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The Pathophysiology of LETM1

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Originally identified as a key element of mitochondrial volume homeostasis through regulation of K^+–H^+ exchange (KHE), the LETM1 protein family is also involved in respiratory chain biogenesis and in the pathogenesis of seizures in the Wolf–Hirschhorn syndrome (WHS). To add further complexity, LETM1 has been recently proposed to catalyze mitochondrial H^+–Ca^{2+} exchange, which would imply a role in mitochondrial Ca^{2+} homeostasis as well. In the following paragraphs, we summarize the current state of the art about the functions of LETM1 and its role in pathophysiology, with some emphasis on whether it is a feasible candidate for regulation of mitochondrial Ca^{2+} homeostasis.

The LETM1 protein family

LETM1 (leucine–zipper–EF hand-containing transmembrane region) encodes a mitochondrial protein conserved in all lower eukaryotes, animals, and plants. All members of the LETM1 family share the same protein features and architecture, with a hydrophobic N-terminal portion spanning the inner membrane and a large hydrophilic portion including the C terminus located in the matrix. The primary amino acid sequence of the C-terminal part predicts at least two coiled-coil domains and two Ca^{2+}-binding EF hand–like motifs (Nowikovsky et al., 2004). However, the EF hands differ from canonical EF hand motifs by several residues, and the α helices are not directly flanking the Ca^{2+}-binding loop. The amino acid sequence of yeast LETM1 proteins lacks these putative Ca^{2+}-binding EF hand motifs. The N-terminal region of the LETM1 protein superfamily displays a mitochondrial targeting sequence and a single transmembrane domain. The latter is highly conserved in all orthologues (Nowikovsky et al., 2004; Schlickum et al., 2004) and rich in proline residues, as usually observed in ion channels. Yeast *Saccharomyces cerevisiae* encodes two LETM1 orthologues, *YOL027c* and *YPR125w*. *YOL027c* codes for proteins of 65 kD and has been referred to as the *MDM38* gene because its deletion alters mitochondrial distribution and morphology (Dimmer et al., 2002). *YPR125w* is also named *MRS7*, as it was initially identified in a genetic screen for multicopy suppressors of *petite* yeast strains lacking the mitochondrial Mg^{2+} transporter *MRS2* (Waldherr et al., 1993) or *YLH47* for yeast LETM1 homologue of 47 kD (Frazier et al., 2006).

Besides the LETM1 protein of 83.4 kD, the human genome also encodes the LETM1-like protein LETM2, the product of a related open reading frame that originated by gene duplication. Studies on the rat LETM1 homologue revealed that it is ubiquitously expressed, whereas LETM2 is only expressed in testis and sperm (Tamai et al., 2008). The *Caenorhabditis elegans* LETM1 is encoded by the F58G11.1 gene and shares 79% amino acid identity with human LETM1 (Hasegawa and van der Bliek, 2007). The *Drosophila melanogaster* homologue is the largest member of the LETM1 protein family with 113.6 kD and is encoded by the open reading frame annotated as CG4589. This protein shares 42% sequence identity with human LETM1 (McQuibban et al., 2010).

Biochemical analyses of Mdm38, Mrs7, and human LETM1 proteins indicated that they are components of high molecular weight complexes (Schlickum et al., 2004; Hasegawa and van der Bliek, 2007; Dimmer et al., 2008; Tamai et al., 2008; Zotova et al., 2010). In blue native PAGE, Mrs7p appeared within two major protein complexes of ∼130 and 250 kD, respectively, as well as in a minor complex of ∼500 kD; Mdm38 appeared within complexes of ∼140, 230, and 500 kD; and LETM1 appeared within a major complex of 300 and a minor complex of ∼500 kD (Hasegawa and van der Bliek, 2007; Dimmer et al., 2008; Zotova et al., 2010). In fact, Mdm38p and LETM1 were found as homodimers (Hasegawa and van der Bliek, 2007; Zotova et al., 2010) and may exist in homomultimers or interact with other proteins. In one study, LETM1 and BCS1L, an AAA-ATPase chaperone

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Abbreviations used in this paper: KHE, K^+–H^+ exchange; MCU, mitochondrial Ca^{2+} uniporter; PTP, permeability transition pore; RR, ruthenium red; WHS, Wolf–Hirschhorn syndrome.

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with a function in assembling the respiratory complex III, were both identified within a protein complex of ~300 kD (Tamai et al., 2008). BCS1L is associated with several human disorders including the GRACILE and the Björnstad syndromes (Morán et al., 2010; DiMauro and Garone, 2011). Interestingly, in BCS1L mutant fibroblasts, the LETM1 protein levels were increased but shifted from the 300-kD complex toward higher molecular weight species (>500 kD) (Tamai et al., 2008), suggesting that LETM1 may require wild-type BCS1L to be inserted into the 300-kD complex. Although there is a general consensus on the molecular size of the Mdm38/LETM1 protein complexes, the identity of the interaction partners of the LETM1 protein family members is still a matter of debate, likely reflecting the different pull-down and protein-tagging approaches (Frazier et al., 2006; Zotova et al., 2010) and the discrete Mdm38p/LETM1-containing complexes.

Recently, structural analyses were performed to solve the crystal structure of the soluble part of Mdm38p from the C terminus of the transmembrane domain to the C terminus of the protein residues 159–573 (Lupo et al., 2011). Although the first highly conserved 23 amino acids could not be resolved, data revealed that the amino acid stretch between residues 182 and 408 formed a 14-3-3–like domain. Classical 14-3-3 proteins share nine antiparallel α helices, with helices 1 and 2 forming the dimerization domain and helices 3, 5, 7, and 9 forming the concave substrate site. These proteins are involved in a large number of regulatory cellular processes (Lupo et al., 2011). As revealed by the structural analysis, Mdm38 exposes a hydrophobic cavity within the 14-3-3–like domain reminiscent of the substrate-binding site of the canonical 14-3-3 proteins and conserved in the LETM1 protein family. However, the 14-3-3–like domain of Mdm38 lacks the first two α helices (Lupo et al., 2011). From the available structural evidence and membrane topology (Dimmer et al., 2008), the Mdm38/LETM1 superfamily thus comprises an N-terminal domain facing the intermembrane space, a transmembrane domain that is likely to regulate dimerization, and a C-terminal domain facing the mitochondrial matrix, which bears a substrate-binding site, coiled-coil domains, and, with the exception of yeast, putative Ca^{2+}-binding EF hand sites (Fig. 1).

LETM1 and mitochondrial potassium homeostasis

In the process of characterizing yeast genes encoding putative mitochondrial cation transporters, Nowikovsky et al. (2004) identified Mdm38p as an essential element both for mitochondrial KHE activity and for growth of yeast cells on nonfermentable substrates. Passive swelling experiments with isolated mitochondria in K^+ acetate (a sensitive method to detect electroneutral KHE; see Bernardi, 1999) demonstrated that Mdm38p/Yol027cp is essential for mitochondrial KHE in yeast, an activity that was lacking in *mdm38* knockout mutants (Nowikovsky et al., 2004; Froschauer et al., 2005). Direct KHE assays in submitochondrial particles with entrapped K^+- and H^+-sensitive fluorescent dyes PBFI and BCECF, respectively, which allow continuous recording of changes in free K^+ and H^+ concentrations over time, indicated that Mdm38p-mediated K^+ and H^+ fluxes were obligatorily coupled and electroneutral (Froschauer et al., 2005). These results are compelling evidence that Mdm38p/Yol027cp is an essential component of the mitochondrial KHE (Nowikovsky et al., 2004; Froschauer et al., 2005), a hypothesis that is strongly supported by the finding that nigericin (an ionophore that catalyzes electroneutral KHE) restored aerobic growth of

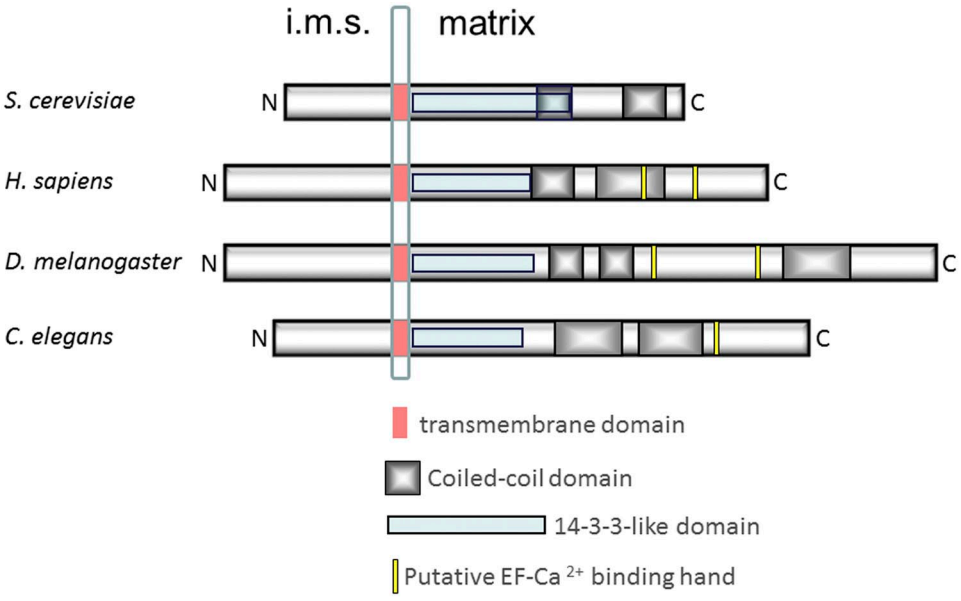

Figure 1. The LETM1 protein superfamily. Schematic representation of selected LETM1 family members aligned on the highly conserved transmembrane region. Specific domains are represented, and bar lengths are proportional to the length of the corresponding amino acid sequences. i.m.s., intermembrane space.

Mdm38p/Yol027cp-null yeast strains and reverted mitochondrial swelling in situ (Nowikovsky et al., 2007). In contrast to *MDM38*, deletion of *MRS7* had no severe effect on growth or KHE activity (Frazier et al., 2006; Zotova et al., 2010). However, reversion to normal K^+ homeostasis and growth features could also be achieved by overexpression of the Mdm38p yeast homologues Ypr125wp/Mrs7p and LETM1 or DmLETM1, the human or *Drosophila* orthologues, confirming the functional homology. As mentioned above, the LETM1 protein family bears only one transmembrane domain, suggesting that its members may not constitute the KHE itself (at least as a monomer) but rather an essential component that allows the exchange activity to take place. Biochemical data confirming that these proteins are part of a higher molecular weight complex suggest that the KHE might consist of discrete subunits. To identify other components of the KHE, a genetic screen for multi-copy suppressors of the *petite* phenotype of *mdm38*Δ cells was performed and led to the identification of the unknown gene *YDL183c*. Overexpression of *YDL183c* was able to restore the KHE activity, confirming a functional homology to *MDM38* and MRS7. *YDL183c* encodes a 37-kD mitochondrial membrane protein with a single predicted transmembrane domain that contains several proline residues but otherwise appears not to be related to Mdm38p. Disruption of *YDL183c* had a weak *petite* phenotype at high growth temperatures. However, the triple deletion of *MDM38*, *MRS7*, and *YDL183c* exhibited a complete negative phenotype on nonfermentable substrates, strongly reduced growth on glucose, and complete block of residual K^+ and H^+ fluxes. All synthetic phenotypes of the triple mutant were fully restored by nigericin in vitro and in vivo (Zotova et al., 2010).

Atomic absorption measurements revealed a significant increase of mitochondrial K^+ in the absence of Mdm38p and, conversely, mitochondrial K^+ depletion under overexpression of Mdm38p (Nowikovsky et al., 2004). Consistent with a role of K^+ in controlling mitochondrial volume and morphology, Mdm38p or LETM1 depletion resulted in extreme matrix swelling and cristae loss, whereas Mdm38p or LETM1 overexpression caused mitochondrial contraction and cristae swelling (Hasegawa and van der Bliek, 2007; Nowikovsky et al., 2007). Interestingly, nigericin fully reverted the phenotype of LETM1 ablation in HeLa cells (Dimmer et al., 2008). Moreover, considerable mitochondrial depolarization was caused upon *MDM38* or *LETM1* deletion (Nowikovsky et al., 2004; Frazier et al., 2006; McQuibban et al., 2010). Surprisingly, studies performed in yeast and mammalian cell cultures led to the observation of ongoing selective autophagy (mitophagy) as a result of shutoff of KHE activity (Nowikovsky et al., 2007; McQuibban et al., 2010). To dissect the phenotypes by depleting Mdm38p in a controlled fashion as a function of time, a conditional *MDM38* shutoff system was generated (Nowikovsky et al., 2007). The primary effect of *MDM38* down-regulation was the absence of mitochondrial KHE activity, confirming the essential function of Mdm38 in regulating K^+ homeostasis. Electron microscopy revealed the presence of swollen and fragmented mitochondria closely associated with and/or ingested by vacuoles. These data provided the first evidence that mitochondrial dysfunction and osmotic swelling caused by excess matrix K^+ leads to mitophagy. Importantly, the addition of nigericin reverted mitochondrial swelling and ongoing mitophagy, confirming the causal link between the two events (Nowikovsky et al., 2007). Down-regulation of DmLETM1 in *Drosophila* cell lines also led to increased mitophagy, as revealed under confocal or electron microscopy (McQuibban et al., 2010). A schematic representation of the role of LETM1 in mitochondrial K^+ (hence volume) homeostasis and of the consequences of its absence is presented in Fig. 2, which highlights how K^+ influx via K^+ channels (K_C) must be counterbalanced by activity of the KHE to maintain volume homeostasis; in the absence of LETM1, mitochondria cannot compensate electrophoretic K^+, resulting in matrix swelling causing, in turn, increased mitophagy and/or cell death.

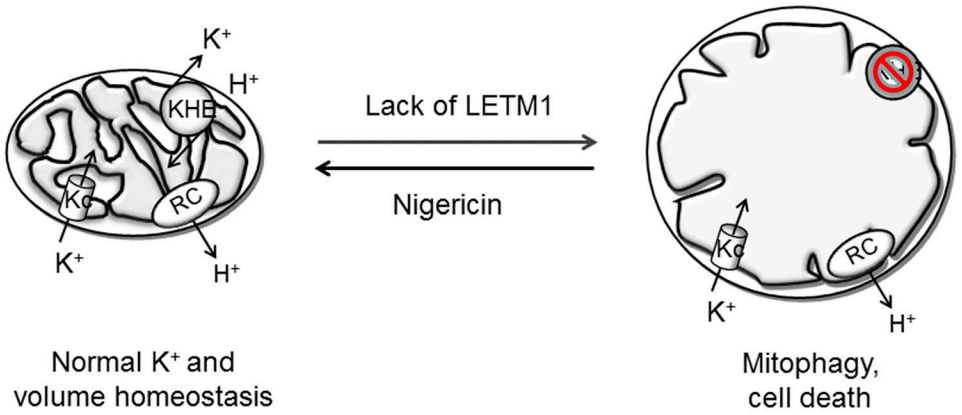

Figure 2. Mitochondrial K^+ and volume homeostasis. RC, respiratory chain; K_C, potassium channels; KHE, K^+–H^+ exchange. For further explanation, see text.

LETM1 in respiratory chain biogenesis

Frazier et al. (2006) reported that lack of Mdm38p caused reduced steady-state levels of the mitochondrially encoded proteins and decreased insertion of cytochrome b and Atp6 into the respiratory complexes. Destabilized interaction of Mdm38 with mitochondrial ribosomes was proposed to account for the defective assembly of the respiratory chain (Frazier et al., 2006). However, time course studies revealed that these defects were secondary to the Mdm38 deletion, impaired KHE, and inner membrane depolarization, as they were rescued by the addition of low concentrations of nigericin to the growth media (Nowikovsky et al., 2007). Hence, *mdm38*Δ-induced K^+ overload or membrane depolarization may destabilize the protein assembly of respiratory complexes.

To further explore the function of LETM1 as a ribosome-associated protein, a double deletion of *MDM38* and of the ribosome receptor *MBA1* was performed that led to a synthetic translational defect causing the complete loss of Cox1 and Cytb. This synthetic defect could not be reverted by nigericin (Bauerschmitt et al., 2010), similarly to the damage caused by deletion of the soluble part of Mdm38 (Lupo et al., 2011), thus supporting the notion that LETM1 has a role in mitochondrial biogenesis in addition to that in ion homeostasis. In human cells, lack of LETM1 was associated with a decreased number of cristae, depolarization, and impaired assembly of respiratory chain supercomplexes (Tamai et al., 2008). In another study, LETM1 was shown to bind the mitochondrial ribosome protein L36 and cause an L36-dependent drop of ATP production (Piao et al., 2009a). However, silencing of *LETM1* in HeLa cells failed to affect defects in complex III assembly, or to cause overt or latent mitochondrial dysfunction (Dimmer et al., 2008), further complicating the picture. The role of Mdm38/LETM1 in the biogenesis of respiratory complexes is essentially based on binding to ribosomal proteins. Furthermore, the extreme mitochondrial swelling observed in the absence of Mdm38/LETM1 was never seen in cells with inactivated components of the respiratory complexes or in ρ_0 cells (Hasegawa and van der Bliek, 2007; Tamai et al., 2008).

LETM1 in mitochondrial calcium transport

In a genome-wide RNAi screening of Drosophila for proteins involved in Ca^{2+} uptake in mitochondria in situ, a Drosophila homologue of the human LETM1 gene was identified as strongly affecting mitochondrial Ca^{2+} and H^+ fluxes. A drastic reduction of mitochondrial Ca^{2+} uptake was observed when protein expression was suppressed; purification and reconstitution of LETM1 in liposomes allowed detection of coupled Ca^{2+} and H^+ fluxes in opposite directions, which were inhibited by ruthenium red (RR) (Jiang et al., 2009). These findings led to the conclusion that LETM1 is a H^+–Ca^{2+} exchanger mediating RR-sensitive Ca^{2+} uptake in energized mitochondria (Jiang et al., 2009). We note that the finding that Ca^{2+} fluxes are mirrored by H^+ fluxes in liposomes reconstituted with LETM1 is certainly compatible with an obligatory H^+–Ca^{2+} antiporter mechanism but does not prove it; indeed, Ca^{2+} flux could be electrophoretic (as is the case for transport via mitochondrial Ca^{2+} uniporter [MCU]), and H^+ movement could occur independently of LETM1 to provide charge compensation.

The mechanisms through which energized mitochondria take up and release Ca^{2+} have been the subject of a large number of studies over the years (Rizzuto et al., 2000; Drago et al., 2011). The pathways characterized so far (Fig. 3) are: (a) the MCU, which in energized mitochondria mediates Ca^{2+} uptake via electrophoretic transport across the inner membrane (Baughman et al., 2011; De Stefani et al., 2011) through a specific channel (Kirichok et al., 2004) that is inhibited by RR (Moore, 1971) and is modulated by a protein component with a single transmembrane domain, mitochondrial Ca^{2+} uptake 1 (Perocchi et al., 2010); (b) the Na^+–Ca^{2+} exchanger, an RR-insensitive system mediated by NCLX (Palty et al., 2010) that in energized mitochondria catalyzes Ca^{2+} release (Carafoli et al., 1974; Crompton et al., 1976); (c) the RR-insensitive putative H^+–Ca^{2+} exchanger, which mediates Ca^{2+} release and whose activity is favored by the membrane potential (Bernardi and Azzone, 1982, 1983) suggesting a stoichiometry of more than $2H^+$–Ca^{2+}; and (d) the permeability transition pore (PTP), a high conductance channel (Kinnally et al., 1989; Petronilli et al., 1989) that plays an important role in cell death (Rasola et al., 2010); owing to its large size

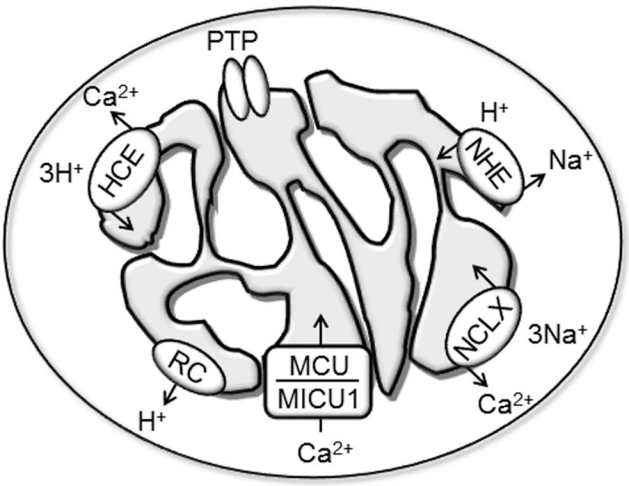

Figure 3. Pathways for Ca^{2+} transport in mammalian mitochondria. RC, respiratory chain; MCU, mitochondrial Ca^{2+} uniporter; MICU1, mitochondrial Ca^{2+} uptake 1; NCLX, Na^+–Ca^{2+} exchanger; HCE, H^+–Ca^{2+} exchanger; PTP, permeability transition pore. For explanation, see text.

allowing depolarization, the PTP could provide mitochondria with a fast Ca^{2+} release channel preventing matrix Ca^{2+} overload (Bernardi and Petronilli, 1996; Bernardi, 1999), an idea supported by some studies (Altschuld et al., 1992; Elrod et al., 2010; Barsukova et al., 2011) and by the recent identification in *Drosophila* of a H^+-permeant Ca^{2+} release channel with features intermediate between the PTP of yeast and mammals (von Stockum et al., 2011).

As already mentioned, LETM1 is a single transmembrane domain protein, a structure that is unlikely to directly mediate Ca^{2+} transport either as a channel or by mediating H^+–Ca^{2+} antiport. Indeed, all known exchangers and channels possess multiple transmembrane domains. Are the features reported for LETM1-mediated Ca^{2+} transport consistent with its modulation of any of the known Ca^{2+} influx/efflux pathways? We tend to exclude both a mechanism involving the Na^+–Ca^{2+} exchanger, because this mechanism can only catalyze Ca^{2+} efflux in coupled respiring mitochondria, and the PTP, because its opening causes depolarization, which prevents Ca^{2+} uptake and favors Ca^{2+} release (Bernardi, 1999). A modulation of the MCU, on the other hand, is not fully consistent with the reconstitution experiments of Jiang et al. (2009), where only LETM1 was apparently present. Although the possibility that LETM1 modulates previously unidentified Ca^{2+} transport systems cannot be ruled out, its proposed role as a H^+–Ca^{2+} exchanger is discussed below.

If LETM1-mediated (or modulated) Ca^{2+} transport is obligatorily coupled to H^+ translocation via an exchange mechanism, assessment of the H^+–Ca^{2+} stoichiometry is essential for predictions to be made about the direction of Ca^{2+} flux it mediates in energized mitochondria. If the H^+–Ca^{2+} stoichiometry is 1, Ca^{2+} uptake is favored by the $\Delta\tilde{\mu}Ca$ and opposed by the $\Delta\tilde{\mu}H$; net charge translocation is 1, and the predicted Ca^{2+} accumulation at equilibrium is 10^2 if the ΔpH is 1 unit, alkaline inside (Fig. 4 B and Appendix). This mode of Ca^{2+} uptake would have to compete with the MCU, whose driving force is 10,000-fold larger (Fig. 4 A and Appendix). Jiang et al. (2009) have suggested that LETM1 catalyzes Ca^{2+} accumulation in energized mitochondria only at low cytoplasmic Ca^{2+} concentrations, whereas MCU predominates at higher Ca^{2+} levels. The possibility that Ca^{2+} is taken up with a single charge by energized mitochondria (either in symport with anions, e.g., Pi or OH^-, or via a H^+–Ca^{2+} antiporter) has been discussed in the field (Heaton and Nicholls, 1976; Azzone et al., 1977; Moyle and Mitchell, 1977) but is inconsistent with all the measurements of net charge translocation during Ca^{2+} transport, which has consistently been found to be 2 (Scarpa and Azzone, 1970; Rottenberg and Scarpa, 1974; Wingrove et al., 1984). Furthermore, mitochondrial Ca^{2+} uptake is almost completely suppressed by MCU-specific siRNAs (Baughman et al., 2011; De Stefani et al., 2011), findings that strongly argue against the existence of a H^+–Ca^{2+} antiporter mediating mitochondrial Ca^{2+} uptake.

Transport mediated by a $2H^+$–Ca^{2+} antiporter is electroneutral, and therefore the direction of Ca^{2+} flux will depend on the proton and Ca^{2+} chemical gradients; given the existence of an alkaline-inside pH difference, such a mechanism cannot mediate Ca^{2+} uptake unless cytosolic $[Ca^{2+}]$ becomes more than 100-fold higher than matrix $[Ca^{2+}]$, with a predicted equilibrium accumulation of 10^{-2} (Fig. 4 C and Appendix). Thus, in respiring coupled mitochondria under physiological conditions, a $2H^+$–Ca^{2+} exchanger should catalyze Ca^{2+} efflux.

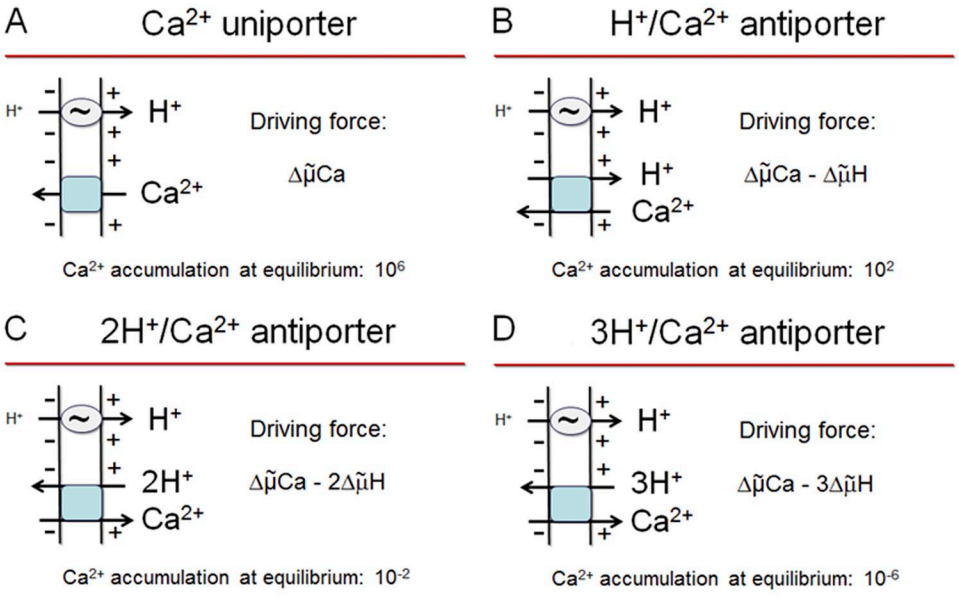

Figure 4. Predicted equilibrium Ca^{2+} accumulation depending on mode of Ca^{2+} transport in energized mitochondria. The scheme highlights the net direction of Ca^{2+} flux for transport via a Ca^{2+} uniporter (A), a H^+–Ca^{2+} exchanger (B), a $2H^+$–Ca^{2+} exchanger (C), and a $3H^+$–Ca^{2+} exchanger (D) in respiring mitochondria. The predicted equilibrium Ca^{2+} accumulation was calculated as described in the Appendix and refers to the matrix/cytosol concentration ratio so that a negative figure means that Ca^{2+} is more concentrated in the cytosol. The squiggle denotes the redox-coupled H^+ pumps. For further explanation, see text and Appendix.

If the transport is mediated by a $3H^+$–Ca^{2+} antiporter, Ca^{2+} efflux is greatly favored, as easily seen by the predicted equilibrium Ca^{2+} distribution, which would be 10^{-6} (i.e., 1,000,000 higher in the cytosol than in the matrix) (Fig. 4 D and Appendix). This is indeed a likely mechanism for mitochondrial Na^+-independent Ca^{2+} release, which is insensitive to RR and slowed down by depolarization (Bernardi and Azzone, 1982, 1983).

Whatever the mechanism of LETM1-catalyzed Ca^{2+} transport, it should be mentioned that the Ca^{2+}-transporting activity of LETM1 in reconstituted systems has so far been studied in K^+-free media (Nowikovsky et al., 2004; Jiang et al., 2009), and that the relevant question of whether Ca^{2+} is actually transported at physiological K^+ concentrations has not been addressed. A change of selectivity of channels and transporters is often observed in the absence of the physiologically transported species. For example, in the absence of divalent cations, the MCU conducts large Na^+ currents that can be inhibited by RR and disappear as soon as Mg^{2+} or Ca^{2+} is added (Kirichok et al., 2004). Although the experiments of Jiang et al. (2009) in living and permeabilized cells were performed at physiological K^+ and Mg^{2+} concentrations, in our view, whether the uptake of Ca^{2+} under those conditions was actually mediated by LETM1 remains to be established.

A recent study addressed the putative role of LETM1 and UCP2/3 in mitochondrial Ca^{2+} transport in endothelial cells (Waldeck-Weiermair et al., 2011). The role of UCP2/3 as MCUs (Trenker et al., 2007) has been a matter of debate (Brookes et al., 2008; Trenker et al., 2008), and although this topic in not within the scope of our review, we feel that such a role for UCP2/3 has been strongly undermined by the subsequent discovery of mitochondrial Ca^{2+} uptake 1 (Perocchi et al., 2010) and MCU (Baughman et al., 2011; De Stefani et al., 2011). As for LETM1, it has been reported that its knockdown strongly diminished the transfer of Ca^{2+} into mitochondria, resulting in subsequent reduction of store-operated Ca^{2+} entry (Waldeck-Weiermair et al., 2011). Although knockdown of LETM1 was reported not to impact cellular ATP levels and membrane potential, its impact on K^+ homeostasis has not been considered or discussed in this study (Waldeck-Weiermair et al., 2011), which in our view will need to be confirmed. We wish to stress that in most other investigations with comparable levels of LETM1 suppression, significant changes of mitochondrial membrane potential were observed (Nowikovsky et al., 2004; Frazier et al., 2006; McQuibban et al., 2010).

In summary, we think that the occurrence and mode of LETM1-dependent Ca^{2+} transport need further study to establish whether, besides its undisputable role in KHE (Nowikovsky et al., 2009), LETM1 can also support or modulate Ca^{2+} uptake in energized mitochondria.

LETM1 and the WHS

The WHS (MIM 194190) is a complex disease involving the central nervous system (Hirschhorn et al., 1965; Wolf et al., 1965) caused by partial heterozygous deletion of the terminal portion of the short arm of chromosome 4 involving the 4p16.3 region (Endele et al., 1999; Zollino et al., 2003). A critical region of 165 kb has been defined (WHSCR-1), whose deletion causes the clinical hallmarks of the disease, i.e., severe growth and mental retardation, hypotonia, midline fusion defects, and typical facial dysmorphism (Johnson et al., 1976; Wilson et al., 1981). A subset of cases of WHS is characterized by seizures, which start during the first year of life and are a frequent cause of death (Zollino et al., 2000). The *LETM1* gene has been localized less than 80 kb distal to the WHSCR-1 region, and it is invariably deleted in WHS patients with seizures and preserved in those without epilepsy (Schlickum et al., 2004) regardless of the WHSCR-1 deletion. Indeed, haploinsufficiency of the WHSCR-1 region results in an atypical WHS phenotype with no seizures, whereas involvement of the WHSCR-2, which includes LETM1, causes the typical WHS phenotype with psychomotor retardation and epilepsy. Thus, LETM1 haploinsufficiency is a potential causative event for WHS with seizures and possibly also in other forms of epilepsy. Given the key role of LETM1 in mitochondrial K^+ homeostasis, it may be predicted that reduced activity of the KHE is followed by osmotic matrix swelling, outer membrane rupture, cytochrome c release, and impaired ATP production. In keeping with this prediction, depletion of LETM1 in wild-type cells caused matrix swelling and fragmentation that, like in yeast, could be rescued by proper amounts of nigericin (Hasegawa and van der Bliek, 2007; Nowikovsky et al., 2007; Dimmer et al., 2008). Overexpression of LETM1 instead resulted in mitochondrial matrix contraction (Dimmer et al., 2008). Collectively, these findings critically support a role of the mitochondrial K^+ cycle in pathophysiological swelling–contraction events of mitochondria. Impaired mitochondrial ATP production after mitochondrial swelling may affect the neurons' ability to maintain the plasma membrane potential and thus increase excitability to the threshold required to trigger seizures, a hypothesis that has not been tested so far. Fibroblastoid cells from one WHS patient showed a detectable decrease in LETM1 protein content, but there was no obvious phenotypic effect on mitochondria. Only stronger reduction in LETM1 expression caused mitochondrial changes and finally cell death (Dimmer et al., 2008). Yet it is well possible that in other types of cells, e.g., neurons, the LETM1 protein contents of WHS patients is below the threshold required to maintain mitochondrial cation homeostasis, and that the basis for cell dysfunction in WHS may be lowered KHE activity, which could be treated with pharmacological agents.

To understand the function of LETM1 in development, *Drosophila* was used as an animal model. Drosophila is a powerful model organism, and the availability of the genome-wide library of Drosophila RNAi transgenes combined with a wide range of *GAL4* drivers allows for the targeting of conditional inactivation of a gene function in a tissue-specific manner (Dietzl et al., 2007). DmLETM1 knockdown recapitulated several important hallmarks of WHS, as ubiquitous or muscle-specific down-regulation significantly delayed development and resulted in small body size and early death. Down-regulation of DmLetm1 in the ommatidia caused deformations of the receptor cells and cell death. Down-regulation in the neuronal system resulted in reduced locomotor activity, and electrophysiological analyses revealed reduced synaptic neurotransmitter release (McQuibban et al., 2010).

A growing list of functions for LETM1

A role for LETM1 as a regulator of the mitochondrial-shaping protein OPA1 has been proposed based on LETM1 overexpression studies, where it was found that mitochondria fragment and OPA1 are differently cleaved, s-OPA1 rather than l-OPA1 being detected after overexpression of LETM1 (Piao et al., 2009b). It is worth recalling that *MDM38*, the yeast homologue of LETM1, was originally identified together with a series of other genes in a screen for mutants affecting mitochondrial morphology (Dimmer et al., 2002). Some of these genes did turn out to encode proteins required for mitochondrial fusion or fission, yet a large number of these genes encode proteins involved in different functions, including lipid metabolism and cation transport (Dimmer et al., 2002; Altmann and Westermann, 2005). Because LETM1 has complex effects on mitochondrial volume, membrane potential, and ATP levels (Nowikovsky et al., 2009), it is presently hard to tell whether the reported effects on OPA1 cleavage are primary or secondary to other events discussed in the preceding paragraphs.

It has recently been found that ER Ca^{2+} uptake in permeabilized neurons and cardiomyocytes was strongly inhibited by replacing K^+ with Na^+ or tetraethylammonium, or by treatment with quinine or propranolol (Kuum et al., 2012), well-known inhibitors of the mitochondrial KHE (Nakashima and Garlid, 1982). The inhibitory effects of propranolol were relieved by nigericin, indicating that proton–potassium exchange is essential for ER Ca^{2+} uptake; strikingly, fluorescence microscopy and Western blot analysis revealed the presence of LETM1 in the ER (Kuum et al., 2012). These data are consistent with a key role of LETM1 in maintaining ER pH homeostasis and exerting an indirect regulatory role on Ca^{2+} uptake in specific cell types (Kuum et al., 2012). An ER retention signal is found at the C terminus of LETM1 in some organisms; it will be interesting to assess the mechanisms through which LETM1 can be sorted to the ER or to the mitochondria, and whether this involves posttranscriptional modifications, as in the case of NADH–cytochrome b_5 reductase (Borgese et al., 1996; Colombo et al., 2005).

Recent work has explored the potential role of LETM1 in cell death as related to carcinogenesis. LETM1 overexpression induced necrotic cell death, which was matched by decreased mitochondrial biogenesis and ATP production, and by AMPK activation (Piao et al., 2009a; Hwang et al., 2010); yet the expression levels of LETM1 were found to be significantly increased in multiple human cancer tissues (Piao et al., 2009a). It is not clear what mechanism(s) mediates the effects of enforced LETM1 overexpression, however, and we think that this issue must be addressed before safe conclusions can be made on the role of LETM1 in tumorigenesis.

Conclusions and perspectives

In summary, studies in yeast, human cell cultures, and Drosophila underscore the prominent role of LETM1 in maintaining mitochondrial K^+ and volume homeostasis and respiratory chain assembly; genetic studies on WHS patients identify the deletion of LETM1 as a most likely causative event of seizures associated with the disease. Understanding the pathophysiology of LETM1 deficiency in vivo may thus lead to important advances toward the therapy of WHS and possibly of other forms of central nervous system diseases with seizures, which are often linked to mitochondrial defects (DiMauro and Schon, 2008). On the other hand, in our view, the potential role of LETM1 in mitochondrial Ca^{2+} homeostasis demands further scrutiny, with specific emphasis on the H^+–Ca^{2+} stoichiometry and on assessing whether this protein can catalyze Ca^{2+} transport at physiological K^+ concentrations. However, it is easy to forecast that the growing interest in LETM1 and the discovery of additional functions for this protein will rapidly lead to substantial advances in our understanding of its roles in pathophysiology.

APPENDIX

Predicted equilibrium Ca^{2+} accumulation ratio according to the mechanism of transport

For each of the four transport mechanisms depicted in Fig. 4, assuming that $\psi_m - \psi_c$ ($\Delta\psi$) is -180 mV, 2.303 RT/F = 60 mV, and $[H^+]_m/[H^+]_c = 0.1$, the ratio between the concentrations of Ca^{2+} in the mitochondrial matrix ($Ca^{2+}{}_m$) and in the cytoplasm ($Ca^{2+}{}_c$) at equilibrium can be calculated by considering the corresponding driving forces ($\Delta\tilde{\mu}$ is the electrochemical, and μ is the chemical potential) equal to zero as follows:

Ca^{2+} uniporter

$$\Delta\tilde{\mu}Ca = \Delta\mu Ca + 2F\Delta\psi = RT\ln([Ca^{2+}]_m/[Ca^{2+}]_c) + 2F\Delta\psi = 0$$

$$\log([Ca^{2+}]_m/[Ca^{2+}]_c) = -\Delta\psi 2F / 2.303\ RT$$

$$\log([Ca^{2+}]_m/[Ca^{2+}]_c) = -\Delta\psi / 30 = 6$$

$$[Ca^{2+}]_m/[Ca^{2+}]_c = 10^6$$

H^+–Ca^{2+} antiporter

$$\Delta\tilde{\mu}Ca - \Delta\tilde{\mu}H = \Delta\mu Ca + 2F\Delta\psi - \Delta\mu H - \Delta\psi$$
$$= RT\ln([Ca^{2+}]_m/[Ca^{2+}]_c) - RT\ln([H^+]_m/[H^+]_c) + F\Delta\psi = 0$$

$$\log([Ca^{2+}]_m/[Ca^{2+}]_c) = -\Delta\psi F / 2.303\ RT + \log([H^+]_m/[H^+]_c)$$

$$\log([Ca^{2+}]_m/[Ca^{2+}]_c) = -\Delta\psi / 60 - 1 = 2$$

$$[Ca^{2+}]_m/[Ca^{2+}]_c = 10^2$$

$2H^+$–Ca^{2+} antiporter

$$\Delta\tilde{\mu}Ca - 2\Delta\tilde{\mu}H = \Delta\mu Ca + 2F\Delta\psi - 2\Delta\mu H - 2F\Delta\psi$$
$$= RT\ln([Ca^{2+}]_m/[Ca^{2+}]_c) - 2RT\ln([H^+]_m/[H^+]_c) = 0$$

$$\log([Ca^{2+}]_m/[Ca^{2+}]_c) = 2\log([H^+]_m/[H^+]_c)$$

$$\log([Ca^{2+}]_m/[Ca^{2+}]_c) = -2$$

$$[Ca^{2+}]_m/[Ca^{2+}]_c = 10^{-2}$$

$3H^+$–Ca^{2+} antiporter

$$\Delta\tilde{\mu}Ca - 3\Delta\tilde{\mu}H = \Delta\mu Ca + 2F\Delta\psi - 3\Delta\mu H - 3F\Delta\psi$$
$$= RT\ln([Ca^{2+}]_m/[Ca^{2+}]_c) - 3RT\ln([H^+]_m/[H^+]_c) - F\Delta\psi = 0$$

$$\log([Ca^{2+}]_m/[Ca^{2+}]_c) = \Delta\psi F / 2.303\ RT + 3\log([H^+]_m/[H^+]_c)$$

$$\log([Ca^{2+}]_m/[Ca^{2+}]_c) = \Delta\psi / 60 - 3 = -6$$

$$[Ca^{2+}]_m/[Ca^{2+}]_c = 10^{-6}$$

This Perspectives series includes articles by Sheu et al., Zhang et al., Balaban, Santo-Domingo and Demaurex, Wei and Dirksen, O-Uchi et al., and Galloway and Yoon.

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