

## Chapter 2

# Molecular Players of Mitochondrial Calcium Signaling: Similarities and Different Aspects in Various Organisms

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### 2.1 Introduction

$\text{Ca}^{2+}$  acts as a second messenger in every cell type, controlling processes as diverse as secretion, cell death, and survival. The versatile and universal nature of calcium as intracellular messenger is guaranteed by a cell-specific  $\text{Ca}^{2+}$  signaling toolkit: several components (e.g.,  $\text{Ca}^{2+}$  channels, pumps, and  $\text{Ca}^{2+}$ -binding proteins) can cooperate and generate a wide range of signals, where changes in intracellular  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) vary in both spatial and temporal patterns. These specific patterns can then be decoded into specific cellular events (Berridge et al. 2003). Compartmentalization of  $[\text{Ca}^{2+}]$  dynamics in the different organelles represents a further level of complexity. Mitochondria are thought to play an integral part that goes beyond acting as passive supporters by providing the ATP required for cellular readjustment of  $[\text{Ca}^{2+}]_i$  following stimulation. These organelles are able to quickly and transiently accumulate  $\text{Ca}^{2+}$  upon cytosolic transients, and thus, they can a priori contribute to shaping cytosolic  $\text{Ca}^{2+}$  transients.

Only during the last decade, significant advances have been achieved regarding the identification of the molecular players of the mitochondrial  $\text{Ca}^{2+}$ -handling machinery. Here, we summarize our current knowledge on the main player, i.e., the

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MCU complex (MCUC) in different organisms including protozoa (*Trypanosoma*), fungi, plants, and animals. In addition, the physiology of mitochondrial calcium homeostasis will be discussed also in light of what we learned from studies in organisms where MCU complex components have been genetically targeted.

## 2.2 The Role of Mitochondrial Calcium Within the Organelle

During the last three decades, considerable experimental work has been carried out using either pharmacological or indirect genetic tools to alter mitochondrial calcium homeostasis and to dissect its pathophysiological role.  $\text{Ca}^{2+}$  inside mitochondria plays a pleiotropic role, with different cellular outcomes that depend on the investigated cell type, the metabolic state, and the concomitant presence of other stress signals. Not only calcium plays a regulatory role within the organelle itself ranging from the regulation of ATP production to the release of apoptotic cofactors with consequent cell death, but in most organisms, mitochondrial calcium can importantly impact cation homeostasis, both locally and globally (Rizzuto et al. 2012).

In respiring mitochondria, the major component of the electrochemical proton gradient ( $\Delta\mu\text{H}^+$ ), the membrane potential difference ( $\Delta\Psi$ ), represents a very large driving force for  $\text{Ca}^{2+}$  accumulation. The inner mitochondrial membrane (IMM) is impermeable for cations (including  $\text{Ca}^{2+}$ ), and passage strictly requires channels/transporters. Early  $\text{Ca}^{2+}$  uptake studies with mammalian mitochondria revealed that transport of calcium required respiration (Vasington and Murphy 1962; Deluca and Engstrom 1961) and was accompanied by  $\text{P}_i$  transport (Greenawalt et al. 1964). The underlying transporter was proposed to be an electrophoretic  $\text{Ca}^{2+}$  uniporter that does not require ATP hydrolysis but is driven by the steep electrochemical gradient across the IMM (Rottenberg and Scarpa 1974). Similarly, studies using isolated mitochondria from different plant species evidenced that these organelles take up  $\text{Ca}^{2+}$  (Akerman and Moore 1983; Dieter and Marme 1980). Uptake strictly required energization (Dieter and Marme 1980).  $\text{Ca}^{2+}$  import in most cases was shown to require inorganic phosphate ( $\text{P}_i$ ) (Hodges and Hanson 1965; Chen and Lehninger 1973), leading to a hypothesis that  $\text{Ca}^{2+}$  is imported by a symport mechanism together with  $\text{P}_i$  (Silva et al. 1992; Day et al. 1978). However, for example, in some studies, isolated plant mitochondria were not found to accumulate calcium (Moore and Bonner 1977; Martins and Vercesi 1985), casting doubt on the existence of a general mechanism of calcium handling by these organelles in plants. In mammals, once mitochondrial  $\text{Ca}^{2+}$  uptake could be monitored directly in intact cells using mitochondria-targeted aequorin as calcium-sensing probe (Rizzuto et al. 1992), it became evident that  $\text{Ca}^{2+}$  concentrations in mitochondria can reach up to hundred  $\mu\text{M}$  in some cell types. The speed and amplitude of  $\text{Ca}^{2+}$  uptake was shown to exceed the values that had been predicted from classical bioenergetic experiments in isolated mitochondria. Subsequent work in mammalian cells suggested an interaction of mitochondria with microdomains of high  $\text{Ca}^{2+}$  concentrations generated

by localized release from the ER and the extracellular space, allowing highly efficient uptake (see, e.g., Rizzuto et al. (2012) also for historical overview).

In mammals,  $\text{Ca}^{2+}$  elevations in the mitochondrial matrix stimulate respiration and ATP synthesis to cover temporarily high-energy needs of cells, e.g. (Denton 2009). Increased biosynthesis rates of ATP rely on the activation of mitochondrial dehydrogenases by  $\text{Ca}^{2+}$  (McCormack et al. 1990). In addition, electron transfer chain (ETC) complexes as well as the ATP synthase are positively regulated by  $\text{Ca}^{2+}$ . Among the dehydrogenases, pyruvate dehydrogenase (PDH) (Denton et al. 1972), NAD-isocitrate dehydrogenase (NAD-ICDH) (Denton et al. 1978), and  $\alpha$ -ketoglutarate (oxoglutarate) dehydrogenase (McCormack and Denton 1979) are activated by physiologically relevant  $\text{Ca}^{2+}$  concentrations (between 100 nM and 1  $\mu\text{M}$ ) in mitochondria isolated from mammalian tissues (Denton and McCormack 1980; Denton et al. 1980). The latter two enzymes do not contain any typical  $\text{Ca}^{2+}$ -binding motifs, such as EF-hands, but are directly, allosterically, regulated by  $\text{Ca}^{2+}$  (McCormack et al. 1990). Instead, both in animal and plants, the former enzyme is activated through  $\text{Ca}^{2+}$ -controlled PDH phosphatase: PDH activity is regulated through reversible phosphorylation (Holness and Sugden 2003; Tovar-Mendez et al. 2003) with activity being enhanced through a dephosphorylation step. These events in turn increase NADH availability and consequently the electron flow through the respiratory chain. In addition to matrix dehydrogenases, aspartate/glutamate exchangers of the inner membrane (aralar1, citrin, and the ATP-Mg/Pi carrier SCaMC-3) also seem to be regulated by  $\text{Ca}^{2+}$  via EF-hand  $\text{Ca}^{2+}$ -binding sites which are exposed to the intermembrane space (Rueda et al. 2015). Direct evidences in favor of regulation of metabolism by calcium are multiple: (i) an increased resting state level of  $\text{Ca}^{2+}$  in the mitochondrial matrix was shown to alter the PDH phosphorylation state in cultured cells (Mallilankaraman et al. 2012b) and (ii) an increase in mitochondrial and then cytosolic ATP was reported to occur upon cell stimulation and to depend on the  $[\text{Ca}^{2+}]_{\text{mt}}$  rise (Jouaville et al. 1995), in order to match ATP synthesis to the increased demand of a stimulated cell. However, several findings argue against an universal conservation of  $\text{Ca}^{2+}$  regulation of mitochondrial metabolism in all organisms: (i) while the activity of TCA cycle enzymes NAD-ICDH and  $\alpha$ -ketoglutarate dehydrogenase from various vertebrates is increased in the presence of  $\text{Ca}^{2+}$ , their homologs from *Escherichia coli*, yeast, insect flight muscle, and potato are insensitive to calcium (Nichols et al. 1994; McCormack and Denton 1981) and (ii) PDH phosphatase is not activated by  $\text{Ca}^{2+}$  in vitro or in intact mitochondria in all organisms (Miernyk and Randall 1987; Budde et al. 1988).

In addition to the regulation of mitochondrial metabolism, calcium plays an important role also in the context of cell death. Indeed, an excessive increase in mitochondrial  $\text{Ca}^{2+}$  concentration under certain stimuli may be harmful: when a certain threshold level is exceeded, it may result in long-lasting opening of the permeability transition pore (Bernardi and von Stockum 2012). The general consensus is that mitochondrial  $\text{Ca}^{2+}$  loading has a permissive role, allowing a variety of toxic challenges to cause the release of caspase cofactors from the organelle and thereby trigger apoptotic cell death. In turn, alteration of this cellular response has a role in

the pathogenesis of human disorders such as neurodegenerative disorders and cancer (Bernardi et al. 2015; Szabo and Zoratti 2014).  $\text{Ca}^{2+}$  transfer from intracellular stores to mitochondria is also emerging as a site of pivotal importance in the regulation of both cell death and cell survival pathways as well as for autophagy (for details, see Filadi et al. in this book). Interestingly, a recent work highlights that a novel, cardiac mitochondrial cAMP-dependent pathway controls mitochondrial  $\text{Ca}^{2+}$  entry through the MCUC in order to prevent PTP opening (Wang et al. 2016).

### 2.3 The Role of Mitochondrial Calcium in Shaping the Cytosolic Calcium Signaling

Mitochondria play a pivotal role in shaping cytosolic  $\text{Ca}^{2+}$  signals. This has been demonstrated experimentally, since mitochondrial  $\text{Ca}^{2+}$  buffering was shown to influence cellular  $\text{Ca}^{2+}$  signals and consequently cell function in many different cell types (Rizzuto and Pozzan 2006). Indeed, the observation that mitochondria rapidly accumulate  $\text{Ca}^{2+}$  upon stimulation allowed to design experiments to prove that these organelles contribute to the buffering of either the whole cytoplasm or of specific cellular domains. Mitochondria were shown to be able to rapidly remove  $\text{Ca}^{2+}$  from the mouth of the ER-located  $\text{Ca}^{2+}$  release channel inositol trisphosphate receptor ( $\text{IP}_3\text{R}$ ) and hence to modify the total amount of  $\text{Ca}^{2+}$  released from intracellular stores to the cytosol.  $\text{Ca}^{2+}$  released across this channel exerts a feedback regulatory action, either activating, inhibitory, or biphasic, depending on the local  $[\text{Ca}^{2+}]$ . This effect is a consequence of the bell-shaped relationship between cytosolic calcium concentration ( $[\text{Ca}^{2+}]_c$ ) and  $\text{IP}_3\text{R}$  opening, where low and high  $[\text{Ca}^{2+}]_c$  inhibit channel activity, whereas intermediate  $[\text{Ca}^{2+}]_c$  increases cation release (Bezprozvanny et al. 1991). Accordingly, removal of  $\text{Ca}^{2+}$  from the proximity of  $\text{IP}_3\text{Rs}$  on one hand reduces the stimulus to opening, and on the other, it relieves  $\text{Ca}^{2+}$ -dependent inhibition of open channels, thus promoting  $\text{Ca}^{2+}$  release. This was first demonstrated in *Xenopus* oocytes, where mitochondrial energization and the resulting increase in mitochondrial  $\text{Ca}^{2+}$  uptake was shown to coordinate  $\text{IP}_3$ -induced  $[\text{Ca}^{2+}]_c$  rises into single propagating waves of low frequency and high amplitude (Jouaville et al. 1995). Regulation of the spatiotemporal patterning of cytosolic  $\text{Ca}^{2+}$  waves by mitochondria has been observed then in numerous different cell types. Instead, in plants, although our current knowledge points to mitochondrial calcium uptake occurring in vivo, this event does not seem to correlate with shaping of the cytosolic calcium signaling (Wagner et al. 2015).

Mitochondrial  $\text{Ca}^{2+}$  buffering could also participate to the local accumulation of a large amount of cations in a defined cell region, thanks to the precise positioning of the organelles. For example, redistribution of mitochondria to the immunological synapse was shown to be necessary to maintain  $\text{Ca}^{2+}$  influx across the plasma membrane and for  $\text{Ca}^{2+}$ -dependent helper T cell activation (Quintana et al. 2007). Further work highlighted that calcium-dependent inactivation of the calcium influx-mediating ORAI channels was prevented by localizing mitochondria close to ORAI

channels. Thus, the redistribution of mitochondria following the formation of immunological synapse maximized the efficiency of calcium influx through ORAI channels, but it also decreased calcium clearance by the exit pathway (calcium ATPase of the plasma membrane), resulting in a more sustained NFAT activity and subsequent activation of T cells (Quintana et al. 2011).

Overall, mitochondria appear to be efficient, high capacity  $\text{Ca}^{2+}$  buffers that shape cytosolic  $\text{Ca}^{2+}$  transients by either regulating the properties of  $\text{Ca}^{2+}$ -releasing channels or limiting the wide spreading of  $[\text{Ca}^{2+}]_c$  rises, at least in animals. The precise positioning of the organelle is critical for shielding defined cell portions in specific cells or for regulating calcium-mediated feedback mechanisms. While these statements are in general valid for most mammalian cell types, the role of mitochondrial calcium buffering is much less clear in other cell types, for example, in plants (Wagner et al. 2016).

## 2.4 $\text{Ca}^{2+}$ Import into Mitochondria

### 2.4.1 *Calcium Flux Across the Outer Mitochondrial Membrane*

Similarly, to other small molecules,  $\text{Ca}^{2+}$  is thought to freely pass the outer mitochondrial membrane (OMM) through VDACs (voltage-dependent anion channels, also called porins). VDACs allow flux of metabolites and ions including  $\text{Ca}^{2+}$ , for which mammalian VDAC also possesses binding sites, as demonstrated both in vitro and in vivo (Bathori et al. 2006; De Stefani et al. 2012; Gincel et al. 2001; Israelson et al. 2007; Rapizzi et al. 2002; Shoshan-Barmatz et al. 2010; Rizzuto et al. 2009). In mammals and plants, functionally distinct protein isoforms have been found in the OMM (for recent reviews, see Shoshan-Barmatz et al. 2010; Rostovtseva 2012; Takahashi and Tateda 2013). VDACs, although defined as anion channels, can conduct a substantial flow of  $\text{Ca}^{2+}$ , as demonstrated both in vitro and in vivo for the mammalian protein. The importance of calcium flux across VDACs is highlighted by recent studies addressing different aspects. For example, efsevin was shown to bind to VDAC2, to potentiate mitochondrial  $\text{Ca}^{2+}$  uptake, and to accelerate the transfer of  $\text{Ca}^{2+}$  from intracellular stores into mitochondria. In cardiomyocytes, efsevin inhibited  $\text{Ca}^{2+}$  overload-induced erratic calcium waves, demonstrating that VDAC2-dependent mitochondrial  $\text{Ca}^{2+}$  uptake plays a critical role in the regulation of cardiac rhythmicity (Shimizu et al. 2015). Uptake of calcium via VDAC1 seems to be required for inhibition of apoptosis by anti-apoptotic proteins. In particular, the BH4 domain of Bcl-XL, but not that of Bcl-2, was shown to selectively target VDAC1 and to inhibit apoptosis by decreasing VDAC1-mediated  $\text{Ca}^{2+}$  uptake into the mitochondria (Monaco et al. 2015). Not only uptake of calcium via VDACs have profound effect on pathophysiological processes but also its release: recent data indicate that mitochondrial calcium, released through VDAC1, triggers Schwann cell demyelination via a signaling pathway including ERK1/ERK2, p38, JNK, and

c-JUN activation following sciatic nerve injury. Importantly, reduction of mitochondrial calcium release, either by VDAC1 silencing or pharmacological inhibition, prevented demyelination (Gonzalez et al. 2016).

### **2.4.2 *Calcium Flux Across the Inner Mitochondrial Membrane***

Mitochondrial calcium uptake mostly occurs via the mitochondrial calcium uniporter complex MCUC, but other uptake modes, differing from MCUC-mediated  $\text{Ca}^{2+}$  uptake in terms of  $\text{Ca}^{2+}$  affinity, uptake kinetics, and pharmacological control, seem to coexist at least in the mammalian system. Here below, we provide an updated overview of the MCUC components and function in different systems and briefly mention the main characteristics of the other uptake modes as well.

#### **2.4.2.1 The Mitochondrial Calcium Uniporter Complex (MCUC)**

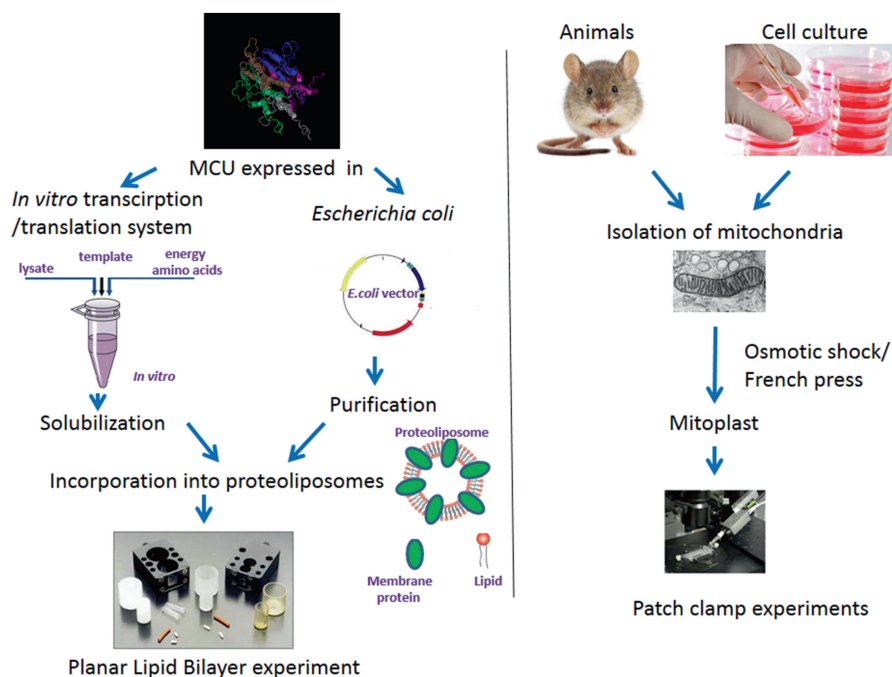
The main bioenergetic properties of the uniporter have been characterized in fine detail, and low concentrations of ruthenium red and Ru360 were shown to lead to a direct inhibition of the uniporter (Reed and Bygrave 1974; Vasington et al. 1972; Moore 1971). The finding that a highly  $\text{Ca}^{2+}$ -selective ion channel recorded in the inner mitochondrial membrane (IMM) (in mitoplasts) by patch clamping (Kirichok et al. 2004) recapitulated the key characteristics observed for the mammalian mitochondrial uniporter in classical bioenergetic experiments, together with the establishment of the MitoCarta database, containing more than 1,000 mitochondrial proteins as identified by subtractive proteomics and GFP-fusion localization studies (Pagliarini et al. 2008), finally led to the molecular identification of the pore-forming component of MCUC. In parallel, regulatory subunits have been shown to affect channel activity and/or mitochondrial calcium uptake in several cell types. While the presence of the pore-forming subunit and of at least one regulatory subunit is a recurrent feature throughout different kingdoms, the actual composition of MCUC greatly varies in different organisms. Interestingly, complexity of MCUC does not necessarily reflect evolutionary order. At the current stage, the mammalian MCUC appears to consist of at least of the pore-forming protein MCU, an MCU paralog (MCUb), the essential MCU regulator (EMRE), the regulatory MICU proteins, and, possibly, the mitochondrial calcium uniport regulator 1 (MCUR1), as discussed below.

#### **The Pore-Forming MCU Component, CCDC109A**

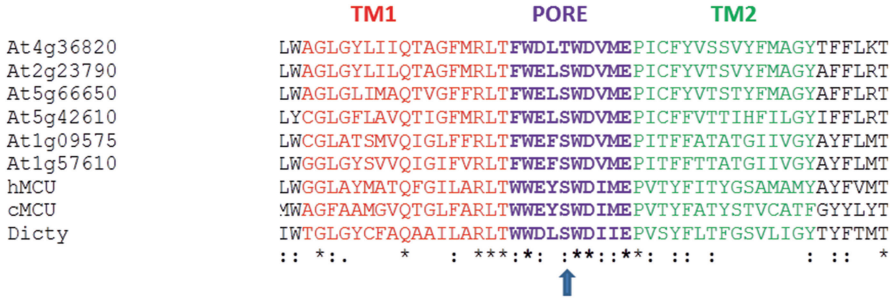
In 2011, the Mootha group and some of us independently identified the 40 kDa coiled-coiled protein CCDC109A that gives rise to  $\text{Ca}^{2+}$ -permeable channel activity. This protein called mitochondrial calcium uniporter (MCU) was proposed to be the

core component of the calcium uniporter of the inner mitochondrial membrane (Baughman et al. 2011; De Stefani et al. 2011) as it is a phylogenetically ancient molecule present in most eukaryotic taxa (Bick et al. 2012). The channel-forming and regulatory components of MCUC are currently studied by classical electrophysiological techniques, either using recombinant proteins or by direct patch clamping of mitoplasts (Fig. 2.1).

In the first paper where CCDC109A was shown to give rise to MCU channel activity, the protein was studied in planar lipid bilayers and displayed electrophysiological properties and inhibitor sensitivity of the uniporter (De Stefani et al. 2011), previously identified as a  $\text{Ca}^{2+}$ -permeable ion channel in patch-clamp experiments on mammalian mitoplasts (Kirichok et al. 2004). The channel was recorded in both works in 100 mM calcium gluconate solution displaying a conductance of 6–7 pS, an increased open probability with increasing negative voltages (on matrix side), and sensitivity to ruthenium red (RR) and gadolinium. The pore-forming nature of MCU was further proven by the following observations: (i) siRNA against the MCU



**Fig. 2.1** Electrophysiological studies on the components of the MCU complex. The pore-forming protein MCU has been investigated in various studies either using the bilayer system with recombinant proteins (*left part*) or by direct patch clamping of mitoplasts (*right part*). The effect of regulators can be evaluated with both techniques, e.g., by co-incorporation of the regulator and the channel into proteoliposomes or by patch clamping of mitoplasts from knockout cells/animals lacking regulatory proteins. Shown structure of MCU is from PDB database (5ID3)



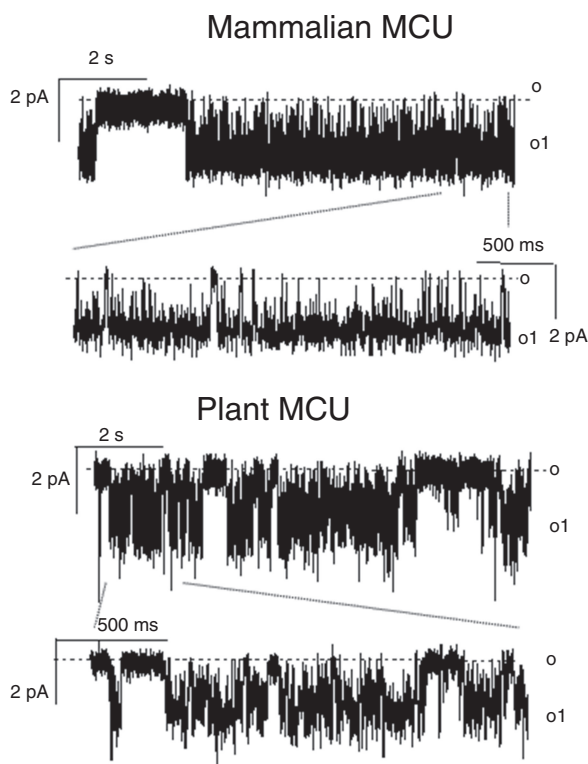
**Fig. 2.2** Amino acid sequence alignment of the transmembrane segments (TM) and the pore region of MCU from different species. Sequences for the six *Arabidopsis* isoforms and MCU from human (hMCU), from *C. elegans* (cMCU), and from *Dictyostelium* (Dicty) are shown. Asterisks show identical residues, while : indicates conservative substitutions. Arrow indicates the serine residue responsible for ruthenium red sensitivity

protein abolished the mitochondrial calcium uptake (De Stefani et al. 2011) (ii) siRNA against MCU abolished the calcium current recorded in mitoplasts (Chaudhuri et al. 2013). (iii) single-point mutations of specific residues in the pore region (see below) abolished channel activity (De Stefani et al. 2011). MCU does not share amino acid sequence similarity with known calcium channels in plants or animals, but its pore region contains several negatively charged amino acids that are crucial for calcium transport, since mutation of these residues abolished mitochondrial calcium uptake as well as calcium-carrying channel formation (De Stefani et al. 2011). In this region, a highly conserved serine residue is involved in binding of the inhibitor RR. These amino acids are highly conserved in the pore region of all species harboring homologs of the mammalian MCU, while much less similarity is present in the predicted transmembrane regions. MCU homologs are found in protozoa from diverse clades including kinetoplasts (*Trypanosoma cruzi*), heterolobosea (*Naegleria gruberi*), oomycetes (*Phytophthora infestans*), and ciliates (*Tetrahymena thermophila*). MCU homologs are also present in many fungi, including many basidiomycetes and *Allomyces macrogynus*, but are absent from yeast (Bick et al. 2012). In the model plant *Arabidopsis thaliana*, six isoforms are present. The amino acid sequences of the highly conserved pore region are shown for some of the MCUs in Fig. 2.2.

Despite the discovery that MCU homologs are present in different organisms and that the primary structure can be very different among species (except for the pore region), direct biophysical characterization of MCUs has been obtained solely for mammalian and plant proteins (Teardo et al. 2017). These studies reveal that the single channel conductance in calcium and the kinetic behavior are similar for mammalian and plant MCUs and that in both cases, sodium can permeate the channel when divalent cations are not present in the recording medium (Fig. 2.3). This aspect is typical of classical calcium channels (Talavera and Nilius 2006).

The consensus view concerning the topology of MCU is that both N- and C-terminal domains face the mitochondrial matrix, with the two membrane-

**Fig. 2.3** Animal and plant MCU display similar activities in bilayer experiments. As mentioned in the text, MCU can conduct sodium in the absence of divalent cations. Current traces were recorded for all cases in 100 mM sodium gluconate, 10 mM Hepes, 5 mM NaEDTA, pH 7.3/NaOH. Applied voltage (on cis side of the chamber) is  $-40$  mV. Current traces on expanded time scale are also shown



spanning domains connected in the intermembrane space (IMS) by the short pore region. The structure of the N-terminal domain has been resolved first, revealing that the N-terminal domain preceding the first coiled domain is essential for the modulation of MCU function: overexpression of MCU lacking this domain had a dominant-negative effect on mitochondrial  $\text{Ca}^{2+}$  uptake (Lee et al. 2015). A more recent structural study suggests that similarly to some other classical ion channels, pentamerization of the two transmembrane helix-containing subunits is required for formation of a functional channel (Oxenoid et al. 2016).

SILAC-based quantitative proteomics showed varying expression level of MCU in different mouse tissues, with cerebellum and gut showing highest level (Murgia and Rizzuto 2015). Several ion channels (including mitochondrial ones) display an altered expression in cancer cells, but this is apparently not the case of MCU (Peruzzo et al. 2016). Interestingly, while only one isoform of MCU is present in most organisms, six homologs of *MCU* were identified in the genomes of maize and *Arabidopsis* (Stael et al. 2012; Meng et al. 2015) (see also Fig. 2.2). The first proteomic evidence from *Arabidopsis* and potato suggests the presence of specific MCU homologs in mitochondrial fractions at low relative abundance, in accordance with MCU being an organellar ion channel (Wagner et al. 2015). The diversification of *MCU* genes in plants may provide regulatory flexibility on the different

levels of gene expression, transcription, translation, and posttranslational organization/regulation. This idea is supported by differential tissue-dependent expression of *MCU* genes (Stael et al. 2012; Meng et al. 2015; Teardo et al. 2017).

#### *The Dominant-Negative Pore-Forming MCU Component, CCDC109B*

Mammalian MCU activity can be regulated through its paralog, CCDC109B/MCUB. MCUB is a 33 kDa protein with a very similar structure to MCU but devoid of calcium-permeable channel activity in bilayer experiments due to the substitution of key amino acids in the pore-forming region. The protein however is still able to form sodium-permeable channel in the absence of divalent cations (Raffaello et al. 2013, Raffaello et al. 2016). MCUB and MCU have been shown to interact and to form hetero-oligomers. When proteoliposomes contained both MCU and MCUB, the presence of MCUB decreased the likelihood of observing calcium-permeable channel activity, strongly indicating that MCUB is a dominant-negative regulatory subunit. In intact cells, overexpression of MCUB reduced the amplitude of calcium uptake into mitochondria, whereas MCUB silencing had the opposite effect, further proving that MCUB incorporates into the uniporter channel oligomer and reduces its activity. The ratio between MCU and MCUB might define the stoichiometry of channel assembly, thus setting a cell-specific baseline of MCU activity in various tissues. Indeed, MCU activity, as recorded by patch clamping of mitoplast, greatly varies among different tissues (Fieni et al. 2012). The molecular basis of this difference may lay in the ratio between MCU and MCUB that, at least at the mRNA level, has been shown to vary in different tissues. Interestingly, tissues characterized by low-amplitude mitochondrial calcium transients, such as the heart, show a relative abundance of MCUB, compared, for example, to skeletal muscle.

#### **MICU Proteins: The EF-Hand Containing Regulatory Subunits**

The mammalian MICU (mitochondrial calcium uptake) protein family consists of three members that share more than 40% sequence identity: (i) MICU1, (ii) MICU2, and (iii) MICU3. MICU1 was the first of the components of the complex to be described: it is a 50 kDa protein with two functional and two pseudo EF-hands that resides in the mitochondrial intermembrane space (Csordas et al. 2013; Patron et al. 2014; Hung et al. 2014; Petrungaro et al. 2015; Wang et al. 2014). MICU2 is a paralog of MICU1 with 27% sequence identity. It was first described as a protein whose silencing resulted in reduced mitochondrial calcium clearance in response to large extramitochondrial calcium pulses. Finally, comparative genomics analyses revealed also the presence of a third protein, MICU3. Whereas MICU1 and MICU2 had wide tissue expression, MICU3 was found to be almost exclusively expressed in neural tissues (Plovanich et al. 2013), and at present, the precise molecular function MICU3 remains unclear. All three proteins as well as their homologs in other organism harbor calcium-binding EF-hand helix-loop-helix motifs. The presence of EF-hands is a typical feature of  $\text{Ca}^{2+}$  sensors in animals in plants, but not every

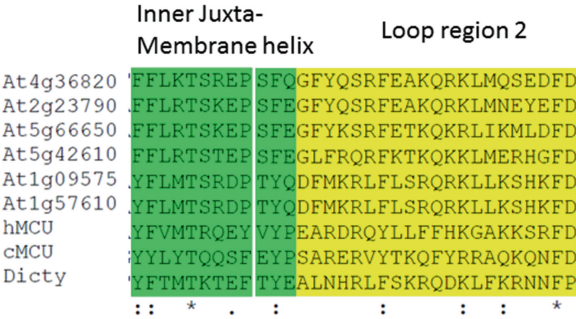
$\text{Ca}^{2+}$ -binding protein carries an EF-hand, and not every EF-hand binds  $\text{Ca}^{2+}$  (e.g., Gelhaye et al. 2004). Recent evidence indicates that increases in cytosolic  $\text{Ca}^{2+}$  are sufficient to induce rearrangement of MICU1 multimers and to trigger activation of mitochondrial  $\text{Ca}^{2+}$  uptake ( $\text{EC}_{50}$  of 4.4  $\mu\text{M}$ ) (Waldeck-Weiermair et al. 2015), in agreement with the emerging hypothesis that at low extramitochondrial  $\text{Ca}^{2+}$  concentrations MICU1 exerts a gatekeeping function, while it activates the channel when surrounding  $\text{Ca}^{2+}$  concentrations are high (Csordas et al. 2013; Mallilankaraman et al. 2012b). According to this hypothesis, MICU1 is sufficient to control calcium flux into mitochondria (Garg and Kirichok 2016; Tsai et al. 2016). Recombinant MICU1 was shown to directly increase MCU activity in planar lipid bilayer experiments in the presence of calcium (Patron et al. 2014). Multiple experimental evidence indicates that in resting conditions, MICU1-MICU2 heterodimers act as the MCU gatekeeper, while increases in calcium concentration, by inducing a conformational change in the dimer, would release MICU2-dependent inhibition and trigger MICU1-mediated enhancement of MCU channel activity (Patron et al. 2014). MICU2 indeed forms a heterodimer with MICU1 through an intermolecular disulfide bond and closes the channel at low extramitochondrial  $\text{Ca}^{2+}$  concentrations (Patron et al. 2014; Petrungaro et al. 2015). The stability of MICU2 depends on MICU1 (Plovanich et al. 2013; Patron et al. 2014), and loss of MICU2 in MICU1-silenced cells renders the difficult assignment of individual MICU1 and MICU2 functions. In summary, currently two models propose either MICU1 (1) to act exclusively as a uniporter activator at high cytosolic  $\text{Ca}^{2+}$  concentrations (Patron et al. 2014) or (2) to gradually disinhibit the uniporter with increasing  $\text{Ca}^{2+}$  concentrations in the cytosol (Csordas et al. 2013). However, hetero-dimerization with the ubiquitous MICU2 should be taken into account, at least in mammals when trying to describe models that best reflect the in vivo situation.

In addition to MICU1, skeletal muscle-specific alternative splice isoform of MICU1, MICU1.1, characterized by the addition of a micro-exon has recently been described (Vecellio Reane et al. 2016). MICU1.1 was shown to bind  $\text{Ca}^{2+}$  one order of magnitude more efficiently than MICU1 and activated MCU-mediated calcium uptake at lower  $\text{Ca}^{2+}$  concentrations than MICU1-MICU2 heterodimers.

MICU protein is conserved also in plants, where typically one or two homologs can be found depending on species (Wagner et al. 2015). *Arabidopsis* possesses only a single *MICU* gene, and knockout strongly affects mitochondrial  $\text{Ca}^{2+}$  dynamics (Wagner et al. 2015). *Arabidopsis* MICU contains an additional, third canonical EF-hand motif, which is conserved among plants and protists but is absent in mammalian MICUs and may open additional complexity of the regulation of MCUC activity by calcium.

## Essential MCU Regulator EMRE

Another proposed core component of the mammalian MCUC is EMRE, a 10 kDa metazoan-specific protein that spans the inner mitochondrial membrane with only one transmembrane motif. Although the recombinant MCU protein when inserted



**Fig. 2.4** Amino acid sequence alignment of the inner juxta membrane helix and of the loop region. Only few amino acids are conserved among different species in the regions proposed to be important for calcium conduction within the channel. Sequences for the six *Arabidopsis* isoforms and MCU from human (*hMCU*), from *C. elegans* (*cMCU*), and from *Dictyostelium* (*Dicty*) are shown. Asterisks show identical residues, while : indicates conservative substitutions. See text for further details

into a bilayer membrane in electrophysiological experiments alone is able to form a functional channel (De Stefani et al. 2011), in vivo EMRE seems to be required for channel formation, at least in mammals. A physical interaction between transmembrane helices between mammalian MCU and EMRE has been shown to take place (Tsai et al. 2016). Homologs of EMRE are not present in plants, fungi, or protozoa, and it has been recently shown that EMRE is required for  $\text{Ca}^{2+}$  uptake in the case of mammalian MCU, but not of MCU from the slime mold *Dictyostelium discoideum*: while expression of MCU from *Dictyostelium* alone was sufficient to import  $\text{Ca}^{2+}$  into yeast mitochondria (which lacks MCUC), human EMRE needed to be expressed alongside MCU to form an active  $\text{Ca}^{2+}$  uniporter system (Kovacs-Bogdan et al. 2014). Similarly to *Dictyostelium*, plants possess a minimal genetic uniporter configuration that lacks EMRE (Wagner et al. 2015, 2016).

Information about the structure of *C. elegans* MCU has recently become available: it was hypothesized that the outer and inner juxtamembrane helices as well as the loop region L2 are unstable regions which may undergo conformational changes upon activation by EMRE in order to create the lateral exit path for  $\text{Ca}^{2+}$  (Oxenoid et al. 2016). In plant and *Dictyostelium* MCUs, relatively few amino acids are conserved in the regions proposed to be important for the regulation of the *C. elegans* channel (Fig. 2.4).

Further research is needed to understand whether differences in these regions might account for the differential participation of EMRE in channel activity, and in general, mutations of the very few highly conserved amino acids in these regions might bring to the fine elucidation of ion permeation through this novel type of calcium channel. EMRE has been proposed to have another role as well, i.e., to bridge MCU and its regulators MICU1/MICU2 and thus to be indispensable for the

activity of the mammalian uniporter in vivo (Sancak et al. 2013). However, when the binding properties of MCU/EMRE with MICU1 and MICU1.1 were investigated by surface plasmon resonance analysis, at least under the used experimental conditions (in vitro), EMRE was not found to be not involved in MCU-MICU1 interaction, in accordance with the electrophysiological data obtained regarding the effect of MICU1 on MCU activity (Vecellio Reane et al. 2016). Recently, EMRE was proposed to regulate MCU channel activity depending on the matrix  $\text{Ca}^{2+}$  concentration (Vais et al. 2016). Altogether, the role of EMRE is far from being clarified, even though MCU together with EMRE and MICU1 has been proposed to correspond to the minimal configuration of MCUC (Tsai et al. 2016). This statement however cannot be true for organisms where EMRE or a homolog is not present in the genome (like in plants and slime mold). It cannot be a priori excluded that a still unidentified protein fulfills the same function in plants and other EMRE-lacking organisms. Likewise, the possibility that EMRE helps the correct membrane insertion/folding of MCU cannot be dismissed as to date yet.

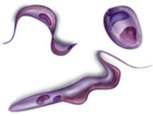



### MCU Regulator 1 (MCUR1)

MCUR1 (mitochondrial calcium uniporter regulator 1)/CCDC90A is a 39 kDa protein with two predicted transmembrane domains that is supposed to interact with MCU (Mallilankaraman et al. 2012a) although later studies were unable to support this interaction (Sancak et al. 2013; Paupe et al. 2015). Paupe et al. (2015) provided evidence that MCUR1 is in fact an assembly factor of cytochrome *c* oxidase and argued that genetic manipulation modulates mitochondrial membrane potential, imposing only a secondary effect on  $\text{Ca}^{2+}$  transport. In support of this notion, MCUR1 has an orthologue in budding yeast which lacks core MCUC components. Although Vais et al. (2015) recently showed that MCUR1 affects MCU activity in patch-clamp experiments, direct regulation of  $\text{Ca}^{2+}$  uniport through MCUR1 is still debated. *Arabidopsis* possesses two MCUR1 homologs that lack functional characterization. Interestingly one of them has been identified as a plant-specific subunit of complex IV by proteome analysis (Millar et al. 2004; Klodmann et al. 2011).

Altogether, functional MCUC has different components in different organisms, with MCU and MICU family members being the only highly conserved constituents. Figure 2.5 shows the composition of MCUC in organisms where characteristics of this complex have been studied in detail.

#### 2.4.2.2 Alternative Calcium Uptake Pathways

Additional  $\text{Ca}^{2+}$  uptake mechanisms in mammalian mitochondria were proposed by several groups still before the discovery of MCU.  $\text{Ca}^{2+}$  transients in mammalian cell culture where MCU expression is knocked down (De Stefani et al. 2011; Baughman

	<i>Trypanosoma cruzi</i>	<i>Dictyostelium discoideum</i>	<i>Arabidopsis thaliana</i>	<i>Mus musculus</i>
MCUC component				
MCU	+	+	+ (6 isoforms)	+
MCUb	-	-	-	+
EMRE	-	-	-	+
MICU	+	+	+	+ (3 isoforms)
MCUR	-	-	+ (2 isoforms)	+

**Fig. 2.5** The presence of MCUC components in different organisms. See text for further details

et al. 2011; Bondarenko et al. 2014) and in the liver of *mcu* animals (Pan et al. 2013) are very efficiently abolished indicating that the MCUC has a dominating role among uptake mechanisms. This of course does not rule out the possibility that other mechanisms make major contributions to  $\text{Ca}^{2+}$  uptake, for example, during a specific developmental stage or in a specific tissue. Indeed, specific mitochondrial  $\text{Ca}^{2+}$  uptake modes (e.g.,  $\text{Ca}^{2+}$ -selective conductance (mCa) 1 and 2 and rapid mode of uptake (RaM)) have been observed in animals, which however currently cannot be ascribed to well-defined molecules. These uptake modes apparently differ from MCUC-mediated  $\text{Ca}^{2+}$  uptake in terms of uptake kinetics, pharmacology, and  $\text{Ca}^{2+}$  affinity (Sparagna et al. 1995; Michels et al. 2009). Potential candidates for these distinct uptake modes include uncoupling proteins 2 and 3 (UCP2/UCP3), the transient receptor potential channel TRPC3, and ryanodine receptor RyR1. UCP2/UCP3 were originally proposed to be an essential components of mitochondrial  $\text{Ca}^{2+}$  uniport (Trenker et al. 2007), but currently it seems more likely that it has indirect effects on  $\text{Ca}^{2+}$  uptake into mitochondria (Brookes et al. 2008; De Marchi et al. 2011; Bondarenko et al. 2015). The mitochondrial ryanodine receptor (mRyR1) belongs to the RyR family that exists as three isoforms (RyR1–3) in animals but has no homologs in plants (Krinke et al. 2007). A low level of RyR1 is detectable in the IMM of heart mitochondria and provides rapid transport of  $\text{Ca}^{2+}$  that is insensitive to ruthenium red (Beutner et al. 2001, 2005). A small fraction of TRPC3 was found to be localized to mitochondria. It was then proven by genetic means that a significant fraction of mitochondrial  $\text{Ca}^{2+}$  uptake relies on TRPC3 expression (Wang et al. 2015; Feng et al. 2013). In summary, the abovementioned alternative pathways should be taken into account when interpreting the phenotypes observed in MCU knockdown systems. Finally, the hypothesis that MCUC is responsible also for the different uptake modes, at least in some cell types, cannot be formally excluded.

## 2.5 Mitochondrial $\text{Ca}^{2+}$ Export

Following the transient accumulation of calcium in the matrix, a part of this ion is exported, and a part remains inside as non-free calcium. The exact chemical states of bound  $\text{Ca}^{2+}$  inside the matrix of the living cell and the relative contributions of proteins, metabolites, and  $\text{Pi}$  are largely unclear in both animals and plants.  $\text{Ca}^{2+}$  can be extruded from mitochondria by an antiport mechanisms giving rise to the so-called  $\text{Ca}^{2+}$  cycle (Carafoli 1979). This way matrix  $\text{Ca}^{2+}$  concentrations are regulated in order to avoid overload, which can be deleterious for mitochondrial function (see above). Two known  $\text{Ca}^{2+}$  export systems are two exchangers, namely, the cation/cation exchanger family member  $\text{Na}^+/\text{Ca}^{2+}$  exchanger (Crompton et al. 1977, 1978) and a  $\text{H}^+/\text{Ca}^{2+}$  exchanger (Akerman 1978; Fiskum and Lehninger 1979) of the cation/proton exchanger family.

### 2.5.1 *The Sodium-Calcium Exchanger NCLX and the Proton-Calcium Exchanger CAX*

The mammalian protein NCLX ( $\text{Na}/\text{Li}/\text{Ca}$  exchanger) (Palty et al. 2010) has been proposed to underlie molecular entity of electrogenic  $\text{Ca}^{2+}$  transport against  $\text{Na}^+$ . De Marchi et al. (2014) have provided further relevant evidence that NCLX represents the long-sought mediator of  $\text{Ca}^{2+}$  export from the mitochondrial matrix. As to CAX, this protein was located to mitochondria in *Plasmodium falciparum*, where it mediates  $\text{Ca}^{2+}$  efflux from the mitochondrial matrix (Rotmann et al. 2010). In other organisms the mechanisms of calcium release from mitochondria is less clear.

### 2.5.2 *Leucine Zipper-EF-Hand-Containing Transmembrane Protein1 (LETM1)*

LETM1 is a one-transmembrane segment-containing protein that is located in the mitochondrial inner membrane and is defective in Wolf-Hirschhorn syndrome (Zollino et al. 2003; Endeley et al. 1999; Dimmer et al. 2008). Initially proposed to act as an  $\text{K}^+/\text{H}^+$ -exchanger (Nowikovsky et al. 2004; Dimmer et al. 2008), a genome-wide RNAi screen for proteins mediating mitochondrial  $\text{Ca}^{2+}$  dynamics identified LETM1 as a  $\text{Ca}^{2+}/\text{H}^+$  antiporter (Jiang et al. 2009; Waldeck-Weiermair et al. 2011; Tsai et al. 2014; Doonan et al. 2014). In vitro, LETM1 has been proposed to function as electroneutral  $\text{Ca}^{2+}/\text{H}^+$  antiporter (Tsai et al. 2014). Recent electron microscopy studies reveal a hexameric structure with a central cavity and with two different conformational states under alkaline and acidic conditions (Shao et al. 2016). While a  $\text{H}^+$ -driven  $\text{Ca}^{2+}$  export by LETM1 is plausible, whether

LETM1 in vivo functions as  $\text{Ca}^{2+}/\text{H}^{+}$  antiporter or as a  $\text{K}^{+}/\text{H}^{+}$  exchanger, as proposed by Nowikovsky and Bernardi (2014), is still a highly debated issue. Several arguments point to LETM1 working as potassium/proton antiporter in intact mitochondria. For example, changes in mitochondrial morphology with altered LETM1 expression could be reverted through the ionophore nigericin that specifically mediates  $\text{K}^{+}/\text{H}^{+}$ -exchange (Nowikovsky et al. 2004). Interestingly, high-level expression of LETM1 was found to be an independent poor prognostic factor of breast cancer (Li et al. 2015). In plants, the *Arabidopsis* genome contains two genes with homology to *LETM1* (Zhang et al. 2012). Partial depletion of LETM did not affect mitochondrial morphology. Instead, mitochondrial protein translation was altered, possibly as a secondary effect of disrupted  $\text{K}^{+}$  homeostasis (Hashimi et al. 2013), based on the observation that nigericin rescued the translation phenotype in cultured yeast cells.

### 2.5.3 Permeability Transition Pore

Transient opening of the mitochondrial permeability transition pore (PTP) has been proposed to cause release of  $\text{Ca}^{2+}$  from mammalian mitochondria (Bernardi and von Stockum 2012). At partial loss of membrane potential due to the opening of PTP, a large  $\text{Ca}^{2+}$  gradient (expected only at  $\text{Ca}^{2+}$  overload) would allow  $\text{Ca}^{2+}$  extrusion in a passive way. However, a partially or fully dissipated electrochemical gradient would not only allow  $\text{Ca}^{2+}$  extrusion but also severely interfere with matrix physiology, including ATP/ADP exchange,  $\text{P}_i$  uptake, and metabolite shuttling, which strictly depend on the proton motive force. Thus, it seems likely that such  $\text{Ca}^{2+}$  release via PTP occurs under specific, pathological conditions. Experimental evidence is still missing to either prove or disprove the above hypotheses.

## 2.6 Pathophysiological In Vivo Consequences of Alteration of Mitochondrial Calcium Homeostasis by Genetic Tools

Following the identification of proteins playing fundamental roles in the calcium uptake and exit pathways, the field of mitochondrial calcium signaling experienced a period of “Renaissance.” Finally, fine dissection of the molecular pathways governing mitochondrial calcium homeostasis has become feasible using genetic tools. However, when interpreting the final outcome of knockout or knockdown experiments in terms of calcium levels, of metabolism, and of cell fate, one has to keep in mind that genetic manipulation of one MCUC component might lead to altered expression of (another) component(s) as well (e.g., when MICU1 is silenced, a dramatic reduction also of MICU2 protein occurs (Patron et al. 2014).

A few recent in vivo studies demonstrate that mitochondrial calcium homeostasis is crucial for regulation of metabolism, and its alterations are linked to

pathologies. Genetic manipulation of MCU in lower organisms such as zebra fish (Prudent et al. 2013) and *Trypanosoma brucei* (Huang et al. 2013) resulted in major developmental and energetic defects, although such effect was not accentuated in the knockout mouse model, possibly due to compensatory mechanisms. Low levels of basal matrix calcium in the *MCU*<sup>-/-</sup> mice led to markedly increased levels of PDH phosphorylation (Pan et al. 2013). In another work, postnatal manipulation of MCU levels in mice (by using adeno-associated virus-mediated gene transfer) demonstrated the contribution of MCU to the regulation of skeletal muscle tropism. MCU overexpression or downregulation caused muscular hypertrophy or atrophy, respectively, likely independent of metabolic alterations but dependent on a novel Ca<sup>2+</sup>-dependent mitochondria-to-nucleus signaling pathway via transcriptional regulators (Mammucari et al. 2015). In mice with myocardial MCU inhibition, obtained by transgenic expression of a dominant-negative (DN) MCU, a strong correlation between MCU function, MCU-enhanced oxidative phosphorylation, and correct pacemaker cell function was observed (Wu et al. 2015). In addition, *in vivo* evidence exists in favor of a serine/threonine kinase LKB1-mediated regulation of MCU expression that controls mitochondrial calcium uptake and neurotransmitter release properties in a bouton-specific way through presynaptic Ca<sup>2+</sup> clearance (Kwon et al. 2016). Changes of mitochondrial calcium level in neurons activated by insulin-like growth factor-1 receptor signaling also constitute a critical regulator of information processing in hippocampal neurons by maintaining evoked-to-spontaneous transmission ratio as assessed *in vivo* (Gazit et al. 2016). Furthermore, inhibition of MCU in *Drosophila*, during development in a brain region that is critical for olfactory memory formation, caused memory impairment in adults without altering the capacity to learn (Drago and Davis 2016). Lack of one of the MCU isoforms of *Arabidopsis* with prevalent expression in roots caused a profoundly altered mitochondrial ultrastructure and shortened root length in intact plants (Teardo et al. 2017).

As to the regulator, MICU1, mitochondria in a mouse model of MICU1 deficiency showed altered calcium uptake. Deletion of MICU1 resulted in significant perinatal mortality. MICU1 knockout animals displayed increased resting mitochondrial calcium levels, altered mitochondrial morphology, and reduced ATP. Deletion of one allele of EMRE helped to normalize calcium uptake while simultaneously rescuing the high perinatal mortality observed in young MICU1<sup>-/-</sup> mice (Liu et al. 2016). In humans, homozygous patients carrying a loss-of-function mutation of MICU1 are characterized by myopathy, cognitive impairment, and extrapyramidal movement disorder (Logan et al. 2014), along with an increased agonist-induced mitochondrial Ca<sup>2+</sup> uptake at low cytosolic Ca<sup>2+</sup> concentrations and a decreased cytosolic Ca<sup>2+</sup> signal. However, at least under resting conditions, the fibroblasts from affected individuals do not display defects in overall cellular metabolic function, but chronic elevation of the mitochondrial matrix Ca<sup>2+</sup> load seems to lead to moderate mitochondrial stress, resulting in fragmentation of the mitochondrial network. In addition, homozygous deletion of exon 1 of MICU1 was shown to be associated with fatigue and lethargy in children with normal mitochondrial oxidative phosphorylation enzyme activities in muscle (Lewis-Smith et al. 2016).

## 2.7 Conclusion

As witnessed by the above-reported data, a considerable advancement has been achieved in the field of mitochondrial calcium handling in the last few years. Now, an even more exciting and stimulating era is expected to come. Following the identification of the calcium uptake and exit machineries, research will most probably focus on understanding the fine regulation of these components, e.g., by posttranslational modifications, and on elucidation of their role in different physiological and pathologic situations. Hopefully, the recently obtained information regarding the structure of several components will also prompt smart drug design in order to fully exploit the information arising in the field, in the context of pathologies linked to altered mitochondrial calcium handling.

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