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The machineries, regulation and cellular functions of mitochondrial calcium

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Abstract | Calcium ions (Ca^{2+}) are some of the most versatile signalling molecules, and they have 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 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, which offers the possibility of targeting mitochondrial Ca^{2+} machinery for the treatment of several disorders.

Membrane contact sites
The close apposition between two (or more) organelles in which membranes do not fuse, thereby maintaining their specific characteristics.

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The two adjectives most commonly used to define calcium signalling are universal and versatile[1]. The universality of calcium ions (Ca^{2+}) as signalling molecules derives from their ubiquity as intracellular second messengers that control 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).

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 can take up 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 60 years, intense research in the Ca^{2+} field has defined the basic features of mitochondrial Ca^{2+} handling and has 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- to 20-fold more Ca^{2+} than the cytosolic compartment. The sources of Ca^{2+} required for such $[Ca^{2+}]$ rises are external (the prevailing mechanism in neurons and other excitable cells), whereby Ca^{2+} is taken up from the extracellular milieu ($[Ca^{2+}]$ of ~1 mM) by plasma membrane channels, such as transient receptor potential channels (TRPCs) and Ca^{2+} release-activated Ca^{2+} (CRAC) channel protein 1 (ORAI1), or internal, resulting from the release of Ca^{2+} from internal sources — endoplasmic reticulum (ER) or sarcoplasmic reticulum (SR) in muscle cells, the Golgi apparatus and lysosomes — via different classes of intracellular channel, such as the inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) receptors ($Ins(1,4,5)P_3Rs$), ryanodine receptors (RyRs) and TRPC mucolipin 1 (TRPML1). These intracellular Ca^{2+} stores are loaded with Ca^{2+} ($[Ca^{2+}]$ >300 μM) at the expense of ATP hydrolysis via the activity of Ca^{2+} pumps (sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPases (SERCAs) and the secretory pathway Ca^{2+}-ATPases (SPCAs)). The reduction of ER intraluminal Ca^{2+} results in a massive influx of Ca^{2+} from the extracellular space through the CRAC channels, 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 large number of signalling functions by increasing cytosolic $[Ca^{2+}]$[7] (BOX 1; FIG. 1).

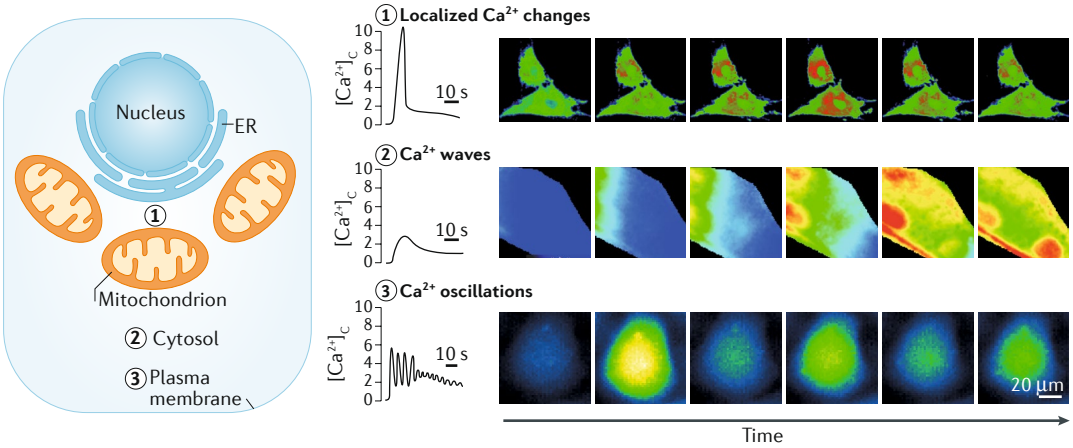
The close proximity of mitochondria to Ca^{2+} stores, owing to the formation of membrane contact sites, in particular, with the ER, and the presence of a highly

Box 1 | **Ca^{2+} 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+} concentration ($[Ca^{2+}]_c$) is elicited by a wide variety of molecules and decoded into very different, sometimes opposite, effects. To fulfil such a complex signalling role, $[Ca^{2+}]_c$ rises are spatially and temporally regulated (see the figure). 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 G protein-coupled receptors, 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, through the specific recruitment of downstream effectors, is decoded into the appropriate cellular effect.

Various mammalian proteins are regulated by Ca^{2+}, and these are classified as buffer or trigger proteins[216]. Ca^{2+} buffers encompass calsequestrin and calreticulin, which are located at the sarcoplasmic and endoplasmic reticulum (ER), respectively, and the cytosolic proteins calbindin and calretinin, as well as the relatively slow buffer parvalbumin. This class of molecules not only cooperates with Ca^{2+} channels, transporters and pumps to shape intracellular Ca^{2+} signals but also displays multiple functions and physiological roles[217]. Trigger proteins include modulators of muscle contraction (troponin C, slow skeletal and cardiac muscles (TN-C)), proteases (calpain), kinases (protein kinase C (PKC)) phosphatases (calcineurin B), transcription factors (members of the nuclear factor of activated T cells (NFAT) and cAMP-responsive element-binding protein (CREB) families) and key mediators of various enzymes (calmodulin). Most of these possess one or multiple EF-hand Ca^{2+}-binding motifs and change their conformation upon binding Ca^{2+}. Overall, Ca^{2+} binding can affect the localization, molecular associations and functions of a multitude of proteins, regulating a vast array of biological processes, such as contraction, transcription and other signalling networks.

The functions of Ca^{2+} in coordinating different cellular events are not limited to variations in its cytosolic levels but can be extended to changes in Ca^{2+} inside organelles. For example, lysosomal Ca^{2+} activates calcineurin, which in turn promotes transcription factor EB (TFEB) translocation into the nucleus and transactivation of its target genes[132]. As another example, after fertilization, mitochondrial Ca^{2+} entry sustains production of reactive oxygen species and cell cycle progression in early *Xenopus laevis* embryos[218].



The images in the figure represent montages from imaging of cytosolic $[Ca^{2+}]$ changes in a single cell, loaded with the Ca^{2+}-sensitive Fura-2/AM probe, acquired with a high speed, wide-field digital microscope. The pseudocoloured images represent $[Ca^{2+}]$, where cold and hot colours indicate low and high $[Ca^{2+}]$, respectively. Images were acquired by S. M. (top and midde panels) and by C. G. (bottom panel).

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

Respiratory chain
Also known as the electron transport chain, a series of proteins in the inner mitochondrial membrane that consists of four complexes that transfer electrons from NADH and $FADH_2$ 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).

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 difference ($\Delta\Psi$) generated by the respiratory chain, which provides the electrochemical force required for positively charged ions to enter the matrix. However, Ca^{2+} does not remain inside mitochondria but instead is 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+} signalling.

In this Review, we 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, discussing the most recent discoveries and the many unanswered questions and conflicting interpretations regarding mechanisms of Ca^{2+} homeostasis. We also outline the physiological role of mitochondrial Ca^{2+} and its deregulation in several pathological contexts.

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

Caspase
An endoprotease involved in cell death and inflammation that contains catalytic cysteine residues in its active site that hydrolyse substrate peptides after specific aspartic acid residues.

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).

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 voltage-dependent anion-selective channel proteins (VDACs), which form pores in the OMM and represent the first molecular interface between mitochondria and Ca^{2+} stores (the ER and/or SR and the extracellular space). VDACs exist as three subtypes (VDAC1, VDAC2 and VDAC3), which are expressed more or less ubiquitously but vary in their isoform ratios and sub-mitochondrial distribution among tissues[8,9] and mediate the flux of ions, nucleotides (ADP and/or ATP) and other metabolites. VDACs 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 determine a high-conductance, anion-selective state, whereas increased voltages (20–40 mV) promote lower conducting conformations, which are assumed to be impermeable to nucleotides but still allow the flow of small cations, including Ca^{2+} (REFS[10,11]). Notably, closed conformations of VDACs show higher selectivity and efficiency of Ca^{2+} transport[12], but the positive charge does not exclude Ca^{2+} from the open state, as the anion selectivity of VDACs is not very high. Of note, it has been proposed that the potential across the IMM might influence the OMM pores, suggesting that VDACs located at the mitochondrial membrane contact sites might assume a closed state, whereas VDACs positioned beyond the contacts should stay in the high-conducting open conformation[13]. Overall, VDACs mediate Ca^{2+} flux in both open and closed conformation, thereby limiting the generation of any $[Ca^{2+}]$ gradient across the OMM. Recent findings have highlighted the key role of VDACs in enhancing the Ins(1,4,5)P3-induced Ca^{2+} signal from the ER in different contexts[14,15], 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, as the depletion of all VDAC isoforms does not affect the sensitivity of mitochondria to Ca^{2+}-driven cell death[16].

After reaching the IMS, Ca^{2+} ions pass the IMM mainly through the mitochondrial Ca^{2+} uniporter (MCU) complex. However, MCU-independent, Ca^{2+} uptake mechanisms have been reported, including an IMM-located pool of RyRs[17], the canonical short transient receptor potential channel 3 (TRPC3)[18], mitochondrial uncoupling protein 2 (UCP2), UCP3[19] and leucine zipper EF-hand-containing transmembrane protein 1 (LETM1)[20]. Nevertheless, the MCU unequivocally represents the dominant mechanism that allows Ca^{2+} accumulation inside the mitochondrial matrix.

It is now firmly established that the uniporter exists in a macromolecular complex, which apart from pore-forming subunits comprises several regulatory proteins[21–23] (FIG. 2b). Molecular characterization of the complex was made possible by studies that simultaneously identified MCU protein (previously known as CCDC109a) as the bona fide channel-forming component[24,25]. The other elements of the holocomplex are the MCU regulator MCUb (also known as CCDC109b)[26] and the IMS-resident protein mitochondrial calcium uptake protein 1 (MICU1) (REF.[27]), which binds to its paralogue MICU2 (REF.[28]) to form a heterodimeric structure[29,30], which is associated with MCU through essential MCU regulator, EMRE (also known as SMDT1)[31]. Additional components of the complex have been described, including

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[219–221], and likewise, agents that are able to release Ca^{2+} from intracellular stores have been shown to be pro-apoptotic[222].

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 the mitochondrial permeability transition pore (mPTP)[138,140]. Indeed, treatment with apoptotic stimuli causes a release of Ca^{2+} from the endoplasmic reticulum (ER) and induces dramatic changes in mitochondrial morphology and in the release of caspase cofactors, leading to caspase activation. If Ca^{2+} changes are prevented, mitochondrial morphology is preserved, and the cells are protected from apoptosis[140].

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. By either controlling Ca^{2+} signals arising from the ER (as is the case for promyelocytic leukaemia protein (PML), PTEN, p53 and AKT, for example) or directly modifying the activity of mitochondrial proteins involved in Ca^{2+} influx and efflux (as is observed, for example, for fragile histidine triad protein (FHIT), signal transducer and activator of transcription 3 (STAT3) and tumour suppressor candidate 2 (TUSC2, also known as FUS1)), these proteins are able to modulate anti-apoptotic or pro-apoptotic signals, preventing or facilitating mitochondrial Ca^{2+} overload and consequently cell death (reviewed in REF.[140]).

In response to different stress signals, tumour suppressors and proto-oncogenes can act at the ER by modulating Ca^{2+} store content and/or Ca^{2+} dynamics (including Ca^{2+} leakage, Ca^{2+} uptake and Ca^{2+} release)[223] and at mitochondria by affecting the expression levels of the components of the mitochondrial Ca^{2+} 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[224,225].

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. 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 pyruvate dehydrogenase. In consequence, pyruvate is utilized for the production of lactate, instead of acetyl-CoA, which would be channelled to the Krebs cycle. Notably, recent evidence has shown that both glycolytic and mitochondrial metabolism are essential for cancer cell proliferation[226]. In addition, higher mitochondrial Ca^{2+} uptake capacity and generation of reactive oxygen species have been recognized as features of metastatic cells, and they promote cell migration and invasiveness but at the same time increase the 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 the survival and augment the proliferative and migratory activity of cancer cells. 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[227,228], 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.

Photodynamic therapy
A clinically approved therapeutic procedure that uses photosensitizing agents that, when exposed to a specific wavelength of light, produce a form of oxygen that kills tumour cells.

Ruthenium red
A polycationic dye that acts as an inhibitor of a wide number of ion channels, including all transient receptor potential channels (TRPCs), voltage-dependent anion-selective channel proteins (VDACs), mitochondrial calcium uniporter (MCU) and ryanodine receptors.

MICU3 (REF.[28]) and MICU1.1, a MICU1 splicing variant with higher Ca^{2+}-binding affinity than MICU1 (REF.[32]), which are tissue-specific members of the uniporter and are expressed in the central nervous system and skeletal muscle, respectively.

The working model of the uniporter holocomplex is the result of extensive research. After passing the OMM, the incoming Ca^{2+} is first handled by MICU1–MICU2 dimers owing to their strategic IMS localization and the presence of two Ca^{2+}-binding EF-hand domains in both MICU1 and MICU2. Loss-of-function studies have definitively demonstrated that MICU1–MICU2 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[33–35].

Dissecting the intrinsic role of both MICU1 and MICU2 is complicated by the observations that MICU2 and MCU protein expression could be affected by the loss of MICU1 or vice versa and that MICU2 is unable to associate with MCU protein in a MICU1-knockout background. One model proposes that MICU1 is a pure stimulatory subunit and identifies the dominant gatekeeping mechanism in MICU2 (REFS[29,30]). 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 activity and requires MICU2 only when the external $[Ca^{2+}]$ is between 500 nM and 1.5 μM[36,37]. Thus, in MICU2-knockout cells, the Ca^{2+} threshold for MCU activation is ~500 nM, which is threefold lower than in the presence of MICU2. Importantly, when the $[Ca^{2+}]$ rises, MICU1 cooperates with MCU to favour extensive Ca^{2+} entry[33,38], whereas MICU2 limits the MICU1-mediated gain of uniporter function[36], suggesting that MICU2 represents an additional layer of control for MCU activation.

Although initial nuclear magnetic resonance (NMR) studies of a truncated version of *Caenorhabditis elegans* MCU protein proposed a pentameric stoichiometry[39], very recent observations showed that MCU assembles into tetrameric structures that form a channel[40–43]. The activity of this channel is strictly dependent on EMRE as mammalian MCU does not transport Ca^{2+} in an EMRE-knockout background[31]. Although it was originally proposed that EMRE might control MCU by sensing the $[Ca^{2+}]$ in the matrix through its carboxy-terminal domain[44], subsequent, and in our opinion more convincing, observations have revealed that the carboxyl terminus of EMRE is located in the IMS rather than in the matrix[45,46], where it connects the MICU1–MICU2 sensors to MCU, thereby regulating Ca^{2+} entry[46]. MCU channel has been shown to be negatively regulated by MCU protein paralogue MCUb[26], although overexpression of MCUb in *Trypanosoma cruzi* did not have a dominant-negative effect on MCU[47], suggesting that in lower organisms, MCUb might have a complementary role to MCU protein.

The characterization of the function of the MCU complex fulfils all the properties that were attributed to the uniporter several years before its molecular discovery, such as sensitivity to inhibition by ruthenium red, high Ca^{2+} selectivity[48], induction of Ca^{2+} uptake only in energized mitochondria and low Ca^{2+} affinity (the apparent dissociation constant (K_d) of MCU is 20–30 μM)[49], 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.

Mitochondrial membrane potential as a driving force for Ca^{2+} uptake. After the wide acceptance of the chemiosmotic theory, it was postulated that the driving force for Ca^{2+} entry into the mitochondria is the proton electrochemical gradient potential generated by the activity of the respiratory electron transport chain. 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 favouring cation entrance) (FIG. 2c). As proof

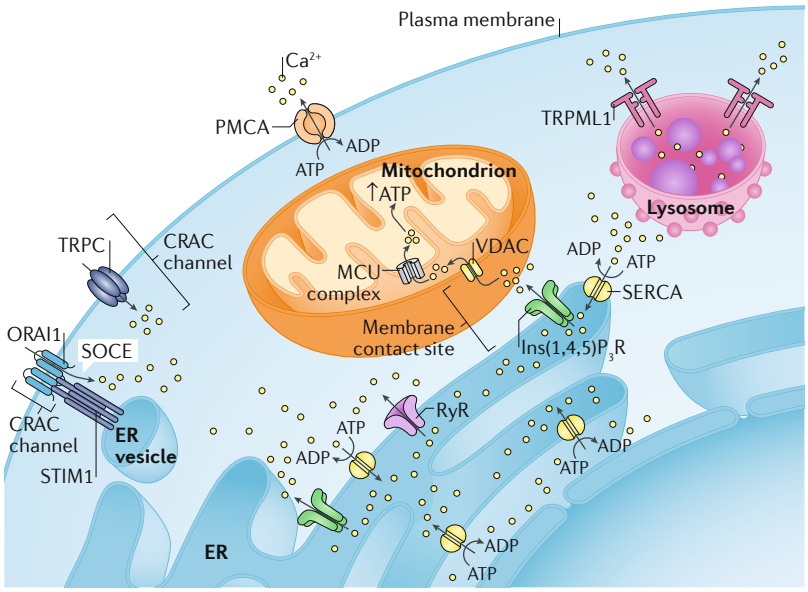


Fig. 1 | **Intracellular Ca^{2+} signalling.** The endoplasmic reticulum (ER) (sarcoplasmic reticulum in muscle cells) is the major intracellular Ca^{2+} storage organelle. The sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPases (SERCAs) actively pump Ca^{2+} into the store. The dynamic release of Ca^{2+} from the ER is mediated by ryanodine receptors (RyRs) and inositol 1,4,5-triphosphate receptors ($Ins(1,4,5)P_3Rs$). Ca^{2+} released from the ER is captured by nearby mitochondria located in close contact with the ER through voltage-dependent anion-selective channel proteins (VDACs) and the mitochondrial Ca^{2+} uniporter (MCU) complex, activating cellular metabolism. Depletion of the ER Ca^{2+} stores 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 Ca^{2+} release-activated Ca^{2+} channel protein 1 (ORAI1) (which functions as a Ca^{2+} release-activated Ca^{2+} channel (CRAC)) for store-operated Ca^{2+} entry (SOCE). The intracellular Ca^{2+} influx is also mediated by transient receptor potential channels (TRPCs), most of which are activated by depletion of Ca^{2+} from the ER and act as CRACs. The plasma membrane Ca^{2+} ATPases (PMCAs) function to export Ca^{2+} from the cytosol and maintain the intracellular Ca^{2+} concentration at the basal value for proper cell signalling. In addition to the ER, lysosomes have recently been recognized as the second largest store of intracellular Ca^{2+}, and they are able to release Ca^{2+} through TRPC mucolipin 1 (TRPML1), which is crucial for maintaining correct lysosomal membrane trafficking.

Box 3 | **Methods of measuring mitochondrial Ca^{2+}**

Two main genetically encoded strategies are currently used to design functional probes that measure mitochondrial Ca^{2+} concentrations: those based on the Ca^{2+}-activated photoprotein aequorin and those based on the use of fluorescent proteins and dyes. Using appropriate mitochondria-targeting signals, aequorin has been directed to both the outer mitochondrial membrane and the intramembrane space, although the most commonly used version is the aequorin chimaera targeted to the mitochondrial matrix by the pre-sequence of subunit VIII of cytochrome *c* oxidase[229]. Recombinant aequorin binds Ca^{2+} with an apparent dissociation constant K_d of 10 μM (K_d value for low-affinity, point-mutated aequorin is ~130 μM). Aequorin provides important benefits, such as a wide dynamic range, a high signal-to-noise ratio and the ability to emit light upon Ca^{2+} binding (without requiring potentially damaging light excitation). However, the use of aequorin displays some pitfalls, including low light emission by the photoprotein, which renders it inappropriate for imaging Ca^{2+} waves at the single-cell level.

These disadvantages have led to the extensive employment of alternative methods, such as fluorescent protein-based approaches, which combine bright fluorescence with efficient targeting to cellular subcompartments through tagging with localization signals or synthetic fluorescent dyes, which can be directly loaded into cells without the need for transfection. Rhod-2 AM (K_d = 0.57 μM) is the most commonly used chemical probe, offering reliable results in saponin-permeabilized cells or isolated mitochondria. However, it cannot be precisely targeted to mitochondria in intact cells, and measurement of its signal exhibits multiple drawbacks[230].

Mitochondrial fluorescent Ca^{2+} indicators are based on Ca^{2+} detection through a Ca^{2+}-sensing protein, such as calmodulin, and they are classified into two families: the first family is represented by the cameleons, which are based on Förster resonance energy transfer (FRET); the second family encompasses engineered single fluorescent proteins, such as GCaMP and pericam. These two classes of sensor allow ratiometric measurement of Ca^{2+} levels. Mitochondrially targeted chameleons include 2mt-D2cpv and its variants (K_d = 0.3–3 μM) and consist of cyan and yellow fluorescent protein pair, linked by calmodulin and the M13 peptide from the myosin light-chain kinase. Mito-pericams (K_d of ~2 μM for the most used variants) are built up by combining a circularly permutated fluorescent protein and a Ca^{2+}-responsive element. The development of mito-GCaMP chimaeras (K_d of the semi-ratiometric, high-affinity 2mt-GCaMP6m chimaera = 0.167 μM) and their derivatives, mito-CEPIAs (K_d of the original CEPIA2mt construct = 0.67 μM) and mito-GECOs (2mt-GEM-GECO1 K_d = 0.34 μM) has expanded the spectra for the analysis of mitochondrial Ca^{2+} concentrations. Intriguingly, by fusing GFP and aequorin, a new class of ratiometric Ca^{2+} probes, termed GAPs, has been generated[231]. GAP indicators have been targeted to various organelles, including mitochondria, but the performance of mito-GAP constructs has not yet been widely tested.

of this concept, dinitrophenol and carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone, two uncouplers of oxidative phosphorylation, were shown to dissipate the membrane potential across the IMM, thereby almost resetting the ΔΨ and abolishing Ca^{2+} entry via the uniporter[50,51].

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 Ca^{2+} store and the formation of ER–mitochondria contact sites. The distance between the ER and the mitochondrion at these sites varies between 10 nm and 60 nm[52], and the ER associates more frequently with mitochondria than with other organelles[53,54]. Upon opening of Ins(1,4,5)P_3Rs, this allows mitochondria to be exposed 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)[55], are small enough to allow contact between proteins on the surface of both organelles and ensure that upon induction of Ca^{2+} mobilization the [Ca^{2+}] on the cytosolic surface of the OMM reaches levels tenfold higher than those in the bulk cytosol[56] (FIG. 2a). Conversely, the [Ca^{2+}] to which the OMM is exposed during SOCE is similar in 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)[57], where mitochondria are exposed to a threefold higher [Ca^{2+}] upon activation of voltage-gated Ca^{2+} channels in the plasma membrane[56].

Mitochondrial Ca^{2+} efflux

Historically, two major systems have been postulated to extrude Ca^{2+} from the mitochondrial matrix: the mitochondrial Na^+/Ca^{2+} exchanger (mNCX) and the mitochondrial H^+/Ca^{2+} exchanger (mHCX). The first appears to be the predominant antiporter in excitable tissues (heart and brain), whereas the latter mainly leads to Ca^{2+} release in non-excitable tissues (liver and kidney). The stoichiometry of mNCX-driven transport is defined as electrogenic, with three (or four) Na^+ for one Ca^{2+} (REFS[58,59]), whereas the exchange ratio of mHCX is electroneutral (two H^+ for one Ca^{2+})[60] (FIG. 2d). Thus, the two Ca^{2+} efflux systems mediate the extrusion of Ca^{2+} from the mitochondrial matrix towards the IMS, reaching the cytosolic compartment by VDACs or additional Ca^{2+}-extruding mechanisms located at the OMM, such as the NCX family member NCX3 (also known as SLC8A3) (REF.[61]). 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 (also known as SLC8B1), a product of mammalian *SLC8B1* (REF.[62]). NCLX mediates not only Na^+/Ca^{2+} exchange but also Li^+-dependent Ca^{2+} transport, which was previously described for NCXs[63]. This property of NCLX, together with its confirmed mitochondrial localization, its sensitivity to the classical NCX inhibitor CGP-37157 and observations in multiple cell types that loss of NCLX alters mitochondrial Ca^{2+} efflux[62,64–66], provides strong indication that NCLX is the mNCX.

Whereas the molecular nature of the mNCX is generally accepted today, the identity of the mHCX is more controversial. In 2009, it was proposed that the IMM protein LETM1 acts as mHCX in both fly and mammalian cells as well as in vitro in proteoliposomes[20]. LETM1 might oligomerize into hexameric structures, thus acting as a transporter, even though it contains only a single transmembrane helix[67]. However, the role of LETM1 in Ca^{2+} release from the mitochondrial matrix has been questioned[68]; indeed, LETM1 was first reported as a K^+/H^+ exchanger[69–71], and some LETM1-related features described in initial studies, such as a stoichiometry of one H^+ for one Ca^{2+} and sensitivity to the MCU inhibitor ruthenium red[20], 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 mHCX function[72]. However, owing to the numerous

Dissociation constant
(K_d). A measure that indicates the strength of the binding interaction between a single biomolecule (for example, a protein) and its ligand or binding partner (for example, Ca^{2+} ions). The smaller the K_d value is, the greater the binding affinity of the ligand for its target.

Chemiosmotic theory
States that 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_1F_0 ATPase enzyme, coupling the electrochemical gradient to ATP production by combining ADP with inorganic phosphate.

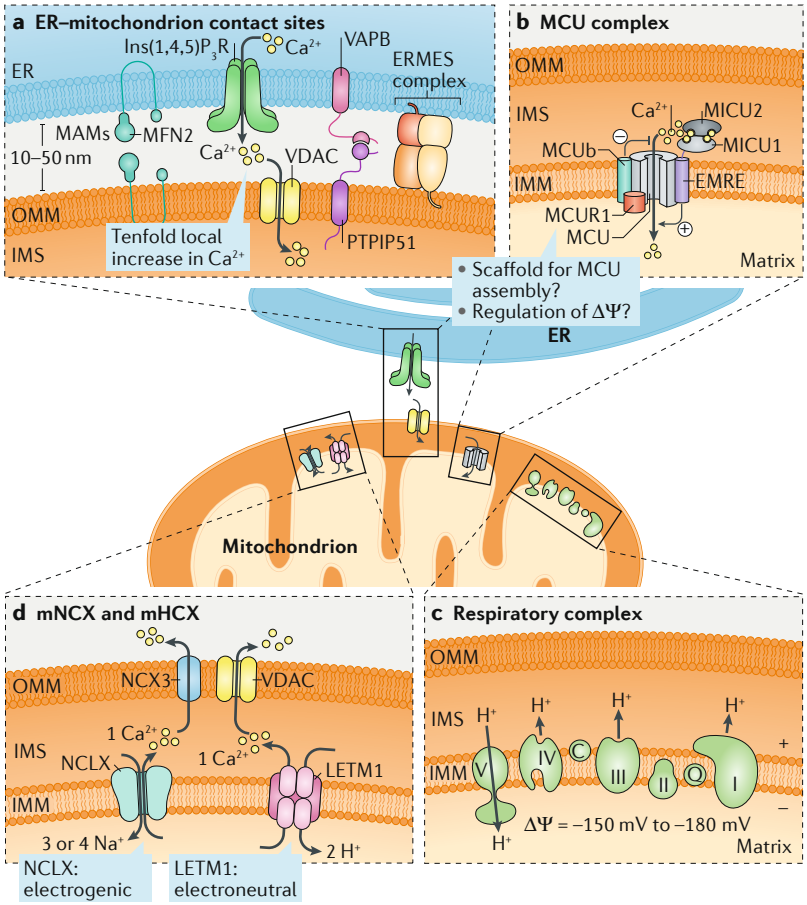


Fig. 2 | **The mitochondrial Ca^{2+} uptake pathway.** The formation of microdomains of high Ca^{2+} concentration between the endoplasmic reticulum (ER) and mitochondria is critical for ensuring proper Ca^{2+} entry into the mitochondrial matrix. **a** | The correct distance between the ER and mitochondria (~50 nm) is preserved by different regulators, including mitofusin 2 (MFN2), the vesicle-associated membrane protein-associated protein B/C (VAPB)–protein tyrosine phosphatase-interacting protein 51 (PTPIP51) complex and, at least in yeast, the ER–mitochondrial encounter structure (ERMES) complex (in mammals PDZ domain-containing protein 8 (PDZD8) has been identified as an orthologue of maintenance of mitochondrial morphology protein 1 (Mmm1) of the complex). Once released by inositol 1,4,5-trisphosphate receptors ($Ins(1,4,5)P_3Rs$), the ER Ca^{2+} enters mitochondria through outer mitochondrial membrane (OMM) voltage-dependent anion-selective channel proteins (VDACs) and thus reaches the intermembrane space (IMS). **b** | Ca^{2+} reaches the mitochondrial matrix via the mitochondrial Ca^{2+} uniporter (MCU) complex, which is located at the inner mitochondrial membrane (IMM). The MCU complex 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 to 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 towards 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 catalysing K^+-independent electrogenic transport. Leucine zipper EF-hand-containing transmembrane protein 1 (LETM1) has been proposed as the mitochondrial H^+/Ca^{2+} exchanger (mHCX). It might act as a transporter by forming hexameric structures, exchanging Ca^{2+} ions for H^+ ions in an electroneutral manner. However, it has been suggested that LETM1 acts as a K^+/H^+ exchanger rather than an mHCX. Very recent findings have shown that LETM1 contributes to Na^+ cycling, thus modulating Ca^{2+} fluxes in an indirect way[232] (not shown). MAMs, mitochondria-associated membranes.

conflicting results with respect to mitochondrial Ca^{2+} levels observed in LETM1-silenced cells[20,64,67,73,74], 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 conditions, such as at low cytosolic $[Ca^{2+}]$ (<1 μM), functioning as a high-affinity Ca^{2+} uptake system alternative to the MCU[20]. This concept has been confirmed by others[74,75], but progress in understanding the mechanisms of the MCU has diminished interest in such observations.

Experimental evidence suggests that mHCX and mNCX cannot be the sole 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) might represent an alternative Ca^{2+} efflux pathway[76,77], although other observations question this hypothesis[78].

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 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).

Owing to their strategic position in the OMM, VDACs are the preferential targets for endogenous proteins located at the interface between mitochondria and the cytoplasm to control Ca^{2+} influx into mitochondria. This group of regulators includes BCL-2 family members[79]. For example, apoptosis regulator BCL-X_L interacts with VDAC1 and VDAC3 (but not VDAC2), shaping mitochondrial Ca^{2+} entry by favouring Ca^{2+} transfer across the OMM[80]. Mechanistically, BCL-X_L was suggested to promote VDAC closure[81], which would enhance its selectivity and permeability for Ca^{2+} (see discussion on VDACs above). However, this aspect remains largely unclear, and several reports contradict this potential mechanism. First, it has been reported that BCL-X_L promotes the open VDAC state rather than the closed configuration[82]. It has also been proposed that VDACs can assume a cation-selective open conformation[83]. Finally, BCL-X_L has been reported to inhibit VDAC1 activity, thus lowering, rather than increasing, the mitochondrial $[Ca^{2+}]$[84].

Several intramitochondrial proteins have been suggested to regulate Ca^{2+} signalling by altering MCU complex assembly or functions. The first, in chronological order, is MCU regulator 1 (MCUR1) (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[85]. The interaction between MCU and MCUR1 has been reported in other studies[86–88], and MCUR1 was recently proposed to function as a scaffold in the assembly of the uniporter complex[89]. Conversely,

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

Regulator	Ca^{2+} regulation	Molecular mechanism	Disease links	Refs
VDACs				
BCL-X_L[a]	Positive	Interacts with VDAC1 and VDAC3, probably promoting VDAC closure and Ca^{2+} permeability	NA	80
BCL-X_L[a]	Negative	Inhibits VDAC1 through its BH4 domain	NA	84
GSK3	Negative	Phosphorylates VDAC at a Thr residue	Liver steatosis	105
miR-7	Negative	Reduces *VDAC1* expression and inhibits mPTP opening	Parkinson disease	233
miR-29a	Probably negative	Reduces *VDAC1* expression and improves survival upon ischaemia	Cerebral ischaemia	234
MCU complex				
MCUR1[a]	Positive	Interacts with the MCU complex, promoting Ca^{2+} entry	Hepatocellular carcinoma	85–88,91
SLC25A23[a]	Positive	Interacts with the MCU complex, promoting Ca^{2+} entry	NA	92
PYK2	Positive	Activated PYK2 translocates to mitochondria, phosphorylating MCU and favouring multimeric channel pore formation	Myocardial cell death	107
CaMKII	Positive	Phosphorylates MCU at Ser57 and Ser92	Myocardial cell death and heart failure	108
miR-25	Negative	Reduces the expression of the pore-forming subunit MCU	• Colon and prostate cancer • Pulmonary arterial hypertension	235–237
miR-138	Negative	Reduces the expression of the pore-forming subunit MCU	Pulmonary arterial hypertension	237
miR-1	Negative	Reduces the expression of the pore-forming subunit MCU	Cardiac hypertrophy	238
miR-340	Negative	Reduces MCU expression and inhibits breast cancer cell migration	Breast cancer	239
PRMT1	Negative	Methylates MICU1, promoting MCU complex-independent Ca^{2+} uptake through UCP2 and/or UCP3	NA	111
AFG3L2–SPG7	Negative	Loss of AFG3L2–SPG7 induces the formation of constitutively active MCU–EMRE complexes	Neurodegeneration	112,113
MIA40	Negative	Ensures the association of MICU1 with the inhibitory subunit MICU2	NA	30
ER–mitochondria tethering				
MFN2[a]	Positive or negative	Loss of MFN2 causes detachment of the ER from mitochondria or increases the ER–mitochondria association	• Charcot–Marie–Tooth neuropathy type 2 A • Obesity and insulin resistance	95,96,240,241
PDZD8	Positive	A structural and functional orthologue of the yeast ERMES protein Mmm1	NA	98
PTPIP51	Positive	Interacts with the ER protein VAPB to regulate ER–mitochondria tethering	• Ischaemia–reperfusion injury • Amyotrophic lateral sclerosis and Parkinson disease	99–101,178
FATE1	Negative	Regulates Ca^{2+} transfer and steroid hormone production	Adrenocortical carcinoma	242
Presenilin 2	Positive	Increases the frequency of Ca^{2+} hot spots at MAMs	Alzheimer disease	243
PACS-2	Positive	Loss causes detachment of the ER from mitochondria	Obesity and insulin resistance	244,245
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	Parkinson disease	103,104
PKA	Negative	Phosphorylates NCLX at Ser258, increasing Ca^{2+} efflux	Parkinson disease	103
Mitochondrial membrane potential				
MCUR1[a]	Positive	Acts as an assembly factor for cytochrome c oxidase	NA	90
SK2 channel	Negative	Activation of mitochondrial SK2 reduces respiration and reactive oxygen species generation	NA	246

AFG3L2, AFG3-like protein 2; BCL-X_L, B cell lymphoma-extra-large; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; EMRE, essential mitochondrial Ca^{2+} uniporter regulator; ER, endoplasmic reticulum; ERMES, ER–mitochondrial encounter structure; FATE1, fetal and adult testis-expressed transcript protein; GSK3, glycogen synthase kinase 3; LETM1, leucine zipper EF-hand-containing transmembrane protein 1; MAMs, mitochondria-associated membranes; MCU, mitochondrial Ca^{2+} uniporter; MCUR1, MCU regulator 1; MFN2, mitofusin 2; MIA40, mitochondrial intermembrane space import and assembly protein 40; miR, microRNA; MICU, mitochondrial calcium uptake protein; Mmm1, maintenance of mitochondrial morphology 1; mPTP, mitochondrial permeability transition pore; NA, not available; NCLX, mitochondrial NCX protein; PACS2, phosphofurin acidic cluster sorting protein 2; PDZD8, PDZ domain-containing protein 8; PINK1, PTEN-induced putative kinase 1; PKA, protein kinase A; PRMT1, protein arginine *N*-methyltransferase 1; PTPIP51, protein tyrosine phosphatase-interacting protein 51; PYK2, proline-rich tyrosine kinase 2; SK2, small conductance Ca^{2+}-activated K^+ channel protein 2; SLC25A23, solute carrier family 25 member 23; SPG7, spastic paraplegia 7; UCP, mitochondrial uncoupling protein; VAPB, vesicle-associated membrane protein-associated protein B/C; VDAC, voltage-dependent anion-selective channel protein.
[a]Protein with a controversial role.

Förster resonance energy transfer
(FRET). A distance-dependent energy transfer process that involves a donor molecule in an excited electronic state that may transfer energy to an acceptor chromophore, leading to a reduction in the donor's fluorescence intensity and excited state lifetime and an increase in the acceptor's emission. Its efficiency depends on the inverse sixth distance between donor and acceptor.

Ratiometric measurement
A measurement based on the use of a ratio between two fluorescence intensities that display a shift in their emission or excitation spectra when they bind to Ca^{2+}. The intensity ratio is calculated at wavelengths for which the difference in fluorescence between bound and free indicator reaches its maximum.

Mitochondrial permeability transition pore
(mPTP). A protein complex that, under certain pathological conditions, including Ca^{2+} overload and oxidative stress, opens in the inner mitochondrial membrane, allowing the free passage of molecules >1,500 Daltons and leading to mitochondrial swelling and cell death through apoptosis or necrosis.

BCL-2 family
A large group of evolutionarily conserved proteins that share BCL-2 homology domains. BCL-2 family members are deeply involved in cell death regulation, consisting of both anti-apoptotic (BCL-2 and apoptosis regulator BCL-X_L (also known as BCL2L1)) and pro-apoptotic (apoptosis regulator BAX and BCL-2 homologous antagonist/killer (BAK)) factors.

ER–mitochondrial encounter structure complex
(ERMES complex). Characterized in yeast, a protein complex consisting of four core components, whose major function is to mechanically link the endoplasmic reticulum (ER) with mitochondria.

when a proteomic assay was used to identify components of the MCU complex, MCUR1 was not recognized[31]. Moreover, the yeast *Saccharomyces cerevisiae*, which lacks any uniporter activity, possesses an MCUR1 orthologue (found in mitochondrial proteome protein 32 (Fmp32)), suggesting a function for MCUR1 outside the MCU complex. Indeed, MCUR1 has been described as a cofactor 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[90]. 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.[91]), indicating that the role of MCUR1 in Ca^{2+} uptake is complex and requires further investigation. Solute carrier family 25 member 23 (SLC25A23) has been recently proposed as a novel regulator of the uniporter[92], although its yeast homologue (suppressor of AAC2 lethality (Sal1)) was originally described as a Ca^{2+}-regulated mitochondrial ATP–Mg/P_i carrier[93,94].

Several regulatory factors, shown in TABLE 1, have been reported to maintain the close association between the ER and mitochondria, thereby ensuring proper Ca^{2+} transfer. Particularly worthy of mention are two proteins, mitofusin 2 (MFN2) and PDZ domain-containing protein 8 (PDZD8). MFN2 was originally characterized as an ER–mitochondria tether[95], but its role was strongly contested[96], and it is still unclear whether MFN2 promotes or inhibits ER–mitochondria contacts. PDZD8, previously known as a regulator of retroviral infection[97], has recently been described as the long-sought mammalian orthologue of yeast maintenance of mitochondrial morphology protein 1 (Mmm1), which is a component of the ER–mitochondrial encounter structure complex (ERMES complex) and coordinates Ca^{2+} exchange exclusively via its ER–mitochondria tethering role[98]. Finally, the molecular duo formed by the OMM protein tyrosine phosphatase-interacting protein 51 (PTPIP51; also known as RMDN3) and its binding partner at the ER, vesicle-associated membrane protein-associated protein B/C (VAPB) has been identified as a structural regulator of ER–mitochondria association[99–101]. Reducing the expression of at least one of these factors results in a strong decrease in mitochondrial Ca^{2+} accumulation.

Post-translational modifications represent additional layers in the regulation of mitochondrial Ca^{2+} handling. The first post-translational modification associated with mitochondrial Ca^{2+} regulation was phosphorylation, with the protein kinase C (PKC) family of kinases shown to be capable of finely tuning mitochondrial Ca^{2+} homeostasis[102]. Recently, a phosphorylation-driven regulatory pathway for Ca^{2+} efflux has been identified[103], whereby two kinases (PTEN-induced putative kinase 1 (PINK1) and protein kinase A (PKA)) positively regulate NCLX[103]. Intriguingly, PINK1 is also able to boost mitochondrial Ca^{2+} extrusion by phosphorylating LETM1 at Thr192 (REF.[104]). Function and interactions of VDACs with other molecular partners such as BCL-2 proteins are affected by multiple phosphorylation events[105]. During hypoxia and inflammatory-mediated oxidative stress, the MCU can be targeted by reactive oxygen species (ROS), which promote *S*-glutathionylation of Cys97 of MCU protein subunits. 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[106]. In addition, the MCU can be subject to phosphorylation events. Proline-rich tyrosine kinase 2 (PYK2) can target the MCU subunits, promoting the formation of multimeric channels[107]. In heart disease, a pool of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) localized to the mitochondrial matrix can phosphorylate MCU protein at two sites (Ser57 and Ser92), resulting in a higher Ca^{2+} response[108]. Interestingly, phosphorylation of Ser92 seems to be crucial for MCU activity in various contexts[86], and the CaMKII–MCU axis has been shown to regulate vascular smooth muscle cell migration and neointimal hyperplasia after endothelial injury[109]. However, subsequent electrophysiological studies have failed to confirm the regulatory effect of CaMKII on MCU[110]. No phosphorylation events have been associated with other uniporter complex components, although MICU1 can be methylated by protein arginine *N*-methyltransferase 1 (PRMT1)[111]. Finally, the mitochondrial matrix AAA proteases have been shown to degrade unassembled EMRE, thus ensuring correct stoichiometry between the different complex subunits and preserving uniporter activity[112,113].

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 remodelling of mitochondrial Ca^{2+} fluxes, which in turn could lead to detrimental changes in cellular Ca^{2+} homeostasis and specific pathological phenotypes (see TABLE 1 and below).

Roles in cellular Ca^{2+} homeostasis

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 that have a strong impact on mitochondrial functions and thus on the whole cellular metabolism, producing some spurious and controversial results[114]. Moreover, the ability of mitochondrial Ca^{2+} uptake to shape the cytosolic Ca^{2+} transient is strongly influenced by the cell type and density of competing Ca^{2+} removal fluxes. For example, adult cardiac myocytes display very high Ca^{2+} transport capacity by SERCA pumps and plasma membrane NCXs, which transport Ca^{2+} out of the myoplasm 20–60 times more quickly than mitochondrial transporters under physiological conditions. Accordingly, in cardiomyocytes, the contribution of mitochondria to decreasing cytoplasmic Ca^{2+} levels has been estimated to be <1%[115,116]. Conversely, in non-muscle cells with modest SERCA and/or NCX functions, mitochondrial Ca^{2+} uptake could be critical in terminating cytosolic Ca^{2+} bursts and transiently buffering the intracellular Ca^{2+}.

PTEN-induced putative kinase 1
(PINK1). A serine/threonine kinase that is imported inside mitochondria in healthy conditions, whereas it accumulates at the outer mitochondrial membrane in dysfunctional mitochondria to promote their degradation through mitophagy. Mutations in *PINK1* cause one form of autosomal recessive early-onset Parkinson disease.

Neointimal hyperplasia
The thickening of the intima layer (tunica) of arteries and veins that results from accumulation of fibroblasts and smooth muscle cells. The result of such excessive cellular deposition is the loss of luminal area.

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.

Acinar cells
The exocrine cells of the pancreas that produce and transport the majority of enzymes required for the digestion of food.

Zymogen
An inactive precursor of an enzyme, also termed a pro-enzyme, which displays no catalytic activity and requires a specific biochemical transformation to become fully active.

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 Ins(1,4,5)P_3-mediated Ca^{2+} release from the ER, the peak amplitude of the cytosolic Ca^{2+} signal is considerably lower in cells in which mitochondrial Ca^{2+} uptake capacity is increased by MCU protein overexpression[25]. 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[98]. Similar findings have been obtained in NCLX-silenced astrocytes[117], although this effect is more pronounced upon SOCE than after emptying ER Ca^{2+} stores. Thus, mitochondria promptly take up cytoplasmic Ca^{2+} regardless of whether it derives from internal stores (ER) or the extracellular space.

Depletion of MCU protein or UCP2 (which, as discussed above, contributes to MCU-independent Ca^{2+} influx into mitochondria), with a consequent reduction of mitochondrial Ca^{2+} uptake, strongly inhibits SOCE by limiting the oligomerization of stromal interaction molecule 1 (STIM1) and activation of ORAI1 (REF.[118]) (FIG. 1). This phenomenon occurs only upon ER Ca^{2+} store depletion through Ins(1,4,5)P_3-generating stimuli, whereas the loss of UCP2 or MCU protein 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 Ins(1,4,5)P_3Rs could represent a pivotal mechanism in the modulation of SOCE, as previously suggested[119].

The cytosolic [Ca^{2+}] affects the function of both ER-resident proteins (such as Ins(1,4,5)P_3Rs)[120] and store-operated Ca^{2+} channels[121], with high cytosolic Ca^{2+} levels in the area surrounding the channels inhibiting their activity and reducing Ca^{2+} release through the Ins(1,4,5)P_3R or Ca^{2+} entry by ORAI1. The strategic positioning of mitochondria in the vicinity of these channels lowers the [Ca^{2+}] locally, preventing negative regulation and sustaining channel activity. Indeed, MCU protein loss impairs mitochondrial Ca^{2+}-buffering capacity, which in turn limits CRAC channel function by enhancing Ca^{2+}-mediated slow inactivation of the channels[122]. Similarly, the number of cytosolic Ca^{2+} oscillations (BOX 1) generated by discharge of Ca^{2+} from Ins(1,4,5)P_3-sensitive stores is significantly lower in MCU protein-depleted cells, which reflects Ins(1,4,5)P_3R inhibition resulting from impaired mitochondrial Ca^{2+} uptake[122]. 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 (BOX 1), thereby regulating specific cellular processes that depend on (or that are regulated by) Ca^{2+} (REF.[123]). For example, in pancreatic acinar cells, mitochondria are distributed as a firewall along the border of the apically located zymogen granules, confining the Ins(1,4,5)P_3-evoked cytosolic Ca^{2+} signals — which are important for granule release — to the apical secretory region through their Ca^{2+}-buffering capacity[124]. A crucial role for mitochondria positioning in the regulation of spatially confined cytosolic Ca^{2+} rises has also been described in neurons[125].

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[126]. 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[127,128].

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. However, several aspects of mitochondrial Ca^{2+}-buffering mechanisms 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 protein depletion has been observed to reduce[129] or marginally increase[130] 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[56,131], and the impact of other organelles in the local buffering of cytoplasmic Ca^{2+} (REFS[132,133]). Moreover, mitochondrial Ca^{2+} transporters 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 the activity of canonical TRPCs at the plasma membrane[134]. 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. Prolonged ROS production by mitochondria leads to SOCE inhibition via the oxidation of ORAI1 at Cys195 (REF.[135]), and thus, NCLX activity contributes to sustaining SOCE through the regulation of ROS production.

Pathophysiology of mitochondrial Ca^{2+}

Mitochondrial Ca^{2+} has an important function in regulating cell fitness through its ability to impact cell energetics by activating oxidative metabolism, mitochondrial respiration and ATP synthesis[120,136,137]. Notably, however, deregulation of intracellular Ca^{2+} and increased mitochondrial Ca^{2+} influx are potent triggers of necrosis, apoptosis and autophagy[138–140]. 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 are 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).

Nutrient secretagogues
Substances that promote secretion.

ER stress
A stressful condition of the endoplasmic reticulum (ER) that triggers a signalling cascade, termed the unfolded protein response, which is aimed to restore ER homeostasis.

Excitation–contraction coupling
(EC coupling). A process whereby the action potential travelling along the plasmalemma evokes initiation of mechanical shortening of the myofibrils through Ca^{2+} release from the sarcoplasmic reticulum.

Excitation–transcription coupling
(ET coupling). A process initiated by Ca^{2+} signals that results in changes in gene expression.

Excitation–metabolism coupling
(EM coupling). A process initiated by Ca^{2+} signals that results in changes in cell metabolism.

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 fibres.

Funny current
(I_f). A mixed Na^+/K^+ inward current with several unusual features.

Troponin C
(TN-C). A component of the troponin complex, together with troponin I and troponin T, which regulates muscle contraction by Ca^{2+} binding. Through its multiple EF-hand domains, TN-C acts as the Ca^{2+} sensor of the troponin complex, initiating the cascade of events that leads to contraction of striated muscle by interacting with troponin I after Ca^{2+} binding.

Other pathological contexts related to deregulation of mitochondrial Ca^{2+}, which are not discussed in this Review, can be found in Supplementary table 1.

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[141], which is secreted on the basis of 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, especially glucose, initiate downstream signals that enable β-cells to break down sugar and release insulin by stimulating mitochondrial energy metabolism[142]. 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[143–145].

The main role of the increase in sub-plasma membrane Ca^{2+} is to permit insulin release[146], involving the activation of PKC β-type II (PKCβII) and its translocation to the surface of secretory vesicles localized in that area, although targets of PKC are not well characterized[147]. 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, where they are able to sense microdomains of high Ca^{2+} concentrations in their proximity, take up Ca^{2+} through the MCU and fuel the exocytotic process by producing ATP, thereby sustaining and amplifying the phase of insulin secretion[144,148,149]. Moreover, the increased cytosolic [Ca^{2+}] consequent on the opening of voltage-gated Ca^{2+} channels promotes ER Ca^{2+} accumulation and can lead to ER Ca^{2+} release[150] through channels including RyRs[151]. This release can be followed by mitochondrial Ca^{2+} uptake at MAMs 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).

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 some of the causal factors in the aetiology of both type 1 and type 2 diabetes[152]. In particular, the ER stress caused by ER Ca^{2+} depletion could represent the main factor contributing to mitochondrial Ca^{2+} dysfunction, reduced ATP production and reduction in glucose-stimulated insulin secretion. Chronic ER Ca^{2+} depletion owing to persistent leaky RyR channels is responsible for decreased mitochondrial Ca^{2+} uptake and β-cell failure[151]. It has been demonstrated that the activity of ER-localized TWIK-related alkaline pH-activated K^+ channel 1 (TALK1) further exacerbates ER stress by increasing Ca^{2+} flow[153], thus contributing to islet dysfunction. Therefore, strategies to restore an efficient mitochondrial Ca^{2+} response in these cells represent promising therapeutic approaches for the treatment of diabetes. Potential therapeutic targets include the MCU[73] and its regulatory partner MICU1 (REF.[154]), which are required for the feedforward mechanism of Ca^{2+} entry into mitochondria and guarantee insulin secretion in β-cells, as well as ER channels (for example, TALK1), the inhibition of which could reduce ER Ca^{2+} handling defects in β-cells during the pathogenesis of diabetes.

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 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[155].

Under physiological conditions, Ca^{2+} signalling in the heart exerts three main functions: controlling the so-called excitation–contraction coupling (EC coupling), excitation–transcription coupling (ET coupling) and excitation–metabolism coupling (EM coupling) 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[156].

In mammals, the cardiac cycle starts with the generation of an automatic action potential in a group of specialized cells, named sinoatrial nodal cells, which autonomously produce the electrical cardiac impulse needed for the subsequent contraction (of note, other cells within the conduction system have similar properties). The action potential is initiated with a change in membrane potential, which becomes more positive, mainly owing to the opening of Na^+ channels and flow of Na^+ into the cell. This depolarization, also called funny current (I_f)[157,158], induces a progressive opening of T type (transient opening Ca^{2+} channels (TTCCs)) and L type Ca^{2+} channels (long-lasting Ca^{2+} channels (LTCCs)), eventually triggering a cytosolic Ca^{2+} influx[159,160]. The cytosolic Ca^{2+} influx through LTCCs is sufficient to regulate and activate mitochondrial functions and thus ATP production[161] through the generation of Ca^{2+} microdomains around nearby mitochondria. Ca^{2+} influx through LTCCs also triggers Ca^{2+} release from the nearby junctional SR (the portion of SR in association with other membrane structures, such as sarcolemma) via intracellular Ca^{2+} release channels in a process known as Ca^{2+}-induced Ca^{2+} release (CICR), which is crucial for muscle contraction[162,163]. The high local cytosolic [Ca^{2+}] generated during CICR initiates contraction (EC coupling) by binding troponin C (TN-C) on myofilaments[164] and boosts mitochondrial metabolism by promoting Ca^{2+} uptake into mitochondria[165]. Only the generation of well-defined mitochondrial Ca^{2+} microdomains, either at the entrance of voltage-gated Ca^{2+} channels at the plasma membrane (sarcolemma) after LTCC opening or

in close proximity to RyRs in the SR during CICR events, can explain mitochondrial Ca^{2+} uptake in cardiac myocytes. 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+} uptake is fundamental for providing the necessary link between the ATP supply and demand during cardiomyocyte contraction (FIG. 3B). In this context, MCU activity functions to increase heartbeat frequency by favouring rapid Ca^{2+} mitochondrial uptake during the cardiac cycle[126]. Oxidative phosphorylation enhanced by MCU activity is also required for reloading SR Ca^{2+} stores and sustaining increased heart rate

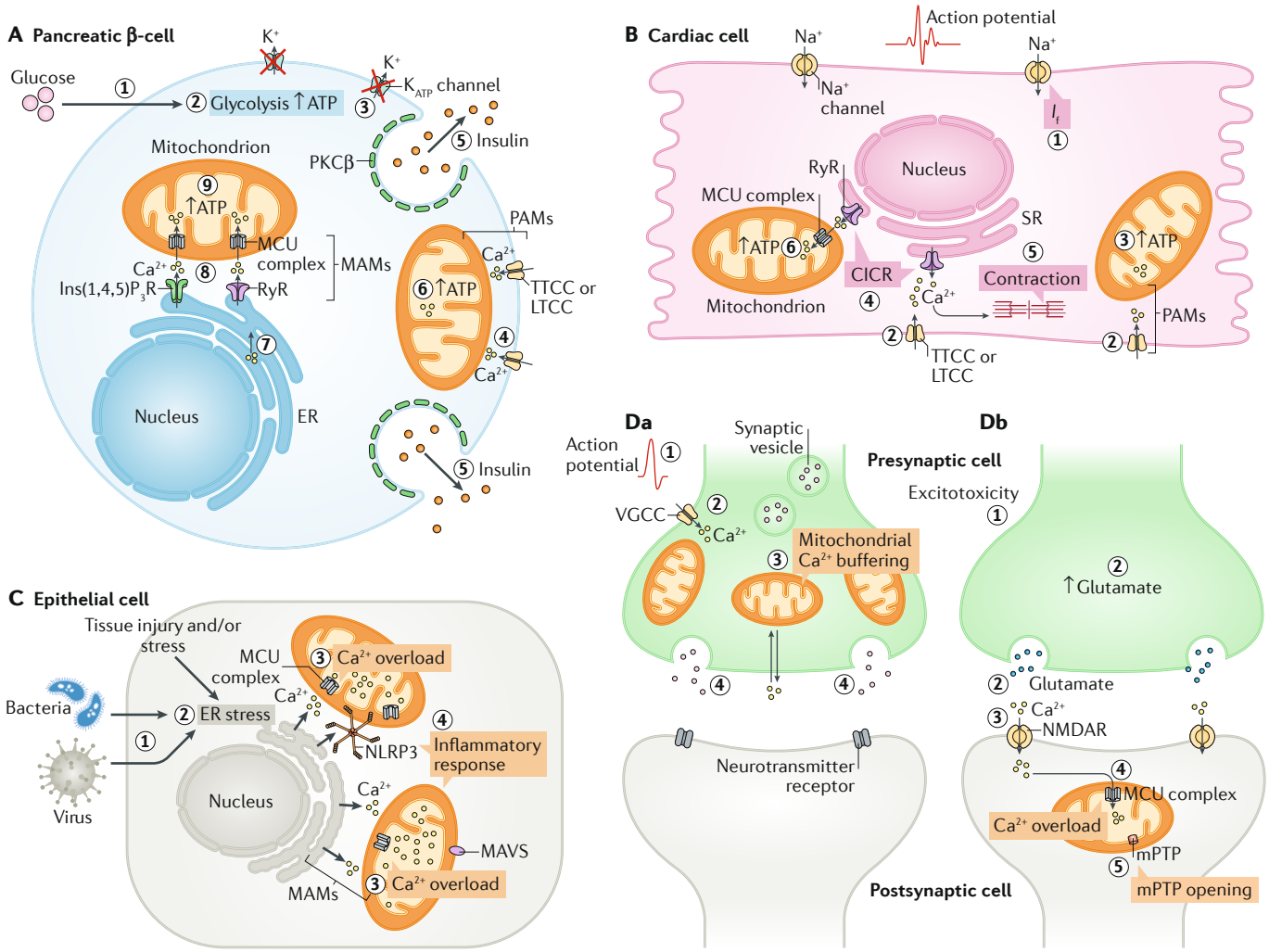


Fig. 3 | **Role of mitochondrial Ca^{2+} in pathophysiological processes.** The generation of high Ca^{2+} microdomains at membrane contact sites. Mitochondria-associated membranes (MAMs) (established between mitochondria and the endoplasmic reticulum (ER)) or plasma membrane-associated membranes (PAMs) are fundamental to permit mitochondrial Ca^{2+} uptake through the mitochondrial Ca^{2+} uniporter (MCU) complex 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) (5). The increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]$) also promotes mitochondrial ATP production from mitochondria at PAMs (6), increased ER Ca^{2+} concentration (7) and, in turn, increased ER Ca^{2+} release by inositol 1,4,5-trisphosphate receptors (Ins(1,4,5)P_3Rs) and/or by ryanodine receptors (RyRs) (8), and mitochondrial ATP production at MAMs (9). **B** | In cardiac cells, during an action potential, the Na^+ inward current (funny current (I_f)) (1) leads to opening of LTCCs and/or TTCCs (2). The cytosolic increase in Ca^{2+} is captured by mitochondria at PAMs 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 the ATP production that supports contraction (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 the NACHT, LRR and PYD domains-containing protein 3 (NLRP3) inflammasome, which is essential for the production of interleukin-1β (IL-1β) and other pro-inflammatory mediators, and by activation of mitochondrial antiviral signalling (MAVS) complexes, which could interact with the MCU complex to positively regulate the release of the pro-inflammatory cytokine interferon-β (IFNβ). **D** | Mitochondria are key for buffering Ca^{2+} in neurons. **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). **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 the mitochondrial permeability transition pore (mPTP) (5), which promotes apoptosis. SR, sarcoplasmic reticulum.

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
The physiological reaction that helps an animal in response to emergency situations such as a harmful event or an attack.

during the fight-or-flight response[166]. 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 (the amount of blood pumped by the left ventricle in one contraction) and that MICU2 loss predisposes to lethal abdominal aortic aneurysms[167].

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[168]. Recent data suggest 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[116,128]. The new mechanistic understanding of MCU complex 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[169]. Acute heart diseases, such as ischaemia–reperfusion injury, are mainly attributed to mitochondrial Ca^{2+} overload, which is evoked by a massive extracellular Ca^{2+} influx, 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 (BOX 2). Thus, targeting the regulatory systems of mitochondrial Ca^{2+} homeostasis, such as the MCU complex and NCLX, as well as limiting ROS generation and mPTP activation, could represent a potential therapeutic strategy to combat these pathologies.

The use of mPTP modulators, such as ciclosporin A (CsA), which is a known inhibitor of the mPTP component cyclophilin-related protein (CYPD; also known as PPID), 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[170,171]. Therefore, more specific and novel mPTP inhibitors, based on new findings regarding the molecular composition of mPTP[172], 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[127,173] show drastic inhibition of acute mitochondrial Ca^{2+} uptake, which correlates with a slowed functional response to an acute increase in cardiac workload, although the basal mitochondrial [Ca^{2+}] appears remarkably normal. These animals are strongly protected from the damage resulting from ischaemia–reperfusion injury[127,173], which is consistent with previous results obtained in isolated hearts treated with the MCU inhibitor Ru360 (REF.[174]). In agreement with this view, the increased MCU current induced by activation of CaMKII during ischaemia–reperfusion injury was shown to promote mPTP opening and myocardial cell death[108]. These data support MCU as a potential new target for cardioprotective drug design. However, the role of MCU in ischaemia–reperfusion injury is highly debated, as is its modulation by CaMKII (see section above). Indeed, animals with whole-body MCU deletion and mice with myocardial MCU inhibition through the expression of a dominant-negative form of MCU protein — generated in a mixed genetic background — are unable to accumulate Ca^{2+} inside the matrix but show no protection against ischaemia–reperfusion injury-driven damage[175–177]. Intriguingly, CsA administration is strongly cardioprotective in wild-type but not in MCU-null hearts[175], suggesting that the myocardial cell death occurring in whole-body MCU-knockout mice subjected to ischaemia–reperfusion injury is independent of canonical Ca^{2+}-dependent mPTP functions. Of note, deletion of MCU in a pure background is embryonic lethal, probably owing to cardiac defects, suggesting that the mixed genetic background, by as yet unknown mechanisms, masks the crucial role of mitochondrial Ca^{2+} in development and cardiovascular homeostasis.

In addition to MCU-mediated Ca^{2+} uptake, it has recently been identified that Ca^{2+} efflux from mitochondria 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 cell death after ischaemia–reperfusion injury[66]. Although NCLX deletion in adult cardiomyocytes elicits sudden death, germline cardiac-specific knockout does not cause heart failure[66]. Moreover, whole-body NCLX-knockout mice are viable, and they do not suffer from severe cardiac failure (I. Sekler, personal communication). However, they show a range of symptoms that includes cognitive impairment, reduced brown fat thermogeneration and defective T cells. Thus, a still unknown mechanism could exist in the newborn animals that compensates for whole-body loss of NCLX, explaining the mild phenotype observed in whole-body knockout mice.

The maintenance of contact sites between the SR and mitochondria also contributes to the damage associated with ischaemia–reperfusion injury. In particular, downregulation of PTPIP51, a crucial regulator of ER–mitochondria and 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[178].

In chronic heart failure, major myocyte dysfunction is related to accumulation of cytoplasmic Ca^{2+}. This defect in failing cells largely results from decreased expression and activity of the SR Ca^{2+} ATPase[179] and increased RyR Ca^{2+} leakage[180] (in particular, RyR2) owing to their redox modifications[181,182] or phosphorylation by CaMKII[183] and PKA[184]. The consequent high amount of Ca^{2+} that persists in the cytoplasm could result in mitochondrial Ca^{2+} overload and dysfunction, thereby contributing to heart failure and myocyte cell death[185,186]. In parallel,

NACHT, LRR and PYD domains-containing protein 3 inflammasome (NLRP3 inflammasome). A complex whose formation leads to the activation of caspase 1, the secretion of pro-inflammatory cytokines and the induction of inflammatory cell death (or pyroptosis).

Mitochondrial antiviral signalling (MAVS). A CARD domain-containing protein located on the outer mitochondrial membrane.

Excitotoxicity
A pathological process by which neurons are damaged or killed by an excessive activation of receptors for excitatory neurotransmitters, such as glutamate. Such hyper-stimulation produces a massive entry of Ca^{2+} inside the cell and induces mitochondrial damage.

N-methyl-D-aspartate receptors (NMDARs). Glutamate-gated cation channels showing high Ca^{2+} permeability. They can be found at most excitatory synapses and exhibit the highest affinity for glutamate among the glutamate receptors.

Purkinje cells
A class of large inhibitory neurons located in the cerebellar cortex of the brain and characterized by an intricate dendritic arbor. They have crucial roles in motor coordination.

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[187]. Induction of SR Ca^{2+} leakage is also sufficient to induce spontaneous action potentials and is therefore considered an important trigger for cardiac arrhythmias[188,189]. In addition, heart failure is frequently associated with an elevation of intracellular Na^+ concentration, which induces higher NCLX activity, thereby limiting the ability of mitochondrial [Ca^{2+}] to rise to levels sufficient to support the robust ATP production required to match the increased energy demand necessary for contraction[190].

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 an effective strategy for the treatment of heart failure.

Inflammatory responses and pathological inflammation. The first evidence that mitochondrial Ca^{2+} is important during inflammation dates back more than 30 years[191], but increasing interest in this area has become evident only since the molecular identity of the MCU complex and identification of the mitochondrial localization of the NACHT, LRR and PYD domains-containing protein 3 inflammasome (NLRP3 inflammasome)[192].

Studies have highlighted how chronic stress increases mitochondrial Ca^{2+} accumulation, 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-induced 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 NLRP3-dependent inflammatory response, which exacerbates the pathology of cystic fibrosis[193]. 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*[193].

MCU-mediated Ca^{2+} overload has also been found to be essential for a virus-induced inflammatory response. Viral infections perturb Ca^{2+} homeostasis, by increasing either ER Ca^{2+} release or extracellular Ca^{2+} influx, thereby promoting Ca^{2+} influx into mitochondria. MCU specifically interacts with mitochondrial antiviral signalling (MAVS) complexes localized on mitochondria and positively regulates the release of the pro-inflammatory cytokine interferon-β (IFNβ) upon viral infection[194]. Chronic viral infection is accompanied by ER stress, inducing mitochondrial Ca^{2+} overload through an MCU–MAVS-dependent pathway, with subsequent sustained IFNβ production that contributes to autoimmune diseases. Knockdown or silencing of MCU (or MAVS) reduces mitochondrial Ca^{2+} uptake capacity and decreases virus-induced IFNβ levels[194] 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 pro-inflammatory response, which is associated with 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[195].

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

Neuronal homeostasis and neurodegeneration. Ca^{2+} is the major signalling molecule connecting neuronal depolarization to synaptic activity. Through their positioning at the mouth of the Ins(1,4,5)P3Rs or close to the plasma membrane to take up Ca^{2+} from the ER or to 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[125] (FIG. 3Da). Therefore, both defects in mitochondrial positioning and toxic changes in Ca^{2+} buffering could contribute to different neurological disorders[196]. In this context, the cell death-inducing sequence of mitochondrial Ca^{2+} overload, ROS formation and mPTP opening could importantly contribute to neuronal pathology, particularly neurodegeneration. For example, during excitotoxicity, postsynaptic neurons are subject to extremely high levels of glutamate, which induces excessive activation of *N*-methyl-D-aspartate receptors (NMDARs), 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[197]. Both pharmacological[198] and genetic[199] inhibition of MCU are able to suppress excitotoxicity, suggesting that this approach could be explored as a neuroprotective strategy in disorders with aberrant NMDAR activity.

A therapeutic strategy based on MCU complex targeting has also been investigated for spinocerebellar ataxia type 28 (SCA28), an autosomal dominantly inherited ataxia caused by mutations in *AFG3L2* and characterized by a loss of Purkinje cells[200]. AFG3-like protein 2 (AFG3L2) depletion induces an accumulation of MCU–EMRE complexes (devoid of MICU regulation), which is concomitant with increased uptake of Ca^{2+} into mitochondria, leading to high mitochondrial Ca^{2+} levels and neuronal apoptosis[112]. Accordingly, MICU1-knockout mice exhibit alterations in the postnatal arborization of Purkinje cells[35]. Furthermore, *Afg3l2*-knockout Purkinje cells display altered mitochondrial Ca^{2+}-buffering capacity — associated with disturbed

Oligodendrocyte
A type of neuroglia that provides support to axons in the central nervous system by producing myelin sheaths. In the peripheral nervous system, the equivalent to oligodendrocytes is Schwann cells.

ultrastructure and intracellular localization of these organelles — and reducing glutamate stimulation in these cells to reduce cellular Ca^{2+} uptake could prevent their degeneration[201]. 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. Thus, although mitochondrial Ca^{2+} overload is not the unique mechanism responsible for neuronal death, the restoration of normal mitochondrial Ca^{2+} dynamics could represent a valuable strategy to limit Purkinje cell degeneration.

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 alteration of the ER–mitochondria connection. In familial Alzheimer disease, mutated presenilin 2 increases ER–mitochondria tethering in an MFN2-dependent manner[203]. The ALS-associated protein TAR DNA-binding protein 43 (TDP-43) disrupts the VAPB–PTPIP51 axis, leading to a decrease in ER–mitochondria contact sites and bioenergetic crisis[101]. A similar molecular mechanism has been described for α-synuclein[100], which forms pathological aggregates in Parkinson disease, although other reports have demonstrated a pro-tethering role for α-synuclein[204]. In addition to its (contested) role in ER–mitochondria tethering, α-synuclein localizes at MAMs and induces mitochondrial damage, such as a loss of membrane potential and morphological alterations[205].

Other mitochondrial aberrations have also been associated with neurodegeneration. In multiple sclerosis, tumour necrosis factor (TNF) exposure affects mitochondrial membrane potential and reduces Ca^{2+} uptake, which limits oligodendrocyte progenitor cell differentiation and myelination of neuronal axons[206]. Reducing Ca^{2+}-mediated mPTP formation by pharmacological inhibition of CYPD confers neuroprotection in an autoimmune encephalomyelitis model of multiple sclerosis[207]. The genetic deletion of CYPD also exhibits beneficial effects in mice expressing ALS-linked mutants of superoxide dismutase-1 (SOD1)[208], which are characterized by increased mPTP opening and aberrant Ca^{2+} transients in astrocyte processes[209].

In Huntington disease, the mutant huntingtin (mHTT), which mediates neuronal degeneration, interacts with Ins(1,4,5)P3Rs, causing a chronic ER Ca^{2+} leak[210]. Notably, early in disease pathogenesis, the negative effects of this leak can be compensated by reduced mitochondrial Ca^{2+} uptake capacity[211], which can alleviate the mHTT-related toxicity by preventing mitochondrial Ca^{2+} overload, mPTP opening and neuronal apoptosis.

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 of a mitochondrial [Ca^{2+}] can affect the energy supply and the cytosolic Ca^{2+}-buffering capacity. Thus, regulating mitochondrial [Ca^{2+}] by re-establishing the proper ER–mitochondria association is essential to preserve neuronal homeostasis.

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+} signalling are linked to many human diseases, including common disorders and various cancers (BOX 2; TABLE 1; Supplementary table 1). 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.

In the future, it will be particularly important to gain a complete understanding of mitochondrial Ca^{2+} regulation and its integration with other processes. This will include a molecular description of the newly discovered accessory proteins and post-translational modifications of mitochondrial Ca^{2+} channels and transporters as well as a determination of a definition of the omic signature of mitochondrial Ca^{2+} signalling by defining the role of mitochondrial Ca^{2+} in genome, proteome and metabolome regulation. 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, such 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, limiting its employment to isolated mitochondria. Very recently, two compounds, DS16570511 (REF.[212]) and mitoxantrone[213], have been proposed as novel MCU inhibitors. The former inhibits mitochondrial Ca^{2+} uptake in intact cells and the 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+} signalling 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 will be proposed. Moreover, the contribution of mitochondrial Ca^{2+} in cancer will be clarified, opening the possibility of modulating mitochondrial Ca^{2+} homeostasis to increase the efficacy of cytotoxic agents.

Finally, the emerging participation of mitochondrial Ca^{2+} in the process of ageing[214] and its importance in stem cell biology[215] will produce new and exciting achievements in the future and will attract scientists from other fields to this fascinating and still vastly unexplored area of mitochondrial biology.

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1. Berridge, M. J., Lipp, P. & Bootman, M. D. The versatility and universality of calcium signalling. *Nat. Rev. Mol. Cell Biol.* **1**, 11–21 (2000).
2. Giorgi, C., Danese, A., Missiroli, S., Patergnani, S. & Pinton, P. Calcium dynamics as a machine for decoding signals. *Trends Cell Biol.* **28**, 258–273 (2018).
3. Carafoli, E. Historical review: mitochondria and calcium: ups and downs of an unusual relationship. *Trends Biochem. Sci.* **28**, 175–181 (2003).
4. Slater, E. C. & Cleland, K. W. The calcium content of isolated heart-muscle sarcosomes. *Biochem J.* **54**, xxii (1953).
This study provides the first historical evidence indicating Ca^{2+} transport inside mitochondria.
5. Deluca, H. F. & Engstrom, G. W. Calcium uptake by rat kidney mitochondria. *Proc. Natl Acad. Sci. USA* **47**, 1744–1750 (1961).
6. Vasington, F. D. & Murphy, J. V. Ca ion uptake by rat kidney mitochondria and its dependence on respiration and phosphorylation. *J. Biol. Chem.* **237**, 2670–2677 (1962).
7. Prakriya, M. & Lewis, R. S. Store-operated calcium channels. *Physiol. Rev.* **95**, 1383–1436 (2015).
8. Shoshan-Barmatz, V. & Mizrachi, D. VDAC1: from structure to cancer therapy. *Front. Oncol.* **2**, 164 (2012).
9. Messina, A., Reina, S., Guarino, F. & De Pinto, V. VDAC isoforms in mammals. *Biochim. Biophys. Acta* **1818**, 1466–1476 (2012).
10. Shoshan-Barmatz, V., Krelin, Y. & Shteinfer-Kuzmine, A. VDAC1 functions in Ca(2+) homeostasis and cell life and death in health and disease. *Cell Calcium* **69**, 81–100 (2018).
11. Colombini, M. VDAC structure, selectivity, and dynamics. *Biochim. Biophys. Acta* **1818**, 1457–1465 (2012).
12. Tan, W. & Colombini, M. VDAC closure increases calcium ion flux. *Biochim. Biophys. Acta* **1768**, 2510–2515 (2007).
13. Adams, V., Bosch, W., Schlegel, J., Wallimann, T. & Brdiczka, D. Further characterization of contact sites from mitochondria of different tissues: topology of peripheral kinases. *Biochim. Biophys. Acta* **981**, 213–225 (1989).
14. De Stefani, D. et al. VDAC1 selectively transfers apoptotic Ca^{2+} signals to mitochondria. *Cell Death Differ.* **19**, 267–273 (2012).
15. Shimizu, H. et al. Mitochondrial Ca(2+) uptake by the voltage-dependent anion channel 2 regulates cardiac rhythmicity. *Elife* **4**, e04801 (2015).
16. Baines, C. P., Kaiser, R. A., Sheiko, T., Craigen, W. J. & Molkentin, J. D. Voltage-dependent anion channels are dispensable for mitochondrial-dependent cell death. *Nat. Cell Biol.* **9**, 550–555 (2007).
17. Ryu, S. Y., Beutner, G., Dirksen, R. T., Kinnally, K. W. & Sheu, S. S. Mitochondrial ryanodine receptors and other mitochondrial Ca^{2+} permeable channels. *FEBS Lett.* **584**, 1948–1955 (2010).
18. Feng, S. et al. Canonical transient receptor potential 3 channels regulate mitochondrial calcium uptake. *Proc. Natl Acad. Sci. USA* **110**, 11011–11016 (2013).
19. Trenker, M., Malli, R., Fertschai, I., Levak-Frank, S. & Graier, W. F. Uncoupling proteins 2 and 3 are fundamental for mitochondrial Ca^{2+} uniport. *Nat. Cell Biol.* **9**, 445–452 (2007).
20. Jiang, D., Zhao, L. & Clapham, D. E. Genome-wide RNAi screen identifies Letm1 as a mitochondrial Ca^{2+}/H^{+} antiporter. *Science* **326**, 144–147 (2009).
This study indicates that the IMM protein LETM1 functions as the HCXs.
21. Kamer, K. J. & Mootha, V. K. The molecular era of the mitochondrial calcium uniporter. *Nat. Rev. Mol. Cell Biol.* **16**, 545–553 (2015).
22. Marchi, S. & Pinton, P. The mitochondrial calcium uniporter complex: molecular components, structure and physiopathological implications. *J. Physiol.* **592**, 829–839 (2014).
23. Foskett, J. K. & Philipson, B. The mitochondrial Ca(2+) uniporter complex. *J. Mol. Cell. Cardiol.* **78**, 3–8 (2015).
24. Baughman, J. M. et al. Integrative genomics identifies MCU as an essential component of the mitochondrial calcium uniporter. *Nature* **476**, 341–345 (2011).
25. De Stefani, D., Raffaello, A., Teardo, E., Szabo, I. & Rizzuto, R. A forty-kilodalton protein of the inner membrane is the mitochondrial calcium uniporter. *Nature* **476**, 336–340 (2011).
26. Raffaello, A. et al. The mitochondrial calcium uniporter is a multimer that can include a dominant-negative pore-forming subunit. *EMBO J.* **32**, 2362–2376 (2013).
27. Perocchi, F. et al. MICU1 encodes a mitochondrial EF hand protein required for Ca(2+) uptake. *Nature* **467**, 291–296 (2010).
This study details the characterization of the first component of the mitochondrial uniporter complex, which paved the way for the identification of the other members, MCU (references 24 and 25), MCUb (reference 26), MICU2 (reference 28) and EMRE (reference 31).
28. Plovanich, M. et al. MICU2, a paralog of MICU1, resides within the mitochondrial uniporter complex to regulate calcium handling. *PLOS One* **8**, e55785 (2013).
29. Patron, M. et al. MICU1 and MICU2 finely tune the mitochondrial Ca^{2+} uniporter by exerting opposite effects on MCU activity. *Mol. Cell* **53**, 726–737 (2014).
30. Petrungaro, C. et al. The Ca(2+)-dependent release of the Mia40-induced MICU1-MICU2 dimer from MCU regulates mitochondrial Ca(2+) uptake. *Cell Metab.* **22**, 721–733 (2015).
31. Sancak, Y. et al. EMRE is an essential component of the mitochondrial calcium uniporter complex. *Science* **342**, 1379–1382 (2013).
32. Vecellio Reane, D. et al. A MICU1 splice variant confers high sensitivity to the mitochondrial Ca^{2+} uptake machinery of skeletal muscle. *Mol. Cell* **64**, 760–773 (2016).
33. Csordas, G. et al. MICU1 controls both the threshold and cooperative activation of the mitochondrial Ca(2)(+) uniporter. *Cell Metab.* **17**, 976–987 (2013).
34. Mallilankaraman, K. et al. MICU1 is an essential gatekeeper for MCU-mediated mitochondrial Ca(2+) uptake that regulates cell survival. *Cell* **151**, 630–644 (2012).
35. Liu, J. C. et al. MICU1 serves as a molecular gatekeeper to prevent in vivo mitochondrial calcium overload. *Cell Rep.* **16**, 1561–1573 (2016).
36. Payne, R., Hoff, H., Roskowski, A. & Foskett, J. K. MICU2 restricts spatial crosstalk between InsP3R and MCU Channels by regulating threshold and gain of MICU1-mediated inhibition and activation of MCU. *Cell Rep.* **21**, 3141–3154 (2017).
37. Kamer, K. J., Grabarek, Z. & Mootha, V. K. High-affinity cooperative Ca(2+) binding by MICU1-MICU2 serves as an on-off switch for the uniporter. *EMBO Rep.* **18**, 1397–1411 (2017).
38. Matesanz-Isabel, J. et al. Functional roles of MICU1 and MICU2 in mitochondrial Ca(2+) uptake. *Biochim. Biophys. Acta* **1858**, 1110–1117 (2016).
39. Oxenoid, K. et al. Architecture of the mitochondrial calcium uniporter. *Nature* **533**, 269–273 (2016).
40. Yoo, J. et al. Cryo-EM structure of a mitochondrial calcium uniporter. *Science* **361**, 506–511 (2018).
41. Nguyen, N. X. et al. Cryo-EM structure of a fungal mitochondrial calcium uniporter. *Nature* **559**, 570–574 (2018).
42. Fan, C. et al. X-ray and cryo-EM structures of the mitochondrial calcium uniporter. *Nature* **559**, 575–579 (2018).
43. Baradaran, R., Wang, C., Siliciano, A. F. & Long, S. B. Cryo-EM structures of fungal and metazoan mitochondrial calcium uniporters. *Nature* **559**, 580–584 (2018).
44. Vais, H. et al. EMRE Is a matrix Ca(2+) sensor that governs gatekeeping of the mitochondrial Ca(2+) uniporter. *Cell Rep.* **14**, 403–410 (2016).
45. Yamamoto, T. et al. Analysis of the structure and function of EMRE in a yeast expression system. *Biochim. Biophys. Acta* **1857**, 831–839 (2016).
46. Tsai, M. F. et al. Dual functions of a small regulatory subunit in the mitochondrial calcium uniporter complex. *Elife* **5**, e15545 (2016).
47. Chiurillo, M. A. et al. Different roles of mitochondrial calcium uniporter complex subunits in growth and infectivity of *Trypanosoma cruzi*. *MBio* **8**, e00574–17 (2017).
48. Kirichok, Y., Krapivinsky, G. & Clapham, D. E. The mitochondrial calcium uniporter is a highly selective ion channel. *Nature* **427**, 360–364 (2004).
49. Bragadin, M., Pozzan, T. & Azzone, G. F. Kinetics of Ca^{2+} carrier in rat liver mitochondria. *Biochemistry* **18**, 5972–5978 (1979).
50. Babcock, D. F., Herrington, J., Goodwin, P. C., Park, Y. B. & Hille, B. Mitochondrial participation in the intracellular Ca^{2+} network. *J. Cell Biol.* **136**, 833–844 (1997).
51. Friel, D. D. & Tsien, R. W. An FCCP-sensitive Ca^{2+} store in bullfrog sympathetic neurons and its participation in stimulus-evoked changes in $[Ca^{2+}]i$. *J. Neurosci.* **14**, 4007–4024 (1994).
52. Rizzuto, R. et al. Close contacts with the endoplasmic reticulum as determinants of mitochondrial Ca^{2+} responses. *Science* **280**, 1763–1766 (1998).
This study demonstrates the crucial role of ER–mitochondria contacts in rapid mitochondrial Ca^{2+} accumulation.
53. Gaigg, B., Simbeni, R., Hrastnik, C., Paltauf, F. & Daum, G. Characterization of a microsomal subfraction associated with mitochondria of the yeast. *Saccharomyces cerevisiae*. Involvement in synthesis and import of phospholipids into mitochondria. *Biochim. Biophys. Acta* **1234**, 214–220 (1995).
54. Willson, V. J. & Tipton, K. F. Purification and characterization of ox brain NAD^{+}-dependent isocitrate dehydrogenase. *J. Neurochem.* **33**, 1239–1247 (1979).
55. Giorgi, C. et al. Mitochondria-associated membranes: composition, molecular mechanisms, and physiopathological implications. *Antioxid. Redox Signal.* **22**, 995–1019 (2015).
56. Giacomello, M. et al. Ca^{2+} hot spots on the mitochondrial surface are generated by Ca^{2+} mobilization from stores, but not by activation of store-operated Ca^{2+} channels. *Mol. Cell* **38**, 280–290 (2010).
57. Suski, J. M. et al. Isolation of plasma membrane-associated membranes from rat liver. *Nat. Protoc.* **9**, 312–322 (2014).
58. Jung, D. W., Baysal, K. & Brierley, G. P. The sodium-calcium antiport of heart mitochondria is not electroneutral. *J. Biol. Chem.* **270**, 672–678 (1995).
59. Dash, R. K. & Beard, D. A. Analysis of cardiac mitochondrial Na^{+}-Ca^{2+} exchanger kinetics with a biophysical model of mitochondrial Ca^{2+} handling suggests a 3:1 stoichiometry. *J. Physiol.* **586**, 3267–3285 (2008).
60. Gunter, K. K., Zuscik, M. J. & Gunter, T. E. The Na(+)-independent Ca^{2+} efflux mechanism of liver mitochondria is not a passive Ca^{2+}/2H+ exchanger. *J. Biol. Chem.* **266**, 21640–21648 (1991).
61. Scorziello, A. et al. NCX3 regulates mitochondrial Ca(2+) handling through the AKAP121-anchored signaling complex and prevents hypoxia-induced neuronal death. *J. Cell Sci.* **126**, 5566–5577 (2013).
62. Palty, R. et al. NCLX is an essential component of mitochondrial Na^{+}/Ca^{2+} exchange. *Proc. Natl Acad. Sci. USA* **107**, 436–441 (2010).
This study identifies NCLX as the mitochondrial NCX.
63. Crompton, M., Kunzi, M. & Carafoli, E. The calcium-induced and sodium-induced effluxes of calcium from heart mitochondria. Evidence for a sodium-calcium carrier. *Eur. J. Biochem.* **79**, 549–558 (1977).
64. De Marchi, U. et al. NCLX protein, but not LETM1, mediates mitochondrial Ca^{2+} extrusion, thereby limiting Ca^{2+}-induced NAD(P)H production and modulating matrix redox state. *J. Biol. Chem.* **289**, 20377–20385 (2014).
65. Kim, B., Takeuchi, A., Hikida, M. & Matsuoka, S. Roles of the mitochondrial Na(+)-Ca(2+) exchanger, NCLX, in B lymphocyte chemotaxis. *Sci. Rep.* **6**, 28378 (2016).
66. Luongo, T. S. et al. The mitochondrial Na(+)/Ca(2+) exchanger is essential for Ca(2+) homeostasis and viability. *Nature* **545**, 93–97 (2017).
This study shows that mitochondrial Ca^{2+} efflux through NCLX is crucial for cardiac homeostasis.
67. Shao, J. et al. Leucine zipper-EF-hand containing transmembrane protein 1 (LETM1) forms a Ca^{2+}/H^{+} antiporter. *Sci. Rep.* **6**, 34174 (2016).
68. Nowikovsky, K., Pozzan, T., Rizzuto, R., Scorrano, L. & Bernardi, P. Perspectives on: SGP symposium on mitochondrial physiology and medicine: the pathophysiology of LETM1. *J. Gen. Physiol.* **139**, 445–454 (2012).
69. Froschauer, E., Nowikovsky, K. & Schweyen, R. J. Electroneutral K^{+}/H^{+} exchange in mitochondrial membrane vesicles involves Yol027/Letm1 proteins. *Biochim. Biophys. Acta* **1711**, 41–48 (2005).
70. Nowikovsky, K. et al. The LETM1/YOL027 gene family encodes a factor of the mitochondrial K^{+} homeostasis with a potential role in the Wolf-Hirschhorn syndrome. *J. Biol. Chem.* **279**, 30307–30315 (2004).
In conflict with reference 20, this study shows that LETM1 mediates mitochondrial K^{+}/H^{+} exchange.
71. Hashimi, H., McDonald, L., Stribrna, E. & Lukes, J. Trypanosome Letm1 protein is essential for mitochondrial potassium homeostasis. *J. Biol. Chem.* **288**, 26914–26925 (2013).
72. Tsai, M. F., Jiang, D., Zhao, L., Clapham, D. & Miller, C. Functional reconstitution of the mitochondrial Ca^{2+}/H^{+} antiporter Letm1. *J. Gen. Physiol.* **143**, 67–73 (2014).
73. Quan, X. et al. Essential role of mitochondrial Ca^{2+} uniporter in the generation of mitochondrial pH gradient and metabolism-secretion coupling in insulin-releasing cells. *J. Biol. Chem.* **290**, 4086–4096 (2015).
74. Doonan, P. J. et al. LETM1-dependent mitochondrial Ca^{2+} flux modulates cellular bioenergetics and proliferation. *FASEB J.* **28**, 4936–4949 (2014).

75. Waldeck-Weiermair, M. et al. Leucine zipper EF hand-containing transmembrane protein 1 (Letm1) and uncoupling proteins 2 and 3 (UCP2/3) contribute to two distinct mitochondrial Ca^{2+} uptake pathways. *J. Biol. Chem.* **286**, 28444–28455 (2011).
76. Elrod, J. W. et al. Cyclophilin D controls mitochondrial pore-dependent Ca(2+) exchange, metabolic flexibility, and propensity for heart failure in mice. *J. Clin. Invest.* **120**, 3680–3687 (2010).
77. Lu, X., Kwong, J. Q., Molkentin, J. D. & Bers, D. M. Individual cardiac mitochondria undergo rare transient permeability transition pore openings. *Circ. Res.* **118**, 834–841 (2016).
78. De Marchi, E., Bonora, M., Giorgi, C. & Pinton, P. The mitochondrial permeability transition pore is a dispensable element for mitochondrial calcium efflux. *Cell Calcium* **56**, 1–13 (2014).
79. Rostovtseva, T. K. & Bezrukov, S. M. VDAC regulation: role of cytosolic proteins and mitochondrial lipids. *J. Bioenerg. Biomembr.* **40**, 163–170 (2008).
80. Huang, H. et al. An interaction between Bcl-xL and the voltage-dependent anion channel (VDAC) promotes mitochondrial Ca^{2+} uptake. *J. Biol. Chem.* **288**, 19870–19881 (2013).
81. Arbel, N., Ben-Hail, D. & Shoshan-Barmatz, V. Mediation of the antiapoptotic activity of Bcl-xL protein upon interaction with VDAC1 protein. *J. Biol. Chem.* **287**, 23152–23161 (2012).
82. Vander Heiden, M. G. et al. Bcl-xL promotes the open configuration of the voltage-dependent anion channel and metabolite passage through the outer mitochondrial membrane. *J. Biol. Chem.* **276**, 19414–19419 (2001).
83. Pavlov, E. et al. The mitochondrial channel VDAC has a cation-selective open state. *Biochim. Biophys. Acta* **1710**, 96–102 (2005).
84. Monaco, G. et al. The BH4 domain of anti-apoptotic Bcl-XL, but not that of the related Bcl-2, limits the voltage-dependent anion channel 1 (VDAC1)-mediated transfer of pro-apoptotic Ca^{2+} signals to mitochondria. *J. Biol. Chem.* **290**, 9150–9161 (2015).
85. Mallilankaraman, K. et al. MCUR1 is an essential component of mitochondrial Ca^{2+} uptake that regulates cellular metabolism. *Nat. Cell Biol.* **14**, 1336–1343 (2012).
86. Lee, Y. et al. Structure and function of the N-terminal domain of the human mitochondrial calcium uniporter. *EMBO Rep.* **16**, 1318–1333 (2015).
87. Vais, H. et al. MCUR1, CCDC90A, is a regulator of the mitochondrial calcium uniporter. *Cell Metab.* **22**, 533–535 (2015).
88. Chaudhuri, D., Artiga, D. J., Abiria, S. A. & Clapham, D. E. Mitochondrial calcium uniporter regulator 1 (MCUR1) regulates the calcium threshold for the mitochondrial permeability transition. *Proc. Natl Acad. Sci. USA* **113**, E1872–E1880 (2016).
89. Tomar, D. et al. MCUR1 Is a scaffold factor for the MCU complex function and promotes mitochondrial bioenergetics. *Cell Rep.* **15**, 1673–1685 (2016).
90. Paupe, V., Prudent, J., Dassa, E. P., Rendon, O. Z. & Shoubridge, E. A. CCDC90A (MCUR1) is a cytochrome *c* oxidase assembly factor and not a regulator of the mitochondrial calcium uniporter. *Cell Metab.* **21**, 109–116 (2015).
91. Ren, T. et al. MCUR1-mediated mitochondrial calcium signaling facilitates cell survival of hepatocellular carcinoma via reactive oxygen species-dependent p53 degradation. *Antioxid. Redox Signal.* https://doi.org/10.1089/ars.2017.6990 (2017).
92. Hoffman, N. E. et al. SLC25A23 augments mitochondrial Ca(2)(+) uptake, interacts with MCU, and induces oxidative stress-mediated cell death. *Mol. Biol. Cell* **25**, 936–947 (2014).
93. del Arco, A. & Satrustegui, J. Identification of a novel human subfamily of mitochondrial carriers with calcium-binding domains. *J. Biol. Chem.* **279**, 24701–24713 (2004).
94. Fiermonte, G. et al. Identification of the mitochondrial ATP-Mg/Pi transporter. Bacterial expression, reconstitution, functional characterization, and tissue distribution. *J. Biol. Chem.* **279**, 30722–30730 (2004).
95. de Brito, O. M. & Scorrano, L. Mitofusin 2 tethers endoplasmic reticulum to mitochondria. *Nature* **456**, 605–610 (2008).
96. Filadi, R. et al. Mitofusin 2 ablation increases endoplasmic reticulum-mitochondria coupling. *Proc. Natl Acad. Sci. USA* **112**, E2174–E2181 (2015).
97. Zhang, S. & Sodroski, J. Efficient human immunodeficiency virus (HIV-1) infection of cells lacking PDZD8. *Virology* **481**, 73–78 (2015).
98. Hirabayashi, Y. et al. ER-mitochondria tethering by PDZD8 regulates Ca(2+) dynamics in mammalian neurons. *Science* **358**, 623–630 (2017).
This study identifies PDZD8 as the mammalian orthologue of Mmm1, the yeast ER component of the ERMES complex, a protein complex that tethers the ER to mitochondria.
99. De Vos, K. J. et al. VAPB interacts with the mitochondrial protein PTPIP51 to regulate calcium homeostasis. *Hum. Mol. Genet.* **21**, 1299–1311 (2012).
100. Paillusson, S. et al. alpha-Synuclein binds to the ER-mitochondria tethering protein VAPB to disrupt Ca(2+) homeostasis and mitochondrial ATP production. *Acta Neuropathol.* **134**, 129–149 (2017).
101. Stoica, R. et al. ER-mitochondria associations are regulated by the VAPB-PTPIP51 interaction and are disrupted by ALS/FTD-associated TDP-43. *Nat. Commun.* **5**, 3996 (2014).
102. Pinton, P., Leo, S., Wieckowski, M. R., Di Benedetto, G. & Rizzuto, R. Long-term modulation of mitochondrial Ca^{2+} signals by protein kinase C isozymes. *J. Cell Biol.* **165**, 223–232 (2004).
103. Kostic, M. et al. PKA phosphorylation of NCLX reverses mitochondrial calcium overload and depolarization, promoting survival of PINK1-deficient dopaminergic neurons. *Cell Rep.* **13**, 376–386 (2015).
104. Huang, E. et al. PINK1-mediated phosphorylation of LETM1 regulates mitochondrial calcium transport and protects neurons against mitochondrial stress. *Nat. Commun.* **8**, 1399 (2017).
105. Martel, C. et al. Glycogen synthase kinase 3-mediated voltage-dependent anion channel phosphorylation controls outer mitochondrial membrane permeability during lipid accumulation. *Hepatology* **57**, 93–102 (2013).
106. Dong, Z. et al. Mitochondrial Ca(2+) uniporter is a mitochondrial luminal redox sensor that augments MCU channel activity. *Mol. Cell* **65**, 1014–1028 (2017).
107. O.-Uchi, J. et al. Adrenergic signaling regulates mitochondrial Ca^{2+} uptake through Pyk2-dependent tyrosine phosphorylation of the mitochondrial Ca^{2+} uniporter. *Antioxid. Redox Signal.* **21**, 863–879 (2014).
108. Joiner, M. L. et al. CaMKII determines mitochondrial stress responses in heart. *Nature* **491**, 269–273 (2012).
This study describes the first post-translational modification (phosphorylation) of the MCU complex and its role in heart failure. This work is in conflict with reference 110.
109. Nguyen, E. K. et al. CaMKII (Ca(2+)/calmodulin-dependent kinase II) in mitochondria of smooth muscle cells controls mitochondrial mobility, migration, and neointima formation. *Arterioscler. Thromb. Vasc. Biol.* **38**, 1333–1345 (2018).
110. Fieni, F., Johnson, D. E., Hudmon, A. & Kirichok, Y. Mitochondrial Ca^{2+} uniporter and CaMKII in heart. *Nature* **513**, E1–E2 (2014).
111. Madreiter-Sokolowski, C. T. et al. PRMT1-mediated methylation of MICU1 determines the UCP2/3 dependency of mitochondrial Ca(2+) uptake in immortalized cells. *Nat. Commun.* **7**, 12897 (2016).
112. Konig, T. et al. The m-AAA protease associated with neurodegeneration limits MCU activity in mitochondria. *Mol. Cell* **64**, 148–162 (2016).
113. Tsai, C. W. et al. Proteolytic control of the mitochondrial calcium uniporter complex. *Proc. Natl Acad. Sci. USA* **114**, 4388–4393 (2017).
114. Pizzo, P., Drago, I., Filadi, R. & Pozzan, T. Mitochondrial Ca(2)(+) homeostasis: mechanism, role, and tissue specificities. *Pflugers Arch.* **464**, 3–17 (2012).
115. Bers, D. M. Cardiac excitation-contraction coupling. *Nature* **415**, 198–205 (2002).
116. Williams, G. S., Boyman, L., Chikando, A. C., Khairallah, R. J. & Lederer, W. J. Mitochondrial calcium uptake. *Proc. Natl Acad. Sci. USA* **110**, 10479–10486 (2013).
117. Parnis, J. et al. Mitochondrial exchanger NCLX plays a major role in the intracellular Ca^{2+} signaling, gliotransmission, and proliferation of astrocytes. *J. Neurosci.* **33**, 7206–7219 (2013).
118. Deak, A. T. et al. IP3-mediated STIM1 oligomerization requires intact mitochondrial Ca^{2+} uptake. *J. Cell Sci.* **127**, 2944–2955 (2014).
119. Gilabert, J. A., Bakowski, D. & Parekh, A. B. Energized mitochondria increase the dynamic range over which inositol 1,4,5-trisphosphate activates store-operated calcium influx. *EMBO J.* **20**, 2672–2679 (2001).
120. Rizzuto, R., De Stefani, D., Raffaello, A. & Mammucari, C. Mitochondria as sensors and regulators of calcium signalling. *Nat. Rev. Mol. Cell Biol.* **13**, 566–578 (2012).
121. Parekh, A. B. Regulation of CRAC channels by Ca^{2+}-dependent inactivation. *Cell Calcium* **63**, 20–23 (2017).
122. Samanta, K., Douglas, S. & Parekh, A. B. Mitochondrial calcium uniporter MCU supports cytoplasmic Ca^{2+} oscillations, store-operated Ca^{2+} entry and Ca^{2+}-dependent gene expression in response to receptor stimulation. *PLOS One* **9**, e101188 (2014).
123. Wacquier, B., Combettes, L., Van Nhieu, G. T. & Dupont, G. Interplay between intracellular Ca(2+) oscillations and Ca(2+)-stimulated mitochondrial metabolism. *Sci. Rep.* **6**, 19316 (2016).
124. Tinel, H. et al. Active mitochondria surrounding the pancreatic acinar granule region prevent spreading of inositol trisphosphate-evoked local cytosolic Ca(2+) signals. *EMBO J.* **18**, 4999–5008 (1999).
This study demonstrates that mitochondrial Ca^{2+}-buffering capacity is essential to shape cytosolic Ca^{2+} signals and to regulate secretion in acinar cells.
125. Billups, B. & Forsythe, I. D. Presynaptic mitochondrial calcium sequestration influences transmission at mammalian central synapses. *J. Neurosci.* **22**, 5840–5847 (2002).
126. Drago, I., De Stefani, D., Rizzuto, R. & Pozzan, T. Mitochondrial Ca^{2+} uptake contributes to buffering cytoplasmic Ca^{2+} peaks in cardiomyocytes. *Proc. Natl Acad. Sci. USA* **109**, 12986–12991 (2012).
127. Kwong, J. Q. et al. The mitochondrial calcium uniporter selectively matches metabolic output to acute contractile stress in the heart. *Cell Rep.* **12**, 15–22 (2015).
128. Lu, X. et al. Measuring local gradients of intramitochondrial [Ca(2+)] in cardiac myocytes during sarcoplasmic reticulum Ca(2+) release. *Circ. Res.* **112**, 424–431 (2013).
129. Tang, S. et al. Mitochondrial Ca(2)(+) uniporter is critical for store-operated Ca(2)(+) entry-dependent breast cancer cell migration. *Biochem. Biophys. Res. Commun.* **458**, 186–193 (2015).
130. Tosatto, A. et al. The mitochondrial calcium uniporter regulates breast cancer progression via HIF-1alpha. *EMBO Mol. Med.* **8**, 569–585 (2016).
131. Quintana, A. et al. T cell activation requires mitochondrial translocation to the immunological synapse. *Proc. Natl Acad. Sci. USA* **104**, 14418–14423 (2007).
132. Medina, D. L. et al. Lysosomal calcium signalling regulates autophagy through calcineurin and TFEB. *Nat. Cell Biol.* **17**, 288–299 (2015).
133. Sbano, L. et al. TFEB-mediated increase in peripheral lysosomes regulates store-operated calcium entry. *Sci. Rep.* **7**, 40797 (2017).
134. Lee, K. P., Yuan, J. P., So, I., Worley, P. F. & Muallem, S. STIM1-dependent and STIM1-independent function of transient receptor potential canonical (TRPC) channels tunes their store-operated mode. *J. Biol. Chem.* **285**, 38666–38673 (2010).
135. Ben-Kasus Nissim, T. et al. Mitochondria control store-operated Ca^{2+} entry through Na^{+} and redox signals. *EMBO J.* **36**, 797–815 (2017).
136. McCormack, J. G. & Denton, R. M. The effects of calcium ions and adenine nucleotides on the activity of pig heart 2-oxoglutarate dehydrogenase complex. *Biochem. J.* **180**, 533–544 (1979).
This study demonstrates that increases in intramitochondrial free Ca^{2+} result in activation of mitochondrial dehydrogenases and stimulation of ATP synthesis.
137. Jouaville, L. S., Pinton, P., Bastianutto, C., Rutter, G. A. & Rizzuto, R. Regulation of mitochondrial ATP synthesis by calcium: evidence for a long-term metabolic priming. *Proc. Natl Acad. Sci. USA* **96**, 13807–13812 (1999).
Reinforces findings presented in reference 136, showing that mitochondrial Ca^{2+} rises stably elevate mitochondrial [ATP].
138. Giorgi, C. et al. Mitochondrial Ca(2+) and apoptosis. *Cell Calcium* **52**, 36–43 (2012).
139. Rimessi, A. et al. Perturbed mitochondrial Ca signals as causes or consequences of mitophagy induction. *Autophagy* **9**, 1677–1686 (2013).
140. Marchi, S. et al. Mitochondrial and endoplasmic reticulum calcium homeostasis and cell death. *Cell Calcium* **69**, 62–72 (2018).
141. Schuit, F. C., In't Veld, P. A. & Pipeleers, D. G. Glucose stimulates proinsulin biosynthesis by a dose-dependent recruitment of pancreatic beta cells. *Proc. Natl Acad. Sci. USA* **85**, 3865–3869 (1988).
142. Wiederkehr, A. & Wollheim, C. B. Mitochondrial signals drive insulin secretion in the pancreatic beta-cell. *Mol. Cell Endocrinol.* **353**, 128–137 (2012).
143. Hoppa, M. B. et al. Chronic palmitate exposure inhibits insulin secretion by dissociation of Ca(2+) channels

from secretory granules. *Cell Metab.* **10**, 455–465 (2009).
144. Tarasov, A. I. et al. The mitochondrial Ca^{2+} uniporter MCU is essential for glucose-induced ATP increases in pancreatic beta-cells. *PLOS One* **7**, e39722 (2012).
145. Gilon, P., Chae, H. Y., Rutter, G. A. & Ravier, M. A. Calcium signaling in pancreatic beta-cells in health and in Type 2 diabetes. *Cell Calcium* **56**, 340–361 (2014).
146. Rutter, G. A., Tsuboi, T. & Ravier, M. A. Ca^{2+} microdomains and the control of insulin secretion. *Cell Calcium* **40**, 539–551 (2006).
147. Pinton, P. et al. Dynamics of glucose-induced membrane recruitment of protein kinase C beta II in living pancreatic islet beta-cells. *J. Biol. Chem.* **277**, 37702–37710 (2002).
148. Wiederkehr, A. et al. Mitochondrial matrix calcium is an activating signal for hormone secretion. *Cell Metab.* **13**, 601–611 (2011).
This study demonstrates a key role of mitochondrial Ca^{2+} in metabolism-driven insulin and aldosterone secretion.
149. Tarasov, A. I. et al. Frequency-dependent mitochondrial Ca(2+) accumulation regulates ATP synthesis in pancreatic beta cells. *Pflugers Arch.* **465**, 543–554 (2013).
150. Varadi, A. & Rutter, G. A. Ca^{2+}-induced Ca^{2+} release in pancreatic islet beta-cells: critical evaluation of the use of endoplasmic reticulum-targeted "cameleons". *Endocrinology* **145**, 4540–4549 (2004).
151. Santulli, G. et al. Calcium release channel RyR2 regulates insulin release and glucose homeostasis. *J. Clin. Invest.* **125**, 1968–1978 (2015).
152. Wright, L. E. et al. Increased mitochondrial calcium uniporter in adipocytes underlies mitochondrial alterations associated with insulin resistance. *Am. J. Physiol. Endocrinol. Metab.* **313**, E641–E650 (2017).
153. Vierra, N. C. et al. TALK-1 channels control beta cell endoplasmic reticulum Ca(2+) homeostasis. *Sci. Signal* **10**, eaan2883 (2017).
154. Alam, M. R. et al. Mitochondrial Ca^{2+} uptake 1 (MICU1) and mitochondrial ca2+ uniporter (MCU) contribute to metabolism-secretion coupling in clonal pancreatic beta-cells. *J. Biol. Chem.* **287**, 34445–34454 (2012).
155. Murgia, M., Giorgi, C., Pinton, P. & Rizzuto, R. Controlling metabolism and cell death: at the heart of mitochondrial calcium signalling. *J. Mol. Cell Cardiol.* **46**, 781–788 (2009).
156. Bers, D. M. Calcium cycling and signaling in cardiac myocytes. *Annu. Rev. Physiol.* **70**, 23–49 (2008).
157. Torrente, A. G. et al. Burst pacemaker activity of the sinoatrial node in sodium-calcium exchanger knockout mice. *Proc. Natl Acad. Sci. USA* **112**, 9769–9774 (2015).
158. Mesirca, P. et al. Cardiac arrhythmia induced by genetic silencing of 'funny' (f) channels is rescued by GIRK4 inactivation. *Nat. Commun.* **5**, 4664 (2014).
159. Perez-Reyes, E. Molecular physiology of low-voltage-activated t-type calcium channels. *Physiol. Rev.* **83**, 117–161 (2003).
160. Mesirca, P., Torrente, A. G. & Mangoni, M. E. Functional role of voltage gated Ca(2+) channels in heart automaticity. *Front. Physiol.* **6**, 19 (2015).
161. Viola, H. M., Arthur, P. G. & Hool, L. C. Evidence for regulation of mitochondrial function by the L-type Ca^{2+} channel in ventricular myocytes. *J. Mol. Cell Cardiol.* **46**, 1016–1026 (2009).
162. Cannell, M. B., Cheng, H. & Lederer, W. J. The control of calcium release in heart muscle. *Science* **268**, 1045–1049 (1995).
163. Santulli, G., Nakashima, R., Yuan, Q. & Marks, A. R. Intracellular calcium release channels: an update. *J. Physiol.* **595**, 3041–3051 (2017).
164. Gambardella, J., Trimarco, B., Iaccarino, G. & Santulli, G. New insights in cardiac calcium handling and excitation-contraction coupling. *Adv. Exp. Med. Biol.* **1067**, 373–385 (2017).
165. Gambardella, J. et al. Functional Role of Mitochondria in Arrhythmogenesis. *Adv. Exp. Med. Biol.* **982**, 191–202 (2017).
166. Wu, Y. et al. The mitochondrial uniporter controls fight or flight heart rate increases. *Nat. Commun.* **6**, 6081 (2015).
167. Bick, A. G. et al. Cardiovascular homeostasis dependence on MICU2, a regulatory subunit of the mitochondrial calcium uniporter. *Proc. Natl Acad. Sci. USA* **114**, E9096–E9104 (2017).
168. O'Rourke, B. & Blatter, L. A. Mitochondrial Ca^{2+} uptake: tortoise or hare? *J. Mol. Cell Cardiol.* **46**, 767–774 (2009).
169. Griffiths, E. J. Mitochondrial calcium transport in the heart: physiological and pathological roles. *J. Mol. Cell Cardiol.* **46**, 789–803 (2009).
170. Piot, C. et al. Effect of cyclosporine on reperfusion injury in acute myocardial infarction. *N. Engl. J. Med.* **359**, 473–481 (2008).
171. Cung, T. T. et al. Cyclosporine before PCI in patients with acute myocardial infarction. *N. Engl. J. Med.* **373**, 1021–1031 (2015).
172. Bonora, M. et al. Mitochondrial permeability transition involves dissociation of F1FO ATP synthase dimers and C-ring conformation. *EMBO Rep.* **18**, 1077–1089 (2017).
173. Luongo, T. S. et al. The mitochondrial calcium uniporter matches energetic supply with cardiac workload during stress and modulates permeability transition. *Cell Rep.* **12**, 23–34 (2015).
This study details the generation of conditional cardiac tissue-specific MCU-knockout mice, highlighting the critical role of mitochondrial Ca^{2+} and the MCU complex in cardiac functions.
174. de Jesus Garcia-Rivas, G., Guerrero-Hernandez, A., Guerrero-Serna, G., Rodriguez-Zavala, J. S. & Zazueta, C. Inhibition of the mitochondrial calcium uniporter by the oxo-bridged dinuclear ruthenium amine complex (Ru360) prevents from irreversible injury in postischemic rat heart. *FEBS J.* **272**, 3477–3488 (2005).
175. Pan, X. et al. The physiological role of mitochondrial calcium revealed by mice lacking the mitochondrial calcium uniporter. *Nat. Cell Biol.* **15**, 1464–1472 (2013).
This study details the generation of whole-body MCU-knockout mice, which display a very mild phenotype. This finding is unexpected to most investigators in the field (see also reference 176).
176. Rasmussen, T. P. et al. Inhibition of MCU forces extramitochondrial adaptations governing physiological and pathological stress responses in heart. *Proc. Natl Acad. Sci. USA* **112**, 9129–9134 (2015).
177. Holmstrom, K. M. et al. Assessment of cardiac function in mice lacking the mitochondrial calcium uniporter. *J. Mol. Cell Cardiol.* **85**, 178–182 (2015).
178. Qiao, X. et al. PTPIP51 regulates mouse cardiac ischemia/reperfusion through mediating the mitochondria-SR junction. *Sci. Rep.* **7**, 45379 (2017).
179. Mercadier, J. J. et al. Altered sarcoplasmic reticulum Ca2(+)-ATPase gene expression in the human ventricle during end-stage heart failure. *J. Clin. Invest.* **85**, 305–309 (1990).
180. Santulli, G., Lewis, D., des Georges, A., Marks, A. R. & Frank, J. Ryanodine receptor structure and function in health and disease. *Subcell. Biochem.* **87**, 329–352 (2018).
181. Terentyev, D. et al. Redox modification of ryanodine receptors contributes to sarcoplasmic reticulum Ca^{2+} leak in chronic heart failure. *Circ. Res.* **103**, 1466–1472 (2008).
182. Xie, W. et al. Mitochondrial oxidative stress promotes atrial fibrillation. *Sci. Rep.* **5**, 11427 (2015).
183. Ai, X., Curran, J. W., Shannon, T. R., Bers, D. M. & Pogwizd, S. M. Ca^{2+}/calmodulin-dependent protein kinase modulates cardiac ryanodine receptor phosphorylation and sarcoplasmic reticulum Ca^{2+} leak in heart failure. *Circ. Res.* **97**, 1314–1322 (2005).
184. Marx, S. O. et al. PKA phosphorylation dissociates FKBP12.6 from the calcium release channel (ryanodine receptor): defective regulation in failing hearts. *Cell* **101**, 365–376 (2000).
185. Santulli, G., Xie, W., Reiken, S. R. & Marks, A. R. Mitochondrial calcium overload is a key determinant in heart failure. *Proc. Natl Acad. Sci. USA* **112**, 11389–11394 (2015).
186. Zhang, T. et al. Phospholamban ablation rescues sarcoplasmic reticulum Ca(2+) handling but exacerbates cardiac dysfunction in CaMKIIdelta(C) transgenic mice. *Circ. Res.* **106**, 354–362 (2010).
187. Gomez, A. M. et al. Defective excitation-contraction coupling in experimental cardiac hypertrophy and heart failure. *Science* **276**, 800–806 (1997).
188. Shiferaw, Y., Aistrup, G. L. & Wasserstrom, J. A. Intracellular Ca^{2+} waves, afterdepolarizations, and triggered arrhythmias. *Cardiovasc. Res.* **95**, 265–268 (2012).
189. Brandenburg, S., Arakel, E. C., Schwappach, B. & Lehnart, S. E. The molecular and functional identities of atrial cardiomyocytes in health and disease. *Biochim. Biophys. Acta* **1863**, 1882–1893 (2016).
190. Maack, C. et al. Elevated cytosolic Na^+ decreases mitochondrial Ca^{2+} uptake during excitation-contraction coupling and impairs energetic adaptation in cardiac myocytes. *Circ. Res.* **99**, 172–182 (2006).
191. Somasundaram, S. & Sadique, J. The role of mitochondrial calcium transport during inflammation and the effect of anti-inflammatory drugs. *Biochem. Med. Metab. Biol.* **36**, 220–230 (1986).
192. Zhou, R., Yazdi, A. S., Menu, P. & Tschopp, J. A role for mitochondria in NLRP3 inflammasome activation. *Nature* **469**, 221–225 (2011).
193. Rimessi, A. et al. Mitochondrial Ca^{2+}-dependent NLRP3 activation exacerbates the *Pseudomonas aeruginosa*-driven inflammatory response in cystic fibrosis. *Nat. Commun.* **6**, 6201 (2015).
This study demonstrates the importance of mitochondrial Ca^{2+} homeostasis and the MCU complex in the modulation of the immune response and inflammation.
194. Cheng, J. et al. Amplified RLR signaling activation through an interferon-stimulated gene-endoplasmic reticulum stress-mitochondrial calcium uniporter protein loop. *Sci. Rep.* **6**, 20158 (2016).
195. Antony, A. N. et al. MICU1 regulation of mitochondrial Ca(2+) uptake dictates survival and tissue regeneration. *Nat. Commun.* **7**, 10955 (2016).
196. Sheng, Z. H. & Cai, Q. Mitochondrial transport in neurons: impact on synaptic homeostasis and neurodegeneration. *Nat. Rev. Neurosci.* **13**, 77–93 (2012).
197. Pivovarova, N. B. & Andrews, S. B. Calcium-dependent mitochondrial function and dysfunction in neurons. *FEBS J.* **277**, 3622–3636 (2010).
198. Abramov, A. Y. & Duchen, M. R. Mechanisms underlying the loss of mitochondrial membrane potential in glutamate excitotoxicity. *Biochim. Biophys. Acta* **1777**, 953–964 (2008).
199. Qiu, J. et al. Mitochondrial calcium uniporter Mcu controls excitotoxicity and is transcriptionally repressed by neuroprotective nuclear calcium signals. *Nat. Commun.* **4**, 2034 (2013).
200. Di Bella, D. et al. Mutations in the mitochondrial protease gene AFG3L2 cause dominant hereditary ataxia SCA28. *Nat. Genet.* **42**, 313–321 (2010).
201. Maltecca, F. et al. Purkinje neuron Ca^{2+} influx reduction rescues ataxia in SCA28 model. *J. Clin. Invest.* **125**, 263–274 (2015).
202. Patron, M., Sprenger, H. G. & Langer, T. m-AAA proteases, mitochondrial calcium homeostasis and neurodegeneration. *Cell Res.* **28**, 296–306 (2018).
203. Filadi, R. et al. Presenilin 2 modulates endoplasmic reticulum-mitochondria coupling by tuning the antagonistic effect of mitofusin 2. *Cell Rep.* **15**, 2226–2238 (2016).
204. Cali, T., Ottolini, D., Negro, A. & Brini, M. alpha-Synuclein controls mitochondrial calcium homeostasis by enhancing endoplasmic reticulum-mitochondria interactions. *J. Biol. Chem.* **287**, 17914–17929 (2012).
205. Grassi, D. et al. Identification of a highly neurotoxic alpha-synuclein species inducing mitochondrial damage and mitophagy in Parkinson's disease. *Proc. Natl Acad. Sci. USA* **115**, E2634–E2643 (2018).
206. Bonora, M. et al. Tumor necrosis factor-alpha impairs oligodendroglial differentiation through a mitochondria-dependent process. *Cell Death Differ.* **21**, 1198–1208 (2014).
207. Warne, J. et al. Selective inhibition of the mitochondrial permeability transition pore protects against neurodegeneration in experimental multiple sclerosis. *J. Biol. Chem.* **291**, 4356–4373 (2016).
208. Parone, P. A. et al. Enhancing mitochondrial calcium buffering capacity reduces aggregation of misfolded SOD1 and motor neuron cell death without extending survival in mouse models of inherited amyotrophic lateral sclerosis. *J. Neurosci.* **33**, 4657–4671 (2013).
209. Agarwal, A. et al. Transient opening of the mitochondrial permeability transition pore induces microdomain calcium transients in astrocyte processes. *Neuron* **93**, 587–605 (2017).
210. Tang, T. S. et al. Disturbed Ca^{2+} signaling and apoptosis of medium spiny neurons in Huntington's disease. *Proc. Natl Acad. Sci. USA* **102**, 2602–2607 (2005).
211. Panov, A. V. et al. Early mitochondrial calcium defects in Huntington's disease are a direct effect of polyglutamines. *Nat. Neurosci.* **5**, 731–736 (2002).
This study demonstrates in human patients and transgenic animal models that mitochondrial Ca^{2+} alterations are a hallmark of early phases of Huntington disease.
212. Kon, N. et al. DS16570511 is a small-molecule inhibitor of the mitochondrial calcium uniporter. *Cell Death Discov.* **3**, 17045 (2017).
213. Arduino, D. M. et al. Systematic identification of MCU modulators by orthogonal interspecies chemical screening. *Mol. Cell* **67**, 711–723 (2017).

214. Uzhachenko, R. et al. Mitochondrial protein Fus1/Tusc2 in premature aging and age-related pathologies: critical roles of calcium and energy homeostasis. *Aging (Albany NY)* **9**, 627–649 (2017).
215. Lee, S. et al. Polo kinase phosphorylates Miro to control ER-mitochondria contact sites and mitochondrial Ca(2+) homeostasis in neural stem cell development. *Dev. Cell* **37**, 174–189 (2016).
216. Clapham, D. E. Calcium signalling. *Cell* **131**, 1047–1058 (2007).
217. Schwaller, B. Cytosolic Ca^{2+} buffers. *Cold Spring Harb Perspect. Biol.* **2**, a004051 (2010).
218. Han, Y. et al. Ca(2+)-induced mitochondrial ROS regulate the early embryonic cell cycle. *Cell Rep.* **22**, 218–231 (2018).
219. Sattler, R. & Tymianski, M. Molecular mechanisms of calcium-dependent excitotoxicity. *J. Mol. Med.* **78**, 3–13 (2000).
220. Hardingham, G. E. & Bading, H. The Yin and Yang of NMDA receptor signalling. *Trends Neurosci.* **26**, 81–89 (2003).
221. Danese, A. et al. Calcium regulates cell death in cancer: roles of the mitochondria and mitochondria-associated membranes (MAMs). *Biochim. Biophys. Acta* **1858**, 615–627 (2017).
222. Mattson, M. P. & Chan, S. L. Neuronal and glial calcium signaling in Alzheimer's disease. *Cell Calcium* **34**, 385–397 (2003).
223. Bittremieux, M., Parys, J. B., Pinton, P. & Bultynck, G. ER functions of oncogenes and tumor suppressors: modulators of intracellular Ca(2+) signalling. *Biochim. Biophys. Acta* **1863**, 1364–1378 (2016).
224. Chakraborty, P. K. et al. MICU1 drives glycolysis and chemoresistance in ovarian cancer. *Nat. Commun.* **8**, 14634 (2017).
225. Huang, B. et al. Suppression of LETM1 by siRNA inhibits cell proliferation and invasion of bladder cancer cells. *Oncol. Rep.* **38**, 2935–2940 (2017).
226. Liberti, M. V. & Locasale, J. W. The Warburg effect: how does it benefit cancer cells? *Trends Biochem. Sci.* **41**, 211–218 (2016).
227. Giorgi, C. et al. Intravital imaging reveals p53-dependent cancer cell death induced by phototherapy via calcium signaling. *Oncotarget* **6**, 1435–1445 (2015).
228. Kuchay, S. et al. PTEN counteracts FBXL2 to promote IP3R3- and Ca^{2+}-mediated apoptosis limiting tumour growth. *Nature* **546**, 554–558 (2017).
This study identifies the ubiquitin E3 ligase (F box/LRR-repeat protein 2 (FBXL2)) responsible for Ins(1,4,5)P_3R isoform 3 (Ins(1,4,5)P_3R3; also known as ITPR3) degradation and demonstrates the importance of the Ins(1,4,5)P_3R3–FBXL2 complex in cancer.
229. Bonora, M. et al. Subcellular calcium measurements in mammalian cells using jellyfish photoprotein aequorin-based probes. *Nat. Protoc.* **8**, 2105–2118 (2013).
230. Fonteriz, R. I. et al. Monitoring mitochondrial [Ca(2+)] dynamics with rhod-2, ratiometric pericam and aequorin. *Cell Calcium* **48**, 61–69 (2010).
231. Rodriguez-Garcia, A. et al. GAP, an aequorin-based fluorescent indicator for imaging Ca^{2+} in organelles. *Proc. Natl Acad. Sci. USA* **111**, 2584–2589 (2014).
232. Austin, S. et al. LETM1-mediated K(+) and Na(+) homeostasis regulates mitochondrial Ca(2+) efflux. *Front. Physiol.* **8**, 839 (2017).
233. Chaudhuri, A. D., Choi, D. C., Kabaria, S., Tran, A. & Junn, E. MicroRNA-7 regulates the function of mitochondrial permeability transition pore by targeting VDAC1 expression. *J. Biol. Chem.* **291**, 6483–6493 (2016).
234. Stary, C. M., Sun, X., Ouyang, Y., Li, L. & Giffard, R. G. miR-29a differentially regulates cell survival in astrocytes from cornu ammonis 1 and dentate gyrus by targeting VDAC1. *Mitochondrion* **30**, 248–254 (2016).
235. Marchi, S. et al. Downregulation of the mitochondrial calcium uniporter by cancer-related miR-25. *Curr. Biol.* **23**, 58–63 (2013).
This is the first report showing epigenetic control of MCU complex expression mediated by microRNA.
236. Pan, L. et al. MiR-25 protects cardiomyocytes against oxidative damage by targeting the mitochondrial calcium uniporter. *Int. J. Mol. Sci.* **16**, 5420–5433 (2015).
237. Hong, Z. et al. MicroRNA-138 and microRNA-25 down-regulate mitochondrial calcium uniporter, causing the pulmonary arterial hypertension cancer phenotype. *Am. J. Respir. Crit. Care Med.* **195**, 515–529 (2017).
238. Zaglia, T. et al. Content of mitochondrial calcium uniporter (MCU) in cardiomyocytes is regulated by microRNA-1 in physiologic and pathologic hypertrophy. *Proc. Natl Acad. Sci. USA* **114**, E9006–E9015 (2017).
239. Yu, C. et al. Mitochondrial calcium uniporter as a target of microRNA-340 and promoter of metastasis via enhancing the Warburg effect. *Oncotarget* **8**, 83831–83844 (2017).
240. Schneeberger, M. et al. Mitofusin 2 in POMC neurons connects ER stress with leptin resistance and energy imbalance. *Cell* **155**, 172–187 (2013).
241. Wang, P. T. et al. Distinct mechanisms controlling rough and smooth endoplasmic reticulum contacts with mitochondria. *J. Cell Sci.* **128**, 2759–2765 (2015).
242. Doghman-Bouguerra, M. et al. FATE1 antagonizes calcium- and drug-induced apoptosis by uncoupling ER and mitochondria. *EMBO Rep.* **17**, 1264–1280 (2016).
243. Zampese, E. et al. Presenilin 2 modulates endoplasmic reticulum (ER)-mitochondria interactions and Ca^{2+} cross-talk. *Proc. Natl Acad. Sci. USA* **108**, 2777–2782 (2011).
244. Simmen, T. et al. PACS-2 controls endoplasmic reticulum-mitochondria communication and Bid-mediated apoptosis. *EMBO J.* **24**, 717–729 (2005).
245. Arruda, A. P. et al. Chronic enrichment of hepatic endoplasmic reticulum-mitochondria contact leads to mitochondrial dysfunction in obesity. *Nat. Med.* **20**, 1427–1435 (2014).
246. Honrath, B. et al. SK2 channels regulate mitochondrial respiration and mitochondrial Ca(2+) uptake. *Cell Death Differ.* **24**, 761–773 (2017).

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