E' ormai chiaro che MCU esiste sotto forma di un complesso, costituito da diverse subunità che compongono la regione del poro (MCU, MCUb, EMRE) e da subunità regolatorie (MICU1, MICU2) [7,10–13].

Tra le varie subunità che compongono il complesso, un ruolo rilevante è svolto da MCUb. Questa subunità è stata identificata nel 2013 nel nostro laboratorio come parte integrante della regione formante il poro del canale [10]. Abbiamo dimostrato che MCUb agisce come dominante negativo di MCU in quanto inibisce l'ingresso di Ca^{2+} nel mitocondrio [10]. Nonostante l'elevata similarità in sequenza e struttura, MCU e MCUb mostrano un diverso grado di espressione nei diversi tessuti (es: rapporto di ~3:1 nel cuore e polmone e di ~40:1 nel muscolo scheletrico). E' stato ipotizzato che queste differenze possano contribuire alla regolazione spazio-temporale dell'ingresso di Ca^{2+} mitocondriale nei diversi tessuti [10]. Coerentemente, il diverso grado di espressione di MCU e MCUb nei vari tessuti correla con dati ottenuti da esperimenti di *patch clamp* in cui è stato misurato l'accumulo di Ca^{2+} in mitocondri isolati da diversi tessuti [14].

Esperimenti di Real Time-PCR da noi condotti hanno dimostrato una elevata induzione dell'espressione di MCUb durante il corso della rigenerazione del muscolo scheletrico 3 giorni dopo il danno indotto da cardiotossina (CTX). Questa induzione è specifica in quanto l'espressione delle altre componenti del canale è inalterata in questa condizione. Dal momento che, in condizioni basali, MCUb è poco espresso nel muscolo scheletrico e che la sua espressione aumenta significativamente e specificamente durante il corso della rigenerazione muscolare, abbiamo ipotizzato che MCUb potesse avere un ruolo fondamentale in questo processo. In aggiunta, MCUb è specificatamente espresso in macrofagi con fenotipo anti-infiammatorio (M2), facendo ipotizzare che l'aumento significativo di MCUb, osservato durante il corso della rigenerazione, potesse essere attribuito alla componente macrofagica. Infatti, i macrofagi di tipo M2, che infiltrano massivamente un muscolo rigenerante, partecipano attivamente durante le ultime fasi che caratterizzano questo processo [15–17].

Risultati ottenuti da esperimenti effettuati *in vitro* hanno dimostrato che il silenziamento di MCUb nei macrofagi impedisce a quest'ultimi di acquisire un fenotipo di tipo M2. Questo dato fa emergere l'ipotesi che MCUb, *in vivo*, possa rivestire un ruolo fondamentale nella polarizzazione dei macrofagi dal tipo M1 al tipo M2 e in questo modo influire sulla rigenerazione del muscolo scheletrico in seguito a danno.

Per confermare le nostre ipotesi, sono stati condotti degli esperimenti di rigenerazione del muscolo scheletrico utilizzando un modello murino in cui MCUb è stato deleto in tutti i tessuti (MCUb *knockout* (KO)). I nostri risultati dimostrano che la mancanza di MCUb, durante il corso della rigenerazione muscolare, impedisce la polarizzazione dei macrofagi da un fenotipo pro-infiammatorio (M1) a quello anti infiammatorio (M2).

E' ormai ampiamente accettato il concetto che i macrofagi di tipo M1 e M2 influenzino il corso della rigenerazione muscolare attraverso il rilascio di citochine e fattori di crescita che facilitano la risoluzione del danno [15]. In particolare, i macrofagi di tipo M1 promuovono l'attivazione e proliferazione delle cellule satellite mentre gli M2 sono coinvolti negli ultimi stadi della rigenerazione promuovendo il differenziamento e la fusione dei precursori delle cellule muscolari (MPCs) [15]. Abbiamo quindi ipotizzato che la mancata transizione dei macrofagi dal profilo pro-infiammatorio (M1) a quello anti-infiammatorio (M2) potesse influenzare negativamente l'espressione di geni codificanti fattori di trascrizione coinvolti nelle varie fasi di riparazione del muscolo scheletrico in seguito a trauma.

Questa ipotesi sembra confermata. Infatti, l'espressione di *Pax7* e di *Myod*, regolatori cruciali del processo di proliferazione e differenziamento delle cellule satelliti [18,19], appare essere drasticamente ridotta nei topi MCUb KO rispetto ai topi di controllo durante il corso della rigenerazione muscolare, suggerendo un'alterazione del processo di rigenerazione muscolare in mancanza di MCUb.

Questi risultati supportano l'ipotesi che l'alterato fenotipo muscolare osservato nei topi KO possa essere ricondotto alla mancata transizione dei macrofagi dal fenotipo di tipo M1 a quello di tipo M2, considerato che quest'ultimi sono dotati di capacità pro-regenerative [15–17]. Ulteriore conferma del fatto che l'assenza di MCUb possa influenzare negativamente la transizione fenotipica dei macrofagi da M1 a M2, deriva dalla dimostrazione che i macrofagi derivanti dal topo MCUb KO, mostrano una riduzione dell'attività fagocitica, funzione essenziale per la polarizzazione dei macrofagi verso un fenotipo di tipo M2 [20].

Un altro parametro attraverso il quale è stato possibile valutare l'efficienza della rigenerazione del muscolo scheletrico, si basa sull'osservazione che il numero

di fibre rigeneranti nei topi KO risulta significativamente ridotto rispetto alle fibre dei topi di controllo 14 giorni dopo l'induzione del danno.

E' noto che, durante il corso della rigenerazione muscolare, le cellule satelliti che non vanno incontro al processo di differenziamento, ritornino ad uno stato di quiescenza andando a ricostituire la nicchia di cellule satelliti originarie che verranno attivate in seguito ad ulteriore trauma [21]. Con lo scopo di valutare se i topi MCUb KO mostrino un'alterazione nella ricostituzione della riserva di cellule satelliti in seguito a danno muscolare, abbiamo condotto degli esperimenti di tripla rigenerazione, come precedentemente effettuato [22], e abbiamo osservato una sostanziale riduzione dell'area delle fibre rigeneranti dei topi KO rispetto a quelle dei topi controllo. Da questi risultati è emersa l'ipotesi che l'assenza di MCUb possa impedire la ricostituzione del pool di cellule satellite, alterando, in questo modo, il normale decorso della rigenerazione. In linea con questi dati abbiamo osservato come l'ablazione di MCUb, impedendo la polarizzazione dei macrofagi di tipo M2, influenzi negativamente la quantità di collagene, componente essenziale della matrice extracellulare [16], depositata durante la fase di riparazione muscolare. Questi risultati sono in linea con dati presenti in letteratura che dimostrano il ruolo dei macrofagi di tipo M2 nella produzione di collagene [16].

La dimostrazione che l'espressione di MCUb sia indotta nei macrofagi di tipo M2 ci ha spinti allo studio dei meccanismi che regolano la trascrizione di MCUb in questa sottopopolazione di cellule. In particolare, abbiamo dimostrato come l'IL-4, citochina di tipo anti-infiammatorio, attivi la trascrizione di MCUb. Dal momento che l'ablazione di MCUb nei macrofagi impedisce la loro polarizzazione verso un fenotipo di tipo M2, abbiamo ipotizzato che la via di segnale mediata dall'asse IL-4-STAT6, nota per essere coinvolta nella polarizzazione dei macrofagi M2 [23], possa essere coinvolta nell'attivazione della trascrizione di MCUb.

In futuro, analizzeremo lo stato di fosforilazione di AMPK, uno dei principali sensori metabolici cellulari, che è stato dimostrato essere un giocatore essenziale nella transizione dei macrofagi dal fenotipo M1 a quello M2 [20]. La nostra ipotesi è che, MCUb, regolando negativamente l'ingresso di Ca^{2+} nel mitocondrio e quindi la produzione di ATP, possa indurre l'attivazione di vie di

segnale mediate da AMPK influenzando, in questo modo, la polarizzazione dei macrofagi.

E’ ormai noto che perturbazioni nella funzionalità e attivazione dei macrofagi possano portare ad alterazioni nel processo rigenerativo e alla deposizione di tessuto fibrotico, come descritto in diverse condizioni patologiche [15]. Questa condizione è una caratteristica comune a diverse patologie come le distrofie muscolari e durante l’invecchiamento [25]. Per questo motivo, la nostra ricerca potrebbe rivelarsi fondamentale per la comprensione delle basi molecolari di malattie croniche del muscolo scheletrico, come la distrofia di Duchenne (DMD) e potrebbe portare all’identificazione di nuovi bersagli terapeutici.

3. Introduction

3.1 Ca^{2+} Signalling

The divalent ion calcium (Ca^{2+}) is an ubiquitous and versatile intracellular messenger [1], required for a wide array of biological processes such as proliferation, gene transcription, post-translational modification of proteins and aerobic metabolism [2]. This versatility allows Ca^{2+} to control crucial processes during early development like fertilization and cell cycle events. Once cells differentiate and become committed to specific functions, Ca^{2+} is required to regulate several physiological mechanisms such as muscle contraction, exocytosis, energy metabolism, chemotaxis and synaptic plasticity [1].

The concept that Ca^{2+} ions are critical for cellular functions dates back to 1883, when Sydney Ringer, discovered that saline solution prepared using tap water, which contained Ca^{2+}, supported the contraction of isolated frog hearts, whereas saline solution prepared using distilled water, which lacked Ca^{2+}, did not induce the same effect [26]. After this seminal observation, several studies led to the discovery of other biological processes controlled by Ca^{2+} signalling. Particularly important was the discovery in 1950 that Ca^{2+}, released from sarcoplasmic reticulum (SR), triggers skeletal muscle contraction by binding to troponin C [26].

Electrical, hormonal and mechanical stimulation of cells can generate Ca^{2+} signals by causing entry of Ca^{2+} through a various classes of plasma membrane Ca^{2+} channels or its release from intracellular stores [27]. The internal stores are held within the membrane systems of the endoplasmic reticulum (ER) or the equivalent organelle in skeletal muscle, the sarcoplasmic reticulum (SR) [1]. Release from these internal stores is controlled by various channels, among which the inositol-1,4,5-trisphosphate receptor (IP_3R) and the ryanodine receptor (RYR) families have been studied most extensively. Ca^{2+} mobilizing second messengers, that are generated when stimuli bind to cell surface receptors, determine whether Ca^{2+} activates these channels [1].

One of the most common intracellular mechanism of Ca^{2+} release from the ER, is represented by the inositol 1, 3, 5 triphosphate (IP_3) that is released as a consequence of a plethora of external stimuli [28]. In detail, many cell stimuli, like hormones and neurotransmitters act on G protein-coupled receptors (GPCR) that are coupled to phospholipase C (PLC) that produces IP_3 after hydrolyses of phosphatidylinosol 4,5-bisphosphate (PIP2). Subsequently, IP_3 interacts with the IP_3R located on the ER membrane and causes the release of Ca^{2+} that is often organized into characteristic spatial, elementary events and waves [28]. The IP_3/Ca^{2+} pathway is a versatile signalling system that has adapted to control processes as diverse as fertilization, proliferation, muscle contraction, cell metabolism, vesicle and fluid secretion and information processing in neuronal cells [28]. To control and orchestrate these different processes, cells have to maintain very low cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_{cyt}$) trough cell type-specific channels and transporters that allow to modulate amplitude, kinetics and subcellular distribution of Ca^{2+} signals [29]. Among these are the plasma membrane Ca^{2+} ATPase (PMCA) that extrudes Ca^{2+} via plasma membrane and the SR/ER Ca^{2+} ATPase (SERCA) transporter that actively pumps Ca^{2+} into the ER. Other important electrogenic regulators of $[Ca^{2+}]_{cyt}$ are the Na^+/Ca^+ exchanger (NCX) and the $Na^+/Ca^{2+}/K^+$ exchangers (NCKX) that exchange one Ca^{2+} ion for three Na^+ ions (NCX) or cotransport one K^+ ion with one Ca^{2+} ion in exchange for four Na^+ ions (NCKX) [29].

Other important key players in the spatio-temporal regulation of $[Ca^{2+}]_{cyt}$ are mitochondria, intracellular organelles that show an enormous capacity to accumulate Ca^{2+} in the very rapid time scale of hundreds of milliseconds (see Introduction, paragraph 3.2.2.3 and [3]).

Dysregulation of $[Ca^{2+}]_{cyt}$ has been associated to necrosis, that is the catastrophic derangement of cell integrity and function following the exposure to different types of cell injury and leading to the activation of Ca^{2+}-activated hydrolysing enzymes [30]. Typical examples are complement-induced cell death and excitotoxicity in neurons, in which glutamate-dependent hyper stimulation leads neurons to the necrotic death [31]. Several studies have also shown that the increases of $[Ca^{2+}]_{cyt}$ occur both at early and late stages of the apoptotic pathway

and both Ca^{2+} release from ER and Ca^{2+} influx through Ca^{2+} release-activated Ca^{2+}channels, have been proposed to be apoptogenic [30]. Thus, the common view is that, while severe Ca^{2+} dysregulation can promote cell death through necrosis, more controlled intracellular [Ca^{2+}] increases, induced by milder insults, promote cell death through apoptosis [30]. Existing evidence implicates Ca^{2+} dysregulation as a common underlying event in different disease such as muscular dystrophies and neurodegenerative disease [32,33].

3.2 Mitochondria

3.2.1 The general framework

Mitochondria are essential organelles for every nucleated cell since they produce energy, required for different cell functions, in the form of adenosine triphosphate (ATP) by the oxidative phosphorylation (OXPHOS) system [34]. Beyond their role as cellular 'power houses', mitochondria are also involved in vital metabolic processes. These processes include the production of the reduced nicotinamide adenine dinucleotide (NADH) and guanosine-5'-triphosphate (GTP) molecules in the citric acid cycle, steroid and heme biosynthesis, intermediary metabolism, Ca^{2+} and iron homeostasis, redox signalling, programmed cell death, innate immunity, and regulation of complex physiological processes [31]. Because of these important functions of mitochondria in cell homeostasis, mitochondrial dysfunction causes a great variety of diseases which can affect almost all tissues and organs in the body [35,36]. Indeed, increasing evidence suggests that mitochondrial dysfunction is involved in aging, cancer, diabetes, and neurodegenerative diseases, such as Alzheimer's, Huntington's, Parkinson's diseases and motoneuron disorders, such as amyotrophic lateral sclerosis (ALS) [35,36].

Mitochondria are highly dynamic organelles that, in the cell, form a tubular network that constantly changes by fission and fusion mechanisms that are processes regulated by a number of dynamin-related GTPases [37].

As ubiquitous, semi-autonomous cellular organelles, mitochondria are separated from the cytoplasm by the outer and the inner mitochondrial membrane

(OMM and IMM, respectively). The OMM is permeable to solutes that are smaller than 5 kDa and thus also to Ca^{2+} [31]. The permeability of the OMM is attributed to the abundant expression of voltage-dependent anion channels (VDACs), whose permeability is controlled by ATP and other regulatory factors [38]. However, larger molecules, in particular proteins, are imported by special translocases [31]. By contrast, the IMM is a tight diffusion barrier to all ions and molecules that can bypass it by specific membrane transporter proteins which are selective for a particular ion and molecule [31].

In vertebrates, as introduced above, most of the energy of a cell is generated in mitochondria by the process of oxidative phosphorylation. During the oxidation of nutrients such as glucose, amino acids or fatty acids, reduced metabolites with low reduction potentials, such as NADH or succinate, are produced in the mitochondrial matrix [39]. These reduced metabolites provide electrons to the respiratory chain that consists of several IMM membrane protein complexes containing redox centers with progressively higher reduction potential, until the final electron acceptor, O_2 [39]. According to the chemiosmotic hypothesis, postulated by Peter Mitchell in 1961, the free-energy obtained by the reactions performed by the respiratory complexes is used to pump protons from the mitochondrial matrix to the intermembrane space (IMS), to create a transmembrane membrane potential ($\Delta\psi$) of about -180 mV [40]. The energy stored by this electrochemical potential is subsequently used by ATP synthase for the synthesis of ATP. This large electrochemical potential (negative inside), gave rise to the hypothesis that not only H^+ but also other cations can be accumulated into the mitochondrial matrix [40].

3.2.2 The contribution of mitochondria to cellular calcium homeostasis

3.2.2.1 Mitochondrial Ca^{2+} uptake: from the past to nowadays

Mitochondria were the first intracellular organelles to be associated with Ca^{2+} handling. This observation dates back to half a century ago when Ca^{2+} uptake was directly measured in energized isolated mitochondria from rat kidney and liver

[41]. In the following 2 decades, the mechanism by which mitochondria accumulate Ca^{2+} have been deeply investigated [6]. As mentioned above, the transfer across the IMM requires the presence of specific transporters [31]. In line with this observation, the basic properties of mitochondrial Ca^{2+} transport were clarified and a rapid electrogenic pathway, known as the mitochondrial calcium uniporter (MCU) was described [6]. It was hypothesized that MCU could rapidly transport Ca^{2+} in the matrix driven by the $\Delta\psi$ established by the respiratory chain. These evidence sustained the general idea that mitochondria could actively and rapidly change their $[Ca^{2+}]$ and contribute to cellular homeostasis [6]. It was also clear that Ca^{2+} accumulation by MCU could not proceed until electrochemical equilibrium due to the activity of two main efflux pathways, the Na^+/Ca^{2+} (mNCX) and H^+/Ca^{2+} (mHCX) exchangers, expressed in excitable and non-excitable tissues, respectively [42]. The existence of a sophisticated machinery for Ca^{2+} handling supported the general consensus that mitochondria could actively and rapidly change their $[Ca^{2+}]$ and participate in cellular homeostasis [6].

This idea was strongly questioned in the 80s by the demonstration that inositol IP_3, generated upon stimulation of receptors coupled to PLC, mobilizes Ca^{2+} from a non-mitochondrial Ca^{2+} store, the ER [6]. Furthermore, when the mitochondrial Ca^{2+} uptake system was functionally characterized, its affinity was found to be too low to accumulate the cation not only in resting conditions, but also during the transient $[Ca^{2+}]_{cyt}$ increases generated by cell stimulation [43]. Therefore, these discoveries made the ER/SR the main research focus in calcium signalling.

3.2.2.2 Mitochondria as active players in cellular Ca^{2+} homeostasis: the microdomain concept

The concept that mitochondria undergo major changes in matrix Ca^{2+} concentration also in physiological conditions, awaited the direct reliable measurement of this parameter in living cells in the early 90s [6]. Indeed, the development of recombinant targeted probes was crucial for the reappraisal of the role of mitochondria in the control of cellular Ca^{2+} homeostasis [6]. The pioneer of these probes was the photoprotein aequorin [44], which was soon followed by the

development of green fluorescent protein (GFP)-based probes, engineered to change fluorescence properties following Ca^{2+} binding [45,46].

The development of these probes allowed to demonstrate that, when a cell is stimulated with a $[Ca^{2+}]$ increasing agonist, mitochondria accumulate Ca^{2+} in the matrix with a speed and an amplitude which greatly exceed those expected from the properties of MCU in isolated mitochondria [44,47].

By using these tools it was shown that, following stimulation with a $[Ca^{2+}]_{cyt}$ raising agonist, the speed and the amplitude of Ca^{2+} accumulation in mitochondria greatly exceed the values that were previously predicted on the basis of MCU properties in isolated mitochondria [47]. The discrepancy with the low affinity of MCU and the efficiency of mitochondrial Ca^{2+} uptake was solved by the evidence that mitochondria are located in close proximity to the Ca^{2+} channels that are responsible for the Ca^{2+} rise including the IP_3 receptors (IP_3Rs) and ryanodine receptors (RyRs) in the ER/SR [48], or different types of channels on the plasma membrane (i.e voltage and store operated Ca^{2+} channels) (Fig. 1 and [49]). Importantly, this observation was recently confirmed [50,51]. Therefore, mitochondria sense microdomains of high $[Ca^{2+}]_{cyt}$ that meet the low affinity of the MCU and that dissipate rapidly, thus preventing mitochondrial Ca^{2+} overload and/or Ca^{2+} cycling across the mitochondrial membrane [52].

The contacts between mitochondria and ER, that can be biochemically purified as MAMs for mitochondria-associated membranes, are enriched of proteins that are involved in the organelles interactions [53]. Among them is Mitofusin 2 (Mfn2), located in both the ER and the mitochondrial membranes that forms homo- and heterotypic interactions, the latter with Mitofusin 1 (Mfn1) (Fig. 1 and [54]). These interactions increase the ER-mitochondria contacts and facilitate mitochondrial Ca^{2+} uptake [55]. However, this model has been questioned by recent data showing that Mfn2-knockout (KO) cells show an increase in ER-mitochondria contacts, demonstrated by quantitative electron microscopy [56]. Along this line, analysis of light microscopy images of cells in which Mfn2 expression is blunted, further supports the increase in the percentage of the mitochondria-ER contacts in this conditions [57]. This observation was confirmed by the increase in mitochondrial Ca^{2+} uptake in these cells [57]. Overall these data [56,57] suggest

that Mnf2 acts as a negative regulator of ER–mitochondrial tethers, by reducing the number of contacts and avoiding toxic Ca^{2+} accumulation [54]. Nevertheless, the role of Mfn2 at ER–mitochondria contacts remains highly debated.

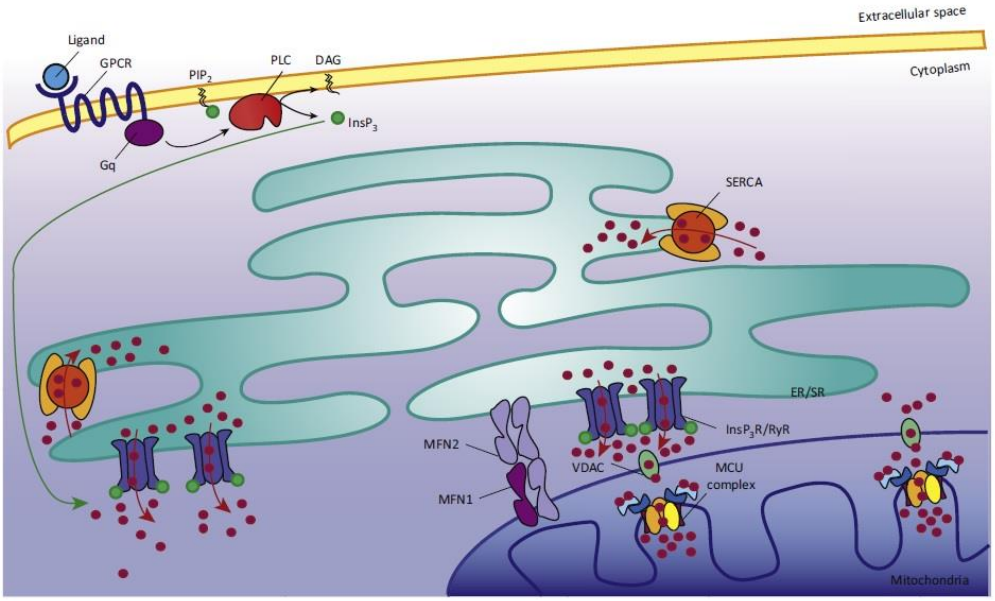


Figure 1. ER Ca^{2+} Store and ER-Mitochondria Contact Sites. (Adapted from A. Raffaello et al., Trends in Biochemical Sciences, 2016).
Following the binding of a ligand to a GPCR, subsequent activation of PLC induces the production of IP_3 and DAG from PIP2. IP_3 binds to IP_3R at the ER membrane thus inducing the release of Ca^{2+} in the cytosol. Ca^{2+} is withdrawn and stored in the ER by SERCA activity. The proximity between mitochondria and ER allows the formation of high [Ca^{2+}] microdomains, allowing a rapid mitochondrial Ca^{2+} uptake. VDCAs are involved in the rapid transfer of Ca^{2+} trough the OMM and the subsequent accumulation of Ca^{2+} into the mitochondrial matrix occurs through the MCU. Numerous regulatory subunits contribute to the regulation of ER- mitochondria contact sites. Red dots indicate Ca^{2+}.

3.2.2.3 Mitochondria as cytosolic Ca^{2+} buffers

The concept that mitochondria respond to the generation of a rapidly dissipating microdomain has major functional implications [6]. Indeed, during cell stimulation, continuous accumulation of Ca^{2+} across the mitochondrial membrane could interfere with ATP production, while a rapid and transient response allows a functional response with limited energy depletion [6]. Another important crucial implication of a microdomain-based signalling mechanism is that the shape and positioning of mitochondria inside the cells is critical for the responsiveness to Ca^{2+} signals [6].

In many cell types, such as in neurons, mitochondria appear to be located to dedicated signalling sites contributing to the accumulation of large amount of Ca^{2+}

in defined subcellular domains, promoting large local cytoplasmic Ca^{2+} rises [53]. Importantly, in neurons, Ca^{2+} sequestration by mitochondria, besides stimulating ATP synthesis through the Ca^{2+}-dependent increase in OXPHOS [58], profoundly affects neurotransmitter release, being strategically located in the proximity of Ca^{2+} channels such as N-methyl-d-aspartate receptor (NMDAR) at the synaptic terminal [59,60]. In general, mitochondria recruitment to neuronal soma, synapses and dendritic spines is crucial for the regulation of nerve activity and any change in the positioning of mitochondria to subcellular domains affects neuron physiology and might contribute to the pathogenesis of neurodegeneration [61].

Striking is also the case of pancreatic acinar cells in which three distinct groups of mitochondria have been identified, i.e. mitochondria located at the peripheral basal area, perigranular and perinuclear mitochondria [62]. Each of these subsets is characterized by specific responses to cytosolic Ca^{2+} signals occurring in their close proximity [62].

Overall, the emerging picture defines mitochondria as efficient, high capacity Ca^{2+} buffers that shape cytosolic Ca^{2+} transients by either regulating the kinetic properties of Ca^{2+} channels or by preventing Ca^{2+} diffusion away from the area where the open channels are located [52].

3.2.2.4 Mitochondrial Ca^{2+} homeostasis and cell death

Mitochondria can be considered as a "firewall" that contribute to the control of $[Ca^{2+}]_{cyt}$ and cytoplasmic microdomains in order to regulate the frequency of oscillatory Ca^{2+} waves and to avoid intracellular Ca^{2+} overload and cell death [63]. Mitochondria are exposed to the risk of Ca^{2+} overload which can induce the permeability transition (PT), a permeability increase of the IMM to ions and solutes with molecular mass up to 1500 Da. This increase is mediated by the permeability transition pore (mPTP), a high-conductance channel mediating mitochondrial swelling [64]. The minimal requirements for pore induction are intact transmembrane electrical potential, matrix pH and matrix Ca^{2+} [65]. Mitochondrial matrix Ca^{2+} is an essential permissive factor for mPTP opening: as $[Ca^{2+}]_{cyt}$ increases beyond a certain value, mitochondrial Ca^{2+} overload ensues [65]. This, together with other causal factors, most notably oxidative stress, high phosphate

concentrations and low adenine nucleotide concentrations, triggers the PT [63]. Consequent dissipation of the mitochondrial $\Delta\Psi$ leads to membrane depolarization, increased mROS generation and decreased ATP production, eventually triggering apoptosis [66]. The function of Ca^{2+} in apoptosis is particularly fascinating, since a small amount of cytochrome c released from mitochondria can bind to and promote Ca^{2+} conductance through IP_3R. The increased cytosolic Ca^{2+} then triggers a massive exodus of cytochrome c from all mitochondria in the cell, thus activating caspase and nuclease enzymes that finalize the apoptotic process [66]. Recently, it has been proposed that dimers of the F_OF_1 ATP synthase form Ca^{2+}-activated channels with the key features of the mPTP [67–69]. However, the detailed mechanisms of mPTP activation are still higly debated [63,70–73].

3.2.3 Molecular identification and characterization of the Mitochondrial Calcium Uniporter

Until six years ago, the study of the physiological role of mitochondrial Ca^{2+} uptake was severely limited by the lack of the molecular identity of the components of the MCU complex [5]. Indeed, in the last 40 years, the search of the molecular identity of the MCU has proven to be unsuccessful [74]. Indeed, most traditional approaches in this molecular search could not be applied or failed. First, no highly specific inhibitor could be utilized in biochemical purifications. Although ruthenium red (RuR) and the related compound Ru360 can inhibit the activity of the uniporter, it shows some major drawbacks because it binds a broad array of glyco-proteins [74]. Second, yeast *Saccaromyces cerevisiae* does not possess a RuR-sensitive mitochondrial Ca^{2+} uptake mechanism [75], thus preventing the use of yeast genetics. Lastly *in silico* comparison with known Ca^{2+} channels proved uninformative.

Novel *in silico* screening techniques, applied to available databases of mitochondrial proteins, led to the identification of the proteins responsible for mitochondrial Ca^{2+} uptake [9]. In detail, Mootha and co-workers reported in 2008 the generation of a mitochondrial "genoteque" (MitoCarta) by performing mass spectrometry analysis on both highly purified and crude mitochondrial preparations

from 14 different mouse tissues to discover genuine mitochondrial proteins, validated by GFP tagging [76]. Thanks to the availability of this database, in 2011, Mootha's and our laboratory identified the mitochondrial Ca^{2+} uniporter (MCU) [7,8]. My laboratory screened the MitoCarta database for the known features of the mitochondrial calcium uniporter:

i. Ubiquitous expression in mammalian tissues;

ii. Sequence compatible with that of a channel (i.e with at least 2 putative transmembrane regions);

iii. Presence in organisms in which mitochondrial Ca^{2+} uptake was reported;

iv. Absence in *Saccaromices cerevisiae* that lacks Ruthenium Red-sensitive mitochondrial Ca^{2+} uptake.

This *in silico* screening generated a list of 13 proteins that have been then analysed in detail. Only one protein (previously known as coiled-coil domain containing protein or Ccdc109A) included a highly conserved domain comprising two transmembrane regions and an intervening loop enriched in acidic residues, suggestive of a possible common role in cation permeation [7]. This gene is well conserved in all eukaryotes except for yeast and encodes a 40 kDa protein. Our group and Mootha's demonstrated that MCU localizes in the IMM and that downregulation of MCU drastically reduces mitochondrial Ca^{2+} uptake both in living cells treated with Ca^{2+} mobilizing agonist and in cells perfused with buffered Ca^{2+} without affecting classical mitochondrial properties [7,8]. MCU was shown to be sufficient *per se* to mediate Ca^{2+} transport into mitochondria [7]. This was demonstrated by electrophysiological experiments in planar lipid bilayer in which reconstitution of MCU was sufficient to generate Ca^{2+} currents with the main electrophysiological properties recorded for the mitochondrial calcium uniporter [7]. Importantly, this current was inhibited by RuR [7].

The molecular identification of the MCU opened a new era in the Ca^{2+} signalling field. The first studies performed after the discovery of the MCU were focused on the characterization of the composition of the complex and on understanding the properties of the channel. Regarding the latter point, soon after MCU discovery it was clear that this protein, by presenting only two transmembrane domains, was not able to form a functional channel but, in order to

form a functional channel, had to form oligomers [10]. Moreover, the MCU exists in a large protein complex of 480 kDa, suggesting the existence of other proteins that participate to the channel formation [9]. Indeed, in the following years it was shown that the MCU complex is composed by pore-forming subunits, MCU itself, MCUb and EMRE and by regulatory subunits (MICU1 and MICU2) [9].

3.2.4 The pore-forming subunits of the mitochondrial calcium uniporter complex

3.2.4.1 MCUb: the dominant-negative regulator of MCU channel activity

In 2013 our laboratory, by sequence analysis of MCU, identified a related gene, named Ccdc109b, located on *Mus musculus* chromosome 3, which was closely related to MCU [10]. The gene is present in vertebrates but absent in other organisms in which MCU is present like plants, kinetoplastids, Nematoda, and Arthropoda [10]. The encoded protein, that was named MCUb, consists of 330 amino acids and is highly conserved among all species and shares a 50% sequence similarity with MCU (Fig. 2A and [10]). MCUb consists of two predicted transmembrane domains that are connected by a loop region containing a DIME motif, similar in sequence to MCU. Although their huge sequence similarity, MCU and MCUb present crucial amino acids substitutions in the loop region (E256V) and in the first helical membrane spanning domain (R251W) that were shown to prevent MCU from forming a Ca^{2+} permeable channel, thus acting as a dominant-negative subunit (Fig. 2A, 2B and [10]). Indeed, it was shown that MCUb overexpression in Hela cells dramatically reduces mitochondrial Ca^{2+} uptake during cell stimulation and that MCUb oligomers, reconstituted in planar lipid bilayers, could not give rise to detectable Ca^{2+} currents [10]. Moreover, reconstituting MCU together with MCUb in planar lipid bilayers demonstrated that the inclusion of MCUb in the MCU oligomer completely inhibited Ca^{2+} permeation through the MCU [10]. Interestingly, expression data on mouse tissues showed that MCUb presents a different expression profile compared with MCU [10]. Intriguingly, MCU/MCUb ratio varies greatly between tissues, suggesting that it might

contribute to the spatiotemporal control of mitochondrial Ca^{2+} uptake in different tissues [10]. Importantly, the ratio MCU/MCUb strictly correlates with Ca^{2+} current measurements by patch clamp of different tissues [14]. Indeed, tissues characterized by low mitochondrial Ca^{2+} transients such as the heart, show a higher MCUb/MCU ratio compared with tissues, such as skeletal muscle, that present an higher mitochondrial Ca^{2+} uptake and a lower MCUb/MCU ratio [9,10,14].

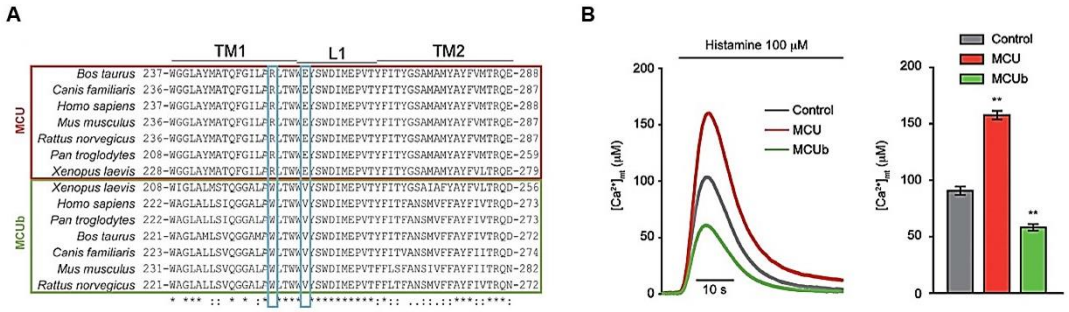


Figure 2. Characterization of the dominant-negative regulator of MCU, MCUb (Adapted from A. Raffaello et al., EMBO Journal, 2013).
*(**A**) Multiple alignment of the transmembrane domain 1 (TM1), loop (L1), and transmembrane domain 2 (TM2) regions of MCU (red) and MCUb (green) in seven different species. Blue boxes show the two critical conserved substitutions. (**B**) MCUb acts as a dominant-negative subunit on MCU. $[Ca^{2+}]_{mt}$ measurements in intact HeLa cells overexpressing MCU or MCUb.*

3.2.4.2 EMRE: the essential MCU regulator

The pore-forming subunit of the MCU complex is also composed by a protein that was named the "essential MCU regulator" (EMRE) [11]. EMRE was identified by SILAC-based quantitative mass spectrometry of affinity purified MCU complex [11]. This protein is a 10 kDa metazoan specific protein of the IMM and it is composed of a transmembrane domain, a short N-terminal domain and a highly conserved acidic C-terminal domain [11]. It has been shown that EMRE is essential for MCU activity, as demonstrated by experiments performed in EMRE KO cells, where mitochondrial Ca^{2+} uptake was abolished [11]. Even though experiments in planar lipid bilayer demonstrated that MCU displays the ability to interact with the regulatory subunits MICU1 and MICU2 without the presence of EMRE [77], this protein has been suggested to have a key role in the interaction between the pore forming subunits and the regulatory subunits [11]. Interestingly, in yeast, which does not show mitochondrial Ca^{2+} uptake, human MCU is able to assemble in a functional channel only when EMRE is present [78]. This gave rise

to the concept that EMRE is essential to assemble a functional MCU channel in metazoan organisms [78].

Furthermore, the acidic C-terminal domain has been proposed to act as a matrix-Ca^{2+} sensor that governs the MCU activity with a mechanism that requires MICU1 and MICU2 [79]. This is in opposition to another report, which proposes a structural role of EMRE and a different topology across the IMM, incompatible with the suggested matrix-Ca^{2+} sensor of the acidic C-terminal domain [80].

3.2.5 The regulatory subunits of the mitochondrial calcium uniporter complex

3.2.5.1 MICU1, MICU2 and MICU3: the gatekeepers of the MCU channel activity

One of the most important features of mitochondrial Ca^{2+} uptake is the sigmoidal response to extra-mitochondrial [Ca^{2+}] [31]. Indeed, in resting conditions mitochondrial Ca^{2+} accumulation is inhibited despite the huge driving force for Ca^{2+} accumulation. This property is essential to protect mitochondria from both a huge energy expenditure and Ca^{2+} overload [31]. On the contrary, in conditions of cell stimulation, when the [Ca^{2+}] in the close proximity of mitochondria reaches values up to 10 µM, mitochondrial Ca^{2+} uptake has to rapidly increase [31], in order to fulfil the energy requirements of a stimulated cell [31]. MCU structure consists of a small loop facing the intermembrane space and the N- and C-termini residing in the matrix and does not present any Ca^{2+} binding domains [7,8]. From this evidence, it is clear that MCU alone is not responsible for the sigmoidal response to extra-mitochondrial [Ca^{2+}]. Therefore, the existence of a highly sophisticated gatekeeping mechanism including both negative modulators acting a low [Ca^{2+}] and activators acting a high [Ca^{2+}] was postulated (Fig. 3 and [9]).

It was demonstrated that the proteins responsible for the sigmoidal response to extra-mitochondrial [Ca^{2+}] belongs to the MICU (as Mitochondrial Calcium Uptake protein) family of mitochondrial EF-hand Ca^{2+}-binding domain containing proteins (Fig. 3 and [34]).

MICU1 (previously known as EFHA3) was the first member of this family to be identified, even before the identification of MCU, as a critical modulator of mitochondrial Ca^{2+} uptake, through an integrative strategy that fuses comparative physiology, evolutionary genomics, organelle proteomics, and RNAi screenings [12]. This 54 kDa protein possesses two EF-hand Ca^{2+} binding domains and a putative transmembrane domain. Several studies demonstrated that MICU1 and the other components of the MICU family localizes in the IMS [11,81,82]. Several hypotheses on MICU1 function have been proposed. Firstly, MICU1 was proposed to be essential for mitochondrial Ca^{2+} uptake, since its silencing was shown to completely inhibit this process [12]. Subsequently, Madesh and co-workers demonstrated that mitochondria lacking MICU1 are constitutive loaded with Ca^{2+} and proposed a gatekeeper role for this protein [83]. Later, Hajnoczky's group confirmed the gatekeeper role of MICU1 at low $[Ca^{2+}]_{cyt}$ and they also showed that, in the absence of MICU1, the cooperativity of mitochondrial Ca^{2+} uptake was lost, suggesting a dual role of MICU1 according to $[Ca^{2+}]_{cyt}$ [84]. The relevance of MICU1 in the regulation of mitochondrial Ca^{2+} uptake was also demonstrated in human patients in which loss of function mutations in MICU1 gene cause myopathy and learning difficulties due to mitochondrial Ca^{2+} overload [85].

The discovery of MICU1 was soon followed by the identification of MICU1 paralogs, MICU2 (Mitochondrial Calcium Uptake protein 2, previously known as EFHA1) and MICU3 (Mitochondrial Calcium Uptake protein 3, previously known as EFHA2), also characterized by the presence of two conserved EF-hand domains (Fig. 3 and [13]). Even if they derived from gene duplication in vertebrates, they show a different expression profile among different tissues [13]. Indeed, MICU3 is specifically expressed in the central nervous system (see below for details) and, at lower levels, in skeletal muscle while MICU2 is ubiquitously expressed with a similar expression pattern of MICU1 [13,86].

As MICU1, MICU2 displays two well-conserved EF-hand domains and several studies demonstrated that it is located in the IMS [82,84]. Interestingly, MICU2 stability depends on the presence of MICU1 and MICU1 knockdown causes also the destabilization of MICU2 protein, without affecting MICU2 mRNA levels [13,77]. Indeed, MICU2 forms obligate heterodimers with MICU1, stabilized

by a disulphide bond through two conserved cysteine residues [77]. Our laboratory demonstrated that the MICU1-MICU2 heterodimer is responsible for one of the most peculiar properties of mitochondrial Ca^{2+} uptake, i.e. the sigmoidal response to increasing $[Ca^{2+}]_{cyt}$ [77]. In detail, in resting conditions, the MCU complex is inhibited to prevent mitochondrial Ca^{2+} overload (Fig. 3, left panel and [77]). However, when $[Ca^{2+}]_{cyt}$ increases, the MCU complex is subjected to a cooperative activation that ensures the prompt response of mitochondria to cell challenge (Fig. 3, right panel and [77]). Overall the regulation of MCU complex activity by MICU1-MICU2 heterodimers is possible thanks to the ability of these proteins to sense Ca^{2+} concentration through their EF-hand domains [77,87].

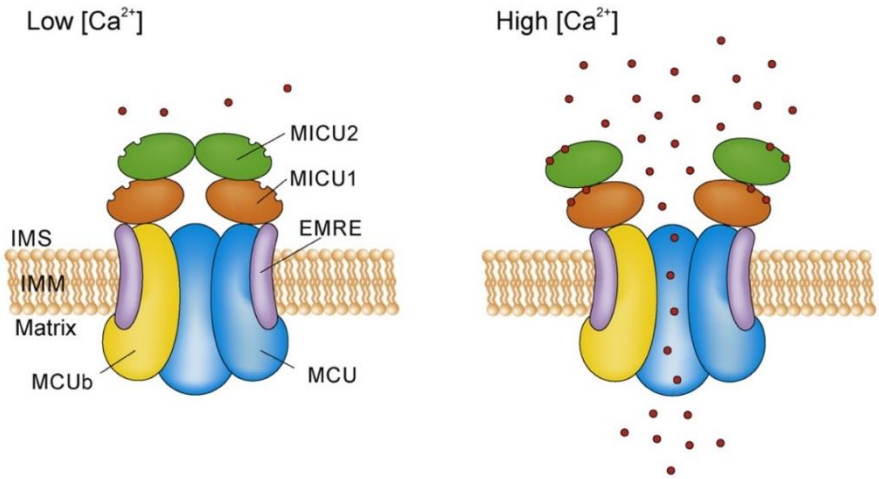


Figure 3. MCU comprises pore-forming and regulatory subunits that are responsible for the sigmoidal response to extra-mitochondrial [Ca^{2+}] (Adapted from C. Mammucari et al., Biochim. Biophys. Acta - Mol. Cell Res, 2016).
MCU is a macromolecular complex composed by pore-forming (MCU, MCUb and EMRE) and regulatory (MICU1 and MICU2) subunits. The regulatory subunits contribute to regulate MCU channel activity at both low (left panel) and high (right paned) $[Ca^{2+}]_{cyt}$ by avoiding a massive energy dissipation and the induction of cell death in resting conditions and to respond to the needs of a stimulated cell. The two main control mechanisms are mediated by MICU1 and MICU2 heterodimers.

3.2.5.2 MICU1.1 and MICU3 regulators of MCU channel activity

The regulation of MCU channel activity is a sophisticated mechanism that requires the presence of different components to avoid a massive energy dissipation

and cell death in resting conditions and to fulfil the needs of a stimulated cell [9]. This concept, beyond the already established role of MICU1 and MICU2 in MCU channel activity, led to the discovery of other regulatory subunits.

It is well known that mitochondrial Ca^{2+} plays a key role in energy metabolism since it stimulates ATP production by regulating oxidative phosphorylation [74]. This regulation is particularly crucial in skeletal muscle, being one of the most ATP consuming organ in the body [9]. Therefore, it is thus not surprising that, compared with other tissues, skeletal muscle mitochondria display high Ca^{2+} conductance [9] and that skeletal muscle expresses a unique MCU Ca^{2+} uptake machinery [88]. Indeed, recently, Vecellio Reane and co-workers discovered an alternative splicing variant of MICU1, that was named MICU1.1 expressed exclusively in skeletal muscles [88], characterized by the addition of a micro-exon coding for four aminoacids that greatly modifies the properties of MCU. Indeed, MICU1.1 binds Ca^{2+} one order of magnitude more efficiently than MICU1 and, when heterodimerized with MICU2, activates MCU current at lower Ca^{2+} concentrations than MICU1-MICU2 heterodimers [88]. *In vivo* injection of antisense oligonucleotides mediating exon skipping of the MICU1.1 extra exon, and thus forced expression of MICU1 instead of MICU1.1, demonstrated that the latter is required for maintaining sufficient levels of mitochondrial Ca^{2+} uptake to provide sufficient levels of ATP needed for contraction [88]. Overall these results demonstrate a novel mechanism of the molecular plasticity of the MCU Ca^{2+} uptake machinery and open the field to the study of other tissue-specific regulatory mechanisms of mitochondrial Ca^{2+} uptake,

3.2.6 The physiological role of mitochondrial Ca^{2+} uptake

3.2.6.1 Role of MCU in non-muscle tissues

The discovery and characterization of the molecular components of the MCU complex opened a new era in the clarification of the physiological relevance of mitochondrial Ca^{2+} uptake in different tissues and physiopathological conditions.

The first study, in chronological terms, examined the role of MCU in glucose-induced ATP production in pancreatic β cells [89]. In these cells, the glucose-dependent stimulation of oxidative metabolism induces an increase in cytosolic ATP concentration that induces the closure of ATP-sensitive potassium channel in the plasma membrane [90]. This event leads to cell membrane depolarization and Ca^{2+} influx via voltage-gated Ca^{2+} channel leading to the first phase of insulin secretion [90]. In addition, beyond this triggering pathway, glucose activates an essential amplifying pathway that increases the action of Ca^{2+} on exocytosis [90]. In this regard, mitochondrial [Ca^{2+}] increases are fundamental for the amplification of glucose-dependent insulin secretion [91]. The discovery of MCU confirmed that Ca^{2+} entry through the plasma membrane induces mitochondrial Ca^{2+} uptake through the MCU complex that, by sustaining oxidative phosphorylation, promotes ATP production and contribute to insulin release [89,92,93]. Interestingly, defects in glucose sensitivity and function of β cells, which characterizes type 2 diabetes (T2D), are accompanied by alterations of β cells Ca^{2+} homeostasis [94]. Since the MCU complex, and thus the regulation of mitochondrial Ca^{2+} uptake, play a key role in this scenario, the modulation of mitochondrial Ca^{2+} uptake through pharmacological intervention on the MCU complex might be considered as a new approach to improve glucose tolerance.

Because of the fundamental role that mitochondrial Ca^{2+} plays in triggering apoptotic cell death [64], the discovery of the molecular identity of MCU and regulators shed new light in the investigation of the role of mitochondrial calcium in cancer progression. The finding that either the overexpression of MCU or the silencing of its regulatory subunits in an immortalized cell line, Hela cells, lead to a huge increase of mitochondrial Ca^{2+} uptake and to cell death is coherent with the notion that an excessive mitochondrial Ca^{2+} accumulation causes cell death [7]. Strikingly, mouse embryonic fibroblasts (MEFs) derived from the total MCU KO mice, proliferated at an indistinguishable rate when compared to wild type MEFs [95] . This suggest that there are either different requirements for MCU between primary and immortalized cell lines, or more likely, there are significant differences between acute versus chronic MCU knockdown [96]. This evidence was also confirmed by the lack of protection from cell death in hearts from the total MCU

KO animals (see Introduction, paragraph 3.2.6.2 and [95]). Another important study demonstrated that increased amount of miR-25 reduces MCU protein levels and confers resistance of cancer cells to apoptotic stimuli [97]. On the opposite, in our laboratory it has been demonstrated that MCU expression correlates with breast cancer progression in triple negative breast cancer that is associated with poor prognosis [98]. Coherently, the same study showed that MCU depletion inhibits *in vivo* tumor growth and metastasis formation with a mechanism that reduces ROS formation and HIF-1α expression [98].

The role of mitochondrial Ca^{2+} homeostasis has been also investigated in cortical neurons. Importantly, neurons are almost exclusively dependent on OXPHOS as main source of ATP and Ca^{2+} entry into mitochondria guarantees activity-dependent regulation of cellular energy metabolism [99]. Neuronal activity not only contributes significantly to ATP consumption, but also rapidly adapts to increased activity by stimulating ATP synthesis through a mitochondrial Ca^{2+}-dependent increase in OXPHOS [58]. Not only mitochondria are responsible for ATP production needed for neuronal activity, but also exerts other neuron-specific functions. Indeed, their cellular distribution contributes to the accumulation of large amount of Ca^{2+} in a defined subcellular domain, promoting large local cytoplasmic Ca^{2+} rises [58]. Importantly, Ca^{2+} sequestration by mitochondria profoundly affects neurotransmitter release, being strategically located in the proximity of Ca^{2+} channels such as NMDAR at the synaptic terminal [60]. In general, mitochondria recruitment to neuronal soma, synapses and dendritic spines is crucial for the regulation of nerve activity and any change in the positioning of mitochondria to subcellular domains affects neuron physiology [61].

Interestingly, MCU overexpression sustains NMDAR-dependent increases in mitochondrial Ca^{2+} and causes loss of mitochondrial membrane potential and cell death [100]. This study demonstrated that the expression of MCU is suppressed by synaptic activity that promotes neuroprotection through a mechanism that involves nuclear Ca^{2+} and calmodulin (CaM) kinase-mediated induction of a specific transcription factor the Neuronal pas domain protein 4 (Npas4) [100]. The importance of MCU channel activity in brain has been also confirmed by the discovery that loss of function mutations of MICU1 gene occurring in patients

affected by a neuromuscular disease are characterized by learning difficulties and extrapyramidal disorder [85].

3.2.6.2 Role of MCU in cardiac physiopathology

Cardiac mitochondria are the major source of ATP, required for heart function. As a consequence, the inhibition of mitochondrial ATP synthesis leads to loss of contractile capacity and cell death [101]. While the importance of mitochondrial metabolism in the heart is undisputed, the role of mitochondrial Ca^{2+} uptake in heart pathophysiology remains controversial [9].

Patch clamp measurements of MCU Ca^{2+} current (I_{MCU}) demonstrated that mitoplasts from mouse heart show a substantially smaller I_{MCU} than other mouse tissues and in particular skeletal muscle [14]. The notion that skeletal muscle and heart mitochondrial Ca^{2+} currents share the same biophysical properties suggested that the differences in current density are not due to differences in the proteins responsible for channel activity but they are probably due to different explanations:

i. In the heart, mitochondria occupy up to 37% of the cell volume and therefore it is reasonable that these organelles accumulate less Ca^{2+} not to abolish the cytosolic Ca^{2+} transients necessary for heart beating;

ii. Frequent elevation of $[Ca^{2+}]_{cyt,}$ if efficiently translated into mitochondrial Ca^{2+} uptake, could lead to futile cycling of Ca^{2+} across the IMM, mitochondrial Ca^{2+} overload and cell death.

Despite the importance of mitochondrial Ca^{2+} buffering in cardiac physiology, the heart phenotype of the first model of MCU KO mouse was surprisingly mild [95,102].

Indeed, cardiac mitochondria derived from total MCU KO animals showed an impairment in mitochondrial Ca^{2+} uptake and Ca^{2+} dependent oxygen consumption was not translated into less ATP production, suggesting that MCU depletion does not affect basal mitochondrial metabolism [102]. More in detail, mice lacking MCU show normal basal cardiac function in terms of ejection fraction, fractional shortening, stroke volume and chamber size, both in adulthood (12-month old mice) and in aged animals (20-month old mice) [102]. In addition, no differences between WT and MCU KO mice were observed in the left ventricular cardiac output at baseline and after isoproterenol stimulus, which mimics the fight-

or-flight response, i.e. an episode of high-energy demand triggered by catecholamine-induced heart acceleration. Also, when mice were subjected to surgical transverse aortic constriction (TAC), as a model of chronic stress, MCU KO hearts showed the same cardiac parameters measured in the WT [102]. Further experiments were carried out to assess the role of MCU during ischemia-reperfusion (I-R) injury. MCU KO hearts show no sign of I-R injury protection [95]. In detail, measurements of the rate pressure product and direct assessment of the infarct area in post-ischemic recovery period, indicate no difference between MCU KO animals and the controls [95]. Interestingly, when treated with cyclosporine A (CsA), that inhibits Ca^{2+}-dependent cell death mediated by the opening of mPTP, WT hearts were protected from I-R injury, while MCU KO hearts were not [95]. This result suggests that an mPTP-independent death pathway occurs in the absence of MCU. Noteworthy, the birth ratio of the MCU KO mice, which are in an outbred strain composed by a mix of CD1 and C57/BL6 backgrounds, is lower than expected [95] and, in a inbred strain, MCU deletion is embryonically lethal [95]. One potential explanation is that in CD1 mice, there is a compensatory gene product that allows for mitochondrial Ca2+ uptake, and that this gene product is absent in the C57/BL6 strain [96].

Importantly, similar results have been obtained from the analysis of a transgenic mouse expressing a dominant-negative MCU isoform, $MCU^{D260Q,E263Q}$ (DN-MCU), in the same mixed background of the constitutive MCU KO model [103]. When overexpressed in cultured cells, the DN-MCU does not completely abolish organelle Ca^{2+} accumulation, although mitochondria from DN-MCU-expressing hearts have no measurable mitochondrial Ca^{2+} uptake [103]. DN-MCU mice show normal heart rate, however they display an impairment in the "fight or flight" response. Additionally, oxygen consumption rate (OCR) is increased in DN-MCU isolated perfused heart, but not in permeabilized fibers or isolated mitochondria [104]. Moreover, DN-MCU heart present a higher diastolic cytosolic $[Ca^{2+}]$, consistent with the loss of mitochondrial buffering. This cytosolic Ca^{2+} increase is partially rescued by the addition of ATP, suggesting that these cardiomyocytes display an extramitochondrial adaptation that depends on the

reduced ATP availability. Importantly, similarly to MCU total KO model, DN-MCU hearts are not protected against I-R injury [104].

Next, to exclude phenomena of adaptations to embryonic long-term loss of mitochondrial Ca^{2+} uptake, an inducible mouse model in which MCU was specifically deleted in cardiomyocytes was developed. As opposed to total MCU KO and DN-MCU mouse models [95,103], in the inducible cardiac-specific model, MCU ablation strongly protects hearts from I-R injury [105,106]. Finally, studies on the cardiac-specific inducible MCU KO mouse, that allows the MCU ablation in adults, confirmed the impairment in the fight-or-flight response triggered by β-adrenergic stimulation [105,106], as observed in the DN-MCU transgenic mouse model [103]. In this contest, MCU has been identified as a target of miRNA 25 and MCU protein levels inversely correlates with miR-25 expression [97,107]. Expression of miR-25 in cardiomyocytes is increased upon oxidative stress and overexpression of miR-25 decreases mitochondrial Ca^{2+} uptake and protects cardiomyocytes from oxidative stress and thus from cell death, suggesting an MCU-dependent protective role of miR-25 in cardiac pathology [107].

3.2.6.3 Role of MCU in skeletal muscle homeostasis

In contrast to heart tissue, only 5% of skeletal muscle cell volume is occupied by mitochondria [9]. Skeletal muscle mitochondria display much higher I_{MCU} compared to the heart, possibly because they are not exposed to frequent Ca^{2+} elevations [14]. As mentioned above (see Introduction, paragraph 3.2.6.2), MCU KO mice show a mild phenotype, although skeletal muscle is the most affected tissue [95]. Indeed, stimulation of O_2 consumption by Ca^{2+} is suppressed in MCU KO muscle mitochondria although matrix levels were reduced only of about the 75% [95]. In addition, in MCU KO animals, the phosphorylation of pyruvate dehydrogenase (PDH) was increased and thus its activity inhibited, in line with the Ca^{2+} dependent regulation of pyruvate dehydrogenase phosphatase 1 (PDP1) [95]. Importantly, since mitochondrial Ca^{2+} stimulate ATP production which is necessary for muscle function, MCU KO mice present a reduced maximal power output [95].

In order to overcome the possible compensatory effects of the MCU KO mouse during embryonic development and to better characterize the

physiopathological role of MCU in skeletal muscle homeostasis, our laboratory studied the effects of the overexpression and silencing of MCU in skeletal muscle through infection with adeno-associated viral vectors (AAVs) of skeletal muscle of neonatal and adult mice [108]. Interestingly, we demonstrated that MCU overexpression triggers skeletal muscle hypertrophy while, coherently, MCU silencing causes muscle atrophy both during post-natal growth and in adulthood [108]. In addition, MCU overexpression protects muscles from the loss of muscle mass occurring during denervation-induced atrophy in mouse muscles, indicating a potential therapeutic role of MCU modulation in muscle atrophy [108]. Interestingly, MCU, and thus mitochondrial Ca^{2+}, promotes muscle hypertrophy by activating the IGF-1-Akt/PKB-mediated pathway, that is known to induce protein synthesis and thus the growth of muscle [109]. Furthermore, the modulation of MCU expression controls global gene expression by, for example, modulating the expression of peroxisome proliferator-activated receptor gamma coactivator 1-alpha 4 (PGC-1α4) [108]. The latter is a PGC-1α isoform that is not involved in mitochondrial biogenesis and whose overexpression has been shown to induce muscle hypertrophy [110]. Moreover, the concept that in skeletal muscle mitochondrial Ca^{2+} uptake is fundamental for tissue homeostasis was sustained by the evidence that a loss-of-function mutations of MICU1 gene in patients causes a neuromuscular disorder characterized by proximal myopathy [85].

Recently, in our laboratory, it has been demonstrated that skeletal muscle mitochondria express a unique MCU complex containing an alternative splice isoform of MICU1, named MICU1.1, demonstrating the molecular plasticity of the MCU Ca^{2+} uptake machinery of skeletal muscle mitochondria (see Introduction, paragraph 3.2.5.2 and [88]).

Altogether, these findings clearly demonstrate that mitochondrial Ca^{2+} signalling in skeletal muscle is fundamental in human pathophysiology. In this regard, the regulation of mitochondrial Ca^{2+} uptake, mediated by the MCU complex, might have an active role during pathophysiological processes. Indeed, recently, we found that the dominant-negative subunit of the MCU complex, MCUb, is highly and specifically upregulated during skeletal muscle regeneration that occurs after muscular injury (see Results paragraph and fig. 10A). Since

alteration in skeletal muscle regenerative capacity characterizes several muscular diseases such as Duchenne Muscular Dystrophy (DMD) [111], the understanding of the role of mitochondrial Ca^{2+} uptake in the regeneration process might be fundamental for understanding the pathogenesis of these diseases and for the development of novel therapeutic interventions.

3.3 Characterization of skeletal muscle regeneration process

Skeletal muscle is the most abundant tissue in the body and is essential for breathing, posture maintenance and locomotion but it also plays important homeostatic and metabolic roles such as heat production and carbohydrate and amino acid storage [112]. Coherently, loss of muscle functionality, occurring in acute and chronic conditions, results in diminishes mobility and strength and leads to metabolic disorders that can have lethal consequences [112]. Compared to other tissues which cannot regenerate after injury, skeletal muscle is able to fully regenerate after mechanical trauma, exposure to toxins or infections [113]. When muscle regenerative capacity is altered, a persistent myofibers degeneration and/or inflammation and excessive extracellular matrix (EMC) deposition is observed, leading to the substitution of the normal muscle architecture by fibrotic tissue [114].

Importantly, skeletal muscle repair is compromised during ageing and in patients affected by muscular dystrophy such as DMD [25]. The ability of muscle to regenerate primarily depends on a specific population of normally quiescent muscle stem cells called satellite cells that are intimately associated with muscle fibers (Fig. 4 and [115]). Satellite cells are myogenic precursor cells (MPCs) that reside in a quiescent state on the surface of fully differentiated myofibers, beneath the plasma membrane and basal lamina that surround muscle fibers [115].

The regeneration of skeletal muscle relies on processes that reuse programmes of gene expression and signalling pathways that characterize the embryonic growth of muscle [115]. In detail, soon after muscle damage, satellite cells escape quiescence and start to proliferate [115]. The pool of proliferating satellite cells follows different fate. Indeed, some daughter cells differentiate

whereas others go back to quiescence in order to reconstitute the pool of satellite cells that will be indispensable for further rounds of skeletal muscle regeneration (Fig. 4 and [115]).

Proliferating MPCs, derived from activated satellite cells and called myoblasts, continue to differentiate and then will form long, cylindrical and multinucleated myotubes. The latter then undergo terminal differentiation to become mature muscle fibers (Fig. 4 and [115]). The process of skeletal muscle regeneration is accompanied by changes in the expression of myogenic transcription factors, PAX7, MYOD, MYF5 and MYOG (Fig. 4 and [116–118]). PAX7 targets many genes implicated in satellite cell function, including genes involved in cell growth, cell adhesion and signalling pathways, whereas it represses genes involved in differentiation [116]. MYF5, MYOD, myogenin and MRF4 are transcription factors that regulate satellite cells proliferation and differentiation into myofibers [118]. Indeed, the MRF family of proteins controls the transcription of important muscle-specific proteins such as myosin heavy chain and muscle creatine kinase [118].

Satellite cells are myogenic precursor cells (MPCs) that reside in a quiescent state on the surface of muscle fibers. During the quiescence MPCs are characterized by the expression of specific myogenic regulatory genes, they express paired box transcription factor protein 7 (PAX7) but they do not express myoblast determination protein 1 (MYOD) and myogenin (MYOG) and may or may not express myoblast determination factor 5 (MYF5) so they are identified as $PAX7^{+}MYOD^{-}MYOG^{-}MYF5^{+/-}$ (Fig. 4 and [119]).

After muscular injury, exercise or muscular perturbations, MPCs become activated and start to proliferate to give rise to daughter cells that can have the same developmental fate (symmetrical division) or cells that does not have identical developmental path (asymmetrical division) [115]. In the latter case, many of the activated and proliferative cells start to express MYOD and these MPCs can be identified as $PAX7^{+}MYOD^{+}$ cells (Fig. 4). Other daughter cells return to quiescence and are characterized as $PAX7^{+}MYOD^{-}MYOG^{-}$ cells (Fig. 4). If the cells continue to differentiate and lose their ability to self-renew, they downregulate the expression of PAX7 and start to express MYOG, that is a transcription factor

required for further differentiation and survival [115]. These MPCs, identified as $PAX7^{-}MYOD^{+}MYOG^{+}MYF5^{+/-}$ cells have the capacity to fuse with other MPCs to form multinucleated myotubes that start to grow and to express genes required for terminal differentiation (Fig. 4). Many of these genes are regulated by MYOG that has been shown to promote the last stages of differentiation and consequent formation of new muscle fibers [115]

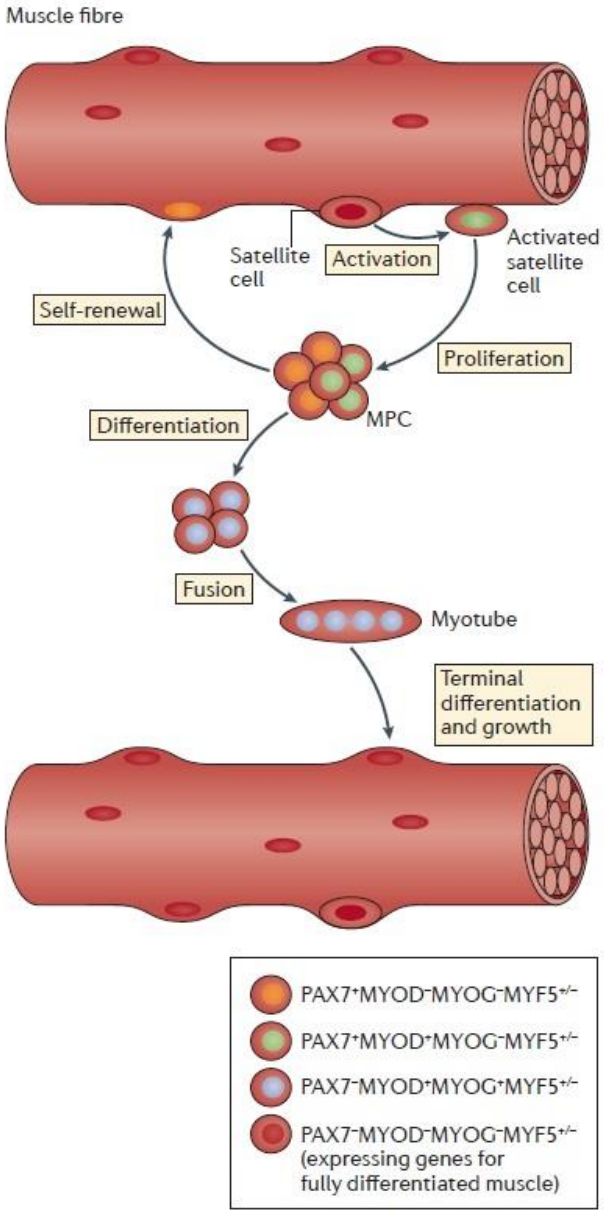


Figure 4. Key events in myogenesis and skeletal muscle regeneration (Adapted from J.G. Tidball et al., Nature Reviews Immunology, 2017).

3.3.1 Regulation of muscle growth and regeneration by the immune system

Satellite cells and their progeny are essential for skeletal muscle regeneration since it has been shown that their depletion abolishes the regeneration of injured muscles [18,120]. However, the presence of satellite cells is not sufficient for an efficient skeletal muscle repair and many additional cell types play an active role to promote tissue repair. Among these are resident cells within the skeletal muscle niche such as mesangioblasts, fibro/adipogenic progenitors (FAPs) and other ECM-associated cells [121,122]. In particular, recent findings demonstrated the relevance of the interplay between satellite cells and fibroblasts and/or FAPs as a determinant factor for the efficiency of the skeletal muscle repair process [120]. Indeed, specific deletion of fibroblast, using genetic approaches, causes an impaired regeneration due to the lack of proliferation of satellite cells and their premature differentiation, suggesting a paracrine effect of fibroblasts on muscle cells [120]. Furthermore, FAPs that are activated to the fibrogenic phenotype, are the primary producers of connective tissue, the major component of the ECM. The latter surrounds the existing muscle fibers providing a structural support for muscle contraction and acts as a scaffold in which new myofibers will be formed during skeletal muscle repair [123]. The amount of ECM is crucial for muscle repair since on one hand correct remodelling and reorganizing of muscle ECM after damage is necessary for the correct spatial organization of the newly formed myofibers but on the other hand excessive ECM deposition leads to fibrosis provoking a defective regenerative outcome [123].

Other key players in the complex scenario of skeletal muscle regeneration are the inflammatory cells that infiltrate the injured muscle and appear to be the most critical cell type, together with satellite cells, for successful regeneration [24]. Among the inflammatory cells, monocytes/macrophages play the major role in this repair process [24].

It is known that the healing process consists of overlapping phases of inflammation, tissue formation, and remodelling with reorganization of the ECM [15]. Macrophages participate in all the different phases of tissue repair by promoting the phagocytosis of myofiber debris and apoptotic neutrophils and by releasing growth factors, cytokines and inflammatory mediators that influence satellite cells behaviour during the repair process [124].

3.3.2 Macrophages: the general framework

Macrophages were formerly identified as phagocytic cells responsible for pathogen elimination and housekeeping functions in a wide range of organisms [125]. They were included in the mononuclear phagocyte system, a population of cells derived from bone marrow progenitors, the myeloid progenitors, that enter the blood as monocytes and once they reach the peripheral tissues can differentiate into macrophages or antigen presenting cells [126]. Monocyte half-life in the blood is about one day and this sustains the notion that blood monocytes replenish macrophage or dendritic cells (DC) pools in peripheral tissues to maintain their homeostasis [127]. Macrophages participate to the immune cellular response to invading pathogens by creating an active link between innate and adaptive immunity by regulating T lymphocyte activation and by modulating their phenotype and functions [16]. Macrophages recognizes epitopes expressed by apoptotic cells through dedicated pattern-recognition receptors (PPRs) [16]. PPRs allow the identification of molecular structures shared by several classes of microbes known as pathogen-associated molecular patterns (PAMPs) [128]. Once activated, PPRs promote the activation of a cascade of coordinated events that culminate in the activation of innate and adaptive responses [16].

Another role that can be ascribed to macrophages is associated to matrix remodelling and neoangiogenesis [16]. Furthermore, they have been implicated in conditions in which neonatal angiogenesis is potentially deleterious such as cancer [129], but also in non-neoplastic conditions such as rheumatoid arthritis or

endometriosis [130–132]. Overall, these evidence suggest the ability of macrophages to respond to injury of various tissues and to activate homeostatic programs that, through the clearance of apoptotic cells, the organization of neovessel formation, the reconstitution of extracellular matrix and activation of T-lymphocyte response, promote an effective tissue healing [133]. Nevertheless, several studies demonstrated a phenotype heterogeneity in macrophages that might reflect peculiar properties and functions of macrophages sub-populations within specific microenvironments [133].

3.3.2.1 Macrophage polarization state in tissue repair: pro (M1) and anti (M2) inflammatory phenotypes

The classification between inflammatory and anti-inflammatory macrophages represents an over-simplification of a more sophisticated array of functions exerted by macrophage populations in injured tissues [134]. Polarized macrophages have been recently classified as either M1 or M2 that respectively reflect the classical or alternative activation [134,135]. The pro-inflammatory M1 macrophages become activated after exposure to the T-helper 1 cytokines interferon γ (IFNγ) and tumor necrosis factor-α (TNF-α), in addition to lypopolysaccaride (LPS) or endotoxin [134]. Macrophage polarization into an anti-inflammatory M2 phenotype is a more complex process than M1 polarization. Indeed, it is possible to distinguish three possible subtypes, currently defined according to the different physiological roles [15,24]. In detail, alternatively activated or M2a macrophages participate to the last stages of tissue repair and wound healing after damage and arise from exposure to T-helper 2 cytokines like interleukin 4 (IL-4) and interleukin 13 (IL-13) [24]. M2c macrophages are activated by macrophage polarization with IL-10 and have an anti-inflammatory function. Similarly to M2c macrophages, M2b macrophages play an anti-inflammatory role and can release large amounts of IL-10 [24]. In the context of skeletal muscle repair, pro-inflammatory macrophages participate to the early stages after skeletal muscle injury by promoting a pro-inflammatory response [24]. On the opposite, the anti-inflammatory macrophages dominate the last stages of skeletal muscle repair by dampening the pro-inflammatory response and sustaining muscle repair after damage [24]. Details

about the role of macrophage subpopulations in skeletal muscle repair will be discussed in the next paragraph.

3.3.2.2 Role of macrophages in skeletal muscle repair

Several mouse models of acute muscle injury represent an attractive system for exploring the interactions between the immune system and tissue regeneration, since the onset of tissue damage is well defined and the time course of inflammation and regeneration is predictable [16]. The most used models include intense eccentric contraction, that causes structural damage in a large portion of fibers, muscle freezing, crushing or injection of snake toxins that cause a huge amount of damage that destroys almost all fibers at the site of injury [22]. Importantly, skeletal muscle sterile injury (i.e after toxin injection) triggers a potent inflammatory response characterized by a rapid and sequential invasion of leukocyte populations that persist during muscle repair, regeneration and growth [15].

Neutrophils are the first leukocyte population to invade the damaged tissue [15]. Indeed, they appear within 2 hours from muscle damage, reaching the maximum number between 6 and 24 hours post injury and then rapidly decrease [15]. The early invasion of injured muscle by neutrophils is a generic and fundamental response to acute muscle damage and their arrival influences the inflammatory environment by influencing the activation state of subsequent immune cell populations [16].

Soon after neutrophils invasion, macrophages begin to accumulate and become the dominant leukocyte population present at the site of injury [15]. The release of cytokines and chemokines, especially CC-chemokine ligand 2 (CCL2) and CXC-chemokine ligand 1 (CXCL1) from neutrophils and resident macrophages promotes the recruitment of immune cells to an inflammatory response [16]. Circulating monocytes extravasate and enter into muscle enriched by pro-inflammatory cytokines including IFNγ and TNF-α released by neutrophils [16]. These cytokines activate macrophages to a pro-inflammatory phenotype [16]. These macrophages are therefore called pro-inflammatory macrophages or M1 macrophages [16]. M1 macrophages reach their maximum peak 2 days post injury and release pro-inflammatory cytokines such as TNF-α, IL-1β and also insulin-like

growth factor 1 (IGF1), which increase MPCs proliferation and further expand the MPCs population [16]. Indeed, the inflammatory response during the early stages of skeletal muscle regeneration occurs in parallel with the initial stages of myogenesis, when satellite cells are activated and begin to proliferate and differentiate [16]. A common marker of M1 macrophages is the inducible nitric oxide synthase (iNOS), which is required to efficiently metabolize L-arginine into nitric oxide (NO), necessary for killing intracellular pathogens during infection [16]. Phagocytosis of apoptotic neutrophils suppresses the expression of TNF-α and phagocytosis of apoptotic and necrotic neutrophils and myogenic cells increases the expression of TGF-β which causes the polarization of macrophages towards an anti-inflammatory phenotype [15,16].

The anti-inflammatory, commonly known as M2 macrophages, are also activated by several ligands like fibrinogen amphiregulin (AREG), IL-10 produced by T regulatory cells (T_{reg}), the IL-10 and IGF-1 produced by macrophages [16]. M2 macrophages, that reach peak number approximately 3 to 5 days post injury, suppress the pro-inflammatory response by releasing TGF-β and IL-10 and, at the same time, sustain fiber reconstitution by releasing cytokines that have trophic functions [15,16]. Among these, IL-10 plays a key role in sustaining cell viability and allows the differentiation and fusion of MPCs into terminal differentiated myofibers [15–17]. Common surface markers of M2 macrophages are mannose receptor type 1 (MRCI), commonly known as CD206 and CD163 [16,136]. The latter was identified as a transmembrane glycoprotein that is constitutively expressed by the M2c subpopulation, although its expression can by induced by specific cytokines such as IL-10 [16]. CD163 has been demonstrated to regulate macrophage phenotype and to promote muscle regeneration [137,138]. The M2a subpopulation also expresses arginase 1 (ARG1), an enzyme that has an important role in fibrosis through the metabolism of arginine, that produces polyamine which stimulate fibroblast proliferation and ornithine, which produces proline required for collagen production [139,140]. These evidence demonstrate that M2 macrophages support the production of connective tissue, the major component of ECM, thus playing a key role in the reconstitution of muscle structure. The key steps of the

time course of skeletal muscle regeneration process and the interplay between MPCs and macrophages are summarized in Fig 5.

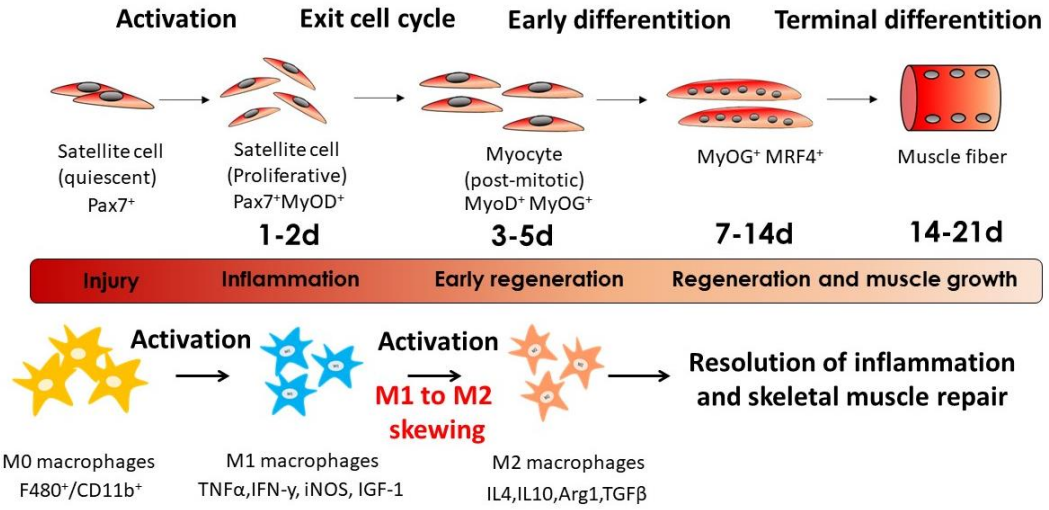


Figure 5. Schematic view of the changes in macrophage phenotype and stages of myogenesis during skeletal muscle regeneration after injury (Adapted from J.G. Tidball et al., Development, 2014).

3.3.2.3 Cellular and molecular pathways involved in the regulation of macrophage phenotypic transition

The mechanisms involved in the phenotypic transition of macrophages from an M1 to an M2 phenotype are still under investigation. The discovery of a possible mechanism by which IL-10 mediates changes in macrophage phenotype came from studies on the involvement of metabolic changes on the M1 to M2 polarization [141]. In detail, it was shown that AMP-activated protein kinase (AMPK) plays regulatory roles in inflammation since it blocks ATP consuming pathways in cells where ATP levels are low [141]. Since M1 macrophages are more glycolytic and M2 macrophages are more oxidative, AMPK levels differ accordingly to the polarization state [142]. Indeed, the anti-inflammatory cytokines IL-10 and TGF-β phosphorylate and thus activate AMPK while pro-inflammatory stimuli inhibit AMPK [143]. Furthermore, it has also been demonstrated that AMPK promotes M1 to M2 macrophage polarization [20]. Indeed, it has been shown that knockdown of AMPK increases the expression of pro-inflammatory cytokines whereas overexpression of the AMPK catalytic subunit α1 in macrophages reduces TNF-α but increases IL-10 expression [144]. Moreover, myeloid cell-specific deletion of

AMPK α1, in a CTX-induced muscle injury, impairs M2 macrophages polarization and phagocytic capacity and affects skeletal muscle regeneration [20]. These evidence clearly sustain the idea that phagocytosis of tissue debris, occurring during the first stages of skeletal muscle repair, might contribute to the M1 to M2 macrophage skewing through an AMPK-dependent mechanism [20] .

3.3.2.4 Calcium signalling in macrophage functions and plasticity

Beyond the fundamental role in cellular bioenergetics, ATP plays a crucial role in the extracellular space as a signalling molecule [145]. ATP and its metabolites are ligands of a family of receptor known as purinergic receptors that are ubiquitously expressed [145]. In the immune system, purinergic signals are involved in cell migration and activation of immune cells and can be involved in the resolution of inflammation [146]. Signalling pathways that are induced after activation of purinergic receptors are coupled with Ca^{2+} signalling. In particular, it has been demonstrated that ATP binds purinergic metabotropic P2Y receptors (G-protein coupled receptors) and ionotropic P2X receptors (Ca^{2+} channel) located on macrophage plasma membrane and can induce Ca^{2+} elevations in myeloid cells [147]. P2Y receptors have more affinity for ATP than P2X and can lead to Ca^{2+} mobilization from intracellular stores at low micromolar concentration of extracellular ATP. The ATP dependent increases in $[Ca^{2+}]_{cyt}$ are also associated with the secretion of pro-inflammatory cytokines such as IL-β and IL-18 [146]. In parallel, it has been demonstrated that adenosine, generated by the action of the membrane-bound ectonucleotidase that converts ATP and ADP into adenosine, by binding its metabotropic receptor, transmits a signal that might orchestrate the resolution of inflammation [146]. Indeed, extracellular adenosine has been demonstrated not only to increase the polarization of macrophages towards the M2 phenotype but also to inhibit the secretion of pro-inflammatory cytokines like TNF-α or IL-6 and IL-8 [148]. Studies in both human patients and mice demonstrated that purinergic signals play a major role in inflammation and tissue injury [146]. Since purinergic signals and the resulting Ca^{2+} dynamics play a key role in macrophage polarization state, it would be interesting to provide new therapeutic approaches to regulate macrophage polarization and function.

3.3.2.5 Mitochondrial Ca^{2+} uptake in M1 and M2 macrophage skewing

The evidence that in skeletal muscle mitochondrial Ca^{2+} uptake not only stimulates the rate of ATP production, by regulating the rate of oxidative phosphorylation, but also controls trophism [108], suggests that mitochondrial Ca^{2+} might play key roles that overcome its metabolic function. Recently, in line with these observation, we found that mitochondrial Ca^{2+} uptake can be implicated in macrophage polarization. Many studies have been focused on the characterization of cytosolic Ca^{2+} signal and its role in the differentiation potential of macrophages [149–151], but nothing is known on the involvement of mitochondrial Ca^{2+} in macrophage plasticity.

Interestingly, by taking advantage of bioinformatics tools, we observed that the dominant-negative subunit of the MCU complex, MCUb, is highly expressed in macrophages (Fig. 6). In detail, MCUb shows a specific expression pattern in M2 macrophages, compared with the other components of the MCU complex, suggesting that MCUb might play a peculiar role in this specific macrophage subpopulation. More importantly, we observed that MCUb expression is specifically upregulated during skeletal muscle regeneration (see Results, Fig 10A). These observations gave rise to the hypothesis that MCUb, might play an important role in skeletal muscle repair.

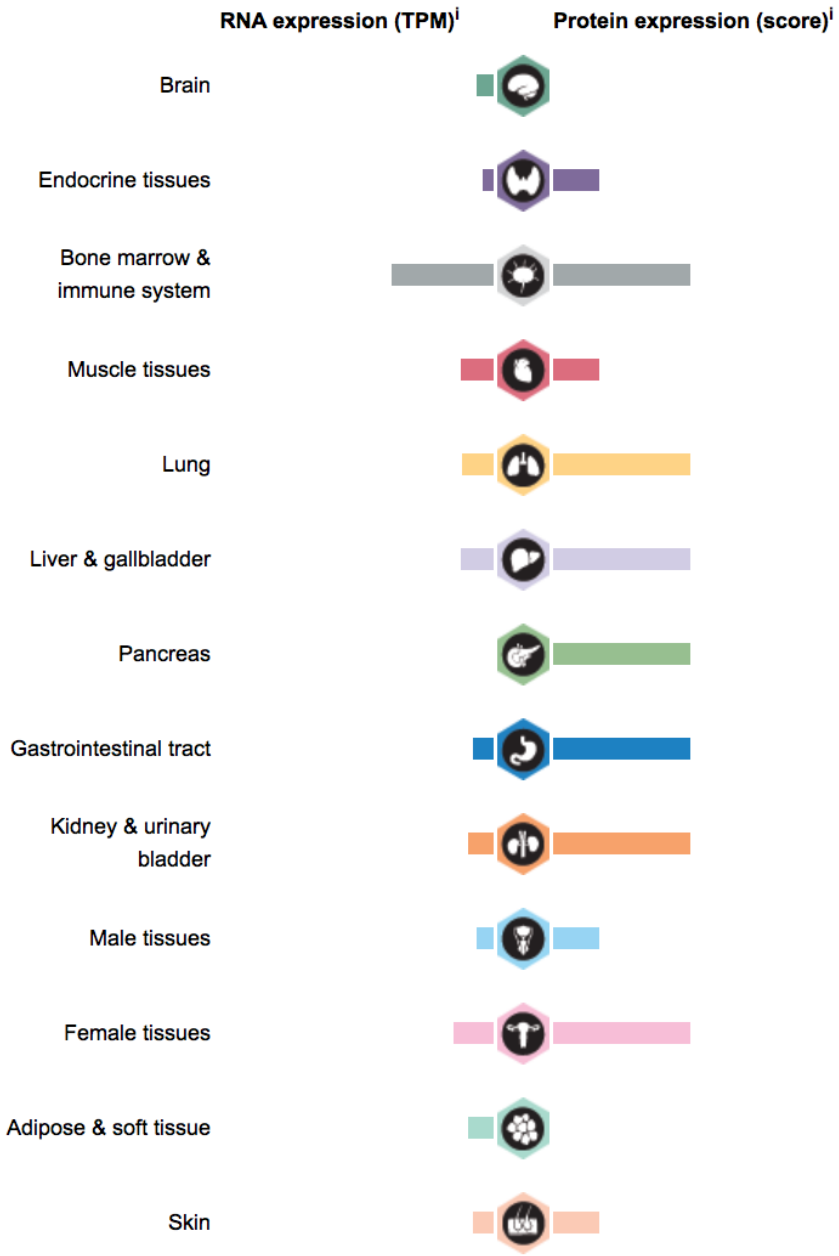


Figure 5. Expression of MCUb in different human tissues (Human protein Atlas, https://www.proteinatlas.org).

4. AIM

The regulation of mitochondrial Ca^{2+} uptake controls a wide array of cellular functions ranging from cell survival to cell death. The study of the role of mitochondrial Ca^{2+} was severely limited by the lack of the molecular identity of the channel responsible for mitochondrial Ca^{2+} uptake, the mitochondrial Ca^{2+} uniporter (MCU). The discovery of the MCU molecular identity gave an important contribution to the understanding the mechanisms controlled by mitochondrial Ca^{2+}.

It is now clear that the MCU is a macromolecular complex formed by pore-forming and regulatory subunits that show a different expression pattern among tissues. These differences in expression have a great impact on the spatio-temporal regulation of mitochondrial Ca^{2+} uptake in different tissues. Recently, in our laboratory it has been demonstrated that the regulation of MCU-mediated mitochondrial Ca^{2+} uptake is not only involved in the control of the rate of ATP production, but it also controls skeletal muscle trophism through a still uncharacterized mitochondria-to-nucleus signal. This finding increases the interest in characterizing the role of MCU complex in skeletal muscle homeostasis in physiological and pathological conditions.

In this regard, the aim of my PhD project was to characterize the physiopathological role of the dominant-negative subunit of MCU, MCUb, during the progression of skeletal muscle regeneration. I decided to study this aspect since I found that MCUb is highly induced in the first phases of skeletal muscle regeneration after damage and this induction occurs in parallel with the increase in the number of the anti-inflammatory (M2) macrophages, important effectors of the later stages of tissue repair. Interestingly, high MCUb expression levels have been detected in M2 macrophages and MCUb silencing in these cells *in vitro* affects macrophages polarization towards an anti-inflammatory phenotype. Moreover, I demonstrated *in vivo* that MCUb ablation, during the progression of skeletal muscle regeneration, affects the skewing from a pro-inflammatory (M1) to an M2 phenotype in regenerating skeletal muscles.

My final goal was to study the role of mitochondrial Ca^{2+} uptake on macrophage plasticity and on skeletal muscle repair capacity. This study could be helpful to clarify the molecular basis of pathological conditions like Duchenne Muscular Dystrophy where skeletal muscle regenerative capacity is strongly affected.

5. Results

5.1 Characterization of MCUb expression

We have previously analysed the mRNA expression of the mitochondrial Ca^{2+} uniporter dominant-negative subunit, MCUb, in different mouse tissues and we have found that it is highly expressed in the immune system (see Introduction, paragraph 3.3.2.5 and Fig. 5). We then asked whether MCUb was expressed in a specific subpopulation of immune cells. Thus, we performed Real Time-PCR (RT-PCR) experiments on different subgroups of immune cells. Interestingly, we found that MCUb is highly expressed in macrophages, one of the three types of phagocyte in the immune system, and in particular in a specific macrophage subpopulation, the anti-inflammatory M2 macrophages (Fig. 7). These cells participate to parasite clearance, damping of inflammation, promotion of angiogenesis and tissue remodelling (see Introduction paragraph 3.3.2 and [152]). To perform this experiment, bone marrow-derived macrophages (BMDM) were either polarized with a pro-inflammatory cytokine, interferon-gamma (IFN-γ), to induce the pro-inflammatory M1 phenotype, or with an anti-inflammatory cytokine, interleukine-4 (IL-4), to induce the anti-inflammatory M2 phenotype. Intriguingly, while *Mcub* expression is highly and specifically induced in M2 macrophages (Fig. 7), the expression of the mitochondrial Ca^{2+} uniporter (*Mcu*) did not change in M1 and M2 macrophages (Fig. 7).

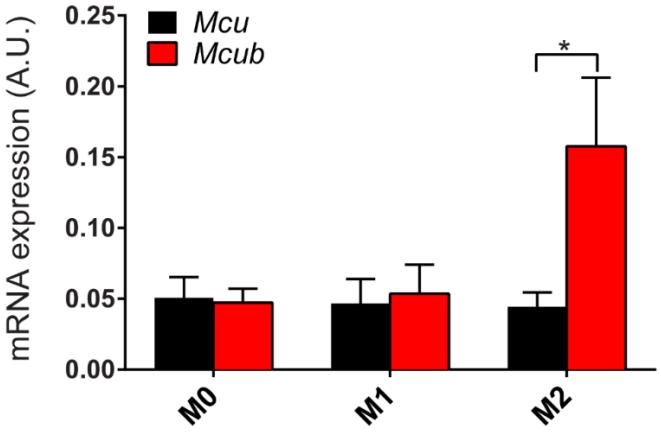


Figure 7. MCUb mRNA is induced in alternatively activated M2 macrophages.
*Murine BMDM were treated with 100 ng/ml IFN-γ (for polarization toward an M1 phenotype) or 20 ng/ml IL-4 (for polarization toward an M2 phenotype) for 24 hours. 24 hours after polarization, total mRNA was extracted and subjected to RT-PCR. The expression of a marker of Mcu and Mcub was assessed. RT-PCR results were normalized to control cells at time 0 and to GAPDH. Data are expressed as mean ± SD. For data analysis, two-way ANOVA was used with post hoc Bonferroni's multiple comparison test for each sample. * $p<0.05$. $n=3$.*

The differences that we observed in MCUb expression between M1 and M2 macrophages were also coherently paralleled by mitochondrial Ca^{2+} measurements. For this measurement, we used the acetoxymethyl (AM) ester of rhodamine-2-based indicator (Rhod-2 AM), that is a cationic fluorescent indicator and therefore it shows a potential-driven uptake into mitochondria [153]. For this reason, Rhod-2 AM can be considered as a selective indicator for mitochondrial Ca^{2+} [153]. Murine BMDM were purified and treated as in Fig. 7. In line with the dominant-negative role of MCUb (see Introduction, paragraph 3.2.4.1, Fig. 2B and [10]) and the expression data showing an higher MCUb expression level in M2 macrophages compared to M1 (Fig. 7), we found that M2 macrophages show a decrease in mitochondrial Ca^{2+} uptake compared to the M1 macrophage subpopulation (Fig. 8).

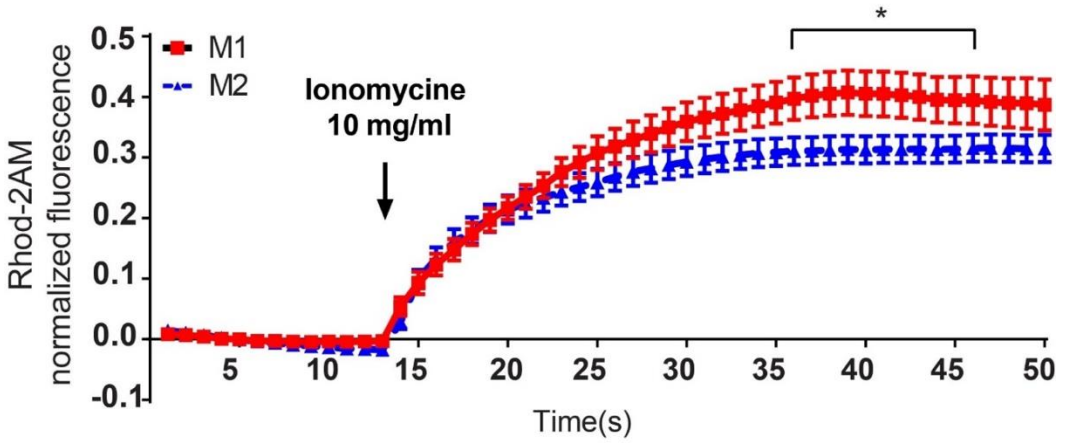


Figure 8. M2 macrophages show a decrease in mitochondrial calcium uptake compared to M1 macrophages.
*BMDM were polarized with 100 ng/ml IFN-γ towards the M1 phenotype or 20 ng/ml IL-4 towards the M2 phenotype for 24 hours. Cells were then loaded with the Ca^{2+} indicator Rhod-2 AM for 20 minutes. Ca^{2+} measurements were performed stimulating cells with 10 μg/ml ionomycin and the analysis was performed by confocal microscope. For data analysis Mann-Whitney-t-test for each sample was used. *p<0.05. n=3.*

Based on these preliminary results showing that the polarization with the anti-inflammatory cytokine, IL-4, induces specifically MCUb expression and does not affect MCU expression (Fig. 7), we wondered whether MCUb, and thus the regulation of mitochondrial Ca^{2+} uptake, were involved in M2 macrophages differentiation. To answer to this question, we silenced MCUb by transfecting murine BMDM with a specific siRNA and we concomitantly treated these cells with the anti-inflammatory stimulus IL-4. Intriguingly, we found that MCUb silencing reduces the expression of a classical marker of anti-inflammatory macrophages, arginase 1 (*Arg1*) (Fig. 9A). On the contrary, MCUb silencing does not affect the expression level of a marker of pro-inflammatory macrophages, the inducible nitric oxide synthase (iNOS), after exposure to a pro-inflammatory polarizing cytokine IFN-γ (Fig. 9B). These results suggest that, in macrophages, MCUb, by regulating the entry of Ca^{2+} into mitochondria, might be fundamental for M2 polarization.

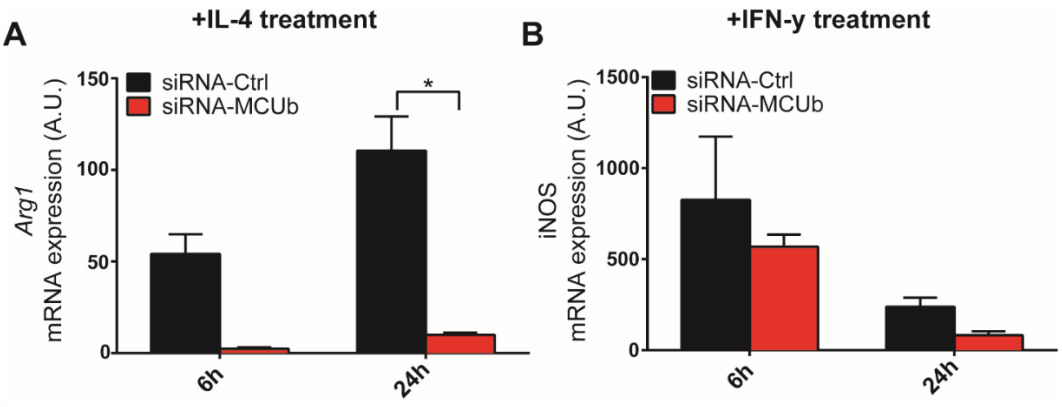


Figure 9. MCUb silencing strongly reduces the expression level of the anti-inflammatory marker ARG1.
*Murine BMDM were transfected using either a control siRNA (scrambled, siRNA-Ctrl) or a siRNA specifically designed to silence MCUb (siRNA-MCUb). 24 hours after transfection, cells were exposed to either 20 ng/ml IL-4 (**A**) or 100 ng/ml IFN-γ (**B**) respectively for polarization towards an M2 and M1 phenotype. 24 hours after polarization, total mRNA was extracted and subjected to RT-PCR as in Fig. 7. The expression of a marker of M2 macrophages, Arg1 (A), and of M1 macrophages, iNOS (B) was assessed. RT-PCR results were normalized to control cells at time 0 and to GAPDH. Data are expressed as mean ± SD. For data analysis, two-way ANOVA was used with post hoc Bonferroni's multiple comparison test for each sample. *p<0.05. n=3.*

5.2 Role of MCUb in skeletal muscle regeneration

Our preliminary results clearly demonstrate that MCUb is highly expressed in macrophages and is induced in the M2 macrophage subpopulation (Fig. 7). Beyond their role in innate immunity, macrophages orchestrate the healing of various injured tissues (see Introduction, paragraphs 3.3.2.1, 3.3.2.2 and [15]). Indeed, macrophages participate to all the different phases of tissue repair: i) phagocytosis of cellular debris and of apoptotic neutrophils; ii) cytokines production that orchestrates the healing response mediated by stem cells or progenitor cells (see Introduction, paragraph 3.3.2.2 and [15]). Noteworthy, unlike many tissues which do not or only partially repair after an injury, skeletal muscle is capable to fully regenerate [123].

It is well accepted that macrophages sustain muscle repair during all the time course of skeletal muscle regeneration process (see Introduction, paragraph 3.3.2.2 and [16]). In detail, M1 macrophages participate to the first stage of skeletal muscle

repair by promoting a pro-inflammatory response, while M2 macrophages take part to the later stage of tissue repair (see Introduction, paragraph 3.3.2.2 and [15–17]). Indeed, the latter promote the deactivation of the pro-inflammatory response and stimulate the terminal differentiation of myogenic precursor cells (MPCs) that derive from the proliferation of satellite cells, their fusion into myotubes and the growth of the newly regenerating myofibers (see Introduction, paragraph 3.3.2.2 and [15–17]).

The evidence that MCUb is highly expressed in M2 macrophages (Fig. 7), and the fundamental role that these cells acquire during the progression of skeletal muscle regeneration, made us hypothesize that MCUb, and thus the regulation of mitochondrial Ca^{2+} homeostasis, might be important for the progression of skeletal muscle regeneration. To confirm our hypothesis, we injected tibialis anterior (TA) muscles of C57/BL6N mice (wild type (WT) mice) with cardiotoxin (CTX). This treatment provides an useful model for sterile inflammation and causes a homogeneous damage in the whole muscle [20]. We analyzed the mRNA expression levels of the components of the MCU complex during the progression of skeletal muscle regeneration by performing RT-PCR experiments. Interestingly, we found that MCUb expression is highly induced 3 days after CTX injection (Fig. 10A). We also analyzed the expression of the pore-forming subunit MCU and the regulatory subunits of the MCU complex, MICU1 and MICU2, to check whether also the expression of the other complex components was modulated during the progression of skeletal muscle regeneration. This experiment clearly shows that MCU and MICU1 expression does not change during the progression of skeletal muscle regeneration (Fig. 10B and 10C). MICU2 expression is slightly induced 3 days after CTX injection (Fig. 10D), but the huge induction of MCUb expression made us concentrate on this protein.

Altogether these results suggested that MCUb could play a specific role in this process.

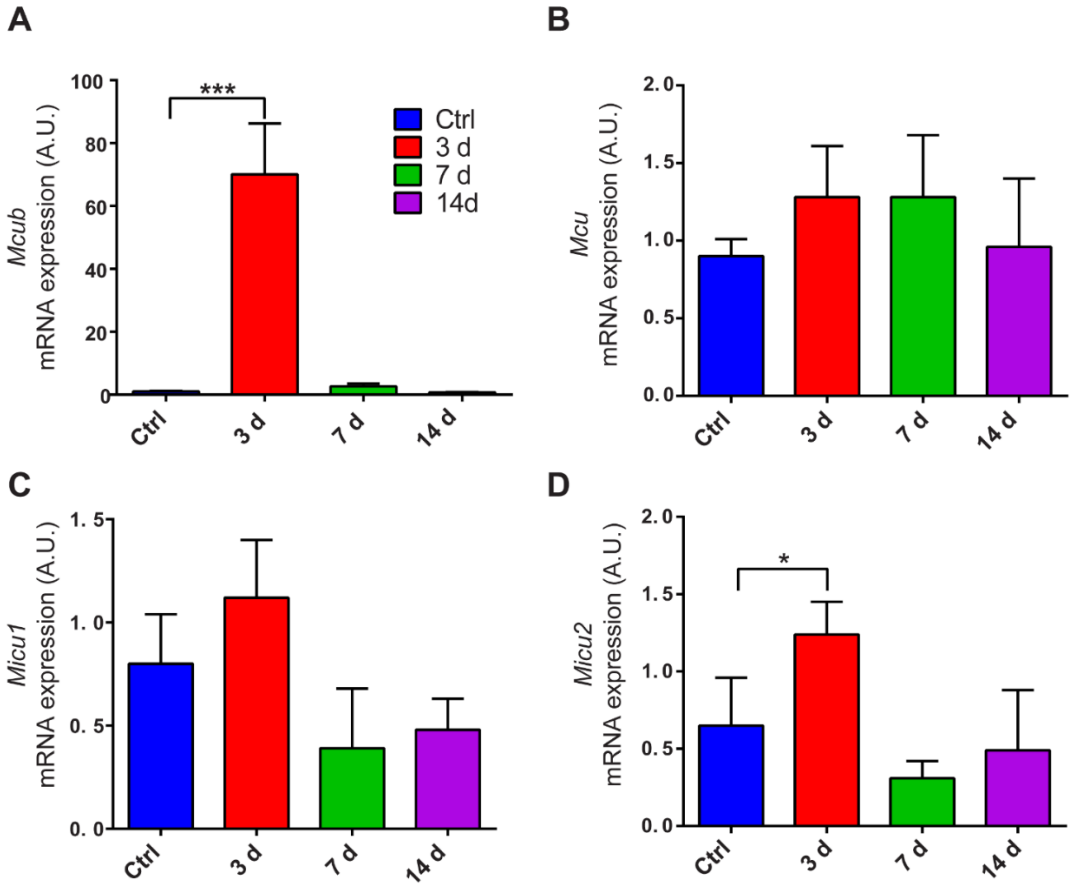


Figure 10. MCUb mRNA expression is highly induced during the progression of skeletal muscle regeneration.
*Adult WT C57/BL6N male animals were used. 50 µl of 10 µM CTX were injected in TA muscles. Muscles were harvested for expression analysis at different time points post-injury (3, 7 and 14 days). Mcub (**A**), Mcu (**B**), Micu1 (**C**), and Micu2 (**D**) expression levels were evaluated by RT-PCR and normalized to GAPDH. Data are expressed as mean ± SD. For data analysis, one–way ANOVA was used with post hoc Bonferroni's multiple comparison test for each sample. * p≤0.05; **, p≤0.001. n=4.*

Since MCUb is poorly expressed in skeletal muscle in basal conditions (Fig. 10A and [10]), and based on our *in vitro* results showing that MCUb is highly expressed in M2 macrophages (Fig. 7), we hypothesized that the induction of MCUb expression 3 days after the injection of CTX (Fig. 10A) could be due to the infiltration of macrophages, that typically invade regenerating muscles at this stage [15]. To confirm this hypothesis, we performed fluorescence activated cell sorting (FACS) on the macrophage population sorted from regenerating TA muscles of WT animals 3 days post CTX injection, time point at which MCUb is maximally

induced (Fig. 10A). We analysed the expression levels of MCUb and MCU (Fig. 11). Cells purified from regenerating muscles, 3 days after CTX injury, were labelled with a specific flow cytometer antibody against EGF-like module-containing mucin-like hormone receptor-like 1, commonly known as F4/80, a membrane protein considered as a classic macrophage marker [154].

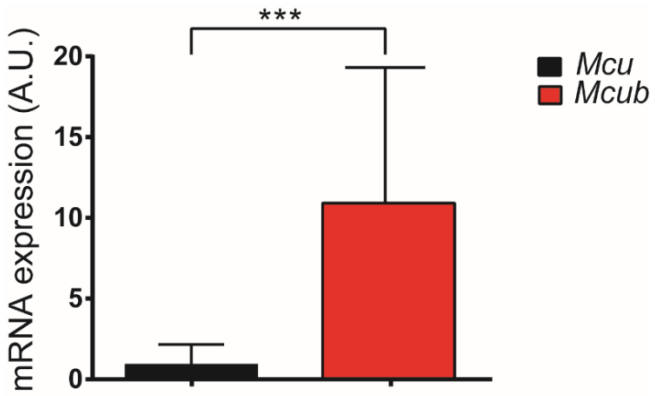


Figure 11. MCUb is highly expressed in F4/80 positive cells 3 days post CTX injection
*For this experiment, adult female WT C57/BL6N were used. Cells purified from regenerating muscles 3 days post CTX injection were labelled with an α-F4/80 specific antibody. After flow cytometric analysis, macrophages were sorted, their RNA was extracted and the expression levels of MCU and MCUb were evaluated by RT-PCR and normalized to GAPDH. Data are expressed as mean ± SEM. For pairwise comparison of means, t tests were used. ***p≤0.001. n=4.*

This experiment clearly demonstrates that MCUb is highly expressed in macrophages extracted from regenerating muscles 3 days post CTX injection. Overall, we have demonstrated that MCUb is highly induced during the progression of skeletal muscle regeneration compared with the other components of the MCU complex (Fig. 10A-D), and that MCUb is highly expressed in macrophages extracted from regenerating muscles 3 days post CTX injection, where macrophages reach the maximum peak (Fig. 11 and [15]).

5.3 Characterization of the total MCUb KO mouse model

To evaluate the effects of MCUb ablation and thus to better characterize the physiopathological role of MCUb, we generated a total MCUb KO mouse model. For this purpose, we crossed mice containing an IRES:lacZ trapping cassette and a floxed promoter-driven neo cassette inserted into the intron 2 of the MCUb gene,

thus disrupting gene function, following the 'knockout-first' technology [155]. In contrast to standard conditional designs, the initial unmodified allele generates a null allele by splicing the cDNA to the LacZ trapping element contained in the targeting cassette (Fig. 12 and [155]). This trapping cassette includes the mouse enhancer-2 splice acceptor and the SV40 polyadenylation sequences that have been demonstrated to be highly effective in creating null alleles in mice [155]. The advantage of the 'knockout-first' allele is that it can crossed with transgenic *FLP* and *cre* mice thus generating a conditional allele [155]. We crossed heterozygotes mice for the MCUb gene ($Tm1a^{+/-}$ mice) to obtain a total MCUb KO mouse model ($Tm1a^{+/+}$). These mice are fertile and viable and they do not show phenotypic abnormalities.

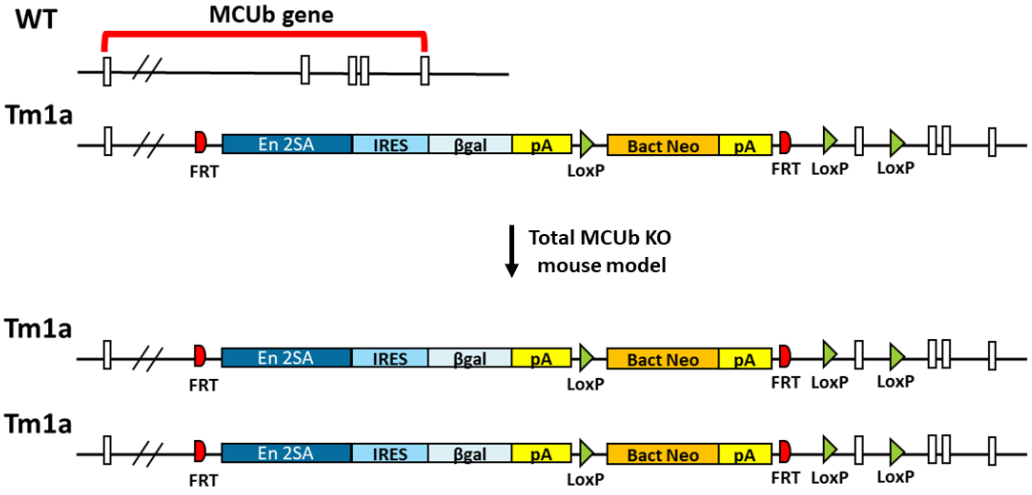


Figure 12. Schematic view of the MCUb 'knockout-first' allele.
The 'knockout-first' allele (Tm1a) contains an IRES: LazZ trapping cassette and a floxed promoter-driven neo cassette inserted into the intron of a gene by causing the disruption of gene function [155]. *The crossing with Flp and Cre transgenic mice allows the generation of different conditional alleles (Tm1c,b and d), by respectively acting on FRT and loxP binding sites* [155].

5.4 Characterization of the skeletal muscle regeneration in MCUb KO animals

To confirm our *in vitro* results suggesting that MCUb could be required for M2 polarization (Fig. 9A), we analysed the expression of markers of M1 and M2 macrophages in both WT and MCUb KO animals during the time course of skeletal muscle regeneration. We thus analysed the expression levels of tumor necrosis

factor-alpha (TNF-α), a typical M1 marker, and mannose receptor type 1 (MRCI or commonly known as CD206), a typical M2 marker, by RT-PCR on regenerating TA muscles of both WT and MCUb KO mice at 3, 7 and 14 days post CTX injection. We compared the results also with non-injected muscles of WT and MCUb KO mice. Interestingly, in MCUb KO mice we found a significant reduction in the expression levels of CD206 3 days after CTX injection (Fig. 13), suggesting that MCUb ablation *in vivo* might affect macrophages polarization into an anti-inflammatory M2 phenotype, coherently with our *in vitro* results. On the contrary, we observed a huge induction of TNF-α expression in regenerating muscles from MCUb KO mice 3 days after CTX injection (Fig. 13B), suggesting that MCUb ablation impairs the capacity of macrophages to adopt an anti-inflammatory M2 phenotype during the time course of skeletal muscle regeneration, thus causing the accumulation of M1 macrophages.

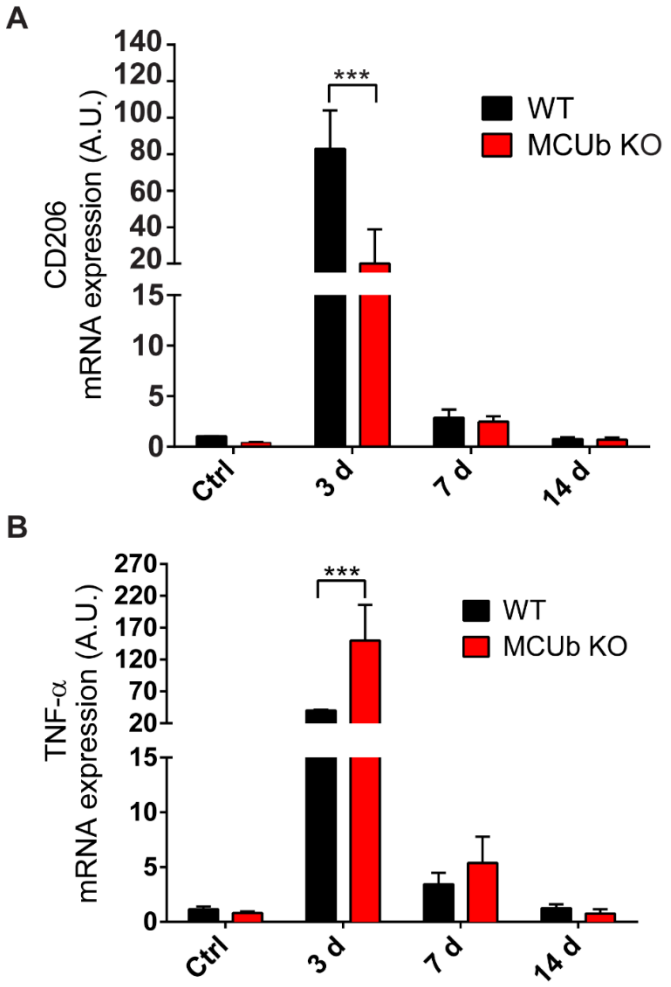
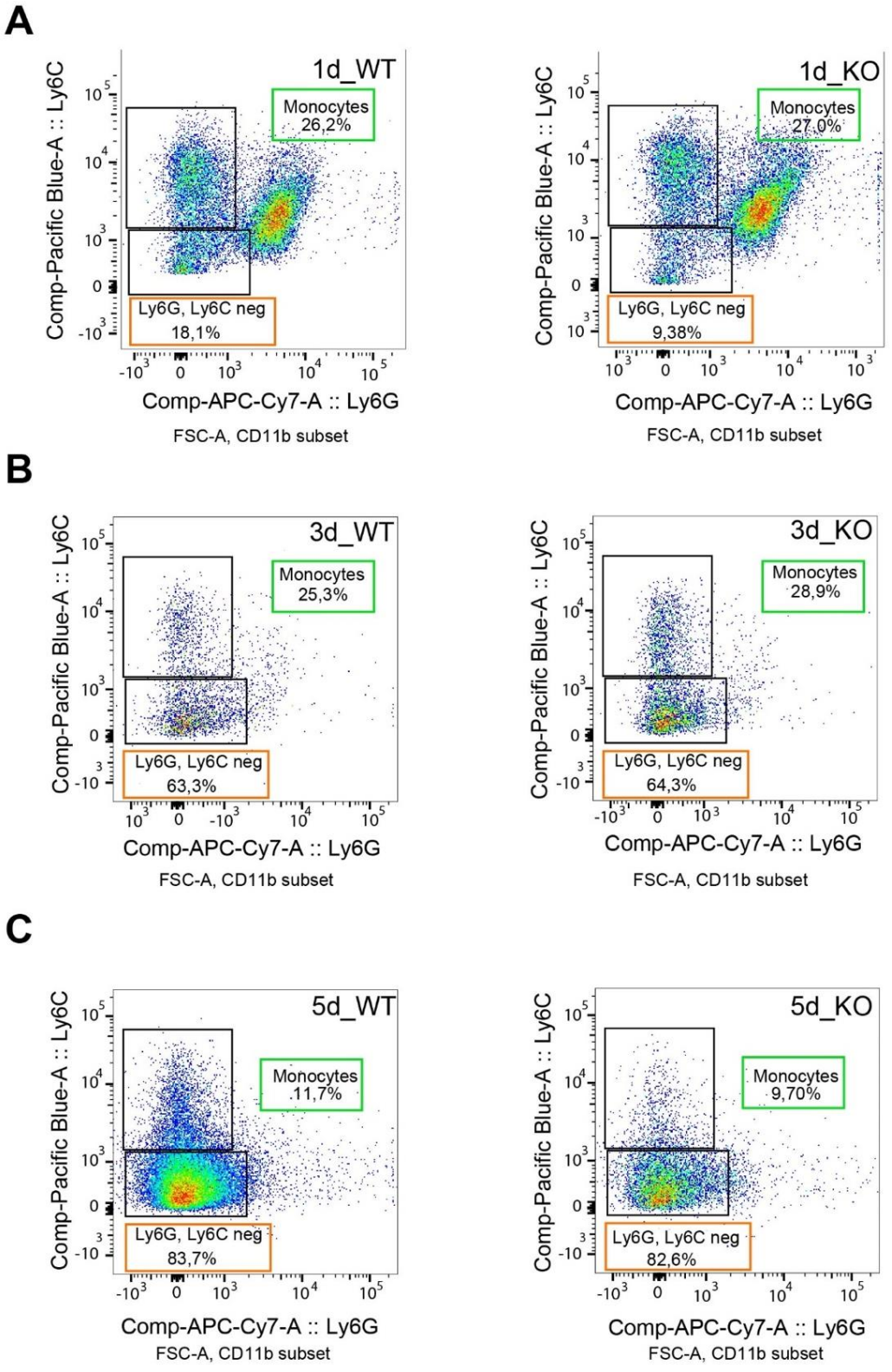


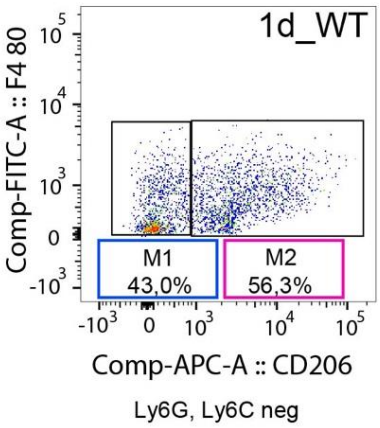
Figure 13. Regenerating TA muscles of MCUb KO mice display a reduction in the expression levels of an anti-inflammatory M2 marker and an induction of a pro-inflammatory M1 marker.
*Adult male animals from WT and MCUb KO mouse lines were used. TA muscles were harvested for analysis at different time points post-injury (3, 7 and 14 days after CTX injection). The mRNA expression levels of markers of both anti-inflammatory (CD206) (**A**), and pro-inflammatory (TNF-α) (**B**) macrophages was assessed by RT-PCR and normalized to GAPDH. Data are expressed as mean ± SD; For data analysis, two-way ANOVA was used with post hoc Bonferroni's multiple comparison test for each sample. *** $p \leq 0.001$. n=4.*

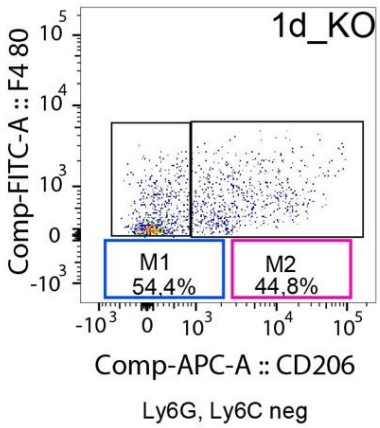
To strengthen our result showing a decrease in the expression levels of a marker of M2 macrophages in MCUb KO mice (Figure 13A), we decided to characterize the inflammatory mononuclear cellular infiltrate during the early stages of skeletal muscle regeneration. We performed this experiment since it is known that the first phase of muscle regeneration (1-7 days post-injury) is predominantly characterized by the infiltration of a huge number of inflammatory

cells [15]. We thus injured TA muscles of both WT and MCUb KO mice by CTX injection, we collected and dissociated regenerating muscles 1, 3 and 5 days post-injury. We then purified cells from regenerating muscles and we labelled them by using specific flow cytometer antibodies to analyse and then isolate monocyte and macrophage cell populations. We first incubated the isolated cellular infiltrate with a specific antibody against a specific membrane protein, named CD11b, also known as Mac-1 α or integrin αM chain [156]. The CD11b antibody allows to isolate the myeloid lineage cells, including monocytes, neutrophils, natural killer cells, granulocytes and macrophages [156]. We then performed an incubation with specific antibodies against membrane surface proteins, the Lymphocyte antigen 6 complex locus G (Ly6G) and Lymphocyte antigen 6 complex locus C (Ly6C), which are suited for identifying neutrophils, eosinophils, and monocytes [156]. To specifically select the macrophage cell population, we incubated the isolated cellular infiltrate with the antibody against F4/80, as described above (Fig. 11 and [154]). Since our goal was to evaluate whether MCUb ablation could affect macrophages skewing from an M1 to M2 phenotype, cells purified from regenerating muscles were also incubated with an antibody against a specific membrane protein expressed by M2 macrophages, CD206 [17]. Labelled cells were then subjected to FACS analysis (Fig.14). We first tested the number of monocytes recruited in regenerating muscles 1, 3 and 5 day after damage. We observed no difference in the number of monocytes, ($Ly6G^+/Ly6C^+$) and macrophages ($Ly6G^-/LY6C^-$) recruited in regenerating muscles of both WT and MCUb KO animals at all the time points considered (Fig. 14A-C and Table 14G). Importantly, 1 and 3 days after the induction of the damage, we observed a reduction of M2 macrophages ($CD206^+$) in MCUb KO mice compared to WT animals (Fig. 14D, 14E and Table 14G) that occurs in parallel with an increase in the number of M1 macrophages ($CD206^-$) (Fig. 14D, 14E and Table 14G), although these differences decreased 5 days after injury (Fig. 14F and Table 14G). Overall these results suggest that MCUb ablation does not affect monocytes/macrophages recruitment in the regenerating area of the muscles while it alters or delays macrophage capacity to acquire an anti-inflammatory M2 phenotype (Fig. 14D-F and Table 14G).

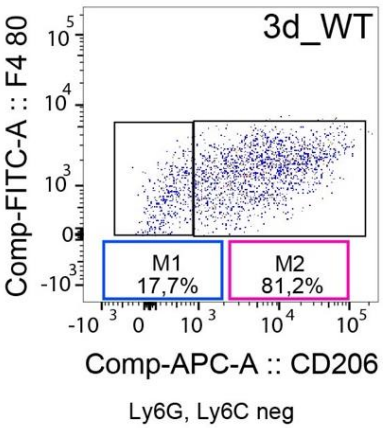

A
1d_WT
Monocytes
26,2%
Ly6G, Ly6C neg
18,1%
Comp-Pacific Blue-A :: Ly6C
Comp-APC-Cy7-A :: Ly6G
FSC-A, CD11b subset
1d_KO
Monocytes
27,0%
Ly6G, Ly6C neg
9,38%
Comp-Pacific Blue-A :: Ly6C
Comp-APC-Cy7-A :: Ly6G
FSC-A, CD11b subset
B
3d_WT
Monocytes
25,3%
Ly6G, Ly6C neg
63,3%
Comp-Pacific Blue-A :: Ly6C
Comp-APC-Cy7-A :: Ly6G
FSC-A, CD11b subset
3d_KO
Monocytes
28,9%
Ly6G, Ly6C neg
64,3%
Comp-Pacific Blue-A :: Ly6C
Comp-APC-Cy7-A :: Ly6G
FSC-A, CD11b subset
C
5d_WT
Monocytes
11,7%
Ly6G, Ly6C neg
83,7%
Comp-Pacific Blue-A :: Ly6C
Comp-APC-Cy7-A :: Ly6G
FSC-A, CD11b subset
5d_KO
Monocytes
9,70%
Ly6G, Ly6C neg
82,6%
Comp-Pacific Blue-A :: Ly6C
Comp-APC-Cy7-A :: Ly6G
FSC-A, CD11b subset

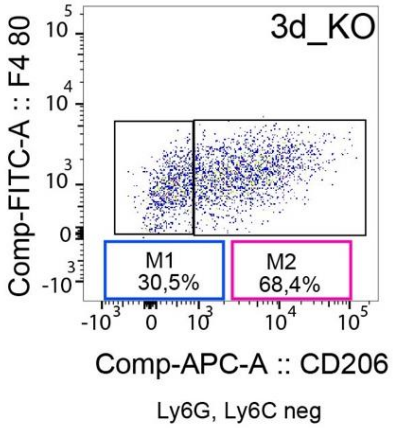
D


1d_WT
Comp-FITC-A :: F4 80
Comp-APC-A :: CD206
M1
43,0%
M2
56,3%
Ly6G, Ly6C neg

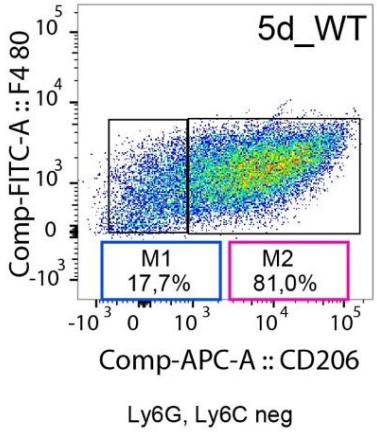

1d_KO
Comp-FITC-A :: F4 80
Comp-APC-A :: CD206
M1
54,4%
M2
44,8%
Ly6G, Ly6C neg

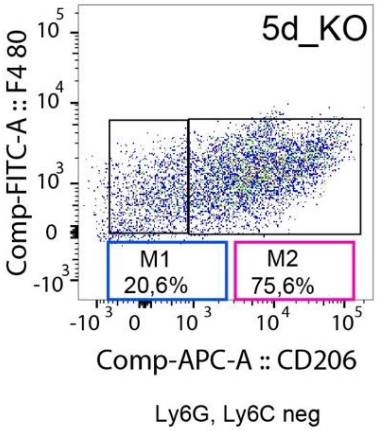
E


3d_WT
Comp-FITC-A :: F4 80
Comp-APC-A :: CD206
M1
17,7%
M2
81,2%
Ly6G, Ly6C neg


3d_KO
Comp-FITC-A :: F4 80
Comp-APC-A :: CD206
M1
30,5%
M2
68,4%
Ly6G, Ly6C neg

F


5d_WT
Comp-FITC-A :: F4 80
Comp-APC-A :: CD206
M1
17,7%
M2
81,0%
Ly6G, Ly6C neg


5d_KO
Comp-FITC-A :: F4 80
Comp-APC-A :: CD206
M1
20,6%
M2
75,6%
Ly6G, Ly6C neg

G

	WT	KO	WT	KO	WT	KO	WT	KO
1 d	26,2%	27,2%	18,1%	9,38%	43,0%	54,4%	56,3%	44,8%
3 d	25,3%	28,9%	63,3%	64,3%	17,7%	30,5%	81,2%	68,4%
5 d	11,7%	9,70%	83,7%	82,6%	17,7%	20,6%	81,0 %	75,6%
	$Ly6C^+/Ly6G^+$ Monocytes		$Ly6C^-/Ly6G^-$ Macrophages		$CD206^-$ M1 macrophages		$CD206^+$ M2 macrophages	

Figure 14. MCUb ablation causes a delay in M2 macrophages polarization.
*(**A-F**) Cellular infiltrate was isolated from regenerating muscles of WT and MCUb KO mice 1, 3 and 5 days after CTX injection. Four cell populations were separated from treated muscles using cytometer antibodies: myeloid lineage cells - α-CD11b, granulocyte neutrophils, eosinophils monocytes - α-Ly6C and α-Ly6G, macrophages - α-F4/80 and M2 macrophages - α-CD206. Each panel represents a representative dot plot at day 1,3 and 5 post-injury. Percentages of monocytes ($Ly6C^+/Ly6G^+$), macrophages ($Ly6C^-/Ly6G^-$) (**A**, **B** and **C**) and percentage of M1 ($CD206^-$) and M2 macrophages ($CD206^+$) (**D**, **E** and **F**) after CTX injection are indicated and were calculated within the gate of positive cell ($F4/80^+$). (**G**) Data table of the percentages of monocytes ($Ly6C^+/Ly6G^+$), macrophages ($Ly6C^-/Ly6G^-$), M1 macrophages ($CD206^-$) and M2 macrophages ($CD206^+$) in regenerating muscles 1,3 and 5 days post CTX injection.*

To deepen our knowledge on this aspect, we analysed the phagocytic capacity of macrophages extracted from MCUb KO mice. We performed this experiment since in macrophages the signalling activated by phagocytosis can promote a shift from an M1 to M2 phenotype (see Introduction, paragraph 3.3.2.3 and [20]). Therefore, M2 macrophages are characterized by an higher phagocytic activity than M1 macrophages. We thus hypothesized that MCUb, and thus mitochondrial Ca^{2+} uptake, could also influence macrophage phagocytic capacity. To asses this hypothesis, we collected TA muscles of WT and MCUb KO mice and phagocytosis activity was assessed at the indicated time points by flow cytometry (Fig. 15). Phagocytosis is the process by which phagocytes, including macrophages, neutrophils and monocytes, engulf and kill invading pathogens, remove foreign particles, and clear cell debris [157]. In particular, once the material has been phagocyted, it is digested through the auto-phagolysosome system [157].

In our experimental setting, we used fluorescent bio particles sensitive to pH, as already performed [158]. Therefore, a decrease in pH results in an increased fluorescent signal which is related to the phagocytic capacity of macrophages.

BMDM were incubated with the fluorescent bio particles and collected at different time points (Fig. 15). Macrophage phagocytic capacity was assessed by flow cytometry and the results show a strong reduction in the phagocytic capacity of BMDM derived from MCUb KO (Fig. 15), supporting the data demonstrating that MCUb ablation leads to impaired M1 to M2 skewing.

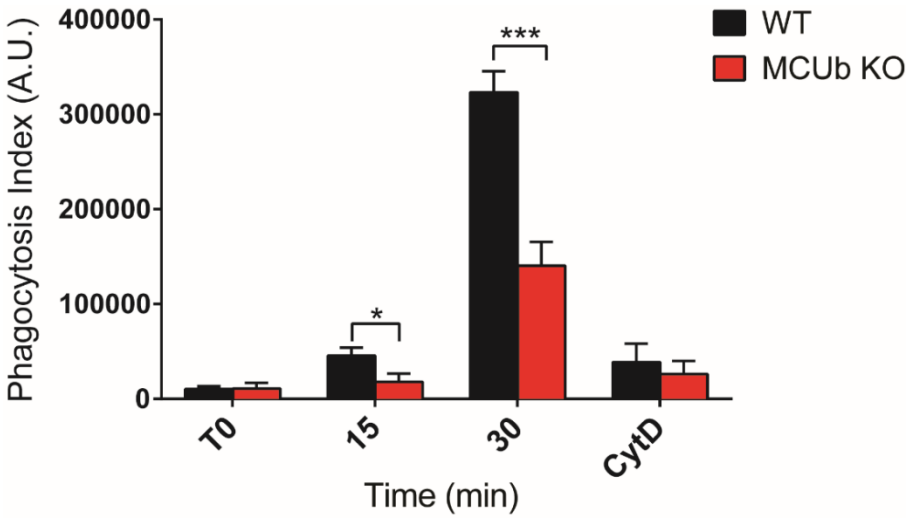


Figure 15. MCUb ablation affects phagocytic capacity of macrophages.
*BMDM derived from WT and MCUb KO mice were differentiated with 40 ng/ml of macrophage colony-stimulating factor (M-CSF) for 7 days. At day 7, cells were collected and phagocytic capacity was assessed. BMDM were incubated with 1 mg/ml of pHrodo™ Green S. Aureus Bio Particles®. As negative control, cells were pre-treated with 10 mg/ml of an actin polymerization inhibitor Cytochalsin D (Cyt D). At the indicated time points, cells were collected and phagocytosis was assessed by flow cytometry. A specific gate was created for cells positive for green fluorescence (i.e. cells engulfed with beads), and the M.F.I. (Mean Fluorescence Intensity) was measured using FlowJo analysis software. Phagocytic Index (P.I.) was determined by multiplying the percentage of cells that had ingested the beads and the M.F.I. of the phagocytic gate. For data analysis, two-way ANOVA was used with post hoc Bonferroni's multiple comparison test for each sample. * $p \leq 0.05$; *** $p \leq 0.001$. $n=3$.*

5.5 MCUb influences macrophage plasticity, thus affecting skeletal muscle repair

The capacity of skeletal muscle to regenerate relies on a specific cell population of quiescent muscle stem cells, satellite cells, that become committed to adult myogenesis. These cells activate after damage and expand before differentiating and fusing to form new myofibers (see Introduction, paragraph 3.3

and [123]). It is widely accepted that macrophages provide a microenvironment for satellite cells proliferation and activation, orchestrating adult myogenesis during regeneration of damaged skeletal muscle [16]. Indeed, soon after injury, infiltrating macrophages are mainly pro-inflammatory M1 macrophages secreting cytokines such as TNF-α, interleukin-6 (IL-6), interleukin-beta (IL1-β) and growth factors such as insulin-like growth factor (IGF-1) that have been shown to orchestrate the proliferation of satellite cells [15–17]. Subsequently, anti-inflammatory M2 macrophages, secreting cytokines with trophic function, such as interleukin-10 (IL-10) and transforming growth factor-beta (TGF-β), are the primary effectors of later stages of tissue repair associated with satellite cells differentiation and fusion [15–17].

Since our *in vitro* and *in vivo* results showed that MCUb plays a key role in M2 polarization (Fig. 9A, 13, 14D-F), we asked whether MCUb ablation could impinge on skeletal muscle repair after damage. To answer to this question, we first analysed whether in MCUb KO animals the expression levels of the different myogenic regulatory factors (MRFs), that are involved in satellite cell proliferation and differentiation [118], could be affected during the progression of skeletal muscle regeneration. Intriguingly, MCUb KO mice, 3 days after CTX injection, present a significant reduction of the mRNA expression level of the paired-box transcription factor (*Pax7)* (Fig. 16A), that marks both quiescent and activated satellite cells [116]. We also evaluated the mRNA expression level of the myoblast determination protein 1 (*Myod*), which marks both activated and proliferating satellite cells which are committed to differentiate (Fig. 16B and [118]). Interestingly, 3 days after CTX injection, we observed a strong reduction in *Myod* expression in MCUb KO mice. Overall, these data strongly suggest an impairment of the capacity of satellite cells to proliferate and become committed to differentiation.

It is widely accepted that, after damage, satellite cells enter cell cycle generating daughter cells that can either replenish the original pool of satellite cells, necessary for future rounds of skeletal muscle repair, that will therefore express PAX7 but not MYOD ($PAX7^+$; $MYOD^-$), or can differentiate and lose the capacity of self-renewal. In the latter case, satellite cells will express both PAX7 and MYOD

($PAX7^+$; $MYOD^+$) [16]. Our results suggest that MCUb ablation, by affecting the expression level of both *Pax7* (Fig. 16A) and *Myod* (Fig. 16B), might both affect the reconstitution of the pool of satellite cells and impinge on the ability of satellite cells to become activated and committed to differentiate into new myofibers. Another important transcription factor which is required for full differentiation and survival is myogenin (MYOG), which is principally involved in the last stages of myogenesis [118]. We observed an increase in *Myog* expression in MCUb KO mice in untreated muscles and no changes were detected at later time points (Fig. 16C). Overall, the alteration in the expression level of *Pax7* and *Myod* (Fig. 16A and 16B), but not of *Myog* (Fig. 16C), caused by MCUb ablation, strongly suggests an impairment in the early phases of muscle repair.

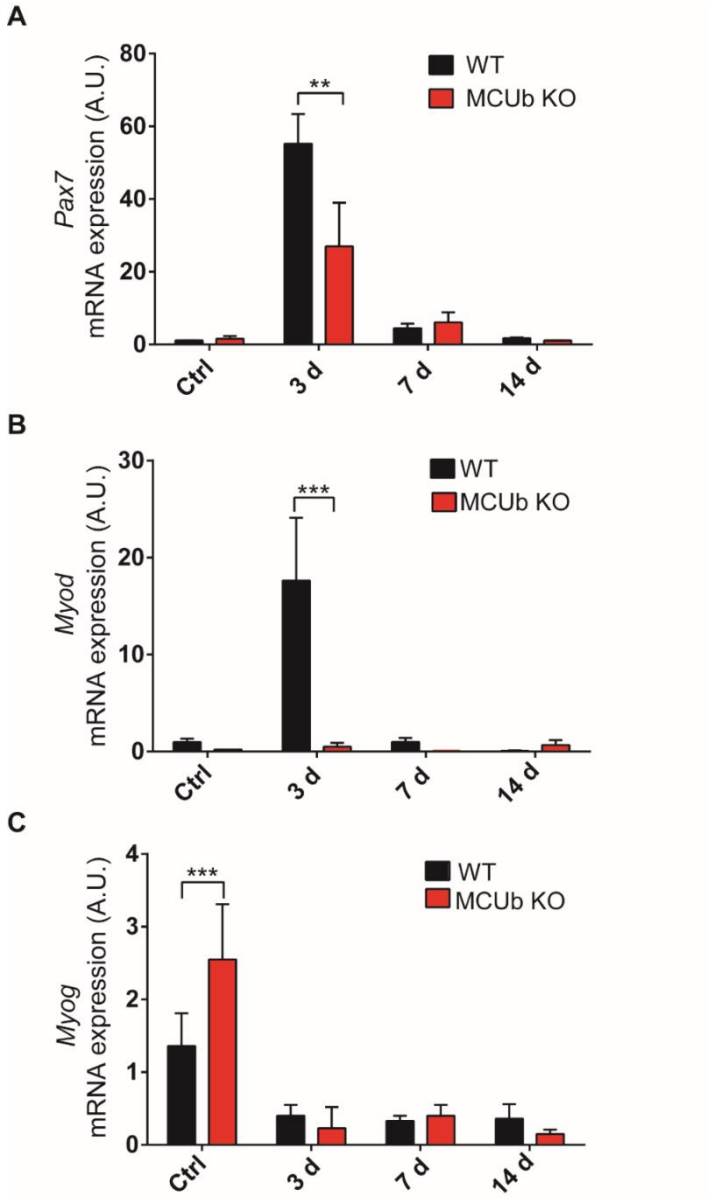


Figure 16. The mRNA expression of early MRFs is altered in regenerating muscles of MCUb KO mice.

*Adult male animals from WT and MCUb KO mouse lines were used. TA muscles were harvested for analysis at different time points post-injury (3, 7 and 14 days after CTX injection). The mRNA expression levels of markers of myogenic precursor cells Pax7 (**A**), Myod (**B**), and Myog (**C**) were analysed by RT-PCR and normalized to GAPDH. Data are expressed as mean ± SD. For data analysis, two-way ANOVA was used with post hoc Bonferroni's multiple comparison test for each sample. ** p≤0.005; *** p≤0.001. n=4.*

We then asked whether the alterations that we observed in the expression levels of the different MRFs could affect skeletal muscle repair after damage in MCUb KO animals (Fig. 17). We first performed Hematoxylin & Eosin (H&E) staining on cryosections of regenerating muscles derived from both WT and MCUb KO animals. Regenerating TA muscles from MCUb KO mice clearly show a

persistent inflammation and the presence of necrotic cells 7 days after CTX injury compared with WT mice (Fig. 17). Moreover, we observed a delay in the disappearance of the cellular infiltrate and an alteration of muscle structure in MCUb KO mice 14 days after CTX injection, suggesting a delay in the progression of skeletal muscle regeneration (Fig. 17).

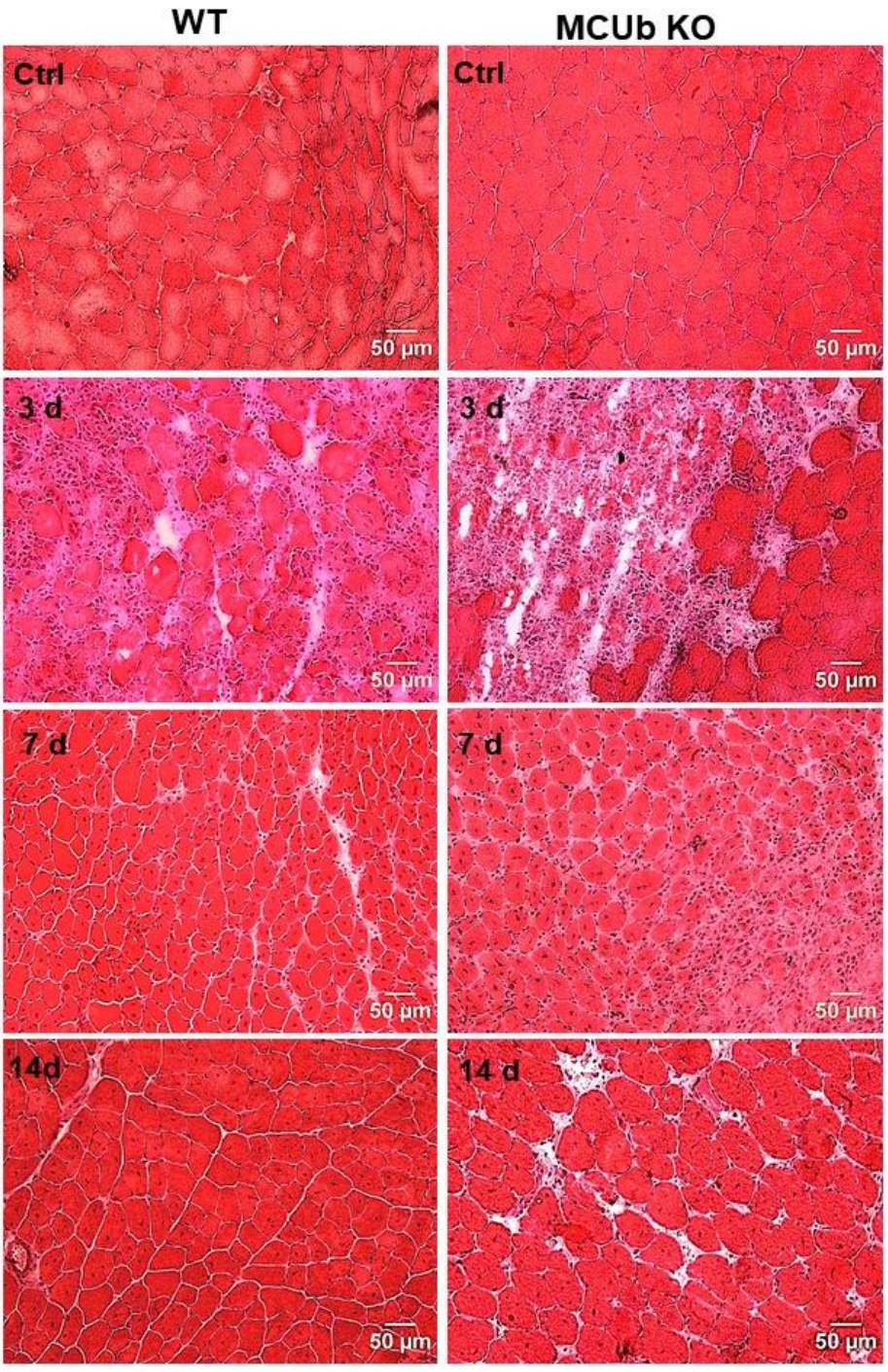


Figure 17. Effects of MCUb ablation on skeletal muscle regeneration.
TA from regenerating muscles 3, 7 and 14 days after CTX injection and control muscles of WT and MCUb KO mice were dissected, frozen in liquid nitrogen-cold isopentane. 6 µm-thick cryosections were prepared for H&E staining.

Since an impairment in skeletal muscle regeneration can influence the number and size of the newly formed myofibers, we analysed the cross-area (CSA), the fiber size distribution and the number of regenerating muscle fibers in WT and MCUb KO regenerating muscles. Firstly, we analysed the area of muscle fibers derived from untreated WT and MCUb KO animals, observing no statistically significant differences (Fig. 18A). We performed this experiment to exclude any difference in basal condition between WT and MCUb KO animals that could lead to misleading interpretation of the results of the regeneration experiments. We then measured the CSA of regenerating fibers, characterized by the presence of central nuclei, from WT and MCUb KO mice 14 days post CTX injection and, again, we did not find any significant difference (Fig. 18B). As expected, no difference in mean CSA was also observed in non-regenerating fibers within regenerating muscles 14 days post CTX injection (Fig. 18C).

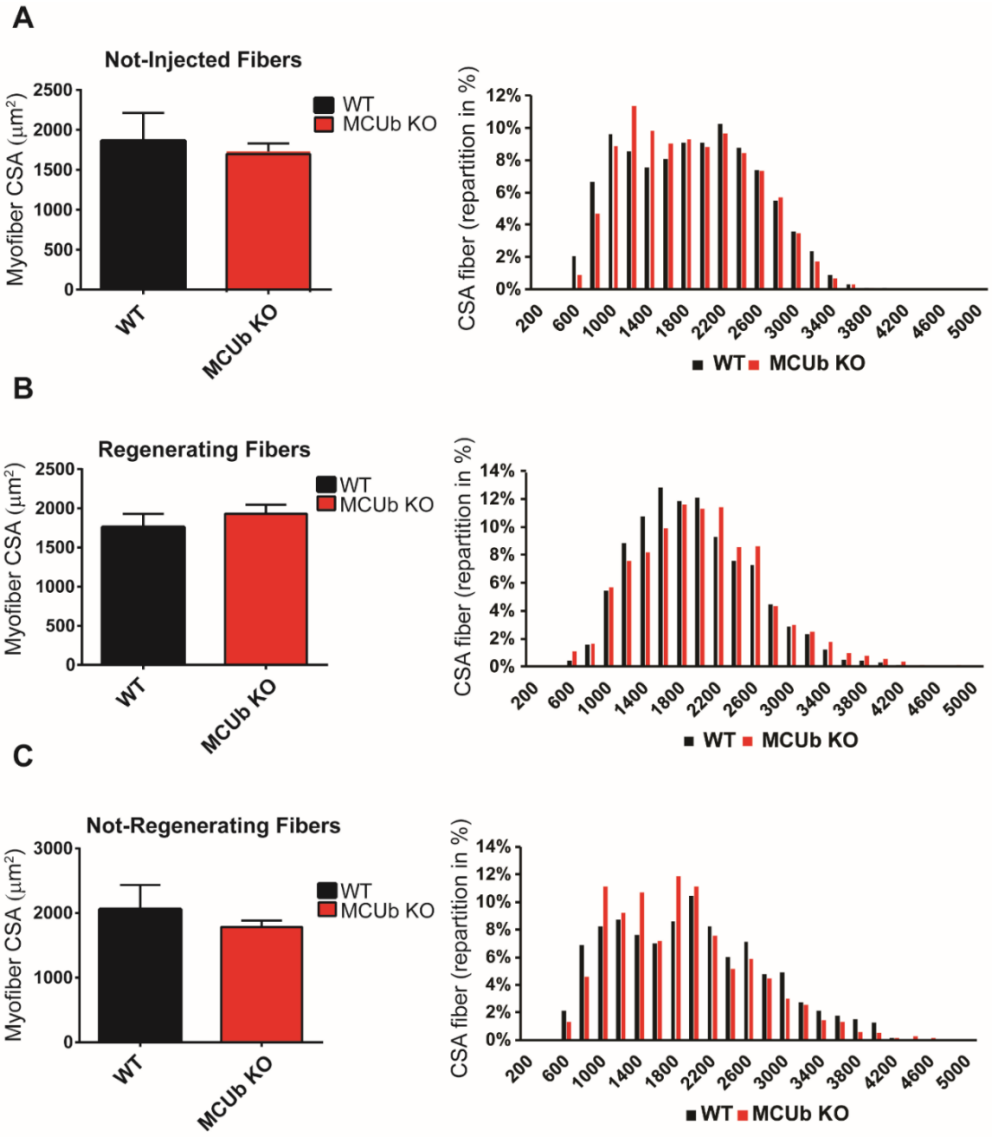
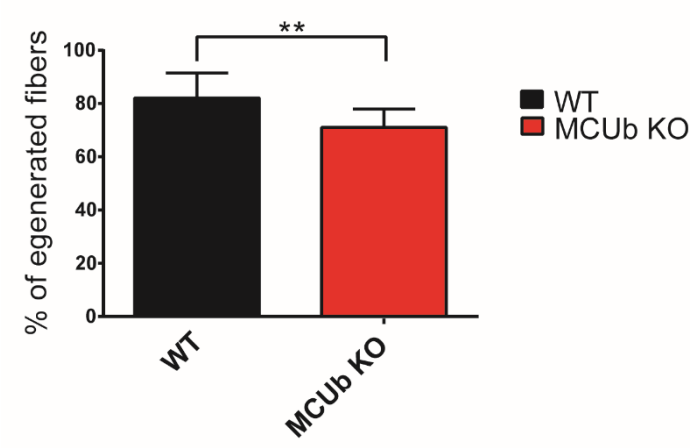


Figure 18. Effects of MCUb ablation on skeletal muscle regeneration.
*WT and MCUb KO TA muscles were injected with CTX and analysed 14 days after injury. Left panels: bar diagrams showing the mean myofiber CSA of not-injected (**A**), regenerating (**B**), and not-regenerating muscles (**C**) 14 days post CTX injection. Right panels: frequency histograms showing the distribution of cross-sectional areas of ~500 not-injected (A), regenerating (B), and not-regenerating fibers (C) 14 days after CTX injection. Results are means ± SEM. n=4.*

We then wondered whether WT and MCUb KO regenerating muscles differ in the number of the newly formed myofibers. This is a crucial parameter, highly related to the efficiency of skeletal muscle repair capacity [159]. Intriguingly, in MCUb KO mice, 14 days post CTX injection, we observed a decrease in the number of myonuclei per fiber (Fig. 19) compared to WT mice. This result suggests that MCUb deficiency, by affecting M2 macrophages polarization, causes an

impairment in the regeneration process by altering the number of newly formed myofibers.



Fig***ure 19. Effects of MCUb ablation on the number of central nuclei in regenerating muscles.***
*WT and MCUb KO TA muscles were injected with CTX and analysed 14 days after injury. The bar diagram represents the quantification of centro-nucleated myofibers expressed as the percentage of regenerating fibers on the total number of myofibers. Results are expressed as mean ± SEM. For data analysis, t-test was used. **p≤ 0,01. n=4.*

We then performed triple regeneration experiments to analyse whether MCUb ablation could cause a depletion of the satellite cells pool, as already performed [22]. We injured TA muscles with CTX three times every 16 days. We then collected muscles and performed immunofluorescence analysis on regenerating muscle fibers (Fig 20A) to highlight the boundary of the fibers. We thus measured the CSA of regenerating fibers. Intriguingly, we observed a strong reduction in the CSA of regenerating muscle fibers of MCUb KO animals compared with WT mice (Fig. 20B). This result suggests that the exhaustion of satellite cells pool occurs in regenerating muscle of MCUb KO animals, thus inducing an impairment in the correct reconstitution of muscle fibers after damage. Since MCUb ablation affects M2 macrophage polarization *in vitro* (Fig. 9A), but also *in vivo* (Fig. 13A and Fig. 14D-F), we hypothesized that the strong decrease that we observed in the CSA of muscle fibers in MCUb KO animals in the triple regeneration experiment was due to a decrease in the number of pro-regenerative M2 macrophages that stimulate fiber growth during skeletal muscle regeneration [16].

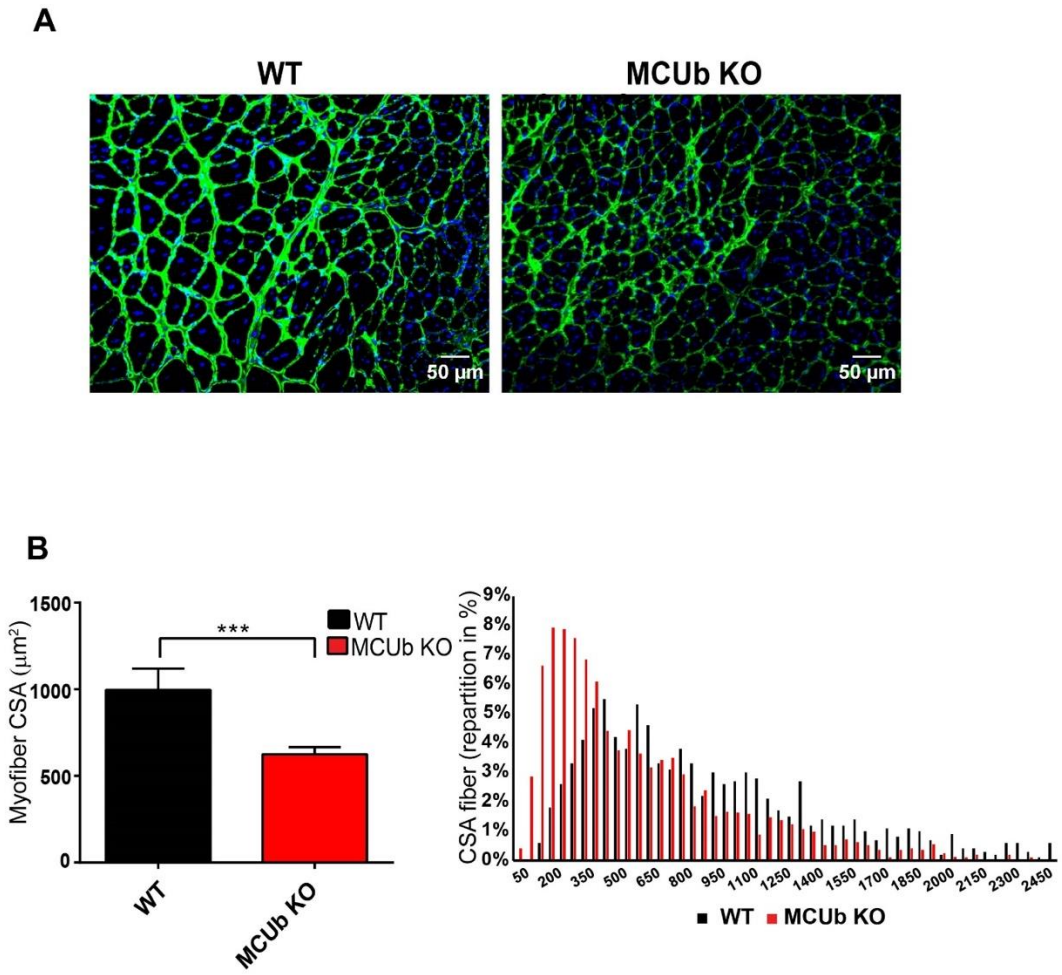


Figure 20. Effects of MCUb ablation on the CSA of regenerating fibers after multiple injuries.
*WT and MCUb KO TA muscles were injected with CTX three times every 16 days. After 16 days from the last injection muscles were collected and immunofluorescence analysis was performed with an antibody against laminin to detect muscle fibers (green) and the nuclei were labelled with Hoechst dye (blue) (**A**). The bar diagram represents the quantification of the CSA of regenerating fibers of the WT and MCUb KO mice (**B**). Right panels: frequency histograms showing the distribution of CSA of ~1000 regenerating fibers. Results are expressed as means ± SEM. For data analysis, t-test was used. ***p≤ 0,001. n=4.*

It is known that pro-inflammatory M1 macrophages exert a positive role on myoblast proliferation while repressing myoblast differentiation (see Introduction, paragraph 3.3.2.2 and [24]). At later stages of skeletal muscle regeneration, they switch their phenotype by acquiring an anti-inflammatory M2 profile, thus releasing anti-inflammatory cytokines such as IL-10 or TGF-β that can dampen the initial inflammatory response (see Introduction, paragraph 3.3.2.2 and [24]). These cytokines have not only a key role in promoting wound healing, thus supporting myogenesis, but they also can stimulate the transient deposition of the extracellular

matrix (ECM) (see Introduction, paragraph 3.3.2.2 and [24]). Importantly, the correct remodelling and reorganization of muscle ECM is of fundamental importance for providing a solid scaffold in which the nascent myofibers will be formed and, in addition, it acts as an additional support for muscle contraction (see Introduction, paragraph 3.3.2.2 and [16]). It is known that, in skeletal muscle, M2 macrophages stimulate ECM deposition by exerting both an autocrine and paracrine effect [16]. In detail, they release metabolites like proline required for collagen production, the major component of ECM, and cytokines, such as TGF-β, that regulate the quantity and quality of collagen produced by the fibro adipogenic precursor cells (FAPs) (see Introduction, paragraph 3.3.2.2 and [16]). Moreover, a particularly delicate interplay between macrophages and FAP cells regulates the production of the ECM [16]. TGF-β, which is released by M2 macrophages, prevents TNFα mediated induction of FAP cell apoptosis by allowing their expansion (see Introduction, paragraph 3.3.2.2 and [16]). Therefore, it is clear that, since FAP cells are the primary producers of connective tissue in injured muscles, the macrophage-mediated effects play a key role in promoting the correct deposition of connective tissue (see Introduction paragraph 3.3.2.2 and [16]). Based on these evidence, we quantified the amount of collagen deposited in WT and MCUb KO animals by performing Syrius Red Staining (Fig. 21A and 21B). We observed no significant differences in the rate of collagen deposition in WT and MCUb KO mice at basal condition but, intriguingly, we observed a significant decrease in collagen content in MCUb KO mice compared to WT mice 14 days after CTX injection (Fig. 21A and 21B). This result indicates that MCUb, by promoting macrophage skewing from an M1 to M2 phenotype (Fig. 9A), sustains M2 macrophages function to support the production of connective tissue. Overall these results further confirm the key role that MCUb, and thus mitochondrial calcium uptake, exerts in the polarization toward an M2 phenotype and, consequently, in the complete structural and functional recovery of injured muscles.

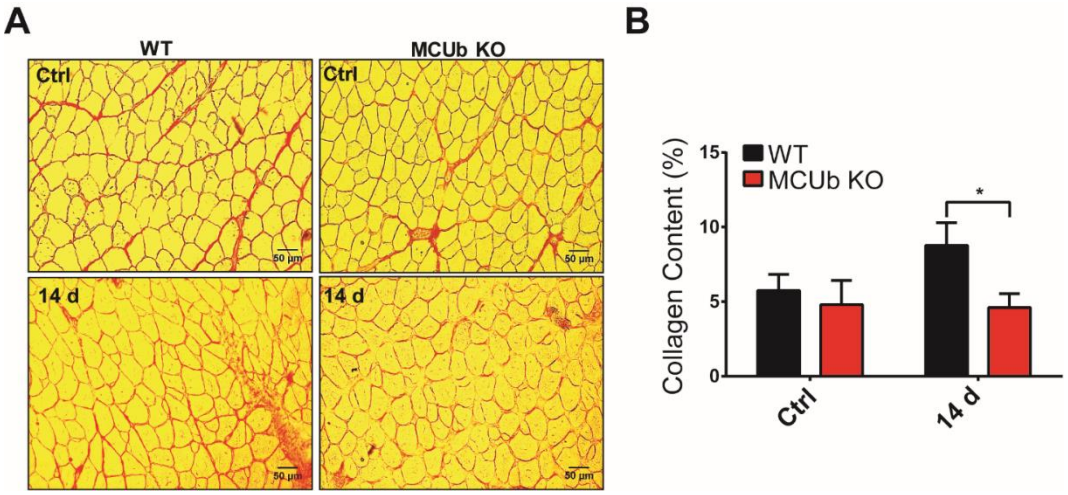


Figure 21. MCUb ablation affects collagen deposition.
*TA muscles from regenerating and control muscles from WT and MCUb KO mice 14 days after CTX injection were analysed. (**A**) Representative images of muscle sections, prepared as in Fig. 17, and stained with Syrius Red. (**B**) Bar diagram representing the percentage of collage content calculated as percent of red pixels in three to six 20X fields Syrius Red stained sections per muscle. Results are represented as means ± SEM. For data analysis, t-test was used. **p<0.01. n=4.*

5.6 Characterization of the signalling pathways promoting MCUb induction in anti-inflammatory M2 macrophages: study of the MCUb promoter

We demonstrated that MCUb shows a peculiar expression pattern in macrophage subpopulation of immune cells, being highly expressed in anti-inflammatory M2 macrophages (Fig. 7). We also demonstrated that MCUb silencing in BMDM polarized with an anti-inflammatory stimulus *in vitro* (IL-4) prevents M1 macrophages to switch to an anti-inflammatory M2 phenotype (Fig. 9A). Overall, these data might imply the activation of a specific transcriptional program mediated by IL-4. Based on these results, we wondered whether IL-4, upon binding on its specific receptor on macrophages, might activate a signaling cascade that culminates with the activation of MCUb transcription.

Intriguingly, it has been already demonstrated that the IL-4-signal transducer and activator of transcription 6 (STAT6) axis is involved in M2 polarization [23], but nothing is known on the regulation of MCUb transcription by this signalling pathway. We thus asked whether the activation of MCUb

transcription could be mediated by STAT6. We first performed a bioinformatics analysis of MCUb promoter looking for STAT6 binding sites. Interestingly, we found a region of 0.5 kilo bases (kb) containing one STAT6 site. We then cloned this region upstream to the luciferase gene. To study the activation of MCUb promoter, we used a human immortalized monocytic cell line, the THP-1 cell line, since it is relatively easier to transfect than primary BMDM. These cells were cultured and then differentiated into macrophages by phorbol-myristate-acetate (PMA). After differentiation, macrophages were transfected with a plasmid coding for firefly luciferase gene under the control of the portion of 0.5 kb of the MCUb promoter (MCUb prom) together with a plasmid coding for renilla luciferase under the control of a constitutive promoter (Fig. 22). The latter plasmid is essential to normalize the luciferase signal to the efficiency of transfection. We used, as control, cells transfected with the empty plasmid coding for firefly luciferase (Ctrl) (Fig. 22). After transfection, mature transfected macrophages were incubated with anti-inflammatory (IL-4) and pro-inflammatory (IFN-γ) polarizing stimuli for 24 hours. We observed a strong increase in bioluminescent signal, corresponding to an increase in luciferase activity, in THP-1 macrophages transfected with the vector containing luciferase under control of MCUb promoter and treated with IL-4 (Fig. 22). This activation is specific, since it cannot be observed in untreated macrophages or cells polarized with a pro-inflammatory stimulus, IFN-γ (Fig. 21).

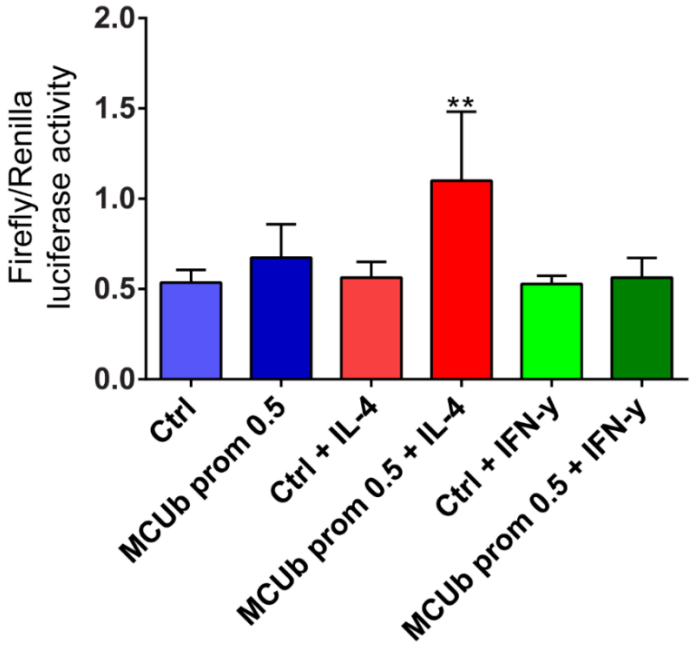


Figure 22. MCUb transcription is induced by the anti-inflammatory cytokine IL-4. *THP-1 monocytes were differentiated by adding to the medium 10 ng/ml PMA for 48 hours and transfected by electroporation with a total of 3 µg of DNA. The ratio used for either the empty vector (Ctrl) or the vector containing the 0.5 kb promoter of MCUb (MCUb prom) with the plasmid coding for renilla luciferase under the control of a constitutive promoter was 5:1. After transfection, differentiation was continued for another 24 hours. Mature transfected macrophages were incubated with anti-inflammatory (IL-4) and pro-inflammatory (IFN-γ) polarizing stimuli for 24 hours. The activation of MCUb promoter was evaluated by Dual Luciferase Reporter Assay kit. Results are represented as means ± SEM. For data analysis, one-way ANOVA was used with post hoc Bonferroni's multiple comparison test for each sample. ** p≤0.01. n=4.*

Altogether, our results support the hypothesis that MCUb transcription in macrophages is activated by the IL-4-mediated cascade. Our hypothesis is that, once activated, MCUb might promote macrophages skewing from an M1 to M2 phenotype. In the future, to evaluate whether MCUb transcription is activated by the IL-4-STAT6 axis, we will perform mutagenesis experiment on the STAT6 binding site on the MCUb promoter to analyse whether the effect of IL-4 on MCUb transcription is specific and mediated by STAT6. The goal of this study is to understand the mechanisms by which macrophages operate the transition from the M1 to the M2 phenotype and how the regulation of mitochondrial Ca^{2+} uptake, mediated by MCUb, might influence this process.

6. Discussion

Mitochondrial Ca^{2+} regulates a wide array of cell functions, from stimulation of aerobic metabolism and thus of ATP in physiological settings, to induction of cell death in pathological conditions [9]. Indeed, many studies demonstrated that mitochondrial Ca^{2+} uptake represents a highly sophisticated process, whose modulation has multiple consequences for the cells [9].

The main physiological role of mitochondrial Ca^{2+} rises occurring in stimulated cells is the induction of Ca^{2+}-sensitive dehydrogenases of the Krebs cycle and thus ATP production [9]. In addition, the regulation of mitochondrial Ca^{2+} uptake prevents cell death which is causally linked to organelle Ca^{2+} overload [9]. The discovery of the molecular identity of the MCU complex, the highly selective channel responsible for mitochondrial Ca^{2+} entry, gave rise to an explosion of discoveries aimed to clarify not only the composition and the regulation of the MCU complex but also the physiopathological role of mitochondrial Ca^{2+} uptake [9].

The discovery of the molecular identity of the pore-forming subunit MCU [7,8], led to the identification of other proteins that participate to the formation of the channel complex [9]. Among these is the other pore-forming subunit, MCUb, that acts as an endogenous dominant-negative subunit within the complex, allowing an intrinsic regulatory mechanism [10]. Importantly, MCUb shares a high sequence and structure similarity with MCU, especially in the transmembrane domain of the protein (see Introduction, paragraph 3.2.4.1, Fig. 2A and [10]). The finding that MCUb acts as a dominant-negative regulator of the MCU complex was first hypothesized observing that, in living cells, the overexpression of MCUb reduces mitochondrial Ca^{2+} uptake and, coherently, MCUb silencing exerts the opposite effect [10]. This hypothesis was then confirmed by experiments performed in reconstituted lipid bilayers that showed that MCUb displays no channel activity and it abolishes the Ca^{2+} channel activity of the co-expressed MCU [10]. Up to date, the role of MCUb in physiopathology is poorly understood. It was hypothesized that the MCU/MCUb expression ratio, which varies greatly between tissues, from ~3:1 in heart to ~40:1 in skeletal muscle, might contribute to the differences in the

amplitude of mitochondrial Ca^{2+} uptake in different tissues, as recently demonstrated by patch-clamp analysis of isolated mitochondria from several mouse tissues [14].

Here we found that MCUb shows a peculiar expression pattern compared with the other components of the MCU complex. Indeed, bioinformatic studies showed that MCUb is highly expressed in immune cells (see Introduction, paragraph 3.3.2.5 and Fig. 6) and our results demonstrated that MCUb is highly expressed in the anti-inflammatory (M2) macrophage subpopulation (Fig. 7). In detail, we found that BMDM, polarized with an anti-inflammatory cytokine, IL-4, show an upregulation of *Mcub* mRNA level (Fig. 7). This induction specifically occurs after IL-4 stimulation since *Mcub* expression is not altered by stimulating cells with a pro-inflammatory cytokine, IFN-γ, a cytokine that induces the differentiation of BMDM in pro-inflammatory (M1) macrophages (Fig. 7). Importantly, *Mcu* expression appears not to be regulated in all these conditions (Fig. 7). Coherently with the expression data, M2 macrophages show a decrease in mitochondrial Ca^{2+} uptake compared to the M1 macrophage subpopulation (Fig. 8).

Furthermore, MCUb silencing in BMDM strongly prevents them from acquiring an M2 phenotype (Fig. 9A), measured by the evaluation of the expression of *Arg1*, a marker of M2 macrophages, in BMDM cells polarized with IL-4. Interestingly, MCUb silencing does not affect M1 polarization, as demonstrated by the lack of significant changes in the expression levels of iNOS, a marker of M1 macrophages, in BMDM cells polarized with the pro-inflammatory INF- γ (Fig. 9B).

Studies both *in vivo* and *in vitro* have demonstrated that macrophages can undergo dynamic transitions from the M1 and M2 states of activation, process that is called polarization skewing [160,161]. However, although recently different regulatory pathways have been associated with macrophage skewing from an M1 to M2 phenotype [20,161], the signals modulating macrophage activation states *in vivo* are still undefined. Importantly, alterations of macrophage responses have been observed in several human chronic diseases [15,162]. While the involvement of cytosolic Ca^{2+} in macrophages differentiation has been described under different physiological and pathological conditions [149,151], nothing is known on the

involvement of mitochondrial Ca^{2+} in the differentiation potential of macrophages. Our results suggest that the MCU complex, by regulating the entry of Ca^{2+} into mitochondria through the modulation of the expression of MCUb, contributes to the proper M1 to M2 skewing (Figure 9A).

Furthermore, the M1 to M2 transition is fundamental for sustaining tissue regeneration [15]. This process is particularly relevant in skeletal muscle, since this tissue shows a remarkable capacity to fully regenerate after mechanical trauma [123]. It is widely accepted that, in skeletal muscle, macrophages play a key role in both the orchestration and the resolution of inflammation and the restoration of the tissue integrity and function [15]. In detail, it has been shown that macrophages accompany skeletal muscle regeneration by sequentially adopting at least two main inflammatory profiles [15]. Indeed, soon after damage, both muscle associated macrophages and macrophages derived from circulating monocytes exhibit a pro-inflammatory M1 profile and can stimulate MPCs proliferation [15]. 24 to 72 hours later, these macrophages skew into an anti-inflammatory M2 phenotype by promoting the terminal differentiation of MPCs, their fusion into myotubes and the growth of the newly regenerated myofibers [15].

Based on our findings strongly suggesting that MCUb plays a key role in promoting M2 macrophage polarization (Fig. 9A), we performed skeletal muscle regeneration experiments on WT C57/BL6N mice. We chose this model since skeletal muscle regeneration post-injury provides an excellent paradigm for studying the resolution of inflammation and tissue repair [20]. We injected tibialis anterior (TA) muscles with CTX and we collected regenerating muscles at 3, 7 and 14 days after CTX injection. CTX treatment is the most common method to induce skeletal muscle regeneration [20]. Indeed, this treatment induces a sterile inflammation and, importantly, it causes a homogeneous damage of the whole muscle [20]. We performed RT-PCR experiments on regenerating muscles and we observed a huge increase in the expression level of *Mcub* 3 days post CTX injection (Fig. 10A). Interestingly, no differences were observed in the expression levels of the pore forming subunit *Mcu* (Fig. 10B) and the positive regulator *Micu1* (Fig. 10C). We observed only a mild upregulation of the gatekeeper of the channel *Micu2*

(Fig. 10D). The impressive induction of MCUb 3 days after CTX injection suggests a peculiar role of this protein in the healing process after damage.

Since *Mcub* is poorly expressed in skeletal muscle in basal conditions (Fig. 10A and [10]), while it is highly expressed in M2 macrophages compared to the M1 subpopulation (Fig. 7 and 7), we hypothesized that the strong increase of MCUb expression, 3 days post CTX injection (Fig. 10A), could be due to the infiltration of macrophages, that are known to reach a maximum peak 3 days post injury [20].

To confirm that *Mcub* is selectively induced in macrophages and not in skeletal muscle fibers, we performed RT-PCR experiments on macrophages purified by FACS from regenerating muscles of WT mice. This experiment clearly shows that *Mcub* expression is induced in macrophages (Fig. 11). The coexpression of MCUb and a typical marker of anti-inflammatory M2 macrophages, 3 days post CTX injection (Figure 13A), make us hypothesize that MCUb expression is induced in the M2 subpopulation of macrophages.

Based on our *in vitro* results, demonstrating that MCUb silencing affects macrophages polarization into an anti-inflammatory M2 phenotype (Fig. 9A), and by considering the importance of M2 macrophages in skeletal muscle repair [15,16], we analysed the effects of MCUb ablation *in vivo.* We generated a total MCUb knockout (KO) mouse model using the 'knockout-first' strategy [155] and we performed skeletal muscle regeneration experiments on both WT and MCUb KO animals. Importantly, the MCUb KO mice are fertile and do not present any phenotypical gross alteration. We collected TA muscles 3, 7 and 14 days after CTX injury and we analysed the expression levels of classical M1 and M2 markers (Fig. 13A and 13B). Interestingly, we found that MCUb ablation *in vivo* causes a significant decrease in the expression level of CD206, a marker of the anti-inflammatory M2 macrophages [17], compared to WT mice (Fig. 13A). On the contrary, in the MCUb KO animals, we observed a significant increase in the expression level of TNF-α, a marker of pro-inflammatory M1 macrophages (Fig. 13B and [17]). To further confirm our findings, we performed FACS analysis on immune cells purified from regenerating muscles of both WT and MCUb KO animals. To better characterize the inflammatory mononuclear cellular infiltrate, we decided to analyse the early stages of skeletal muscle regeneration process, since it

is known that they are characterized by the infiltration of a huge number of inflammatory cells [20]. We thus collected and purified immune cells from TA muscles of WT and MCUb KO animals 1, 3 and 5 days post CTX injury. We used specific flow cytometer antibodies to detect and isolate the monocytes and macrophages subpopulations. Interestingly, we did not find significant differences in the total number of monocytes and total macrophages between WT and MCUb KO animals (Fig. 14A-C and Table 14G). Coherently with our *in vitro* results showing that MCUb silencing affects the ability of macrophages to acquire an M2 phenotype (Fig. 9A), we observed a decrease in the number of M2 macrophages in MCUb KO mice compared with WT mice 1 and 3 days after CTX injury (Fig. 14D, 14E, and Table 14G). Interestingly, these differences were blunted 5 days post-injury (Figure 14F and Table 14G).

Altogether, these results confirm our hypothesis that MCUb ablation *in vivo* might cause a delay or a defect in M2 macrophage polarization, thus affecting skeletal muscle regeneration.

To evaluate whether MCUb ablation can also affect the phagocytic capacity of macrophages, which is the ability to engulf and kill invading pathogens and to clear cell debris [157], we tested the phagocytic capacity of BMDM derived from WT and MCUb KO animals. Phagocytes and their ability to phagocytose are important part of the innate immune system and an impairment in phagocytosis has been associated with numerous diseases and disorders [163]. We thus tested the phagocytic capacity of BMDM derived from WT and MCUb KO animals *in vitro* and we observed a significant decrease in macrophage phagocytic activity in KO mice compared to WT mice (Fig. 15).

Overall, our results show that MCUb ablation negatively influences the phagocytic capacity of macrophages and this alteration might have consequences on skeletal muscle repair *in vivo*.

Importantly, it is known that inflammatory cells influence muscle regeneration by releasing crucial cytokines that can affect gene expression of MPCs, thus impinging on proliferation, differentiation and growth of the newly formed myofibers [16,17]. Thus, since macrophages sustain skeletal muscle regeneration [15–17], any alteration in macrophage polarization from M1 to M2

subtypes can differently affect the functionality of the skeletal muscle stem cell, satellite cells [15].

In order to evaluate the progression of the regeneration process in WT and MCUb KO animals, we analysed the expression of different MRFs, crucial players of satellite cells proliferation and differentiation [118], during the time course of skeletal muscle regeneration in both WT and MCUb KO animals. RT-PCR experiments demonstrated that, 3 days after CTX injection, MCUb KO mice show a significant reduction of the expression of *Pax7* compared to WT mice (Fig. 16A), a marker of both quiescent and activated satellite cells [117]. This data strongly suggests that the regeneration of TA muscles from MCUb KO mice might be impaired. This was also confirmed by the blunted expression of the transcription factor *Myod*, in MCUb KO mice (Fig. 16B). Indeed, *Myod* is a crucial player of the commitment of satellite cells to the differentiation process [118] and the strong decrease we observe in MCUb KO mice, 3 days post-CTX injection (Fig. 16B), suggests an impairment in satellite cells differentiation. Surprisingly, we observed no differences in the expression of *Myog* (Fig. 16C), a marker of fully differentiated myofibers that participate to the latest stages of tissue repair [118]. These results suggest that MCUb ablation, by influencing macrophages phenotype, might have an impact on the early stages of skeletal muscle repair after damage.

An impairment in skeletal muscle structure of MCUb KO animals has been also observed by H&E staining performed on regenerating TA muscles from WT and MCUb KO animals 2, 7 and 14 days after CTX injection (Fig. 17). Indeed, regenerating muscles of MCUb KO animals show a persistent mononuclear cellular infiltrate and a clear alteration of muscle structure 7 and 14 days post CTX injection (Fig. 17).

In order to test the efficiency of skeletal muscle regenerative capacity of MCUb KO mice, we also measured the CSA and the number of regenerating muscle fibers 14 days after CTX injection, as previously performed [20]. To exclude differences in the CSA area of muscle fibers between WT and MCUb KO animals in basal conditions, we measured the area of fibers from muscles not subjected to CTX-mediated regeneration, finding no differences (Fig. 18A). We performed this

experiment to avoid misleading results in the analysis of the differences in CSA in regenerating fibers of WT and MCUb KO animals.

We then analysed the area of regenerating fibers 14 days post CTX injection (Fig. 18B), finding, also in this case, no significant differences between WT and MCUb KO animals (Fig. 18B). As expected, no differences were also detected between WT and MCUb KO animals in the CSA of not-regenerating fibers within regenerating muscles 14 days post CTX injection (Fig. 18C).

Lastly, we analysed the number of the newly formed myofibers by counting the number of fibers with central nuclei in WT and MCUb KO regenerating muscles. This data is another crucial parameter that is used to determine skeletal muscle regeneration capacity after damage [159]. Intriguingly, in MCUb KO mice, 14 days post CTX injury, we observed a decrease in the number of myonuclei per fiber, compared to regenerating muscles of WT mice (Fig. 19). This decrease strongly indicates that the MCUb KO animals present a depletion of the pool of satellite cells. This data is supported by our finding that, in regenerating muscles of MCUb KO, the expression levels of *Pax7* and *Myod*, that respectively mark the pool of quiescent/activated and proliferating/differentiating satellite cells [116,118,164] are strongly reduced (Fig. 16A and 16B). Altogether these findings suggest that MCUb deficiency, by influencing macrophage plasticity and their polarization into an M2 phenotype, might cause an altered proliferation and differentiation of satellite cells.

To further prove that MCUb ablation could impinge on the pool of quiescent satellite cells, we performed a triple regeneration experiment, as already performed [22]. In this regard, it is known that satellite cells represent the major regenerative cell population in adult skeletal muscle and can support multiple rounds of skeletal muscle regeneration while maintaining intact the satellite cell pool [165,166]. Based on these observations and on our *in vivo* data showing no major differences in the CSA of regenerating fibers of WT and MCUb KO animals (Fig. 18B), we hypothesize that the effect of MCUb ablation was not appreciable after one round of regeneration. Furthermore, multiple rounds of damage and regeneration is a hallmark of several muscular pathologies, such as muscular dystrophies [167]. Therefore, to test the ability of skeletal muscle to regenerate after injury and to

evaluate the satellite cells capacity to react to multiple injuries reconstituting the satellite cells reservoir, we performed triple skeletal muscle regeneration experiment by inducing three consecutive damage every 16 days, as already performed [22]. Intriguingly, we observed that MCUb KO mice show a strong reduction of the area of regenerating muscle fibers compared with WT mice (Fig. 20B).

This result, together with the data showing the reduction in the number of regenerating fibers after a single muscle damage (Fig. 19) and the decrease in *Myod* and *Pax7* expression in regenerating MCUb KO muscles (Fig. 16A and 16B), indicates an alteration in the reconstitution of muscle structure after injury that might be due to the exhaustion of the satellite cells pool due to the reduction of trophic factors, released by the M2 pro-regenerative macrophage population.

It is well established that inflammatory cells influence muscle regeneration, not only by directly acting on MPCs, but also by promoting the reconstitution of the myogenic environment [16]. In this regard, the interplay between macrophages and fibro adipogenic progenitors (FAPs) plays a crucial role [16]. This crosstalk regulates the production of the extracellular matrix (EMC), composed mainly by collagen that is a fundamental component of the extracellular matrix and acts as a scaffold for the formation and maintenance of the newly formed fibers [16]. Importantly, M2 macrophage subpopulation stimulates collagen deposition by secreting molecules that participate to its synthesis and by stimulating FAPs to secrete collagen [16]. We therefore compared the amount of collagen produced by regenerating muscles from WT and MCUb KO animals 14 days post CTX injection. We observed that regenerating muscles from KO animals showed a significant decrease in collagen content 14 days post-injury (Fig. 21B). Altogether, these findings show that MCUb ablation, by affecting M1 to M2 skewing *in vivo*, impinges on M2 macrophages function by causing a reduction in collagen content in MCUb KO animals and an impairment in the reconstitution of the architecture of muscle structure.

The observation that BMDM treated with an anti-inflammatory cytokine, IL-4, show an induction in MCUb expression (Fig. 7), and that MCUb silencing in BMDM affects macrophage capacity to acquire an M2 phenotype after IL-4

treatment (Fig. 9A), made us hypothesize that MCUb transcription could be regulated by IL-4. Indeed, it is widely accepted that the IL-4-STAT6 pathway is involved in M2 polarization [23]. We thus wondered whether the activation of MCUb transcription could be mediated by STAT6. For this purpose, we first performed a bioinformatic analysis of the MCUb promoter. Intriguingly, we found a conserved STAT6 binding site on the MCUb promoter and we cloned a portion of 0,5kb containing this site upstream to the luciferase gene. To study the regulation of the MCUb promoter, we performed a Dual Luciferase Assay on a human immortalized monocytic cell line, the THP-1 cell line, after differentiation to either M1 or M2 macrophages. The results we obtained demonstrated that MCUb promoter activity is induced in macrophages treated with the anti-inflammatory cytokine IL-4 (Fig. 22). Interestingly, the activation of MCUb promoter, mediated by IL-4, is specific, since it could not be observed in untreated or macrophages polarized with a pro-inflammatory stimulus, IFN-γ (Fig. 22). Overall these results strongly indicate that the activation of MCUb transcription in macrophages is mediated by an IL4 mediated pathway. To strengthen our findings on the IL-4-STAT6 mediated regulation of MCUb transcription, we will perform mutagenesis experiments on STAT6 binding site to see whether the effect of IL-4 on MCUb transcription is specifically mediated by STAT6.

In conclusion, our results strongly suggest that MCUb is necessary for M2 macrophage differentiation (Fig. 9A, 13A, 14D-F and 15). We also demonstrated that MCUb ablation *in vivo* negatively affects M1 to M2 macrophages skewing by causing an impairment in satellite cell proliferation and differentiation (Fig. 16A and 16B, 19, 20B).

In the future, we will deepen our knowledge on the mechanisms by which macrophages derived from MCUb KO animals show altered satellite cells proliferation and differentiation. To address this point, we will perform co-culture experiments in which co-conditioned media from WT- and MCUb KO-derived macrophages will be added to cultured satellite cells from WT animals. This experiment will give a clear indication on the involvement of MCUb KO altered macrophage population to skeletal muscle differentiation without the confusing effects of other cell populations and of skeletal muscle itself on this process.

Moreover, since our preliminary histochemical analysis on regenerating muscles from MCUb KO animals highlighted an alteration in the reconstitution of muscle structure after damage (Fig. 17), we will investigate this aspect in depth by analysing several parameters to precisely quantify the efficiency of skeletal muscle regeneration. We will thus analyse the number of necrotic and phagocytic muscle fibers after injury, other parameters highly correlated with macrophages functionality [17,20]. We will also detect the number of $PAX7^+$ and $MYOD^+$ cells, which will give a precise quantification of the population of satellite cells committed to the generation of new fibers. This analysis, together with the data on the CSA of regenerating fibers after multiple rounds of CTX-induced regeneration (Fig. 20), will strongly determine whether the lack of MCUb impairs the maintenance of the pool of satellite cells.

It is well established that dynamic changes in macrophage populations and activation states within the damaged muscle tissue contribute to its efficient regeneration [24]. Indeed, skewing in macrophage phenotype, from M1 to M2 subtypes, can differentially affect satellite cells function and lead to aberrant regeneration and fibrosis deposition, as described in several pathological diseases [15]. In this context, we will determine the expression of MCUb in muscles biopsies from patients affected by Duchenne muscular dystrophy. Our hypothesis is that, since the regeneration program is heavily compromised in patients affected by this neuromuscular disease caused by the continuous damage-regeneration cycles [111] we will find altered MCUb expression. In the long run, we shall study the role of MCUb in the pathogenesis of this disease by crossing MCUb KO animals and the animal model of Duchenne muscular dystrophy, the MDX mice, and evaluating the muscular phenotype of these animals.

We believe that our research might be fundamental for uncovering the molecular basis of chronic muscular diseases, such as the Duchenne muscular dystrophy, and in pathological conditions where muscle repair system is compromised such as during ageing [25,111]. We strongly believe that MCUb, and thus mitochondrial calcium, could represent a new pharmacological target to combat these debilitating conditions.

7. Materials and Methods

7.1 Legend of Abbreviation

µl = microliters
µM = micromolar
BM = Bone marrow
BMDM = Bone marrow derived macrophages
bp = base pair
cDNA = DNA complementary
CTX = cardiotoxin
CytD = Cytochalsin D
DMEM = Dulbecco's modified Eagle's medium
FACS = fluorescent activated cell sorting
FBS = fetal bovine serum
H&E = haematoxylin & eosin
h = hours
KO = knockout
M-CSF = macrophage colony-stymulating factor
min = minute
mM = millimolar
MM = Mus Musculus
NH_4Cl = ammonium chloride
o.n. = over night
PBS = phosphate buffered saline
PCR = polymerase chain reaction
PFA = paraformaldehyde
r.t. = room temperature
RNA = ribolucleic acid
RPMI = Roswell Park Memorial Institute
TA = tibialis anterior
WT = wild type
IMDM = Iscove's Modified Dulbecco's Medium

7.2 Animals

Adult male C57/BL6N mice (8 weeks old) from WT and MCUb KO mouse strains were used. For the generation of the total MCUb KO mouse model, the strategy provided by the European Conditional Mouse Mutagenesis (EUCOMM) and the National Institute of Health Knockout Mouse (KOMP) was used. EUCOMM uses promoterless and promoter-driven targeting cassettes for the generation of a 'knockout-first' allele [155] in C57BL/6N embryonic stem cells [168]. In detail, the 'knockout-first' allele (Tm1a) contains an IRES:lacZ trapping cassette and a floxed promoter-driven neo cassette inserted into the intron of a gene, disrupting gene function. For the generation of a total MCUb KO mouse model, mice containing a promoterless selection cassette was used. Mice were provided in heterozygous form and were bred to obtain the homozygous genotype. As, controls, age and sex matched WT C57BL/6N were used. *In vivo* experiments were performed in accordance with the Italian law D. L.vo n.26/2014.

7.3 Mouse Cardiotoxin Muscle Regeneration Model

Myonecrosis was induced by the intramuscular injection of CTX (Sigma-Aldrich), as already performed [17,20]. CTX destroys muscle fibers without altering the muscle fiber basal lamina, nerves, blood vessels, and satellite cells. In brief, mice were anaesthetized with isoflurane and 50 µl of 10 µM CTX were injected in TA muscle. Muscles were collected for analysis at different time points post-injury (3, 7, 14 days). In the triple regeneration protocol, TA muscles of both WT and MCUb KO animals were injected three times every 16 days with 50 µl of 10 µM CTX and muscles were collected 16 days after the last injury.

7.4 Histological and Immunohistochemical Analysis

Regenerating and control TA muscles of both WT and MCUb KO mice were dissected 3, 7 and 14 days post CTX injection, frozen in nitrogen-cold

isopentane and kept at –80° C until use. 6 µm-thick muscle cryosections were prepared and stained by H&E staining kit (Bio-Optica), following manufacturer instructions. For collagen quantification muscle cryosections were stained by Sirius Red staining kit (Sigma-Aldrich), following manufacturer instructions. For immunofluorescence analysis, 20 µm-thick muscle cryosections were fixed in 4% PFA for 20 min, quenched with 50 Mm NH_4Cl in PBS and blocked in PBS containing 10% goat serum and 0.5% BSA for 20 min, as already performed [108]. Sections were then incubated with α-Laminine primary antibody (Sigma-Aldrich) at the dilution of 1:100 in PBS with 0,5% BSA and 2% goat serum o.n. at 4° C to label the sarcolemma of muscle fibers. Muscle cryosections were washed 3 times with PBS. The sections were incubated for 1 hour at r.t. with an anti-rabbit Alexa Fluor 488-conjugated secondary antibody (Thermo Fisher Scientific) at the dilution of 1:500 in PBS with 0,5% BSA and 2% goat serum. Myonuclei were identified by Hoechst 33342 (Sigma-Aldrich), as already performed [108].

7.5 Fiber size measurements

20 µm-thick muscle cryosections from controls and TA regenerating muscles of WT and MCUb KO animals were labelled with α-Laminine primary antibody following the protocol described in the previous paragraph. CSA and fiber size distribution of regenerating (centronucleated myofibers), non-regenerating and control TA muscles was manually measured by using IMAGE software (Scion, Frederick, MD). More than 500 fibers were measured for each muscle.

7.6 RNA extraction, reverse transcription and quantitative Real-Time PCR

The expression levels of the MCU complex components (MCU, MCUb, MICU1 and MICU2), myogenic regulatory factors (PAX7, MYOD, MYOG and MRF4) and markers of pro-inflammatory M1 (iNOS, TNF-α) and anti-inflammatory M2 macrophages (ARG1, CD206) were analysed in regenerating and control TA muscles and BMDM from adult male C57/BL6N WT and MCUb KO animals.

Total RNA was extracted trough mechanical tissue homogenization in TRIZOL reagent (Thermo Fisher Scientific), following manufacturer instructions. The RNA was quantified with Nanodrop (Thermo Fisher Scientific) and 1 µg, for each muscle, and 500 ng of RNA, for each cellular sample, was retro-transcribed with the cDNA synthesis kit SuperScript II (Thermo Fisher Scientific), following manufacturer instructions. Oligo $(dT)_{12-18}$ primer (Thermo Fisher Scientific) was used as primer for first stand cDNA synthesis with reverse transcriptase together with dNTPs (Thermo Fisher Scientific), following manufacturer instructions. The obtained cDNA was analysed by RT-PCR using the IQ5 thermocycler and SYBR green chemistry (Bio Rad), following manufacturer instructions. The primers were designed and analysed with Primer 3 and their efficiency was between 95 and 100%. The housekeeping genes GAPDH was used for cDNA normalization in cDNA samples derived from both BMDM and TA muscles of WT and MCUb KO animals. For quantification, expression levels were calculated by using the $2^{-\Delta\Delta CT}$ Method [169]. Primers for RT-PCR were used at the final concentration of 20 µM and the sequences are reported in the table below:

Gene	Forward	Reverse
mm_GAPDH	5'-CACCATCTTCCAGGAGCGAG-3'	5'-ACAGTTCCGAGCGTCAAAGACC-3'
mm_MCUb	5'-CTGGCTTACTTGGTGGGTGT-3	5'-CGCTGCGATTTCTTGTGGAA-3
mm_MCU	5'- AAAGGAGCCAAAAAGTCACG-3'	5'- AACGGCGTGAGTTACAAACA-3'
mm_MICU1	5'- AAGGCAGCATCTTCTACAGCC-3'	5'- CCTGCTCAAACTCCTCCATGT-3'
mm_MICU2	5'- TGGAGCACGACGGAGAGTAT-3'	5'- GCCAGCTTCTTGACCAGTGT-3'
mm_PAX7	5'-GGGACTTGTGTTGTGGGGAA-3'	5'- CCACTGGAAATCGGCCTTCT-3'
mm_MYOD	5'-CTCTGATGGCATGATGGATT-3'	5'-GTGGAGATGCGCTCCACTAT-3'
mm_MYOG	5'-TGCAAAATTGGCTCAAACAG-3'	5'-GCAGTCAAGCCCAAAGTCTC-3'
mm_MRF4	5'-AATTCTTGAGGGTGCGGATT-3'	5'-GAAGACTGCTGGAGGCTGAG-3'
mm_TNF-α	5'-CACAGAAAGCATGATCCGCG-3'	5'- ACTGATGAGAGGGAGGCCAT-3'
mm_CD206	5'-ATCCACTCTATCCACCTTCA-3'	5'-TGCTTGTTCATATCTGTCTTCA-3'
mm_ARG1	5'- ACAAGACAGGGCTCCTTTCAG -3'	5'-GGCTTATGGTTACCCTCCCG-3'
mm_iNOS2	5'-GCACATTTGGGAATGGAGACTG-3'	5'-GGCCAAACACAGCATACCTGA-3'

7.7 Macrophage cell culture and transfection

Macrophages were obtained from BM precursor cells isolated from 8-10 weeks C57/BL6N WT and MCUb KO mice. Briefly, total BM was obtained from mice by flushing femur and tibiae BM with DMEM (Thermo Fisher Scientific). BM precursor cells were cultured and differentiated into macrophages in a DMEM-containing conditioned medium enriched with 40 ng/ml of M-CSF (Sigma-Aldrich). After 7 days, where indicated, BMDM were activated with cytokines to obtain various activation states. Macrophages were treated either with IFN-y (100 ng/ml) or IL-4 (20 ng/ml) for 24 h to trigger respectively the polarization into pro-inflammatory (M1) or anti-inflammatory (M2) macrophages, in DMEM containing 10% FBS and cells were used for RT-PCR analysis. Where indicated, MCUb was silenced in BMDM by transfection of a specific siRNA using the Amaxa™ Nucleofector™ Technology. 24 h post transfection, macrophages were polarized into an M1 or M2 polarization state and cells were used for RT-PCR analysis.

7.8 siRNA

siRNA-MCUb: nucleotides 848–866 of the corresponding mRNA:

5′-UUUCUUCAGUUCUUCCACAtt-3′

3′-ttAAAGAAGUCAAGAAGGUGU-5′

The non-targeting siRNA (scrambled) is the following:

5′-GCCUAAGAACGACAAAUCAtt-3′

3′-ttCGGAUUCUUGCUGUUUAGU-5′

7.9 Isolation of leukocytes and macrophages from skeletal muscle

TA mouse muscles were enzymatically and mechanically digested using the skeletal muscle dissociation kit (Miltenyi Biotec) and the gentleMACS™ Dissociator (Miltenyi Biotec), following manufacturer instructions. After

dissociation, the samples were applied firstly to a filter with 70 µm pore size (Miltenyi Biotec) and subsequently to a filter with 30 µm pore size to remove any remaining larger particles from the single-cell suspension. Cell suspension was magnetically labelled with CD11b MicroBeads (Miltenyi Biotec). Subsequently, the cell suspension was loaded onto a MACS® LS Column (Miltenyi Biotec) and placed in the magnetic field of a MACS Separator (Miltenyi Biotec), following manufacturer instructions. The magnetically labelled $CD11b^+$ cells were retained on the LS column. The unlabeled cells run through and this cell fraction were depleted of $CD11b^+$ cells. After removal of the column from the magnetic field, the magnetically retained $CD11b^+$ cells were eluted as the positively selected cell fraction. $CD11b^+$ cells were labelled with secondary antibodies conjugated with specific fluorescent dyes and incubated for 15 minutes at 4−8 °C. Fluorescent antibodies against markers of monocytes, Ly6C (Ly6C-efluor 450) (BD Bioscences), granulocytes, Ly6G (APC-Cy7) (BD Bioscences), macrophages, F4/80 (F4/80-fitc) (BD Bioscences) and anti-inflammatory macrophages (M2), CD206 (CD206-Alexa Fluor 647) (BD Bioscences) were used. Cells were analyzed by fluorescence activated cell sorter (FACS) (BD Bioscences FACSCanto^{TM}).

7.10 Phagocytosis Assay

BMDM were differentiated with 40 ng/ml of M-CSF for 7 days. After 7 days, cells were harvested for phagocytosis assay using PBS 2 mM EDTA. Briefly, 10^5 cells were starved in (IMDM) 0,2% BSA for 30 min at 37° C, then seeded into pre-chilled 96 well and kept at 4°C for 20 min. BMDM were then incubated with 1 mg/ml of pHrodo™ Green *S. aureus* BioParticles® (Thermo Fisher Scientific). As negative control, BMDM were pre-treated with 10 µg/ml of an actin polymerization inhibitor Cytochalsin D (CytD). At the indicated time points, cells were collected and washed in cold PBS and phagocytosis were assessed by flow cytometry. A specific gate was created for cells positive for green fluorescence (i.e. cells engulfed with beads), and the M.F.I. (Mean Fluorescence Intensity) was measured using FlowJo analysis software. Phagocytic Index (P.I.) is determined by multiplying the % of cells that had ingested the beads and the M.F.I. of the phagocytic gate.

7.11 Reporter assay on THP-1 macrophages

Human immortalized THP-1 monocytes were cultured in RPMI-1640 (Thermo Fisher Scientific) medium supplemented with 10% (v/v) FBS 1% (v/v) penicillin /streptomycin (Thermo Fisher Scientific) in a CO_2 incubator at 37° C. 1.0 x 10^7. THP-1 cells were seeded into a 75 cm^2 culture flask and differentiated into macrophages by using RPMI-1640 supplemented medium added with 10 ng/ml phorbol-12myristate 13-acetate (PMA) for 48 h. Subsequently, cells were detached by using Accutase (Thermo Fisher Scientific) and centrifuged at 300 xg at r.t. Cell pellet was resuspended in 1 ml of RPMI medium and cell number was determined. For transfection 1.000.000 cells were used for each condition. 3 µg of DNA were used for each transfection. The transfection was performed using a Nucleofector 2b device for electroporation of the cells. The 0,5 kb human promoter sequence of MCUb was amplified by PCR from human genomic DNA with the following primers:

hsMCUb_0.5kb:

Fw 5'-GGTACCGGGGAAAACAAACTTCCTGA-3'

Rv 5'- CTCGAGCCTCCCGCCCCAGGCGCGCG-3'

The MCUb 0,5 promoter region was then cloned in PGL4.17 [luc2/Neo] vector (Promega) between the KpnI and the XhoI restriction sites. As control, the PGL4.17 vector, lacking the MCUb promoter sequence was used. To normalize the luciferase signal, a renilla luciferase vector pGL4.74 [hRluc/TK] Vector (Promega) was used. After transfection, macrophages were incubated with either anti-inflammatory (IL-4) and pro-inflammatory (IFN-γ) polarizing stimulus for 24 h. Luciferase reporter assay was performed in THP-1 macrophages with Dual-Luciferase Reporter Assay System (Promega), according to manufacturer instructions.

7.12 Calcium measurements

BMDM were polarized with 100 ng/ml IFN-γ towards the M1 phenotype or 20 ng/ml IL-4 towards the M2 phenotype for 24 hours. Cells were then loaded with 4 mM Rhod-2, AM (Thermo Fisher Scientific) for 20 min. Ca^{2+} measurements were performed after stimulating cells with 10 μg/ml Ionomycine and the analysis was performed by confocal microscope.

7.13 Statistical analysis

All *in vitro* experiments were replicated at least three times and at least four animals for each condition were used for *in vivo* analyses. Isolation of macrophages from regenerating muscle required three mice for each condition (3, 7, 14 days post CTX injury). Statistical data are presented as mean ± SD, unless otherwise specified. Depending on the experiments, a Student's t test, Mann-Whitney-t-test, one or two-way ANOVA and Bonferroni post hoc tesst were applied. $p < 0.05$ was considered significant.

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