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Molecular regulation of MCU: Implications in physiology and disease

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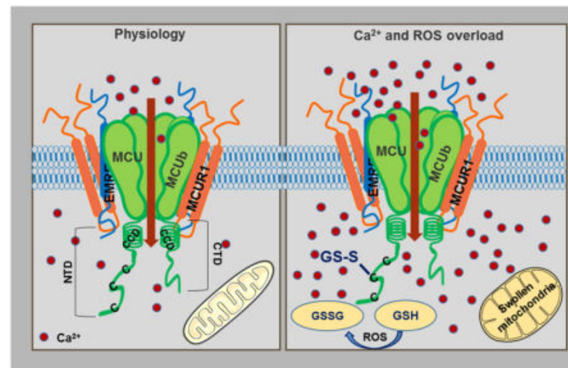
Abstract

Ca^{2+} flux across the inner mitochondrial membrane (IMM) regulates cellular bioenergetics, intra-cellular cytoplasmic Ca^{2+} signals, and various cell death pathways. Ca^{2+} entry into the mitochondria occurs due to the highly negative membrane potential (Ψ_m) through a selective inward rectifying MCU channel. In addition to being regulated by various mitochondrial matrix resident proteins such as MICUs, MCUb, MCUR1 and EMRE, the channel is transcriptionally regulated by upstream Ca^{2+} cascade, post transnational modification and by divalent cations. The mode of regulation either inhibits or enhances MCU channel activity and thus regulates mitochondrial metabolism and cell fate.

Graphical abstract



Ca^{2+} ion is a versatile second messenger essential for a variety of kinetically different cellular processes from fertilization to cell death [1]. While some processes like endocytosis occur in seconds, other processes such as gene transcription take up to hours and how Ca^{2+}

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regulates these diverse processes is a question still being studied [2]. A rise in cytosolic Ca^{2+} ($_cCa^{2+}$) can occur either by Ca^{2+} entry through plasma membrane channels (voltage gated/ receptor mediated/second messenger mediated channels/store operated channels) or release from intracellular Ca^{2+} stores [3]. One ubiquitous mode of receptor-regulated Ca^{2+} entry is capacitative calcium entry, first proposed by Putney [4]. At any given point, resting $_cCa^{2+}$ is kept low (~ 100nM) and this is achieved by Ca^{2+} efflux mechanisms of pumps (PMCA), exchangers (NCX) and organelles such as ER and the mitochondria that act as Ca^{2+} sinks. $_cCa^{2+}$ transients are defined and shaped by the mitochondria. The first observation of mitochondrial Ca^{2+} ($_mCa^{2+}$) uptake was evidenced five decades ago when several groups witnessed isolated mitochondria to buffer Ca^{2+} [5]. Since then, $_mCa^{2+}$ has been extensively studied. Three main roles have been attributed to Ca^{2+} uptake by the mitochondria: 1) Ca^{2+} is utilized by the dehydrogenases of the TCA cycle (pyruvate dehydrogenase, iso-citrate dehydrogenase and α-ketoglutarate dehydrogenase) for ATP generation. 2) To maintain $_cCa^{2+}$ dynamics. 3) Activation of various cell death pathways of apoptosis and necrosis [6–10].

Ca^{2+} entry into the mitochondria is due to the high electrochemical gradient (~ −180 mV) and occurs without the transport of any other ions hence making it a uniporter. Studies on isolated mitochondria estimated a flux of more than 10,000 Ca^{2+} ions per second through the channel [5]. Although the properties of the uniporter were established, its molecular identity remained elusive. The identification of the mitochondrial calcium uniporter (MCU) was made possible with the advances in integrative genomics [11, 12]. Progress in the field has been made in identifying and characterizing MCU and its regulatory molecules.

Here, in this review we summarize the architecture of MCU channel, its mitochondrial resident regulators, regulation of the channel by ions and redox molecules and go onto explore the activity of the channel in pathological conditions along with therapeutic insights.

Architecture of MCU

Early work on $_mCa^{2+}$ uptake revealed that Ca^{2+} uptake into the mitochondria was Ψ_m dependent with no transport of anions hence making it a uniporter. The transport of Ca^{2+} followed second order kinetics and was sensitive to Ruthenium Red (RR) (an inorganic cationic dye that binds acidic mucopolysaccharides and phospholipids)[13, 14]. Electrophysiological studies defined the channel to be an inward rectifying current with high specificity for divalent cations Ca^{2+} ~Sr^{2+} ≫ Mn^{2+} ~ Ba^{2+} and to be inhibited at Nano molar concentrations of RR and its analog Ru360 [15]. Although the biophysical properties of the channel were characterized as being 1. Electrogenic dependent 2. Selective and 3. Low affinity for Ca^{2+}, it was not until 2011 when two independent groups discovered the molecular identity of the channel. Whole genome phylogenetic profiling, RNA co-expression analysis and organelle wide protein co-expression analysis revealed an RR sensitive transmembrane protein resident of the IMM that is part of a large complex called the MCU [11, 12]. Reconstitution of MCU in planar lipid bilayer produced channel recordings with conductance similar to previously findings. Additionally, presence of RR failed to produce Ca^{2+}-permeable channel activity indicating sensitivity to ruthenium, a characteristic of the $_mCa^{2+}$ transport [12]. Consistent with the finding that all vertebrate

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mitochondria take up Ca^{2+}, the expression of MCU is conserved across eukaryotes except in yeast. In contrast to their evolutionary sister group Amoebozoa that have a single homolog of MCU and MICU1, yeast do not exhibit uniporter activity with no homologs of the uniporter components [16]. Because of this property yeast serve as an excellent heterologous expression system. Reconstitution of DdMCU (MCU from Dictyostelium discoideum) in yeast was sufficient to evoke a $_mCa^{2+}$ uptake response that was otherwise absent, further suggesting that MCU was the pore-forming subunit of the uniporter complex. MCU, the pore forming channel has been identified to be a two transmembrane, Ca^{2+} selective, ruthenium sensitive channel with its selectivity filter in the inter-membrane space (IMS) and N and C terminal domains resting in the mitochondrial matrix [16].

The structure of N-terminal domain (NTD) of MCU spanning exons 3 and 4 adopts a β-grasp like fold that entails an α–helix and six β-strands that form the central core with two highly conserved leucine rich loops [17]. Atomic resolution structure of NTD revealed a cluster of negatively charged residues called the MCU-regulating acidic patch (MRAP) in the β-grasp fold domain that binds divalent cations. Either interaction of Ca^{2+} or Mg^{2+} with the MRAP domain or mutations in the MRAP domain destabilizes MCU and shifts the self-association equilibrium to monomer with a loss of $_mCa^{2+}$ uptake. This study for the first time shows that like most Ca^{2+} channels that are regulated by Ca^{2+} feedback mechanisms, MCU is autoregulated by matrix Ca^{2+} and Mg^{2+} binding to the MRAP domain in the NTD of MCU [18]. Expression of MCU ^{NTD} localizes to the mitochondria, and forms MCU oligomers with intact Ψ_m. Deletion of the NTD showed a significant reduction in $_mCa^{2+}$ uptake with intact $_cCa^{2+}$ dynamics. Co-immuno precipitation assay revealed that loss of NTD had diminished interaction with MCUR1 (a positive regulator of MCU) but not MICU1 and MICU2 [17]. The C-terminal domain (CTD) harbors two transmembrane domains and two coiled-coiled domains that are required for interaction with its regulators. Nuclear magnetic resonance (NMR) in combination with electron microscopy revealed the architecture of the MCU channel to be a pentamer. The second transmembrane (TM2) and the coiled-coil helix (CCH) form the inner cores of the pentamer that are wrapped by the first TM helix and CCH respectively. TM1 and TM2 domains are connected by a loop consisting of conserved acidic residues called the DIME motif loop that forms the pentameric barrel at the mouth of the pore. Asp240 and Glu243 are positioned inside the barrel to form two carboxylate rings that make up the selectivity filter of the channel [19]. Although this finding for the first time reported the structure of the *C.elegans* MCU pore, no proof of channel activity was performed in the pentameric state.

Regulators of MCU channel

MCU exists as part of a heteromeric complex that consist of MICU1, MICU2, MCUR1, EMRE, MCUb and SLC25A23 [20–24]. Sequence analysis of MCU identified a gene with 50% similarity to MCU called MCUb. MCUb is conserved across all vertebrates and absent in species of plants, kinetoplastids, Nematoda, and Arthropoda where MCU is present. Immuno-precipitation and foster resonance energy transfer (FRET) analysis revealed interaction of MCU and MCUb. However, electrophysiological studies of purified MCUb inserted into planar lipid bilayers showed no channel activity. While RNAi mediated silencing of MCUb in HeLa cells demonstrated an increase in $_mCa^{2+}$ uptake upon

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stimulation with histamine, over-expression of MCUb resulted in a marked decrease in $_mCa^{2+}$ uptake. These pieces of data indicate that MCUb functions as a dominant negative MCU sub-unit [25].

The activity of the channel is determined by its negative regulator MICU1 and its positive regulator MCUR1. Like most ion channels that open upon agonist stimulation, MCU channel opens only when $_cCa^{2+}$ rises above 1–2 μM. The threshold is set by its gatekeeper MICU1. The EF hands of MICU1 sense the rise in $_cCa^{2+}$ (> 3 μM) and physically dissociate from the channel thus transforming MCU to its open confirmation [26]. MICU1 was the first MCU uniplex component to be identified using targeted RNAi screen based on clues from comparative physiology, evolutionary genomics and organelle proteomics. MICU1 was found to be an IMS resident protein with its EF hand domains facing the cytosol. It was also shown that silencing MICU1 abrogated $_mCa^{2+}$ uptake without dissipating Ψ_m [21, 27]. Contrary to the previous findings that MICU1 is an IMS resident protein, authentic biochemical and confocal validations from our laboratory reveal MICU1 to be a soluble matrix protein that senses rise in $_cCa^{2+}$ or $_mCa^{2+}$ with its EF hands and opens MCU channel. Serial mutation analysis revealed that the evolutionary conserved poly basic region of MICU1 (99 –110 aa) directly interacts with the coiled-coiled domains of MCU. Mutations in polybasic region but not EF hand domain results in loss of interaction with MCU [28]. Structural analysis of MICU1 reveals that under Ca^{2+}-free conditions MICU1 forms a hexamer that interacts with MCU and inhibits its activity through the c-helix [29]. Complementary to our findings, genetic deletion of MICU1 exhibits increased Ca^{2+} uptake at low $_cCa^{2+}$ with a concomitant $_mCa^{2+}$ overload. This matrix overload resulted in altered mitochondrial morphology particularly in the skeletal muscles [30]. Autosomal recessive mutations in MICU1 presents with proximal skeletal muscle weakness with neurological features of chorea, tremors or ataxia [31, 32]. MICU1$^{-/-}$ animals also exhibit similar abnormalities to that seen in humans. Crispr-Cas9 mediated deletion of *Micu1* yielded significant perinatal lethality suggesting that MICU1 is dispensable for embryonic development but essential post-natal [30]. Along these lines it was also observed that MICU1 plays a key role in liver regeneration post partial hepatectomy (PHx). Loss of MICU1 results in elevated inflammatory state post PHx with failure of the hepatocytes from entering the cell cycle due to $_mCa^{2+}$ overload mediated necrosis of hepatocytes [33].

Bio-informatic analysis revealed two paralogues of MICU1 that evolved in the vertebrates possibly by a gene duplication event. MICU2 and MICU3 were found to contain conserved domains as MICU1 along with two canonical EF hand domains with different expression patterns. While MICU1 and MICU2 were expressed in almost all tissues, MICU3 was specific to the central nervous system. The first study that identified the protein demonstrated mitochondrial localization of MICU2 and interaction with MICU1. RNAi mediated silencing of MICU2 revealed a reduced rate of $_mCa^{2+}$ uptake and premature release of matrix calcium. Hence it was concluded that MICU2 played a role in $_mCa^{2+}$ uptake [22]. Subsequently, gene knock out studies using transcription activator-like effector nuclease (TALEN) technology was utilized to generate MICU1 and MICU2 KO in HEK293T cells to functionally distinguish the roles of the two proteins. Addition of 1μM bolus of Ca^{2+} revealed a rapid uptake by MICU1 KO but not control mitochondria due to the loss of gate-keeping effect. MICU2 KO cells also exhibited similar gate-keeping

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characteristic albeit with reduced rate. Importantly, the impaired calcium handling was rescued upon re-expression of the ablated protein signifying that the phenotype is not because of off-target effects of gene KO [34]. It is now accepted that under low $_cCa^{2+}$, MICU1 and MICU2 function as gate-keepers of MCU. Controversy in the role of MICU1 and MICU2 under conditions of high $_cCa^{2+}$ exists and further studies are warranted. Genetic deletion of *Micu2* in animals produced off-springs at Mendelian ratio with comparable sizes to wild-type littermates that survived > 18 months. Liver mitochondria from MICU2$^{-/-}$ animals took up low-regime Ca^{2+} which otherwise was inhibited by liver mitochondrial from wild-type animals, indicating gate-keeping effect of the protein. On the other hand, 25 μM Ca^{2+} pulse was taken up at a slower rate, a phenotype attributed to the reduction in MCU complex proteins [35]. In a recent finding, MICU1-MICU2 hetero-dimers were shown to exhibit steep co-operativity at sub-micromolar $_cCa^{2+}$, thus functioning as ON/OFF switches for MCU-mediated $_mCa^{2+}$ uptake [36].

A positive regulator of the channel, MCU regulator 1 (MCUR1) was identified in an RNAi screen of 45 mitochondrial proteins. Of the 45 genes, only one RNAi resulted in the inhibition of mitochondrial calcium uptake. MCUR1 directly interacts with the channel and promotes Ca^{2+}-uptake into the mitochondria. Silencing of this ubiquitously expressed protein resulted in blunted $_mCa^{2+}$ uptake under basal and activated conditions, with no changes in $_cCa^{2+}$ dynamics. The reduced $_mCa^{2+}$- uptake then resulted in perturbed bioenergetics in MCUR1 KD cells [20, 37]. Findings from another laboratory suggest that MCUR1 mediates assembly of complex IV cytochrome oxidase (COX) and fibroblasts from patient mutations in COX exhibit similar phenotype to loss of MCUR1. Their study defines MCUR1 as an assembly factor of complex IV, thus loss of which results in impaired oxidation phosphorylation (Ox-phos) leading to reduced Ψ_m [38]. A decrease in Ψ_m, decreases the driving force for Ca^{2+} and hence reduced $_mCa^{2+}$ uptake in the absence of MCUR1. Later *in vivo* studies in MCUR1 addressed this controversy. To understand the role of MCUR1 in high and moderate ox-phos demanding tissues, heart (MCUR1$^{fl/fl}$α-MHCCre; cMCUR1 KO) and vasculature (MCUR1$^{fl/fl}$VE-Cad-Cre; MCUR1 ^{EC}) specific deletion of MCUR1 were generated [39]. While germ line deletion of MCUR1 in endothelial cells (ECs) did not show any discernable phenotype, cMCUR1 KO animals were smaller and died 3 weeks after birth. Electrophysiological and biochemical studies from tissue specific KOs demonstrated a significant decrease in MCU current due to the disruption of MCU super complex in the absence of MCUR1. Blunting of MCU current when the transmembrane voltage is clamped suggests a direct effect of MCUR1 on $_mCa^{2+}$ uptake and not an indirect effect of decreased Ψ_m. MCUR1's ability to serve as MCU scaffolding factor stems from i) it being a transmembrane protein with coiled-coil domains essential for protein interaction ii) failure of MCU complex to assemble in the absence of MCUR1 and iii) considerable reduction in I_{MCU} [39]. The role of MCUR1 in $_mCa^{2+}$ uptake may not be consistent for all species. Drosophila cells do not show a difference in $_mCa^{2+}$ uptake in the absence of MCUR1 [40].

MCU proteome analysis revealed essential MCU regulator (EMRE) in addition to MICU1, MICU2 and MCUb. EMRE is a 10 KDa, single pass transmembrane protein with aspartate rich C-terminus. EMRE was shown to be essential for MCU-mediated $_mCa^{2+}$ uptake [23]. Although MCU protein was present in EMRE KO cells, I_{MCU} was significantly reduced,

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suggesting that EMRE was required for the open configuration of the channel. Bioinformatics sequence analysis revealed that EMRE is absent in lower organisms including yeast and is only specific to metazoans. Although reconstitution of MCU alone was sufficient to conduct Ca^{2+} in lipid bi-layers, EMRE along with MCU was required in human mitoplasts, suggesting that Ca^{2+} entry into the metazoan mitochondria depended on EMRE. Using the yeast model system that lack mitochondrial Ca^{2+} uptake machinery, it was demonstrated that DdMCU can be reconstituted using a single genetic component, while the minimum metazoan uniplex components are MCU along with EMRE [16]. EMRE has been proposed to be the matrix sensor regulating MCU activity by sensing Ca^{2+} through the acidic patch at its carboxy terminal and mediating interaction of MICU1 and MICU2 with MCU [41]. However, deletion of the acidic amino acids did not affect $_mCa^{2+}$ uptake [42]. Furthermore, co-immunoprecipitation and bimolecular fluorescence complementation (BiFC) analysis revealed a direct interaction of MICU1 with MCU without EMRE bridging [39].

Transcriptional Regulation of MCU

Regulation of $_cCa^{2+}$ is critical for cell survival. The ability of mitochondria to shape cytosolic Ca^{2+} transients is well documented and hence one might predict that cytosolic Ca^{2+} may stimulate the genes responsible for mitochondrial Ca^{2+} uptake [43–45]. In most non-excitable cells cytosolic Ca^{2+} is released from (inositol 1,4,5-trisphosphate) IP3-induced ER store release and store-operated Ca^{2+} entry (SOCE) [46–53]. Ca^{2+} released from ER and SOCE is rapidly cleared by the plasma membrane components and the mitochondria thus underscoring the interdependence of the upstream cytosolic Ca^{2+} signaling with the downstream mitochondrial Ca^{2+} transport mechanisms. Any perturbation in the anterograde Ca^{2+} signaling including loss of IP3Rs or STIM1 or Orai1 has been shown to alter mitochondrial Ca^{2+} uptake, reducing basal matrix Ca^{2+} and current through the channel by a reduction in MCU protein. This decrease in protein levels of MCU in the absence of proximal Ca^{2+} signals was shown to be mediated by Ca^{2+}-regulated transcription factor CREB (cyclic adenosine monophosphate response element). Chromatin immune-precipitation (Chip) assay revealed the binding of CREB onto the MCU promoter. Lymphocytes lacking IP3R, or SOCE had undetectable levels of phosphorylated CREB, thus decreased MCU abundance and mCa2+ uptake [54]. This study was the first to reveal a mechanistic link of regulation of mitochondrial transport by proximal Ca^{2+} signaling components.

Multiple layers of regulation exist in cells, in the event that one fails, other mechanisms in place take over. One such mode of regulation is by microRNAs (miRNAs). miRNAs are small, non-coding nucleotides that regulates gene expression by sequence complementarity based binding of the target mRNA. Binding to the target mRNA results in either degradation of the target mRNA or inhibition of translation [55]. Target prediction algorithms identified five miRNAs (miR-15, miR-17, miR-21, miR-25 and miR-137) that have sequence complementarity to the 3′UTR of MCU. Of these, only miR-25 shows a 100% seed sequence match to the 3′UTR of MCU. In vitro luciferase assays reveal a down-regulation of MCU RNA and protein by miR-25 but not that of other MCU complex components. Immuno-histochemistry confirmed the inverse regulation of MCU by miR-25 in mucosal

tissues of colon cancer. The increased levels of miR-25 in colon cancer down-regulated MCU levels thus protecting the tumor cells from cell death [56]. This study was the first to propose MCU regulation by miRNAs and progression of cancer due to protection from MCU-mediated tumor cell death but it lacked in vivo data to show tumor cell protection by miR-25. Later studies show that in pulmonary arterial hypertension (PAH) patients where the pulmonary smooth muscle cells (PSMCs) proliferate uncontrollably (cancer-like phenotype), migrate and are resistant to apoptosis, MCU expression is down-regulated without any changes on the MCU complex components. This study demonstrates the post-transcriptional regulation of MCU by miR-25 and miR-138 in PSMCs from PAH patients. The authors validate their findings by using MCU over-expression and anti-miR25 and anti-miR138 that restores $_{m}Ca^{2+}$ homeostasis and reverses PAH [57]. To further corroborate the regulation of MCU by miRNA in vivo, a lung metastasis model of animals was generated. Nude mice were injected via tail vein with estrogen receptor-negative breast cancer cells MDA-MB-231, MDA-MB-231 cells with down regulated MCU expression (MDA-MB-231 with shRNA MCU) or cells over expressing miR-340. Hemoxylin and eosin staining of the lung tissue from each animal revealed significantly fewer metastatic lung nodules in mice that had been injected with MCU-downregulated or miR-340–overexpressing MDA-MB-231 cells than in the control group, indicating that miR-340 silencing of MCU reduces cell migration and metastasis [58]. An interplay of $_{m}Ca^{2+}$ and miRNAs has been implicated in other pathological conditions. An example of this is the down-regulation of MCU by miR-1 in cardiac hypertrophy by interfering with protein translation. miR-1 is well characterized miRNA that plays a key role in cardiac development and stress conditions. Analysis of mice subjected to exercise induced-cardiac hypertrophy or transaortic constriction (TAC), a condition that corresponds to the initial, compensatory response to a hypertrophying stimulus with cardiomyocyte growth accompanied by no contractile failure demonstrated decreased miR-1 with concomitant increase in MCU levels. Thus, the initial cardiomyocyte adaptation to increased heart work is characterized by increased MCU levels that are specifically regulated by miR-1 [59]. Collectively, these findings suggest new modes of regulation of MCU under various physiological and pathological conditions.

Regulation of MCU by Anions and Nucleotides

The primary driving force for mitochondrial Ca^{2+} uptake is the highly negative proton gradient generated by the pumping of the H^{+} ions into the IMS. Ca^{2+} uptake into the mitochondria requires active anions like Pi, acetate, β-hydroxybutyrate, glutamate, bicarbonate that can provide an H^{+} source. Transport of active anions makes the matrix more negative, thus generating a pulling force for the uptake of cations. Early studies on isolated mitochondria postulated two modes of Ca^{2+} uptake: i) limited Ca^{2+} uptake in the absence of Pi and ii) massive uptake in the presence of Pi [60]. The phosphate facilitated Ca^{2+} uptake was explained by the formation of calcium-phosphate precipitates in the matrix to maintain the electrochemical gradient for Ca^{2+} uptake and also to balance the charge [61]. The major carrier of Pi into the matrix is through PiC encoded by the nuclear gene SLC25A3 with two isoforms PiC A and PiC B. PiC B is ubiquitously expressed while PiC A is specific to the skeletal muscle [5, 62–65]. PiC belongs to the super family of mitochondrial carriers characterized by six transmembrane domains with both the C and N-terminal domains in the

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IMS. Inhibitor stop assay revealed the flux of an estimate 50,000 Pi ions per second at 25°C with H^+ symport. The first functional studies on PiC were performed in yeast where genetic deletion of MIR1, yeast homologues of PiC1 failed to grow on non-fermentable carbon source [66]. Patients harboring frame-shift mutation (c.158–9A→G and c.215G→A) in the gene have been identified and present with hypertrophic cardiomyopathy, muscular hypotonia and lactic acidosis [66–68]. Assessment of skin fibroblasts from control and PiC mutation revealed a decrease in oxygen consumption capacity in the mutants compared to the control [69] Although the fundamental requirement of Pi for calcium uptake and ox-phos was long established, a direct involvement of Pi in Ca^{2+} uptake in vivo has not been shown. An attempt to answer this question was made in cardiac specific knock-out of slc25A3. 2 weeks post tamoxifen administration resulted in significant reduction in Pi with ~ 45% decrease in cardiac mitochondrial ATP while total cardiac tissue ATP levels remained unaltered with minimal cardiac phenotype. Additionally, MCU-mediated Ca^{2+} overload was reduced in cardiomyocytes deleted from PiC [70, 71]. Possible explanations for the limited phenotype are either decreased mitochondrial Ca^{2+} uptake due to the absence of Pi transport or compensation of Pi transport through other mitochondrial solute carriers.

${}_mCa^{2+}$ is known to generate ATP and vice-versa ATP is essential for ${}_mCa^{2+}$ uptake. It is known that ATP is required to maintain mitochondrial ion homeostasis which is key for MCU-mediated Ca^{2+} uptake [72]. The ATP-Mg solute carriers (SLC25A23, SLC25A24 and SLC25A25) transport adenine nucleotides to the matrix of the mitochondria in response to cytosolic Ca^{2+} [73–77]. Silencing of SLC25A23 exhibited a significant decrease in ${}_mCa^{2+}$ uptake and rate of ${}_mCa^{2+}$ with sustained ${}_cCa^{2+}$. In complement, SLC25A23 KD cells displayed reduced I_{MCU}. This decrease in ${}_mCa^{2+}$ uptake was not a result of decreased Ψ_m and SLC25A23 KD cells showed no changes in Ψ_m. SLC25A23 was shown to facilitate ${}_mCa^{2+}$ uptake and mitochondrial reactive oxygen species (mROS) production by directly interacting with MCU possibly through hydrophobic interactions [24]. One key factor by which SLC25A23 contributes to ${}_mCa^{2+}$ uptake could be by Ca^{2+}-activated of Pi flow that balances the net matrix charge.

Oxidative Regulation of MCU

Post translational modifications are key mechanisms utilized by cells to fine-tune and expand a proteins function. Two predominant forms of PTM are oxidation and phosphorylation and MCU has been shown to undergo both [78, 79]. MCU has three conserved cysteines containing free thiols in the NTD that get oxidized. Of the three conserved cysteines (C67, C97 and C191), Cys97 was susceptible to nucleophilic reaction. Oxidative stress causes S-glutathionylation of C97 and addition of glutathione to MCU remodels the NTD conformation promoting persistent MCU activity and increased rate of ${}_mCa^{2+}$ entry. Size exclusion chromatographic analysis of MCUC97A or S-glutathionated MCU revealed higher order oligomers or MCU super complex formation in the mutant. To further study the detailed assembly and regulation mechanisms upon oxidative stress photo-activated localization microscopy (PALM) was utilized. PALM imaging revealed well organized, clustered distribution of the channel in MCUC97A, in contrast with the randomly distributed MCU along the IMM under un-oxidative conditions without altering its interactions with the regulatory proteins. Thus, both biochemical and super resolution

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microscopy reveal the increased rate of mCa2+ uptake to be due to MCU super complex formation. It is intriguing that though MICU1, MCUR1, MCUb contain several conserved cysteines, the comprehensive biochemical gel shift assay revealed only MCU as the luminal mROS sensor. Thus, conditions of inflammation or I/R injury that promote mROS production in the matrix of the mitochondria tipping the GSH/GSSG balance resulting in mROS-mediated modification of MCU. S-glutathione conjugated MCU exhibits sustained channel activity leading to $_{m}Ca^{2+}$overload-mediated death [18].

A second PTM that MCU has been proposed to undergo is phosphorylation by Calmodulin kinase II (CaMKII) at S57 and S92 of the NTD of MCU [80]. It has been demonstrated that $_{m}Ca^{2+}$ overload during IR can be prevented by inhibiting phosphorylation of S92 in MCU by CaMKII. Electrophysiological studies on mitoplasts from WT and CaMKIIN (transgenic mice expressing CaMKII inhibitor) mice reveal reduced I_{MCU} from CaMKII heart mitochondria compared to the WT mitochondria. Furthermore, I_{MCU} reversed when constitutively active form of CaMKII was added to the pipette solution only in the presence of ATP. Absence of ATP or addition of catalytically incompetent CaMKII (K43M) failed to increase $I_{MCU,}$ indicating that catalytically active CaMKII was required to increase I_{MCU}. Structural analysis of MCU revealed that S92 is a conserved residue in the L2 loop of NTD that stabilizes by hydrogen bond formation with D119. Either phosphorylation of S92 by CaMKII or mutating S92 to alanine abrogates $_{m}Ca^{2+}$ uptake but does not destabilize the MCU complex and does not inhibit interaction with the regulatory molecules. S92A modulates channel activity by changing the conformation of L2-L4 loops such that S92 no longer forms hydrogen bond with D119 [17]. Although mutation analysis demonstrates the significant of S92, a direct phosphorylation assay by CaMKII needs to be investigated.

MCU in Ca^{2+} Overload and PTP Opening

Having established that Ca^{2+} that enters through MCU channel shapes cytosolic Ca^{2+} transients, a major question arises. What may be the implications of impaired $_{c}Ca^{2+}$ signaling? Sudden elevation of $_{c}Ca^{2+}$ due to sustained release from the stores as well as acidic endo-lysosomal compartments through two-pore channels (TPC1 and TPC2) upon nicotinic acid adenine dinucleotide phosphate (NAADP) agonist stimulation under conditions of ischemia-reperfusion (IR) injury results in persistent $_{m}Ca_{2+}$ uptake [81]. This sustained elevation of $_{m}Ca^{2+}$ disrupts the physiological Ca^{2+} cycling of influx and efflux leading to the activation of permeability transition pore (PTP) [45, 82, 83]. PTP is a voltage dependent, high conductance pore comprising of proteins spanning the OMM, IMS, IMM and matrix. Two known triggers of the pore are permissive amounts of matrix Ca^{2+} and ROS. Opening of the pore results in dissipation of the proton gradient, inhibition of the ETC and ATP hydrolysis, rapid influx of water and solutes leading to swelling of the mitochondria and eventually cell death by necrosis [84–86]. Hence, it was thought that preventing Ca^{2+} uptake by the mitochondria under conditions of Ca^{2+} overload would offer protection from PTP-mediated cell death. To test this hypothesis, mice genetically deleted for *Mcu* were generated. Mice were born in Mendelian ratio and developed without any gross phenotypic changes. A loss of $_{m}Ca^{2+}$ uptake was observed in various cell types thus confirming the global KO. Surprisingly, WT and $MCU^{-/-}$ MEFs subjected to various stressors that trigger apoptotic and necrotic pathways including ceramide, H_2O_2,

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doxorubicin, thapsigargin and tunicamycin failed to offer any protection from Ca^{2+}-overload induced death. In complement, $MCU^{-/-}$ animals subjected to IR injury exhibited unchanged post ischemic recovery as that of the control animals, although the in vitro indices of PTP opening were absent. Similar infarct size, magnitude of ischemic contracture and cell death was observed between the WT and $MCU^{-/-}$ mice following global IR injury [87]. In support, acute conditional deletion of MCU ($MCU^{fl/fl}$ x αMHC-MCM) in the heart exhibited no difference in basal NADH production and oxygen consumption capacity but a metabolic failure under acute stress. In contrast to the previous finding, $MCU^{fl/fl}$ x αMHC-MCM animals demonstrated protection from IR injury and PTP-mediated cell death [88]. The discrepancy in the studies has been attributed to acute vs chronic deletion of the channel where global germ line deletion of MCU animals do not display phenotypic changes possibly due to compensation by other mitochondrial solute carriers. Additionally, differences in the genetic backgrounds of the animals may be a contributing factor to the discrepancy in the two studies observed. Although both the findings report the absence of $_mCa^{2+}$ uptake, upstream cytosolic components were not assessed.

Role of MCU in Mitochondrial shape transition (MiST)

There is long standing evidence supporting the interdependence of mitochondrial function and its shape. Tubular long filamentous network of mitochondria are known to be actively respiring and channel ATP to different part of the cell [89–92]. On the other hand, short circular fragmented mitochondria are often associated with various pathological conditions [93]. The canonical opinion in the field is that mitochondria undergo changes in shape in response to insults such as fragmentation or Ca^{2+} overload mediated mitochondrial swelling. However, recent findings from two independent groups show a novel mechanism by which mitochondria transition from long filamentous to short circular [94, 95]. The authors term this phenomenon of mitochondrial transition from long spaghetti-like to short donut-like mitochondrial shape transition (MiST). They demonstrate that MiST is triggered by sustained elevation of $_cCa^{2+}$ but not $_mCa^{2+}$. MiST occurs in hepatocytes isolated from MCU liver specific knock out animal (MCU ^{HEP}) at the same rate as WT hepatocytes even though MCU-mediated Ca^{2+} overload was absent. MiST was shown to be temporally separate from PTP-mediated mitochondrial swelling, where HeLa cells exhibited a change in mitochondrial shape ~200 s before mitochondrial swelling observed as release of calcein from mitochondria. These data were further validated in genetic deletion of components of OMM ($Bax^{-/-}Bak^{-/-}$ MEFs) and PTP ($SPG7^{-/-}$ and $VDAC1/3^{-/-}$ MEFs). Ca^{2+}-induced MiST was then demonstrated to be independent of mitochondrial fragmentation. MiST was observed in multiple cells that were genetically, RNAi and pharmacologically inhibited of the mitochondrial fission machinery ($Drp1^{-/-}$, $MFF^{-/-}/Fis1^{-/-}$, Dyn2 KD, Mid49 KD and Mid51 KD MEFs). The molecular factor mediating MiST in response to $_cCa^{2+}$ changes was identified to be an OMM tethered EF hand containing atypical Rho GTPase, Miro1. Mutations in EF hand 1 of Miro1 that limit Ca^{2+} binding drastically limit Ca^{2+}-induced MiST. Miro1 senses rise in $_cCa^{2+}$ with its EF hands and depending on the $_cCa^{2+}$ transients (sustained elevation by Ca^{2+} stressor or transient rise by GPCR agonists) relays either an irreversible MiST or reversible MiST. The K_d for MiST was determined to be > 1 μM $_cCa^{2+}$. Miro1EF1-mediated MiST was revealed to be a pre-requisite for mitochondrial quality

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control. Long filamentous mitochondria need to transition to sterically small entities to be engulfed by the autophagosomes and inhibiting this change in shape halts autophagosome formation. This was confirmed in Miro1EF1 mutants that do not undergo MiST and thereby do not get engulfed by the autophagosome or degraded by the lysosome [95]. A second group report the same mitochondrial transition independent of mitochondrial fission and swelling to be necessary for mitochondrial DNA release during an inflammatory response. In an attempt to elucidate how mtDNA located in the matrix of the mitochondria releases into the cytosol and activates the cGAS STING pathway that causes cells to secrete type 1 interferon, the authors observed a dynamic change in mitochondrial phenotype from filamentous to globular upon treatment with Bcl2 inhibitor. Herniation of IMM releases mtDNA through OMM pores formed by Bax Bak oligomers [94]. It is possible that other triggers of MiST exist to function under varied pathological conditions and remain to be explored.

Perspectives

A large body of evidence on the molecular identity, stoichiometry, its regulatory proteins has accumulated since the discovery of the mitochondrial calcium uniporter. The generation of germline and tissue specific MCU KO animals permitted understanding the physiological significance of the channel. A lot remains to be explored on the role of MCU under pathological conditions.

Binding of Mg^{2+} to the MRAP region in the NTD of MCU has been shown to close MCU channel activity. Studies on supplementing Mg^{2+} to limit MCU channel activity under conditions of Ca^{2+} overload may be warranted. Studies for the first time have shown like most ion channels, MCU also undergoes oxidative modifications, based on this studying if MCU can serve as matrix ROS set point and understanding the molecular mechanisms of MCU oxidation in inflammatory diseases may open therapeutic avenues. Finally, genetic ablation of MCU did not offer protection from ${}_{m}Ca^{2+}$ overload-mediated death. A prospective reason is that MCU KO cells triggered MiST due to elevated Ca^{2+} leading to MiST-mediated cell death. Future studies on MiST mediated pathways need to be explored.

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Highlights

- The discovery of the molecular identity and structure of the N- terminal domain of mitochondrial calcium uniporter (MCU) has identified MCU to be the pore forming subunit.
- MCU is regulated on many levels from protein-protein interaction with its regulatory components (MCUb, MICUs, MCUR1, EMRE and SLC25A23), transcriptional regulation by CREB and micro RNAs, post translational modifications of oxidation and phosphorylation to divalent cations of Ca^{2+} and Mg^{2+}.
- Loss of mitochondrial Ca^{2+} uptake in the absence of MCU promotes elevated cytosolic Ca^{2+}-induced mitochondrial shape change (MiST)

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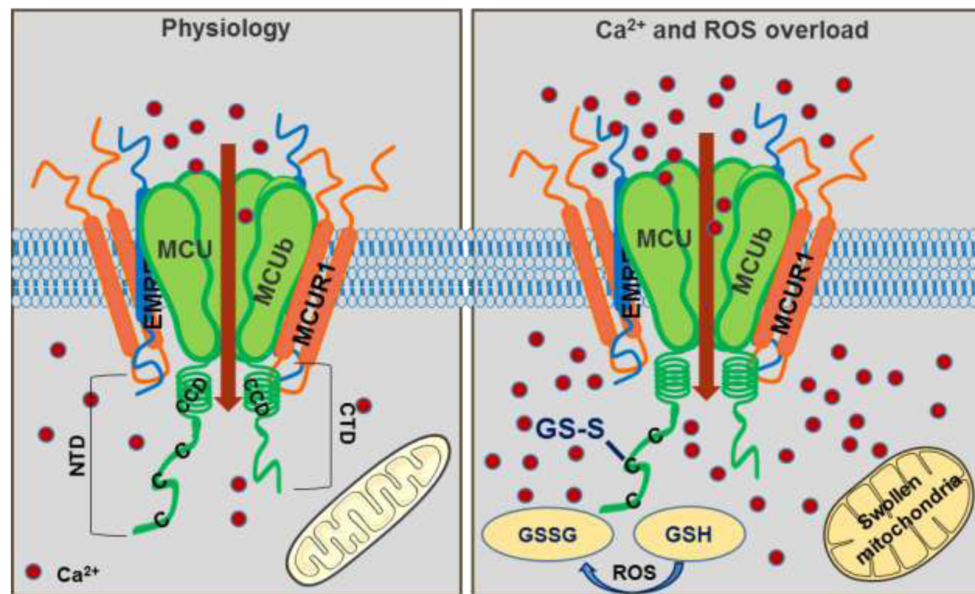

Figure 1.

MCU super complex in physiology and pathology.

Ca^{2+} enters the mitochondria through MCU channel. MCU, the pore-forming subunit is a pentamer with N and C terminal domains in the matrix. MCUR1 and EMRE are transmembrane proteins that interact with MCU and regulate $_mCa^{2+}$ uptake by the channel. Under conditions of oxidative stress, C97 at the NTD of MCU gets S-glutathionylated promoting MCU oligomerization and increasing $_mCa^{2+}$ uptake leading to Ca^{2+} overload and swelling of the mitochondria.

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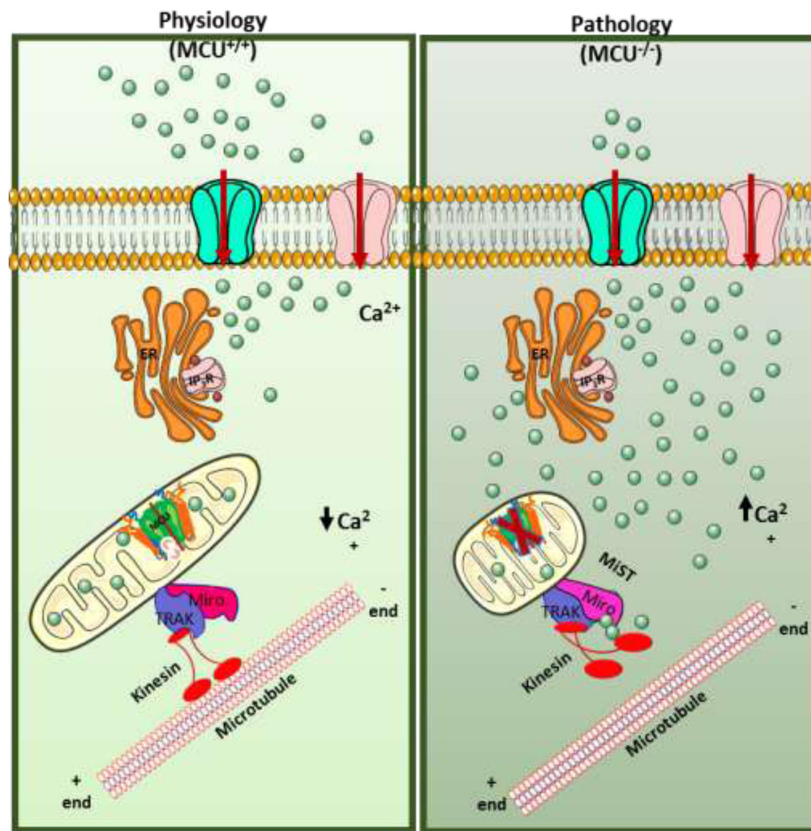

Figure 2.

Loss of MCU elicits elevated $[Ca^{2+}]_c$ – induced MiST.

Actively respiring filamentous mitochondria that are tethered to microtubules through interactions of Miro and kinesin, take up $_cCa^{2+}$ in response to agonist stimulation through the MCU channel for bioenergetic output. Loss of $_mCa^{2+}$ uptake in the absence of MCU causes elevated $_cCa^{2+}$. Sustained elevated $_cCa^{2+}$ binds the EF hand of Mior1 and releases the mitochondria from microtubules thus causing a change in mitochondrial shape.