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SYMPOSIUM REVIEW

Pharmacological modulation of mitochondrial calcium homeostasis

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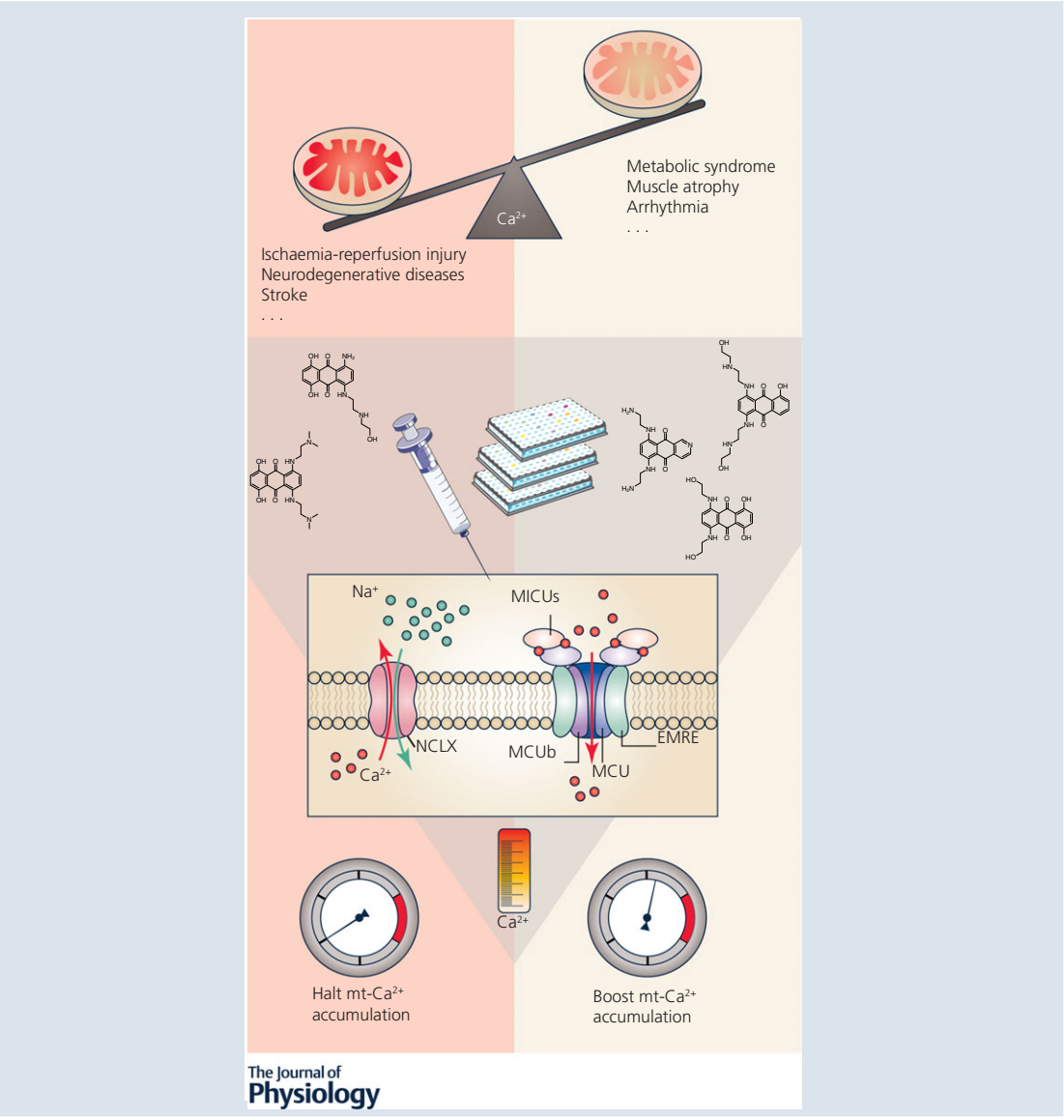

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Abstract Mitochondria are pivotal organelles in calcium (Ca^{2+}) handling and signalling, constituting intracellular checkpoints for numerous processes that are vital for cell life. Alterations in mitochondrial Ca^{2+} homeostasis have been linked to a variety of pathological conditions and are critical in the aetiology of several human diseases. Efforts have been taken to harness mitochondrial Ca^{2+} transport mechanisms for therapeutic intervention, but pharmacological compounds that direct and selectively modulate mitochondrial Ca^{2+} homeostasis are currently lacking. New avenues have, however, emerged with the breakthrough discoveries on the genetic identification of the main players involved in mitochondrial Ca^{2+} influx and efflux pathways and with recent hints towards a deep understanding of the function of these molecular systems. Here, we review the current advances in the understanding of the mechanisms and regulation of mitochondrial Ca^{2+} homeostasis and its contribution to physiology and human disease. We also introduce and comment on the recent progress towards a systems-level pharmacological targeting of mitochondrial Ca^{2+} homeostasis.

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Abstract figure legend A chemical biology approach for the identification of specific modulators of mitochondrial calcium homeostasis. Unbalanced mitochondrial Ca^{2+} levels are a common pathological hallmark of several human diseases. Examples of diseases associated with mitochondrial Ca^{2+} overload (mitochondria with red matrix) are ischaemia–reperfusion injury, neurodegenerative diseases and stroke. Diseases associated with low mitochondrial Ca^{2+} levels (mitochondria with pastel pink matrix) are metabolic syndrome, muscle atrophy and arrhythmia. Therefore, there is an urge to screen for novel pharmacological modulators of mitochondrial Ca^{2+} homeostasis that target either the mitochondrial Ca^{2+} uniporter complex or mitochondrial Ca^{2+} efflux pathways (NCLX). Meters illustrate the main therapeutic interventions (halt or boost mitochondrial Ca^{2+} accumulation) that could be followed to counteract mitochondrial Ca^{2+} deregulation according to the disease phenotype.

Daniela Arduino obtained her PhD in 'Biomedical Sciences' from CNC-UC, Portugal. Her studies in the laboratories of Prof. Cardoso and Prof. Cuervo uncovered a main role for mitochondrial metabolism in the regulation of autophagy–lysosomal pathways in Parkinson's disease. She is currently a postdoctoral fellow in Fabiana Perocchi's laboratory, where she has been developing novel high-throughput chemical screening approaches to identify specific modulators of mitochondrial Ca^{2+} homeostasis as well as characterizing mitochondrial signalling networks and elucidating their potential roles in cell physiology and disease. **Fabiana Perocchi** is group leader at LMU and HMGU in Munich. Throughout her graduate studies at EMBL and postdoctoral training at Harvard, she became an expert in the area of functional genomics applied to the study of mitochondrial biogenesis and calcium signalling. Her research interests have been on the development of integrative strategies that combine systematic approaches with genetic, biochemical and physiological studies of mitochondrial function.

Introduction

In virtually all eukaryotic cells, mitochondria are primed organelles for the regulation of intracellular Ca^{2+} signalling. They can take up and release Ca^{2+} ions through the concerted action of influx and efflux pathways to shape the spatio-temporal dynamics of cytosolic Ca^{2+} transients (Nicholls, 1978; Nicholls & Scott, 1980; Zoccarato & Nicholls, 1982). In addition, mitochondrial Ca^{2+} entry is coupled to an increase in energy production (Denton & McCormack, 1980) to meet the rising energy demand of signalling cells (Szabadkai & Duchen, 2008). Mitochondrial Ca^{2+} homeostasis has been extensively studied for decades and functionally linked to the regulation of diverse physiological processes such as energy metabolism, tissue growth and development, neurotransmitter release, muscle contraction, autophagy, and cell death. More than half a century ago, it was shown that Ca^{2+} uptake by isolated mammalian mitochondria was a rapid, electrogenic and relatively selective process that was driven by the negative potential (~ -180 mV) across the inner mitochondrial membrane (IMM) and inhibited by lanthanide cations and ruthenium red (RuR) (Deluca & Engstrom, 1961; Vasington & Murphy, 1962; Chance, 1965; Brand *et al.* 1976). At the same time, it was demonstrated that the presence of Na^+ in the medium and changes in pH were able to stimulate the efflux of Ca^{2+} accumulated in isolated mitochondria (Carafoli *et al.* 1974). It was thus concluded that both mitochondrial Ca^{2+} influx and mitochondrial Ca^{2+} efflux occurred through specific carriers located in the IMM, a putative and ubiquitous mitochondrial Ca^{2+} uniporter and either a Na^+/Ca^{2+} or a H^+/Ca^{2+} exchanger, in excitable and non-excitable tissues, respectively. However, although

much progress has been made to elucidate the biophysical properties of mitochondrial Ca^{2+} entry and release, the molecular identities of these transporters remained elusive until 2010. Then, the mitochondrial Ca^{2+} uniporter 1 (MICU1; Perocchi *et al.* 2010) was discovered as the founding member of the mitochondrial Ca^{2+} uniporter complex and the Li-permeable Na^+/Ca^{2+} exchanger (NCLX; Palty *et al.* 2010) as a new member of the Na^+/Ca^{2+} exchanger superfamily, opening a new era in the mitochondria and Ca^{2+} signalling fields. These findings allowed targeted genetic loss- and gain-of-function analyses *in vitro* and *in vivo* towards a comprehensive assessment of the physiological and pathophysiological role of mitochondrial Ca^{2+} homeostasis in cells, tissues and whole organisms (De Stefani *et al.* 2016). Moreover, the identification of MICU1 loss-of-function mutations in human patients with neuromuscular disorders (Logan *et al.* 2014; Lewis-Smith *et al.* 2016) has confirmed that modulating mitochondrial Ca^{2+} homeostasis might constitute a promising therapeutic approach.

This review attempts to provide an overview of the latest knowledge on mitochondrial Ca^{2+} homeostasis and its modulation for therapeutic intervention in human diseases. First, we summarize the findings that led to the identification and characterization of the molecular components involved in mitochondrial Ca^{2+} uptake and efflux. We then discuss the relevance of mitochondrial Ca^{2+} homeostasis in physiology and disease and the so far available pharmacological approaches to modulate mitochondrial Ca^{2+} uptake and release pathways. Finally, we address recent progresses on pharmacological targeting of mitochondrial Ca^{2+} homeostasis and present a newly established high-throughput chemical screening approach for the systematic identification of modulators of the mitochondrial Ca^{2+} uniporter (Arduino *et al.* 2017).

The mitochondrial Ca^{2+} uniporter

Many attempts have been made to identify the components of the mitochondrial Ca^{2+} uniporter, for example by purifying Ca^{2+}-binding proteins from isolated mitochondria and then reconstituting Ca^{2+} uptake *in vitro*. The group of Lehninger reported on the purification of a soluble heat-labile protein of over 150 kDa from rat liver mitochondria subjected to osmotic shock (Lehninger, 1971). This factor was able of binding Ca^{2+} with high affinity and it was sensitive to lanthanides. Prompted by the serendipitous finding that mitochondria were stained by the polysaccharide stain RuR and that this completely inhibited mitochondrial Ca^{2+} uptake, Sottocasa and colleagues focused on the extraction of glycoproteins as possible active centres of Ca^{2+} binding and transport (Sottocasa *et al.* 1972). From ox and rat liver mitochondria they isolated a major band with a molecular mass of approximately 30 kDa by PAGE, which was stained by murexidine, a Ca^{2+} dye, and whose high-affinity binding (K_d of approximately 100 nM) was affected by both lanthanides and RuR. This proteinaceous factor, named 'calvectin', was found primarily in the intermembrane space in a free soluble form, but could become membrane bound in a Ca^{2+}-dependent manner. Calvectin was able to increase Ca^{2+} conductance in artificial lipid bilayers and its antisera specifically inhibited Ca^{2+} transport by rat liver mitoplasts (mitochondria stripped of their outer membrane) without affecting respiration. This protein was therefore suggested to represent a major component of the mitochondrial Ca^{2+} uniporter. Other laboratories also suggested that the components responsible for Ca^{2+} transport in mitochondria were not of protein but rather of lipid nature. A lipid moisture isolated from the Ca^{2+}-transported glycoprotein was shown to form channels in lipid bilayers with an electroconductivity between 100 and 200 times higher in the presence of Ca^{2+} and the ability to transport Ca^{2+} was lost in delipidated mitochondria (Medvedev *et al.* 1982). Similarly, Sokolove and Brenza (1983) demonstrated that cardiolipin, an abundant lipid of mitochondrial membranes, binds Ca^{2+} with an apparent affinity of 700 nM in the absence of other competitive divalent cations such as Mn^{2+}, Zn^{2+} and Mg^{2+}. Calcium binding to cardiolipin was also inhibited by lanthanides and RuR. After these pioneering reports, various Ca^{2+} binding glycoproteins and peptides were isolated, all of which were able to bind Ca^{2+} and were inhibited by lanthanides and RuR (Mironova & Utesheva Zh, 1989; Zazueta *et al.* 1998). Several of these factors have been dismissed as components of the Ca^{2+} uniporter, as they were then found to be either cytosolic or microsomal contaminants and the majority of those observations faded away with lack of follow-up studies.

Much later, electrophysiological measurements of Ca^{2+} currents in single mitoplasts demonstrated that the mitochondrial Ca^{2+} uniporter is an inward rectifying channel, highly selective for Ca^{2+}, and with a remarkable high capacity for Ca^{2+} transport (half-saturation at ~20 mM) (Kirichok *et al.* 2004), and several proteins, such as the mitochondrial type 1 ryanodine receptor (mRyR1) (Beutner *et al.* 2005) and the isoforms 2 and 3 of the ion-transporting uncoupling protein UCP (UCP2 and UCP3) (Trenker *et al.* 2007), were initially proposed to form this channel. However, the direct involvement of these proteins in mediating RuR-sensitive Ca^{2+} uptake has been questioned. Thus, even after so much effort spanning several decades of research and when the majority of the new genomics and proteomics approaches were already available, the molecular nature of the uniporter had remained a mystery to molecular biologists, biochemists and physiologists alike. In 2010, Perocchi *et al.* identified MICU1 as the first genuine member of the uniporter channel based on the conceptual integration of physiological and biophysical clues from

50 years of literature on mitochondrial Ca^{2+} homeostasis in different organisms with comparative genomics and mitochondrial proteomics analyses (Perocchi *et al.* 2010). Analyses performed during the 1960s and '70s had shown that uniporter-dependent Ca^{2+} uptake is not a prerogative of all eukaryotic organisms, as it can be measured in mitochondria of mammals and kinetoplastids but not in fungi. Therefore, human genes encoding core components of the uniporter should be conserved in the mitochondrial proteome of organisms that are competent for Ca^{2+} uptake but not in other species devoid of such ability. Of the thousand plus human proteins localized to mitochondria, the authors narrowed down the search to just 18 that fulfilled the evolutionary signature of the uniporter, were associated with the IMM and were widely expressed in mitochondria from several mouse tissues. RNA interference (RNAi) against these 18 genes highlighted one, *CBARA1*, which was thereafter renamed *MICU1* (Perocchi *et al.* 2010). Silencing *MICU1* did not disrupt mitochondrial respiration or membrane potential but impaired mitochondrial Ca^{2+} entry in intact and permeabilized cells, as well as in mitochondria isolated from mouse liver. It also attenuated the metabolic coupling between cytosolic Ca^{2+} transients and stimulation of energy metabolism. MICU1 had two predicted EF hands that were found to be essential for regulating the activity of the uniporter, suggesting a role in Ca^{2+} sensing. Building on this discovery, the same authors and the group of Rizzuto (Baughman *et al.* 2011; De Stefani *et al.* 2011) identified a 40 kDa coiled-coiled protein, known as CCDC109A, that coevolved with MICU1 and had two transmembrane domains, which they proved to be the *bona fide*, pore-forming subunit of the mitochondrial calcium uniporter and threrefore renamed it mitochondrial Ca^{2+} uniporter (MCU). First, RNAi-mediated silencing of *MCU* was found to strongly inhibit mitochondrial Ca^{2+} uptake in cultured cells and in purified mouse liver mitochondria, whereas *MCU* overexpression enhanced RuR-sensitive mitochondrial Ca^{2+} uptake in intact and permeabilized cells. Single-point mutations of conserved acidic residues (E257A, D261A and E263A) within the short loop linking the two transmembrane domains of MCU on the intermembrane space side, termed the DIME motif, abrogated the ability of mitochondria to take up Ca^{2+}, indicating these residues were required for Ca^{2+} transport (Baughman *et al.* 2011; De Stefani *et al.* 2011; Chaudhuri *et al.* 2013). Expression of S259A (Baughman *et al.* 2011; Chaudhuri *et al.* 2013) and D261A MCU (Arduino *et al.* 2017) mutants conferred reduced RuR sensitivity, highlighting a possible role in RuR-dependent MCU inhibition. Second, expression of pure MCU in planar lipid bilayers was shown to be sufficient to reconstitute ion channel activity, generating a single channel Ca^{2+} current consistent with that of the uniporter (De Stefani *et al.* 2011). Finally, heterologous expression of the MCU orthologous protein from *Dictyostelium dyscodium* was sufficient to reconstitute mitochondrial Ca^{2+} uptake capacity in the yeast *Saccharomyces cerevisiae*, which lacks all uniporter components (Kovacs-Bogdan *et al.* 2014). Altogether, these observations confirmed that MCU is the Ca^{2+}-conducting pore of the long-sought mitochondrial uniporter. Moreover, recent studies on the structural biology (Oxenoid *et al.* 2016), coordination chemistry and molecular dynamics (Cao *et al.* 2017) of MCU from *Caenorhabditis elegans* suggested that a functional channel results from the pentameric assembly of MCU multimers stabilized by a coiled-coil motif protruding into the mitochondrial matrix. In this structural model, the DIME motif forms the selectivity filter of the channel, whereby ion permeation and inhibitor binding occur. These insights have been recently exploited for *in silico* modelling of MCU and binding of mitoxantrone, a novel inhibitor of the uniporter channel (Arduino *et al.* 2017).

MCU was initially found to reside in the IMM as a large complex of roughly 450 kDa (Baughman *et al.* 2011). Over the past few years, the macromolecular nature of this channel was confirmed and several additional subunits have been characterized. MCU can form hetero-oligomers with its paralogue, MCUb, a 33 kDa protein with very similar amino acid sequence and topological features (Raffaello *et al.* 2013). Co-expression studies of MCU and MCUb in planar lipid bilayers and intact cells demonstrated that the presence of MCUb decreases MCU-dependent Ca^{2+} uptake activity, suggesting that MCUb is a dominant-negative pore-forming component of the uniporter. The regulation of MCU activity by extramitochondrial Ca^{2+} is dictated by a duet of EF-hand containing proteins, MICU1 and its paralogue EFHA1 (termed MICU2), interacting with MCU from the mitochondrial intermembrane space (Plovanich *et al.* 2013; Patron *et al.* 2014; Liu *et al.* 2016; Kamer *et al.* 2017). A related protein termed MICU3 was also proposed to be a member of the uniporter based on its sequence similarity to MICU2, but neither its mitochondrial localization nor its involvement in Ca^{2+} uptake have been confirmed so far. Following a similar strategy to Perocchi *et al.* (2010), Mallilarankaraman and colleagues identified the mitochondrial Ca^{2+} uniporter regulator 1 (MCUR1), formerly known as CCDC90A, as an essential membrane component of the uniporter (Mallilankaraman *et al.* 2012; Vais *et al.* 2015). Interestingly, MCUR1 was shown to have an opposite membrane topology to MCU, whereby both N- and C-termini project into the intermembrane space, while the majority of the protein, including its coil-coil domain, faces the mitochondrial matrix, suggesting that unlike MCU and MCUb, MCUR1 does not act as a pore-forming subunit. In this regard, MCUR1 was recently suggested to function as a scaffold factor for the assembly of the uniporter channel (Tomar *et al.* 2016),

although other independent roles have also been ascribed to it, such as the assembly of complex IV (Paupe *et al.* 2015) and the regulation of Ca^{2+}-induced mitochondrial permeability transition (Chaudhuri *et al.* 2016). Finally, a 10 kDa integral IMM protein termed essential MCU regulator (EMRE) was found to be indispensable for uniporter-mediated Ca^{2+} uptake and together with MCU was sufficient to reconstitute Ca^{2+} uptake in yeast mitochondria (Sancak *et al.* 2013; Kovacs-Bogdan *et al.* 2014). EMRE is thought to be a multifunctional element of the uniporter that is required for inwardly rectifying Ca^{2+} currents, bridging of MCU and its regulators MICU1 and MICU2 (Sancak *et al.* 2013; Kovacs-Bogdan *et al.* 2014; Petrungaro *et al.* 2015; Tsai *et al.* 2016) and sensing of mitochondrial matrix Ca^{2+} (Vais *et al.* 2016). In contrast to its essential role in higher eukaryotes, EMRE expression is absent in some organisms where MCU is conserved and functional, such as *D. discoideum* (Kovacs-Bogdan *et al.* 2014). To date, the precise role of this protein in both function and regulation of the uniporter remains to be clearly elucidated. In addition to this plethora of structural and regulatory subunits, both uniporter activity and composition can also vary across tissues through the expression of tissue-specific components. For example, MICU2 and MICU3 were found to be mostly expressed in visceral organs and central nervous system, respectively (Plovanich *et al.* 2013; Murgia & Rizzuto, 2015), whereas a recently found MICU1 alternative splice variant, MICU1.1, is skeletal muscle-specific (Vecellio Reane *et al.* 2016). The discovery of the major molecular players of mitochondrial Ca^{2+} homeostasis constitutes a great accomplishment for the mitochondrial biology and Ca^{2+} fields, and additional elements might be on the research horizon.

Mitochondrial Ca^{2+} efflux

Outward Ca^{2+} flux from mitochondria is mainly mediated by two transport systems, mitochondrial Na^+/Ca^{2+} (mNCLX) and H^+/Ca^{2+} (mHCX) exchangers. By a genome-wide siRNA screen in *Drosophila* cells, the leucine zipper and EF-hand containing transmembrane protein 1 (LETM1) was found to regulate RuR-sensitive mitochondrial Ca^{2+} uptake at cytosolic concentrations below, but not above, 1 uM and to couple the movement of Ca^{2+} in exchange for H^+ both in intact cells and in proteoliposomes (Jiang *et al.* 2009). Thus, LETM1 was proposed to function as the mitochondrial electroneutral H^+/Ca^{2+} antiporter. However, previous studies had characterized the same protein as a member of the mitochondrial K^+/H^+ exchanger and shown that it was essential to control mitochondrial volume (Nowikovsky *et al.* 2004; McQuibban *et al.* 2010). The hypothesis that LETM1 could mediate both extrusion and uptake of Ca^{2+} independently of K^+ was also put forward (Jiang *et al.* 2013; Tsai *et al.* 2014) and later challenged based on evidence for electroneutral exchange of H^+ with K^+ *in vivo*, thus disclaiming the contribution of LETM1 to mitochondrial Ca^{2+} efflux (De Marchi *et al.* 2014; Nowikovsky & Bernardi, 2014). Despite the renowned relevance of mitochondrial outward shuttling mechanisms for cell physiology, the molecular identity of mHCX still remains unclear.

In a seminal study by Palty *et al.* (2010), it was found that the gene *FLJ22233* (also known as *SLC8B1*) encodes an IMM-located protein that mediates both Li^+- and Na^+-dependent Ca^{2+} clearance from the mitochondrial matrix. The authors named this protein mNCLX and demonstrated that it constitutes the mitochondrial Ca^{2+} antiporter. Similarly to MCU-dependent mitochondrial Ca^{2+} uptake, the action of mNCLX is electrogenic, with 3 Na^+/1 Ca^{2+} ions per transport cycle. Consequently, mNCLX constitutes the main pathway for Na^+ influx into mitochondria, and its activity is finely tuned to sense changes in intracellular Na^+ concentrations within the physiological range. The discovery of the molecular identity of mNCLX (Palty *et al.* 2010) was instrumental for understanding the molecular mechanisms of mitochondrial Ca^{2+} homeostasis. Accordingly, a functional crosstalk between mNCLX and other Na^+ and Ca^{2+} transporters located on both mitochondrial and plasma membranes has been described (Nita *et al.* 2015; Ben-Kasus Nissim *et al.* 2017), underscoring the significant physiological role of mNCLX for the regulation of both Ca^{2+} and Na^+ signalling pathways.

Physiopathological role of mitochondrial Ca^{2+} homeostasis

Genetic and biochemical strategies modulating mitochondrial Ca^{2+} homeostasis have already demonstrated that privileged functional relationships exist between mitochondrial Ca^{2+} homeostasis, cell bioenergetics and cell fate-determination pathways. Calcium accumulation in functional mitochondria, mediated by the MCU complex (MCUC) and modulated by mNCLX, regulates mitochondrial ATP generation as well as cytosolic NAD^+/NADH metabolism, thus sustaining the energy requirements of the cell. Ca^{2+} loading in the mitochondrial matrix stimulates mitochondrial generation of NADH, which can be shuttled to the cytosol where it initiates retrograde signalling, resulting in inhibition of sirtuins activity and alterations in protein acetylation (Marcu *et al.* 2014). Moreover, Ca^{2+} constitutively released from the ER to mitochondria is essential for efficient oxygen consumption, maintenance of cell bioenergetics and inhibition of autophagy (Cardenas *et al.* 2010). Conversely, mitochondrial Ca^{2+} overload, or perturbations in mitochondrial Ca^{2+} homeostasis, can sensitize cells to distinct modes of

cell death induced by different stimuli, and in a cell type-specific way (Curry *et al.* 2013; Qiu *et al.* 2013; Liao *et al.* 2015; Cardenas *et al.* 2016).

A large body of evidence from experimental models and human subjects corroborates the notion that mitochondrial Ca^{2+} deregulation is a relevant feature of several human disorders. As a remarkable example, its role in cardiovascular pathologies has been supported by several reports demonstrating that in heart failure, elevated cytosolic levels of Na^{+}, which stimulates mitochondrial Ca^{2+} efflux via the mNCLX, reduce mitochondrial bioenergetic responses and promote mitochondrial oxidative stress in cardiomyocytes (Maack *et al.* 2006). In contrast, mitochondrial Ca^{2+} overload caused by sarcoplasmic reticulum (SR) Ca^{2+} leakage elicits mitochondrial dysfunction and contributes to impaired cardiac function in postischaemic heart failure (Santulli *et al.* 2015). Also in this pathological context, loss-of-function mutations and genetic ablation of the major players in mitochondrial Ca^{2+} influx and efflux pathways in *in vivo* models have demonstrated that mitochondrial Ca^{2+} homeostasis is a determining factor in cardiac physiology. Cardiac-specific deletion of mNCLX was recently found to elicit severe heart failure, predisposing mice to sudden death (Luongo *et al.* 2017). Moreover, adult inducible cardiac-specific deletion of MCU and transgenic expression of a dominant-negative MCU (DN-MCU) isoform impaired the ability of the heart to adapt to certain stresses that require increases in mitochondrial metabolism, for example the heart's 'fight-or-flight' stress response associated to an increased cardiac contractile performance (Kwong *et al.* 2015; Luongo *et al.* 2015; Wu *et al.* 2015). In addition, inducible MCU ablation conferred significant protection against ischaemia–reperfusion injury in adult heart (Kwong *et al.* 2015), as expected from the anticipated role of mitochondrial Ca^{2+} overload on induction of cell death. In contrast to these findings, mice constitutively lacking MCU or expressing the DN-MCU did not exhibit marked cardiac deficits, but a pathological response to ischaemia–reperfusion injury and only a slightly impaired skeletal muscle metabolism and peak performance (Pan *et al.* 2013; Holmstrom *et al.* 2015; Rasmussen *et al.* 2015). The discrepancies observed between the phenotypes of different MCU knockout mouse models as well as between *in vivo* and *in vitro* cellular models may result from both timing and duration of the genetic perturbation (e.g. acute *versus* chronic MCU deletion). Moreover, with regard to the mouse models, while MCU deletion at the whole-organism level is embryonically lethal in a pure C57BL/6 mouse strain, an MCU knockout mouse model could, however, be established in a mixed C57BL/6xCD1 line (Pan *et al.* 2013). This paradox can be explained by an increase in the genetic variability of the outbred C57BL/6xCD1 strain that may result in compensatory mechanisms for the lack of MCU, and thus in milder phenotypes. However, the nature of those compensatory mechanisms is still unknown. Moreover, whole-body deletion of *MICU1* in mice was perinatally lethal, though no major anatomical deficiencies were observed. Instead, conditional *MICU1* knock down in liver resulted in extensive inflammation, tissue damage and failure of the liver's regeneration ability (Antony *et al.* 2016). In an independent mouse model established in a different strain with a slightly different background, homozygous *MICU1* deletion caused a significant but partial postnatal mortality (Liu *et al.* 2016). Similar to human patients carrying loss-of-function *MICU1* mutations, viable *MICU1* knockout mice revealed a strong phenotype, including atypical mitochondrial morphology, reduced muscle ATP levels and features of neuromuscular disorders such as ataxia (Logan *et al.* 2014). Reminiscent of these features, silencing of *MCU* and *MICU1* in *Drosophila melanogaster* during development in a brain region essential for memory resulted in impaired memory formation in adulthood without affecting the learning ability (Drago & Davis, 2016). In contrast to the lethality phenotype observed for the *MICU1*-null mice, constitutive loss of *MICU2* in pure C57BL/6 background mice was recently found to be compatible with life and development, although leading to alterations in cardiovascular homeostasis associated with diastolic heart failure (Bick *et al.* 2017). All the above-mentioned findings together with further evidence from other studies (Table 1) suggest that mitochondrial Ca^{2+}-handling proteins are relevant for drug targeting.

Pharmacological modulation of mitochondrial Ca^{2+} homeostasis

For more than 30 years, the only approaches and tools available to address the physiopathological importance of mitochondrial Ca^{2+} homeostasis relied on the use of pharmacological compounds that impinge on mitochondrial Ca^{2+} uptake and release processes.

Since the late 1960s, pioneer reports have demonstrated that lanthanides (particularly La^{3+}) and the transition metal derivative RuR are powerful inhibitors of Ca^{2+}-associated responses in mitochondria (Mela, 1968, 1969). Studies by Vasington *et al.* (1972) and Reed & Bygrave (1974) established RuR and lanthanides as tightly binding inhibitors of Ca^{2+} transport in mitochondria, competing for different binding sites, which differ from the Ca^{2+}-transport site. Years later, an oxygen-bridged dimeric Ru amine complex, purified from RuR, which absorbs light at 360 nm (named Ru360), was reported to exert a more specific inhibition of mitochondrial Ca^{2+} uptake *in vitro* (Ying *et al.* 1991; Matlib *et al.* 1998; Zazueta *et al.* 1998) and *in situ* in intact cardiomyocytes (Matlib *et al.* 1998). Other

Table 1. Phenotypic analysis of the mitochondrial Ca^{2+} uniporter complex components after genetic manipulation in the context of a whole organism or a specific tissue

Gene	Specie	Organ/tissue	Genetic perturbation	Phenotype	Refs/source
Mitochondrial Ca^{2+} influx					
MCU	*M. musculus*	Whole organism (C57BL/6)	KO	Embryonic mortality	IMPC
		Whole organism (C57BL/6xCD1)	KO	Small size Intolerance to strenuous work	Pan *et al*. (2013)
		Whole organism	KO, single allele	Decreased cardiac stroke volume Increased fasted circulating glucose levels	IMPC
		Skeletal muscle	OE	Skeletal muscle hypertrophy	Mammucari *et al*. (2015)
			KD	Skeletal muscle atrophy	
		Heart	DN – MCU expression	Impaired cathecolamine-induced heart rate increase	Wu *et al*. (2015)
			KO	Absent responsiveness to acute stress and "fight-or-flight" response Protection from ischaemia/reperfusion injury	Luongo *et al*. (2015) Kwong *et al*. (2015)
	D. melanogaster	Mushroom body neurons	KD (during pupation)	Impaired adult memory formation	Drago & Davis (2016)
	D. rerio	Whole organism	KD	Alterations during gastrulation	Prudent *et al*. (2013)
	C. elegans	Whole organism	KO	Impaired wound healing	Xu & Chisholm (2014)
	T. brucei	Whole organism	KD/KO OE	Defective growth Decreased infectivity to mice	Huang *et al*. (2013)
MCUb	*M. musculus*	Whole organism	KO	Impaired righting response Absent vibrissae Increased mean corpuscular volume	IMPC

(*Continued*)

Table 1. Continued

Gene	Specie	Organ/tissue	Genetic perturbation	Phenotype	Refs/source
MCUR1	*M. musculus*	Heart	KO	Partial perinatal mortality	Tomar *et al*. (2016)
		Vasculature	KO	Small size Postnatal mortality	
MICU1	*H. sapiens*	Whole organism	Loss-of-function mutations	Features of neuromuscular disease Cognitive impairment	Logan *et al*. (2014)
		Whole organism	Deletion (exon 1)	Early-onset proximal muscle weakness Exercise-induced lethargy Confusion	Lewis-Smith *et al*. (2016)
	M. musculus	Whole organism (C57BL/6)	KO	Perinatal mortality	Antony *et al*. (2016)
		Whole organism (C57BL/6N or C57BL/6NxJF1)	KO	Partial perinatal mortality Severe neurological and myophatic complications (in surviving mice)	Liu *et al*. (2016)
		Liver	KD	Extensive liver inflammation and damage Impaired liver regeneration	Antony *et al*. (2016)
MICU2	*M. musculus*	Whole organism	KO	Abnormal active cardiac relaxation Lethal abdominal aortic aneurisms (in hypertensive mice)	Bick *et al*. (2017)
EMRE	*M. musculus*	Whole organism	KO	Embryonic mortality	IMPC
	M. musculus (MICU1 $^{-/-}$*)*	Whole organism	KO, single allele	Survival (rescue of high perinatal mortality)	Liu *et al*. (2016)
Mitochondrial Ca^{2+} efflux					
NCLX	*M. musculus*	Heart	KO	Significant postnatal mortality Cardiac hypertrophy (in surviving mice)	Luongo *et al*. (2017)

C. elegans, *Caenorhabditis elegans*; *D. melanogaster*, *Drosophila melanogaster*; DN-MCU, dominant negative MCU; *D. rerio*, *Danio rerio*; EMRE, essential MCU regulator; *H. sapiens*, *Homo sapiens*; IMPC, International Mouse Phenotyping Consortium; KD, knockdown; KO, knockout; MCU, mitochondrial calcium uniporter; MCUb, mitochondrial calcium uniporter b; MCUR1, mitochondrial calcium uniporter regulator 1; MICU1, mitochondrial calcium uptake 1; MICU2, mitochondrial calcium uptake 2; *M. musculus*, *Mus musculus;* NCLX, Li-permeable Na^+/Ca^{2+} exchanger; OE, overexpression; *T. brucei*, *Trypanosoma brucei*.

compounds that directly target additional mitochondrial functions, by dissipating the $\Delta\Psi_m$ (e.g. uncouplers such as carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP)/carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) or dinitrophenol), inhibiting the ability to synthetize or transport ATP (oligomycin), and blocking the respiratory chain complexes (e.g. rotenone, antimycin A, potassium cyanide), have also an inhibitory effect on mitochondrial Ca^{2+} uptake. Several compounds are also known to inhibit mitochondrial Ca^{2+} extrusion through the mNCLX. Among those are benzothiazepines and benzodiazepines, including diltiazem, clonazepam and amiloride, as well as related compounds such as semotiadil, verapamil, trifluoperazine, tetraphenilphosphonium and cyclosporine A (Jurkowitz *et al.* 1983; Matlib *et al.* 1983; Wolkowicz *et al.* 1983; Wingrove & Gunter, 1986; Wei *et al.* 2011). Of this class, the benzothiazepine CGP-37157 was shown to be the most potent and selective inhibitor of mNCLX (Matlib *et al.* 1983; Chiesi *et al.* 1988), with a 10-fold higher affinity than any other inhibitor.

The inhibitory properties of these compounds on mitochondrial Ca^{2+} dynamics are especially relevant in pathological situations where mitochondrial Ca^{2+} overload has been shown to be detrimental. In fact, protective effects of approaches that moderate mitochondrial Ca^{2+} accumulation have been demonstrated for different disease contexts. Chemical inhibition by RuR and Ru360 improved the functional recovery of hearts after ischaemia and suppressed arrhythmias and haemodynamic dysfunction elicited by reperfusion *in vivo* (Garcia-Rivas Gde *et al.* 2006). Similarly, modest depolarization of $\Delta\Psi_m$, by transient pharmacological uncoupling of mitochondrial oxidative phosphorylation, was also found to be a potential therapeutic strategy for several human disorders that involve metabolic and mitochondrial oxidative stress, including Parkinson's and Alzheimer's diseases (Wu *et al.* 2011; Geisler *et al.* 2017), cerebral ischaemia (Korde *et al.* 2005), heart failure (Brennan *et al.* 2006), and metabolic diseases such as diabetes and obesity (Parascandola, 1974; Perry *et al.* 2013; Tao *et al.* 2014). Pharmacological inhibition of mNCLX with CGP-37157 conferred protection in hippocampal slices against veratridine-induced Ca^{2+} and Na^+ overload by regulating oxidative stress and p38 mitogen-activated protein kinase-linked activation of cell death (Nicolau *et al.* 2010). In addition, by enhancing mitochondrial oxidative metabolism, CGP-37157 promoted ATP generation and increased glucose-stimulated insulin secretion in rat islets and INS-1 cells (Lee *et al.* 2003). These initial results illustrated the potential utility of drugs that inhibit mitochondrial Ca^{2+} release as neuroprotective tools and insulin secretagogues, underscoring mNCLX as a novel drug target in neurological and metabolic disorders.

However, all of the pharmacological approaches and tools to modulate mitochondrial Ca^{2+} homeostasis listed above have major drawbacks when applied *in vivo*. Classical MCU and mNCLX inhibitors (1) lack specificity, as they also inhibit other channels and transporters in the cell; (2) show several side effects, interfering with other organelle functions and/or several extra-mitochondrial targets; and (3) reveal suboptimal cellular targeting, as most of them are poorly or not cell-permeant. The most commonly used mitochondrial Ca^{2+} uptake inhibitors, RuR, Ru360 and its derivatives (Nathan *et al.* 2017), are potent and effective on isolated mitochondria, but their use in intact cells or *in vivo* is inadequate due to their limited permeance across the plasma membrane (Hajnoczky *et al.* 2006). In addition, RuR, due to its affinity for ryanodine receptors, was also shown to inhibit Ca^{2+} release from the SR (Chamberlain *et al.* 1984). Moreover, positive and negative inotropic responses to RuR were observed in isolated rat hearts in a dose-dependent manner. These effects were attributed to its concomitant capacity to inhibit SR Ca^{2+} release or sarcolemmal Na^+/Ca^{2+} exchange (Gupta *et al.* 1988).

Mitochondrial Ca^{2+} uptake inhibition is often indirectly achieved by depolarizing the $\Delta\Psi_m$. Nevertheless, in most cells types, compounds that inhibit the electron transport chain (ETC) also increase mitochondrial reactive oxygen species production (rotenone, antimycin, myxothiazol) and inhibit the plasma membrane Na^+/K^+-ATPase (oligomycin) (Plesner & Plesner, 1991). In addition, the protonophore activity of uncouplers (FCCP/CCCP or dinitrophenol) is not limited to mitochondria, as they are also able to dissipate proton gradients across the plasma membrane and other membranous organelles at concentrations comparable to the ones used for mitochondria. Therefore, these chemical strategies lead to numerous undesired side effects, including plasma membrane depolarization, inhibition of ATP production, alterations in intracellular pH, and ultimately cytotoxicity (Juthberg & Brismar, 1997; Buckler & Vaughan-Jones, 1998; Park *et al.* 2002).

Major concerns were also raised regarding the use of the known inhibitors of mNCLX owing to their additional interaction with many Ca^{2+} channels and transporters in the cell. The benzodiazepine and benzothiazepine analogues diltiazem and clonazepam were found to inhibit the L-type Ca^{2+} channel in the plasma membrane (Koidl *et al.* 1997) and other receptor proteins in mitochondria (Kinnally *et al.* 1993). Even the classical blocker CGP-37157, at low micromolar concentration, was more recently shown to affect Ca^{2+} fluxes through the ryanodine receptor and block the sarco/endoplasmic reticulum Ca^{2+}-ATPase pump in striated muscle (Neumann *et al.* 2011). Many other studies also demonstrated the pleiotropic nature of this compound in different cell types. Resembling the effects

of the other described inhibitors, CGP37157 also inhibits the L-type Ca^{2+} channel in β-cells at concentrations considered specific for mNCLX (Luciani *et al.* 2007), and modulates intracellular Ca^{2+} entry through voltage gated Ca^{2+} channels in cortical neurons, attenuating NMDA-induced cytosolic and mitochondrial Ca^{2+} overload (Ruiz *et al.* 2014). As these channels constitute an integral part of the cellular machinery that regulates Ca^{2+} homeostasis, this constitutes a confounding factor to inhibition of mitochondrial Ca^{2+} efflux mechanisms. Furthermore, this evidence further compromised the role of mNCLX in the regulation of insulin secretion and prevention of neuronal cell death.

Taken together, these comprehensive studies demonstrated that caution should be taken regarding the use of these chemical inhibitors of mitochondrial influx and efflux pathways, especially in an *in vivo* context. Therefore, over the past years there has been an urge to find drugs and therapeutic strategies that directly and specifically modulate mitochondrial Ca^{2+} homeostasis.

Novel drug-screening strategies to identify direct modulators of mitochondrial Ca^{2+} homeostasis

The molecular characterization of mitochondrial Ca^{2+} pathways has opened the possibility of systematic and large-scale drug screenings to identify novel means to target MCUC and mNCLX. The right approach to drug discovery depends on the availability of a robust, affordable and highly selective assay compatible with high-throughput screening (HTS) (Walters & Namchuk, 2003). Common methods used to monitor dynamic changes in mitochondrial Ca^{2+} levels, such as cell-based Ca^{2+} imaging assays and patch-clamp recordings in mitoplasts, have not been optimized so far for HTS. Furthermore, the close functional interconnection between intracellular Ca^{2+} signalling, energy production, $\Delta\Psi_m$ and Ca^{2+} uptake and release constitute a major challenge in the design of robust and effective high-throughput assays for the discovery of specific modulators of MCUC and mNCLX. As mentioned above, the entry and exit of Ca^{2+} in mitochondria is dependent on the same $\Delta\Psi_m$ used to produce ATP and, therefore, any compound with inhibitory effects on mitochondrial bioenergetics and ion gradients will indirectly affect mitochondrial Ca^{2+} homeostasis. Moreover, MCUC and mNCLX are intracellular targets and their activities depend on increases of cytoplasmic ionic concentrations by signalling events upstream of mitochondria. Accordingly, there is the potential in cell-based assays for numerous false-positive hits.

Very recently, a novel systematic chemical biology approach was developed by our group to identify small molecule drugs that directly modulate MCU (Arduino *et al.* 2017). We used mitochondria isolated from the yeast *S. cerevisiae*, wherein mitochondrial Ca^{2+} uptake activity was reconstituted by heterologous expression of the human MCU and its essential regulator EMRE, and MCU-mediated Ca^{2+} uptake was quantitatively measured by the Ca^{2+}-sensitive photoprotein aequorin, stably expressed in the mitochondrial matrix. This approach takes advantage of several key properties of yeast mitochondria. First, they do not possess any intrinsic mitochondrial Ca^{2+} permeability (Carafoli & Lehninger, 1971). Second, they have a simplified OXPHOS system lacking complex I but including a D-lactate:cytochrome *c* oxidoreductase (DLD), which is sensitive to D-lactate and enables the direct transfer of electrons to cytochrome *c* oxidase (complex IV). Thus, when mitochondria are energized with D-lactate there is a bioenergetic shunt pathway that bypasses the majority of the ETC complexes and NADH generating pathways while it is still sufficient to build up $\Delta\Psi_m$ (Gregolin & D'Alberton, 1964; Pajot & Claisse, 1974). In our study, Ca^{2+} uptake in mitochondria fuelled with succinate remained sensitive to blockers of all the ETC complexes, whereas the use of D-lactate as a respiratory substrate rescued the inhibitory effects of ETC blockers upstream of complex IV (Arduino *et al.* 2017). Likewise, yeast mitochondria developed and maintained a CCCP-insensitive $\Delta\Psi_m$ when assayed in an isosmotic mannitol–sucrose medium. In contrast, in a nearly isotonic KCl-based medium, CCCP treatment resulted in a dramatic reduction of mitochondrial Ca^{2+} uptake (Arduino *et al.* 2017). Finally, yeast mitochondria reconstituted with coelenterazine can be frozen without losing the ability to generate a $\Delta\Psi_m$ that is sufficiently high to allow Ca^{2+} fluxes as well as other mitochondrial processes such as, for example, protein import (Hartl & Neupert, 1990; Koll *et al.* 1992; Izawa & Unger, 2017). Altogether, these properties make the use of reconstituted yeast mitochondria a powerful high-throughput drug screening strategy to identify specific and direct modulators of MCU activity, by minimizing false discovery rate due to confounding effects of drug-mediated inhibition of $\Delta\Psi_m$ and bioenergetics, ETC and signalling events upstream of mitochondria.

In a screen of ~700 Food and Drug Administration (FDA)-approved drugs, our group identified mitoxantrone as a specific inhibitor of MCU (Arduino *et al.* 2017). We confirmed that mitoxantrone inhibits mitochondrial Ca^{2+} uptake in mammalian cells without affecting mitochondrial bioenergetics. In addition, patch-clamp electrophysiology in human mitoplasts treated with mitoxantrone showed a direct and reversible inhibition of MCU-dependent Ca^{2+} currents when the drug was applied on the cytosolic but not on the matrix side. Although Ca^{2+} channel blockers are often reported to have off-target effects on other ion channels, mitoxantrone exhibited an exquisite selectivity for MCU when tested on ER and plasma membrane channels. Based

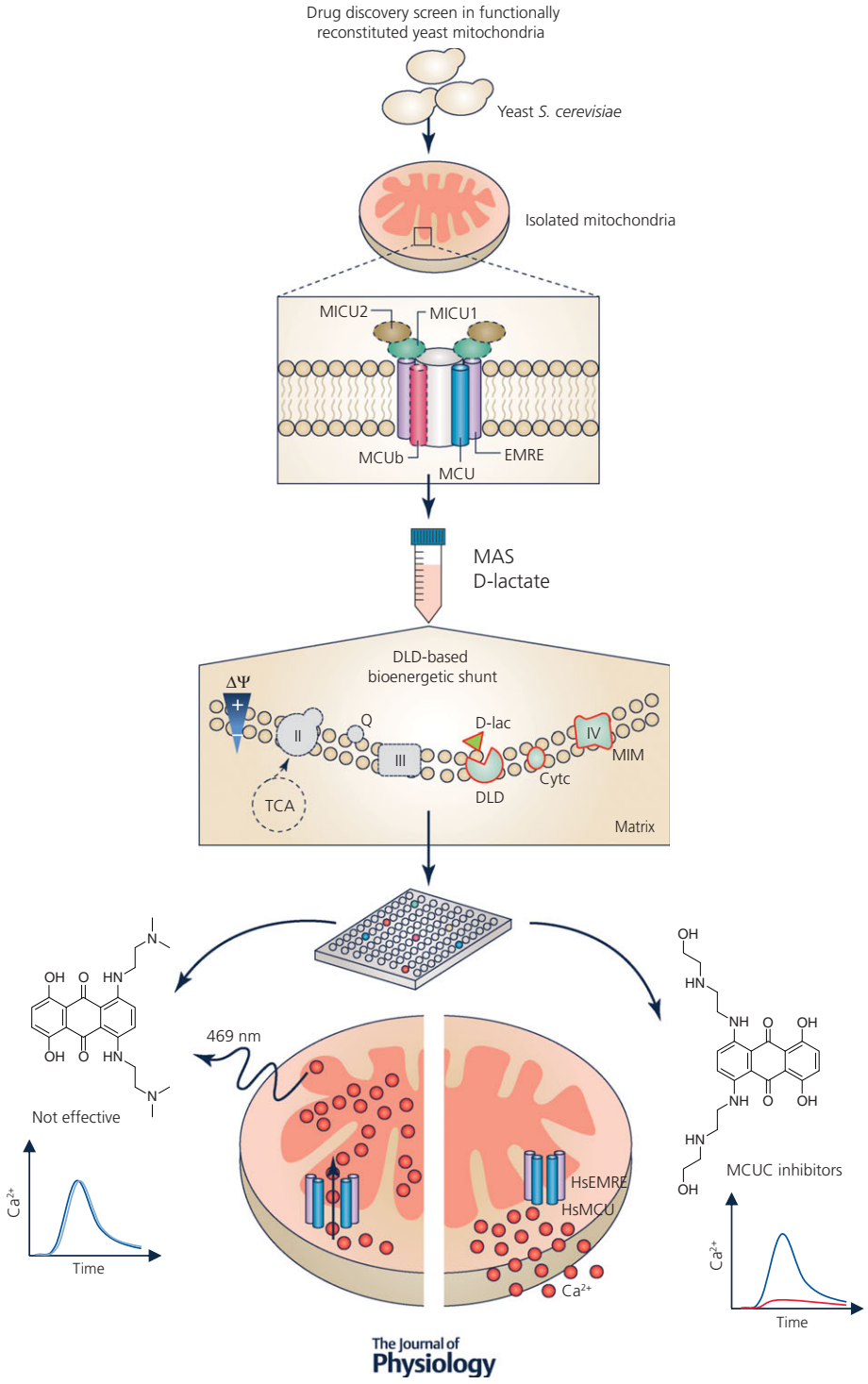

Figure 1. Workflow of the drug screen assay in reconstituted yeast mitochondria
The yeast *S. cerevisiae* constitutes a versatile system that can be reconstituted with different components of the MCUC machinery (e.g. MCU and EMRE and additionally MCUb, MICU1 and/or MICU2, as represented with dashed lines). When D-Lactate (D-Lac) is supplied to mitochondria as the energy source, it provides a bioenergetic shunt pathway that minimizes the detection of false-positive hits. This drug screen platform allows the quantification of mitochondrial Ca^{2+} uptake kinetics based on mitochondria-targeted-aequorin luminescence emitted at 469 nm. MCUC modulators are accurately identified based on their effects on mitochondrial Ca^{2+} uptake kinetics. MAS, mannitol-sucrose buffer; DLD, D-lactate:cytochrome c oxidoreductase; TCA, tricarboxylic acid cycle; Q, coenzyme Q; Cytc, cytochrome c; II, succinate dehydrogenase; III, coenzyme Q:cytochrome c-oxidoreductase; IV, cytochrome c oxidase.

on our structure - activity relationship (SAR) analysis, we found that the antineoplastic and anti-MCU properties of mitoxantrone are not interrelated. Accordingly, the quinizarin moiety, which is common to other related chemotherapeutic drugs and mediates DNA intercalation and topoisomerase II binding, is dispensable for the inhibition of MCU. Instead, the positively charged side chains of mitoxantrone at positions 5 and 8 played a key role as they mediate the binding to highly conserved aspartate residues in the selectivity filter of the uniporter channel (Arduino *et al.* 2017).

Altogether, these results validate the yeast mitochondria-based screening strategy as a tool to discover drug molecules directly targeting MCUC. Indeed, the assay represents a flexible, cost-effective HTS solution, which can be exploited to reconstitute and target other subunits of the uniporter, such as tissue-specific regulators (Fig. 1). This would address the need to tailor the modulation of MCUC activity to the tissue's physiology as well as to pathological consequences of loss or gain of function mutations. Additionally, drug delivery should ensure specificity to an intended tissue or cell type while simultaneously minimizing cytotoxicity by reducing side effects due to undesired drug accumulation on peripheral healthy tissues. This is normally achieved by active targeting, i.e. targeting unique receptors in specific cell types or tissues of interest by ligands present on the surface of drug delivery nanocarriers (e.g. polymeric nanoparticles or liposomes) (Cheng *et al.* 2015). Nowadays, the application of these drug delivery platforms constitutes a promising approach of demonstrated efficacy even in disease contexts where pharmacological targeting of MCUC has been proven to be beneficial, such as myocardial infarction and ischaemia–reperfusion injury (Takahama *et al.* 2009; Magruder *et al.* 2017).

In the near future, in order to enhance the translational value of differently regulated proteins involved in mitochondrial Ca^{2+} homeostasis as biomarkers or disease-targets, it is essential to integrate hints from different disciplines. Those can comprise results from drug screenings, detailed information from clinical studies (e.g. disease endophenotypes) and prediction of novel drug–target interactions. This strategy will not only allow the identification of novel drug molecules but also provide a broader knowledge on their application to treat or halt human diseases in which mitochondrial Ca^{2+} homeostasis deregulation and mitochondrial dysfunction are pathological hallmarks.

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Additional information

Competing interests

None of the authors have any conflict of interests to declare.

Author contributions

Both authors have approved the final version of the manuscript and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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