**The EMBO Journal (2011) 30,** 4119–4125 | © 2011 European Molecular Biology Organization | All Rights Reserved 0261-4189/11
www.embojournal.org

THE EMBO JOURNAL

Review

# After half a century mitochondrial calcium in- and efflux machineries reveal themselves

**Ilaria Drago[1], Paola Pizzo[1] and Tullio Pozzan[1,2,3,*]**

[1]Department of Biomedical Sciences, University of Padova, Padova, Italy, [2]Neuroscience Institute, National Research Council (CNR), Padova, Italy and [3]Venetian Institute of Molecular Medicine, Padova, Italy

**Mitochondrial $Ca^{2+}$ uptake and release play a fundamental role in the control of different physiological processes, such as cytoplasmic $Ca^{2+}$ signalling, ATP production and hormone metabolism, while dysregulation of mitochondrial $Ca^{2+}$ handling triggers the cascade of events that lead to cell death. The basic mechanisms of mitochondrial $Ca^{2+}$ homeostasis have been firmly established for decades, but the molecular identities of the channels and transporters responsible for $Ca^{2+}$ uptake and release have remained mysterious until very recently. Here, we briefly review the main findings that have led to our present understanding of mitochondrial $Ca^{2+}$ homeostasis and its integration in cell physiology. We will then discuss the recent work that has unravelled the biochemical identity of three key molecules: NCLX, the mitochondrial $Na^{+}/Ca^{2+}$ antiporter, MCU, the pore-forming subunit of the mitochondrial $Ca^{2+}$ uptake channel, and MICU1, one of its regulatory subunits.**

*The EMBO Journal* (2011) **30,** 4119–4125. doi:10.1038/emboj.2011.337; Published online 20 September 2011
*Subject Categories*: membranes & transport; signal transduction
*Keywords*: calcium; MCU; MICU1; mitochondria; NCLX

## Introduction

All membrane enclosed organelles (with the notable exception of nucleus and peroxisomes that appear to be in rapid, passive, equilibrium with the cytosol) are endowed with mechanisms that allow an energy-dependent $Ca^{2+}$ accumulation and a release dependent on the cation concentration gradient between the organelle lumen and the cytosol (for review see Rizzuto and Pozzan, 2006). In most cases, the uptake mechanism is due to $Ca^{2+}$ pumps (i.e. vectorial enzymes that utilize the energy liberated by ATP hydrolysis to drive $Ca^{2+}$ accumulation into the organelle lumen); the release channels, on the contrary, are controlled by different second messengers and allow $Ca^{2+}$ efflux into the cytoplasm. Sparse—and debated—evidence has been provided, indicating that lysosomes and other acidic organelles may use ATP indirectly, by generating a $H^{+}$ gradient (acid inside) through ATP hydrolysis, followed by $Ca^{2+}$ accumulation via a $H^{+}/Ca^{2+}$ exchanger (Rizzuto and Pozzan, 2006). Mitochondrial $Ca^{2+}$ accumulation and release are based on an opposite strategy: not only they do not need ATP for uptake, but they utilize gated channels for $Ca^{2+}$ uptake and exchangers ($Na^{+}$ or $H^{+}/Ca^{2+}$ exchangers) for release. This apparent paradox depends on the fact that the driving force for $Ca^{2+}$ accumulation in the mitochondrial matrix is the membrane potential (negative inside) ($\Delta\Psi$) across the inner membrane; the exchangers use the concentration gradient of $Ca^{2+}$, $H^{+}$ and $Na^{+}$ across the inner membrane to cause the release of $Ca^{2+}$ back into the cytosol (Nicholls, 2005). This unique mitochondrial toolkit ensures the maintenance of a low matrix $Ca^{2+}$ concentration ($[Ca^{2+}]$) in resting cells and a rapid $Ca^{2+}$ accumulation by the organelle when cytosolic $Ca^{2+}$ is elevated during activation. In turn, mitochondrial $Ca^{2+}$ uptake and release is central not only for the regulation of cellular $Ca^{2+}$ homeostasis, but is vital also for the regulation of intramitochondrial enzymes concerned with the utilization of oxidizable substrates. Finally, excess $Ca^{2+}$ accumulation by mitochondria is a common event in the process of cell death, by both necrosis and apoptosis (Rizzuto and Pozzan, 2006; Giacomello *et al*, 2007).

Despite the importance and the general interest for this problem, and over 40 years of intense and frustrating research on the topic, the molecular nature of the components of the mitochondrial $Ca^{2+}$ homeostatic machinery has remained mysterious. In the last year, however, three novel proteins have been identified that appear to fulfil all the characteristics expected by the mitochondrial $Ca^{2+}$ uptake channels and by the $Na^{+}/Ca^{2+}$ exchanger (Palty *et al*, 2010; Baughman *et al*, 2011; De Stefani *et al*, 2011). We here briefly summarize the historical development of the research on this topic, the key findings that have unravelled the physiopathological role of mitochondrial $Ca^{2+}$ homeostasis and finally we describe the latest findings concerned with the molecular identification of the key players of the mitochondrial $Ca^{2+}$ accumulation and release machinery.

## Historical background

Mitochondria were identified as the powerhouse for energy production in eukaryotic cells; thanks to decades of extensive biochemical work on carbohydrate metabolism and organelle morpho-functional characterization carried out, in the first half of the 20th century, by leading scientific figures such as Krebs, Corey, Claude, Palade and many others. It soon became clear that $Ca^{2+}$ played a special role in mitochondrial

*Corresponding author. Department of Biomedical Sciences, University of Padova, Viale G Colombo, 3, Via Trieste 75, Padova 35121, Italy.
Tel.: +39 049 827 6070; Fax: +39 049 827 6049;
E-mail: tullio.pozzan@unipd.it

**Received: 10 August 2011; accepted: 26 August 2011; published online: 20 September 2011**

physiology and, in particular, $Ca^{2+}$ was demonstrated to behave as a unique uncoupler of oxidative phosphorylation, OXPHOS (reviewed in Carafoli, 2010). The reason for this uncoupling effect of $Ca^{2+}$ remained, however, mysterious for some time, until in 1961 and 1962 two groups demonstrated that it was due to the ability of mitochondria to efficiently take up $Ca^{2+}$ from the medium (and accumulate it in the matrix) at the expenses of energy consumption (Deluca and Engstrom, 1961; Vasington and Murphy, 1962). Two of the basic characteristics of $Ca^{2+}$ accumulation by mitochondria were immediately highlighted: (i) $Ca^{2+}$ uptake by the organelle requires an energy source in the form of either oxidizable substrates or ATP and (ii) $Ca^{2+}$ can uncouple oxidation of substrates from ATP synthesis. A number of groups quickly jumped on the topic and many aspects of the $Ca^{2+}$ uptake system of mitochondria were rapidly clarified; most of these findings still remain undisputed. In particular, it was shown that (i) the $Ca^{2+}$ uptake system can take up also other divalent cations ($Mn^{2+}$ and $Sr^{2+}$ in particular), while $Mg^{2+}$ acts as a competitive inhibitor; (ii) $Ca^{2+}$ entry is blocked by uncouplers of OXPHOS such as dinitrophenol or FCCP (carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone); (iii) this $Ca^{2+}$ entry does not require ATP hydrolysis, although it can be supported by ATP if the respiratory chain (RC) is blocked; and (iv) the uptake is saturable (suggesting the existence of a 'carrier') and is accompanied by stoichiometric $H^+$ extrusion from the matrix.

In the beginning, the driving force for such $Ca^{2+}$ accumulation was unclear: the main idea was that it depended on the generation of a high energy phosphorylated chemical intermediate (denomitated X∼P; Chance, 1965), but with the general acceptance of the chemiosmotic hypothesis, it became clear that the driving force for $Ca^{2+}$ accumulation is the $\Delta\Psi$ across the mitochondrial inner membrane (Mitchell and Moyle, 1967). Thus, $Ca^{2+}$ enters the mitochondrial matrix down its electrochemical gradient, that can be generated either by the electron flow in the RC or by reversal of the ATP synthase. Indeed, in those years, one of the most convincing pieces of evidence that supported the chemiosmotic hypothesis came from the study of mitochondrial $Ca^{2+}$ accumulation: it was demonstrated that, in mitochondria with both the RC and the ATP synthase blocked, $Ca^{2+}$ uptake could be activated by the $K^+$ diffusion potential generated by adding valinomycin in the presence of low $K^+$ in the medium (Scarpa and Azzone, 1970).

For some time, the net positive charges (2 or 1) transported across the membrane during $Ca^{2+}$ accumulation was a matter of debate. Two models were proposed: in the first case (two positive charges), the $Ca^{2+}$ carrier was defined mitochondrial $Ca^{2+}$ 'uniporter', MCU (Brand *et al*, 1976) while in the second (one positive charge), it was define as a $H^+/Ca^{2+}$ antiporter (Moyle and Mitchell, 1977). The controversy was eventually settled (but has surfaced again recently; see below) and the general agreement was that $Ca^{2+}$ accumulation results in the net transfer of two positive charges into the matrix; that is, it is mediated by the MCU. $Ca^{2+}$ influx, in turn, results in the drop of $\Delta\Psi$ that is regenerated by the extrusion (through the RC or ATP hydrolysis) of $2H^+$ per $Ca^{2+}$ accumulated. Thus, to maintain gross electroneutrality, the $H^+/Ca^{2+}$ stoichiometry is 2.

An obvious consequence of $Ca^{2+}$-dependent $H^+$ extrusion is the alkalinization of the mitochondrial matrix that eventually blocks further $Ca^{2+}$ uptake. Indeed, addition of permeant anions such as inorganic phosphate ($P_i$) or acetate collapses the $\Delta$pH and allows massive $Ca^{2+}$ accumulation into the matrix. When $P_i$ is used, $Ca^{2+}$ tends to precipitate in the matrix as hydroxiapatite, a process favoured by the alkaline pH inside the organelle, with damage of the mitochondrial integrity and irreversible uncoupling. Such $Ca^{2+}$–$P_i$ precipitates were observed in cells of damaged tissues, suggesting that this may occur also *in vivo*.

If $Ca^{2+}$ accumulation depends solely on the $\Delta\Psi$ across the inner membrane, it should reach electrochemical equilibrium. For a value of 180 mV (negative inside) this would imply, according to the Nernst equation, about 1 million fold accumulation of $Ca^{2+}$ in the matrix, clearly incompatible not only with experimental observations in living cells (Somlyo *et al*, 1979, 1985), but also with general cell physiology concepts. The solution of this paradox came from the observation that, in isolated mitochondria, a slow and complete release of $Ca^{2+}$ is observed if, after $Ca^{2+}$ accumulation, the MCU is blocked by Ruthenium Red (RR). The conclusion was therefore that an 'electroneutral $Ca^{2+}$ efflux' must exist, whose activity prevents attainment of electrochemical equilibrium. Steady-state $Ca^{2+}$ accumulation is reached when the rate of $Ca^{2+}$ influx through the MCU equals that of the efflux (antiport). In non-excitable tissues (liver, kidney), such an antiport appears to be predominantly an $H^+/Ca^{2+}$ antiport, while in excitable tissues (heart, brain) it appears to be primarily a $Na^+/Ca^{2+}$ exchanger (Puskin *et al*, 1976; Brierley *et al*, 1994). The kinetic equilibrium between MCU-dependent influx and antiport-dependent efflux thus results in a futile (energy consuming) cycle of $Ca^{2+}$ across the mitochondrial inner membrane. As to the stoichiometry of such antiporters, the question remained unsettled for some time, but a general consensus has been reached, at least for the $Na^+/Ca^{2+}$ antiport, that catalyses the exchange of $3(4)Na^+$ per $(1)Ca^{2+}$, that is, similarly to its counterpart in the plasma membrane (PM). In coupled mitochondria, the highly negative (inside) membrane potential favours the efflux of $Ca^{2+}$ and the influx of $Na^+$ and prevents its reverse functioning. No consensus has yet been reached on the stoichiometry for the $H^+/Ca^{2+}$ antiport, that is, whether it is electroneutral ($2H^+$ per $Ca^{2+}$) or electrogenic ($\geqslant 3$ $H^+$ per $Ca^{2+}$).

## Mitochondrial $Ca^{2+}$ uptake and cell physiology

A second major question was apparently settled at the end of the 1970s, that is, the participation of mitochondria in the physiological control of $Ca^{2+}$ in living cells. Not only it was demonstrated that in healthy cells the mitochondrial $Ca^{2+}$ content is low (Somlyo *et al*, 1985), but it was also shown that the apparent affinity of the MCU for $Ca^{2+}$, under physiological conditions (i.e. 1 mM $Mg^{2+}$), is very low (apparent Kd of 20–30 μM) and the influx rate only becomes substantial when the extramitochondrial [$Ca^{2+}$] reaches values above 5–10 μM, that is, concentrations rarely (or never) observed in the cytosol of healthy cells (Rizzuto and Pozzan, 2006). This conclusion drastically reduced the interest for the mitochondrial $Ca^{2+}$ handling process and, for a long time, many investigators considered it simply an interesting laboratory artefact. Most remaining interest was

 

further abated by the discovery of the second messenger IP3 (inositol trisphosphate) and the demonstration that the $Ca^{2+}$ store that is mobilized during cell activation is the endoplasmic reticulum (ER) and not mitochondria (Streb *et al*, 1983). The last question concerning the role of $Ca^{2+}$ uptake in cell physiology was primarily focused on the role of matrix [$Ca^{2+}$] in the regulation of NADH dehydrogenases (Denton and McCormack, 1980; Denton, 2009). However, the affinity of these enzymes for $Ca^{2+}$ is quite high and, accordingly, it was argued that even a slow and inefficient $Ca^{2+}$ uptake by mitochondria was sufficient to fulfil this function.

Mitochondrial $Ca^{2+}$ returned to the limelight in 1992 when our group generated a novel, genetically encoded chemiluminescent indicator, aequorin. This probe, specifically targeted to the mitochondrial matrix, allowed dynamic, accurate and specific monitoring of the [$Ca^{2+}$] within the matrix of mitochondria in living cells (Rizzuto *et al*, 1992). With this new tool, we could show that mitochondria *in situ* are capable not only of taking up $Ca^{2+}$ (even for normal physiological cytoplasmic $Ca^{2+}$ rises), but also that mitochondria in living cells undergo very fast and large increases in their matrix $Ca^{2+}$ levels, reaching peaks one or two orders of magnitude higher than those in the cytoplasm (Rizzuto *et al*, 1993; Rizzuto and Pozzan, 2006). The discrepancy between the low $Ca^{2+}$ affinity of the MCU observed *in vitro* and the high efficiency discovered *in vivo* was explained on the basis of the microheterogeneity of cytoplasmic $Ca^{2+}$ rises during stimulation. In particular, we suggested that microdomains of high [$Ca^{2+}$] (10–20 μM) can be transiently formed in regions of close apposition between mitochondria and $Ca^{2+}$ channels of the ER/SR (sarcoplasmic reticulum) or of the PM (Rizzuto *et al*, 1998). These high $Ca^{2+}$ microdomains rapidly dissipate (due to diffusion) insuring that mitochondria do not overload with $Ca^{2+}$. This hypothesis received a number of indirect confirmations in the last 20 years by different groups; more recently, such microdomains in selected regions of contact between ER and mitochondria were finally measured directly (Giacomello *et al*, 2010; Csordas *et al*, 2010). The interest of the scientific community in mitochondrial $Ca^{2+}$ handling was further stimulated when it was demonstrated that excess $Ca^{2+}$ uptake by mitochondria could initiate the apoptotic process through the opening of the so-called 'permeability transition pore', PTP (Bernardi, 1999; Zamzami and Kroemer, 2001). A large number of data have thus been published in the recent years concerned with the physiopathological role of mitochondrial $Ca^{2+}$ in processes as different as ischaemic death, excitotoxicity and cancer (Contreras *et al*, 2010). The field of mitochondrial $Ca^{2+}$ homeostasis has undergone a significant revival and nowadays hundreds of papers on this topic are published every year.

## The molecular nature of the mitochondrial $Ca^{2+}$ handling machinery: a 40-year long story

In spite of all the renewed interest, a key element in the mitochondrial $Ca^{2+}$ saga continued to elude the scientific community. Indeed, until very recently, the molecular identification of the players in this choreography (schematized in Figure 1) thwarted all the groups that tried to elucidate this issue. Importantly, without this information it was impossible to try any genetic manipulation of this key aspect of cell physiopathology. Many attempts were made to identify the molecular nature of the MCU and antiporters, starting in the early 1970s, that is, soon after the discovery of this mitochondrial function. As far as the MCU is concerned, the early 1970s saw the purification of the so-called mitochondrial glycoprotein that, when added to lipid bilayers, induced the appearance of a $Ca^{2+}$ current. The finding raised significant interest since the best-known inhibitor of the MCU was RR, a generic stain for glycoproteins, thus consistent with the proposal that this might indeed be the MCU (Sottocasa *et al*, 1972). Even more striking was the observation that an antibody raised against this glycoprotein was capable of

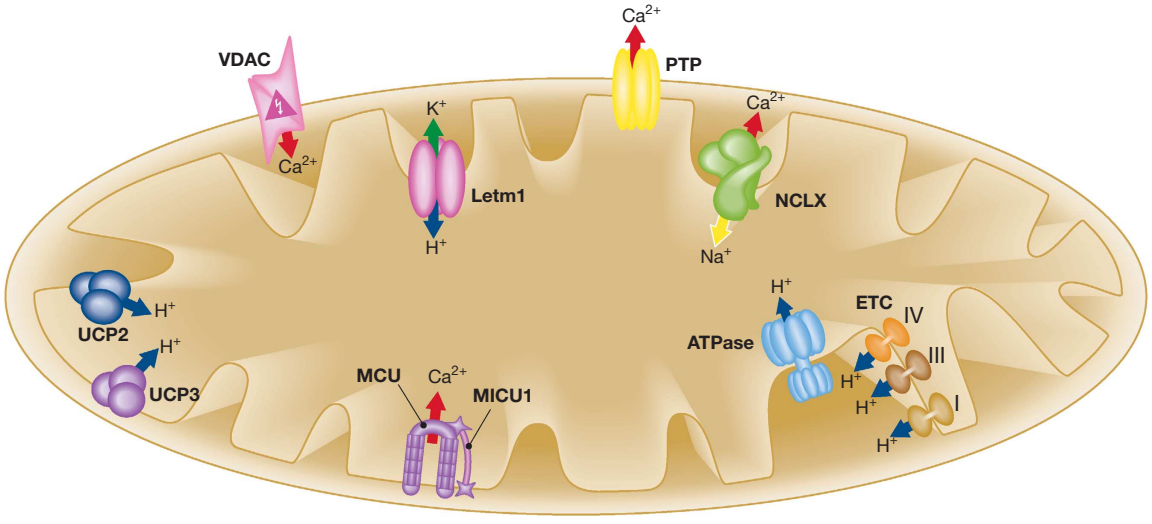


**Figure 1** Schematic representation of the mitochondrial $Ca^{2+}$, $Na^+$ and $H^+$ handling machinery. Ion fluxes are indicated by arrows. Red arrow, $Ca^{2+}$; blue arrow, $H^+$; green arrow, $K^+$; yellow arrow, $Na^+$. ETC, electron transport chain; Letm1, Leucine-zipper EF-hand containing transmembrane protein 1; NCLX, $Na^+/Ca^{2+}$ exchanger; PTP, permeability transition pore; UCP2/3, uncoupling protein 2/3; VDAC, voltage-dependent anion channel. See text for details.

inhibiting energy-dependent mitochondrial $Ca^{2+}$ uptake in mitoplasts (mitochondria without the outer membrane) and even in intact organelles (Panfili *et al*, 1976). Soon after, however, it became clear that the glycoprotein was far from pure and that the glycosylated peptide was a contaminant. The specificity of the antibodies was also dubious and the story quietly and rapidly faded away. A new attempt at the purification of the MCU was made in the mid-1990s by the group of Saris. A 40-kDa protein was purified that catalysed RR-sensitive electrogenic $Ca^{2+}$ uptake in lipid bilayers and reconstituted liposomes. Again, however, also this potential candidate soon disappeared from the major journals (Saris *et al*, 1993).

The purification of the $Na^+/Ca^{2+}$ or the $H^+/Ca^{2+}$ antiporters has been even more frustrating. In spite of a few remarkable reports identifying the stoichiometry of the $Na^+/Ca^{2+}$ exchanger (3 or 4 $Na^+$ ions per $Ca^{2+}$) (Brierley *et al*, 1994), their molecular identity remained, until very recently, completely mysterious. Two papers (in 1992 and 2004) reported the partial purification of a 110-kDa protein capable of reconstituting a $Na^+/Ca^{2+}$ exchange process in liposomes (Li *et al*, 1992; Paucek and Jaburek, 2004). Similarly, another protein of 66 kDa with $H^+/Ca^{2+}$ exchanger characteristics in reconstituted liposomes was partially purified by the group of Satrustegui (Villa *et al*, 1998). Surprisingly, none of these observations was successfully pursued, leading to the cloning of the relevant genes.

In very recent years, the search for the molecular identity of the MCU was intensified. A key innovative finding was published in 2004 by Kirichok *et al* (2004): by patch clamping, the authors provided the direct electrophysiological demonstration that the MCU is a gated, $Ca^{2+}$ selective, ion channel. It is noteworthy that the channel nature of the MCU had been originally proposed 25 years before by one of us (TP), on the basis of indirect data (Bragadin *et al*, 1979), though it was not followed by more direct evidence. In 2007, Graier and co-workers proposed that an essential component of the uniporter-mediated $Ca^{2+}$ uptake system, though not the MCU itself, is represented by the isoforms 2 and 3 of the uncoupling protein, UCP, but these data have not been confirmed by other investigators and still await clarification (Trenker *et al*, 2007). In 2009, Clapham and co-workers, using a siRNA genome-wide screen in Drosophila, concluded that the ubiquitously expressed mitochondrial protein Letm1 fulfils the criteria for being a 1 $H^+/Ca^{2+}$ antiport (Jiang *et al*, 2009). Because of this stoichiometry, however, Letm1 should allow $Ca^{2+}$ uptake in coupled mitochondria, unlike the classical view of $H^+$ (or $Na^+$)/$Ca^{2+}$ antiporters that, in coupled mitochondria, catalyse $Ca^{2+}$ efflux from the matrix. It is also noteworthy that the proposal by Clapham's group is somehow a rediscovery of the original idea of P Mitchell (Moyle and Mitchell, 1977), that is, that $Ca^{2+}$ uptake by mitochondria depends not on the MCU (with the net translocation of two positive charges per $Ca^{2+}$ taken up), but rather by an antiport with the net transfer of only one positive charge per $Ca^{2+}$. Clapham and co-workers did not go as far as claiming that all $Ca^{2+}$ uptake depends on Letm1, but suggested that Letm1 allows $Ca^{2+}$ uptake only at low cytosolic [$Ca^{2+}$], while for higher $Ca^{2+}$ levels they admitted that the still unknown MCU is the responsible channel. A similar conclusion was very recently reached also by Waldeck-Weiermair *et al* (2011). Jiang *et al* (2009) also showed that Letm1, when overexpressed, caused a substantial augmentation of agonist-dependent mitochondrial $Ca^{2+}$ accumulation, though this finding was not confirmed in endothelial cells (Waldeck-Weiermair *et al*, 2011). Most importantly, they reported that $Ca^{2+}$ accumulation, when the purified protein is incorporated in liposomes, is favoured by the generation of a $K^+$ diffusion potential and it is completely blocked by RR, as expected for the classical MCU. In our biased opinion, a number of experimental and conceptual considerations make it unlikely that Letm1 is a component of the $Ca^{2+}$ uptake system, in particular: (i) the $H^+/Ca^{2+}$ stoichiometry in respiring mitochondria has been firmly and unequivocally established many years ago ($2H^+$ per $Ca^{2+}$) and, when $Ca^{2+}$ uptake is driven by $K^+$ diffusion potential, no $H^+$ extrusion has ever been measured; (ii) importantly, Letm1 is structurally analogous to the yeast protein Mdm38p (Schlickum *et al*, 2004) and, when transfected, it can rescue the phenotype of Mdm38p knockout yeasts cells (Nowikovsky *et al*, 2004); (iii) it is notorious that yeast do not possess a RR-sensitive mitochondrial $Ca^{2+}$ uptake system (Carafoli *et al*, 1970); (iv) in mammalian cells, the phenotype of Letm1 knockout cells can be rescued by addition of the $H^+/K^+$ ionophore nigericin (Dimmer *et al*, 2008). This latter evidence represents a very strong—and, in our opinion, conclusive—argument in favour of Letm1 being itself a $K^+/H^+$ antiporter. The data of the mitochondrial $Ca^{2+}$ uptake inhibition by Letm1 knockout can be explained by an effect on the $\Delta\Psi$. Although the authors claimed that they found no appreciable difference in $\Delta\Psi$ between control and Letm1 KO cells, we suspect that the methodology used, rhodamine 123 uptake, is insufficiently sensitive to reveal such drop. On the contrary, the increase, upon Letm1 overexpression, of mitochondrial $Ca^{2+}$ uptake can be easily explained by a $H^+/K^+$ antiport catalysed by Letm1. Indeed, an artificial $H^+/K^+$ antiport, nigericin, causes a drop in $\Delta$pH and an increase in $\Delta\Psi$, thus augmenting the capacity of mitochondria to take up $Ca^{2+}$ via the classical MCU. The reported RR sensitivity of $Ca^{2+}$ uptake in liposomes reconstituted with Letm1 isolated from mitochondria could be, on the other hand, due to a contaminant protein in the preparation. Clearly, the problem of Letm1 and $Ca^{2+}$ uptake is at the moment unsolved and more experiments need to be performed in order to clarify the issue. The last data on MCU (see below), however, have somehow reduced the interest for this topic.

## The discovery of the mitochondrial $Na^+/Ca^{2+}$ antiporter and of an MCU component

In the last year, good news finally emerged regarding the molecular identities of the major players in mitochondrial $Ca^{2+}$ handling. In January 2010, Sekler and co-workers published a paper where they demonstrated that NCLX (until then considered an isoform of the PM $Na^+/Ca^{2+}$ exchanger family) fulfils the criteria to be the elusive mitochondrial $Ca^{2+}/Na^+$ antiport (Palty *et al*, 2010). NCLX had been previously identified by the same group and by others (Cai and Lytton, 2004; Palty *et al*, 2004) and had been considered a novel PM $Na^+/Ca^{2+}$ exchanger, the only mammalian member of a phylogenetically ancestral branch of the $Na^+/Ca^{2+}$ exchanger superfamily. Two spliced

 

variants of the NCLX (a long and a short isoform) are known to be ubiquitously expressed and the different roles of these isoforms are not presently known: indeed, the discovery was a serendipitous bonus, as the authors were looking for a specific $Zn^{2+}$ transporter (Sekler, personal communication). The interest was triggered by the observation that NCLX, when overexpressed, was partially mis-targeted to the PM and could mediate not only a $Na^+/Ca^{2+}$ exchange, but also an efficient $Li^+/Ca^{2+}$ exchange, a feature that is known to be a unique characteristic of the (at that time) unidentified mitochondrial $Na^+/Ca^{2+}$ exchanger. They could also show that (i) practically all endogenous NCLX is recovered in the mitochondrial fraction; (ii) NCLX is sensitive to the classical mitochondrial $Na^+/Ca^{2+}$ exchanger inhibitor CGP-37157; (iii) KO of NCLX drastically reduced $Na^+$-dependent $Ca^{2+}$ efflux in isolated mitochondria; (iv) a catalytically inactive mutant of NCLX transfected in cells blocks the $Na^+/Ca^{2+}$ exchange in isolated mitochondria, thus acting as dominant negative; and (v) last, but not least, the PM mis-targeted NCLX appears to mediate an electrogenic transport of 3–4 $Na^+$ ions per $Ca^{2+}$ transported. Admittedly, the data obtained by Sekler's group have yet to be reproduced by others and the other team that initially identified NCLX concluded that the protein is expressed in both the PM and ER/SR. We do not know the reasons for this important discrepancy that still awaits clarification. However, it is again our biased opinion that the data reported by Sekler's team (and some of their more recent unpublished results) are very convincing and strongly support the idea that NCLX is indeed the $Na^+/Ca^{2+}$ antiporter of the mitochondrial inner membrane. In addition, the mitochondrial NCLX is remarkably similar in size to the mitochondrial protein that, when purified and reconstituted, exhibited $Na^+/Ca^{2+}$ exchange activity, see above (Li *et al*, 1992; Paucek and Jaburek, 2004).

A few months after the publication of the NCLX paper, another new protein involved in mitochondrial $Ca^{2+}$ handling, this time the MCU, was identified by V Mootha's group. In this case, the protein was named MICU1, acronym for mitochondrial $Ca^{2+}$ uptake 1 (Perocchi *et al*, 2010). The identification of MICU1 came from the establishment of the so-called MitoCarta in which about 1000 proteins have been identified (many of them with unknown functions) that are specifically present in mitochondria (Zhang *et al*, 2010). Using bioinformatics followed by a selective siRNA screening, Perocchi *et al* (2010) identified a protein of unknown function, ubiquitously expressed in mammalian cells that possess two classical EF-hand $Ca^{2+}$-binding domains. When MICU1 is down-regulated, it results in a drastic reduction of the mitochondrial $Ca^{2+}$ uptake of intact cells challenged with IP3-generating agonists. Most relevant, MICU1 does not have an orthologue in yeast (see above). MICU1 is a 54-kDa protein, with only one putative transmembrane domain, which makes it unlikely that it can function as a $Ca^{2+}$ channel. MICU1 down-regulation did not affect $\Delta\Psi$, $O_2$ consumption or ATP synthesis by mitochondria, indicating that the inhibition of its activity does not grossly compromise the overall functionality of the organelle, but rather specifically affects the $Ca^{2+}$ uptake mechanism. Two observations, which suggest an ancillary role of MICU1 in the MCU functions, need to be stressed: (i) when overexpressed it does not increase the $Ca^{2+}$ uptake of mitochondria in intact cells (Rizzuto, personal communication) and (ii) when studied in MICU1 down-regulated permeabilized cells, apparently mitochondria could initially take up $Ca^{2+}$, but this capacity was soon lost upon further $Ca^{2+}$ additions to the medium (Perocchi *et al*, 2010). The authors did not exclude that this residual $Ca^{2+}$ uptake was independent of mitochondria, but taken together the above-mentioned characteristics suggest that MICU1 is not the channel-forming subunit of MCU itself, but rather an associated key subunit. The identification of MICU1 appears, however, a fundamental step in the molecular understanding of mitochondrial $Ca^{2+}$ uptake machinery, but the channel itself still managed to escape molecular identification. Fortunately, we did not have to wait long for the final discovery, carried out in parallel by the groups of Mootha and Rizzuto on opposite sides of the Atlantic Ocean.

## The $Ca^{2+}$ uniporter, at last!

Two papers came out, back to back, in the same issue of *Nature* a few weeks ago reporting the identification of another protein, this time called MCU, that possesses all the characteristics expected by the elusive $Ca^{2+}$ uniporter of the mitochondrial membrane (Baughman *et al*, 2011; De Stefani *et al*, 2011). The two papers addressed the problem with slightly different approaches, but their conclusions are remarkably similar and, accordingly, they will be discussed together. The differences and the few remaining discrepancies between the two groups will be also pointed out and discussed. The key characteristics of MCU, agreed upon by both groups, are as follows: (i) MCU is a 40-kDa protein (previously known as NP_001028431, coiled-coil domain-containing protein 109A) ubiquitously expressed in all mammalian tissues and in most eukaryotes, but missing a yeast orthologue; (ii) MCU possesses two transmembrane domains and this characteristic makes it reasonable that it forms (through oligomerization) a gated ion channel; (iii) down-regulation of MCU drastically reduces mitochondrial $Ca^{2+}$ uptake both in living cells treated with $Ca^{2+}$ mobilizing agonists, in permeabilized cells perfused with buffered $Ca^{2+}$ and in isolated mitochondria; (iv) transfection with the native channel rescues the phenotype of the specific siRNA-treated cells; and (v) the other classical properties of mitochondria are not affected by MCU down-regulation, that is, organelle shape and ER–mitochondrial interactions, $O_2$ consumption, ATP synthesis and $\Delta\Psi$. Whether or not complete knockdown of MCU had deleterious effects on cell or organ functions is still unknown. The group of Rizzuto also showed that overexpression of MCU drastically increases the mitochondrial $Ca^{2+}$ accumulation in intact cells while, contemporarily, reducing the amplitude of the cytosolic $Ca^{2+}$ peaks (due to mitochondrial $Ca^{2+}$ buffering); on the opposite site, down-regulation of MCU slightly increases the cytosolic $Ca^{2+}$ peaks. Most important of all, De Stefani *et al* showed that bacterial expressed MCU reconstituted in lipid bilayers results in the appearance of a $Ca^{2+}$ current; the single channel activity not only has electrophysiological characteristics similar to those reported by Clapham's group in patched clamped mitoplasts (Kirichok *et al*, 2004), but the channel activity is blocked by well-known inhibitors of the MCU, such as RR and $La^{3+}$. Finally, Mootha's group showed that infecting *in vivo* the liver with an adenoviral vector encoding a siRNA against MCU results in an almost complete block of $Ca^{2+}$ uptake by mitochondria isolated from the organ.

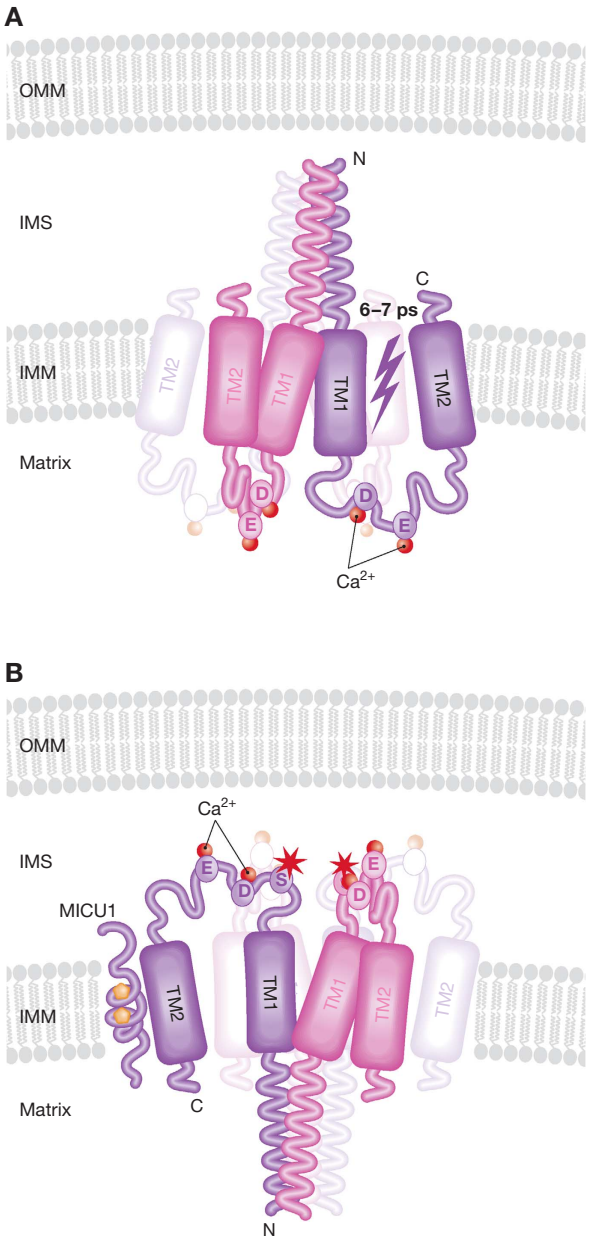


**Figure 2** Main features of the recently identified MCU. The figure schematizes the main properties of the MCU according to De Stefani *et al* (**A**) and Baughman *et al* (**B**). MCU has two transmembrane domains (TM1 and 2) that spans the inner mitochondrial membrane (IMM) with the N- and C-termini facing the intermembrane space (IMS), according to De Stefani *et al* (**A**) or the matrix (Baughman *et al* (**B**)). When reconstituted in lipid bylayers, MCU can mediate a $Ca^{2+}$ current of 6–7 ps (**A**). The amino acids that, when mutated, drasticaly reduce $Ca^{2+}$ uptake (D and E) or the one that confers RR sensitivity to the MCU (S, red star in panel **B**) are also shown. MICU1 that is reported to physically interact with MCU is also shown in (**B**). Its two EF-hand domains are indicated by the orange pentagons. MCU most likely oligomerizes in the IMM, and evidence from De Stefani *et al* (personal communication) suggests that it forms a tetramer (shadowed subunits in panels (**A**, **B**)).

Either group carried out site-directed mutagenesis of MCU, the group of Mootha demonstrating that mutation of S259 resulted in a functional $Ca^{2+}$ accumulation, while RR sensitivity was lost; De Stefani *et al* showed that a mutation in the region of the putative pore results not only in an inactive channel, but the mutant protein behaves as dominant negative when expressed in HeLa cells. This latter property suggests (together with the existence of only two putative transmembrane domains) that the active uniporter is made by oligomers of MCU. Modelling the MCU structure with classical algorithms suggests indeed that the channel is a tetramer (Rizzuto *et al*, unpublished data). Mootha's group also showed that MCU co-precipitated with MICU1 in a supramolecular complex. The only real discrepancy between the two groups concerns the topology of MCU: according to Baughman *et al* (2011) the C- and N-terminal domains face the mitochondrial matrix and the linker between the two transmembrane domains faces the intermembrane space, while the opposite is true in the model of Rizzuto, C- and N-terminal in the intermembrane space and the linker facing the matrix (Figure 2). This contrasting conclusions were reached by different approaches: the data of the former group result from a biochemical study of the endogenous MCU in isolated mitochondria and its sensitivity to proteases; the model of the second group was based on evidence obtained using a N-terminal GFP-tagged version of MCU. This latter, when transiently expressed in cells, is perfectly functional in terms of $Ca^{2+}$ uptake capacity. De Stefani *et al* showed that the GFP fluorescence of MCU, in PM permeabilized cells, is insensitive to proteinase K (unlike a GFP expressed on the cytosolic side of the outer mitochondrial membrane), but it is rapidly quenched by Trypan Blue that permeates the outer mitochondrial membrane (in fact Trypan Blue quenches the fluorescence of a GFP-cyt.C, Pozzan, unpublished data), but does not quench a GFP localized in the matrix. We are confident that this remaining discrepancy can be rapidly solved.

## Conclusions

After 50 years of intense and frustrating experimentation, two of the major players in the mitochondrial $Ca^{2+}$ saga—the MCU and the $Na^{+}/Ca^{2+}$ antiport—have now been molecularly identified. We can now expect a strong acceleration in the search for the functional role of this property of mitochondria, in both physiology and physiopathology. The key molecular targets have been revealed and new tools, such as siRNA and dominant-negative constructs to inhibit their functions in a highly specific ways, are available. It is easy to predict that we will soon have KO mice for both proteins. With the identification of the molecular nature of the proteins, we have also more promising ways for starting a journey in search of pharmacological inhibitors with important medical applications in a variety of cell processes. Mitochondrial $Ca^{2+}$ handling, from its humble state of 'interesting laboratory artefact' (as it was labelled in the 1980s), has evolved in the last decade—and even more now—into a process of major interest for a large group of investigators with a plethora of promising medical applications.

## Conflict of interest

The authors declare that they have no conflict of interest.

## References

Baughman JM, Perocchi F, Girgis HS, Plovanich M, Belcher-Timme CA, Sancak Y, Bao XR, Strittmatter L, Goldberger O, Bogorad RL, Koteliansky V, Mootha VK (2011) Integrative genomics identifies MCU as an essential component of the mitochondrial calcium uniporter. *Nature* **476:** 341–345

Bernardi P (1999) Mitochondrial transport of cations: channels, exchangers, and permeability transition. *Physiol Rev* **79:** 1127–1155

Bragadin M, Pozzan T, Azzone GF (1979) Activation energies and enthalpies during $Ca^{2+}$ transport in rat liver mitochondria. *FEBS Lett* **104:** 347–351

Brand MD, Chen CH, Lehninger AL (1976) Stoichiometry of $H^+$ ejection during respiration-dependent accumulation of $Ca^{2+}$ by rat liver mitochondria. *J Biol Chem* **251:** 968–974

Brierley GP, Baysal K, Jung DW (1994) Cation transport systems in mitochondria: Na+ and K+ uniports and exchangers. *J Bioenerg Biomembr* **26:** 519–526

Cai X, Lytton J (2004) Molecular cloning of a sixth member of the $K^+$-dependent $Na^+/Ca^{2+}$ exchanger gene family, NCKX6. *J Biol Chem* **279:** 5867–5876

Carafoli E (2010) The fateful encounter of mitochondria with calcium: how did it happen? *Biochim Biophys Acta* **1797:** 595–606

Carafoli E, Balcavage WX, Lehninger AL, Mattoon JR (1970) $Ca^{2+}$ metabolism in yeast cells and mitochondria. *Biochim Biophys Acta* **205:** 18–26

Chance B (1965) The energy-linked reaction of calcium with mitochondria. *J Biol Chem* **240:** 2729–2748

Contreras L, Drago I, Zampese E, Pozzan T (2010) Mitochondria: the calcium connection. *Biochim Biophys Acta* **1797:** 607–618

Csordas G, Varnai P, Golenar T, Roy S, Purkins G, Schneider TG, Balla T, Hajnoczky G (2010) Imaging interorganelle contacts and local calcium dynamics at the ER-mitochondrial interface. *Mol Cell* **39:** 121–132

Deluca HF, Engstrom GW (1961) Calcium uptake by rat kidney mitochondria. *Proc Natl Acad Sci USA* **47:** 1744–1750

Denton RM (2009) Regulation of mitochondrial dehydrogenases by calcium ions. *Biochim Biophys Acta* **1787:** 1309–1316

Denton RM, McCormack JG (1980) On the role of the calcium transport cycle in heart and other mammalian mitochondria. *FEBS Lett* **119:** 1–8

De Stefani D, Raffaello A, Teardo E, Szabo I, Rizzuto R (2011) A forty-kilodalton protein of the inner membrane is the mitochondrial calcium uniporter. *Nature* **476:** 336–340

Dimmer KS, Navoni F, Casarin A, Trevisson E, Endele S, Winterpacht A, Salviati L, Scorrano L (2008) LETM1, deleted in Wolf-Hirschhorn syndrome is required for normal mitochondrial morphology and cellular viability. *Hum Mol Genet* **17:** 201–214

Giacomello M, Drago I, Bortolozzi M, Scorzeto M, Gianelle A, Pizzo P, Pozzan T (2010) $Ca^{2+}$ 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

Giacomello M, Drago I, Pizzo P, Pozzan T (2007) Mitochondrial $Ca^{2+}$ as a key regulator of cell life and death. *Cell Death Differ* **14:** 1267–1274

Jiang D, Zhao L, Clapham DE (2009) Genome-wide RNAi screen identifies Letm1 as a mitochondrial Ca2+/H+ antiporter. *Science* **326:** 144–147

Kirichok Y, Krapivinsky G, Clapham DE (2004) The mitochondrial calcium uniporter is a highly selective ion channel. *Nature* **427:** 360–364

Li W, Shariat-Madar Z, Powers M, Sun X, Lane RD, Garlid KD (1992) Reconstitution, identification, purification, and immunological characterization of the 110-kDa $Na^+/Ca^{2+}$ antiporter from beef heart mitochondria. *J Biol Chem* **267:** 17983–17989

Mitchell P, Moyle J (1967) Chemiosmotic hypothesis of oxidative phosphorylation. *Nature* **213:** 137–139

Moyle J, Mitchell P (1977) Electric charge stoichiometry of calcium translocation in rat liver mitochondria. *FEBS Lett* **73:** 131–136

Nicholls DG (2005) Mitochondria and calcium signaling. *Cell Calcium* **38:** 311–317

Nowikovsky K, Froschauer EM, Zsurka G, Samaj J, Reipert S, Kolisek M, Wiesenberger G, Schweyen RJ (2004) 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

Palty R, Ohana E, Hershfinkel M, Volokita M, Elgazar V, Beharier O, Silverman WF, Argaman M, Sekler I (2004) Lithium-calcium exchange is mediated by a distinct potassium-independent sodium-calcium exchanger. *J Biol Chem* **279:** 25234–25240

Palty R, Silverman WF, Hershfinkel M, Caporale T, Sensi SL, Parnis J, Nolte C, Fishman D, Shoshan-Barmatz V, Herrmann S, Khananshvili D, Sekler I (2010) NCLX is an essential component of mitochondrial Na+/Ca2+ exchange. *Proc Natl Acad Sci USA* **107:** 436–441

Panfili E, Sandri G, Sottocasa GL, Lunazzi G, Liut G, Graziosi G (1976) Specific inhibition of mitochondrial $Ca^{2+}$ transport by antibodies directed to the $Ca^{2+}$-binding glycoprotein. *Nature* **264:** 185–186

Paucek P, Jaburek M (2004) Kinetics and ion specificity of Na(+)/Ca(2+) exchange mediated by the reconstituted beef heart mitochondrial Na(+)/Ca(2+) antiporter. *Biochim Biophys Acta* **1659:** 83–91

Perocchi F, Gohil VM, Girgis HS, Bao XR, McCombs JE, Palmer AE, Mootha VK (2010) MICU1 encodes a mitochondrial EF hand protein required for Ca(2+) uptake. *Nature* **467:** 291–296

Puskin JS, Gunter TE, Gunter KK, Russell PR (1976) Evidence for more than one Ca2+ transport mechanism in mitochondria. *Biochemistry* **15:** 3834–3842

Rizzuto R, Brini M, Murgia M, Pozzan T (1993) Microdomains with high $Ca^{2+}$ close to $IP_3$-sensitive channels that are sensed by neighboring mitochondria. *Science* **262:** 744–747

Rizzuto R, Pinton P, Carrington W, Fay FS, Fogarty KE, Lifshitz LM, Tuft RA, Pozzan T (1998) Close contacts with the endoplasmic reticulum as determinants of mitochondrial $Ca^{2+}$ responses. *Science* **280:** 1763–1766

Rizzuto R, Pozzan T (2006) Microdomains of intracellular $Ca^{2+}$: molecular determinants and functional consequences. *Physiol Rev* **86:** 369–408

Rizzuto R, Simpson AW, Brini M, Pozzan T (1992) Rapid changes of mitochondrial $Ca^{2+}$ revealed by specifically targeted recombinant aequorin. *Nature* **358:** 325–327

Saris NE, Sirota TV, Virtanen I, Niva K, Penttila T, Dolgachova LP, Mironova GD (1993) Inhibition of the mitochondrial calcium uniporter by antibodies against a 40-kDa glycoproteinT. *J Bioenerg Biomembr* **25:** 307–312

Scarpa A, Azzone GF (1970) The mechanism of ion translocation in mitochondria. 4. Coupling of $K^+$ efflux with $Ca^{2+}$ uptake. *Eur J Biochem* **12:** 328–335

Schlickum S, Moghekar A, Simpson JC, Steglich C, O'Brien RJ, Winterpacht A, Endele SU (2004) LETM1, a gene deleted in Wolf-Hirschhorn syndrome, encodes an evolutionarily conserved mitochondrial protein. *Genomics* **83:** 254–261

Somlyo AP, Bond M, Somlyo AV (1985) Calcium content of mitochondria and endoplasmic reticulum in liver frozen rapidly *in vivo*. *Nature* **314:** 622–625

Somlyo AP, Somlyo AV, Shuman H (1979) Electron probe analysis of vascular smooth muscle. Composition of mitochondria, nuclei, and cytoplasm. *J Cell Biol* **81:** 316–335

Sottocasa G, Sandri G, Panfili E, De Bernard B, Gazzotti P, Vasington FD, Carafoli E (1972) Isolation of a soluble $Ca^{2+}$ binding glycoprotein from ox liver mitochondria. *Biochem Biophys Res Commun* **47:** 808–813

Streb H, Irvine RF, Berridge MJ, Schulz I (1983) Release of $Ca^{2+}$ from a nonmitochondrial intracellular store in pancreatic acinar cells by inositol-1,4,5-trisphosphate. *Nature* **306:** 67–68

Trenker M, Malli R, Fertschai I, Levak-Frank S, Graier WF (2007) Uncoupling proteins 2 and 3 are fundamental for mitochondrial $Ca^{2+}$ uniport. *Nat Cell Biol* **9:** 445–452

Vasington F, Murphy JV (1962) $Ca^{2+}$ uptake by rat kidney mitochondria and its dependence on respiration and phosphorylation. *J Biol Chem* **237:** 2670–2677

Villa A, Garcia-Simon MI, Blanco P, Sese B, Bogonez E, Satrustegui J (1998) Affinity chromatography purification of mitochondrial inner membrane proteins with calcium transport activity. *Biochim Biophys Acta* **1373:** 347–359

Waldeck-Weiermair M, Jean-Quartier C, Rost R, Khan MJ, Vishnu N, Bondarenko AI, Imamura H, Malli R, Graier WF (2011) Leucine zipper EF hand-containing transmembrane protein 1 (Letm1) and uncoupling proteins 2 and 3 (UCP2/3) contribute to two distinct mitochondrial Ca2+ uptake pathways. *J Biol Chem* **286:** 28444–28455

Zamzami N, Kroemer G (2001) The mitochondrion in apoptosis: how Pandora's box opens. *Nat Rev Mol Cell Biol* **2:** 67–71

Zhang X, Cui J, Nilsson D, Gunasekera K, Chanfon A, Song X, Wang H, Xu Y, Ochsenreiter T (2010) The Trypanosoma brucei MitoCarta and its regulation and splicing pattern during development. *Nucleic Acids Res* **38:** 7378–7387