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TOPICAL REVIEW

The mitochondrial calcium uniporter complex: molecular components, structure and physiopathological implications

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Abstract Although it has long been known that mitochondria take up Ca^{2+}, the molecular identities of the channels and transporters involved in this process were revealed only recently. Here, we discuss the recent work that has led to the characterization of the mitochondrial calcium uniporter complex, which includes the channel-forming subunit MCU (mitochondrial calcium uniporter) and its regulators MICU1, MICU2, MCUb, EMRE, MCUR1 and miR-25. We review not only the biochemical identities and structures of the proteins required for mitochondrial Ca^{2+} uptake but also their implications in different physiopathological contexts.

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Abbreviations $[Ca^{2+}]_m$, mitochondrial $[Ca^{2+}]$; EMRE, essential MCU regulator; ER, endoplasmic reticulum; IMM, inner mitochondrial membrane; IMS, intermembrane space; IP_3, inositol 1,4,5-trisphosphate; MCU, mitochondrial calcium uniporter; mPTP, mitochondrial permeability transition pore; OMM, outer mitochondrial membrane.

Introduction

The publication of back-to-back papers reporting the molecular identification of the mitochondrial calcium uniporter (MCU) complex in the June 19, 2011, issue of *Nature* (Baughman *et al.* 2011; De Stefani *et al.* 2011) represented a definitive culmination of 50 years of intensive research in this field.

Mitochondria rapidly transport Ca^{2+} across their membranes and accumulate it in the mitochondrial matrix, where several Ca^{2+} effectors are located (Rizzuto *et al.* 2012). The driving force of Ca^{2+} uptake is the mitochondrial membrane potential ($\Delta\Psi$), which is present throughout the inner mitochondrial membrane (IMM) and is generated by the respiratory chain. The pumping of protons by the respiratory complexes toward the intermembrane space (IMS) generates an electrochemical gradient (−180 mV) inside the matrix. In response to this gradient, two strong uncouplers of oxidative phosphorylation, dinitrophenol and carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP), are typically involved in preventing Ca^{2+} entry. Thus, mitochondria take up Ca^{2+} electrophoretically via the Ca^{2+} uniporter. To export Ca^{2+} from the mitochondrial matrix, mitochondria release Ca^{2+} via an antiporter by exchanging Ca^{2+} with Na^+ (in excitable tissues, such as the brain and heart) or H^+ (in the liver and many other tissues) (Nicholls & Crompton, 1980), with what

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is considered an electroneutral stoichiometry for Ca^{2+} efflux (Ca^{2+}–$2H^+$ antiport) (Brand, 1985). However, the identification of leucine zipper-EF-hand containing transmembrane protein 1 (Letm1) as the Ca^{2+}–H^+ antiporter suggests a different stoichiometry (Jiang *et al.* 2009). Letm1 seems to act as a Ca^{2+} extrusion mechanism when the $[Ca^{2+}]$ is elevated in the matrix, whereas it may contribute to non-linear Ca^{2+} uptake at low mitochondrial Ca^{2+} levels (Jiang *et al.* 2009). Letm1 has also been proposed to act as a mitochondrial K^+–H^+ exchanger (Dimmer *et al.* 2008). Consequently, the role of Letm1 awaits further confirmation.

The recent discovery of NCLX as the mitochondrial Na^+–Ca^{2+} exchanger (Palty *et al.* 2010) confirms the general understanding that the mitochondrial Na^+–Ca^{2+} antiport is electrogenic (exchanging 3 or 4 Na^+ per Ca^{2+}). Nevertheless, the mitochondrial permeability transition pore (mPTP), a key effector of cell death, has been indicated as a putative component of Ca^{2+} efflux machinery (Altschuld *et al.* 1992). However, this topic is still controversial because other observations suggest a minimal contribution of mPTP to Ca^{2+} release (Wei *et al.* 2011), and further studies are needed to reach a definitive conclusion.

The activity of these Ca^{2+} efflux pathways shows that mitochondrial Ca^{2+} accumulation by the MCU complex does not proceed to electrochemical equilibrium, a biological scenario that is incompatible with every cell physiology concept (a $\Delta\Psi$ of 180 mV implies a $[Ca^{2+}]$ of $\sim$1 M, based on the Nernst equation).

One of the main properties of the MCU complex is its very low affinity for Ca^{2+} (K_D of 20–30 μM under physiological conditions). Thus, the intracellular (cytosolic) Ca^{2+} concentration should be approximately 5–10 μM for considerable mitochondrial Ca^{2+} influx, but such values have never been observed in live, healthy cells. This riddle was solved through the demonstration that mitochondria are juxtaposed with the endoplasmic reticulum (ER) membrane (Rizzuto *et al.* 1998). The ER is the major intracellular Ca^{2+} store (Somlyo, 1984; de la Fuente *et al.* 2013), and the release of the Ca^{2+} content from the ER into the cytosol is due to the presence of inositol 1,4,5-trisphosphate (IP_3), which is generated upon the stimulation of receptors coupled to phospholipase C (Streb *et al.* 1983). Therefore, microdomains with high Ca^{2+} concentrations ($[Ca^{2+}]>10$ μM) can form transiently in regions of close apposition between the mitochondria and the Ca^{2+} channels of the ER (Patergnani *et al.* 2011), ensuring a prompt accumulation of Ca^{2+} inside the mitochondria (see schematization in Fig. 1). However, higher affinity mitochondrial Ca^{2+} uptake has been observed in many studies (Sparagna *et al.* 1995; Santo-Domingo & Demaurex, 2010), and patch-clamp experiments have suggested that the uniporter pore has high Ca^{2+} affinity (dissociation constant of <2 nM; Kirichok *et al.* 2004).

The development of the Ca^{2+}-sensitive photoprotein aequorin, which targets the mitochondrial matrix (Bonora *et al.* 2013), together with other GFP-based fluorescent probes (Rudolf *et al.* 2003), has enabled the direct visualization and measurement of $[Ca^{2+}]$ variations in imaging experiments. Using these tools, we and other independent groups have shown that mitochondria undergo large increases in their Ca^{2+} levels, reaching >100 μM in some cell types. In summary, during resting conditions, the mitochondrial Ca^{2+} content is low (Somlyo *et al.* 1985), but when a cell is stimulated with a cytosolic $[Ca^{2+}]$-increasing agonist (e.g. histamine or ATP), the mitochondria accumulate high amounts of Ca^{2+} via the MCU complex (Fig. 1).

Research examining the molecular nature of the MCU complex began in the 1970s, when a soluble Ca^{2+}-binding glycoprotein was isolated from liver mitochondria; this protein was found to be capable of inducing a Ca^{2+} current after reconstitution in lipid bilayers (Sottocasa *et al.* 1972). Interestingly, Ca^{2+} import is blocked by both Ruthenium Red, a polycationic stain used to visualize glycoproteins and a well-known inhibitor of MCU, and antibodies directed against this Ca^{2+}-binding glycoprotein (Panfili *et al.* 1976). Several years later, the idea that a glycoprotein could serve as the mitochondrial Ca^{2+} uniporter was revived through the isolation of a 40-kDa protein that forms Ca^{2+}-conducting channels in black-lipid membranes from beef heart mitochondria (Saris *et al.* 1993) and the description of Ruthenium Red-sensitive Ca^{2+} uptake by proteoliposomes containing mitochondrial proteins with a similar molecular weight (>35 kDa; Zazueta *et al.* 1991). However, the lack of strong experimental evidence, as well as doubts about the purity of the preparations, gradually dismissed these observations. More recently, some candidates were pinpointed as important components for the mitochondrial Ca^{2+} uptake machinery (Trenker *et al.* 2007), but only 3 years ago, the identification of CCDC109A as the channel-forming subunit of the MCU complex and the identification of different MCU regulators provided the key players that fulfil all of the properties predicted for the mitochondrial Ca^{2+} uptake machinery (Table 1).

MCU

The inventory of the 1098 mouse (1013 human) nuclear and mtDNA genes that encode proteins with mitochondrial localization, the so-called MitoCarta (Pagliarini *et al.* 2008), is the foundation for the identification of the MCU components. Among these

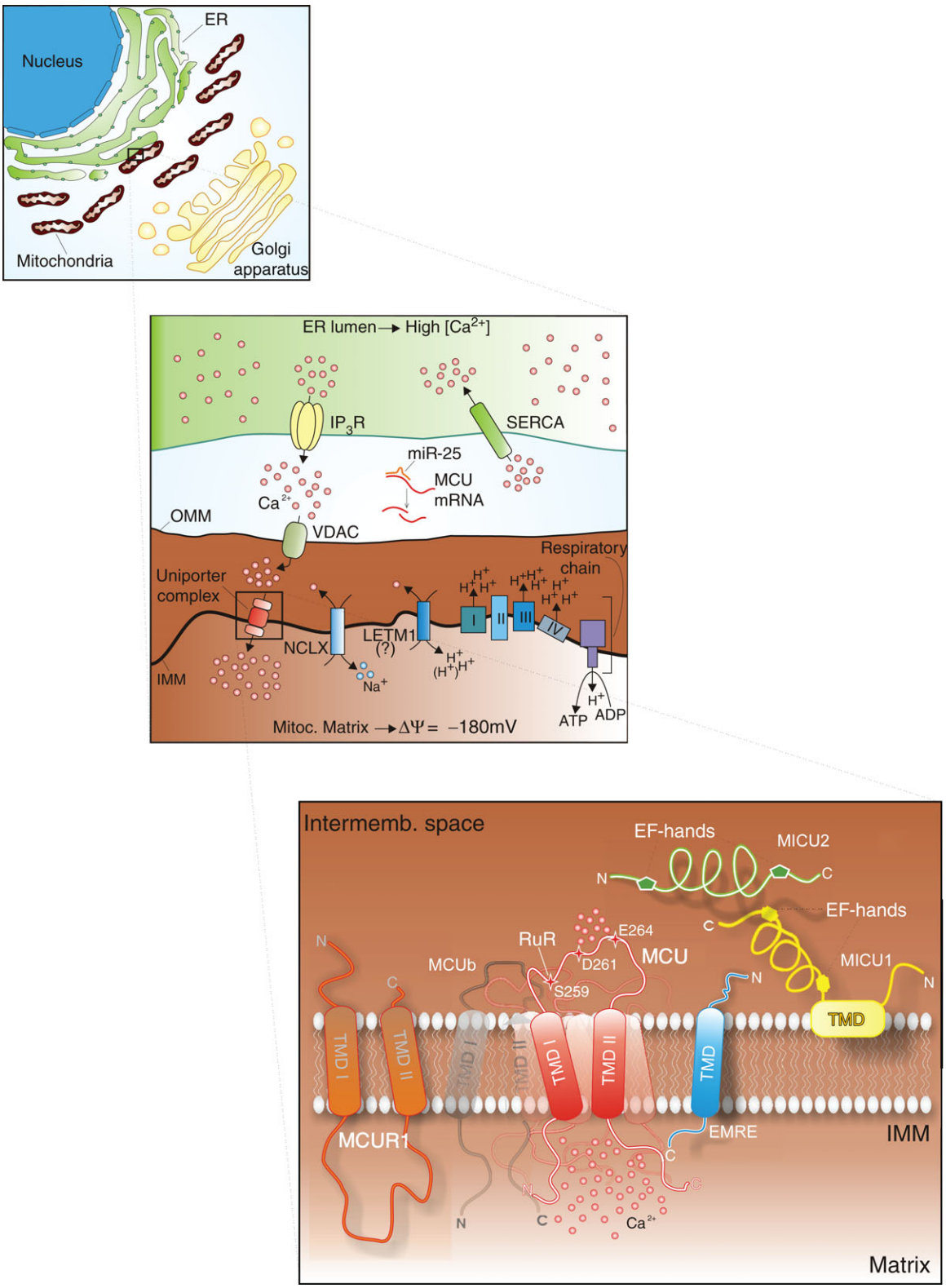

Figure 1. Schematic representation of the mitochondrial Ca^{2+} homeostasis machinery
Ion fluxes are indicated by arrows. The lower magnification represents the different components of the uniporter complex. MCU Oligomerization at IMM forms a tetramer (shadowed subunits). MCUb is represented as the black shadowed subunit. SERCA, sarcoendoplasmic reticulum calcium transport ATPase; VDAC, voltage-dependent anion channel; TMD, transmembrane domain; RuR, Ruthenium Red. See text for further details.

Table 1. Molecular nature of the components of the mitochondrial Ca^{2+} uniporter complex

Name	Also known as:	MW (kDa)	Topology	Functions	References
MCU (Mitochondrial Calcium Uniporter)	C10orf42 CCDC109A	40→35	Two transmembrane domains. N- and C-termini span into the matrix	Channel-forming subunit of the uniporter. MCU silencing abolishes Ca^{2+} entry; MCU overexpression strongly enhances Ca^{2+} entry.	(Baughman *et al.*, 2011) (De Stefani *et al.*, 2011)
MICU1 (Mitochondrial Calcium Uptake 1)	CALC CBARA1 EFHA3	54→50	Single-pass membrane protein. Two EF-Hands span into the IMS	MICU1 interacts with MCU. Thresholding function on MCU. MICU1 silencing enhances the $[Ca^{2+}]_m$ under resting conditions or during small cytosolic $[Ca^{2+}]$ elevations.	(Perocchi *et al.*, 2010) (Mallilankaraman *et al.*, 2012b) (Csordas *et al.*, 2013)
MICU2 (Mitochondrial Calcium Uptake 2)	EFHA1	50→45	Two conserved EF-Hands, probably faced on IMS	MICU2 associates with the MICU1/MCU complex. MICU2 silencing lowers the $[Ca^{2+}]_m$.	(Plovanich *et al.*, 2013)
MCUb (Mitochondrial Calcium Uniporter b)	CCDC109B	40→37	Two transmembrane domains. N- and C-termini span into the matrix	Paralog of MCU. Lower expression than MCU. No channel activity. MCUb overexpression reduces the $[Ca^{2+}]_m$.	(Raffaello *et al.*, 2013)
MCUR1 (Mitochondrial Calcium Uniporter Regulator 1)	C6ORF79 CCDC90A	40→37	Two transmembrane domains. N- and C-termini span into the IMS. Large portion of the protein faced on the matrix.	MCUR1 interacts with MCU but not with MICU1. MCUR1 silencing abrogates Ca^{2+} uptake. MCUR1 overexpression enhances the $[Ca^{2+}]_m$.	(Mallilankaraman *et al.*, 2012a)
EMRE (Essential MCU REgulator)	C22ORF32 SMDT1	12→10	Single-pass membrane protein, with highly conserved aspartate-rich tail.	EMRE silencing abolishes Ca^{2+} uptake. EMRE is required for interaction of MCU with MICU1/MICU2.	(Sancak *et al.*, 2013)

The lower molecular weights are consistent with predicted cleavable amino-terminal mitochondrial targeting sequences.

mitochondrial proteins, coiled-coil domain-containing protein 109A (CCDC109A), renamed MCU, shows all of the typical characteristics of a Ca^{2+} uniporter:

(1) Using patch-clamp electrophysiology of mitoplasts (mitochondria without an OMM), CCDC109A/MCU has been demonstrated as a unique, gated Ca^{2+}-selective channel in the IMM (Kirichok *et al.* 2004). The reconstitution of purified CCDC109A/MCU into a planar lipid bilayer generates a Ca^{2+} current with similar properties to those previously reported by the Clapham group (De Stefani *et al.* 2011). This result was very recently corroborated and updated with another patch-clamp experiment, which showed that a reduction in MCU transcripts via knock-down and their enhancement via overexpression produce parallel changes in the mitochondrial Ca^{2+} current (Chaudhuri *et al.* 2013). Moreover, a single point mutation (S→A at position 259) abolishes the sensitivity of MCU to Ruthenium Red (Baughman *et al.* 2011; Chaudhuri *et al.* 2013). These analyses establish that MCU encodes the pore-forming subunit of the uniporter and that Ruthenium Red acts directly on the channel.

(2) All ion channels require at least two transmembrane domains to exert their activity. CCDC109A/MCU possesses two transmembrane α-helixes, which are highly conserved among different species.

Moreover, CCDC10A/MCU is ubiquitously expressed in mammals.

(3) CCDC109A/MCU has no orthologue in the yeast *S. cerevisiae* (De Stefani *et al.* 2011), which lacks a Ruthenium Red-sensitive mitochondrial Ca^{2+} uptake system (Carafoli *et al.* 1970). Nevertheless, CCDC109A/MCU is conserved in trypanosomatidae, a group of parasites possessing a Ca^{2+} uptake system with properties similar to those described in mammalian mitochondria (Docampo & Lukes, 2012). Interestingly, the essential role of MCU in the regulation of cell bioenergetics in *Trypanosoma brucei* has been recently reported (Huang *et al.*, 2013).

(4) The overexpression of CCDC109A/MCU almost doubles the mitochondrial Ca^{2+} content in both intact and permeabilized cells, causing a significant decrease in the cytosolic Ca^{2+} content due to an enhanced mitochondrial buffer activity (De Stefani *et al.* 2011). Accordingly, the down-regulation of CCDC109A/MCU strongly inhibits mitochondrial Ca^{2+} entry (Baughman *et al.* 2011; De Stefani *et al.* 2011), and the re-introduction of the wild-type protein in MCU knock-down cells fully rescues Ca^{2+} uptake (Baughman *et al.* 2011). Nevertheless, the other classical features of mitochondria, such as $\Delta\Psi$, organelle shape, O_2 consumption and ATP synthesis, appear unchanged after MCU down-regulation. Thus, MCU is essential for high-capacity Ca^{2+} transport into the mitochondria but does not alter any other mitochondrial parameters.

The nuclear *MCU* gene, which is located on chromosome 10, encodes a 40-kDa protein that loses its cleavable target sequence during mitochondrial import, resulting in a 35-kDa mature form (Baughman *et al.* 2011). Curiously, among the different attempts to identify the mitochondrial Ca^{2+} uniporter, two studies described MCU-like glycoproteins with similar molecular weights (Zazueta *et al.* 1991; Saris *et al.* 1993).

Although the topology of MCU was initially a matter of debate (reviewed in Drago *et al.* 2011), it is now clear that its N- and C-terminal domains span into the mitochondrial matrix and that its 9-aa linker (the DIME domain) between the two transmembrane domains faces the intermembrane space (Baughman *et al.* 2011). The orientation of MCU has been definitively solved through the development of APEX, a monomeric 28-kDa peroxidase that is used as an electron microscopy tag, which is active in all cellular compartments and does not require light. The fusion of APEX to the N- and C-termini of MCU clearly stains the mitochondrial matrix but not the intermembrane space (Martell *et al.* 2012).

The existence of only two putative transmembrane domains strongly suggests that an active and functional uniporter channel could be formed by oligomers of MCU. The predicted quaternary structure is compatible with a tetramer, in which eight helices line the putative pore region, and the clustering of charged residues in proximity of the pore generates a negative electrostatic potential that favours the flux of a cation (Raffaello *et al.* 2013). In addition, the mutation of two negatively charged residues inside the DIME motif (D261/E264 in human MCU, D260/E263 in the mouse orthologue) abolishes MCU activity (Baughman *et al.* 2011; De Stefani *et al.* 2011).

Blue native polyacrylamide gel electrophoresis experiments have confirmed that that MCU oligomerizes in the mitochondrial inner membrane as part of a larger complex, migrating at an apparent molecular weight of ~480 kDa (Baughman *et al.* 2011). Thus, the uniporter complex includes different regulatory subunits (Fig. 1), and one of these subunits is represented by the MICU1 protein.

MICU1

The discovery of mitochondrial calcium uptake 1 (MICU1) preceded the identification of MCU by only few months (Perocchi *et al.* 2010). The Mootha group used MICU1 as a molecular (and computational) bait to identify the core component of the uniporter MCU. Not only do MCU and MICU1 display the same evolutionary pattern of expression and similar RNA expression in a variety of mouse tissues (Bick *et al.* 2012), but they also physically interact (Baughman *et al.* 2011).

MICU1 (previously known as CBARA1 and EFHA3) is a 54-kDa single-pass membrane protein that contains two highly conserved EF-hand Ca^{2+}-binding domains. MICU1 was identified through the selective siRNA screening of IMM proteins that are expressed in the majority of mammalian tissues and have homologues in vertebrates and kinetoplastids but not in the yeast *S. cerevisiae*. The down-regulation of MICU1 drastically reduces the mitochondrial Ca^{2+} content in an EF-hand-dependent manner without significantly impairing mitochondrial respiration or membrane potential (Perocchi *et al.* 2010). However, only 2 years after the identification of MICU1, its role as a positive regulator of mitochondrial Ca^{2+} entry was re-examined. The Foskett and Madesh laboratories proposed an essential role for MICU1 as a gatekeeper for MCU-dependent Ca^{2+} accumulation (Mallilankaraman *et al.* 2012*b*). Contrary to the previous work, MICU1 knock-down cells displayed unchanged histamine-induced mitochondrial Ca^{2+} uptake but dramatically increased basal Ca^{2+} content. Thus, MICU1 limits Ca^{2+} entry via MCU when the intracellular $[Ca^{2+}]$ is low, under resting conditions or during weak agonist stimulation. When MICU1 is lost, the mitochondria become constitutively loaded with Ca^{2+}, suggesting that MICU1 is not required for

uniporter-dependent Ca^{2+} uptake (Mallilankaraman *et al.* 2012*b*).

The initially proposed role of MICU1 is radically different from its currently understood role: MICU1 acts as a high-affinity Ca^{2+} brake on MCU-mediated Ca^{2+} uptake, and this scenario concurs with the so-called 'rapid mode of Ca^{2+} uptake (RaM),' a phenomenon through which isolated mitochondria seem to sequester Ca^{2+} very rapidly at the beginning of each Ca^{2+} pulse in a sequence (Sparagna *et al.* 1995). Following this model, upon Ca^{2+} addition, MCU initially takes up Ca^{2+} very rapidly, but when $[Ca^{2+}]$ inevitably increases inside the matrix, MICU1 binds Ca^{2+} through its EF-hand domains, exerting an inhibitory role on MCU-dependent Ca^{2+} entry.

These findings imply that the MICU1 EF-hands must face the matrix to enable them to sense the mitochondrial $[Ca^{2+}]$ ($[Ca^{2+}]_m$). Indeed, it has recently been showed as MICU1 might compartmentalize in the mitochondrial matrix side of the IMM, and that MICU1 binding with MCU is defined by a MICU1 N-terminal polybasic domain and two interacting coiled-coil domains of MCU (Hoffman *et al.*, 2013). However, only few months ago, both the topology and functions of MICU1 were reassessed (Fig. 2).

First, using proteinase K digestion with increasing concentrations of detergent to differently permeabilize the OMM and IMM, MICU1 was shown to be localized to the outer surface of the IMM, facing the intermembrane space rather than the matrix (Csordas *et al.* 2013). Nevertheless, proteomic mapping of the mitochondrial matrix, based on a combination of mass spectrometry and the employment of matrix-targeted APEX (Rhee *et al.* 2013; see the 'MCU' section), identified a matrix proteome of ∼500 proteins, and MICU1 was absent from this list. Thus, the localization of MICU1 at the IMS suggests a primary response to changes in the cytosolic $[Ca^{2+}]$ rather than the $[Ca^{2+}]_m$. Notably, the existence of a putative intermembrane component that is involved in regulating the activity of the uniporter was described more than 20 years ago (Igbavboa & Pfeiffer, 1991).

Second, Csordas *et al.* (2013) showed that MICU1 contributes to the cooperative activation of the uniporter at high cytosolic $[Ca^{2+}]$, whereas the same phenomenon was not observed in the previous paper published in *Cell*. The reason for this difference might be related to the composition of the experimental buffer, as several seminal studies reported that Mg^{2+} controls the allosteric activation of the uniporter (Bragadin *et al.* 1979). The effect of MICU1 deletion on MCU cooperativity has been detected exclusively in the presence of Mg^{2+}, and similar measurements by Mallilankaraman *et al.* were performed in a Mg^{2+}-free bath.

Third, although both of these studies agree that MICU1 is critical in keeping MCU closed (Mallilankaraman *et al.* 2012*b*; Csordas *et al.* 2013), Csordas and co-workers showed that the resting $[Ca^{2+}]_m$ is unaltered and that the IP_3-dependent Ca^{2+} uptake is mostly abolished in MICU1 knock-down cells (Csordas *et al.* 2013), in agreement with previous findings (Perocchi *et al.* 2010). Importantly, unchanged mitochondrial Ca^{2+} levels in MICU1-silenced cells have been detected using a non-ratiometric fluorescent dye-based Ca^{2+} indicator (Rhod-2; Mallilankaraman *et al.* 2012*b*), whereas a dramatic decrease in the $[Ca^{2+}]_m$ has been measured upon agonist stimulation using genetically encoded Ca^{2+} sensors in calibrating aequorin-based assays (Perocchi *et al.* 2010) or ratiometric pericam (Csordas *et al.* 2013). Thus, the use of different Ca^{2+} tools is a possible explanation for these discrepancies with regard to the silencing approach. Both papers showed no difference

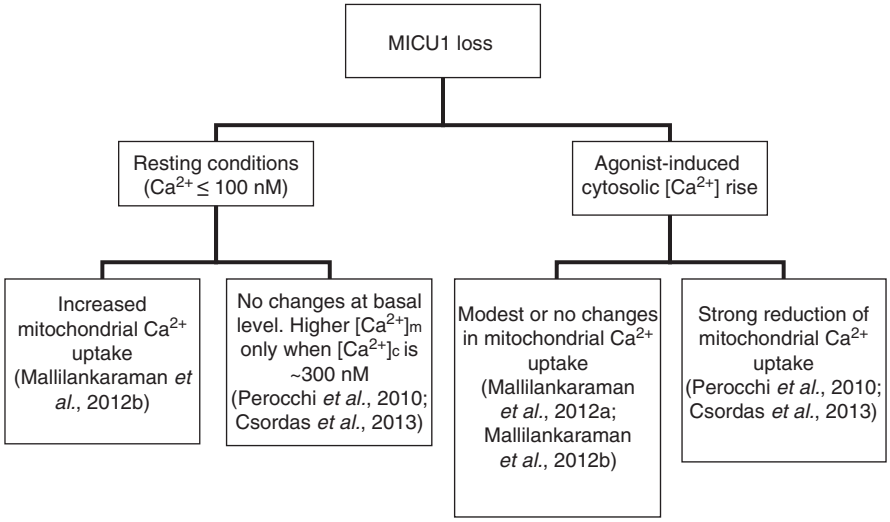

Figure 2. Dynamic diagram showing Ca^{2+} changes in the different states of cytosolic Ca^{2+} upon MICU1 knock-down

in the MCU protein levels in MICU1-silenced cells (Mallilankaraman *et al.* 2012*b*; Csordas *et al.* 2013). However, the silencing of MICU1 in the mouse liver (based on the same strategy used in Csordas *et al.* 2013) appeared to have a major impact on the abundance of the MCU protein (but not its mRNA; Plovanich *et al.* 2013), and impaired mitochondrial Ca^{2+} handling might reasonably be correlated to the decrease in MCU stabilization.

In summary, on one hand, MICU1 stabilizes the closed state of the MCU complex, limiting mitochondrial Ca^{2+} entry under resting conditions or during small $[Ca^{2+}]$ elevations through a mechanism that requires its Ca^{2+}-binding EF-hand domains (Mallilankaraman *et al.* 2012*b*) or is independent of its EF-hands (Csordas *et al.* 2013). On the other hand, MICU1 most likely cooperates with MCU to allow Ca^{2+} accumulation inside the matrix, but the extent of this cooperation is still a matter of debate (Fig. 2). However, the identification of new components of the uniporter complex, such as MICU2, the MCU paralogue MCUb and the regulator MCUR1, introduces additional players in the control of mitochondrial Ca^{2+} dynamics.

MICU2

MICU1 has two paralogues, namely, the protein products of the human genes *EFHA1* and *EFHA2*, which share 25% sequence identity with MICU1. Both proteins possess N-terminal mitochondrial targeting sequences and are detected in multiple mouse tissues; thus, these proteins have been renamed MICU2 and MICU3, respectively (Plovanich *et al.* 2013). However, MICU3 does not display a strong or exclusive mitochondrial localization, leading to its exclusion from the MitoCarta list (Pagliarini *et al.* 2008). In contrast, MICU2 is a mitochondrially localized protein and, similar to MICU1, possesses highly conserved EF-hand domains. MICU2 interacts with MICU1 and MCU (Plovanich *et al.* 2013) and should reside at the IMS due to its analogy with MICU1 and its absence from the matrix protein list (Rhee *et al.* 2013). The *in vivo* silencing of MICU2 does not affect $\Delta\Psi$ or mitochondrial respiration (Plovanich *et al.* 2013) but reduces mitochondrial Ca^{2+} clearance upon the addition of multiple Ca^{2+} spikes (Plovanich *et al.* 2013). Moreover, the silencing of both MICU1 and MICU2 shows additive Ca^{2+} defects, and MICU2 overexpression in MICU1-silenced HeLa cells restores the wild-type phenotype.

MICU1, MICU2 and MCU have been shown to reside within a complex (Plovanich *et al.* 2013). The interrelation of the expression levels of MICU1, MICU2 and MCU have been analysed in three cellular contexts:

(1) In the mouse liver, both MICU1 and MICU2 siRNAs lead to a decrease in MCU protein expression and a shift in the size of the MCU complex (from ∼480 kDa to ∼350 kDa).
(2) In HEK293 cells, MICU1 knock-down leads to a reduction in the level of MICU2 but not vice versa. Moreover, MCU overexpression increases the expression of both MICU1 and MICU2, and MICU1 up-regulation results in a higher level of MICU2.
(3) In HeLa cells, MICU1 down-regulation leads to a reduction in the level of the MICU2 protein (and vice versa). Of note, MCU expression seems to be inversed compared to MICU2 expression.

Further studies are required to clarify whether MICU1–2 and MCU regulate each other with respect to protein expression. Indeed, the silencing approach used might confound the results, changing the indirect activities for specific molecular features.

MCUb

CCDC109B, which was renamed MCUb, is an MCU paralogue/isogene. MCUb is a 33-kDa protein that shares 50% similarity with MCU. Its protein topology and structure is very similar to that of MCU, as it has two transmembrane domains with its N- and C-termini facing the IMS, but MCUb has a lower expression level and a different expression profile than MCU (Raffaello *et al.* 2013). MCU and MCUb can interact, and the reconstitution of MCUb in the lipid bilayer does not result in channel activity. Accordingly, MCUb overexpression in intact cells reduces mitochondrial Ca^{2+} uptake (Raffaello *et al.* 2013). Thus, MCUb might act as an endogenous dominant-negative isoform, and the insertion of one or more MCUb subunits in the multimer might alter Ca^{2+} permeation. The MCUb primary sequence differs from that of MCU by two residues in the putative pore-forming region. Indeed, the introduction of the same amino acid substitutions in MCU (R251W, D256V) blunted the increase in mitochondrial Ca^{2+} uptake (Raffaello *et al.* 2013).

However, the mRNA levels of MCUb are very low compared to those of MCU. MCUb mRNA is highly expressed in the heart and lung and minimally expressed in skeletal muscle. Interestingly, variations in tissue-dependent mitochondrial Ca^{2+} uptake have been recently reported, with a recorded skeletal muscle Ca^{2+} influx that is 28-fold greater than that in cardiac mitoplasts (measured in 100 μM $[Ca^{2+}]$ at −160 mV; Fieni *et al.* 2012).

MCUR1

Mitochondrial Ca^{2+} uniporter regulator 1 (MCUR1; previously known as CCDC90A) was identified as an essential regulator of Ca^{2+} uptake in an siRNA screen of 45 mitochondrial proteins that were predicted to be integral to the IMM or that have a well-documented role

in mitochondrial Ca^{2+} homeostasis (Mallilankaraman *et al.* 2012*a*). MCUR1 silencing resulted in a dramatic reduction in the $[Ca^{2+}]_m$ (approximately −85% in Rhod-2 fluorescence compared to the control) without modifying the cytosolic Ca^{2+} content. Interestingly, under these experimental conditions, MICU1 knock-down in HEK293 cells caused only minor Ca^{2+} changes (approximately −15% compared to the control siRNA).

MCUR1 interacts with MCU but not with MICU1, and pull-down assays suggested that these three proteins do not exist in the same complex (Mallilankaraman *et al.* 2012*a*). Importantly, MCUR1 overexpression in HeLa cells enhances $[Ca^{2+}]_m$, but this increase is strongly diminished by MCU knock-down. Accordingly, MCU overexpression fails to restore the Ca^{2+} levels in MCUR1-silenced cells, suggesting that both MCU and MCUR1 are required for efficient Ca^{2+} uptake through the uniporter. Notably, both MCU mRNA and protein expression are up-regulated in MCUR1-depleted cells, confirming the deep interrelation between these two proteins.

As for MCU and MICU1, MCUR1 itself has a paralogue, CCDC90B, whose function is still not clear. MCUR1 is 40-kDa protein that contains two transmembrane domains and one coiled-coil region, with N- and C-termini facing the same compartment. The topology of MCUR1 has been solved using a proteinase K-based biochemical assay, which showed that its N- and C-terminal residues are projected into the IMS (Mallilankaraman *et al.* 2012*a*). Thus, a large portion of the protein (∼250 aa) should span into the matrix, explaining the classification of MCUR1 as a mitomatrix protein (Rhee *et al.* 2013).

EMRE

Using stable isotope labelling by amino acids in cell culture (SILAC), a mass spectrometry-based proteomic approach, Mootha and co-workers identified EMRE (essential MCU regulator, previously known as C22ORF32) as a component of the uniporter complex (Sancak *et al.* 2013). EMRE is 10-kDa single-pass membrane protein, located at IMM, with a highly conserved aspartate-rich C-terminal region. EMRE is a member of the MitoCarta list (Pagliarini *et al.* 2008) and, notably, homologues have not been found in any plants, protozoa and fungi, indicating that EMRE could represent a metazoan innovation (Sancak *et al.* 2013).

EMRE interacts with MICU1 at IMS and with MCU oligomers in the inner membrane, thus EMRE seems to act as a bridge between the Ca^{2+}-sensing activity of MICU1/MICU2 and the channel properties of MCU. Loss of EMRE induces a reduction of Ca^{2+} entry to the same extent as MCU depletion, suggesting that MCU requires EMRE for *in vivo* Ca^{2+} conductance (Sancak *et al.* 2013). MCU over-expression in EMRE-silenced cells failed to restore mitochondrial Ca^{2+} uptake. Interestingly, EMRE protein expression is strictly dependent on MCU levels, a partnership that could be analogous to that of MICU1/MICU2. Indeed, in MCU-depleted cells, EMRE abundance is drastically decreased (but not vice versa), despite no alteration in mRNA levels.

With the identification of EMRE and its 'bridging activity', all the members of the uniporter complex should be now defined. Based on SILAC results, the uniplex (uniporter complex) seems to be composed of MCU holomers, MCUb, MICU1, MICU2 and EMRE (Sancak *et al.* 2013). MCUR1 has not been found using this experimental approach, suggesting a role of this protein in Ca^{2+} handling outside the uniplex.

Physiopathological implications of the MCU complex

Mitochondrial Ca^{2+} uptake plays a critical role in the regulation of aerobic metabolism (Bonora *et al.* 2012) and cell survival (Giorgi *et al.* 2012). Several oncogenes and tumour suppressors manipulate Ca^{2+} to exert their anti/pro-apoptotic activities, and mitochondrial Ca^{2+} overload has been associated with apoptosis or necrosis in many pathological states (Giorgi *et al.* 2012). Accordingly, upon pro-apoptotic stimuli, MCU-expressing cells display an enhanced sensitivity to apoptosis, confirming that increased Ca^{2+} loading correlates with a predisposition for cell death (De Stefani *et al.* 2011). MCU expression and apoptosis are regulated by miRNA (Marchi *et al.* 2013). The screening of putative MCU-targeting miRNAs showed that miR-25 affects mitochondrial Ca^{2+} uptake through the specific down-regulation of MCU, conferring reduced mitochondrial Ca^{2+} content and resistance to Ca^{2+}-dependent apoptotic challenges (Marchi *et al.* 2013). The alteration of the miRNA expression pattern could lead to a variety of human disorders, including cancer. Thus, miRNAs may function as oncogenes or tumour suppressors. The cancer-related miRNA miR-25 is up-regulated in various human cancers, including prostate and colon carcinomas. Indeed, colon adenocarcinoma samples with high miR-25 levels display low MCU expression (Marchi *et al.* 2013).

In addition to cancer, fundamental roles for MCU and mitochondrial Ca^{2+} uptake have been identified in specific cellular processes, which range from the regulation of gastrula morphogenesis in zebrafish (Prudent *et al.* 2013) to the control of excitotoxicity (Qiu *et al.* 2013). In cardiomyocytes, MCU silencing amplifies the bulk cytosolic $[Ca^{2+}]$ and is associated with increased contractile responses (Drago *et al.* 2012). Moreover, Ca^{2+}–calmodulin-dependent protein kinase II (CaMKII), which is highly activated in ischaemia reperfusion and myocardial infarction, promotes myocardial death by increasing the current through the MCU complex (Joiner *et al.* 2012). CaMKII resides in the matrix, interacts with

MCU and promotes mitochondrial Ca^{2+} entry, most likely by catalysing the phosphorylation of serines 57 and 92 (Joiner *et al.* 2012).

In pancreatic β-cells, MCU- and MICU1-dependent Ca^{2+} accumulation regulate the ATP level, glucose metabolism and insulin secretion (Alam *et al.* 2012; Tarasov *et al.* 2013). Interestingly, MCU silencing impairs the Ca^{2+}-dependent phase of glucose-induced ATP increase and essentially eliminates secretion stimulated by tolbutamide, a potassium channel blocker used in the management of type II diabetes (Tarasov *et al.* 2013). Regulation of exocytosis by mitochondrial Ca^{2+} accumulation could involve both K_{ATP}-dependent or -independent hormone secretion. However, the lack of evidence for a role for mitochondrial Ca^{2+} uptake in the regulation of plasma membrane electrical dynamics might suggest a predominant involvement of the K_{ATP}-independent pathway (Tarasov *et al.* 2012).

The down-regulation of MICU1 dramatically elevates the basal levels of reactive oxygen species (ROS), particularly superoxide anion, and sensitizes the cells to apoptosis (Mallilankaraman *et al.* 2012*b*). As the thresholding activity of MICU1 plays a critical role in the regulation of mitochondrial oxidant signalling, the critical roles of MCU and MCUR1 in mitochondrial Ca^{2+} uptake affect various bioenergetic parameters. The absence of Ca^{2+} transfer from the ER to the mitochondria results in reduced O_2 consumption and ATP levels and the activation of AMP kinase (AMPK), which, in turn, triggers pro-survival autophagy (Cardenas *et al.* 2010). Furthermore, the knock-down of MCU or MCUR1 induces bioenergetic stress, which is reflected by an increased AMP/ATP ratio and diminished oxidative phosphorylation, and the activation of the autophagic pathway (Mallilankaraman *et al.* 2012*a*,*b*).

However, interesting results have been obtained through the characterization of an MCU-deficient mouse model (Pan *et al.* 2013). As expected, the drastic reduction in mitochondrial Ca^{2+} uptake correlates with higher pyruvate dehydrogenase (PDH) phosphorylation and consequent minor PDH activity in knock-out (KO) skeletal muscle mitochondria. MCU-null mice perform less efficiently under situations that require a rapid increase in skeletal muscle work load and a high expenditure of energy (Pan *et al.* 2013). These findings agree with the widely accepted view that the activation of matrix-located dehydrogenases is crucial for ATP supply under conditions of increased ATP demand. However, the other results of this study were highly unexpected. Surprisingly, no difference in oxygen consumption and autophagy levels were detected in MCU KO cells or tissues, suggesting that basal metabolism is not altered by MCU loss. Nevertheless, the absence of MCU expression does not confer any protection from cell death, although MCU KO mitochondria did not show Ca^{2+}-induced mPTP opening (Pan *et al.* 2013). Similar effects have also been observed *in vivo*, using a model of ischaemic-reperfusion injury of the heart. Thus, in the absence of MCU, alternative mPTP- or Ca^{2+}-independent cell death pathways might emerge, acting as predominant mechanisms of death in this scenario. Notably, MCU loss does not reset the matrix $[Ca^{2+}]$ at resting conditions (Pan *et al.* 2013); therefore, the activity of Ca^{2+} players that do not reside inside the uniporter complex might be enhanced to compensate for MCU deficiency, ensuring a slow, but continuous, Ca^{2+} influx inside the matrix.

In conclusion, we have summarized the recent findings on the molecular identities of all of the known members of the mitochondrial calcium uniporter complex.

Since the discovery of MICU1, which paved the way for the identification of MCU, the search for the functional roles of these key players has intensified. We are aware that the molecular study of mitochondrial Ca^{2+} signalling is just getting underway, and we can expect a rapid increase in the body of knowledge on the mitochondrial Ca^{2+} uptake machinery. In addition, it will be important to consider the link between the Ca^{2+} uniporter and other players, including Letm1 (Jiang *et al.* 2009), uncoupling proteins (Trenker *et al.* 2007), TRPC3 (Feng *et al.* 2013) and NCLX (Palty *et al.* 2010), that may influence the properties of the mitochondria with respect to both physiology and physiopathology.

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Additional information

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None declared.

Author contributions

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