The Mitochondrial Calcium Channel Discovery

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The notion of the active role of mitochondria in the decoding and shaping of intracellular Ca2+ signals dates back at the end of the 19th century. However, the identity of the molecule(s) involved in Ca2+ ion transport into mitochondria remained elusive for decades. Only in the last ten years, the factors, and the relative coding genes, mediating Ca2+ entry in mitochondria started to be genetically and biochemically described. The gene for the pore-forming unit of the mitochondrial Ca2+ channel was discovered in 2011, and its product was named mitochondrial Ca2+ uniporter or MCU. The mitochondria Ca2+ uptake regulator 1 gene, MICU1, was cloned one year before, in 2010. The increasing interest of the scientific community towards mitochondrial Ca2+ signaling and metabolism in the subsequent years led to the identification of many other MCU components and to the description of their 3D structure and physiological role. Here, we will present a brief overview of the land marking discoveries in the history of mitochondrial Ca2+ studies.

MCU mitochondrial Ca2+ uniporter Ca2+ signaling mitochondrial metabolism

1. Introduction

Every cell type, in every tissue and at any evolutionary level, can communicate with the surrounding environment and with neighboring cells. Both intercellular and extracellular communication play fundamental roles in shaping cell behavior and driving cell fate decisions. Cell-to-cell and environmental signals are normally conveyed by distinct extracellular mediators (hydrophilic or hydrophobic compounds, mechanical, ionic, cell–cell interactions, etc.) that are normally perceived by cells through surface receptors. These receptors convey them into a limited number of intracellular molecules, which are referred to as ‘second messengers’, which, in turn, forward the message to intracellular effectors finally activating the ultimate cellular responses. Despite the plethora of different signals and stimuli that cells may receive, only a few molecules to date have been described as second messengers of intracellular communication. Among them, the most common and, definitively, the most extensively studied is Ca^{2+}.

Ca^{2+} ions participate in the decoding of a vast range of stimuli and the variety of cellular components involved in the Ca^{2+} signal transduction is extremely wide, including basically all kinds of components, organelles, and molecules [1][2][3]. The research studies on Ca^{2+} second messenger started more than one hundred years ago, with the initial recognition of the role of Ca^{2+} in muscle cell contraction [4]. Since then, the understanding of Ca^{2+} signaling regulation and dynamics has progressively increased leading to the definition of the concept of Ca^{2+} compartmentalization and to the demonstration of the existence of microdomains of local high Ca^{2+} concentration [5], which are crucial for the fine-tuning and correct triggering of the Ca^{2+}-dependent cellular effects [1].

Mitochondria play a fundamental and multifaceted role in the orchestration of cellular Ca^{2+} signals. Indeed, mitochondria are not a store of rapidly releasable Ca^{2+} (such as the ER), but rather they efficiently accumulate Ca^{2+} upon Ca^{2+} entry from the extracellular space or upon release from ER Ca^{2+} stores [6]. Upon cytosolic Ca^{2+} elevation, the entry of Ca^{2+} into mitochondria exerts a central function in the modulation of cell metabolism. Mitochondria host the enzymes and complexes of the TCA cycle, fatty acid oxidation (FAO), and oxidative phosphorylation (OXPHOS) thus representing the site of the major metabolic pathways and enzymes for cell energy supply, which deserved them the name of 'cellular powerhouses'. Interestingly, Ca^{2+} entry and oxidative activity are two strictly intertwined aspects of mitochondrial physiology. The increase of the mitochondrial matrix Ca^{2+} level stimulates both Ca^{2+}-sensitive dehydrogenases [7][8][9] and respiratory chain complexes [10][11] resident in the organelles, fueling the TCA cycle activity as well as aerobic respiration and thus boosting the overall oxidative metabolism. This makes mitochondria the central hubs for the rapid and effective adaptation of cell metabolism to the changes in energy requirements that are typically decoded as variations of intracellular Ca^{2+} concentration.

In addition, mitochondria also actively participate in the tuning of global Ca^{2+} signals thanks to their ability to take up Ca^{2+} during intracellular Ca^{2+} elevation with a net result of buffering the cytosolic cation concentration thus modulating the overall cellular Ca^{2+} response. This buffering capacity is due to two crucial characteristics of the mitochondria: (i) their strategic position in close contacts to the Ca^{2+} release channels of the ER store [6] and the plasma membrane in immune cells [12] (ii) the presence on their inner membrane of highly selective and efficient machinery for taking up Ca^{2+}, the MCU complex.

Finally, the large buffering capacity of mitochondria can protect cells from Ca^{2+} overload. Indeed, an excessive accumulation of the cation in the mitochondrial matrix triggers the permeability transition pore (PTP) opening, the release of pro-apoptotic factors, and finally, induction of programmed cell death [13]. Given the strong association between mitochondrial Ca^{2+} overload and apoptosis induction, the maintenance of mitochondrial Ca^{2+} homeostasis is thus a crucial aspect for ensuring cell survival [14][15].

Given the extreme relevance of mitochondria Ca^{2+} signaling for cell physiology, the unveiling of the molecular factors mediating mitochondrial Ca^{2+} entry and the mechanism(s) of their regulation has been one of the scientific challenges of recent years. In this review, we aim to summarize some of the milestone achievements in the history of mitochondrial Ca^{2+} research with a particular focus on the recent findings of the mitochondrial Ca^{2+} uniporter and its role in organ physiology. We will briefly describe the early studies leading to the demonstration of the Ca^{2+} accumulation capacity of mitochondria, then we will go through the historical chronicle of the discoveries of the mitochondrial Ca^{2+} uniporter genes and multiple regulators (**Figure 1**) and we will conclude with an excursus on the physiological relevance of mitochondrial Ca^{2+} uptake in the context of skeletal muscle tissue.

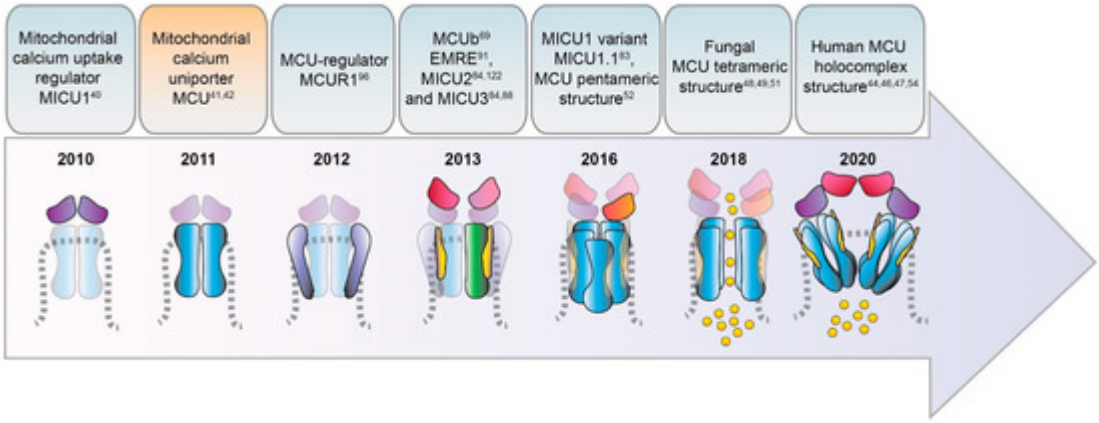

Figure 1. Timeline of the identification of MCU complex components. The most relevant findings on the structure and composition of the MCU complex discovery are summarized and chronologically presented along a timeline covering the last 10 years. Schematic cartoons show the different components of the MCU complex according to the date of their discovery along the timeline.

2. Timeline of MCU Identification

It is suggestive to recall that the notion of Ca^{2+} ions being relevant to organ physiology dates back to more than one century ago when the first report on the physiological action of Ca^{2+} ions appeared in 1883 [4]. At that time, Ringer described the effects of Ca^{2+} addition to isolated frog hearts and demonstrated that the supplementation of Ca^{2+} in the perfusion solution actively induces and sustains the contraction of the organ ex vivo [4]. This seminal observation revealed that Ca^{2+} is a fundamental messenger within cells, a concept that then extended to virtually every cell type and physiological and pathological process, giving rise to a broad field of study commonly referred to as the field of intracellular Ca^{2+} signaling. The intrinsic ability of contracting myocytes to operate ex vivo and to rapidly and effectively respond to environmental condition changes made them the ideal experimental system for the investigation of the role of Ca^{2+} in organ and cell physiology and was extensively exploited by researchers in the following years.

The original concept of the existence of intracellular compartments acting as Ca^{2+} stores to accumulate the cation required to sustain muscle contraction has been later postulated and demonstrated in 1947 by Heilbrunn [16]. However, although surprising, the identification of the sarco/endoplasmic reticulum (SR/ER) as the principal cellular Ca^{2+} store came only 20 years later. It was in the 1960′s, with the identification of Ca^{2+} pumping machinery on intracellular membranes (in particular the calcium pump of the sarcoplasmic reticulum, better known as SERCA) by three independent scientists [17][18][19][20] and the advent of new methodologies for the measurement of intracellular Ca^{2+} concentration [21] that the ER and its specialized counterpart in muscle cells (the SR) were recognized the main cellular reservoir of Ca^{2+}.

Before that, the pioneering work of Slater & Cleland on cardiac myocyte preparations from rat hearts firstly described some subcellular compartments, called "sarcosomes" at that time, as the entities actively accumulating Ca^{2+} [22]. Interestingly, these “sarcosomes” did not consist of ER but, instead, they corresponded to isolated

mitochondria, to which the addition of Ca^{2+} caused the block of their oxidative phosphorylation activity. Thus, Ca^{2+} ions behaved as mitochondria uncouplers. Despite that, Ca^{2+} appeared to exert a peculiar inhibitory action on mitochondrial OXPHOS activity, which differed from the other irreversible uncouplers known at that period (dinitrophenol, dicoumarol, rotenone, antimycin A, azide, or cyanide), due to the reversibility of its action [23]. This assigned to Ca^{2+} ions a functional role in mitochondria activity.

After that, a series of land-marking works in the early 60′s experimentally revealed that energized mitochondria can actively take up Ca^{2+} [24][25][26]. Interestingly, these results anticipated the clear demonstration of a driving force for Ca^{2+} accumulation in mitochondria, i.e., the chemiosmotic theory, postulated and validated by Peter Mitchell [27]. This theory is based on the following concepts: (i) the activity of the respiratory chain complexes is linked to the extrusion of protons from the matrix to the intermembrane space (IMS) across the inner membrane of mitochondria (IMM). (ii) The accumulation of protons in the IMS generates a difference in the charges across the IMM of around −150 ÷ −180 mV (negative inside) establishing the so-called mitochondrial membrane potential ($\Delta\Psi_m$). (iii) This steep $\Delta\Psi_m$ represents the main driving force for the proton gradient-sustained synthesis of ATP and Ca^{2+} cation entry into the matrix.

Shortly after, at the University of Bristol, Denton and his group made important discoveries on the Ca^{2+}-dependent modulation of three critical oxidative enzymes resident in mitochondria [7][8][9]: pyruvate dehydrogenase phosphatase (the enzyme that dephosphorylates and relieves pyruvate dehydrogenase (PDH) activity allowing the conversion of NAD^+, coenzyme A (CoA) and pyruvate into NADH, CO_2, and acetyl-CoA, providing substrates to the citric acid (TCA) cycle and cellular respiration), NAD-isocitrate dehydrogenase and oxoglutarate dehydrogenase. The fact that these mitochondrial rate-limiting enzymes are under the control of Ca^{2+} definitively sets the cation at the center of the cell oxidative metabolism. Moreover, the evidence that mitochondria can take up Ca^{2+} in response to elevation of cytosolic Ca^{2+} levels, as shown in insulin-treated epididymal adipose tissue [28], established an active role of mitochondrial Ca^{2+} entry in shaping cell oxidative metabolism to match the increased cellular energy demands thus tailoring the metabolic outcomes according to the different environmental cues.

Despite all these conceptual advancements, two major issues were destined to puzzle the scientific community for decades. On one end, there is the apparent paradox between the physiological concentration of cytosolic Ca^{2+}, which was estimated in the submicromolar range [29][30][31], and the low affinity of the mitochondrial Ca^{2+} uptake, whose half-maximal rate (Km) was measured in the order of several μM (reviewed in [32]). On the other end, the fundamental question about the molecular identity of the IMM apparatus responsible for the entry of Ca^{2+} into mitochondria was still without an answer.

It took around 30 and 50 years, respectively, to find solutions to those two enigmas. The answer to the former came with the advent of innovative and sophisticated technologies allowing the assessment of Ca^{2+} distribution at the sub-cellular and sub-organellar levels, including mitochondria. Indeed, thanks to the development of Ca^{2+}-sensitive genetic probes and recombinant fluorescent proteins targeted to specific intracellular microdomains [33][34], it was possible to measure variations of Ca^{2+} concentration in defined and limited areas of the cell (such as the cytosolic face of plasmalemma [35] or the surface of outer mitochondrial membrane [36], or the Golgi cisternae [37])

as well as the relative positioning of the intracellular organelles. These studies were pivotal in the field of cell biology for two main reasons: i) they allowed the observation of the intrinsic heterogeneity of cellular Ca^{2+} distribution, definitively demonstrating that large variations in Ca^{2+} concentration are highly regionalized within the cell cytoplasm and allowing the direct measure of Ca^{2+} levels in the mitochondria matrix as well as in the ER lumen; ii) they pinpointed the fact that organelles, including ER and mitochondria, are in close contact with each other through macromolecular structures involving proteins from both the compartments. In the case of ER-mitochondrial contacts, these structures are biochemically isolated as mitochondria-associated membranes (MAMs) and are formed by membrane channels, as the IP_3R and VDAC, respectively, and adaptor proteins of both organelles, such as Grp75, mitofusins, PACS [38]. Upon cell stimulation, the massive release of Ca^{2+} through the ER membrane clusters of IP_3Rs generates microdomains of high Ca^{2+} concentration right at the mouth of the channel pores, exactly where mitochondria are located. This allows mitochondria to perceive a local cation concentration sufficient to meet the low affinity of the mitochondrial Ca^{2+} uptake machinery [36][39]. Thus, their strategic position in proximity of ER Ca^{2+} release channels and their ability to take up Ca^{2+} with high conductance make mitochondria the ideal operator for cushioning the sudden Ca^{2+} rise in the cytosol of stimulated cells, thus behaving as an instrumental Ca^{2+} buffer [6]. The fact that Ca^{2+} entry into mitochondria stimulates the TCA cycle, respiration, and ATP production then places mitochondrial Ca^{2+} uptake as a key element for the prompt modulation of cell metabolism to rapidly and efficiently adapt to a variety of environmental cues and energy demands.

Another fundamental advancement in mitochondrial signaling occurred when scientists found the answer to the second big question, i.e., the molecular identity of the mitochondrial Ca^{2+} uniporter (MCU) machinery. The chronicle of MCU discoveries actually started in 2010, with the identification of the first gene required for the uptake of Ca^{2+} by mitochondria, *CBARA1*, coding for the mitochondrial Ca^{2+} uptake 1 protein (MICU1) [40], then followed by the identification of the mitochondrial channel and the elucidation of its interactors, as described below. The search of the other mitochondrial Ca^{2+} channel components has been proceeding expansively in the last decade (see next paragraph for a detailed timeline) and it is presently still actively ongoing. The discoveries of many different groups worldwide have been indeed instrumental to provide cell biologists with new knowledge on the functional role of mitochondrial Ca^{2+} and with new tools for the genetic and molecular intervention on global Ca^{2+} signaling and cell energetics.

3. Discovery and Characterization of the MCU Complex Components

3.1. MCU

The chronicle of MCU discovery starts with two pioneering studies published in 2011 [41][42] (**Figure 1**) that finally identified and cloned the long-sought MCU pore-forming unit gene, *CCDC109A*. MCU is a highly conserved 40 kDa protein ubiquitously expressed in plants, metazoans, protozoans, and fungi but not present in yeast [43]. This pore-forming unit oligomerizes to form the active channel within the inner mitochondrial membrane (IMM) and it directly interacts with the channel regulator MICU1, which was identified one year earlier [40]. These reports clearly demonstrated that the transient downregulation of MCU inhibits the mitochondrial accumulation of Ca^{2+} that follows

the IP_3-generating agonist in stimulated cells. Of note, the blunted mitochondrial Ca^{2+} uptake response occurs without changes in the mitochondria morphology or membrane potential of the MCU-silenced cells [41]. On the contrary, MCU overexpression enhances agonist-induced mitochondrial Ca^{2+} uptake in mammalian cells. In addition, in vitro experiments, in which recombinant MCU proteins were inserted in a planar lipid bilayer, showed that this pore-subunit alone is sufficient to form the channel. Indeed, in this setting, the MCU electrophysiological activity is completely abolished by the addition of the known inhibitor Ruthenium Red, firmly pointing at MCU as the genuine core component of the mitochondrial Ca^{2+} machinery.

Sequence and topology analyses revealed that both the *N*- and *C*-termini of MCU are located in the mitochondrial matrix and that MCU is endowed with two transmembrane domains, linked by a short highly conserved acidic loop exposed in the intermembrane space (IMS) which contains the so-called acidic “DIME” motif. The acidic residues present in this stretch (in particular E257, D261, E264) are critical for the Ca^{2+} transport since, if substituted with uncharged residues, MCU mutants failed to rescue the mitochondrial Ca^{2+} uptake in MCU-silenced cells [41][42]. However, the definitive description of MCU 3D structure had to wait till the very last years, when cryo-EM and X-ray diffraction analyses finally allowed the resolution of full-length MCU structure [44][45][46][47][48][49][50][51]. These studies coherently confirmed that purified MCU from different sources (fungi and metazoan) arranges in a tetramer, confuting previous assumptions on a putative pentameric MCU architecture [52]. Notably, the cryo-EM data also unveiled the exact position of the MCU channel selectivity filter, in which the DIME motif is fundamental for the coordination of Ca^{2+} ions and which was definitively shown to reside at the beginning of the second transmembrane α-helix [50] and not in the linker region between the two transmembrane helices, as previously suggested [52].

More recently, the structure of the human MCU together with its auxiliary component EMRE was obtained [53]. Each human MCU arranges in tetramers and each subunit complexes with one EMRE peptide. Differently from the three described for fungal MCU, human MCU appears organized in four domains, which are: (i) the *N*-terminal domain (NTD), (ii) the linker helix domain (LHD) —absent in fungi—, (iii) the coiled-coil domain (CCD), and iv) the transmembrane domain (TMD) (**Figure 2**).

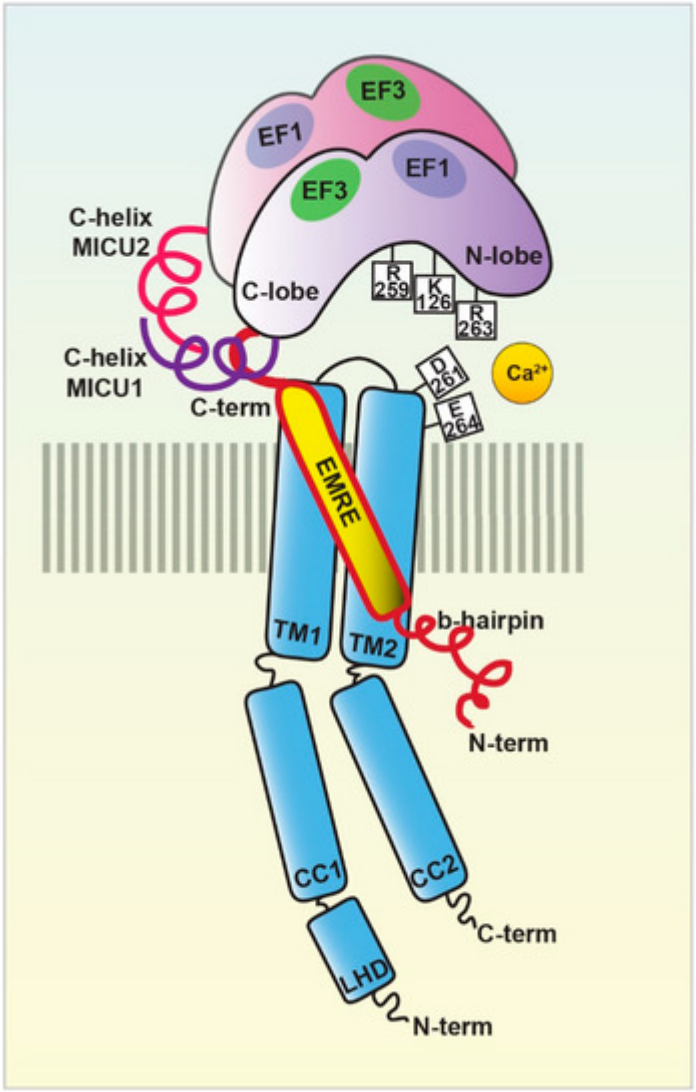

Figure 2. The MCU holocomplex structure. Schematic representation of the MCU holocomplex (uniplex) components and their relevant domains: the pore-forming subunit MCU (light blue) with the two transmembrane (TM) and coiled-coil (CC) domains and the linker helix domain (LHD); the essential mitochondrial Ca^{2+} uniporter regulator EMRE (yellow); the mitochondrial Ca^{2+} uptake proteins MICU1 (violet) and MICU2 (purple), with the EF-hands relevant for the MICU dimer interaction highlighted. The critical residues of the MCU DIME motif forming the Ca^{2+} selectivity filter are indicated, together with the MICU1 residues of the K-R ring coordinating the MCU acidic region.

Moreover, in the very last year, further insight into MCU channel modulation and function has been gained thanks to the achievement of the human MCU-MICUs holocomplex structure in both the Ca^{2+}-free and Ca^{2+}-bound state by several independent reports. The gating mechanism by which MICU1 regulates the uniporter activity via the conformational change triggered by Ca^{2+} was finally unveiled [44]. Furthermore, the precise description of the molecular interactions between MCU-EMRE-MICU1-MICU2 in the human MCU supercomplex (MEMMS) has been also obtained [54] (**Figure 2**). Indeed, MEMMS appears as a 480 kDa integral unit where EMRE coordinates the matrix gate of the MCU channel and MICU proteins interact with the *C*-terminus of EMRE in the IMS thus enhancing Ca^{2+} influx through the MCU pore in high [Ca^{2+}] conditions [54] (**Figure 3**). Finally, the distinct Ca^{2+}-

dependent assembly conformations of the beetle and human MCU holocomplexes with human MICUs have also been detailed [46][47]. In the presence of Ca^{2+}, the multiprotein complex shows a two-fold symmetry and consists of two V-shaped MCU-EMRE tetrameric subcomplexes and two MICU1-MICU2 heterodimers that bridge the tops of the subcomplexes (**Figure 1**). In this setting, the assembly of the MICU1-MICU2 heterodimers to the MCU-EMRE subcomplexes is ensured by the interaction between MICU1 and EMRE [47] (**Figure 2**). Differently, in the absence of Ca^{2+}, the holocomplex adopts alternative less stable conformations with both monomeric and dimeric forms of the MCU-EMRE tetramers, where the MICU1-MICU2 heterodimer block the channel entrance formed by MCU transmembrane domains [47] (**Figure 2** and **Figure 3**).

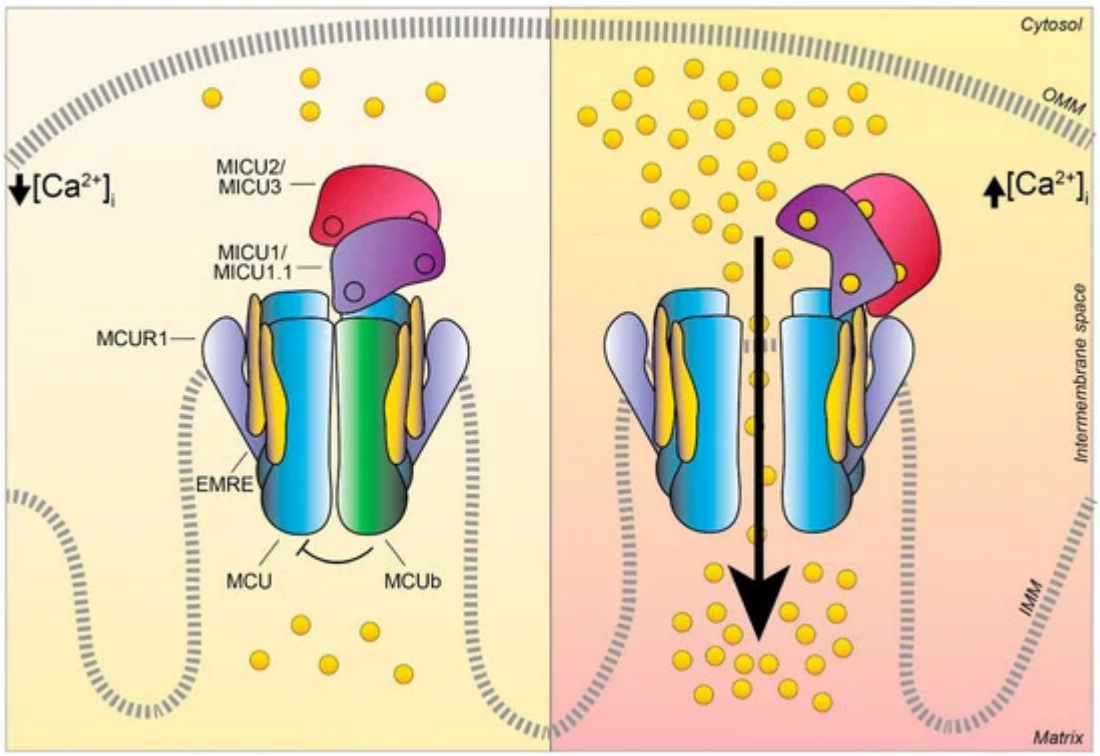

Figure 3. The MCU complex activity at low and high intracellular Ca^{2+} concentration. Schematic representation of the proteins involved in the MCU complex-mediated mitochondrial Ca^{2+} uptake: the pore-forming subunits MCU (light blue) and MCUb (green), the essential mitochondrial Ca^{2+} uniporter regulator EMRE (yellow), the mitochondrial Ca^{2+} uptake proteins MICU1 / MICU1.1 (violet), MICU2/ MICU3 (purple), and the MCU regulator 1 MCUR1 (light violet). The EF-hand Ca^{2+} binding domains of MICU proteins are indicated as little circles. At low intracellular Ca^{2+} concentration, the cation does not permeate through the MCU channel since the heterodimers formed by MICU1/MICU1.1–MICU2/MICU3 block the channel pore, thus preventing Ca^{2+} flux in resting conditions. Differently, at high intracellular Ca^{2+} concentration, MICU proteins undergo conformational changes relieving the inhibition on MCU and positively regulating channel activity, leading to an efficient mitochondrial Ca^{2+} uptake. OMM, outer mitochondrial membrane; IMM, inner mitochondrial membrane.

A large body of experimental evidence on the MCU complex functional role has accumulated since the discovery of the MCU. The genetic manipulation of the MCU led to the generation of germline and tissue-specific transgenic

models [55][56][57][58][59][60][61][62], which provided pivotal tools for understanding the pathophysiological implications of the mitochondrial Ca^{2+} signaling in vivo that would have otherwise remained unexplored.

In 2013, three independent groups showed that the *MCU* silencing/knockout or its overexpression affect survival in different in vivo models [55][63][64]. In *Trypanosoma brucei*, for instance, the downregulation or the conditional knockout of the uniporter augments the AMP/ATP ratio, thus affecting the parasite growth in vitro. On the contrary, when MCU is overexpressed, mitochondrial Ca^{2+} accumulation produces a high concentration of ROS and sensitizes trypanosomes to apoptotic stimuli [63]. In zebrafish (*Danio rerio)*, instead, the knockdown of MCU has shown crucial alterations not only during the early step of gastrulation, where the blastomere convergence and extension are altered, but also at later stages of development as the maturation of the notochord and anteroposterior axis formation were strongly impaired in morphant fish [64]. A series of recently published works and our unpublished observation in the zebrafish knockout model obtained with CRISPR/Cas9 gene ablation gave further insight into the role of MCU during development. For example, the ablation of the *MCU* gene in *Danio rerio* inhibits mitochondrial Ca^{2+} influx, reduces oxidative phosphorylation, and induces lipid accumulation, a phenotype that was also observed in the hepatic tissue of liver-specific KO mouse [65]. Notably, the inactivation of *MCU* has shown to be protective in neurons of Parkinson Disease zebrafish genetic model (namely in *pink*$^{-/-}$ fish), suggesting the crucial role of the MCU-dependent mitochondrial Ca^{2+} load on neuronal fitness [66].

As for mammals, the MCU$^{-/-}$ mouse [55] develops normally and displays minor defects without signs of impaired cell survival. Under stress conditions, relatively mild metabolic alterations were observed, such as increased plasma lactate levels in line with impaired exercise performance. However, the same group soon after those findings, showed that embryos from MCU$^{-/-}$ mice in a pure C57BL/6 background were not viable, displaying embryonic lethality at around E 11.5–E 13.5, thus suggesting a major involvement of mitochondrial Ca^{2+} uptake in organ metabolism and organism development that was compensated in a mixed genetic background [67].

Of note, some years later, it has been reported that the block of MCU-dependent Ca^{2+} uptake affects *Drosophila melanogaster* development. In particular, the inhibition of the uniporter activity has been shown to be detrimental for memory establishment during the papulation stage. Indeed, during the development of adult flies, alterations in the structural and functional neuronal substrates, crucial for memory formation, occur in the MCU deficient fly [58].

The genetic manipulation of MCU in *C. elegans* model also provided additional interesting notions on its role in organism physiology. Indeed, MCU$^{-/-}$ *C. elegans* is viable and grossly normal, mirroring what was found in the first MCU$^{-/-}$ mouse model described [55], even though it presents some defects in the epidermal wound repair mechanisms. More recently, the characterization of *C. elegans* deficient of a functional MCU [68] suggested that uniporter activity is essential for mitochondrial Ca^{2+} transfer during high-intensity stimulation of the worm pharynx muscle. However, a lot still remains to be explored on the role of MCU in this model and most of the knowledge on mitochondrial Ca^{2+} regulation of muscle physiology has been achieved using the mammalian mouse model where MCU expression was genetically targeted, which we will briefly review in the following paragraphs.

3.2. MCUb

In 2013, Raffaello and co-authors discovered that MCU is not the only pore-forming subunit of the mitochondrial Ca^{2+} uniporter, since an alternative MCU isoform exists, named MCUb, which crosses the IMM and associates to MCU to form the calcium channel [69] (**Figure 1** and **Figure 3**). The MCUb protein is encoded by the MCU *CCDC109a* paralog gene *CCDC109b*. Interestingly, this gene is found in vertebrates, but it is not present in other organisms in which MCU is expressed, such as plants, Nematoda, and Arthropoda. Despite the high structural similarity with MCU, MCUb sequence presents two critical aminoacidic substitutions in the loop region and in the TM1 domain, which explains its inability to transport Ca^{2+}. Indeed, MCUb acts as a negative regulator of MCU activity, drastically reducing mitochondrial Ca^{2+} currents in vitro in planar lipid bilayer experiments and also when overexpressed in mammalian cells [69]. On the contrary, in other organisms, such as trypanosomatid species, the ortholog of MCUb is capable to conduct the cation and its overexpression facilitates mitochondrial Ca^{2+} uptake [70].

Interestingly, MCUb displays different expression levels in the different mammalian tissues, and the MCUb:MCU proportion appears also the distinctive feature ensuring the appropriate mitochondrial Ca^{2+} current to each cell type [69][71]. For instance, a high MCUb/MCU ratio (3:1) is typical of cells with low mitochondrial Ca^{2+} transients, such as adult cardiomyocytes (**Figure 4**). In fact, MCUb can be described as a protective gene in cardiac myocytes since i) its expression is transiently induced after ischemia-reperfusion injury and ii) transgenic mice overexpressing MCUb have a reduced mitochondrial Ca^{2+} uptake ability, thus preventing Ca^{2+} overload, which is sufficient to protect myocytes from ischemia-reperfusion injury and to decelerate their ongoing necrosis [72][73]. A low MCUb/MCU ratio (1:40) is instead a characteristic of tissues with an extremely high capacity of mitochondrial Ca^{2+} accumulation, such as skeletal muscle [69][74] (**Figure 4**).

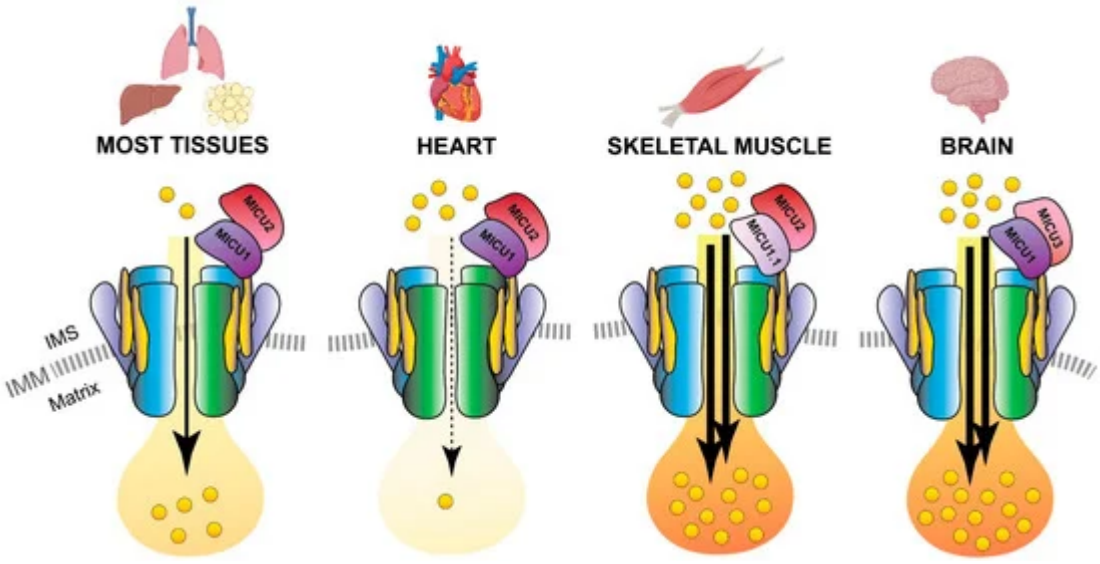

Figure 4. MCU holocomplex composition in different tissues. Schematic representation of the tissue-specific components of the MCU holo-complex. The presence of a relatively high MCUb:MCU ratio in the heart ensures a re-duced Ca^{2+} load in cardiomyocyte mitochondria. On the contrary, the expression of the MICU1.1 variant and MICU3 determines an elevated Ca^{2+} flux in the mitochondria of skeletal muscle fibers and neurons, respectively. IMS, inter-membrane space; IMM, inner mitochondrial membrane.

These lines of evidence highlight the importance of MCU:MCUb proportion in the control of mitochondrial Ca^{2+} uniporter activity and further investigation will be of fundamental relevance for the understanding of its role in the pathophysiology of different tissues.

3.3. MICU1

MICU1 was actually the first component to be described as the regulator of the long-sought MCU channel and its identification even anticipated that of the pore-forming subunit MCU (**Figure 1**). Indeed, in 2010, an integrative strategy combining comparative physiology, evolutionary genomics, and organelle proteomics revealed the 54 kDa protein, encoded by the *CBARA* gene and residing in the IMS, to be the mitochondrial calcium uptake 1 (MICU1), which does not take part to the pore-forming domain of the channel, but it strongly regulates its activity in a Ca^{2+}-dependent way [40][75]. Indeed, after the *N*-terminal mitochondrial targeting sequence, MICU1 shows two canonical EF-hand Ca^{2+} binding domains that confers the Ca^{2+}-sensitivity (**Figure 2** and **Figure 3**). In the last decade, several lines of evidence confirmed the initial hypothesis about the role of MICU1 as both MCU gatekeeper at low Ca^{2+} concentration and MCU positive regulator at high Ca^{2+} concentration, thus explaining the sigmoid cooperative effect of the MCU activation curve [75].

The downregulation of MICU1 was initially shown to abolish mitochondrial Ca^{2+} influx in intact and permeabilized HeLa cells [40]. Later studies also demonstrated that the absence of MICU1 also leads to an adaptive Ca^{2+} accumulation inside the mitochondria matrix, triggering excessive ROS production and the consequent higher sensitivity to apoptotic stress. In addition, the ability of MICU1 to sense cytosolic Ca^{2+} levels confers to MICU1 the capacity to set the threshold for the activation of mitochondrial Ca^{2+} uptake. Nevertheless, this occurs without altering the overall kinetics of the channel [75][76].

Two in vivo studies, performed in $MICU1^{-/-}$ mouse models have strengthened this concept and gave additional insight into the physiological role of this MCU regulator. In more detail, the characterization of $MICU1^{-/-}$ transgenic mice reveals that, despite partial postnatal mortality, the viable animals show marked ataxia and muscle weakness [77], a phenotype which is reminiscent of that of human patients bearing MICU1 genomic mutation [78]. Moreover, the $MICU1^{-/-}$ mice display several biochemical defects, including an increase in resting mitochondrial Ca^{2+} levels, altered mitochondrial morphology, and a decreased ATP production [77]. Interestingly, the loss of MICU1 triggers a sustained pro-inflammatory response after partial hepatectomy and failure of liver regeneration in MICU1-deficient mice. In this scenario, the lack of MICU1 enhances mitochondrial permeability transition pore (PTP) opening in hepatocytes, thus leading to massive necrosis [79].

Interestingly, more recently, evidence of a new role of MICU1 in the regulation of crucial metabolic steps of cell metabolism has emerged. Indeed, MICU1 was shown to inhibit the mitochondrial transport of pyruvate and fatty acids and, interestingly, this MICU1 function appears independent of Ca^{2+} and MCU core-complex composition [80]. This study reveals a mechanism that controls the MCU-mediated Ca^{2+} flux machinery and that relies on TCA cycle substrate availability. According to this view, the MICU1 regulatory axis acts as a metabolic homeostatic circuit to protect cells from the risk of bioenergetic crisis and mitochondrial Ca^{2+} overload during periods of nutrient stress.

Altogether these findings [77][79][80] highlight once more the crucial importance of the fine-tuning of mitochondrial Ca^{2+} uptake by Ca^{2+} and MICUs, especially in the context of promotion of cell survival under stress conditions.

In addition to that, new important functions of MICU1 in the control of mitochondrial cristae junctions have been revealed by the recent super-resolution structured illumination microscopy (SIM) and electron microscopy studies of Gottschalk and collaborators [81]. According to the authors, MICU1 appears to act as a Ca^{2+}-dependent regulator of cristae junctions' integrity and cytochrome c localization. Another intriguing feature of MICU1 regulatory function on the determination of uniporter cation selectivity has recently emerged [82]. MICU1 was revealed as the primary responsible for conferring and ensuring the stringent MCU selectivity for Ca^{2+} over Mn^{2+} since. Indeed, when present, MICU1 impedes Mn^{2+} ions to cross the MCU channel pore; on the contrary, in the absence of MICU1, both Ca^{2+} and Mn^{2+} cations can enter the mitochondrial matrix. This additional MICU1 checkpoint is of fundamental importance to guarantee cell survival of cells sensitive to Mn^{2+} such as neurons, thus setting MICU1 as a crucial safeguard against cellular toxicity due to manganese in neurodegenerative diseases [82].

3.4. MICU1.1

A variant of MICU1, named MICU1.1, has been discovered by our group as an alternative splicing product of the *MICU1* mRNA [83] (**Figure 1**). MICU1.1 is well-conserved among species and expressed almost exclusively in skeletal muscle tissue, where it is by far the most abundant MICU moiety (**Figure 4**), although limited but still appreciable levels are found also in the brain [83]. Similar to MICU1, MICU1.1 behaves as a positive regulator of MCU. Indeed, MICU1.1 expression increases mitochondrial Ca^{2+} uptake upon stimulation in HeLa cells and skeletal muscle in vivo, and this increase is even higher than that observed after expression of the conventional MICU1 isoform [83]. This behavior is explained by the fact that the muscular MICU1.1-MICU2 heterodimer (see next paragraph for a detailed description of MICU2), binds Ca^{2+} more efficiently than the canonical MICU1-MICU2 pair, thus activating the uniporter at lower Ca^{2+} concentrations [83]. This peculiar feature is of critical importance for the physiology of skeletal muscle tissue, and the presence of MICU1.1 is functional to ensure the Ca^{2+} uptake required for the matching of ATP production to the energy expenditure of muscle contractile activity [83]. These findings shed light on a novel modality by which MCU machinery can be modulated in skeletal muscle mitochondria and widen the scenario of the possible endogenous regulators of mitochondrial Ca^{2+} uptake, opening the path for future investigations on other tissue-specific isoforms and mechanisms controlling mitochondrial Ca^{2+} uptake.

3.5. MICU2

The first information about the existence of other genuine MCU regulators, in addition to MICU1, was provided by human genome sequencing studies a few years later [84] (**Figure 1**). Initially known as EF-hand domain-containing family member A1 (EFHA1), the mitochondrial calcium uptake protein 2 or MICU2, was found to be a paralog of MICU1 [84]. MICU2 resides in the IMS, contains two EF-hand Ca^{2+}-binding domains, and interacts with both MICU1 and MCU [84] (**Figure 2**). The analysis of *MICU2* transcript expression shown that this regulator has a peculiar cell-type distribution: it is present at high levels in the intestine, prostate, and cardiac tissues [84]. Biochemical data evidenced that MICU2 stability is strictly dependent on the presence of MICU1: indeed, silencing of MICU1 leads to loss of also MICU2 protein, while MICU1 (or MCU) overexpression stabilizes both MICUs in mammalian cells [84].

Moreover, the recently reported human MICU1-MICU2 crystal structure [45][85] revealed interesting details on the architecture of the heterodimer and of MICU2 in both the Ca^{2+}-bound and Ca^{2+}-free condition. The MICU1-MICU2 interaction sites have been identified and correspond to Glu242 in MICU1 and Arg352 in MICU2 in the Ca^{2+}-free state, while Phe383 in MICU1 and Glu196 in MICU2 contribute to the interaction of the two proteins in the Ca^{2+}-bound state.

Although the functional role of MICU2 is still controversial, recent findings clarify some key aspects of uniporter modulation by this regulator. In mammalian cells, for example, it has been shown that MICU2 positively regulates MCU activation by controlling the cytosolic [Ca^{2+}] threshold for the relief of MICU1-mediated inhibition of MCU. This function allows MICU2 to restrict the spatial Ca^{2+} crosstalk between inositol 1,4,5-trisphosphate receptor (InsP3R) and MCU channels [86].

Subsequently, the generation and characterization of the $MICU2^{-/-}$ mouse model highlighted other important properties of this regulator [87]. MICU2 genetic ablation produces a decrease in the threshold for mitochondrial Ca^{2+} uptake due to loss of the gatekeeping activity and overall loss of MCU-dependent Ca^{2+} influx due to the destabilization of the entire uniporter complex. These findings lead to the conclusion that the amount of MCU and MICUs proteins is crucial to maintain the stability of the whole complex. Moreover, some phenotypic features of the $MICU2^{-/-}$ mice are in common with models of cardiac pathologies, suggesting that MICU2 may act as a possible cardioprotective factor.

3.6. MICU3

MICU3, previously known as EFHA2, is another MICU1 paralog, originally identified by the same genetic sequence analysis that described MICU2 [84] (**Figure 1**). This finding added another level of complexity to the regulation of the MCU machinery. *MICU3* is an evolutionarily conserved gene since it is present in plants and in vertebrates, with a peculiar tissue-specific distribution: indeed, it is mainly expressed in the brain, much less expressed in skeletal muscle, and virtually absent in other tissues [88][89]. The *MICU3* gene encodes a 55 kDa protein that shares 34% and 47% protein sequence similarity with MICU1 and MICU2, respectively [84]. Like the other MICUs, also MICU3 has an *N*-terminal mitochondrial targeting sequence and binds Ca^{2+} thanks to the presence of EF-hand domains. The crystal structure of human MICU3 has been recently characterized in both Ca^{2+}-free and Ca^{2+}-bound conditions [90]. This crystallographic analysis revealed a MICU3 3D structure very similar to that of MICU2, in line with the role of these factors as MCU channel gatekeepers at low intracellular Ca^{2+} levels. Upon cytosolic Ca^{2+} increase, MICU heterodimers, including those containing MICU3, undergo a conformational change that releases the latch formed upon the uniporter mouth, thus allowing Ca^{2+} flux through the MCU pore [90] (**Figure 3** and **Figure 4**).

Regarding its functional role, our group showed that MICU3 acts as a positive regulator of mitochondrial Ca^{2+} uptake through MICU1 [88]. Indeed, MICU3 forms heterodimers exclusively with MICU1, but not with MICU2, and the MICU1-MICU3 interaction leads to a significant increase of mitochondrial Ca^{2+} uptake, demonstrating the stimulatory action of MICU3 on uniporter activity [88]. Moreover, MICU3 downregulation blocks Ca^{2+} influx elicited by synaptic activity in primary cortical neurons, suggesting a specific role of this MCU regulator on neuronal

function [88]. This line of evidence lets to hypothesize that the primary role of MICU3 is to enhance MCU opening to ensure mitochondrial Ca^{2+} uptake in response to both small and fast cytosolic Ca^{2+} rises, typical of synaptic neuronal stimulation (**Figure 4**).

3.7. EMRE

The Essential MCU REgulator (EMRE) is an additional constituent of the uniporter, discovered by quantitative mass spectrometry analysis of affinity-purified MCU complex components [91] (**Figure 1**). EMRE is a metazoan-specific protein of 10 kDa, ubiquitously expressed in all mammalian tissue, with one transmembrane domain, a mitochondrial targeting sequence, and a highly conserved *C*-terminus [91]. Moreover, it is required for the binding of MICU1 to MCU (**Figure 2**). Initial biochemical and cellular studies revealed that it is required for MCU function [91] [92]. Indeed, in yeast cells, reconstituted with human MCU protein, the expression of MCU alone is not sufficient for uniporter activity, because the MCU channel is active only when also EMRE is co-expressed with the MCU pore-forming unit [92]. Interestingly, the knockdown of EMRE led to the loss of mitochondrial Ca^{2+} uptake to a similar extent to what was observed in *MCU*-silenced HEK-293T and HeLa cells [91]. The following studies tried to give a more detailed explanation of the EMRE function within the MCU complex [93]. EMRE was shown to control MCU activity by sensing Ca^{2+} elevation inside the matrix via its *C*-terminal domain and coordinating the other MCU regulators. Indeed, when EMRE acidic *C*-terminus was either deleted or substituted with neutral residues, mitochondrial Ca^{2+} permeation through the uniporter increased and the Ca^{2+} concentration inside the matrix consistently augmented [93]. After that, a dual-mode of action for the small MCU regulator has been proposed, in contrast with the previously described model [94]. According to this view, EMRE stimulates MCU channel activity via the interaction of transmembrane helices from both proteins, proposing a different EMRE orientation in which the *N*-terminus is present inside the matrix, while its *C*-terminal portion faces the IMS. In addition, EMRE was proposed to exert its MCU-regulation activity by binding MICU1 via its conserved *C*-terminal poly-aspartate tail (**Figure 2**). In the same year, another interesting aspect of EMRE-dependent MCU regulation has been revealed: the m-AAA protease-mediated degradation of EMRE is an essential event to guarantee the correct MCU-MICU proteins' assembly [95]. Indeed, the deficiency of m-AAA leads to EMRE accumulation, which prevents the MCU-MICUs association by competing for MCU binding. This generates a constitutively active MCU-EMRE channel that finally induces mitochondrial Ca^{2+} overload and eventually death of neuronal cells.

Very recently, the cryo-EM structure of the human MCU complex added new insights into the interaction of EMRE within the complex and defined the structural elements by which EMRE exerts its action on the channel [53]. In particular, it confirmed that EMRE crosses the IMM with its *N*-terminus facing the mitochondrial matrix. Secondly, it proposed that EMRE interaction with MCU occurs along with three major contact points of EMRE *N*-terminus, thus allowing Ca^{2+} ions to exit the channel vestibule. Finally, EMRE seems also to be crucial for MCU dimerization since, in its absence, the MCU channel is found as a monomer [53].

These new findings added important notions on the mechanisms of action and regulation of the MCU complex and highlighted the need for a precise orchestration of the holocomplex assembly to ensure its fundamental role in regulating cellular Ca^{2+} signals, cell metabolism, and cell survival.

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