Mitochondrial calcium: mechanisms of transport, regulation and cellular functions [Au: OK? Or 'Mitochondrial calcium: machineries, regulation and cellular functions'?]

Formattato: Colore carattere: Blu

Carlotta Giorgi[1*], Saverio Marchi[1*] and Paolo Pinton[1,2,3]

[1]Department of Morphology, Surgery and Experimental Medicine, Section of Pathology, Oncology and Experimental Biology, Laboratory for Technologies of Advanced Therapies (LTTA), University of Ferrara, Ferrara, Italy; [2]Cecilia Hospital, GVM Care & Research, E.S: Health Science Foundation, Cotignola, Italy; [3]CNR Institute of Cell Biology and Neurobiology, Monterotondo, Italy. [*]Co-first authors

Correspondence to:

Paolo Pinton,

email: paolo.pinton@unife.it

Abstract

Calcium ions (Ca^{2+}) constitute one of the most versatile signaling molecules with many physiological functions, prominently including muscle contraction, neuronal excitability, cell migration and cell growth. By sequestering and releasing Ca^{2+}, mitochondria serve as important regulators of cellular Ca^{2+}. Mitochondrial Ca^{2+} also has other important functions, such as regulation of mitochondrial metabolism, ATP production and cell death. In recent years, identification of the molecular machinery regulating mitochondrial Ca^{2+} accumulation and efflux has expanded the number of (patho)physiological conditions that rely on **[Au:OK?]** mitochondrial Ca^{2+} homeostasis. Thus, expanding the understanding of the mechanisms of mitochondrial Ca^{2+} regulation and function in different cell types is an important task in biomedical research, offering the possibility of targeting mitochondrial Ca^{2+} machinery for the treatment of several disorders. **[Au: Edit OK?]**

Keywords: Calcium (Ca^{2+}) signaling, homeostasis, MCU complex, cell death, inflammation, cancer, ion channels, heart, metabolism

[H1] Introduction

The two adjectives most commonly used to define calcium (Ca^{2+}) signalling are "universal" and "versatile"[1]. The universality of Ca^{2+} as a signalling molecule derives from its ubiquity as an intracellular second messenger that controls a wide range of critical processes, whereas the versatility of Ca^{2+} depends on its ability to generate signals with largely different spatial and temporal forms[2] (Box 1). Among the many organelles, mitochondria have major roles as both regulators and decoders of Ca^{2+} inputs. Owing to their intrinsically dynamic nature, mitochondria can localize at specific positions throughout the cell, thereby shaping the cellular Ca^{2+} response. Mitochondria also act as Ca^{2+}-dependent effectors of a vast range of processes, such as energy production and cell death (Box 2). **[Au: I think it would be beneficial to exchange the order of Box 2 and 3.]**

As described by Ernesto Carafoli in a fascinating historical review[3], the first indirect evidence of Ca^{2+} transport inside mitochondria dates back to 1953 (REF [4]), but the concept that isolated mitochondria could uptake high levels of Ca^{2+} by using ATP-derived energy emerged only in the 1960s with observations made by two independent groups[5,6]. Since then, mitochondria have been thought of as well-defined structures capable of accumulating large amounts of Ca^{2+} ions inside their matrix. Over the past sixty years, intense research in the Ca^{2+} field has defined the basic features of mitochondrial Ca^{2+} handling and clearly established the role of mitochondria in the regulation of cellular Ca^{2+} homeostasis, as well as specific functions of mitochondrial Ca^{2+} uptake. Under resting conditions, the Ca^{2+} concentration ($[Ca^{2+}]$) inside mitochondria approaches the values measured in the bulk cytoplasm (100-200 nM), but during stimulation with $[Ca^{2+}]$-increasing agents, mitochondria accumulate 10–20-fold more Ca^{2+} than the cytosolic compartment. The sources of Ca^{2+} required for such $[Ca^{2+}]$ rises are external, represented by the extracellular milieu ($[Ca^{2+}]$ of ~1 mM) from which Ca^{2+} is taken up by plasma membrane channels **[Au: Perhaps it would be beneficial to briefly mention here the plasma membrane channels shown in Fig. 1. Below you discuss the channels for ER, for example.]** (the prevailing mechanism in neurons and other excitable cells), or internal by the release of Ca^{2+} from internal sources — endoplasmic reticulum (ER) or sarcoplasmic reticulum (SR) in muscle cells and the Golgi apparatus **[Au: In current figure 1 ER and lysosomes are shown as major intracellular stores of calcium. Here you discuss ER and Golgi. Please revise for consistency.]** — via different classes of intracellular channels (e.g., the inositol 1,4,5 trisphosphate receptor (IP3Rs) or ryanodine receptors (RyRs)). These intracellular Ca^{2+} stores are loaded with Ca^{2+} ($[Ca^{2+}]$: >500 μM) at the expense of ATP hydrolysis via the activity of Ca^{2+} pumps (sarco/endoplasmic Ca^{2+} ATPase, or SERCA, and the secretory pathway Ca^{2+}-transport

ATPases, or SPCAs). The reduction of ER intraluminal Ca^{2+} results in a massive influx of Ca^{2+} from the extracellular space, a mechanism known as capacitative Ca^{2+} influx or store-operated Ca^{2+} entry (SOCE), to provide Ca^{2+} for refilling the ER and to regulate a wide number of signalling functions by increasing cytosolic $[Ca^{2+}]$[7] (Box 1, **[Au: OK to refer to Box 1 here?]** Fig. 1).

The close proximity of mitochondria to Ca^{2+} stores, in particular the ER, and the presence of a highly Ca^{2+}-selective channel located at the inner mitochondrial membrane (IMM), explain how large amounts of Ca^{2+} could enter these organelles. Ca^{2+} uptake is driven by a membrane potential **[G] [Au: Please leave the G marks in the text; these will facilitate the work of your production team during styling]** difference ($\Delta\Psi$) generated by the respiratory chain **[G]** , which provides the electrochemical force required for positively charged ions to enter the matrix. However, Ca^{2+} does not remain inside mitochondria, but is rather rapidly extruded into the cytoplasm through a complex system of Ca^{2+} antiporters, restoring the basal state. Thus, the coordination of this highly sophisticated Ca^{2+} machinery, which consists of different pumps, channels and auxiliary proteins, is crucial for the maintenance of mitochondrial Ca^{2+} homeostasis, which in turn further demonstrates the impact of the mitochondrial compartment in the regulation of cellular Ca^{2+} signaling.

In this Review, we will describe the molecular details of the different Ca^{2+} transporters and provide mechanistic insight into the related regulatory pathways of mitochondrial Ca^{2+} uptake and efflux **[Au:OK?]** , discussing the most recent discoveries and the many unanswered questions and conflicting interpretations regarding mechanisms of Ca^{2+} homeostasis. We will also outline the physiological role of mitochondrial Ca^{2+} and its deregulation in several pathological contexts.

[H1] Mitochondrial Ca^{2+} entry

The continuous development of methods for measuring $[Ca^{2+}]$, based on either luminescent or fluorescent probes (Box 3), has enabled the characterization of intrinsic mechanisms regulating mitochondrial Ca^{2+} handling. To reach the mitochondrial matrix, cytosolic Ca^{2+} has to cross two membranous systems, the outer mitochondrial membrane (OMM) and the IMM, both of which harbour protein pores enabling regulated Ca^{2+} uptake (Fig. 2).

[H2] Mitochondrial Ca^{2+} channels

The first barrier, the OMM, is considered highly permeable to Ca^{2+} ions, and this permeability is ensured by high expression of the OMM protein voltage-dependent anion channel (VDAC), which forms membrane pores and represents the first molecular interface between

mitochondria and Ca^{2+} stores (the ER/SR and the extracellular space). VDAC porins exist as three subtypes (from 1 to 3), which are expressed more or less ubiquitously but vary in their isoform ratios and sub-mitochondrial distribution among tissues[8,9]. VDAC porins can assume multiple structural conformations, and the transition between open (diameter of the channel pore: 2.5 nm) and closed (pore size: 0.9 nm) states occurs in a voltage-dependent manner. Low transmembrane potentials **[Au: It would be helpful to clarify when/in what conditions low versus high transmembrane potentials are observed]** determine a high-conductance, anion-selective state, whereas increased voltages (20-40 mV) promote lower conducting conformations, which are assumed to be impermeable to ADP/ATP **[Au: Are these channels also transporting ATP/ADP? Unclear. Please clarify this.]** but sill allow the flow of small cations, including Ca^{2+} (REFs [10,11]). Notably, closed conformations of VDAC show higher selectivity and efficiency of Ca^{2+} transport[75] **[Au: Edit OK? Ref 75 OK?]** Overall, VDACs mediate Ca^{2+} flux in both open and closed conformation, **[Au: Edit OK?]** thereby limiting the generation of any OMM [Ca^{2+}] gradient **[Au: What do you mean by OMM[Ca2+] gradient? What is this gradient? Unclear.]** . Recent findings have highlighted the key role of VDACs in enhancing the IP3-induced Ca^{2+} signal from the ER **[Au:OK?]** in different contexts[12,13], facilitating Ca^{2+} entry into the intermembrane space (IMS) and its accumulation inside the matrix (Fig. 2a). However, other unidentified pathways might be involved in the control of Ca^{2+} permeation across the OMM, since the depletion of all VDAC isoforms does not affect the sensitivity of mitochondria to Ca^{2+}-driven cell death[14].

After reaching the IMS, Ca^{2+} ions pass the IMM mainly through the mitochondrial calcium uniporter (MCU) channel. However, MCU-independent, Ca^{2+} uptake pathways have been reported, including an IMM-located pool of RyRs[15], the canonical transient receptor potential 3 (TRPC3)[16], the uncoupling proteins 2-3 (UCP2-3)[17], and LETM1 (leucine zipper EF-hand containing transmembrane protein 1)[18]. Nevertheless, the MCU channel unequivocally represents the dominant mechanism that allows Ca^{2+} accumulation inside the mitochondrial matrix.

It is now firmly established that the MCU is a macromolecular complex composed of pore-forming subunits and regulatory proteins[19-21] (Fig. 2b). Molecular characterization of the entire complex was made possible by studies that simultaneously identified the *bona fide* channel component MCU (previously known as CCDC109a)[22,23]. The other elements of the holocomplex are the MCU regulator MCUb (also known as CCDC109b)[24] and the IMS-resident protein MICU1 (REF [25]), which binds to its paralogue MICU2 (REF [26]) to form heterodimeric structures[27,28]

associated with MCU through the essential MCU regulator (EMRE, also known as single-pass membrane protein (SMDT1))[29]. Additional components of the complex have been described, including MICU3 (REF [26]) and MICU1.1, a MICU1 splicing variant with higher Ca^{2+}-binding affinity than MICU1 (REF [30]), which are tissue-specific members of the uniporter and are expressed in the central nervous system and skeletal muscle, respectively.

The working model for MCU complex **[Au:OK?]** is the product of extensive research: after passing the OMM, the incoming calcium is first handled by MICU1-2 dimers due to their strategic IMS localization and the presence of two Ca^{2+}-binding EF-hand domains in both the MICU1 and MICU2. Loss-of-function studies have definitively demonstrated that MICU1-2 dimers act as gatekeepers of the uniporter, setting the $[Ca^{2+}]$ threshold for MCU activation and allowing mitochondrial Ca^{2+} uptake exclusively at a high $[Ca^{2+}]$, thereby limiting the detrimental accumulation of Ca^{2+} inside the matrix under basal (unstimulated) conditions[31-33]. **[Au: Edit OK?]**

Dissecting the intrinsic role of both MICU1 and 2 is complicated by the observations that MICU2 and MCU expression could be affected by the loss of MICU1 or vice versa, and thatMICU2 is unable to associate with MCU in a MICU1 knockout background. One model proposes that MICU1 is a pure stimulatory subunit and identifies the dominant gatekeeping mechanism in MICU2[27,28]. However, recent findings have provided new insights into the MICU regulatory mechanism, showing that at low cytosolic $[Ca^{2+}]$ (< 500 nM), MICU1 alone is sufficient to repress MCU channel activity and requires MICU2 only when the external $[Ca^{2+}]$ is between 500 nM and 1.5 μM[34,35]. Thus, in MICU2 knockout cells, the Ca^{2+} threshold for MCU activation is ~500 nM, which is 3-fold lower than in the presence of MICU2. **[Au: Edit OK?]** Importantly, when the $[Ca^{2+}]$ rises, MICU1 cooperates with MCU to favour extensive Ca^{2+} entry[31,36], whereas MICU2 limits the MICU1-mediated gain of uniporter function[34], suggesting that MICU2 represents an additional layer of control for MCU activation.

The pore-forming MCU subunit forms pentameric structures *in vitro*[37], and its activity is strictly dependent on EMRE since mammalian MCU does not transport Ca^{2+} in an EMRE knockout background[29]. Although it was originally proposed that EMRE might control MCU by sensing the $[Ca^{2+}]$ in the matrix through its C-terminal domain[38], subsequent, and in our opinion more convincing, observations have revealed that the C-terminus of EMRE is located in the IMS rather than in the matrix[39,40], connecting the MICU1–2 sensors to MCU and thus regulating Ca^{2+} entry[40]. MCU has been shown to be negatively regulated by its paralogue MCUb[24], although overexpression of MCUb in *Trypanosoma cruzi* did not have a dominant-negative effect on MCU[41] **[Au: What could this suggest? Could you perhaps briefly elaborate?]** .

This characterization of the function of the MCU complex fulfills all the properties that were attributed to the uniporter several years before its molecular discovery, such as sensitivity to ruthenium red **[G] [Au: Please add to Glossary]** inhibition, high Ca^{2+} selectivity[42], induction of Ca^{2+} uptake only in energized mitochondria and low Ca^{2+} affinity (apparent dissociation constant **[G] [Au: Please define in Glossary]** K_D of MCU is 20-30 μM)[43], implying that the function of the MCU complex relies completely on two main parameters: the mitochondrial membrane potential and the [Ca^{2+}] in the area surrounding the channel.

[H2] Mitochondrial membrane potential as a driving force for Ca^{2+} uptake

After the acceptance of the chemiosmotic theory **[G]** , it was postulated that the driving force for Ca^{2+} entry is the proton electrochemical gradient potential generated by the activity of the respiratory electron transport chain (ETC). The reductive transfer of electrons through respiratory complexes I-IV produces the energy required to pump H^+ ions against their concentration gradient in the IMS, resulting in a $\Delta\Psi$ of 150-180 mV (negative inside, thus favoring cation entrance) (Fig. 2c). As proof of this concept, dinitrophenol and carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), two uncouplers of oxidative phosphorylation, were shown to dissipate the membrane potential across the IMM, thereby almost resetting the $\Delta\Psi$ and abolishing Ca^{2+} entry via the uniporter[44,45].

[H2] Role of ER–mitochondria tethering in mitochondrial Ca^{2+} uptake

The role of mitochondria in many Ca^{2+} signalling pathways depends on close interactions with the ER calcium store and the formation of ER–mitochondria contact sites. The distance between the ER and the mitochondrion at these sites varies between 10 and 60 nm[46], and the ER associates more frequently with mitochondria than with other organelles[47,48]. This allows mitochondria to be exposed upon opening IP3R to microdomains of high [Ca^{2+}] that are necessary to induce Ca^{2+} accumulation through the low-affinity MCU complex.

These synaptic-like, inter-organelle associations, called mitochondria-associated membranes (MAMs)[49], are small enough to allow contact between proteins on the surface of both organelles and ensure that upon induction of **[Au:OK? Otherwise, which agonist do you mean here?]** Ca^{2+} mobilization, the [Ca^{2+}] on the cytosolic surface of the OMM reaches levels 10-fold higher than those in the bulk cytosol[50] (Fig. 2a). Conversely, the [Ca^{2+}] to which the OMM is exposed during SOCE is similar between mitochondria located near the plasma membrane and those located in other intracellular areas. However, mitochondria can also form associations with the plasma membrane called plasma membrane-associated mitochondria (PAM)[51], where

mitochondria are exposed to a 3-fold higher [Ca^{2+}] upon activation of voltage-gated Ca^{2+} channels in the plasma membrane[50].

[H1] Mitochondrial Ca^{2+} efflux

Historically, two major systems have been postulated to extrude Ca^{2+} from the matrix: the sodium (Na^+)/Ca^{2+} exchanger (mNCX) and the H^+/Ca^{2+} (mHCX) exchanger. The first appears to be the predominant antiporter in excitable tissues (heart, brain), whereas the latter mainly leads to Ca^{2+} release in non-excitable tissues (liver, kidney). The stoichiometry of mNCX-driven transport is defined as electrogenic, with 3 (or 4) Na^+ for 1 Ca^{2+} (REF [52,53]), whereas the exchange ratio of mHCX is electroneutral (2 H^+ for 1 Ca^{2+})[54] (Fig. 2d). Thus, the two Ca^{2+} efflux systems mediate the extrusion of Ca^{2+} from the mitochondrial matrix **[Au:OK?]** toward the IMS, reaching the cytosolic compartment by VDAC channels or additional Ca^{2+}-extruding mechanisms located at the OMM, such as the NCX family member NCX3 (REF [55]). Although Na^+-dependent and Na^+-independent Ca^{2+} exit mechanisms have been described since the 1970s, the molecular identities of the different components of mitochondrial Ca^{2+} efflux were revealed only a few years ago.

In 2010, mNCX function was ascribed to NCLX, a product of mammalian *SLC8B1* (REF [56]). NCLX catalyzes not only Na^+/Ca^{2+} exchange but also lithium (Li^+)-dependent Ca^{2+} transport, which was previously described for mNCX[57]. This property of NCLX, together with its confirmed mitochondrial localization, its sensitivity to the classical mNCX inhibitor CGP-37157, and observations in multiple cell types that loss of NCLX alters mitochondrial Ca^{2+} efflux[56,58-60], provide strong indication **[Au:OK? Is it really undisputable that NCLX is the Na/Ca exchanger?]** that NCLX is the mitochondrial Na^+/Ca^{2+} exchanger.

While the molecular nature of the mNCX is generally accepted today, the identity of the H^+/Ca^{2+} antiporter is more controversial. In 2009, it was proposed that the IMM protein leucine zipper EF-hand containing transmembrane protein 1 (LETM1) acts as a H^+/Ca^{2+} exchanger in both fly and mammalian cells as well as in vitro in proteoliposomes **[Au:OK?]** [18]. LETM1 might oligomerize into hexameric structures, thus acting as a transporter, even though it only contains a single transmembrane helix[68]. However, the role of LETM1 in Ca^{2+} release from the mitochondrial matrix has been questioned[63]; indeed, LETM1 was first reported as a K^+/H^+ exchanger[64-66], and some LETM1-related features described in initial studies, such as a stoichiometry of 1 H^+ for 1 Ca^{2+} and sensitivity to the MCU inhibitor ruthenium red[18], conflict with those originally described for mHCX. Novel findings obtained with a highly purified LETM1-containing liposome system have partially clarified these issues, suggesting an

electroneutral transport of Ca^{2+} and insensitivity to ruthenium red and CGP-37157 and thereby reaffirming LETM1 as a strong candidate for **[Au:OK?]** mHCX function **[Au:OK?]** [67]. However, owing to the numerous conflicting results with respect to mitochondrial Ca^{2+} levels observed in LETM1-silenced cells[18,58,62,68,69], additional experimental evidence is required to firmly establish the functional role of LETM1 as a component of the mitochondrial Ca^{2+} efflux machinery. In addition, it has been proposed that LETM1 might promote mitochondrial Ca^{2+} entry under particular **[Au: What do you mean y particular? What are the conditions exactly?]** conditions, functioning as a high-affinity Ca^{2+} uptake system alternative to the MCU complex[18]. This concept has been confirmed by others[61,62], but progress in understanding the mechanisms of MCU has diminished interest in such observations. **[Au: Edit OK? Please note a slight change in the order of references]**

Importantly, experimental evidence suggests that mHCX and mNCX cannot be the sole **[Au:OK?]** molecular pathways aimed to extrude Ca^{2+} from the mitochondrial matrix. It has also been proposed that under certain conditions, the transient opening of the mitochondrial permeability transition pore (mPTP) **[G]** **[Au: Please add to Glossary]** might represent an alternative Ca^{2+} efflux pathway[70,71], although other observations question this hypothesis[72].

[H1] Regulation of mitochondrial Ca^{2+}

Molecular studies have revealed that the heterogeneity of the machinery driving mitochondrial Ca^{2+} exchanges is associated with an equally complex regulatory system, which operates at multiple levels to maintain physiological Ca^{2+} homeostasis. Many proteins have been reported to be genuine regulators of Ca^{2+} uptake by acting on specific molecular components of the influx–efflux machinery, controlling mitochondrial membrane potential or regulating the association of mitochondria with Ca^{2+} stores (Table 1). **[Au: Edit OK? The sentence was vague.]**

Owing to its strategic position in the OMM, VDAC is the preferential target for endogenous proteins located at the interface between mitochondria and the cytoplasm to control Ca^{2+} influx into mitochondria. This group of regulators includes the Bcl-2 family **[G]** members[73]. For example, Bcl-xL interacts with VDAC1 and VDAC3 (but not VDAC2), shaping mitochondrial Ca^{2+} entry by favouring Ca^{2+} transfer across the OMM[74]. **[Au: OK to include this information on page 5?]** Mechanistically, Bcl-xL was suggested to promote VDAC closure[76], which would enhance its selectivity and permeability for Ca^{2+} (see discussion on VDAC above). However, this aspect remains largely unclear and several reports contradict this potential mechanism. First,

it has been reported that Bcl-xL promotes the open VDAC state, rather than the closed configuration[77]. It has also been proposed that VDAC can assume a cation-selective open conformation[78]. Finally, Bcl-xL has been reported to inhibit VDAC1 activity, thus lowering, rather than increasing, the mitochondrial [Ca^{2+}][79]. **[Au: Edit OK?]**

Several intramitochondrial proteins have been suggested to regulate Ca^{2+} signalling by altering MCU complex assembly or **[Au:OK?]** functions. The first, in chronological order, is MCUR1 (MCU regulator 1, also known as CCDC90a; Fig. 2b). It has been shown that MCUR1 binds to MCU at the matrix interface and that MCUR1 knockdown abolishes Ca^{2+} uptake in intact cells[80]. The interaction between MCU and MCUR1 has been reported in other studies[81-83], and MCUR1 was recently proposed to function as a scaffold in the assembly of the uniporter complex[84]. Conversely, when a proteomic assay was used to identify components of the MCU complex, MCUR1 was not recognized[29]. Moreover, the yeast *Saccharomyces cerevisiae*, which lacks any uniporter activity, possesses an MCUR1 orthologue named fmp32, suggesting a function for MCUR1 outside the MCU complex. Indeed, MCUR1 has been described as a co-factor in the assembly of the respiratory chain, rather than the essential component of the uniporter, indicating that the reduction of Ca^{2+} uptake observed in MCUR1-depleted cells may be due to an alteration of the mitochondrial membrane potential[85]. Notably, in hepatocarcinoma cells, MCUR1 was shown to regulate Ca^{2+} entry in an uniporter-dependent manner, whereas forced MCUR1 silencing induced a decrease in $\Delta\Psi$ (REF. [86]), indicating that the role of MCUR1 in Ca^{2+}uptake s complex and requires further investigation **[Au:OK?]**.

SLC25A23 has been recently proposed as a novel regulator of the uniporter[87] **[Au: OK to remove?]**

Several regulatory factors, shown in Table 1, have been reported to maintain the close association **[Au:OK?]** between the ER and mitochondria, thereby ensuring proper Ca^{2+} transfer. Particularly worthy of mention are two OMM proteins, mitofusin-2 (MFN2) and PDZD8 **[Au: Briefly mention the role of PTPIP51 in ER-mitochondria tethering?]**. MFN2 was originally characterized as an ER–mitochondria tether[90], but its role was strongly contested[91], and it is still unclear whether MFN2 promotes or inhibits ER–mitochondria contacts. PDZD8, previously known as a regulator of retroviral infection[92], has recently been described as the long-sought mammalian orthologue of the yeast Mmm1 protein, which is a component of the ER–mitochondrial encounter structure (ERMES) complex **[G] [Au: Please add to Glossary.]** and coordinates Ca^{2+} exchange exclusively via its ER-mitochondria tethering role[93].

Posttranslational modifications represent additional layers in the regulation of mitochondrial Ca^{2+} handling. The first example of phosphorylation events capable of finely tuning

mitochondrial Ca^{2+} homeostasis is attributed to the PKC family[94]. Recently, a regulatory pathway for Ca^{2+} efflux has been identified[95]: both PINK1 **[G] [Au: Please add to Glossary]** and PKA positively regulate NCLX, and inhibition of Ca^{2+} release in PINK1 knockout cells can be reverted by PKA-mediated NCLX phosphorylation[95]. Intriguingly, PINK1 is also able to boost mitochondrial Ca^{2+} extrusion by phosphorylating LETM1 at Thr192 (REF [96]). Both VDAC function and its interactions with other molecular partners such as BCL-2 proteins are affected by multiple phosphorylation events[97]. **[Au: OK to shorten?]**

During hypoxia and inflammatory-mediated oxidative stress MCU channel can be targeted by reactive oxygen species (ROS), which promote S-glutathionylation of Cys97 of MCU. This modification does not affect the interaction of MCU with other uniporter subunits, but it increases the stability of the complex, thereby promoting Ca^{2+} accumulation in mitochondria and augmenting the susceptibility to cell death[98]. **[Au: Edit OK?]** In addition, the MCU complex can be subjected to phosphorylation events. The proline-rich tyrosine kinase 2 (Pyk2) can target MCU, promoting the formation of multimeric channels[99]. During heart disease, a pool of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) localized to the mitochondrial matrix can phosphorylate MCU at two sites (Ser57 and Ser92), resulting in a higher Ca^{2+} response[100]. Interestingly, phosphorylation of Ser92 seems to be critical for MCU activity in various contexts **[Au:OK?]** [81], and the CaMKII–MCU axis have been shown to regulate vascular smooth muscle cell migration and neointimal hyperplasia **[G] [Au: Please add to Glossary]** after endothelial injury[101]. However, subsequent electrophysiological studies have failed to confirm the regulatory effect of CaMKII on MCU[102]. No phosphorylation events have been associated with other uniporter complex components, although MICU1 can be methylated by protein arginine methyl transferase 1 (PRMT1)[103]. Moreover, the m-AAA protease (AFG3L2/ **[Au: What is the meaning of the solidus character here? Unclear.]** SPG7) has been shown to degrade unassembled EMRE, thus ensuring correct stoichiometry between the different complex subunits and preserving uniporter activity[104,105].

Overall, these observations demonstrate that mitochondrial Ca^{2+} uptake and efflux are regulated at multiple levels. An aberration in a single regulatory mechanism could result in the harmful remodeling of Ca^{2+} mitochondrial Ca^{2+} fluxes, which in turn could lead to changes in cellular Ca^{2+} homeostasis and specific pathological phenotypes (see Table 1 and next sections). **[Au: OK to remove? This has been clearly outlined in the Table 1, together with other examples. Please note a change in the reference order.]**

[H1] Roles in cellular Ca^{2+} homeostasis [Au:OK? Please note our main headings can be 38 characters maximum, including spaces.]

One of the main functional roles attributed to mitochondrial Ca^{2+} uptake is the ability to spatially remodel intracellular Ca^{2+} signalling. Numerous correlative studies performed in different cellular types have suggested that mitochondria shape the intracellular Ca^{2+} response both locally and in the bulk cytoplasm. However, these observations were obtained using chemical compounds with low specificity or having a strong impact on mitochondrial functions and thus on the whole cellular metabolism, producing some spurious and controversial results[113]. Moreover, the ability of mitochondrial Ca^{2+} uptake to shape the cytosolic Ca^{2+} transient **[Au: concentration instead of transient?]** is strongly influenced by the cell type and density of competing Ca^{2+} removal fluxes. For example, adult cardiac myocytes display very **[Au: or 'uniquely']** high Ca^{2+} transport capacity by SERCA pumps and plasma membrane Na^{+}/Ca^{2+} exchangers, which transport Ca^{2+} out of the myoplasm 20-60 times faster than mitochondrial transporters **[Au:OK?]** under physiological conditions. Accordingly, in cardiomyocytes the contribution of mitochondria to decreasing cytoplasmic Ca^{2+} levels **[Au: Edit OK?]** has been estimated to be less than 1% (REF [114,115]). Conversely, in non-muscle cells with modest SERCA and/or NCX functions, mitochondrial Ca^{2+} uptake could be critical in terminating cytosolic Ca^{2+} signals **[Au: bursts instead of signals?]** and transiently buffering the intracellular Ca^{2+}.

The molecular characterization of Ca^{2+} influx and efflux pathways has provided new evidence supporting the concept that mitochondria can act as bulk cytosolic Ca^{2+}-buffering systems. During IP3-mediated ER Ca^{2+} release, the peak amplitude of the cytosolic Ca^{2+} signal **[Au:OK?]** is significantly lower in cells in which mitochondrial Ca^{2+} uptake capacity is increased by MCU overexpression[23]. By contrast, in PDZD8-depleted neurons, in which ER–mitochondria tethering is disrupted, cytoplasmic Ca^{2+} levels are elevated and this increase can be related to reduced mitochondrial Ca^{2+} buffering activity[93]. Similar findings have been obtained in NCLX-silenced astrocytes **[G]** [116], although this effect is more pronounced upon SOCE rather than after emptying ER Ca^{2+} stores. **[Au: Edit OK?]** Thus, mitochondria promptly take up cytoplasmic Ca^{2+}, regardless of whether it derives from internal stores (ER) or the extracellular space.

The knockdown of both **[Au: simultaneously? or any of the two?]** MCU and UCP2 (which as discussed above contributes to MCU-independent Ca^{2+} influx into mitochondria), **[Au: Edit OK?]** with a consequent reduction of mitochondrial Ca^{2+} uptake, strongly inhibits SOCE by limiting the aggregation of STIM1 and activation of ORAI1 (see Fig. 1)[117]. **[Au: I think it would help if CRACs were briefly discussed already in the introduction. OK?]** This phenomenon

only occurs upon ER Ca^{2+} depletion through IP3-generating stimuli, whereas the loss of UCP2 or MCU does not affect STIM1 oligomerization and SOCE activation when Ca^{2+} is mobilized by SERCA inhibition, which induces minimal and delayed mitochondrial Ca^{2+} uptake. Thus, the Ca^{2+} buffering capacity of mitochondria positioned at the opening of IP3Rs could represent a pivotal mechanism in the modulation of SOCE, as previously suggested[118].

The cytosolic [Ca^{2+}] affects the function of both ER-resident proteins (such as IP3Rs)[119] and store-operated Ca^{2+} channels[120], with high cytosolic Ca^{2+} levels in the area surrounding the channels inhibiting their activity and reducing Ca^{2+} release through the IP3R or Ca^{2+} entry by ORAI1. **[Au: Edit OK?]** The strategic positioning of mitochondria in the vicinity of these channels (at MAMs) **[Au:OK?]** lowers the [Ca^{2+}] locally, preventing negative regulation and sustaining channel activity. Indeed, MCU loss impairs mitochondrial Ca^{2+} buffering capacity, which in turn limits CRAC function by enhancing Ca^{2+}-mediated slow inactivation of the channels[121]. Similarly, **[Au:OK? Is this another example for the importance of local calcium buffering?]** the number of cytosolic Ca^{2+} oscillations (see Box 1) **[Au: OK to refer to Box?]** , generated by discharge of Ca^{2+} from IP3-sensitive stores, is significantly lower in MCU knockdown cells, which reflects IP3R inhibition resulting from impaired mitochondrial Ca^{2+} uptake[121]. Overall, by controlling Ca^{2+} channel functions and collecting large amounts of Ca^{2+} in specific subcellular areas, mitochondria have the ability to preclude the propagation of Ca^{2+} waves (see Box 1) **[Au: OK to refer to Box?]**, thereby regulating specific cellular processes that depend (or that are regulated) by Ca^{2+} (REF [122]). For example, in pancreatic acinar cells **[G] [Au: Please add to Glossary]** , mitochondria are distributed as a firewall along the border of the apically located zymogen **[G] [Au: Please add to Glossary]** granules, preventing the spread of cytosolic Ca^{2+} — which is important for granule release — **[Au:OK? Otherwise, what is the role for calcium waves apically and why it is restrained basolaterally in acinar cells?]** to the basolateral region through their Ca^{2+} buffering capacity[123]. A crucial role for mitochondria positioning in the regulation of spatially confined cytosolic Ca^{2+} rises has also been described in neurons[124].

In neonatal cardiomyocytes, reducing mitochondrial Ca^{2+} uptake results in a prominent increase in the amplitude of beat-to-beat cytosolic Ca^{2+} oscillations, which in turn contributes to extending the contraction of the cardiac muscle **[Au:OK?]** [125]. However, in adult cardiac myocytes, inhibition of MCU using either a pharmacological approach (Ru360) or genetic knockout has almost no effect on cytosolic Ca^{2+} transients[126,127].

Overall, there is strong evidence that mitochondria contribute to the regulation of cellular Ca^{2+} both in the bulk cytoplasm and locally, in spatially defined cellular subcompartments. **[Au: OK**

to add?] However, several aspects of mitochondrial Ca^{2+} buffering mechanisms **[Au:OK?]** require more clarification, including the complex relationship between mitochondrial Ca^{2+} uptake and SOCE. For example, in breast cancer cell lines, abolition of mitochondrial Ca^{2+} accumulation by MCU depletion has been observed to reduce[128] or marginally increase[129] SOCE. Although different experimental conditions might explain some contradictory results, other important factors should be taken into account, including the number of mitochondria and their subcellular distribution, which can vary substantially depending on the cell type and condition[50,130], or the impact of other organelles in the local buffering of cytoplasmic Ca^{2+} (REF [131,132]). Moreover, mitochondrial Ca^{2+} proteins **[Au: What are Ca^{2+} proteins? Do you mean Ca^{2+} transporters? Regulators? Please clarify.]** can also regulate SOCE independently of their contribution to mitochondrial Ca^{2+} buffering capacity. For example, upon ER Ca^{2+} depletion, cytosolic Na^+ levels rapidly increase owing to **[Au: How is this increase in Na linked to Ca depletion from the WR? Could you please elaborate briefly?]**. This increase in cytosolic Na^+ promotes NCLX activity and drives mitochondrial Ca^{2+} efflux, which lowers $[Ca^{2+}]$ in the mitochondrial matrix, thereby reducing the activity of the respiratory chain and in consequence lowering generation of ROS. **[Au: Edit OK?]** Prolonged ROS production by mitochondria leads to SOCE inhibition via the oxidation of Orai1 at Cys195 (REF [133]), and thus, NCLX activity contributes to sustaining SOCE through the regulation of ROS production. **[Au: Edit OK?]**

[H1] Pathophysiology of mitochondrial Ca^{2+}

Mitochondrial Ca^{2+} has an important function in regulating cell fitness **[Au:OK? Or 'cell survival'?]** through its ability to impact cell energetics by activating oxidative metabolism, mitochondrial respiration and ATP synthesis[134]. Notably however, deregulation of intracellular Ca^{2+} and increased mitochondrial Ca^{2+} influx are potent triggers of necrosis, apoptosis and autophagy[135-137]. Thus, mitochondrial Ca^{2+} homeostasis is intimately linked to both cell growth and survival, and cell death. Bearing in mind this dual role, it is perhaps not surprising that mitochondrial Ca^{2+} dynamics and their regulation have been implicated in various pathophysiological processes, including insulin secretion and diabetes, cardiomyocyte contraction and heart failure, inflammatory responses and pathological inflammation and neuronal homeostasis and neurodegeneration (Table 1). These examples will be discussed in more detail below. In addition, recent preclinical and clinical data have indicated that mitochondrial Ca^{2+} deregulation is a novel feature of cancer pathology (Box 2). Other pathological contexts related to deregulation of mitochondrial Ca^{2+}, which are not discussed in

this Review can be found in Supplementary Table S1. **[Au: Edit OK? Please note that taking into account the extended length of the current article, we will not be able to accommodate 8 main display items for your article. Thus, I propose moving current Table 2 to the supplement as Supplementary Table S1. This table will be readily available in the online version of the article.]**

[H2] Insulin secretion and associations with diabetes

Pancreatic β cells are the body's sole source of circulating insulin. β cells are specifically designed to synthesize and store large amounts of insulin[138], which is secreted based on the demand of target tissues. In healthy individuals, β cells sense changes in plasma glucose concentration and respond by releasing corresponding amounts of insulin into the bloodstream. Despite decades of research, the molecular mechanisms underlying the activation of β cells are not yet fully defined.

Nutrient secretagogues **[G] [Au: Please add to Glossary]** , especially glucose, initiate downstream signals that enable β cells to break down sugar and release insulin by stimulating mitochondrial energy metabolism[139].

Glucose uptake induces glycolysis-dependent ATP increase. The resulting shift in the cytosolic ATP:ADP ratio leads to the closure of ATP-sensitive K^+ (K_{ATP}) channels on the plasma membrane of β cells, eliciting plasma membrane depolarization. Once a threshold potential is reached, voltage-gated Ca^{2+} channels in the plasma membranes of β-cells open, generating individual Ca^{2+} microdomains beneath the plasma membrane[140-142].

The main role of the increase in sub-plasma membrane Ca^{2+} is to permit insulin release[143], possibly through the activation of protein kinase C (PKC) β-II **[Au: What is the role of PKC in insulin secretion? Is it known? Could you please briefly elaborate?]** and its translocation to the surface of secretory vesicles localized in that area, although the details of the underlying mechanism are still not fully understood[144]. Insulin secretion is further promoted by mitochondria. In β cells a pool of mitochondria is strategically situated close to plasma membrane Ca^{2+} channels forming PAMs, **[Au:OK?]** where they are able to sense microdomains of high Ca^{2+} concentrations in their proximity, take up Ca^{2+} through the MCU complex and fuel the exocytotic process by producing ATP, thereby sustaining and amplifying the phase of insulin secretion[141,145,146]. Moreover, the increased cytosolic [Ca^{2+}], consequent of the opening voltage-gated Ca^{2+} channels, promotes ER [Ca^{2+}] accumulation and, under some conditions, **[Au: what are these condition? This phrasing is vague]** can lead to ER Ca^{2+} release[147] through channels including RyRs[148]. This release can be followed by mitochondrial Ca^{2+} uptake

at MAMs **[Au:OK?]** with consequent ATP production further enhancing insulin secretion. Thus, the mitochondrial Ca^{2+} machinery has a fundamental physiological role in glucose-mediated insulin secretion, by supplying energy for the process (Fig. 3A). **[Au:OK?]**

Defects in mitochondrial Ca^{2+} homeostasis within pancreatic β cells, with a consequent reduction of mitochondrial ATP production and thus impaired insulin secretion, are considered one of the causal factors in the aetiology of both type 1 and type 2 diabetes[149].

For example, **[Au: OK? Is it one of examples of mitochondrial Ca dysfunction in this context?]** chronic ER Ca^{2+} depletion owing to leaky RyR channels is responsible for decreased mitochondrial Ca^{2+} uptake and β cell failure[148]. Therefore, strategies to restore an efficient mitochondrial Ca^{2+} response in these cells represent a promising therapeutic approach for the treatment of diabetes. Potential therapeutic targets include MCU channel[69] and its regulatory partner MICU1 (REF[150]), which are required for feed-forward mechanism of Ca^{2+} entry into mitochondria and guarantee insulin secretion in β cells. Recently, the enhancement of K^+ flux across the ER membrane through ER-localized TALK-1 channels has been shown to facilitate Ca^{2+} release from the ER, improving mitochondrial ATP production. Thus,.TALK-1is capable of reducing ER Ca^{2+}-handling defects and its activation could be used to restore mitochondrial Ca^{2+} homeostasis in diabetic β cells [151]. **[Au: Edit OK?]**

[H2] Cardiac cell functions and heart failure

Ca^{2+} is of vital importance for maintaining cardiac cell function as it is a key modulator of cardiac functional **[Au:OK?]** cycle (excitation, contraction or diastole, and relaxation or systole). Moreover, it also has a key role in the pathology of heart failure, being responsible for cardiac cell death via apoptotic and necrotic pathways[152].

Under physiological conditions, Ca^{2+} signalling in the heart exerts three main functions: controlling the so-called excitation-contraction coupling **[G]** (*EC coupling*), excitation-transcription coupling **[G]** (*ET coupling*) and excitation-metabolism coupling **[G]** (*EM coupling*) **[Au: I think it would be useful to define these mechanisms in the Glossary]** mechanisms. While EC and ET coupling are governed essentially by cytosolic Ca^{2+} transients that drive contraction and cardiac muscle gene activation or inactivation, mitochondrial Ca^{2+} contributes to the local control of oxidative metabolism (EM coupling), generating the ATP needed to power cardiac excitation and contraction during every heartbeat[153].

In mammals, the cardiac cycle starts with the generation of an automatic action potential **[G]** in a group of specialized cells, named sinoatrial nodal cells, which autonomously produce **[Au:OK?]** the electrical cardiac impulse needed for the subsequent contraction (of note other

cells within the conduction system have similar properties). **[Au:OK?]** The action potential is initiated with a change in membrane potential, **[Au: Edit OK?]** which becomes more positive, mainly due to the opening of sodium channels and flow of Na^+ into the cell. This depolarization, also called "funny" current **[G] [Au: Maybe provide details of the funny current in the Glossary]** (or I_f) [154-156] **[Au: Are these references required?]** , induces a progressive opening of T-type (Transient opening Ca^{2+} channels, TTCCs) and L-Type Ca^{2+} channels (Long-lasting Ca^{2+} channels, LTCC), eventually triggering a cytosolic Ca^{2+} influx[157,158].

[Au: OK to simplify?] The cytosolic Ca^{2+} influx through LTCCs is sufficient to regulate and activate mitochondrial functions and thus ATP production[163] through the generation of Ca^{2+} microdomains around nearby mitochondria. Calcium influx through LTCCs also triggers Ca^{2+} release from the nearby junctional SR via intracellular Ca^{2+} release channels, in a process known as Ca^{2+}-induced Ca^{2+} release (CICR), a crucial process for muscle contraction[164,165]. The high local cytosolic [Ca^{2+}] generated during the CICR process initiates contraction (EC coupling) by binding troponin C **[G]** on myofilaments **[G]** [159] and boosts **[Au:OK?]** mitochondrial metabolism, by promoting Ca^{2+} uptake into mitochondria[166]. A rapid increase in mitochondrial Ca^{2+} is essential for telegraphing the enhanced metabolic demand for ATP necessitated by muscle contraction to increased production of ATP by oxidative phosphorylation. Thus, mitochondrial Ca^{2+} is fundamental for providing the necessary link between the ATP supply and demand during cardiomyocyte contraction (Fig. 3B).

In this context, the MCU activity functions to increase **[Au:OK?]** heartbeat frequency by favoring rapid Ca^{2+} mitochondrial uptake during the cardiac cycle[125]. **[Au: OK to remove? Sounds like repetition of the points above.]** MCU-enhanced oxidative phosphorylation is also required for reloading SR Ca^{2+} stores and sustaining increased heart rate during the fight-or flight-response **[G] [Au: I propose adding this term to the Glossary]** [167]. The crucial role of MCU-mediated Ca^{2+} uptake in preserving cardiovascular homeostasis is supported by the observation that $MCU^{+/-}$ female mice exhibit a decreased cardiac stroke volume **[Au: What is cardiac stroke volume? This is unclear to naïve reader. Please explain or rephrase]** and that MICU2 loss predisposes to lethal abdominal aortic aneurysms[168].

Undoubtedly, mitochondria in cardiomyocytes are far from being only passive Ca^{2+} sinks — they are able to sense cytosolic Ca^{2+} signals and transform them into mitochondrial energy production. It remains controversial, though, whether variations in the mitochondrial [Ca^{2+}] occur quickly in a beat-to-beat fashion, taking place synchronously with cytosolic Ca^{2+} fluctuations, or whether Ca^{2+} uptake can occur slowly[169]. Recent data suggests the existence of

subpopulations of mitochondria exposed to high levels of [Ca^{2+}] with the ability to take up Ca^{2+} on a beat-to-beat basis[115,127]. The new mechanistic understanding of MCU functions and advancements in Ca^{2+} measurement technologies (Box 3) will be instrumental in solving this issue.

As mitochondrial Ca^{2+} is fundamental for maintaining the ATP supply in myocardial cells, disrupted cardiomyocyte Ca^{2+} homeostasis is recognized as a major contributor to the heart failure phenotype[170]. Acute heart diseases, such as ischaemia–reperfusion injury, are mainly attributed to mitochondrial Ca^{2+} overload together with increased production of ROS caused by excessive mitochondrial respiratory chain activity, which by driving the activation of the mPTP, leads to necrotic and apoptotic cardiac cell death (see also Box 2). Thus, limiting mPTP activation, could represent a potential therapeutic strategy to combat these pathologies. The use of mPTP inhibitors, such as cyclosporin A (CsA), has been reported in experimental studies to reduce myocardial infarction size and to preserve cardiac function. However, in clinical trials, opposite effects were observed by the same group upon administration of CsA, thus failing to demonstrate a final and conclusive benefit for clinical outcomes[171,172]**.** Therefore, more specific and novel mPTP inhibitors, based on new findings regarding the molecular composition of mPTP[173], are required to translate mPTP inhibition as a cardioprotective strategy into clinical practice.

In parallel, other strategies targeting the regulatory systems of mitochondrial Ca^{2+} homeostasis are being explored. Cardiac-specific inducible MCU knockout mice[126,174] show drastic inhibition of acute mitochondrial Ca^{2+} uptake, which correlates with a slowed functional response to an acute increase in workload **[Au: What do you mean by 'increase in workload'? Stimulation? Fight-or-flight response?]** , although the basal mitochondrial [Ca^{2+}] appears remarkably normal. These animals are strongly protected from the damage resulting from ischaemia–reperfusion injury[126,174], **[Au: Edit OK?]** consistent with previous results obtained in isolated hearts with the MCU inhibitor Ru360 (REF [175]). In agreement with this view, the increased MCU current induced by activation of CaMKII during ischaemia–reperfusion injury promotes mPTP opening and myocardial cell death[100]. These data support MCU as a potential new target for cardioprotective drug design. However, the effect of CaMKII on MCU is highly debated (see section above). Furthermore, animals with whole-body **[Au:OK?]** MCU deletion[176] and mice with myocardial MCU inhibition through the expression of a dominant-negative form of MCU[177] are unable to accumulate Ca^{2+} inside the matrix but show no protection against ischaemia–reperfusion injury-driven damage REFS **[Au: Which refs?]**[178]. **[Au: This method will not be known by non-specialists. OK to remove?] [Au: I think this is too much off topic.]**

Intriguingly, CsA administration is strongly cardioprotective in wild type but not in MCU-null hearts[176], suggesting that the myocardial cell death **[Au:OK?]** occurring in whole-body MCU knockout mice subjected to ischaemia–reperfusion injury might be independent from canonical Ca^{2+}-dependent mPTP functions.

In addition to MCU, it has recently been identified that Ca^{2+} efflux is essential for maintaining cardiac cellular function. Targeting NCLX by increasing its expression is sufficient to prevent mitochondrial Ca^{2+} overload and to limit mPTP opening, with a consequent reduction in myocardial infarction size and decreased **[Au:OK?]** cell death after ischaemia–reperfusion injury[60]. **[Au: I think this is also off the main topic.]**

The maintenance **[Au:OK? Or establishment?]** of contact sites between the SR and mitochondria also contributes to the damage associated with ischaemia–reperfusion injury. **[Au:OK?]** In particular, down-regulation of the tyrosine phosphatase protein tyrosine phosphatase-interacting protein 51 (PTPIP51), **[Au: Briefly discuss already on page 11?]** a crucial regulator of ER/SR–mitochondria contacts (Table 1), protects cardiomyocytes from mitochondrial Ca^{2+} overload and cell death, highlighting its potential as a new therapeutic target for alleviating heart damage after ischaemia–reperfusion injury[179].

In chronic heart failure, a major myocyte dysfunction is related to stasis **[G]** **[Au: Please add to Glossary]** and accumulation of cytoplasmic Ca^{2+}. This defect in failing cells largely results from decreased expression and activity of the SR Ca^{2+}-ATPase[180] and increased RyR Ca^{2+} leakage[181] (in particular the type 2 receptor RyR2), owing to their redox modifications[182,183] or phosphorylation by CaMKII[184] and PKA[185]. In addition, heart failure is frequently associated with an elevation of intracellular [Na^+], which induces higher NCLX activity, thereby limiting the ability of mitochondrial [Ca^{2+}] to rise to levels sufficient to support robust ATP production required to match the increased energy demand necessary for contraction[186].

Ca^{2+} leakage from SR can have various effects on mitochondrial Ca^{2+} levels and mitochondrial functions. **[Au: Sentence OK?]** Extensive, short-term **[Au:OK to add short-term here to differentiate between this scenario and prolonged leakage like in the case of diabetes, which leads to decreased Ca uptake by mitochondria?]** Ca^{2+} leak from the SR could induce mitochondrial Ca^{2+} overload[187] and consequent cell death[188]. Induction of SR Ca^{2+} leakage is also sufficient to induce spontaneous action potentials and is therefore considered an important trigger for cardiac arrhythmias[190,191]. Deprivation of SR Ca^{2+} stores also means that less Ca^{2+} can be released from the SR upon LTCC activation, resulting in a decreased magnitude of Ca^{2+} transients and reduced mitochondrial Ca^{2+} uptake upon stimulation, which in turn reduces ATP supply leading to contractile dysfunction[189]. **[Au: Edit of last sentence OK?]**

[Au: Edit OK? I thought that in this organization the flow of information is more logical.]

Overall, these findings suggest that targeting both MCU and NCLX to prevent mitochondrial Ca^{2+} overload in combination with systems to reduce SR Ca^{2+} mishandling could represent effective strategies for the treatment of heart failure. **[Au: OK? Or is this only applicable to chronic heart failure?]**

[H2] Inflammatory responses and pathological inflammation

The first evidence that mitochondrial Ca^{2+} is important during inflammation dates back more than 30 years[192], but increasing interest in this area has only become evident since the molecular identity of the MCU complex and localization of the pyrin domain-containing 3 (NLRP3) inflammasome **[G]** in the mitochondria[193].

Studies have highlighted how chronic stress enhances mitochondrial Ca^{2+} accumulation **[Au: How does ER stress link to these mechanisms? It is depicted in Fig. 3C but not discussed here.]** , which in turn induces excessive and sustained inflammation. Mitochondrial Ca^{2+} homeostasis has been reported to be disrupted in infectious diseases, where MCU seems to be the main player in the regulation of bacteria- and virus-induced activation of inflammation (Fig. 3C).

Pseudomonas aeruginosa infection of airway epithelial cells from patients with cystic fibrosis drives excessive MCU-mediated mitochondrial Ca^{2+} accumulation, which is critical for the activation of a heightened NLRP3-dependent inflammatory response, which exacerbates the pathology of cystic fibrosis[194]. **[Au: Edit OK?]** Loss of MCU *in vitro* has been shown to reduce mitochondrial Ca^{2+} uptake and to blunt activation of the NLRP3 inflammasome induced by *P. aeruginosa*[194].

MCU-mediated Ca^{2+} overload has also been found to be essential for a virus-induced inflammatory response. Viral infections perturb Ca^{2+} homeostasis, either increasing ER Ca^{2+} discharge **[Au: Do you mean release?]** or extracellular Ca^{2+} influx, thereby promoting Ca^{2+}influx into mitochondria. **[Au:OK?]** MCU **[Au: Channel or complex?]** specifically interacts with mitochondria antiviral signaling (MAVS) complexes **[G] [Au: please add to Glossary]** localized on mitochondria and positively regulates the release of the proinflammatory cytokine IFN-β upon viral infection[195]. Chronic viral infection is accompanied by ER stress, inducing mitochondrial Ca^{2+} overload through a MCU–MAVS-dependent pathway, with subsequent sustained IFN-β production that contributes to autoimmune diseases. Knockdown or silencing of MCU (or MAVS) reduces mitochondrial Ca^{2+} uptake capacity and significantly decreases virus-induced IFN-β levels[195] and thus the inflammatory response.

The importance of regulating MCU under stress conditions, when the risk of Ca^{2+} overload is elevated, has also been reported in a model of the inflammatory response induced after tissue injury in the liver. During liver regeneration after partial hepatectomy, loss of MICU1, the Ca^{2+}-sensing regulator of MCU, leads to an enhanced and sustained proinflammatory response, which is associated with a mitochondrial Ca^{2+} overload. The mitochondrial Ca^{2+} overload response is followed by mPTP opening, sensitizing MICU1-deficient hepatocytes to cell death instead of permitting cell proliferation and regeneration[196].

These findings all support the notion that increased Ca^{2+} flux through the uniporter complex fuels important pathways related to inflammatory responses, identifying this channel as a potential target in the treatment of inflammation-associated diseases.

[H2] Neuronal homeostasis and neurodegeneration

Ca^{2+} ions are the major signalling molecule connecting neuronal depolarization to synaptic activity. Through their positioning at the mouth of the IP3Rs or close to the plasma membrane to take up Ca^{2+} from the ER or modulate extracellular Ca^{2+} entry, respectively, mitochondria have a primary function in shaping cytosolic Ca^{2+} oscillations. Moreover, Ca^{2+} sequestration by mitochondrial buffering systems located at synaptic regions affects neurotransmitter release[124] (Fig. 3Da). Therefore, defects in both mitochondrial orientation and toxic changes in Ca^{2+} buffering could contribute to different neurological disorders[197]. In this context, the cell death-inducing sequence: mitochondrial Ca^{2+} overload–ROS formation–mPTP opening, could importantly contribute to neuronal pathology, particularly neurodegeneration. For example, during excitotoxicity **[G] [Au: Please add to Glossary]** postsynaptic neurons are subject to extremely high levels of glutamate, which induces excessive activation of N-methyl-$_D$-aspartate receptors (NMDARs), **[G] [Au: Please add to Glossary]** increased Ca^{2+} uptake and necrotic or apoptotic-like excitotoxic cell death (Fig. 3Db). Moderate NMDAR over-activity has been linked to apoptotic damage in many neurodegenerative diseases, and higher concentrations of glutamate and necrotic cell death have been observed in the ischaemic core after a stroke[198]. Both pharmacological[199] and genetic[200] inhibition of MCU are able to suppress excitotoxicity, suggesting that this approach could be explored as a neuroprotective strategy in disorders with aberrant NMDARs activity.

A therapeutic strategy based on MCU-targeting has also been investigated for spinocerebellar ataxia type 28 (SCA28), an autosomal dominantly inherited ataxia caused by mutations in the *AFG3L2* gene and characterized by a loss of Purkinje cells **[G] [Au: Please add to Glossary]** [201]. AFG3L2 depletion induces an accumulation of MCU–EMRE complexes (devoid of **[Au:OK?]**

MICU regulation), concomitant with increased uptake of Ca^{2+} into mitochondria, leading to high mitochondrial Ca^{2+} levels and neuronal apoptosis[104]. However, simultaneous deletion of *Mcu* together with *Afg3l2* specifically in mouse Purkinje cells does not promote their survival[202], suggesting that increased mitochondrial Ca^{2+} is not sufficient to trigger the loss of Purkinje cells in SCA28. **[Au: Edit OK?]** However, previous studies have shown that MICU1 knockout mice exhibit alterations in the postnatal arborization of Purkinje cells[33], and reducing glutamate stimulation could prevent degeneration of AFG3L2 knockout Purkinje cells[203], suggesting that. **[Au:How can these findings be integrated with the discussion above on the links between mitochondrial calcium and Purkinje cell death?]**

Other neurodegenerative disorders, including amyotrophic lateral sclerosis (ALS), Alzheimer disease, Parkinson disease and Huntington disease, display dysregulation of Ca^{2+} homeostasis, which in most cases has been attributed to rearrangement **[Au: What do you mean by 'rearrangement'? Maybe 'alteration' would be more suitable here?]** of the ER–mitochondria connection. In familial Alzheimer disease, mutated presenilin 2 increases ER–mitochondria tethering in a MFN2-dependent manner[204]. The ALS-associated protein TDP-43 disrupts the vesicle-associated membrane protein-associated protein B (VAPB)-PTPIP51 axis, leading to a decrease in organelle **[Au: Between which organelles? Please clarify]** contact sites and bioenergetic crisis[205]. A similar molecular mechanism has been described for α-synuclein[206], which forms pathological aggregates in Parkinson disease, although other reports have reported a pro-tethering role for α-synuclein[207]. Notably, α-synuclein localizes at MAMs and induces mitochondrial damage, such as a loss of membrane potential and morphological alterations[208].

In addition to alteration in ER–mitochondria contacts, other mitochondrial aberrations have been associated with neurodegeneration. In multiple sclerosis, TNF-α exposure affects mitochondria membrane potential and reduces Ca^{2+} uptake, limiting oligodendrocyte **[G]** **[Au: Please add to Glossary]** progenitor cell (OPC) differentiation **[Au: How does this link to neurodegeneration? This is unclear. Please clarify.]** [209]. Reducing the Ca^{2+}-mediated mPTP formation by pharmacological inhibition of Cyclophilin D (CypD) **[Au: What is CypD? How is it linked to mPTP formation?]** confers neuroprotection in an autoimmune encephalomyelitis model of multiple sclerosis[210]. The genetic deletion of CypD exhibits beneficial effects also in mice expressing ALS-linked mutants of superoxide dismutase-1 (SOD1) (REF [211]), which are characterized by enhanced mPTP opening and aberrant **[Au:OK?]** Ca^{2+} transients in astrocyte processes[212].

In Huntigton disease, the mutant huntingtin protein (mHTT), which mediates the neuronal degeneration, interacts with IP3R, causing a chronic ER Ca^{2+} leak[213]. Notably, early in disease pathogenesis the negative effects of this leak can be compensated by reduced mitochondrial Ca^{2+} uptake capacity[214], which can alleviate the mHTT-related toxicity by preventing mitochondrial Ca^{2+} overload, mPTP opening and neuronal apoptosis. **[Au: OK to move this paragraph here?]**

Overall, these findings support a central role for mitochondrial Ca^{2+} in neurodegeneration: enhanced Ca^{2+} uptake can promote mPTP opening and apoptosis, whereas too low mitochondrial [Ca^{2+}] can affect the energy supply and contribute to mitochondrial derangements. **[Au: The latter mechanism has not been discussed in this section. Could you please add this?]** Thus, regulating mitochondrial [Ca^{2+}] is essential to maintain neuronal homeostasis. **[Au: Sentence OK?]**

[H1] Conclusions and perspectives

A large body of evidence has accumulated regarding the molecular basis of mitochondrial Ca^{2+} homeostasis, a pivotal regulator of many cellular functions. Mitochondrial Ca^{2+} has a crucial role in controlling mitochondrial functions and adapting mitochondrial activity to cellular needs, but it is also tightly coupled to the cytosolic Ca^{2+} changes induced by a variety of stimuli. Importantly, genetic or environmental alterations in intracellular Ca^{2+} signaling are linked to many human diseases, including common disorders and various cancers (Box 2, Table 1, Supplementary Table S1). A complete understanding of the pathways allowing mitochondrial Ca^{2+} entry and release will be crucial for characterization of the molecular pathways linked to mitochondrial Ca^{2+} dynamics. **[Au: Edit OK?]**

In the future it will be particularly important to gain a complete understanding of mitochondrial Ca^{2+} regulation and its integration with other processes. **[Au:OK?]** This will include molecular description of the newly discovered accessory proteins and posttranslational modifications of mitochondrial Ca^{2+} channels and transporters as well as obtaining a definition of the "omic" signature of mitochondrial Ca^{2+} signalling through genomic, proteomic and metabolomics analyses **[Au:OK? It was unclear to me what you exactly meant by 'omic signature'. did you mean to express the regulation of mitochondrial Ca by the genome, proteome and metabolome or how Ca signalling affects these? This is unclear.]** It will also be important to develop new drugs targeting mitochondrial Ca^{2+} pathways. Classical inhibitors of MCU (ruthenium red and its derivatives) and of mNCX (benzothiazepines as CGP-37157) lack full specificity and could affect other important cellular functions, thereby confounding the

experimental findings. Ruthenium red also displays very low cell permeability, restricting its employment to isolated mitochondria. Very recently, two compounds, DS16570511 (REF [215]) and mitoxantrone[216], have been proposed as novel MCU inhibitors. The former inhibits mitochondrial Ca^{2+} uptake in intact cells and perfused heart, but requires further validation, and the latter has been extensively characterized but exhibits high cytotoxicity that could hamper its use *in vivo*.

Building on this molecular description of mitochondrial Ca^{2+} regulation, it will be possible to better define the role of mitochondrial Ca^{2+} signalling in many human disorders through the identification of specific mutations in the proteins responsible for the maintenance of mitochondrial Ca^{2+} homeostasis. For example, considering the role of Ca^{2+} signaling and mitochondria in neurons, the mechanisms of neurodegenerative diseases, as well as neuronal aberrations with more complex phenotypes, such as psychiatric disorders, will be better understood and new therapeutic approaches proposed. Moreover, the contribution of mitochondrial Ca^{2+} in cancer will be clarified, opening the possibility of modulating mitochondrial Ca^{2+} homeostasis to enhance the efficacy of cytotoxic agents.

Finally, the emerging participation of mitochondrial Ca^{2+} in the process of ageing[217] and its importance in stem cell biology[218] will produce new and exciting achievements in the future and will attract scientists from other fields to this fascinating and still vastly unexplored **[Au:OK?]** area of mitochondrial biology.

BOX 1 [Au: The content of the box was slightly too long (we would not be able to fit the figure), so I aimed to shorten it. Please check if my changes are OK.]

Calcium as a second messenger

Calcium ions (Ca^{2+}) are ubiquitous second messengers that translate information delivered by extracellular and intracellular signals into an intracellular effect. A rise in cytoplasmic [Ca^{2+}] ($[Ca^{2+}]_c$) is elicited by a wide variety of molecules and decoded into very different, sometimes opposite effects. To fulfill such a complex signalling role, $[Ca^{2+}]_c$ rises are spatially and temporally regulated (see figure) **[Au:OK?]**. The localized rises can remain confined, thereby preventing the inappropriate stimulation of different cellular domains, or gradually diffuse, as in the case of "Ca^{2+} waves" — orderly propagations of $[Ca^{2+}]_c$ rises throughout the cell. Agonists of receptors that serve as Ca^{2+} channels, **[Au:OK? Otherwise, which agonists?]** such as histamine, ATP or carbachol, can induce, in many cell types, a series of repetitive $[Ca^{2+}]_c$ increases, commonly referred to as "Ca^{2+} oscillations". Each of these signalling patterns,

783 through the specific recruitment of downstream effectors, is decoded into the appropriate
784 cellular effect.
785 A wide range of mammalian proteins are regulated by Ca^{2+}, classified as buffer or trigger
786 proteins[219]. Ca^{2+} buffers encompass calsequestrin and calreticulin, located at the sarcoplasmic
787 and endoplasmic reticulum, respectively, and the cytosolic proteins calbindin and calretinin, as
788 well as the relatively slow buffer parvalbumin. This class of molecules not only cooperates with
789 Ca^{2+} channels, transporters and pumps to shape intracellular Ca^{2+} signals but also displays
790 multiple functions and physiological roles[220]. Trigger proteins include modulators of muscle
791 contraction (troponin C), proteases (calpain), kinases (protein kinase C, PKC) phosphatases
792 (calcineurin B), transcription factors (NFAT, CREB), **[Au:OK?]** or key mediators of different
793 enzymes (calmodulin). Most of them possess one or multiple EF-hand calcium-binding motif
794 and change their conformation upon binding Ca^{2+}. Overall, Ca^{2+} binding can affect localization,
795 molecular associations and functions of a multitude of proteins, regulating a vast array of
796 biological processes, such as contraction, transcription and other signalling networks
797 **[Au:OK?]**.
798 Functions of Ca^{2+} in coordinating different cellular events are not limited to variations in its
799 cytosolic levels, but can be extended to changes in Ca^{2+} inside organelles. For example,
800 lysosomal Ca^{2+} activates calcineurin, which in turn promotes transcription factor EB (TFEB)
801 translocation into the nucleus and transactivation of its target genes[131]. As another example,
802 after fertilization, mitochondrial Ca^{2+} entry sustains production of reactive oxygen species and
803 cell cycle progression in early *Xenopus laevis* embryos[221].
804
805 **BOX 3 [Au: I propose exchanging the order of Boxes; please amend the order of**
806 **references accordingly]**
807 **Methods for measuring mitochondrial Ca^{2+}**
808 Two main genetically encoded strategies are currently used to design functional probes that
809 measure mitochondrial Ca^{2+} concentrations: those based on the Ca^{2+}-activated photoprotein
810 aequorin, and those based on the use of fluorescent proteins and dyes. Using appropriate
811 mitochondria-targeting signals, aequorin has been directed to both the outer mitochondrial
812 membrane and intramembrane space (IMS), although the most commonly used version is the
813 aequorin chimera targeted to the mitochondrial matrix by the pre-sequence of subunit VIII of
814 cytochrome c oxidase[222]. Recombinant aequorin binds Ca^{2+} with apparent dissociation constant
815 K_d of 10 μM (K_d value for low-affinity, point-mutated aequorin is approximately 130 μM). **[Au:**
816 **Edit OK? Otherwise, what did you mean by Kd here?]** Aequorin provides important benefits,

817 such as a wide dynamic range, a high signal-to-noise ratio and the ability to emit light upon Ca^{2+}
818 binding (without requiring potentially damaging light excitation). However, the use of aequorin
819 displays some pitfalls, including low light emission by the photoprotein, which renders it
820 inappropriate for imaging Ca^{2+} waves at the single-cell level.
821 These disadvantages have led to the extensive employment of alternative methods, such as
822 fluorescent protein-based approaches, **[Au: How do fluorescent proteins enable Ca**
823 **detection? This is unclear. Please clarify]** which combine bright fluorescence with efficient
824 targeting to cellular subcompartments through tagging with localization signals **[Au: OK?**
825 **Otherwise, what did you mean by 'efficient targeting'?]** , or synthetic fluorescent dyes,
826 which can be directly loaded into cells, without the need for transfection. **[Au: Edit OK?]** Rhod-
827 2 AM (K_d = 0.57 μM) is the most commonly used chemical probe, offering reliable results in
828 saponin-permeabilized cells or isolated mitochondria. However, it cannot be precisely targeted
829 to mitochondria in intact cells, and measurement of its signal **[Au:OK?]** exhibits multiple
830 drawbacks[223].
831 Mitochondrial fluorescent Ca^{2+} indicators are based on Ca^{2+} detection through a Ca^{2+}-sensing
832 protein, such as calmodulin **[Au:OK?]** , and they are classified into two families: the first
833 represented by the Förster resonance energy transfer (FRET) **[G] [Au: Please add to Glossary]**
834 -based cameleon type, and the second by an engineered single fluorescent protein type, such as
835 GCaMP and pericam. Mitochondrial-targeted cameleons, include 2mt-D2cpv and its variants
836 (K_d = 0.3/3 μM), consisting of cyan and yellow fluorescent protein pair **[Au:OK?]**, linked by
837 calmodulin and the M13 peptide from the myosin light-chain kinase, and mito-pericam (K_d of
838 approximately 2 μM for the most used variants), **[Au: Does mito-pericam belong to the**
839 **cameleon type of sensors? Above it is stated otherwise]** built up by combining a circularly
840 permutated fluorescent protein and a Ca^{2+}-responsive element. These sensors allow
841 ratiometric measurements **[G] [Au: Please add to Glossary]** of Ca^{2+}levels. The development
842 of mito-GCaMP chimeras (K_d of the semi-ratiometric, high-affinity 2mt-GCaMP6m chimera:
843 0.167 μM) and their derivatives mito-CEPIAs (K_d of the original CEPIA2mt construct: 0.67 μM)
844 and mito-GECOs (2mt-GEM-GECO1: K_d = 0.34 μM) has expanded the spectra for the analysis of
845 mitochondrial Ca^{2+} concentrations. Intriguingly, by fusing GFP and aequorin, a new class of
846 ratiometric Ca^{2+} probes, termed GAPs, has been generated[224]. GAP indicators have been
847 targeted to various organelles, including mitochondria, but the performance of mito-GAP
848 constructs has not yet been widely tested. **[Au:OK?]**
849

BOX 2

Mitochondrial Ca^{2+} in cell death and cancer

There is no doubt that cell death belongs to the numerous cell functions in which Ca^{2+} exerts a complex regulatory role. It has long been known that in neurons and other cell types, an uncontrolled increase in cytoplasmic Ca^{2+} concentration $[Ca^{2+}]_c$ can trigger apoptosis[225-227], and likewise, agents that are able to release Ca^{2+} from intracellular stores have been shown to be pro-apoptotic[228]. **[Au: OK to remove? I think this study supports the observations that Ca influx can lead to neuronal death.]**

Mitochondria have emerged as a critical site for the action of the "apoptotic" Ca^{2+} signal. Whereas transient mitochondrial Ca^{2+} oscillations stimulate metabolism and constitute a pro-survival signal, prolonged mitochondrial Ca^{2+} overload is a fundamental trigger to initiate apoptosis through the opening of mitochondrial permeability transition pore (mPTP)[135,137]. **[Au: Maybe you could briefly explain what mPTP is and how it contributes to apoptosis?]** Indeed, treatment with apoptotic stimuli causes a release of Ca^{2+} from the ER and induces dramatic changes in mitochondrial morphology and in the release of caspase **[G] [Au: Please add to Glossary]** co-factors, leading to caspase activation **[Au:OK?]**. If Ca^{2+} changes are prevented, mitochondrial morphology is preserved, and the cells are protected from apoptosis[137].

The mitochondrial Ca^{2+} machinery thus represents a key decoding station for cell fate decisions. Several proto-oncogenes and tumour suppressors critically control these decisions by modulating mitochondrial Ca^{2+} dynamics. **[Au:OK?]** By either controlling Ca^{2+} signals arising from the ER (as is the case for PML, PTEN, p53, AKT, for example) or directly modifying the activity of mitochondrial proteins involved in Ca^{2+} influx and efflux **[Au:OK?]** (as is observed, for example, for FHIT, STAT, Fus1), these proteins are able to modulate anti- or pro-apoptotic signals, preventing or facilitating mitochondrial Ca^{2+} overload, and in consequence cell death (for a review, see [137]).

In response to different stress signals, tumour suppressors and proto-oncogenes can act at the ER by modulating Ca^{2+} store contents and/or Ca^{2+} dynamics (including Ca^{2+} leakage, Ca^{2+} uptake and Ca^{2+} release)[230], and at mitochondria, by affecting the expression levels of the components of the mitochondrial calcium uniporter (MCU) complex or the expression of leucine zipper EF-hand containing transmembrane protein 1 (LETM1), which can drive MCU-independent Ca^{2+} uptake, thereby regulating mitochondrial Ca^{2+} levels[231,232]. **[Au: Edit OK?]**

Mitochondrial Ca^{2+} levels can also regulate the metabolic shift from oxidative phosphorylation to aerobic glycolysis (Warburg effect) that generally occurs in cancer cells and fuels their

proliferation **[Au:OK?]**. More specifically, lowering the levels of mitochondrial Ca^{2+} stimulates the activity of pyruvate dehydrogenase phosphatase, which is inhibited by Ca^{2+}, thereby inducing the phosphorylation, and hence inactivation, of the pyruvate dehydrogenase. In consequence, pyruvate is utilized for the production of lactate, instead of acetyl-CoA, which would be channeled to the Krebs cycle. **[Au: Edit OK?]** Notably, recent evidence has shown that both glycolytic and mitochondrial metabolism are essential for Warburg effects and **[Au:OK?]** cancer cell proliferation[233]. In addition, higher mitochondrial Ca^{2+} uptake capacity and generation of reactive oxygen species has been recognized as a feature of metastatic cells, which promotes cell migration and invasiveness, but at the same time increases vulnerability of cancer cells to Ca^{2+}-mediated apoptosis.

Overall, it has become evident that the loss of mitochondrial Ca^{2+} homeostasis is a hallmark of tumorigenesis and that it can favour their survival and can augment their proliferative and migratory activity. Restoring proper mitochondrial Ca^{2+} signalling could therefore be a promising avenue for cancer treatment. For example, by modulating mitochondrial Ca^{2+} cancer cells could be re-sensitized to pro-apoptotic signalling. Indeed, many chemotherapeutic agents, as well as photodynamic therapy **[G] [Au: Please add to Glossary]** [234,235], exert their cytotoxic effects via Ca^{2+} signalling at ER–mitochondria contact sites, and therefore, their actions are completely abolished in cancer cells with altered Ca^{2+} kinetics and could be improved by combinatorial treatment with drugs targeting mitochondrial Ca^{2+} transport machinery. **[Au: Edit OK?]**

Figure Legends

Figure 1: Intracellular Ca^{2+} signalling

The endoplasmic reticulum (ER) (sarcoplasmic reticulum (SR) in muscle cells) is the major intracellular Ca^{2+} storage organelle. The sarco/endoplasmic reticulum Ca^{2+}-ATPase SERCAs actively pump Ca^{2+} into the store. The dynamic release of Ca^{2+} from the ER is mediated by the ryanodine receptor (RyR) and the inositol 1,4,5-triphosphate receptor (IP3R). Ca^{2+} released from the ER is captured by nearby mitochondria located in close contact with the ER through the voltage-dependent anion channel (VDAC) and the mitochondrial Ca^{2+} uniporter complex (MCU_c), activating cellular metabolism.

Depletion of the ER Ca^{2+} stores **[Au:OK?]** results in the activation of Ca^{2+} sensor protein, stromal interaction molecule 1 (STIM1), at the junctions between the ER and the plasma membrane, where it binds to and activates the Ca^{2+} channel protein ORAI1 (channel protein of Ca^{2+} release-activated Ca^{2+} channels (CRACs)) **[Au:OK?]** for store-operated Ca^{2+} entry (SOCE). The

intracellular Ca^{2+} influx is also mediated by TRP channels (TRPCs), most of which are activated by depletion of Ca^{2+} from the ER, whereas the plasma membrane Ca^{2+} ATPase (PMCAs) function to export Ca^{2+} from the cytosol and maintain the intracellular Ca^{2+} concentration at the basal value for proper cell signaling. In addition to the ER, lysosomes have recently been recognized as the second largest store of intracellular Ca^{2+} that are able to release Ca^{2+} through the transient receptor potential mucolipin 1 (TRPML1) **[Au: Nomenclature OK?]** channel, which is crucial for maintaining correct lysosomal membrane trafficking.

Figure 2: The mitochondrial Ca^{2+} uptake pathway

The formation of microdomains of high Ca^{2+} concentration between the endoplasmic reticulum (ER) and mitochondria is critical for ensuring proper Ca^{2+} entry into the mitochondrial matrix. **a)** The correct distance between the ER and mitochondria (~50 nm) is preserved by different regulators, including mitofusin-2 (MFN2), the VAPB (vesicle-associated membrane protein-associated protein B)–PTPIP51 (protein tyrosine phosphatase-interacting protein 51) complex and, at least in yeast the ER–mitochondrial encounter structure (ERMES) complex (in mammals PDZD8 has been identified as an orthologue of the MM1 protein of the complex). **[Au: Edit OK?]** Once released by inositol 1,4,5 trisphosphate receptors (IP3Rs), the ER Ca^{2+} enters mitochondria through the outer mitochondrial membrane (OMM) protein voltage-dependent anion channel (VDAC) and thus reaches the intermembrane space (IMS). **b)** Ca^{2+} reaches the mitochondrial matrix via the mitochondrial Ca^{2+} uniporter complex (MCU_c), located at the inner mitochondrial membrane (IMM). **[Au: Edit OK?]** The MCU_c consists of the pore-forming subunit MCU and the transmembrane proteins: MCU regulatory subunit b (MCUb) and the essential MCU regulator (EMRE) in association with the IMS proteins: mitochondrial calcium uptake protein 1(MICU1) and MICU2. MCU regulator 1 (MCUR1) might regulate Ca^{2+} entry from the matrix, but its role as a specific MCU complex component is highly controversial. **c)** The activity of the electron transport chain, a series of enzymes and coenzymes located in the cristae, results in the pumping of H^+ ions outside the mitochondrial matrix, thereby generating an electrochemical proton gradient. This gradient consists of two components: the difference between the cytosolic and matrix pH and the membrane potential difference ($\Delta\Psi$), which is maintained at approximately -180 mV and represents the driving force for mitochondrial Ca^{2+} uptake. **d)** Mitochondria contain both Na^+-dependent and Na^+-independent mechanisms for Ca^{2+} extrusion toward the cytoplasm. The molecular nature of the Na^+/Ca^{2+} exchanger (mNCX) has been identified in NCLX, an IMM protein containing 13 transmembrane domains and catalyzing K^+-independent electrogenic transport. Leucine zipper EF-hand containing

transmembrane protein 1 (LETM1) has been proposed as H^+/Ca^{2+} exchanger (mHCX). It might act as a transporter by forming hexameric structures, exchanging Ca^{2+} ions for H^+ ions **[Au:OK?]** in an electroneutral manner. However, it has been suggested that LETM1 acts as a K^+/H^+ exchanger rather than the H^+/Ca^{2+} exchanger (mHCX). Very recent findings have shown that LETM1 contributes to Na^+ cycling, thus modulating Ca^{2+} fluxes in an indirect way[236] (not shown).

Figure 3: Role of mitochondrial Ca^{2+} in pathophysiological processes

The generation of high Ca^{2+} microdomains at membrane contact sites: mitochondria associates membranes (MAMs; established between mitochondria and the endoplasmic reticulum (ER)) or plasma membrane-associated membranes (PAMs) is fundamental to permit mitochondrial Ca^{2+} uptake through the mitochondrial Ca^{2+} uniporter complex (MCU_c) and for many cellular functions.

A) In pancreatic β cells, glucose uptake [1] induces ATP production via glycolysis [2]. The increased metabolism inhibits ATP-sensitive K^+ channels (K-ATP channels) [3] and permits the opening of transient opening Ca^{2+} channels (TTCCs) and long-lasting Ca^{2+} channels (LTCCs) on the plasma membrane [4] and in turn insulin release assisted by protein kinase C β type (PKCβ) **[Au:OK?]** [5]. The increase of cytosolic [Ca^{2+}] also promotes mitochondrial ATP production from mitochondria at PAMs [6] and increased ER Ca^{2+} concentration **[Au: Edit OK?]** [7] and in turn enhanced ER Ca^{2+} release by inositol 1,4,5 trisphosphate receptors (IP3Rs) and/or by ryanodine receptors (RyRs) [8] and mitochondrial ATP production at MAMs [9]. **[Au: Edit OK?]**

B) In cardiac cells **[Au: Or in sinoatrial nodal cells specifically?]** , during action potential, the sodium inward current (funny current) [1] leads to opening of LTCC and/or TTCCs **[Au:OK?]** [2]. The cytosolic increase in Ca^{2+} is captured by mitochondria at PAMs **[Au:OK?]** to produce ATP [3]; it also induces Ca^{2+}-induced Ca^{2+} release (CICR) from the ER through RyRs [4] that permits cardiac muscle contraction [5] and sustains mitochondrial metabolism and ATP production that supports contraction **[Au:OK?]** [6].

C) In epithelia, different chronic stress situations, including tissue damage or infection [1], induce ER stress [2] with a consequent, prolonged Ca^{2+} transfer towards mitochondria at MAMs and mitochondrial Ca^{2+} overload [3]. This Ca^{2+} overload induces a strong inflammatory response mediated by the activation of NLRP3 inflammasome, which is essential for the production of Interleukin 1β and other pro-inflammatory mediators and by the mitochondria antiviral signaling (MAVS) complexes, which interact with the MCU to positively regulate the release of the proinflammatory cytokine interferon β. **[Au: Edit OK?]**

D) Mitochondria are key for buffering Ca^{2+} in neurons to regulate neurotransmitter release, which is dependent on Ca^{2+}. **[Au:OK?]** **(Da)** Under normal stimulation, action potential generation [1] promotes Ca^{2+} entry through Voltage-Gated Ca^{2+} Channels (VGCCs) in presynaptic cells [2] and mitochondria efficiently buffer Ca^{2+} [3] to ensure moderate neurotransmitter release[4]. **[Au: Edit OK?]** **(Db)** During excitotoxicity [1] , higher levels of glutamate released from presynaptic cells [2] induce excessive activation of N-methyl-$_D$-aspartate receptors (NMDARs) and Ca^{2+} entry into postsynaptic cells [3], with consequent mitochondrial Ca^{2+} overload [4] and the opening of mitochondrial permeability transition pore (mPTP) [5], which promotes apoptosis. **[Au: Edit OK?]**

Table 1: Regulatory pathways of mitochondrial Ca^{2+}

Regulator	Ca^{2+} Regulation	Molecular Mechanism	Disease links
VDAC channels			
*** Bcl-xL**	Positive	Interacts with VDAC1-3, probably promoting VDAC closure and calcium permeability	- [74]
*** Bcl-xL**	Negative	Inhibits VDAC1 through its BH4 domain	- [79]
GSK3	Negative	Phosphorylates VDAC at a Thr residue	Liver steatosis [97]
miR-7	Negative	Reduces *VDAC1* expression and inhibits mPTP opening	Parkinson disease [106]
miR-29a	- (Probably negative)	Reduces *VDAC1* expression and improves survival upon ischaemia	Cerebral ischaemia [107]
MCU complex			
*** MCUR1**	Positive	Interacts with MCU, promoting Ca^{2+} entry	Hepatocellular carcinoma [80-83,86]
*** SLC25A23**	Positive	Interacts with MCU, promoting Ca^{2+} entry	- [87]
PyK2	Positive	Activated PyK2 translocates to mitochondria, phosphorylating MCU and favoring multimeric channel pore formation	Myocardial cell death [99]
CaMKII	Positive	Phosphorylates MCU at Ser57 and Ser92	Myocardial cell death and heart failure [100]
miR-25	Negative	Reduces the expression of the pore-forming subunit *MCU*	Colon and prostate cancer; pulmonary arterial hypertension **[Au: Use bullet points instead of semi-colon?]** [108-110]
miR-138	Negative	Reduces the expression of the pore-forming subunit *MCU*	Pulmonary arterial hypertension [110]

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miR-1	Negative	Reduces the expression of the pore-forming subunit *MCU*	Cardiac hypertrophy [111]
miR-340	Negative	Reduces *MCU* expression and inhibits breast cancer cell migration	Breast cancer [112]
PRMT1	Negative	Methylates MICU1, promoting MCU-independent Ca2+ uptake through UCP2/3 **[Au: Edit OK?]**	- [103]
AFG3L2/SPG7	Negative	Loss of AFG3L2/SPG7 induces the formation of constitutively active MCU–EMRE complexes	Neurodegeneration [104,105]
Mia40	Negative	Ensures the association of MICU1 with the inhibitory subunit MICU2	- [28]

ER-mitochondria tethering			
*** MFN2**	Positive or negative	Loss of MFN2 causes detachment of the ER from mitochondria or increases the ER–mitochondria association	Charcot-Marie-Tooth neuropathy type 2A; obesity, insulin resistance **[Au: Use bullet points instead of semi-colon?]** [90,91,237,238]
PDZD8	Positive	A structural and functional orthologue of the yeast ERMES protein Mmm1	- [93]
PTPIP51	Positive	Interacts with the ER protein VAPB to regulate ER–mitochondria tethering	Ischaemia–reperfusion injury; amyotrophic lateral sclerosis; Parkinson disease **[Au: Use bullet points instead of semi-colon?]** [179,205,206,239]
FATE1	Negative	Regulates Ca^{2+} transfer and steroid hormone production	Adrenocortical carcinoma [240]
Presenilin-2	Positive	Increases the frequency of Ca^{2+} hot spots at MAMs	Alzheimer disease [241]
PACS-2	Positive	Loss causes detachment of the ER from mitochondria	Obesity, insulin resistance [242,243]

Mitochondrial Ca^{2+} efflux			
PINK1	Negative	• PINK1 knockout cells display reduced mitochondrial Ca^{2+} efflux • PINK1 increases Ca^{2+} release by phosphorylating LETM1 at Thr192 **[Au: Use of bullet points OK?]**	Parkinson disease [95,96]
PKA	Negative	Phosphorylates NCLX at Ser258, increasing Ca^{2+} efflux	Parkinson disease [95]

Mitochondrial membrane potential			
*** MCUR1 (CCDC90a)**	Positive	Acts as assembly factor for cytochrome c oxidase	- [85]
SK2 channel	Negative	Activation of mitochondrial SK2 reduces respiration and reactive oxygen species generation	- [244]

The * indicates proteins with a controversial role

Abbreviations: VDAC (voltage-dependent anion channel), MCU (mitochondrial Ca^{2+} uniporter), Bcl-xL (B-cell lymphoma-extra-large), GSK3 (glycogen synthase kinase 3), mPTP (mitochondrial permeability transition pore), MCUR1 (mitochondrial calcium uniporter regulator 1), MCU (mitochondrial calcium uniporter), SLC25A23 (solute carrier family 25 member 23), Pyk2 (proline-rich tyrosine kinase 2), CaMKII (Ca2+/calmodulin-dependent protein kinase II), PRMT1 (protein arginine N-methyltransferase 1), MICU1 (mitochondrial calcium uptake 1), UCP2/3 (mitochondrial uncoupling protein 2/3), AFG3L2 (AFG3 like matrix AAA peptidase subunit 2), SPG7 (spastic paraplegia 7), Mia40 (mitochondrial intermembrane space import and assembly protein 40), MICU2 (mitochondrial calcium uptake 2), MFN2 (mitofusin 2), ER (endoplasmic reticulum), PDZD8 (PDZ domain-containing protein 8), ERMES (ER–mitochondrial encounter structure), PTPIP51 (protein tyrosine phosphatase-interacting protein 51), VAPB (vesicle-associated membrane protein-associated protein B/C), IP3R (inositol 1,4,5 trisphosphate receptor), FATE1 (fetal and adult testis-expressed transcript protein 1), MAMs (mitochondria-associated membranes), PACS-2 (phosphofurin acidic cluster sorting protein 2), PINK1 (PTEN-induced putative kinase 1), LETM1 (leucine zipper-EF-hand containing transmembrane protein 1), PKA (protein kinase A), NCLX (sodium/calcium/lithium exchanger), CCDC90a (coiled-coil domain-containing protein 90A), SK2 (potassium intermediate/small conductance calcium-activated channel).

Table 2: Other pathological contexts related to deregulation of mitochondrial Ca2+

Pathological Phenomena	Type of Disease	Site of altered mitochondrial signalling	Mitochondrial Ca^{2+} association	REFs
Muscle diseases	Proximal muscle weakness	Chronic activation of the MCU channel	Increased mitochondrial Ca^{2+} load, mitochondrial fragmentation and muscle dysfunction	245
	Muscular dystrophy	Sarcolemma damage and calcium leak	Mitochondrial Ca^{2+} overload, muscle wasting	246,247
Ageing	Neuronal aging	Increased ER–mitochondrial cross talking	Mitochondrial Ca^{2+} overload, neuron cell death, cognitive decline	248
	Muscle aging	Decreased SR–mitochondria interaction	Reduced mitochondrial Ca^{2+} signalling, reduced ATP production, decline of skeletal muscle performance	249

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	Cardiac aging	maladaptive changes in protein levels of the MCU complex (reduced MCU and EMRE, increased MCUb)	Deficient mitochondrial Ca^{2+} handling, reduced mitochondrial metabolism	250
		Defective communication between SR-mitochondria	Reduced mitochondrial Ca^{2+} uptake, energy demand–supply mismatch	251
Pulmonary arterial hypertension		MCU complex dysfunction (decreased MCU and increased MICU1)	Reduced mitochondrial Ca^{2+} uptake, mitochondrial fragmentation reduced apoptosis	110
		UCP2 deficiency	Reduced mitochondrial Ca^{2+} signalling	252
Mitochondrial disorders		Decreased ER–mitochondria contacts	Reduced mitochondrial Ca^{2+} uptake	253,254

Abbreviations:

EMRE, essential MCU regulator; ER, endoplasmic reticulum; MICU1, mitochondrial calcium uptake 1; MCU, mitochondrial Ca^{2+} uniporter;; MCUb, MCU regulator MCUb (also known as CCDC109b); SR, sarcoplasmic reticulum; UCP2, mitochondrial uncoupling protein 2.

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Competing interests

The authors declare no competing interests. **[Au: Correct?]**

Author contributions

All authors researched data for the article, contributed to discussion of the content, wrote the article and edited the manuscript. **[Au:Correct?]**

Glossary [Au: Please note that I added several new terms to the Glossary]

Membrane potential

The difference in electric potential (measured in mV) between the interior and exterior of a biological membrane generated from different concentrations of ions, such as H^+, Na^+, K^+ and Cl^-.

Respiratory chain

The electron transport chain consists of four complexes that transfer electrons from NADH and FADH2 to oxygen, which is reduced to water. Electron flow within these transmembrane complexes leads to the transport of H^+ across the inner mitochondrial membrane, generating an electrochemical proton gradient (negative inside the matrix).

Ruthenium red

Chemiosmotic theory

The energy stored in the form of the transmembrane electrochemical gradient is used to produce ATP inside the mitochondrial matrix. The protons move back across the inner mitochondrial membrane through the F_1 F_o ATPase enzyme, coupling the electrochemical gradient to ATP production by combining ADP with inorganic phosphate.

Mitochondrial permeability transition pore (mPTP)

Bcl-2 family

A large group of evolutionarily conserved proteins that share Bcl-2 homology (BH) domains. Bcl-2 family members are deeply involved in cell death regulation, consisting of both anti-apoptotic (Bcl-2, Bcl-xL) and pro-apoptotic (Bax, Bak) factors.

ER–mitochondrial encounter structure (ERMES) complex

PINK1

Neointimal hyperplasia

Astrocytes

The most numerous and heterogeneous neuroglial cells in the central nervous system, distinguished by a star-like morphology with multiple primary processes originating from the soma. **[Au: OK to shorten?]**

Acinar cells

Zymogen

Nutrient secretagogues

Excitation-contraction coupling (*EC coupling*)

Excitation-transcription coupling (*ET coupling*)

Excitation-metabolism coupling (*EM coupling*)

Action potential

A movement of charge sufficient to generate a large and brief deviation in the membrane potential. It is used to communicate information between neurons and from neurons to muscle fibers.

'Funny' current

Troponin C

A component of the troponin complex, together with troponin I and T, which regulates muscle contraction by Ca^{2+} binding. Through its multiple EF-hand domains, troponin C acts as the Ca^{2+}

sensor of the troponin **[Au:OK?]** complex, initiating the cascade of events that leads to contraction of striated muscle by interacting with troponin I after Ca^{2+} binding.

Myofilaments

The principal molecular regulators of contraction in cardiac and skeletal muscles, responsible for force generation and motion. Myofilaments consist primarily of thick filament myosin and thin filament actin proteins, as well as additional components, including troponin, titin and nebulin.

Fight-or-flight response

Stasis

Pyrin domain-containing 3 (NLRP3) inflammasome

A complex, formation of which leads to the activation of caspase 1, secretion of proinflammatory cytokines and induction of inflammatory cell death (or pyroptosis). **[Au: Edit OK?]**

Mitochondria antiviral signaling (MAVS) complexes

Excitotoxicity

N-methyl-D-aspartate receptors (NMDARs)

Purkinje cells

Oligodendrocyte

Förster resonance energy transfer (FRET)

Ratiometric measurement

Caspase

Photodynamic therapy

[Au: We have now discontinued publishing of Key points.]

References [Au: For references that are particularly worth reading (5-10% of the total), please provide a single bold sentence that indicates the significance of the work. Please add each sentence below the highlighted reference directly in the main reference list.]

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