Chapter 2
Mitochondrial Calcium Handling in Physiology and Disease

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Calcium as Important Second Messenger

In all multicellular organisms, a precise and efficient cell-to-cell communication is required for coordinating the vast majority of physiological processes. Hundreds of diverse molecules (e.g. ions, metabolites, peptides and proteins) can carry information from one cell to another, thus acting as signaling entities that interact with specific receptors generally located on the plasma membrane. Despite this huge complexity, only a small number of intracellular transduction pathways (e.g. Ca^{2+}, cAMP or phosphorylation cascades) can ensure the proper decoding of all these diverse signals. One of the most important intracellular messengers is Ca^{2+}. Indeed, between cytosol and the extracellular environment there are both chemical and electrical gradients that represent the main driving force for changes of intracellular Ca^{2+} concentration ($[Ca^{2+}]$). Cells invest a substantial quota of their energy to guarantee this electrochemical gradient to allow rapid and transient changes in $[Ca^{2+}]$ that can largely differ through space and time [1], thus generating a plethora of signaling patterns that can be differentially decoded. Ca^{2+}-dependent signal transduction requires the binding to buffering proteins, the compartmentalization into intracellular compartments and the extrusion outside the cells [2]. Ca^{2+} binding triggers changes in protein shape and charge and consequently activates or inhibits protein functions. The best known Ca^{2+}-binding protein is calmodulin that, in concert with other Ca^{2+} buffers and intracellular compartments, controls the spatiotemporal patterning of Ca^{2+} signals [3]. These dynamic changes of $[Ca^{2+}]$ thus trigger a number of cellular events, including muscle contraction, hormone secretion, synaptic

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transmission, cellular proliferation, apoptosis and others [4–7]. The universality and versatility of Ca^{2+} signaling is thus guaranteed by a sophisticated machinery of ion channels, pumps, and exchangers that drive the flux of Ca^{2+} ions across the plasma membrane and across the membrane of intracellular organelles, such as the Endoplasmic Reticulum (ER) and mitochondria (see Chap. 10). During last decades, an increasing number of researchers focused their efforts in the identification and characterization of all the import/export mechanisms of Ca^{2+} homeostasis, but the whole scenario is still not complete. Ca^{2+} signaling can be activated through two main mechanisms. The first is the Ca^{2+} entry from the plasma membrane channels, which can be classified into four groups, Store Operated Ca^{2+} Channels (SOCs), which open following intracellular store depletion [8], Receptor Operated Ca^{2+} Channels (ROCs), which open after an external ligand binds the receptor [9], Voltage Operated Ca^{2+} Channel (VOCs), which are sensitive to decreases of membrane potential and Second Messenger Operated Ca^{2+} Channels (SMOCs) which open after binding of second messenger on the inner side of the membrane [10, 11]. The second mechanism implies Ca^{2+} release from intracellular depots. The most important store is the ER, but recent works demonstrated that also other organelles, such as Golgi apparatus, endosome and lysosome are able to participate in Ca^{2+} signaling [12, 13]. In this scenario, the best described pathway involves the release of IP_3 after activation of a G-protein coupled receptor, and the consequent Ca^{2+} release from the ER through the binding to the IP_3R. Other intracellular Ca^{2+}-releasing channels are the Ryanodine Receptor (RyR) [14] or the Two-pore channels (TPCs) activated by NAADP [15]. Termination of Ca^{2+} signals is finally completed through Ca^{2+} extrusion from the cytoplasm by various pumps and exchangers, e.g. SERCA (sarco-endoplasmic reticulum ATPase) or PMCA [16].

This chapter is primarily focused on the role of mitochondria in cellular Ca^{2+} handling. Indeed, many subcellular compartments, including mitochondria, are key regulators of Ca^{2+} signaling, by actively participating to shape global Ca^{2+} waves and determining specific cellular functions.

Biophysical and Structural Basis of Mitochondrial Ca^{2+} Handling

The relationship between mitochondria and Ca^{2+} dates back to more than 50 years ago, when Engstrom's group, in the 1961, demonstrated for the first time that energized mitochondria, when exposed to Ca^{2+} pulses, were able to rapidly and efficiently take up large quantity of this cation [17]. These organelles are characterized by a distinctive structure. They are double membrane-bounded organelles thought to be derived from an proteobacterium-like ancestor, presumably due to a single ancient invasion occurred more than 1.5 billion years ago [18, 19]. The basic evidence of this endosymbiont theory is the existence of the mitochondrial DNA (mtDNA), with structural and functional analogies to bacterial genomes. Mitochondria are defined by two structurally and functionally different membranes,

the outer membrane (OMM) and the inner membrane (IMM), characterized by invaginations called "cristae", which enclose the mitochondrial matrix. The space between these two structures is traditionally called intermembrane space (IMS). Cristae are not random folds, but rather internal compartments formed by profound invaginations, originating from very tiny "point-like structures" in the inner membrane [20]. These narrow tubular structures, called cristae junctions, can limit the diffusion of molecules from the intra-cristae space towards the IMS, thus creating a micro-environment where respiratory chain complexes, and also other proteins, are hosted and protected from random diffusion. The OMM contains high amount of a specific transport protein, VDAC (Voltage-Dependent Anion Channel), which is able to form pores on the membrane, becoming permeable to ions and metabolites up to 5 kDa [21]. Conversely, the IMM is a highly selective membrane that tightly controls the exchange of ions and metabolites through specialized molecular machineries. The chemiosmotic theory of energy transfer was first proposed by Peter Mitchell [22], who suggested that the electrochemical gradient across the IMM is utilized by the F_1/F_0 ATPase to convert the energy of NADH and $FADH_2$, generated by the breaking down of energy rich molecules, such as glucose or lipids, into ATP. This gradient ($\Delta\mu$) is composed by both electrical ($\Delta\Psi$) and chemical components (ΔpH). This potential represents a huge driving force that allows the accumulation of cations into the matrix, and is normally estimated to be approximately −180 mV [23]. Another key feature of mitochondria is their spatial organization in the cells. They are not solitary organelles, but they make contacts with several other structures, especially with the ER. Indeed, the physical and functional coupling of these two organelles in living cells was originally found to determine the Ca^{2+} and lipid transfer between the two organelles. Several works indeed underlined the presence of overlapping regions of two organelles and allowed to estimate the area of the contact sites as 5–20% of total mitochondrial surface [24, 25]. More recently, electron tomography techniques allowed to estimate an even smaller distance (10–25 nm), as well as the presence of trypsin-sensitive tethers between the two membranes [26].

In mammals, many proteins have been identified to be indirectly involved in the regulation of ER–mitochondria functional interaction, such as some chaperones, PACS-2, BAP31 and NOGO-A. Searching for the long-sought direct tether, de Brito and Scorrano and co-workers have recently identified MFN2 as important mammalian protein directly bridging the two organelles. MFN2 is enriched in the ER–mitochondria interface and connects ER with mitochondria via direct interactions between the protein localized in the ER and MFN1 or MFN2 present in the OMM. They also showed that genetic ablation of MFN2 causes an increase in the distance between the two organelles with a consequent impairment of mitochondrial Ca^{2+} uptake [27]. The mechanistic role of MFN2 in tethering the two organelles was confirmed in different systems [28–30], but this notion was also recently challenged [31, 32]. Nevertheless, there is a broad agreement in considering ER/mitochondria contact sites as critical determinant of the rapid transport of Ca^{2+} into the matrix. Indeed, some mitochondria are strategically positioned near the ER Ca^{2+} releasing sites, and are thus exposed to a microdomain where the local [Ca^{2+}] is

significantly higher compared to the bulk cytosol [24, 25, 33–37]. In addition, a specific direct coupling between the IP_3R and Ca^{2+} channels of the OMM has been described [38], thus further underlining the pivotal role that organelle tethering play in cellular Ca^{2+} homeostasis.

Mitochondrial Ca^{2+} Homeostasis

Mitochondrial matrix $[Ca^{2+}]$ ($[Ca^{2+}]_{mt}$) is the result of the dynamic equilibrium between two continuous and opposite processes, i.e. mitochondrial Ca^{2+} influx vs efflux. The basic functional characteristics of mitochondrial Ca^{2+} uptake and release have been firmly established in the 1960s and 1970s. Ca^{2+} influx requires an intact mitochondrial membrane potential (negative inside) and a so called electrogenic Ca^{2+} uniporter (i.e. resulting in the net transport of two positive charges the matrix) [17, 39, 40]. Conversely, the efflux from the matrix depends on two distinct mechanisms, (i) an ubiquitous H^+/Ca^{2+} exchange (likely to be electroneutral) [41] and (ii) a Na^+/Ca^{2+} exchange (likely to be electrogenic, with 3 or 4 Na^+ ions per Ca^{2+}) [42, 43].

Mitochondrial Ca^{2+} Uptake

3.1.1.The Mitochondrial Ca^{2+} Uniporter (MCU)

In the last 50 years, several efforts have been spent to identify the channel responsible for ruthenium red (RuR)-sensitive mitochondrial Ca^{2+} entry. However, only in the last 10 years significant successes have been accomplished. The first fundamental step was indeed obtained in Clapham's lab in the 2004, when the Ca^{2+} channel of the inner mitochondrial membrane was finally directly measured. In this work, single mitoplasts (i.e. mitochondria lacking the outer mitochondrial membrane) were isolated from COS7 cells and patch clamp was performed. Accordingly, it was unambiguously shown that organelle Ca^{2+} entry was mediated by a Mitochondrial Ca^{2+} Uniporter (MCU), here also named MiCa. This channel is inward rectifying, highly Ca^{2+}-selective, sensitive to RuR and characterized by an enormous Ca^{2+} carrying capacity (half-saturation at 19 mM) with no Ca^{2+}-dependent inactivation [44]. Nonetheless, despite the detailed electrophysiological characterization of MCU, its molecular identity remained a mystery. The first step in the right direction came in 2010, when the group of Vamsi Mootha identified a mitochondrial protein, named MICU1, specifically affecting mitochondrial Ca^{2+} uptake [45]. However, it was immediately evident that MICU1 was a regulator rather than the channel per se. Only 1 year later, our group and Mootha's lab independently identified another previously uncharacterized mitochondrial protein acting as the bona fide mitochondrial Ca^{2+} uniporter [46, 47]. The MCU gene (previously known as CCDC109A) is

broadly conserved among metazoans and plants, but absent in yeasts [48, 49], and encodes for a 40 kDa protein (running at 35 kDa due to the cleavage of the organelle localization peptide) with two coiled-coil domains and two transmembrane domains separated by a short loop (EYSWDIMEP). The loop is enriched in acidic residues and exposed to the intermembrane space, while the vast majority of the protein is located inside the matrix [50]. Residues E256, D260 and E263 are critical for Ca^{2+} channeling function, since their replacement with both uncharged and positively charged amino acids result in the loss of Ca^{2+} permeation [47, 51]. Moreover, substitution of Ser^{258} leads to decreased ruthenium red sensitivity [46, 52]. The recent elucidation of the 3D structure revealed that the functional channel is a homopentamer stabilized by the coiled-coil motif protruding into the mitochondrial matrix [53]. However, it is now clear that MCU is the key component of a higher order macromolecular complex, named the MCU complex, that represents the whole molecular machinery mediating the electrophoretic transport of Ca^{2+} cations inside the organelle matrix [54, 55]. MCU alone is necessary and sufficient (at least *in vitro*) for mitochondrial Ca^{2+} uptake, thus indicating that it represents the pore forming subunit [47], but what happens in vivo is still under intense investigation.

The MCU Complex

The first indication that MCU is part of a bigger complex was revealed even before the discovery of the channel itself, with the identification of MICU1 [45]. In the last 5 years, a growing amount of proteins have been shown to interact and modify MCU activity. At the moment, the general consensus is that the MCU complex can be composed by three different membrane components, MCU [46, 47], its isoform MCUb [51] and the recently identified EMRE (essential MCU regulator, previously known as C22orf32) [56], and some associated regulators (see later). The MCUb protein has a high similarity in sequence with MCU, but at least a couple of amino acid substitutions in the loop domain are predicted to be critical for ion permeation and suggest that this protein has a lower efficiency in Ca^{2+} transport. Accordingly, MCUb overexpression drastically reduces the mitochondrial Ca^{2+} uptake, while its silencing exerts the opposite effect, thus indicating that MCUb is the endogenous dominant-negative isoform of MCU [51]. Intriguingly, the MCU/MCUb expression ratio widely varies among tissues, suggesting that this mechanism could account at least in part for the differences in MCU activity displayed by different tissues [57]. On the other hand, EMRE is a small protein of about 10 kDa, with a predicted mitochondrial targeted sequence containing a single transmembrane domain and a highly conserved C-terminus rich in aspartate residues. This protein was identified in the 2013 using a quantitative mass spectrometry approach in cell culture (SILAC) [56]. As to its function, EMRE appears to be required for Ca^{2+} channeling activity in vivo and to mediate the binding of MICU1 to the channel. In the contrast to its essential role, EMRE homologs are not present in plants, fungi or protozoa in with MCU and MICU1 are highly conserved. However, knockout of EMRE is able to abrogate the mitochondrial Ca^{2+} uptake and that the overexpression of MCU in

these conditions was not able per se to restore the normal mitochondrial Ca^{2+} uptake. More recently, Foskett and colleagues proposed that the C-terminal region of EMRE acts as Ca^{2+} sensor and ensures the inhibition of uniporter activity under high matrix [Ca^{2+}] [58]. However, the membrane topology of EMRE is still debated and additional studies will be necessary to carefully dissect its contribution to the whole MCU complex. In addition to these core membrane components, the MCU complex also includes soluble components localized in the intermembrane space that accounts for the so-called sigmoidicity of the mitochondrial Ca^{2+} uptake system.

The Sigmoidicity of Mitochondrial Ca^{2+} Uptake

One well established feature of organelle Ca^{2+} handling is the sigmoidal relationship between extramitochondrial [Ca^{2+}] and Ca^{2+} uptake into the matrix. In resting cells, [Ca^{2+}] in the cytosol ([Ca^{2+}]$_{cyt}$) is approximately 100 nM, thanks to the activity of Ca^{2+}-ATPases (e.g. PMCA, SERCA, SPCA). In this condition, MCU activity is very low even in front of the huge driving force represented by the mitochondrial membrane potential, in order to prevent vicious cycling of the cation and consequent energy drain. As soon as Ca^{2+}-signaling is activated, [Ca^{2+}]$_{cyt}$ rises and MCU gets activated and leads to a very large cation accumulation inside the matrix, thus ensuring rapid mitochondrial Ca^{2+} uptake. This behavior requires both a gatekeeper (i.e. a MCU inhibitor working at resting [Ca^{2+}]$_{cyt}$) as well as cooperative activator (i.e. a MCU enhancer that becomes active after Ca^{2+}-signaling activation). Importantly, these features are not part of the MCU channel per se but they are rather provided by the MICU protein family, that includes three distinct members (MICU1, MICU2 and MICU3). Originally, MICU1 was shown to possess EF-hand domains and be associated with the inner mitochondrial membrane. Its silencing compromises mitochondrial Ca^{2+} uptake in intact cells and isolated organelle, without affecting mitochondrial membrane potential or respiration [45]. Few years later, Madesh and coworkers demonstrated that MICU1 inhibits channel function in unstimulated cells, thus proposing MICU1 as the essential gatekeeper of MCU-mediated mitochondrial Ca^{2+} uptake. Indeed, MICU1 silencing causes an increase of mitochondrial Ca^{2+} levels in basal conditions, sensitizing the cells to excessive ROS production and cell death [59]. In parallel, Hajnoczky's group confirmed the role of MICU1 as MCU gatekeeper, and further demonstrated that MICU1 also act as a cooperative activator of MCU activity at high cytosolic [Ca^{2+}] [60]. Finally, we recently added another level of complexity to this picture. Indeed, we found that MICU1 forms dimers with its isoform MICU2 through a disulfide bridge. Thus, according to our hypothesis, MICU1 and MICU2 represents a unique entity, widely expressed in all tissues, and represent the molecular machinery underlying the sigmoidicity of mitochondrial Ca^{2+} uptake. In this model, MICU1 acts predominantly as an MCU activator, although it can still retain some residual gatekeeping function in some situation. Conversely, MICU2 act as a genuine MCU inhibitor, thus guaranteeing the normal physiological activation threshold. Accordingly, at resting [Ca^{2+}], the prevailing

inhibitory effect of MICU2 ensures minimal Ca^{2+} accumulation in the presence of a very large driving force for cation accumulation, thus preventing the deleterious effects of Ca^{2+} cycling and matrix overload. As soon as extramitochondrial [Ca^{2+}] increases, Ca^{2+}-dependent inactivation of MICU2 and the concomitant activation of MICU1 guarantee the prompt initiation of rapid mitochondrial Ca^{2+} accumulation, thus stimulating aerobic metabolism and increasing ATP production [61].

Finally, other MCU regulators have been proposed, including MCUR1 (also known as CCDC90A) and SLC25A23 (also known as SCaMC-3). They are both are membrane proteins shown to interact with MCU and modulate its activity [62, 63]. However, these two protein are also involved in other processes, such as the assembly of complex IV [64] or ATP/Pi exchange [65–67] respectively. This opens the possibility that the functional effect of these proteins on mitochondrial Ca^{2+} homeostasis could be an indirect secondary effect, as better discussed elsewhere [68].

Mitochondrial Ca^{2+} Efflux

As to the Ca^{2+}-release pathways from mitochondria, their functional existence has been characterized already in the 1970s [41, 43]. Ca^{2+} efflux from mitochondria depends on two different mechanisms, one mediated by a $2H^+/Ca^{2+}$ antiporter (mHCX) and expressed in most of the cells and the other by a 3 (or 4) Na^+/Ca^{2+} exchanger (mNCX), predominant in excitable tissues. The molecular identity of the $2H^+/Ca^{2+}$ exchanger is still a matter of debate, but the recent works by Clapham and coworkers suggests that Letm1 is a strong candidate [69, 70], although this notion is not yet widely accepted [71, 72]. Conversely, a universal consensus exists on the identity of the mitochondrial Na^+/Ca^{2+} exchanger. Indeed, in 2010 Sekler and coworkers demonstrated that the gene NCLX (also known as NCKX6, SLC24A6 or SLC8B1) encodes for a mitochondrial protein necessary for Na^+-dependent Ca^{2+} clearance from the matrix [73]. Interestingly, many years before, an unidentified mitochondrial protein, but similar in molecular weight to NCLX, was reported to exhibit Na^+/Ca^{2+} exchange activity when purified and reconstituted in vitro [74, 75]. The NCLX protein shares only low sequence homology (approximately 20%) to Na^+/Ca^{2+} exchangers of the plasma membrane and the exact stoichiometry of Na^+/Ca^{2+} exchange is still unknown. Based on thermodynamic considerations, it should catalyze the exchange of 3–4 Na^+ every Ca^{2+}, thus causing the net import of one or two positive charges into the matrix. In line with this, molecular dynamics simulation based on the crystal structure of a bacterial Na^+/Ca^{2+} exchanger [76] suggests a 3:1 stoichiometry [77], that should apply also to mNCX. Up to now, the contribution of NCLX to mitochondrial Ca^{2+} extrusion has been confirmed in a variety of cellular models, including B lymphocytes, pancreatic beta cells, cardiomyocyte-like cell lines and astrocytes. Unfortunately, animal models lacking the NCLX gene are still missing at this moment, but in the future they will provide essential information on the physiopathological role of mitochondrial Ca^{2+} homeostasis.

The Pathophysiological Role of Mitochondrial Ca^{2+} Signaling

The molecular identification of the components of the MCU complex finally opened the possibility to address the physiological role of mitochondrial Ca^{2+} uptake by genetic approaches, thus overcoming the limits of classical pharmacological approaches, in particular considering the lack (or, at least, the poor performance) of MCU-targeting drugs suitable for in vivo applications. However, in the last three decades a huge amount of experimental work has been carried out using either pharmacological or indirect genetic tools to target mitochondrial Ca^{2+} homeostasis and dissect its patho-physiological role. According to these studies, the general consensus is that Ca^{2+} inside mitochondria acts a pleiotropic signal, with different cellular outcomes that depend on the investigated cell type, the metabolic state and the concomitant presence of other stress signals. On one hand, Ca^{2+} plays a regulatory role within the organelle itself ranging from the regulation of ATP production to the release of caspases cofactor with consequent cell death. On the other hand, mitochondrial Ca^{2+} can exert its function at the whole cell level, e.g. by regulating cation homeostasis both locally and globally [78]. However, in all the studies that preceded the molecular identification of MCU, the experimental approaches used to address the physiological relevance of mitochondrial Ca^{2+} homeostasis was (i) the use of drugs interfering, more or less specifically, with the Ca^{2+} uptake (e.g. uncouplers, ruthenium red, Ru360) or release (e.g. CGP37157) processes and (ii) genetic manipulations indirectly modifying the amount of Ca^{2+} that mitochondria can accumulate (e.g. regulation of Bcl2 family proteins or MAM-localized proteins). However, none of these approaches is specific, since they either modify other organelle functions (e.g. ATP synthesis), or affect extramitochondrial processes (e.g. Ca^{2+}-depletion of intracellular stores, IP_3R activity).

Even the data obtained with the most specific MCU inhibitors, i.e. ruthenium red and Ru360, should be interpreted with caution. Indeed, it has been shown that in several cellular models, the functional effects of these two compounds are not dependent on their inhibition of MCU [79], in line with the predicted poor membrane solubility of these drugs. Fortunately, the recent identification and elucidation of the MCU complex, although not yet definitive, has opened the field to genetic loss- or gain-of function approaches to unambiguously determine the role of mitochondrial Ca^{2+} uptake in organism pathophysiology. Due to the intrinsic novelty of these discoveries, the picture is still largely incomplete, and apparently contrasting data are being published. While the studies carried out in cultured cells concern both the overexpression and downregulation of all the proteins in the complex, in vivo models (at the moment this chapter was written) are available only for MCU KO and overexpression. Here, we will critically synthetize the main findings on the role of mitochondrial Ca^{2+} transport in both physiological and pathological conditions.

Regulation of Cellular Bioenergetics and Autophagy

In the last 5 years, the genetic manipulation of MCU complex components has been carried out in several cultured cells. Overall, the relationship between mitochondrial Ca^{2+} uptake and the regulation of energy production has been confirmed in most of them. For example, in pancreatic β-cells, glucose-induced insulin release is dependent on the stimulation of mitochondrial oxidative metabolism, and it involves both K_{ATP}-dependent [80] and K_{ATP}-independent [81] regulation of exocytosis. Stimulation of mitochondrial dehydrogenases by organelle matrix [Ca^{2+}] is a critical step critical in this process [82]. Indeed, MCU silencing impairs the Ca^{2+}-dependent phase of glucose-induced ATP increase [83, 84], and hence insulin secretion [85, 86]. In line with this, NCLX silencing temporally enhances the glucose-induced increase in oxidative phosphorylation [87, 88] and cellular ATP levels [84]. Interestingly, also silencing of MICU1 (that increases basal $[Ca^{2+}]_{mt}$ but reduces agonists-induced transients) has a similar effect in this cellular model [85], thus suggesting that tonic rises in $[Ca^{2+}]_{mt}$, and not constitutively high resting $[Ca^{2+}]_{mt}$, are decoded and translated into a boost of ATP production. This is further supported by the observation that, at least in HeLa cells, silencing of MICU1 do not modify basal respiration levels, but it rather dampens agonists-induced increase in oxygen consumption [59]. Overall, these data demonstrated that mitochondrial Ca^{2+} accumulation, mediated by MCU and modulated by NCLX, is thus required for normal glucose sensing by pancreatic β-cells. Moreover, direct coupling of mitochondrial Ca^{2+} transients and oxidative metabolism has also been confirmed in other cellular model (as well as in vivo, see below). Overexpression of MCU in human fibroblasts derived from both healthy subjects and patients affected by mitochondrial disorders can efficiently decrease AMPK phosphorylation levels [89], i.e. the major cellular metabolic sensor of AMP/ATP ratio. Similarly, MCU silencing enhances starvation-induced increase of phospho-AMPK levels in MDA-MB231, a breast cancer derived human cell line [90].

Autophagy is a broadly conserved adaptive response to energetic defects [91], as well as the leading mechanism for eliminating damaged organelles [92, 93]. Recently, a seminal work by Foskett and colleagues clearly demonstrated that mitochondrial Ca^{2+} signaling has a fundamental role in the regulation of autophagy [94]. In addition, the removal of damaged or non-functional mitochondria is regulated by a specific autophagic process named "mitophagy", that acts as a critical organelle quality control pathway [95]. In this context, mitochondrial Ca^{2+} has been recently shown to be a potential specific signal in the control of mitophagy. Indeed, down-regulation of ER to mitochondrial Ca^{2+} transfer can effectively decrease parkin-mediated organelle removal [96]. In addition, we also demonstrated a causal link between mitochondrial Ca^{2+} signaling and autophagy/mitophagy in a cellular model of mitochondrial disease [89]. Mitochondrial disorders are indeed a wide range of clinical syndromes caused by inherited mutations in the mitochondrial DNA (mtDNA). Given the complexity of mitochondrial genetics and biochemistry, the

clinical manifestations of mitochondrial disorders are extremely heterogeneous. They range from lesions of single tissues or structures, such as the optic nerve in Leber's hereditary optic neuropathy (LHON), to more widespread lesions including myopathies, encephalomyopathies, cardiomyopathies, or complex multisystem syndromes with onset ranging from neonatal to adult life, such as MELAS (mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes [97, 98]. In this pathological context, the direct correlation between mitochondrial Ca^{2+} and autophagy is supported by the demonstration that genetically or pharmacological approaches that enhance mitochondrial Ca^{2+} uptake can efficiently slow down the hyperactivated autophagic flux, through an AMPK-dependent mechanism [89]. Overall, the emerging picture consistently shows that the regulation of the autophagy is dependent on the modulation of oxidative metabolism by $[Ca^{2+}]_{mt}$. Accordingly, the silencing of MCU, MICU1 and MCUR1, all conditions that decrease organelle Ca^{2+} accumulation after IP_3-coupled stimuli, increase the formation of autophagosomes and are accompanied by higher AMPK phosphorylation in both HeLa and HEK293 cells [59, 63]. In conclusion, the positive role that $[Ca^{2+}]_{mt}$ exerts on energy production has been well confirmed by genetic approaches targeting the MCU complex in cultured cells.

Regulation of Cell Death

As to the Ca^{2+}-dependent regulation of cell death, the situation is more complex, with apparently slightly contrasting results. As reported above, all conditions that decreases $[Ca^{2+}]$ should in principle protect cells from toxic stimuli. In line with this view, MCU overexpression enhances cell death triggered by a variety of stimuli (including H_2O_2, C2-ceramide and NMDA) in HeLa cells [47, 99], cerebellar granule neurons (CGNs) [99] and primary cortical neurons [100]. Notably, in cortical neurons overexpression of MCU enhances cell death per se, even in the absence of excitotoxic agents [100], thus confirming the idea that different cell types have different susceptibility to mitochondrial Ca^{2+} overload. Accordingly, in the same experimental settings, MCU silencing exerted the opposite effect, i.e. protected cells from apoptosis [99, 100]. However, in one report, primary human epithelial cells (HMEC), but not HeLa or MDA-MB-231 (a breast cancer-derived line), displayed reduced survival after C2-ceramide treatment when MCU was downregulated. MCU was also dispensable for apoptosis triggered by paclitaxel (a cytoskeletal drug) and ionizing radiations in MDA-MB-231 [90]. In the same cell line, MCU silencing was even shown to enhance caspase-independent cell death when moderate dose of ionomycin (3 μM) was used as stimulus, and ineffective at higher doses (10 μM) or after Bcl-2 inhibition with ABT-263 [101]. Conversely, in a similar cell line, the MDA-MB-435S, silencing of MCU slightly protected from a different form of cell death, named paraptosis, induced by celastrol [102]. However, it must be stressed that (i) not all death stimuli depend on organelle Ca^{2+} overload and (ii) mitochondrial Ca^{2+} uptake is not an proapoptotic signal per se, but it rather requires the concomitant activation of other signaling pathways that can be cell-type

specific. It was recently shown that MCU mRNA is one of the targets of miR-25 [103]. Therefore, overexpression of miR-25 leads to a decrease of MCU at protein level and of mitochondrial Ca^{2+} transients. Accordingly, upregulation of miR-25 protects both HeLa [103] and H9c2 (derived from rat myocardium) [104] cells from death induced by H_2O_2 and C2-ceramide, but not by staurosporine (a stimulus that does not strictly rely on mitochondrial Ca^{2+} overload). In addition, treatment of PC3 (derived from prostate cancer) cells with a specific miR-25 antagonist, efficiently enhances cell death [103], thus further reinforcing the notion that mitochondrial Ca^{2+} is a sensitizing element for apoptosis.

Some additional insights into the relationship between mitochondrial Ca^{2+} homeostasis and apoptosis can be obtained by looking at the other components of the MCU complex. Indeed, it has been demonstrated that in HeLa [59, 60, 90], HMEC and primary hepatocytes [60], but not in MDA-MB-231 [90], silencing of MICU1 enhances cell death triggered by C2-ceramide or H_2O_2. Comparable results have been obtained also in primary endothelial cells exposed to lipopolysaccharide and cycloheximide [59]. It is here worth noting that loss of MICU1 leads on one hand to higher mitochondrial Ca^{2+} uptake at low $[Ca^{2+}]_{cyt}$, but on the other it also lowers organelle cation accumulation when $[Ca^{2+}]_{cyt}$ rises [60, 61]. As a consequence, in MICU1 silenced cells, the transfer of low amplitude Ca^{2+} signals should be favored, while large and rapid increases of $[Ca^{2+}]_{mt}$ triggered by temporally restricted Ca^{2+} signals should be impaired. In line with this, Ca^{2+}-dependent apoptotic stimuli generates increases in $[Ca^{2+}]_{cyt}$ that are small in amplitude but prolonged in time [105–107], as opposed to normal physiological agonists (e.g. IP_3-mobilizing agents), which triggers large but transient rises in $[Ca^{2+}]_{cyt}$. Indeed, silencing of MICU1 potentiates the transfer of apoptotic stimuli (i.e. small and prolonged signals) and dampens the burst on oxidative metabolism triggered by IP3-coupled stimuli (i.e. large and fast signals) [59]. Finally, also the knockdown of another putative MCU modulator, SLC25A23, a condition that inhibit mitochondrial Ca^{2+} accumulation, protect from cell death induced by oxidative stress [62]. Regarding the exact cell death mechanism downstream to $[Ca^{2+}]_{mt}$ rises, generation of reactive oxygen species (ROS) is one of obvious candidate, although different results have been obtained. According to Madesh and coworkers, silencing of MICU1 leads to an increase of basal mitochondrial ROS levels that in turn enhances the susceptibility to apoptotic stimuli [59]. Conversely, Hajnoczky and coworkers showed that the basal ROS levels are not affected by MICU1 deficiency. However, during prolonged store operated Ca^{2+} entry, a gradual increase in mitochondrial H_2O_2 production was detected when MICU1 was downregulated, maybe contributing to enhanced cell death [60]. Finally, no differences in ROS levels were detected after regulation of either MICU1 or MCU in MDA-MB-231 cells [90], a model were MCU appears to be dispensable for cell death. However, we also recently reported that the genetic inhibition of MCU in this cell line limits tumor progression both in vitro an in vivo through a ROS/HIF1α-dependent mechanism [108]. Overall, the available data obtained in cultured cells support the idea that mitochondrial Ca^{2+} overload can be a trigger of cell death, although its contribution can largely vary depending on the cell type.

Targeting Mitochondrial Ca^{2+} Uptake In Vivo

The vast majority of studies cited so far agrees on the fact that mitochondrial Ca^{2+} is a pleiotropic signal that regulates many essential aspects of cellular physiology. Starting from these observation, one could easily expect that the genetic ablation of the MCU gene would be detrimental for so many functions at whole organism level to be incompatible with life (i.e. leading to embryonic lethality). However, the first attempt to target MCU in *Mus musculus* showed a very surprising result that puzzled most of the scientists in the field. Indeed, in 2013 Finkel and coworkers published a paper showing a viable MCU knockout (KO) mouse, characterized by the lack of any significant phenotype except for a slight decrease of skeletal muscle peak performance [109]. MCU-KO mice showed no histological aberrations or evident dysfunctions in any organ, neither in high energy demanding tissues such as heart, skeletal muscle and brain. In this model, differences are lacking not only in resting conditions, but also under both physiological (e.g. isoprenaline) or pathological (e.g. ischemia/reperfusion injury in the heart) stimuli [109, 110]. However, it became evident that ablation of MCU in the standard C57BL/6 strain actually leads to embryonic lethality, and this notion has been confirmed in different and independent mouse models (see below). Viable mice could indeed be obtained only in a mixed C57BL/6×CD1 background, and even in this strain the birth ratio of the homozygous MCU null mice is half of the expected [111], thus indicating the requirement for mitochondrial Ca^{2+} uptake during embryo development. How can the genetic background have such a profound impact remains an enigma, although similar results are not new [112]. For example, knockout of the transforming growth factor-β1 (TGF-β1) leads to different outcomes that largely depend on the mouse strain. Embryonic lethality is complete when backcrossed in C57/BL6, 50% in a mixed 129×CF1 and just 20% in BALB/c [113]. Similarly, endothelial growth factor (EGF)-receptor-deficient mice show large variation in survival, ranging from peri-implantation to postnatal lethality, according to the strain of the developing embryos [114, 115]. In any case, the lack of any phenotype of the viable MCU-KO mice is still puzzling. The obvious explanation is that some kind of compensation could take place, for example due to the activation of alternative routes for mitochondrial Ca^{2+} uptake. However, the evidences provided by Finkel and coworker are compelling and demonstrate that MCU is the only mechanism leading to the fast, energy-driven organelle Ca^{2+} uptake in a variety of experimental setups (see Chap. 3). The only concern lies in the observation that in MCU-KO mitochondria, in steady state, matrix Ca^{2+} content, although greatly reduced, is still measurable, maybe suggesting that alternative pathways for slow Ca^{2+} uptake could exist. Again, also the absence of the "expected" phenotype is not new when dealing with loss-of-function models [116]. For example, knockout of creatine kinase, an enzyme that control a critical parameter such as the intracellular ATP levels, leads to no evident phenotype [117]. Even more strikingly, genetic ablation of myoglobin, the oxygen-binding protein of striated skeletal muscle, resulted in no consequences [118].

Interestingly, in myoglobin-KO mice non-obvious multiple compensatory mechanisms take place, all converging to steepen the oxygen pressure gradient to the mitochondria, including higher capillary density, smaller cell width, elevated hematocrit and increased coronary flow and coronary flow reserve [119–121]. Other examples of unpredictable compensations include cGKII- [122] and GDNF-KO [123] mice. More generally, biological robustness should be taken into account when evaluating these models. For example, in yeasts only 13% of genes are essential when mutations in single genes are analyzed [124, 125], but most of the genes are only apparently dispensable, as demonstrated when the functional buffering of biochemical pathway is taken into account [126]. In our biased opinion, a compensation in the downstream signals activated by mitochondrial Ca^{2+} transient (that are still largely unknown) must be present in these mice, a concept that is supported also by results obtained in other related genetic models. Indeed, Anderson and coworkers generated a heart-specific transgenic mouse model expressing a dominant negative MCU isoform, $MCU^{D260Q,E263Q}$ (DN-MCU), in the same mixed C57BL/6×CD1 background of the constitutive MCU-KO model [127]. In cultures cells this mutant is not able to completely abolish organelle Ca^{2+} accumulation [47], but mitochondria from DN-MCU expressing hearts show no measurable Ca^{2+} uptake. In any case, this genetic model shows a clear phenotype, i.e. the lack of isoprenaline-induced fight or flight response. In particular, these mice are incapable of heart rate acceleration induced by β-adrenergic stimulation, an effect completely absent in the constitutive MCU-KO model [110]. The mechanism behind this effect depends on the enhancement of oxidative phosphorylation triggered by $[Ca^{2+}]_{mt}$ increase. Indeed, while MCU appears to be dispensable for normal heart function, under physiological stress, the MCU-dependent boost of ATP production is necessary to sustain the increased demand of SERCA pumps and the proper Ca^{2+} load of the sarcoplasmic reticulum in pacemaker (SAN) cells. In line with this, DN-MCU expressing hearts exhibit impaired performance at increasing workloads (lower ventricular developed pressure at >600 bpm) and a higher oxygen consumption rate (OCR) at any heart rate [127]. Moreover, DN-MCU expressing cardiomyocytes display a clear extramitochondrial adaptation (i.e. higher diastolic $[Ca^{2+}]_{cyt}$) that depends on the reduced ATP availability. However, despite this phenotype, also in this model the susceptibility to ischemia reperfusion injury is comparable to control animals [128]. Conversely, this notion has been recently challenged by a new mouse model developed by Molkentin and coworkers. They indeed generated a conditional MCU knockout model with two LoxP sites between exons 5 and 6 ($MCU^{fl/fl}$) that were crossed with mice expressing a tamoxifen-inducible Cre recombinase (MerCreMer [MCM]) driven by the cardiomyocyte-specific α-myosin heavy chain promoter. MCU gene deletion was induced in adult (i.e. 8 weeks) mice and the cardiac function was evaluated [129]. Interestingly, in a parallel study, Elrod and colleagues crossed the $MCU^{fl/fl}$ animals with mice constitutively expressing Cre recombinase (B6.CMV-Cre). No homozygous $MCU^{-/-}$ mice could be obtained due to complete embryo lethality, thus confirming the requirement for MCU during development [130]. Ablation of MCU in adult heart leads to an 80% decrease of MCU at the protein level, most likely due to the mosaicism of the MCM strain [131,

132]. Therefore, this lead to a drastic, but not complete, reduction of mitochondrial Ca^{2+} uptake has been observed, as can be easily appreciated when looking to intramitochondrial Ca^{2+} dynamics (as opposed to indirect extramitochondrial $[Ca^{2+}]$ measurement in isolated organelle, which is a less sensitive method especially when dealing low mitochondrial Ca^{2+} uptake rates). Cardiac-specific MCU-KO mice show no major defects, even 40 weeks after gene ablation, in both normal conditions and after cardiac pressure overload induced by transverse aortic constriction (TAC) [129]. However, this genetic model not only show the lack of any fight or flight response, as previously reported [127], but it also display a clear additional phenotype. Indeed, MCM-MCU-KO mice are strongly protected from I/R injury [129, 130], in line with the predicted role that mitochondrial Ca^{2+} plays in cell death, but in sharp contrast to other MCU-KO models [109, 128]. In support of this notion, mitochondrial Ca^{2+} overload has been shown to be a key determinant of cardiac cell death also in different models of heart failure [133]. However, these discrepancies are difficult to solve, in particular considering the lack of standardization among these models. On one hand, the mice were generated in two different strains (pure C57BL/6 and mixed C57BL/6×CD1), and on the other inhibition of MCU during development is likely to activate compensatory networks. In order to solve some of these issue, it is worth looking to data produced by the International Mouse Phenotype Consortium (IMPC) with the aim to generate standardized procedures for phenotyping and provide reliable insights into the function of the whole mammalian genome [134–137]. The consortium already generated KO models for MCU (http://www.mousephenotype.org/phenotype-archive/genes/MGI%3A3026965), MCUb (http://www.mousephenotype.org/phenotype-archive/genes/MGI%3A1914065) and EMRE (http://www.mousephenotype.org/phenotype-archive/genes/MGI%3A1916279). Some phenotypic data are already available and will be discussed here, although it must be stressed that overall the picture is not yet complete and some of the data must still undergo to quality control processes, and should be thus taken with caution. According to these data, genetic ablation of MCU or EMRE (both conditions that completely block mitochondrial Ca^{2+} uptake) leads to embryonic lethality, thus further reinforcing the requirement of mitochondrial Ca^{2+} signaling during embryogenesis. Unexpectedly, $MCU^{+/-}$ heterozygous mice show several impaired physiological parameters, including a decreased cardiac stroke volume, abnormal fasting glucose level and fat amount (although for these last two parameters, males and females behave differently, thus indicating sexual dimorphism). $EMRE^{+/-}$ heterozygous mice display instead neurological defects (e.g. lower center distance travelled) and impairment of blood glucose homeostasis near statistical significance. Finally, $MCUb^{-/-}$ homozygous mice are viable (as one would expect) but show vestibular impairment (low contact righting score) and electrocardiogram defects. Interestingly, considering the high expression of MCUb in the immune system, a tendency (although not significant) to higher lymphocytic count is also present. Overall, these data suggest that the modulation of MCU complex components has a broad potential impact on physiology at whole organism level.

In line with this view, mitochondrial Ca^{2+} uptake has been shown to play a central role in skeletal muscle pathophysiology. In this case, in order to avoid potential compensatory effects activated during embryonic development, adeno-associated viral (AAV) particles for both the overexpression and silencing of MCU were used in either newborn or adult mice. MCU overexpression and downregulation causes muscular hypertrophy and atrophy, respectively. Moreover, MCU overexpression also protects from denervation-induced muscle atrophy triggered by sciatic nerve resection. Regarding the mechanisms behind this phenotype, they appear to be independent of the control of aerobic metabolism, since (i) PDH activity, although defective in MCU-silenced muscles was unaffected in MCU-overexpressing muscles, (ii) the hypertrophy was comparable in both oxidative and glycolytic muscles (in the latter the contribution of mitochondrial metabolism should play a relatively marginal role) and (iii) analysis of aerobic metabolism revealed no major alterations. Conversely, this effect rather relies on two major hypertrophic pathways of skeletal muscle, PGC-1α4 and IGF1-Akt/PKB, leading to marked increase in protein synthesis. These results indicate the existence of a Ca^{2+}-dependent mitochondria-to-nucleus signaling route that links organelle physiology to the control of muscle mass. It also should be underlined here that the apparent differences in phenotype between gene knockout and gene silencing approaches are not new [138–140] and are thought to be due to the activation of a compensatory network to buffer against deleterious mutations (i.e. gene knockout), which are not observed after gene knockdown [141].

Genetic manipulation of MCU has also been carried out in other organisms. Indeed, both silencing and conditional knockout of MCU in *Trypanosome brucei* impairs energy production, enhances cellular recycling through autophagy and produces marked defects of growth *in vitro* and infectivity in mice [142]. Similarly, developmental defects are also evident in *Danio rerio* injected with anti-MCU morpholinos [143], underlining the physiological requirement of an efficient organelle Ca^{2+} uptake machinery in simple vertebrates. Conversely, knockout of MCU in *Caenorhabditis elegans* leads to apparently viable and fertile worms, although the productions of ROS and wound healing are impaired [144].

Also in the case of the MCU regulators, the situation is still controversial. Very recently, Hajnoczky and coworkers described the first attempt to target MICU1 in vivo [145]. Embryonic ablation of MICU1 causes perinatal lethality in mice without causing gross anatomical defects. Most importantly, liver-specific MCU deletion determines the striking failure in organ regeneration after partial hepatectomy. MICU1-deficient hepatocytes show exaggerated Ca^{2+} overload-induced mitochondrial permeability transition pore (PTP) opening, and indeed the phenotype is completely reversed in vivo by using the PTP inhibitor NIM811 [145]. Conceptually similar results have been obtained in a different model of MICU1-KO mice generated by Finkel and coworkers (see Chap. 3). However, in this model the perinatal mortality is only partial, and the phenotype of surviving MICU1-KO animals spontaneous improves during aging, most likely due to a compensation through a decreased expression level of EMRE [146].

Finally, the pleiotropic role of mitochondrial Ca^{2+} uptake has also been confirmed in human pathophysiology. Few families carrying a loss-of-function mutation of MICU1 have been identified. Homozygous individuals for this mutation are characterized by early-onset proximal muscle weakness with a static course, moderately or grossly elevated serum creatine kinase levels accompanied by learning difficulties and a progressive extrapyramidal movement disorder. Fibroblasts derived from these patients show the expected impairment of MCU gating accompanied by mitochondrial fragmentation and decreased oxidative metabolism [147, 148]. Although the pathogenesis lying behind this disease is impossible to dissect at the moment, it needs stressing that this finding underlines the importance of mitochondrial Ca^{2+} transport in humans, as a change in one of the regulatory proteins in the complex results in a genetic disease with devastating symptoms.

Overall, it is still difficult at the moment drawing any definitive conclusion on the role of mitochondrial Ca^{2+} handling in pathophysiology. Still, despite the initial setback caused by the lack of significant phenotype of the MCU-KO, the new available models are starting to uncover the real contribution of mitochondrial Ca^{2+} signals to physiological processes. New questions need to be addressed and new cellular pathways are waiting to be discovered, in an unexpectedly exciting exploding field.

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