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# Mitochondrial $Ca^{2+}$ Signaling

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## Abstract

Mitochondrial $Ca^{2+}$ regulation is crucial for bioenergetics and cellular signaling. The mechanisms controlling mitochondrial calcium homeostasis have been recently unraveled with the discovery of mitochondrial inner membrane proteins that regulate mitochondrial $Ca^{2+}$ uptake and extrusion. Mitochondrial $Ca^{2+}$ uptake depends on a large complex of proteins centered around the $Ca^{2+}$ channel protein, mitochondrial $Ca^{2+}$ uniporter (MCU) in close interactions with several regulatory subunits (MCUb, EMRE, MICU1, MICU2). Mitochondrial $Ca^{2+}$ extrusion is mainly mediated by the mitochondrial $Na^{+}/Ca^{2+}/Li^{+}$ exchanger (NCLX). Here, we review the major players of mitochondrial $Ca^{2+}$ homeostasis and their physiological functions.

### Keywords

$Ca^{2+}$ signaling; MCU; NCLX; Mitochondria associated membranes; Reactive oxygen species; Cell metabolism; physiology of mitochondrial $Ca^{2+}$

## Introduction

Mitochondria are vital cellular organelles which are required for adenosine triphosphate (ATP) production and are active participants in cellular $Ca^{2+}$ signaling. Long before the establishment of the endoplasmic reticulum (ER) as the major reservoir of intracellular $Ca^{2+}$ storage within eukaryotic cells, mitochondria were the first intracellular organelles associated with $Ca^{2+}$ handling and sequestering (for review: (Rizzuto, De Stéfani, Raffaello, & Mammucari, 2012)). The high $Ca^{2+}$ capacity of mitochondria is achieved mainly through $Ca^{2+}$ chelation by phosphates in mitochondrial matrix, forming amorphous $Ca_3(PO_4)_2$ precipitates (Lehninger, Carafoli, & Rossi, 1967). The formation of these precipitates is pH dependent and they are reversible into $Ca^{2+}$ and phosphate on collapse of mitochondrial potential by protonophores, which liberate $Ca^{2+}$, leading to its efflux from mitochondria into the cytosol (Chalmers & Nicholls, 2003; Zoccarato & Nicholls, 1982). Pioneering studies from the 1960s and 1970s showed that mitochondrial $Ca^{2+}$ uptake was dependent on the

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Conflict of Interest

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steep mitochondrial membrane potential ($\Delta\psi$ ~−180mV) and was inhibited by the drug Ruthenium Red or its analog Ruthenium 360 (Carafoli & Lehninger, 1971; DeLuca & Engstrom, 1961; Ying, Emerson, Clarke, & Sanadi, 1991). The mitochondrial electron transport chain (ETC) transfers protons across the inner mitochondrial membrane (IMM) into the intermembrane space generating a huge electrochemical proton gradient which drives the synthesis of ATP. This mitochondrial membrane potential also creates a large driving force for cytosolic $Ca^{2+}$ uptake by mitochondria (Rizzuto et al, 2012; Stock, Leslie, & Walker, 1999). Changes in mitochondrial $Ca^{2+}$ concentrations, which can apparently range between 0.1μM to the sub-millimolar (mM) range, are important for coupling receptor activity to bioenergetics and for regulating downstream functions such as exocytosis by controlling the amount of $Ca^{2+}$ available near secretory vesicles (Montera et al., 2000). Indeed, at least three dehydrogenases of the tricarboxylic acid (TCA) cycle are regulated by mitochondrial matrix $Ca^{2+}$ (Hansford, 1994; McCormack & Denton, 1979; McCormack, Halestrap, & Denton, 1990). The cytosolic $Ca^{2+}$ signal typically initiated by activation of plasma membrane receptors to specific agonists propagates into mitochondria, enhancing its respiratory rate, $H^+$ extrusion and ATP synthesis, thus effectively coupling agonist stimulation to mitochondrial metabolism and ATP production. Hence, the strength and pattern of receptor-generated $Ca^{2+}$ signals are decoded by mitochondria to match the energetic demands of the cell.

Despite the huge driving force for $Ca^{2+}$ across the IMM, mitochondria have very low affinity for $Ca^{2+}$ uptake. As such, $Ca^{2+}$ transfer into mitochondria occurs at highly specialized regions of close contacts between mitochondria and ER called mitochondria associated membranes (MAMs). MAMs are the site where phosphatidyl serine (PS) synthase Eli are enriched, and where the newly synthesized PS in the ER gains the mitochondria (Mesmin, 2016). Enzymes required for cholesterol and ceramide synthesis also reside in MAMs. In addition to lipid transfer, MAMs are required for reactive oxygen species (ROS) transfer between ER and mitochondria (Booth, Enyedi, Geiszt, Vârnai, & Hajnoczky, 2016). Metabolic reprograming of $CD8^+$ T cells was shown to be dependent on mTORC2-AKT-GSK3β signaling complex at the MAMs which promotes hexokinase-I binding to voltage-dependent anion channel (VDAC) and facilitate metabolite flux into mitochondria required for rapid production of cytokines (Bantug et al, 2018). Recent studies indicate that MAMs can regulate mitophagy in response to cellular and environmental signals (Gelmetti et al, 2017; Gomez-Suaga et al, 2017; Liu et al, 2012; W. Wu et al., 2016). MAMs are also critical for the unfolded protein response (UPR) and autophagy signaling, insulin signaling, and function as sites for glucose sensing (for review (Rieusset,2018). Miscommunication at MAMs or disruption of MAMs could result in various metabolic diseases such as obesity (Theurey et al., 2016; Tubbs et al., 2018), type 2 diabetes (Thivolet, Vial, Cassel, Rieusset, & Madec, 2017) and leptin resistance (Schneeberger et al., 2013).

Within the MAMs, $Ca^{2+}$ release into the cytosol through the ER $Ca^{2+}$ release channel inositol-1,4,5-trisphosphate receptor ($IP_3R$) creates a cytosolic $Ca^{2+}$ nanodomain that is sufficiently concentrated, within the μM range, to be sensed by adjacent mitochondria (Mannella, Buttle, Rath, & Marko, 1998; Rizzuto et al., 1998; Szabadkai et al., 2006) (Fig. 1). In addition to decoding $Ca^{2+}$ signals to match the energetic demands of the cell, the ability of mitochondria to either shuttle $Ca^{2+}$ between different areas of the cell or to buffer

cytosolic $Ca^{2+}$ through coordinated $Ca^{2+}$ uptake and extrusion allows it to shape cytosolic $Ca^{2+}$ signaling (Fig. 1). By virtue of their mobile nature, mitochondria can buffer $Ca^{2+}$ at the mouth of plasma membrane voltage-gated $Ca^{2+}$channels in excitable cells, thus reducing local cytosolic $Ca^{2+}$ concentrations and exocytosis (Montera et al., 2000). Mitochondria can also buffer $Ca^{2+}$ at the vicinity of store-operated $Ca^{2+}$ entry (SOCE) channels, which are encoded by ORAI proteins and regulated by the $Ca^{2+}$ sensors, stromal interaction molecule (STIM) (for review (Trebak & Putney, 2017)) (Fig. 1). In this case, the consequence is relief of $Ca^{2+}$-dependent inactivation of SOCE and increased $Ca^{2+}$ entry (Hoth, Button, & Lewis, 2000). Recent evidence showed that knockdown of the newly discovered mitochondrial $Ca^{2+}$ uniporter protein (MCU; discussed in detail below) in HeLa cells caused a decrease in STIM1 oligomerization and inhibited SOCE in response to Phospholipase C-coupled agonists, suggesting that mitochondrial $Ca^{2+}$ uptake prevents inactivation of SOCE (Deak et al, 2014). Through their specific cytosolic organization, mitochondria can form a buffering barrier between the apical and basal membranes of polarized pancreatic acinar cells. This subcellular organization effectively divides the cell into two distinct cytosolic compartments with distinct $Ca^{2+}$ signatures (Park, Ashby, Erdemli, Petersen, & Tepikin, 2001) Similarly at the immunological synapse (IS) between T helper cells and antigen presenting cells, mitochondrial $Ca^{2+}$ uptake prevents SOCE inactivation and maintains a sustained $Ca^{2+}$ influx, supporting nuclear factor of activated T-cells (NFAT) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κ B) mediated T cell activation (A. Quintana et al, 2007; Ariel Quintana et al, 2011). In some instances, subplasmalemmal mitochondria can take up $Ca^{2+}$ entering through SOCE and extrude this $Ca^{2+}$ near the Sarco/Endoplasmic Reticulum $Ca^{2+}$ ATPase (SERCA) pumps at ER sites distant from the plasma membrane, thus contributing to refilling of ER stores after their depletion by agonist stimulation (Arnaudeau, Kelley, Walsh, & Demaurex, 2001).

The mechanisms of $Ca^{2+}$ homeostasis in mitochondria have been studied extensively in the past few years, and several molecular players involved in mitochondrial $Ca^{2+}$ uptake and extrusion have been identified. To reach the mitochondrial matrix, $Ca^{2+}$ must cross both outer and inner mitochondrial membranes. The outer mitochondrial membrane (OMM) is permeable to molecules smaller than 5kDa (Madesh & Hajnoczky, 2001; Rapizzi et al, 2002), thus allowing $Ca^{2+}$ to easily diffuse across the OMM. The OMM permeability is mostly the result of abundant expression of VDAC. However, the IMM ion permeability is more stringent, and $Ca^{2+}$ uptake into the mitochondrial matrix is mediated by a highly $Ca^{2+}$ selective channel called the mitochondrial Ca uniporter (MCU). Mitochondrial Ca extrusion is mediated by a Na /Ca exchanger-like protein termed $Na^+/Ca^{2+}/Li^+$ exchanger (NCLX); its name is owed to the fact that unlike members of the plasma membrane $Na^+/Ca^{2+}$ exchangers (NCX), NCLX can function when $Na^+$ is substituted with $Li^+$. Interestingly, a single point mutation of threonine 103 to valine in plasma membrane NCX1 resulted in significant activation of $Li^+/Ca^{2+}$ exchange (Doering et al., 1998). While threonine 103 is conserved in NCX, the $Na^+/Ca^{2+}/K^+$ exchanger (NCKX), and NCLX, only NCLX possesses a valine preceding threonine 103, which could underlie the $Li^+/Ca^{2+}$ exchange activity of NCLX. Nevertheless, it is not yet clear which residues are responsible for the $Li^+/Ca^{2+}$ exchange activity of NCLX. In some instances, the rate of mitochondrial $Ca^{2+}$ extrusion and uptake is

maintained by a $2H^+/Ca^{2+}$ exchanger likely encoded by Letm1(Jiang, Zhao, & Clapham, 2009; Mingn Feng Tsai, Jiang, Zhao, Clapham, & Miller, 2014).

## The Mitochondrial $Ca^{2+}$ Uniporter (MCU)

The mitochondrial $Ca^{2+}$ uniporter (MCU) is present in almost all mammalian tissues, and its activity has been known over 50 years before the discovery of its molecular identity (for review (De Stefani *et al.,* 2016)). In the early 1990, Rizzuto et al. targeted recombinant aequorin to mitochondria and demonstrated that agonist-activated increase in cytosolic $Ca^{2+}$ is rapidly coupled to increases in mitochondrial $Ca^{2+}$ (Rizzuto, Simpson, Brini, & Pozzan, 1992). These authors subsequently showed efficient exposure of mitochondria to $Ca^{2+}$ signals emanating from $IP_3Rs$ owing to the existence of functional sites of close ER-mitochondria contacts (Rizzuto et al., 1998). MCU currents were first recorded in 2004 using patch clamp electrophysiology on isolated mitoplasts which revealed the existence of an inwardly rectifying, highly $Ca^{2+}$ selective channel in the IMM (Kirichok, Krapivinsky, & Clapham, 2004). The first protein of the mitochondrial $Ca^{2+}$ uniporter complex was identified in 2010 and named MICU1 (mitochondrial calcium uptake 1) (Perocchi et al., 2010). Shortly after, two studies from two independent laboratories identified MCU as a 40 kDa protein (known as CCDC109A) that is present in the IMM and is essential for $Ca^{2+}$ uptake into mitochondria (Baughman et al., 2011; De Stefani, Raffaello, Teardo, Szabô, & Rizzuto, 2011). MCU has two transmembrane domains, which are connected by a short loop with both the N- and C-termini located in the mitochondrial matrix (Fig. 2). MCU is present in nearly all vertebrate cells and is critical for mitochondrial $Ca^{2+}$ homeostasis, ATP synthesis and mitochondrial metabolism (Rizzuto et al., 2012). Downregulation of MCU resulted in reduced mitochondrial $Ca^{2+}$ and increased cytosolic $Ca^{2+}$, while overexpression of MCU caused mitochondrial $Ca^{2+}$ accumulation with a reduction in cytosolic $Ca^{2+}$. Reconstitution of MCU proteins in lipid bilayers resulted in a $Ca^{2+}$ current which was blocked by Ruthenium red (Rizzuto et al., 2012). These findings were subsequently confirmed with whole-mitoplast voltage-clamp recordings showing that knockdown of MCU decreased MCU $Ca^{2+}$ currents whereas MCU overexpression led to increase in current (Chaudhuri, Sancak, Mootha, & Clapham, 2013). In the same study Chaudhuri et al, showed MCU current inhibition by ruthenium red and loss of ruthenium red-mediated inhibition in a form of MCU where serine 259 was mutated to alanine (S259A), consistent with the idea that MCU is the pore forming subunit of the uniporter complex (Chaudhuri et al., 2013). A more recent study in *Caenorhabditis elegans* showed that an analog of ruthenium red, Ruthenium 360 blocks MCU by direct interaction with the Asp240-X-X-Glu243 (DXXE) motif (D261 and E264 in human MCU). They proposed that Ruthenium 360 blocks MCU by binding to Asp240 but not to Glu243 (Cao, Wang, Cui, Su, & Chou, 2017). The same group proposed a pore domain structure of MCU from *Caenorhabditis elegans* based on nuclear magnetic resonance and electron microscopy. These authors suggested that MCU forms a pentameric assembly *in vitro* and that the DXXE motif forms the pore gate and selectivity filter (Oxenoid et al., 2016). Both $Ca^{2+}$ selectivity and conductance of MCU were shown to depend on this conserved DXXE sequence. Mutation of D or E within the conserved DXXE sequence of human MCU resulted in complete loss of channel activity (Baughman et al., 2011). While these findings support the idea that MCU is the pore forming unit of the MCU

complex, the idea that functional MCU channels are organized into pentameric structure remain a matter of debate. MCU was proposed to serve as a mitochondrial matrix sensor of reactive oxygen species (ROS). Recently, Dong et al. identified a conserved cysteine (Cys-97) in the N-terminus of MCU located within the mitochondrial matrix. Oxidation of this cysteine promoted oligomerization of MCU and enhanced MCU channel activity leading to enhanced mitochondrial ROS, mitochondrial $Ca^{2+}$ overload, and cell death (Dong et al., 2017). The mechanisms of transcriptional and posttranslational regulation of the MCU protein complex remain scarce. One report suggested that the expression of MCU in chicken DT40 B lymphocytes is dependent on $Ca^{2+}$ signals generated through the $IP_3$/SOCE signaling nexus and controlled by the $Ca^{2+}$-dependent transcription factor, cyclic adenosine monophosphate response element-binding protein (CREB); CREB was shown to interact directly with the MCU promoter to stimulate gene expression. Mitochondrial $Ca^{2+}$ uptake and MCU expression were reduced in DT40 cells lacking either $IP_3$ receptors, STIM1, or ORAI1 (Shanmughapriya et al., 2015), suggesting that SOCE is a potential regulator of MCU

## Regulators of MCU.

Since the discovery of MCU as the pore-forming unit, it is now appreciated that MCU functions as a large multi-molecular complex consisting of MCU proteins, mitochondrial calcium uptake proteins (MICU1, MICU2, and MICU3), and a 10 KDa protein called essential MCU regulator (EMRE); additional MCU partners include MCUR1, SLC25A23, and MCUb (N. E. Hoffman et al., 2014; Mallilankaraman, Cârdenas, et al., 2012; Mallilankaraman, Doonan, et al., 2012; Plovanich et al., 2013; Sancak et al., 2013).

## Mitochondrial $Ca^{2+}$ uptake proteins (MICU1, MICU2, and MICU3)

Mitochondrial $Ca^{2+}$ uptake (MICU1) is a critical regulator of MCU (Perocchi et al., 2010), which appears to have evolved alongside MCU. The discovery of MICU1, which preceded that of MCU, emerged from an RNAi screen on mitochondria-located proteins (Perocchi et al., 2010). The compendium of approximately 1,100 mitochondrial proteins was established few years earlier and termed mitocarta (Pagliarini et al., 2008). Perocchi et al utilized RNAi strategy and identified that MICU1 is required for mitochondrial Ca uptake. Downregulation of MICU1 resulted in reduced mitochondrial $Ca^{2+}$ entry without significantly affecting mitochondrial respiration or membrane potential (Perocchi et al., 2010). They proposed that MICU1 resides in the IMM and has two EF hands which are essential for its function (Perocchi et al., 2010). While most investigators agree on the location of MICU1 in the intermembrane space, one report of time lapse protein flux assay with selective permeabilization of either the OMM or both the OMM and IMM of the mitochondria showed that MICU1 is released only when both membranes are permeabilized, suggesting that MICU1 is located in the mitochondrial matrix (Nicholas E Hoffman et al., 2013). Later, two homologs of MICU1 were identified in humans, MICU2 and MICU3 (Plovanich et al., 2013). Both MICU2 and MICU3 have N-terminal mitochondrial targeting sequence. A proteomic study and microarray analysis showed that MICU1, MICU2 and MICU3 are differentially expressed in different tissues. MICU2 is strongly expressed in visceral organs while MICU3 is expressed in skeletal muscle and neuronal tissues. Similar to MICU1, both

MICU2 and MICU3 have EF hands and since both MICU2 and MICU3 are paralogs of MICU1 (Plovanich et al., 2013), they likely have redundant roles depending on the tissue types where they are expressed.

MICU1 can form a functional heterodimer with MICU2 or MICU3, while MICU2 and MICU3 do not seem to heterodimerize (Patron, Granatiero, Espino, Rizzuto, & De Stefani, 2018).The original role of MICU1 as a positive regulator of $Ca^{2+}$ uptake into mitochondria by MCU stems from the fact that when MICU1 is deleted MCU expression is also reduced. The prevailing model at this juncture is that MICU proteins are gate keepers for mitochondrial $Ca^{2+}$ uptake. The EF hands allow MICU proteins to sense $Ca^{2+}$ at the MCU channel gate and regulate its opening depending on the $Ca^{2+}$ concentration in the intermembrane space (Fig. 2). In the presence of low cytosolic $Ca^{2+}$ concentrations, MICU keeps MCU in a closed state preventing $Ca^{2+}$ flow into the mitochondrial matrix. Increases in $Ca^{2+}$ concentrations are detected by the EF hands of MICUs, which results in the opening of MCU (Csordâs et al., 2013; Nicholas E. Hoffman et al., 2013; Mallilankaraman, Doonan, et al., 2012; Troilo et al., 2014; Ming Feng Tsai et al., 2016). A recent study showed that skeletal muscle possesses a splice variant of MICU1 termed MICU1.1, which binds to $Ca^{2+}$ more efficiently than MICU1. MICU1.1/MICU2 heterodimers activate MCU at lower $Ca^{2+}$ concentrations than MICU1/MICU2 heterodimers (Vecellio Reane et al., 2016). Petrungaro et al. showed an important role for the mitochondrial intermembrane space (IMS) resident oxidoreductase Mia40 in MCU function. Mia40 links MICU1 and MICU2 as a heterodimer through a disulfide covalent bond, which is critical for the gate keeping function of MICU1/MICU2 heterodimer. The divalent bond allows binding of MICU1/MICU2 to MCU at low levels of $Ca^{2+}$ and heterodimer dissociation from MCU at high $Ca^{2+}$ concentrations. Without this MICU1/MICU2 covalent bond, MICUs gatekeeping capability is jeopardized and agonist-activated mitochondrial $Ca^{2+}$ uptake is enhanced (Petrungaro et al., 2015). These studies show that the MCU channel complex is a highly regulated mitochondrial $Ca^{2+}$ uptake pathway, and that its mechanisms of regulation vary in a tissue-dependent manner. The different $Ca^{2+}$ binding capacity of different isoforms of MICUs can serve as a threshold-setting for mitochondrial $Ca^{2+}$ and bioenergetics in different tissues and cell types, expressing different isoforms and different levels of MICU isoforms.

Studies showed that protein expression of MICU1 and MICU2, are dependent upon each other (Patron et al., 2014). Interestingly, protein knockdown of either MICU1 or MICU2 or both also resulted in reduced MCU protein levels (Plovanich et al., 2013). Double knockdown of MICU1 and MICU2 has an additive effect on impaired mitochondrial $Ca^{2+}$ uptake. Differential expression and relative abundance of MICU1, MICU2 and MICU3 in different cell types likely serve to tailor mitochondrial $Ca^{2+}$ uptake and kinetics to the demands of a specific cell type. Two studies proposed that MICU1 can act as an MCU gatekeeper (Csordâs et al., 2013; Mallilankaraman, Doonan, et al., 2012). These studies showed that deletion of MICU1 causes mitochondrial $Ca^{2+}$ accumulation at low basal levels of cytosolic $Ca^{2+}$, suggesting that MICU1 has a dual role in both activating $Ca^{2+}$ uptake at high cytosolic $Ca^{2+}$ concentrations and in inhibiting MCU at low resting cytosolic $Ca^{2+}$ concentrations. A follow up study by Patron et al. proposed that MICU2 is the genuine gatekeeper. They showed that MICU1 and MICU2 within a functional heterodimer have opposing effects on MCU allowing for fine tuning of MCU current activity both in a lipid

bilayer reconstitution system and in intact cells. They proposed that MICU1 stimulates MCU activity at higher $Ca^{2+}$ concentrations, whereas at lower $Ca^{2+}$ concentrations MICU2 inhibits MCU activity (Patron et al., 2014). This suggested that at low $Ca^{2+}$ concentrations the inhibitory effect of MICU2 on MCU is dominant, but when cytosolic $Ca^{2+}$ concentrations increase, MICU1 stimulatory effect permits rapid mitochondrial uptake of cytosolic $Ca^{2+}$ by MCU. Patron et al. showed that when MICU1 expression is eliminated, MICU2 expression is also abrogated and mitochondrial $Ca^{2+}$ gatekeeping ability is impaired, thus in agreement with previous studies (Patron et al., 2014). Kamer et al reported that both MICU1 and MICU2 have high affinities for $Ca^{2+}$, with Kd of recombinant proteins to $Ca^{2+}$ around 300 nM for MICU1 and 620 nM for MICU2. MICU1 and MICU2 form heterodimers *in vitro* in both $Ca^{2+}$-free and $Ca^{2+}$-bound conditions. $Ca^{2+}$ binding to the EF hands of MICU1/MICU2 heterodimers occurs in a cooperative fashion with a Hill coefficient of ~2.1. Under $Ca^{2+}$-free conditions, MICU1/MICU2 heterodimers inhibit MCU while cooperative binding of $Ca^{2+}$ to MICU1/MICU2 heterodimers, at around 620 nM, stabilizes the interaction and induces a conformational change, which disinhibits MCU and allows $Ca^{2+}$ uptake by mitochondria (Kamer, Grabarek, & Mootha, 2017). According to Kamer et al, MICU1 alone (in the absence of MICU2) can function as a gatekeeper to inhibit MCU at low $Ca^{2+}$ concentrations (below ~350 nM). The absence of both MICU1 and MICU2 results in a lower threshold of MCU inhibition (~200 nM). Whether MICU3 is the functional equivalent of MICU2 in different cell types such as neurons and can function as a heterodimer with MICU1 to regulate the $Ca^{2+}$ threshold of MCU activity remains to be determined. A recent study showed that MICU3 can form a heterodimer with MICU1 through a disulfide bond and works as a positive regulator of MCU-mediated mitochondrial $Ca^{2+}$ uptake in primary cortical neurons (Patron et al., 2018).

## Essential MCU regulator (EMRE)

EMRE was discovered through a quantitative mass spectroscopy analysis as a component of the MCU complex. EMRE is a small 10kDa protein with a single transmembrane domain which is present in the inner mitochondrial membrane (Sancak *et al.,* 2013). EMRE is present in metazoans and absent in plants and protozoa. Downregulation or deletion of EMRE resulted in impaired mitochondrial $Ca^{2+}$ uptake despite normal expression of the MCU protein and its oligomerization. Further overexpression of MCU in EMRE knockout cells failed to rescue mitochondrial $Ca^{2+}$ uptake. Interactions between MCU and MICU1/MICU2 require EMRE leading to the proposal that EMRE is essential for MCU current activity through its ability to connect MCU channels to the calcium-sensing proteins MICU1 and MICU2 (Sancak *et al.,* 2013). Interestingly, abrogation of MCU also resulted in significant reduction in EMRE protein levels with no discernible change in mRNA levels, suggesting that without MCU, EMRE proteins become destabilized and are likely degraded (Sancak *et al.,* 2013). This is similar to the dependence of MCU expression on the presence of MICU1 or that of MICU2 on the presence of MICU1(Patron et al., 2014).

Using protease protection assays, Vais et al. proposed that EMRE C-terminus is located in the mitochondrial matrix and serves as a sensor of matrix $Ca^{2+}$, which contributes to MCUgatekeeping activity (Vais et al., 2016). The authors proposed that regulation of MCU by EMRE requires both cytosolic $Ca^{2+}$ and the presence of MICU1 and MICU2 attached to

the MCU complex from the intermembrane side, suggesting that MCU activity is regulated by both cytosolic $Ca^{2+}$ through MICU1/MICU2 and by matrix $Ca^{2+}$ through EMRE. Deletion of the C- terminus of EMRE or neutralization of seven acidic residues within this C-terminus abrogated MCU current regulation by matrix $Ca^{2+}$ without altering the expression, localization or interaction between different proteins of the MCU complex (MCU, MICU1, MICU2, EMRE) (Vais et al., 2016). However, shortly after the publication of this report, two independent studies challenged the findings of Vais et al. (Ming Feng Tsai et al., 2016; Yamamoto et al., 2016). Yamamoto et al. used a yeast expression system of Flag-tagged EMRE either at the N- or C- terminus and concluded that the topology of EMRE consists of the N-terminus (and not the C- terminus as reported by Vais et al) being located in the mitochondrial matrix side (Yamamoto et al., 2016). Tsai et al. used two independent strategies consisting of cysteine modification and mass-tagging and the expression of MCU-EMRE fusion proteins and reached the same conclusion as Yamamoto et al, that EMRE N-terminus is located in the mitochondrial matrix while its C-terminus is in the intermembrane space (Ming Feng Tsai et al., 2016). In the study by Tsai et al. the authors showed that the N-terminus of EMRE physically interacts with MCU while the C terminus interacts with MICU1. This led the authors to propose a model where EMRE has a dual role: 1) EMRE binds to MCU and acts as an essential component of the MCU pore; and 2) EMRE serves as a tether for the $Ca^{2+}$-sensing MICU proteins, which serve as gatekeepers to the MCU channel. According to this model, EMRE transduces MICU1/MICU2 gatekeeping function to ensure that MCU is active only when cytosolic $Ca^{2+}$ levels are high, preventing mitochondrial $Ca^{2+}$ overloaded and cell death (Ming Feng Tsai et al., 2016). However, it is not clear that how interactions between EMRE and MCU or MICU1/MICU2 alter the gating of MCU. Two separate studies showed that the mitochondrial m-AAA protease, an IMM resident protein whose mutations are associated with neurodegenerative and neuromuscular disease, degrades non-assembled EMRE proteins and thus regulates MCU activity. Loss of function of m-AAA protease disrupts the stoichiometry of the MCU complex, leading to increased number of overactive MCU-EMRE channels lacking gatekeeping MICU subunits. The loss of m-AAA protease resulted in mitochondrial $Ca^{2+}$ overload and neurnal death (König et al., 2016; C.-W. Tsai et al., 2017).

## MCU Regulator 1 (MCUR1)

Mitochondrial $Ca^{2+}$ regulator 1 (MCUR1 or CCDC90A) was reported as a member of the MCU complex and was proposed to regulate $Ca^{2+}$ entry through MCU (Mallilankaraman, Cârdenas, et al., 2012). Similar to MCU, MCUR1 is ubiquitously expressed across mammalian tissues. MCUR1 has two transmembrane domains with both N- and C-termini facing the inter-membrane space (Fig. 2). The precise role of MCUR1 in mitochondrial $Ca^{2+}$ uptake and whether MCUR1 directly interacts with the MCU complex are still under debate. Downregulation of MCUR1 in HEK293T, HeLa or human primary fibroblasts reduced mitochondrial $Ca^{2+}$ uptake (Mallilankaraman, Cârdenas, et al., 2012). However, a study from Paupe et al. suggested that MCUR1 does not directly regulate MCU and that reduced MCU activity upon MCUR1 knockdown or deletion is rather due to decreased mitochondrial membrane potential. Instead, they proposed that MCUR1 is important for cytochrome c oxidase (COX) assembly (Paupe, Prudent, Dassa, Rendon, & Shoubridge, 2015). In response

to Paupe at al., Vais et al. Provided direct MCU current measurements on isolated mitoplasts. Under these conditions where membrane potential across the IMM is controlled through the recording electrode, Vais et al. showed that knockdown of MCUR1 reduced MCU inward currents (Vais et al., 2015). Tomar et al. used pulldowns of overexpressed proteins in COS7 cells and bimolecular fluorescence complementation assays and suggested that MCUR1 functions as a scaffold protein for the MCU complex through binding to both MCU and EMRE via conserved coiled-coil domains identified in both MCU and MCUR1 (Tomar et al., 2016). Chaudhuri et al. proposed that MCUR1 confers mitochondrial permeability transition induced by $Ca^{2+}$ while MCUR1 abrogation increased the $Ca^{2+}$ threshold for initiation of permeability transition, thus increasing resistance to $Ca^{2+}$ overload and enhancing cell survival (Chaudhuri, Artiga, Abiria, & Clapham, 2016). In a more recent study, Xing et al reported that MCUR1 expression is commonly increased in human hepatocellular carcinomas. Enhanced MCUR1 expression increases MCU-mediated mitochondrial $Ca^{2+}$ uptake, enhances cell proliferation, and inhibits apoptosis (Xing et al., 2017). Enhanced expression of MCUR1 might confer a proliferative and survival advantage through enhanced mitochondrial $Ca^{2+}$ uptake and bioenergetics. However, it is also conceivable that excessive increases in MCUR1 levels might drive mitochondrial permeability transition and cell death.

## SLC25A23

Solute carrier family 25 member 23 (SLC25A23) is part of a family of mitochondrial proteins containing an N-terminal regulatory domain, four EF-hands, a linker domain, and C-terminal with transporter domain. SLC25A23 works as ATP-Mg/Pi exchanger, it imports adenine nucleotides into the mitochondrial matrix and effluxes inorganic phosphate. Interestingly this function of SLC24A23 is $Ca^{2+}$ dependent, as the $Ca^{2+}$ concentration increases in inner mitochondrial space, it binds with EF hand of SLC25A23 and activates transport of adenine nucleotides (Fiermonte et al., 2004; Harborne, King, Crichton, & Kunji, 2017).. In neurons, SLC25A23 prevents N-Methyl-D-aspartic acid (NMDA) dependent neuronal excitotoxicity (Rueda et al., 2015). SLC25A23 deletion caused a reduction in oxidative phosphorylation and mutation in SLC25A23 resulted in early age bone dysplasia of the skull and fingers (Amigo et al., 2013; Aprille, 1988; Tewari, Dash, Beard, & Bazil, 2012; Writzl et al., 2017). SLC25A23 has also been proposed to regulate mitochondrial $Ca^{2+}$ uptake through the MCU complex. Downregulation of SLC25A23 resulted in reduced mitochondrial $Ca^{2+}$ uptake without affecting either $Ca^{2+}$ efflux or mitochondrial membrane potential. Hoffman et al. proposed that SLC25A23 interacts with MICU1 and MCU and that expression of EF-hand mutants of SLC25A23 has a dominant-negative effect on mitochondrial $Ca^{2+}$ uptake. In the same report, SLC25A23 knockdown also decreased basal mitochondrial ROS and reduced cell death (N. E. Hoffman et al., 2014). Further studies are required to uncover the exact mechanisms by which SLC25A23 regulates mitochondrial $Ca^{2+}$ uptake.

## MCUb

MCU regulatory subunit b (MCUb), also known as CCDC109B, is a paralogue of MCU (~330aa long protein with 50% sequence identity with MCU) which was proposed to

function as a part of the MCU complex (Plovanich et al., 2013; Raffaello et al., 2013). Similar to MCU, MCUb has two predicted transmembrane domains. Unlike MCU, when MCUb is reconstituted in lip bilayers it does not generate functional $Ca^{2+}$ channels (Raffaello et al., 2013). Thus, it has been proposed that MCUb works as a negative regulator of MCU (Plovanich et al., 2013; Raffaello et al., 2013). MCUb physically interacts with other proteins of the MCU complex and seems to negatively regulate MCU channel activity (Fig. 2). Insertion of MCUb in the MCU complex has a dominant-negative effect manifested by inhibition of mitochondrial $Ca^{2+}$ uptake in response to receptor stimulation. Further, reconstitution of MCUb with MCU in lipid bilayers largely reduced the probability of observing channel activity (Raffaello et al, 2013). The mechanisms by which MCUb regulates the MCU complex and MCU channel activity remain unknown.

## Physiological role of MCU

Mitochondrial dehydrogenases such as pyruvate dehydrogenase, a-ketoglutarate dehydrogenase, and isocitrate dehydrogenase are regulated by $Ca^{2+}$. $Ca^{2+}$ activates a-ketoglutarate dehydrogenase and isocitrate dehydrogenase through direct binding. However, $Ca^{2+}$-mediated activation of pyruvate dehydrogenase is indirect and mediated through a $Ca^{2+}$-dependent phosphatase which dephosphorylates its catalytic subunit (Hansford, 1994; McCormack & Denton, 1979; McCormack et al, 1990). Activated mitochondrial dehydrogenases increase NADH level leading to increased electron supply to the ETC which increases ATP generation. Thus, mitochondrial $Ca^{2+}$ uptake directly correlates with ATP production and a reduction in mitochondrial $Ca^{2+}$ would inhibit ATP production. Since the MCU complex is a major regulator of mitochondrial $Ca^{2+}$ uptake, MCU is expected to play a major role in controlling mitochondrial function. It has been shown in a myriad of organisms including plants, that the MCU complex plays a critical role in mitochondrial $Ca^{2+}$ homeostasis by regulating $Ca^{2+}$ uptake into the mitochondria (Teardo et al, 2017). MCU complex also regulates pollen tube germination and growth in Arabidopsis (Selles, Michaud, Xiong, Leblanc, & Ingouff, 2018). In trypanosomes, MCU governs bioenergetics, autophagy, and cell death (Huang, Vercesi, & Docampo, 2013). MCU is also required in the function of mushroom body neurons of *Drosophila* in olfactory memory formation (Drago & Davis, 2016). Accordingly, deletion of MCU in cultured cells causes reduced oxidative phosphorylation, reduced ATP generation and increased autophagy (Rizzuto et al, 2012). Remarkably, MCU global knockout mice generated using gene trap are viable, although mitochondria isolated from their skeletal muscle and heart lack the ability to rapidly uptake $Ca^{2+}$. Although the number, morphology and basal metabolism of purified mitochondria from MCU knockout mice are unaffected, these mice showed reduced ability to perform energy-intensive work (Pan et al, 2013). Skeletal muscle derived from MCU knockout mice showed reduced activity of pyruvate dehydrogenase, and this coincided with muscle hypertrophy of these mice (Mammucari et al, 2015; Pan et al, 2013). A recent study showed that muscle exercise at old age results in improved muscle function and structure which correlated with increased MCU expression (Zampieri et al, 2016). MCU has a critical role in many other tissues and different cells types such as pancreatic-β cells, macrophages, and lung. MCU is critical for glucose sensing by pancreatic β cells (Tarasov et ak, 2012). One study proposed a role for mitochondrial $Ca^{2+}$ in macrophage polarization (Gu, Larson-

Casey, & Carter, 2017). MCU knockdown in a cystic fibrosis cell line model resulted in reduced *Pseudomonas aeruginosa-induced* inflammatory response due to reduced inflammasome activation (Rimessi et al 2015). Further, mitochondrial $Ca^{2+}$ uptake through MCU was proposed to have a role in embryonic development. Specifically, MCU was proposed to regulate notochord axis formationby controlling blastomere movement during gastrulation, likely through regulation of microfilament dynamics (Prudent et al., 2013).

Additional roles were ascribed to MCU in several pathophysiologies including ischemia, myocardial infarction, and neurohumoral injury. Joiner et al reported co-immunoprecipitation of MCU and CaMKII from heart mitochondria, suggesting that MCU and CaMKII interact with each other. They also showed that increased $Ca^{2+}$/calmodulin-dependent protein kinase II (CaMKII) activity enhances MCU currents while CaMKII inhibition reduced currents. CaMKII inhibition in heart mitochondria protected against ischemia-reperfusion injury. The authors concluded that increased MCU current through catalytically active CaMKII in mitochondria resulted in increased myocardial cell death and heart failure (Joiner et al., 2012). Interestingly, MCU knockout mice did not show any defect in basal cardiac function under either normal or stress conditions (Holmström et al., 2015) and hearts from MCU knockout mice were not protected against ischaemia-reperfusion injury (Pan et al., 2013). Inhibition of MCU function by Ru360 in isolated sinoatrial nodal cells resulted in reduced action potential frequency in response to isoproterenol. Similarly, transgenic mice expressing a dominant negative form of MCU in the myocardium have reduced ATP generation and inhibited fight or flight-induced enhancement of heart rate, which can be rescued by dialysis of ATP (Y. Wu et al., 2015). These data suggest that altered MCU function in the heart results in mitochondrial dysfunction and reduced ATP production. It is known that Ru360 and its derivatives are nonspecific MCU inhibitors and results obtained with these inhibitors in physiological experiments should be interpreted with extreme caution. Recently, a yeast-based orthogonal interspecies chemical screen identified Mitoxantrone (an anti-cancer drug) as a specific MCU inhibitor. Mitoxantrone proved promising in blocking MCU $Ca^{2+}$ currents in permeabilized cells, intact cells, and in isolated mitochondria, suggesting that it could represent a potential drug for MCU-related diseases (Arduino et al., 2017).

It is puzzling that MCU knockout and MCU dominant negative mice were viable with no overt phenotype. To address potential adaptation in these two global MCU loss of function models, Luongo et al. generated a tamoxifen-inducible conditional MCU knockout mice (Luongo et al.,2015). Interestingly, germline deletion of MCU in this model resulted in embryonic lethality, suggesting that MCU is critical for embryonic development, whereas MCU knockout after birth or in adults did not result in any significant phenotype. These findings suggest that adaptation likely plays an important role in MCU knockout and MCU dominant negative mice. Another possibility is that the absence of MCU is compensated for by Letm1 or another transporter which have partial permeability to $Ca^{2+}$ and can support $Ca^{2+}$ uptake into the mitochondrial matrix in the absence of MCU. Specific deletion of MCU in adult cardiomyocytes resulted in complete loss of mitochondrial $Ca^{2+}$ uptake without affecting mitochondrial membrane potential. While these mice did not show any inhibition in basal cardiac function or morphology, they displayed reduced heart rate during fight or flight responses. Interestingly, ablation of MCU in adult hearts showed strong

protection from ischaemia-reperfusion mediated injury (Kwong et al., 2015; Luongo et al., 2015), which is at odds with results from Pan et al using global MCU knockout mice (Pan et al., 2013). A recent study showed that diabetic mice have impaired $Ca^{2+}$ handling in heart, due to altered expression of proteins of the MCU complex. MCU and EMRE levels were decreased while MCUb level was increased in mice hearts with age. Cardiac specificoverexpression of MCU improved cardiomyocyte contractility and significantly rescued ischemia reperfusion injury in diabetic mice (Suarez et al., 2018).

Mitochondrial $Ca^{2+}$ overload is associated with apoptosis and necrosis. The current models show that apoptotic signals cause mitochondrial $Ca^{2+}$ overload, resulting in mitochondrial fragmentation and release of pro-apoptotic proteins such as cytochrome C (Giorgi et ah, 2012). Mitochondria play a major role in cancer progression as altered mitochondrial function is one of the main drivers of tumor growth (Hanahan & Weinberg, 2011). Marchi et al showed that a cancer-related microRNA, miR-25 downregulates MCU expression when overexpressed in HeLa cells causing reduced mitochondrial $Ca^{2+}$ uptake. Several human colon cancer cell lines (HCT116, RKO, SW80 and WiDr) have higher levels of miR-25 and reduced MCU levels compared to normal mucosal cells. MCU levels were also reduced in adenocarcinoma samples which have higher miR-25 expression as compared to normal mucosa. These results suggest that cancer cells which have a higher level of miR-25 downregulate MCU as means to protect against apoptosis and promote growth (S. Marchi et al., 2013). However, Zeng et al have challenged these results by showing that receptor-interacting protein kinase 1 (RIPK1) and MCU are upregulated in colorectal cancer samples from patients and that RIPK1 interacts with MCU to increase MCU activity and promote colorectal cancer through enhanced cell proliferation and energy metabolism (Zeng et al., 2018). In agreement with Zeng et al, knockdown of MCU in triple-negative breast cancer cell lines resulted in reduced cell motility, invasion and tumor growth (Tosatto et ah, 2016). Downregulation of MCU resulted in decreased ROS production, decreased expression of hypoxia-inducible factor-la (HIF-la) and reduced breast cancer cell growth and metastasis. This indicates that mitochondrial $Ca^{2+}$ influx through the MCU complex regulates mitochondrial ROS, expression of HIF-la and breast cancer growth (Tosatto et ah, 2015). Another study found a correlation between MCU mRNA expression and metastatic breast cancer. Knockdown of MCU expression with siRNA or pharmacological inhibition with ruthenium red inhibited migration of MDA-MB-231 breast cancer cells in response to serum (Tang et ah, 2015). Overall, these results suggest that MCU promotes metabolism, survival and proliferation in different cancers.

## Mitochondrial $Na^+/Ca^{2+}/Li^+$ exchanger (NCLX)

The Mitochondrial $Na^+/Ca^{2+}/Li^+$ exchanger is also known as solute carrier family 8-member B1 (SLC8B1) or solute carrier family 24 member 6 (SLC24A6). NCLX extrudes one $Ca^{2+}$ from the mitochondria for every three $Na^+$ imported and the estimated turnover rate of NCLX is 1000 cycles per second. NCLX is a ~60kDa protein present in almost all types of tissues and cells. Putative topology modeling using TopPred (von Heijne, 1992) suggested that similar to NCX, NCLX has two domains al and a2 attached by a long loop, each containing six transmembrane segments. This long loop is believed to play a regulatory role in NCLX function although its exact orientation either in the matrix side or the

intermembrane space remains unknown (Kostic et al., 2015). NCLX was first cloned in 2004 and thought to be localized in the ER or plasma membrane (Palty *et al.*, 2004). In 2010, the Sekler group showed that NCLX is enriched in mitochondria and localized in the IMM. Using $Na^{2+}$ and $Ca^{2+}$ fluorescent dyes, they showed that overexpression of NCLX resulted in increased mitochondrial $Ca^{2+}$efflux (Fig. 1) while knockdown of NCLX resulted in reduced mitochondrial $Ca^{2+}$ efflux. Inhibition of NCLX function by a pharmacological benzothiazepine compound called CGP-37157 resulted in reduced mitochondrial-$Ca^{2+}$ efflux (R. Palty et al., 2010). Palty et al. expressed NCLX in SH-SY5Y cells and showed that agonist-induced mitochondrial $Ca^{2+}$ efflux was higher in NCLX-overexpressing cells as compared to wild-type cells (R. Palty et ah, 2010). They also confirmed the $Na^{+}$ dependence of NCLX, by showing strong $Ca^{2+}$ efflux in the presence of 20mM $Na^{+}$ as compared to the absence of $Na^{+}$. Loading mitochondria with the $Na^{+}$ dye CoroNa Red confirmed that the $Ca^{2+}$ efflux is dependent on mitochondrial $Na^{+}$ uptake (R. Palty et al., 2010).

## Regulation of NCLX

Members of the NCX superfamily of exchangers have an allosteric $Ca^{2+}$-binding site within their regulatory region, which mediates NCX regulation by cytosolic $Ca^{2+}$ (Doering et al., 1998). However, NCLX does not possess such $Ca^{2+}$ regulatory site (Sekler, 2015). Sequence analysis of NCLX revealed many phosphorylation sites potentially involved in regulating NCLX function. A previous study showed that PTEN-induced putative kinase 1 (PINK1 ) is one of the regulators of mitochondrial $Ca^{2+}$ efflux. Using primary cortical neurons from PΓNK1 knockout mice and human dopaminergic neuroblastoma cell line (SH-SY5Y) with PINK1 knockdown, Gandhi et al. showed that PINK1 depletion resulted in reduced mitochondrial $Ca^{2+}$ efflux rate (Gandhi et al, 2009). In PINK1 knockdown neurons, neuronal induction by UV caused irreversible increase in mitochondrial $Ca^{2+}$ and this enhanced mitochondrial $Ca^{2+}$ was associated with reduced mitochondrial $Na^{+}$ uptake (Gandhi et al, 2009). A subsequent study by Kostic et al. also confirmed that downregulation of PINK1 in SH-SY5Y cells resulted in significant reduction of mitochondrial $Ca^{2+}$ efflux rate (Kostic et al, 2015). Previous studies have shown that reduced mitochondrial membrane potential was rescued by activation of protein kinase A (PKA) (Dagda et al., 2011), suggesting that PINK1 could regulate NCLX through PKA-mediated NCLX phosphorylation. Kostic et al found that mitochondrial $Ca^{2+}$ efflux rate was significantly increased upon PKA activation with forskolin in PINK1 knockdown SH-SY5Y cells (Kostic et al, 2015). NCLX has a putative PKA phosphorylation site at serine 258 (S258) located in the loop connecting aland a2 domains. A phosphorylation-deficient mutant of NCLX where S258 was mutated to alanine (S258A) failed to rescue mitochondrial $Ca^{2+}$ efflux in NCLX knockdown SH-SY5Y cells while a phosphomimetic mutation (S258D) rescued mitochondrial $Ca^{2+}$ efflux. Interestingly, neurons isolated from PINK1 knockout mice showed reduced $Ca^{2+}$ efflux rate compared to wild- type neurons and overexpression of NCLX S258D, but not NCLX S258A, in neurons from PINK1 knockout mice rescued mitochondrial membrane potential and restored mitochondrial $Ca^{2+}$ efflux. PINK1 depleted neurons are very sensitive to dopamine induced cell death and this was prevented by overexpression of NCLX S258D but not NCLX S258A (Kostic et al, 2015). While NCLX transports $Na^{+}$ into mitochondria, the $Na^{+}/H^{+}$ exchanger (NHE), which is responsible for Na

$^{+}$ efflux from mitochondria into the cytosol, is major regulator of mitochondrial $Na^{+}$ in several cell types (Bemardinelli, Azarias, & Chatton, 2006; Bers, Barry, & Despa, 2003; Murphy & Eisner, 2009). NHE is therefore critical for maintaining $Na^{+}$ gradients across the mitochondrial membrane and its function is critical for NCLX activity and for the maintenance of $Ca^{2+}$ homeostasis in mitochondria (Nita, Hershfinkel, Lewis, & Sekler, 2015). A genome wide *Drosophila* RNAi screen identified CG4589, which is a mammalian orthologue of Letml, as the mitochondrial $Ca^{2+}/H^{+}$ antiporter. Furthermore, downregulation of Letml reduced $Ca^{2+}/H^{+}$ antiporter activity, while overexpression of Letml resulted in increased $Ca^{2+}/H^{+}$ antiporter activity. Reconstitution of purified Letml in lysosomes also resulted in enhanced $Ca^{2+}$transport (Jiang et al., 2009). These results show that in addition to NCLX, Letml can also extrude $Ca^{2+}$ from the mitochondrial matrix into cytosol (Fig. 1). Downregulation of Letml resulted in impaired basal mitochondrial oxygen consumption, increased ROS production, AMP- activated protein kinase (AMPK) activation, autophagy and cell cycle arrest (Doonan et al., 2014), suggesting that Letml plays a crucial role in regulating cellular metabolism. Nevertheless, the identity of Letml as a $Ca^{2+}$ exchanger is uncertain. Originally, Letml has been proposed to be a $K^{+}/H^{+}$ exchanger that regulates mitochondrial osmolarity (Dimmer et al., 2007; Hasegawa & van der Bliek, 2007; Hashimi, McDonald, Stribrna, & Lukes, 2013; Mitchell, 2011; Nowikovsky et al., 2004). Kinetic analysis showed that the turnover rate of Letml for $Ca^{2+}$ is two cycles per second which is in the non-physiological range (Mingn Feng Tsai et ak, 2014). Further, the overexpression of Letml, which is insensitive to the NCLX blocker CGP-37157, in HeLa cells did not increase the $Ca^{2+}$ efflux rate of mitochondria and did not enhance mitochondrial $Ca^{2+}$ efflux in response to physiological stimulation (U. De Marchi et ak, 2014). It is possible that Letml does not work alone to regulate mitochondrial $Ca^{2+}$, and rather works in conjunction with MCU and NCLX to maintain $Ca^{2+}$ homeostasis in the mitochondrial matrix. A detailed study is required to decipher Letml stoichiometry, mechanisms of $Ca^{2+}$ transport, and communication with MCU and NCLX during homeostatic and regulated mitochondrial $Ca^{2+}$ transport.

## Physiological role of NCLX

Nita et al showed that downregulation of NCLX in MIN6 cells or primary pancreatic β-cells inhibited mitochondrial $Ca^{2+}$ efflux induced by either high glucose or by cell depolarization. This coincided with reduced rate and amplitude of the cytosolic $Ca^{2+}$ signal and delayed insulin secretion in response to high glucose or membrane depolarization (Nita et ak, 2012), suggesting that NCLX plays a critical role in the communication between the plasma membrane and mitochondria and in regulating cytosolic $Ca^{2+}$ signals and insulin secretion by pancreatic β-cells. A whole-genome genotyping of single nucleotide polymorphisms and exome sequencing discovered a patient with congenital hyperinsulinemia with a mutation in the NCLX gene. This NCLX mutation is caused by a change of tyrosine at position 564 to histidine (Proverbio et ak, 2013); this residue is located in the highly conserved putative transmembrane domain 12 (Takeuchi, Kim, & Matsuoka, 2015). However, it remained to be established whether this mutation results in altered NCLX function and loss of mitochondrial $Ca^{2+}$ extrusion, thus leading to altered insulin secretion of pancreatic β cells. Kim et al showed that NCLX knockdown from A20 and DT40 B lymphocytes results in

increased random migration and significantly reduced chemotaxis in response to the chemokine C-X-C motif chemokine 12-CXCL12 (Kim, Takeuchi, Hikida, & Matsuoka, 2016). Similarly, B lymphocytes derived from mouse spleen also showed markedly reduced chemotaxis after pharmacological inhibition of NCLX by CGP-37157. In NCLX knockdown cells, F-actin and Racl localization was altered leading to enhanced random cell migration (Kim et al, 2016).

There is growing evidence that astrocytes release neurotransmitters in response to increased cytosolic $Ca^{2+}$ (Navarrete & Araque, 2008; Serrano, 2006). It was suggested that NCLX is enriched in mitochondria of astrocytes and plays an important role in glutamate release, cell proliferation and wound healing (Parnis et al, 2013). Downregulation or pharmacological inhibition of NCLX in primary astrocytes resulted in significantly higher mitochondrial $Ca^{2+}$ levels and decrease in store-operated $Ca^{2+}$ entry (SOCE) when cells were challenged externally by the purinergic agonist ATP. Pamis et al showed that basal glutamate release, proliferation and migration were reduced in astrocytes with NCLX knockdown (Parnis et al, 2013). A study in *Caenorhabditis elegcms* showed that the NCLX like protein NCX-9 (which has similar $Ca^{2+}$ handling properties as NCLX) plays a role in neural circuit development, specifically in axonal guidance (Sharma, Roy, Sekler, & O'Halloran, 2017). In fibroblasts derived from familial Parkinson's disease patients where leucine-rich repeat kinase 2 (LRRK2) is mutated, MCU and MICU1 genes (which are located on the same chromosome in humans) were transcriptionally upregulated. In a mouse model, overexpression of LRRK2 also resulted in upregulation of MCU and MICU1. Interestingly, no change in MICU2 or NCLX levels was observed. Knockdown or pharmacological inhibition of MCU as well as overexpression of a constitutively active NCLX allele appeared to be neuroprotective, showing that tight mitochondrial $Ca^{2+}$ homeostasis is required for neuronal activity and function (Verma et al, 2017). Taken together, these studies suggest that NCLX plays a role in neural development, transmission and plasticity. More detailed studies are required to fully uncover the mechanisms of NCLX regulation of neuronal signaling and plasticity.

Takeuchi et al showed that NCLX modulates action potential and regulates automaticity in HL-1 mouse atrial cardiomyocytes. Downregulation of NCLX resulted in reduced frequency of spontaneous $Ca^{2+}$ oscillations and action potentials due to significantly slow and prolonged cycle length of each action potential. The authors showed that $Ca^{2+}$ content and reuptake in the sarcoplasmic reticulum (SR) was reduced in NCLX knockdown HL-1 cells, likely explaining the prolonged cycle length of spontaneous action potentials (Takeuchi, Kim, & Matsuoka, 2013). Mathematical modeling of HL-1 cardiomyocyte showed that blocking NCLX caused a reduction in SR $Ca^{2+}$ content leading to prolongation of the cycle length of the spontaneous action potential. These findings suggest that functional $Ca^{2+}$ extrusion from mitochondria by NCLX contributes to SR $Ca^{2+}$ refilling during action potentials. While inhibition of NCLX by CGP37157 resulted in slower rabbit sinoatrial (SA) node automaticity (Yaniv et al, 2012), these results are based on an NCLX inhibitor with questionable specificity. Indeed, CGP37157 is also a blocker of L-type $Ca^{2+}$ channels, which are required for automaticity of sinoatrial (SA) cells (Takeuchi et al., 2015).

A recent study showed that tamoxifen-induced deletion of NCLX in adult mouse heart resulted in myocardial dysfunction leading to sudden cardiac arrest and death (Luongo et al, 2017). Adult cardiac myocytes lacking NCLX showed loss of mitochondrial $Ca^{2+}$ efflux and significant reduction of mitochondrial $Ca^{2+}$ uptake. These NCLX knockout cardiomyocytes showed reduced protein levels of EMRE while the expression levels of MCU, MCUb, MICU1, LETM1 or electron transport chain proteins were normal in these cells. No significant changes in cytosolic $Ca^{2+}$ were observed in these NCLX knockout cells. Cardiomyocytes with NCLX knockout showed increased mitochondrial $Ca^{2+}$, mitochondrial swelling (due to opening of mitochondrial permeability transition pore; MPTP), increased mitochondrial superoxide generation and enhanced necrotic cell death within two days of tamoxifen treatment. Luongo et al. also generated a mouse in which NCLX overexpression was specifically induced in cardiomyocytes with doxycycline. Overexpression of NCLX resulted in increased mitochondrial $Ca^{2+}$ efflux by −38%; this enhanced $Ca^{2+}$ efflux activity was sufficient to inhibit MPTP activity. Overexpression of NCLX did not affect basal respiration, ATP-linked respiration and proton leak, but it inhibited myocardial infraction, reduced post infraction fibrosis, inflammation, superoxide generation andcell death caused by mitochondrial $Ca^{2+}$ overload (Luongo et al, 2017). Another study showed that cardiomyocyte-specific knockout mouse for the main transcription factor for mitochondrial DNA (transcription factor A, mitochondrial, Tfam) develop dilated cardiomyopathy associated with altered oxidative phosphorylation and this correlated with enhanced mitochondrial $Ca^{2+}$ due to increased MCU and reduced NCLX expression and activity (Sommakia et al, 2017). There is no obvious reason that could explain the phenotype of cardiomyocyte-specific NCLX KO mice, which is more drastic than that of cardiomyocyte-specific MCU KO mice. One possible explanation is that another $Ca^{2+}$ conducting channel can compensate for the lack of MCU. However, NCLX being a slower transporter and the rate-limiting step in $Ca^{2+}$ efflux from mitochondria, cannot be easily compensated for by another transporter.

Pharmacological inhibition of mitochondrial $Ca^{2+}$ extrusion by the NCLX inhibitor CGP-37157 or the use of mitochondria-depolarizing compounds partially inhibited SOCE, but when both strategies were used together SOCE was completely inhibited (Naghdi et al, 2010). When the authors physically immobilized mitochondria at the plasma membrane using an mAKAP-RFP- CAAX linker, SOCE activity was not altered, questioning the prominence of the proximity- dependent mechanism involving $Ca^{2+}$ buffering by mitochondria in regulating SOCE and suggesting the existence of additional mechanisms independent of buffering (Naghdi et al, 2010). A more recent study by Ben-Kassus Nissim et al showed that molecular downregulation of NCLX proteins in HEK293 cells resulted in partial inhibition of SOCE and its biophysical correlate, the $Ca^{2+}$ release activated $Ca^{2+}$ (CRAC) current (Ben- Kasus Nissim et al, 2017), in agreement with the findings of Naghdi et al, Ben-Kassus Nissim et al used whole cell patch clamp electrophysiology to rule out a role for mitochondrial $Ca^{2+}$ buffering in this mode of SOCE regulation by mitochondria and demonstrated that instead NCLX knockdown causes mitochondrial $Ca^{2+}$ overload which in turn leads to upregulation of mitochondrial reactive oxygen species (ROS) production. ROS act directly on the redox-sensitive Cysteine 195 within the SOCE channel ORAI1 to inhibit SOCE and CRAC currents (Ben- Kasus Nissim et al, thus providing evidence for a

proximity-independent role of mitochondria in regulationg plasma membrane channels through ROS-mediated ORAI1 channel inhibition.

## Future Directions

The discovery of MCU and NCLX has shed light on how mitochondria contribute to shaping cellular $Ca^{2+}$ signaling to control metabolism and growth. This provided a clearer picture on how distinct $Ca^{2+}$ stores within the cell communicate with one another to shuttle $Ca^{2+}$ ions, ensuring precise temporal and spatial $Ca^{2+}$ signaling required for a myriad of cellular processes. In the past decade, much work has been focused on understanding the mechanisms of $Ca^{2+}$ uptake into mitochondria through MCU. Although the discovery of components of the MCU uniporter complex provided valuable insights into the regulatory mechanisms of mitochondrial $Ca^{2+}$ uptake, several questions remain unanswered. For instance, the stoichiometry of the MCU complex and whether this stoichiometry is dynamically regulated within the same cell or whether its components vary between different cell types remain unknown. The heterogeneity of the MCU complex during cell signaling or within different tissue types might be due to the existence of multiple isoforms of MICU regulators. For example, the role of MICU3, which was proposed to be invovled in the central nervous system, remain unclear (Plovanich et al, 2013). Similarly,other regulators of MCU such as SLAC25A23 and MCUb await detailed and thorough investigations. Additional studies are required to unravel the stoichiometry and signaling pathways regulating NCLX activity. The exact orientation of NCLX within IMM remains unknown and whether S258 is located in the matrix or the intermembrane space is also unknown. The mechanisms of PKA phosphorylation of NCLX are also not clear. Regardless of where S258 is located, the presence of PKA in either the mitochondrial matrix or the intermembrane space is a highly contentious idea. NCLX has been recently proposed to have an important role in heart function and neuronal plasticity (Luongo et al, 2017; Parnis et al, 2013). However, the exact mechanisms behind this regulation remain elusive. Future high resolution structures of MCU bound to its regulators in the presence and absence of $Ca^{2+}$ will provide crucial insights on MCU channel gating and regulation. Similarly, structure of NCLX and Letml have not been reported. As we uncover the permeation and regulatory mechanisms of mitochondrial $Ca^{2+}$ transport and identify potential cell-specific heterogeneities in these mechanisms, we will be one step closer to harness this knowledge for the purpose of disease therapy.

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## Abbreviations

| | |
|---|---|
| **AMPK** | AMP-activated protein kinase |
| **ATP** | adenosine triphosphate |
| **CaMKII** | $Ca^{2+}$/calmodulin- dependent protein kinase II |

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|---|---|
| **CREB** | cyclic adenosine monophosphate response element-binding protein |
| **CXCL12** | C-X-C motif chemokine 12 |
| **EMRE** | essential MCU regulator |
| **ER** | endoplasmic reticulum |
| **ETC** | electron transport chain |
| **HIF-lα** | hypoxia-inducible factor-lα |
| **IMM** | inner mitochondrial membrane |
| **$IP_3R$** | inositol-1,4,5-trisphosphate receptor |
| **IS** | immunological synapse |
| **MAMs** | mitochondria associated membranes |
| **MCU** | mitochondrial $Ca^{2+}$ uniporter |
| **MCUb** | MCU regulatory subunit b |
| **MCUR1** | mitochondrial $Ca^{2+}$ regulator 1 |
| **MICU1** | mitochondrial calcium uptake 1 |
| **MPTP** | mitochondrial permeability transition pore |
| **NCLX** | $Na^+/Ca^{2+}/Li^+$ exchanger |
| **NCX** | $Na^+/Ca^{2+}$ exchangers |
| **NCKX** | $Na^+/Ca^{2+}/K^+$ exchanger |
| **NFAT** | nuclear factor of activated T-cells |
| **NF-κB** | nuclear factor kappa-light-chain-enhancer of activated B cells |
| **NHE** | $Na^+/H^+$ exchanger |
| **NMDA** | N-Methyl-D-aspartic acid |
| **OMM** | outer mitochondrial membrane |
| **PINK1** | PTEN-induced putative kinase 1 |
| **PKA** | protein kinase A |
| **PS** | phosphatidylserine |
| **RIPK1** | receptor-interacting protein kinase 1 |
| **ROS** | reactive oxygen species |
| **SERCA** | sarco/endoplasmic reticulum $Ca^{2+}$ ATPase |

| | |
|---|---|
| **SLC8B1** | solute carrier family 8-member Bl |
| **SLC24A6** | solute carrier family 24 member 6 |
| **SLC25A23** | solute carrier family 25 member 23 |
| **SOCE** | store-operated $Ca^{2+}$ entry |
| **SR** | sarcoplasmic reticulum |
| **STIM** | stromal interaction molecule |
| **VDAC** | voltage-dependent anion channel |
| **UPR** | unfolded protein response |

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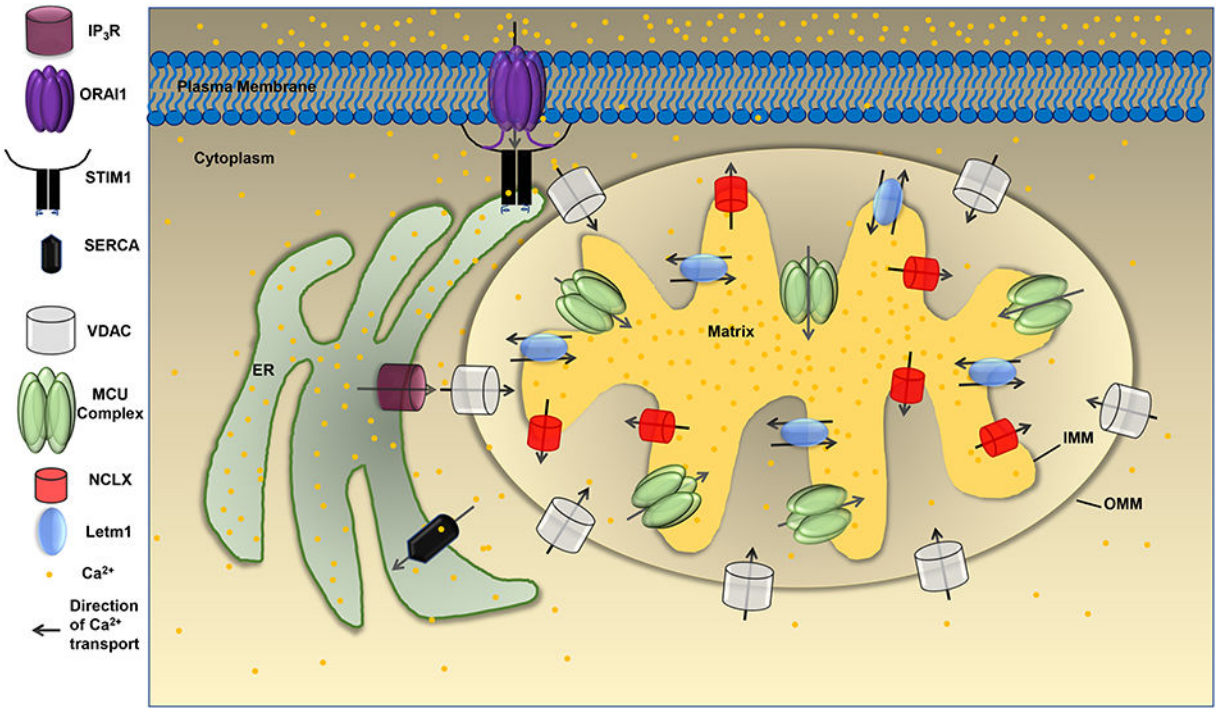


**Figure 1: Mitochondrial $Ca^{2+}$ homeostasis: $Ca^{2+}$ cycling between the cytosol, ER, and mitochondria.**

Activation of phospholipase C (PLC)-coupled agonists produce the soluble cytosolic second messenger inositol-1,4,5 trisphosphate (ΓP3) which binds to its receptor, the $IP_3R$, and cause $Ca^{2+}$ release from the ER to the cytosol. Through mitochondria associated membranes (MAMs), $Ca^{2+}$ released from $IP_3R$ crosses the OMM through VDAC and enters the intermembrane space (IMS) before reaching the mitochondrial matrix through MCU. Depletion of ER $Ca^{2+}$ concentration also triggers the activation of store-operated $Ca^{2+}$ entry (SOCE) into the cytosol across the plasma membrane through ORAI1 channels. $Ca^{2+}$ entry through SOCE is buffered back into the ER by the action of sarcoplasmic/endoplasmic reticulum ATPase (SERCA) pumps or can be taken up by mitochondria through MCU.VDAC resides in the outer mitochondrial membrane (OMM) and brings $Ca^{2+}$ in the intermembrane space (IMS) of mitochondria. Once $Ca^{2+}$ concentration in the IMS is high, it is sensed by the gatekeeper proteins MICU1/MICU2 which disinhibit MCU allowing $Ca^{2+}$ uptake into the mitochondrial matrix. Matrix $Ca^{2+}$ is pumped back to the IMS through the $Na^+/Ca^{2+}$ exchange (NCLX) and Letml.

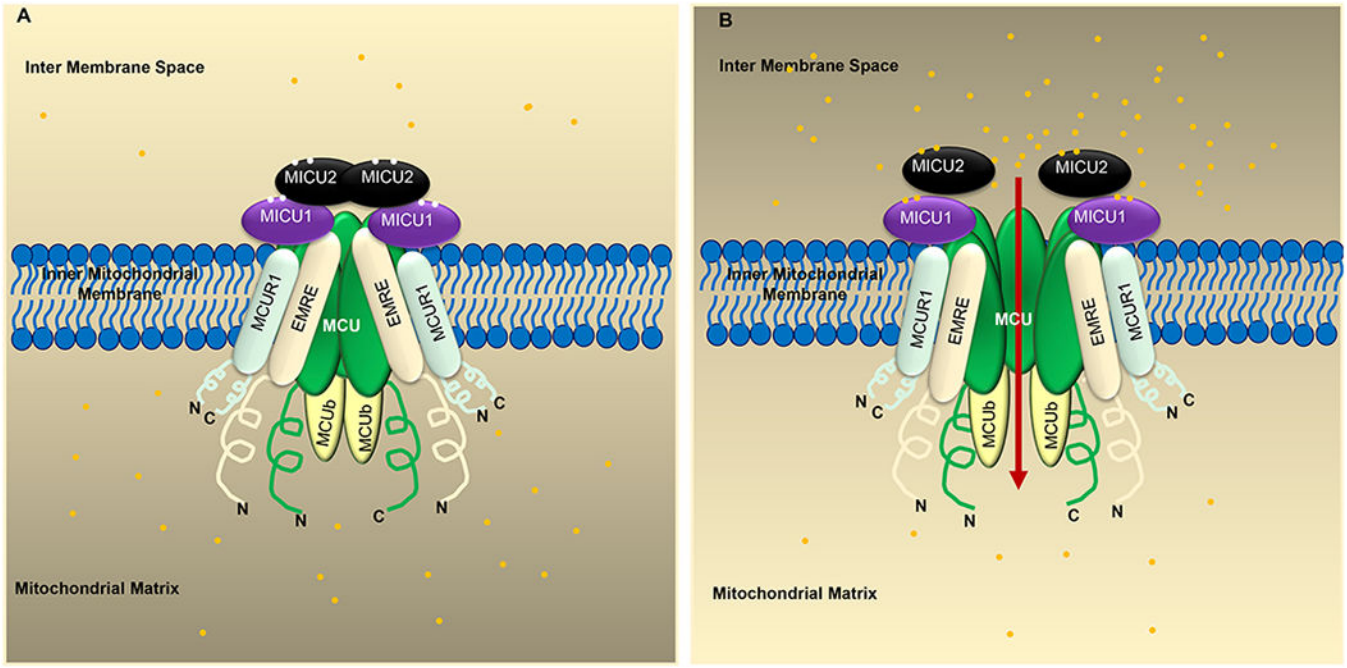


**Figure 2: Components of the MCU complex and its regulation by $Ca^{2+}$.**

**A)** MCU, MICU1/2/3, EMRE, MCUR1, and MCUb form the Uniporter complex. When the $Ca^{2+}$ concentration in mitochondrial intermembrane space (IMS) is low, MICU2 inhibits MCU activity. **B)** When the IMS $Ca^{2+}$ concentration is high, $Ca^{2+}$ binds to MICU1 and activates MCU to cause $Ca^{2+}$ uptake into the mitochondrial matrix. EMRE is an essential subunit that is required for MCU function and helps tether MICU1/MICU2 to the MCU complex. The yellow dots represent $Ca^{2+}$.