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The mysteries of LETM1 pleiotropy

Sami E.M. Mohammed , Karin Nowikovsky *

Department of Biomedical Sciences and Pathobiology, Centre of Biomedical Sciences, Institute of Physiology, Pathophysiology and Biophysics, Vetmeduni, Vienna, Austria

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ABSTRACT

LETM1 is a nuclear-encoded protein located in the inner mitochondrial membrane, playing a critical role in regulating mitochondrial cation and volume homeostasis. However, numerous studies on functional features, molecular interactions, and disease-associated effects of LETM1 revealed that LETM1 is also involved in other metabolic functions including glucose utilization, mitochondrial DNA and ribosome organization, cristae architecture and respiratory complex stability. Undisputedly, osmoregulatory processes are essential for mitochondrial functionality, but the pleiotropic aspects of LETM1 challenges us to understand the core function of LETM1, which still remains elusive. In this review, we provide an overview of the current knowledge and latest developments regarding the activities involving LETM1. We highlight various findings that offer different functional perspectives and ideas on the core function of LETM1. Specifically, we emphasize data supporting LETM1's role as a mitochondrial translational factor, K^+/H^+ exchanger, or Ca^{2+}/H^+ exchanger, along with recent findings on its interaction with ATAD3A and TMBIM5. We also present the severe clinical implications of LETM1 deficiency. Finally, we discuss emerging questions raised by the different views on LETM1, which need to be addressed to guide future research directions and ultimately resolve the function of this essential protein and develop targeted therapeutic strategies.

1. The essentiality of ion homeostasis

1.1. Role of mitochondrial ion homeostasis

Metal ions play essential roles in cellular functions across all living organisms, acting as enzymatic cofactors, signaling molecules, and regulators of metabolic networks and osmotic pressure. Consequently, maintaining ionic balance is critical for numerous physiological processes, including electrical signal conduction, cell differentiation, and inflammatory responses [1–4]. Mitochondria are integral to these activities as they play a crucial role in maintaining ion homeostasis [5,6]. Ancestrally equipped to adapt to fluctuating external metabolic conditions, osmotic pressures, and ionic environments, mitochondria have evolved into dynamic organelles that reflect the intracellular landscape of metal ions and control ion homeostasis through their capacity for ion sensing, buffering, and cycling [7,8]. While regulating cellular ion homeostasis, mitochondria must also maintain their internal ionic balance. The homeostasis of mitochondrial monovalent cations such as K^+ and Na^+, which control osmotic flexibility, is fundamental to mitochondrial volume and functions; divalent cations like Mg^{2+}, Ca^{2+}, and Fe^{2+} must also be balanced as they are involved in enzymatic, redox, and signaling activities [9]. These boundaries, however, are not sharply defined. For instance, mitochondrial Na^+ has emerged as a second messenger that can regulate inner membrane fluidity [10], and K^+ as a cofactor for mitoribosomes and kinases involved in pyruvate and branched-chain α-keto acid dehydrogenases, influencing their activity. Or, Ca^{2+}, known as a signaling molecule, modulates osmotic changes by regulating Na^+ and K^+ levels [9].

Among all metal ions, the importance of mitochondrial Ca^{2+} or K^+ homeostasis has been amply reviewed [7,11–14]. Mitochondrial Ca^{2+} homeostasis has a key function in the regulation of ATP synthesis. However, mitochondrial Ca^{2+} overload has far-reaching consequences on the activation of ROS, free fatty acids [15], inflammatory molecules [6,16] and the initiation of cell death by inducing the permeability pore transition [17]. K^+ is known to maintain the mitochondrial membrane architecture and the functional stability required for oxidative phosphorylation [7,18]. The osmolytic nature of K^+, which obligatorily moves together with water, places K^+ homeostasis in a special position for controlling the mitochondrial matrix volume, preventing excessive matrix contraction or matrix swelling, and the mitochondrial integrity [7]. As mitochondrial swelling has likely consequences on the membrane potential and substrate oxidation [19], maintaining the proper

* Corresponding author.
E-mail address: Karin.nowikovsky@vetmeduni.ac.at (K. Nowikovsky).

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mitochondrial K^+ homeostasis is also crucial for preserving the electrochemical gradient and managing ROS [18].

1.2. Inner mitochondrial membrane and ion transport

Central to cation homeostasis, osmotic flexibility and signal transmission are the mitochondrial membranes [7,20,21]. At the interface between cytosol and mitochondria, the outer membrane (OM) serves as a platform for interorganellar communication. The inner membrane (IM) is where cytosolic and mitochondrial ions are exchanged and oxidative phosphorylation takes place. The IM, with the densest protein content of all cellular membranes and the signature lipid cardiolipin [22], surrounds the matrix and can fold and create deep invaginations named cristae. In contrast to OM, which has a porous and permissive nature to ions and small and uncharged molecules, and a low stretch capacity [23], the IM forms a highly selective barrier to all ions and molecules, which can only cross the membrane through dedicated transport proteins.

The nature of mitochondrial ions is similar to that of cytosolic ions, but adjustments of cytosolic to mitochondrial levels need to be dynamically regulated by uni- and bidirectional transport systems. The tight barrier function of the IM enables the adjacent matrix to maintain a pH of about 7.8–8, well above that of the intermembrane space, essentially generating the electrochemical gradient required for ATP production [24]. As the movement of cations through the inner membrane is a function of the electrochemical gradient, cation homeostasis is especially challenged by the negative membrane potential ($-\Delta\Psi_m$), created by the electron transport chain. $-\Delta\Psi_m$ can drive the accumulation of cations into the matrix at concentrations dictated by the electrochemical equilibrium, which exceed the cytosolic concentrations. At a $\Delta\Psi_m$ is ~-180 mV, the electrochemical equilibrium of a monovalent cation would be reached after the accumulation of a mitochondrial concentration a thousand times higher than that of the cytosolic and that of a divalent cation with a concentration a million times higher [25]. Such an unphysiological equilibrium calls for compensation of electrophoretic cation entry by cation efflux systems. In his chemiosmotic hypothesis of energy conservation, Peter Mitchell [26,27] dealt with the problem of cation leakage along the electrical gradient and postulated the presence of exchange diffusion systems in the coupling membrane to prevent excess cation accumulation. Mitchell's hypothesis stimulated numerous studies on cation transport across the mitochondrial IM, which provided a wealth of information on mitochondrial cation channels/uniporters and exchangers/carriers, and in the 70ies and 80ies on their physiological aspects (reviewed by [28]) and later on molecular identities and structures.

1.3. Impact of mitochondrial K^+ and Ca^{2+} homeostasis on cristae shape

The architecture of cristae features membrane infoldings, which host the respiratory complexes of oxidative phosphorylation (OXPHOS) and are relevant for the formation of supercomplexes and ATP synthase dimers, and "pockets" (lumen) between the cristae membranes. The pockets serve as proton pools, creating a pH-specific microenvironment, store cytochrome c (Cytc), and are held together at the tip by a junction (the Mitochondrial contact site and Cristae Organizing System (MICOS) complex) that separates the cristae lumen from the IMS. Cristae can rapidly adjust to the inner matrix volume, fold or unfold under matrix contraction or swelling. Swelling occurs proportionally to osmotic pressure when water accumulates. This happens when mitochondrial uptake of the osmolyte K^+ is not matched by the KHE, or when excess Ca^{2+} triggers PTP opening, causing water influx [29]. K^+ and Ca^{2+} homeostasis is therefore important for cristae architecture and thus for OXPHOS organization. Cristae remodeling involves cardiolipin, a facilitator of membrane curvatures, the MICOS complex and the ATP synthase dimers. The regulation of cristae junction width by MICOS is key to the release of Cytc into the IMS, which initiates cell death upon OM rupture. MICU1, the regulator of the mitochondrial Ca^{2+} uniporter, was revealed to interact with MICOS and functions as a Ca^{2+}-sensitive regulator of cristae and transmits Ca^{2+}-dependent signals for un/folding and hence for the efficiency of the electron transfer chain [30]. This interplay perfectly links Ca^{2+} homeostasis, cristae architecture and bioenergetics. An intriguing question arises as to how signals are transmitted to yeast cristae, as yeast lacks MICU1 and whether the sensing mechanism for cristae un/folding is taken over by a different Ca^{2+} sensor or a sensor for a different cation.

1.4. The mitochondrial KHE and CHE and their intricate molecular identities

The presence of a mitochondrial KHE, that exchanges K^+ but also Na^+ and Li^+ against H^+ was longtime proposed. The first evidence for the existence of KHE comes from the swelling experiments of isolated mitochondria in KOAc media of Mitchell and Moyle's (the interested readers are referred for more details to [27,28]). Extensive research has been conducted to define the properties of KHE. However, only one targeted study has identified its putative molecular structure using the irreversible KHE inhibitor dicyclohexylcarbodiimide [13]. This study identified an 82 kDa protein of unknown identity, which after subsequent extraction and reconstitution in proteoliposomes demonstrated electroneutral KHE activity [31]. The molecular identity of this KHE candidate was however never solved [32]. The KHE remained molecularly unidentified [7,28,33], until LETM1 was proposed as an essential KHE component.

While mitochondrial K^+ influx is mediated by various potassium channels [21], electroneutral KHE is the only mitochondrial K^+ efflux pathway. In contrast to K^+, Ca^{2+} efflux is facilitated by two mitochondrial Ca^{2+} exchangers, the Na^+/Ca^{2+} exchanger identified as NCLX which operates in an electrogenic way [34] and a putative Na^+-independent CHE also proposed to be electrogenically regulated [35–38]. The only CHE candidate ever proposed was LETM1 [39], as discussed below, leading to controversy as to whether LETM1 was the CHE, KHE or both, which eventually may be resolved by the recent identification of TMBIM5 as the long-sought mitochondrial CHE [40,41].

2. The essentiality of the mitochondrial inner membrane protein LETM1

Leucine zipper-EF-hand containing transmembrane protein, LETM1 is so far the best-known mitochondrial osmoregulator. The evolutionarily conserved gene *LETM1* is present in all sequenced eukaryotic organisms [42] and ubiquitously expressed and encodes the founder member of the LETM1-like family, which regroups LETM1, LETM2 and LETMD1/alias HCCR1. LETM1 family members are mitochondrial membrane proteins and share the conserved features of a transmembrane domain and a site for phosphorylation by protein casein kinase II (CK2) and protein kinase C (PKC) [42]. Recently, these proteins were included in the Solute Carrier superfamily (SLC) as SLC55A1, SLC55A2 and SLC55A3, in line with the idea that they functionally have a role in ion transport. Yet, a role in ion transport (KHE and/or CHE) has only been evidenced for LETM1. Plants, mammals, worms flies and yeast express a paralog of LETM1 [43–45]. Mammalian *LETM2* evolved by gene duplication and there is sparse knowledge on its functional role and tissue distribution. It has been suggested that rat *LETM2* is preferentially expressed in testes and sperm [44]. Human LETMD1/HCCR1 was shown to rescue the growth phenotype on fermentable and non-fermentable media of yeast strains depleted for genes controlling mitochondrial K^+/H^+ exchange [46]. Whether the phenotypic rescue is through restoring K^+/H^+ exchange has not been explored. Mouse LETMD1/HCCR1 revealed to be involved in thermogenesis of brown adipocytes, but whether by mediating ion transport is unstudied.

Two independent studies identified *LETM1* as one of the

approximately 2000 essential genes [47,48]. *LETM2* or *LETM1D* were not among the essential genes. Thus, according to the definition of gene essentiality, cell and organismal growth, survival, development and reproduction are not possible in absence of *LETM1*. In line with its essentiality, gene mutations are associated with considerable human pathogenic effects, and decreased gene dosage is associated with the development of Wolf-Hirschhorn syndrome (WHS). [49,50]. This highlights the potential clinical significance of *LETM1*. Studies in fly, worm and mouse animal models confirmed that loss of LETM1 is not viable, suggesting that it is not compensated for. Unexpectedly, a compensatory mechanism bypassing essentiality has been revealed in letm1-/- zebrafish [51]. While the majority of *letm1-/-* offspring were malformed, carried massively compromised mitochondria and only survived a few days after fertilization, a small group of *letm1-/-* offspring escaped the lethality and displayed wildtype-like appearance and mitochondria with partly healthy morphology, indicating a potential compensation in the essential function of LETM1, highlighting the importance of understanding the nature of this essential function.

3. Discovery of LETM1 and emerging functions

The congenital gene deletion syndrome WHS results from the loss of a variable-sized region on the short arm of chromosome 4. *LETM1* emerged as one of the lost genes and was described as "encoding a putative member of the EF-hand family of Ca($^{2+}$)-binding proteins" [52]. Though the function of LETM1 was completely unknown at this time, the coincidence of seizures, growth, motor and speech delay and *LETM1* haploinsufficiency suggested that *LETM1* may contribute to the neuromuscular clinical presentations of WHS [52]. The first molecular functional clues came from yeast studies. First, the Schweyen lab identified the yeast *LETM1* orthologue encoded by the open reading frame *YOL027c* as a multicopy suppressor gene for the mitochondrial Mg^{2+} transporter Mrs2p [53], and characterized it in follow-up studies as a mitochondrial IM protein and essential component of the mitochondrial KHE [54]. About the same time, the Westermann lab identified *YOL027c* in a screen for Mitochondrial Distribution and Morphology deficiency, henceforth the yeast *LETM1* ortholog is named *MDM38* (the gene and protein nomenclature for yeast is kept accordingly) [55]. Functional studies demonstrated that depletion of *MDM38* impedes growth, causes massive mitochondrial swelling and abolishes mitochondrial K^+/H^+ exchange, and that these phenotypes are rescued by overexpression of human *LETM1*. The function of the mitochondrial KHE is consistent with its roles in maintaining mitochondrial volume homeostasis and cellular K^+ cycling, and with the notion that osmoregulation is a cornerstone of mitochondrial functionality [7,26]. The subcellular localization and functional features of LETM1 were confirmed to be conserved in human [54,56], worm [57], fly [58] and trypanosome [59], suggesting an evolutionary conserved osmoregulatory function of LETM1.

The groundbreaking discovery that Ca^{2+} signaling is closely linked to mitochondrial-induced apoptosis shifted the mitochondrial Ca^{2+} field to the center of interest. The field of mitochondrial Ca^{2+} transport systems developed rapidly, culminating in solving the long molecular mystery of mitochondrial Ca^{2+} transporters. The mitochondrial Ca^{2+} uniporter MCU and regulatory subunits [60–62] and the Na^+-dependent mitochondrial Ca^{2+} exchanger NCLX [34] were identified. A basic idea driving the search for the Ca^{2+} uniporter was that the transporters are encoded by genes that are not conserved in yeast such as *S. cerevisiae* [60,61] being known to lack mitochondrial Ca^{2+} transport systems. However, as mentioned before, the identity of a CHE responsible for the Na^+-independent Ca^{2+} release pathway remained elusive. The search for it proved to be more complicated until LETM1, although conserved in yeast, became a perennial favorite candidate for CHE based on a Drosophila genome-wide screen [39]. LETM1 was then described as a high-affinity Ca^{2+} transporter [63], or Ca^{2+} channel or CHE [64]. Yeast data would argue against *Mdm38p* being a Ca^{2+} exchanger. However, the presence of a putative Ca^{2+} EF binding hand motif in human LETM1, and its involvement in Ca^{2+} transport [65] favored the idea that human LETM1 functions in Ca^{2+} sensing and transport, but still can compensate for the absence of Mdm38p. Proponents of LETM1 as a CHE suggested that LETM1 has different functions depending on whether mitochondria are equipped with a Ca^{2+} transport system or not. An increasing number of data still emerge supporting a function of LETM1 in the regulation of K^+ release and homeostasis or fiercely defending its function as a CHE. Unbiased studies that compare LETM1-mediated Ca^{2+}/H^+ versus K^+/H^+ exchange in cell and cell free systems are missing. However, they would be necessary for a consensus on whether LETM1 is a cation exchanger or an essential component of a cation exchanger, and whether the cation exchanger transports K^+ or Ca^{2+} against H^+ or both, depending on the organism.

There is also ample evidence for the connection between LETM1 and crista formation. Mitochondria with too little LETM1 had fewer cristae and swollen matrix [44,51,54,56,58] while with too much LETM1 swollen cristae and condensed matrix [43,57]. In addition, recombinant LETM-domain induces crista-reminiscent membrane invaginations [43]. However, in the context of these observations across animals and also *in vitro* systems, we note the vital role of LETM1 in different organisms as well as different tissues from the same organism, although these can have very different crista shapes [66].

Pointing to another direction, some studies conducted in yeast found that Mdm38p cofractionates with mitochondrial ribosomes and the mitochondrial insertase Oxa1p or with the mitochondrial ribosome MRPL39 and the ribosomal receptor Mba1p, and accordingly proposed a role of *MDM38* in mitochondrial translation or cotranslational protein insertion in the IM [67,68]. A transient interaction between Mba1 and Letm1, which yet remains functionally unexplored, has been reported in trypanosome, suggesting a likely evolutionary conservation of this relationship [69]. Mitochondrial translation of mitochondrial-encoded genes occurs in a membrane-attached manner and translated proteins are directly inserted into the IM and assembled into the respiratory subunits that create the electrochemical gradient. The discovery of a 14–3–3-like fold within a conserved region between the transmembrane domain and the C-terminal end of Mdm38p, which represents the site of ribosomal interaction led to the naming of this region "ribosomal binding domain (RBD)" [70]. The deletion of RBD in yeast resulted in the absence of the mitochondrial-encoded subunits of respiratory chain complexes III and IV.

Other observations were however made on RBD, and in a subsequent independent study, it was renamed as LETM domain and showed that it is crucial for LETM1 oligomerisation to high molecular weight complexes and that mutations of the most conserved residues of the LETM domain were associated with loss of LETM1 oligomerization and cristae structures. Furthermore, recombinant LETM domain was shown to promote the formation of membrane invagination. Altogether, this led to the conclusion that the LETM domain facilitates membrane bending at the junctions and rims of cristae structures [43]. In a recent study, the yeast *LETM1* orthologue *MDM38* was expressed in an *E. coli* strain deficient for the Na^+/H^+ antiporter. While confirming the K^+, Na^+ and Li^+/H^+ exchange activity of Mdm38p and excluding CHE activity, the study also presents mutational analyses which indicate that RBD/LETM domain is essential for KHE [71]. Thus, in line with the pleiotropy of LETM1, the conserved LETM domain itself may have additional functions. Future studies will be needed to evaluate whether the LETM domain is functionally conserved in connecting crista membrane bending, KHE activity and ribosomal binding. Though the functional interaction of Mdm38p with ribosomes is not yet well understood it has been suggested to be involved in the biogenesis of the respiratory chain [67]. Consistent with this idea, patient-derived cells with mono-allelic loss of LETM1 exhibited impaired mitochondrial translation and reduced respiratory capacity, suggesting LETM1 being a translational "facilitator" [72], a still vague concept lacking a model that conceptualizes the molecular role of LETM1 as translational factor.

There would be several ways to consider LETM1 as a factor required

for mitochondrial translation or membrane insertion. The aspect of a dual function of the LETM domain in ribosomal binding and cristae membrane bending would link cotranslational insertion of nascent membrane proteins in respiratory complexes and distribution of respiratory complexes in cristae. This vision implies that LETM1 could control mitochondrial translation in function of cristae architecture. From another perspective, mitochondrial protein translation could be linked to the building of an electrochemical gradient. This vision would suggest that LETM1 couples mitochondrial translation to the need of ATP production, which however may also depend on cristae formation. Experimental evidence is needed to test these views. On the other hand, the loss of osmotic balance, matrix swelling and cristae unfolding have spatial consequences that affect the maintenance and distribution of ribosomes, RNA, proteins and functions and alter membrane properties, which in turn affect the insertion of membrane-spanning proteins and membrane receptors. From this view, osmotic homeostasis seems to control the biogenesis of OXPHOS subunits and the chemiosmotic process of ATP production, suggesting that LETM1 deficiency affects translation, but secondarily to osmotic imbalance [59]. This view is supported by the facts that mitochondrial translational defects upon LETM1 depletion are reverted when osmotic homeostasis is re-established by nigericin [59,73], and that ribosomes disassociate at high K^+ level [59]. The idea that mitochondrial swelling is not downstream of mitochondrial translation and OXPHOS biogenesis is supported by the mitochondrial morphology which showed no signs of enlargement and cristae depletion in rho^0 HeLa cells that are devoid of mitochondrial genome and mitochondrial protein synthesis (rho^0) [44]. Further doubts about a primary function in mitochondrial translation come from a study on LETM1 in the evolutionary ancient protozoan *Trypanosoma brucei*, which is oxidatively active in the procyclic stage or inactive in the bloodstream stage and rho^0 in the subspecies *T. brucei evansi*. In all three types of cells, depletion of LETM1 inhibits growth and causes massive mitochondrial swelling [59]. This highlights the role of LETM1-mediated mitochondrial volume and ion homeostasis in supporting cellular metabolism. In the context of the latter study, a role of LETM1 in shaping cristae upstream of matrix swelling can also be ruled out, as *T. brucei evansi* has cristae-devoid mitochondria that are slender in the presence of LETM1 and swollen in the absence of LETM1 [59].

Despite important papers published on this topic [68,70,72], the idea that LETM1's primary function is to facilitate translation of mitochondrial transcripts has been overshadowed by the debate about the function of LETM1 in Ca^{2+} or K^+ release. The debate is still ongoing, and clarifying data from parallel approaches on LETM1-mediated K^+ or Ca^{2+} transport in proteoliposomes are missing. Unfortunately, there is only one experiment on K^+ transport mediated by reconstituted LETM1. It explored the electroneutral radiolabeled rubidium transport (Rubidium is a surrogate of K^+) into proteoliposomes that already contained a 1000-fold higher concentration of K^+ and in absence of a pH gradient. This setting was doomed to fail, so a conclusive liposome experiment on K^+ is still missing [64,74]. Residues found to activate Ca^{2+} transport were not studied for K^+ transport. Besides the unresolved cation nature that is primary transported, effects on other metabolic functions are also increasingly being observed. As such, effects on glucose utilization, pyruvate dehydrogenase activity, reactive oxygen production, respiratory complex stability and NAD^+ biosynthesis have been described [51, 63,65,70,72,75], but remained underinvestigated and therefore are not discussed here. However, it illustrates how importantly a consensus on the core function of LETM1 is needed in order to distinguish between function and effects.

Altogether, considering the debate about the core function of LETM1 from the perspective of gene essentiality speculations can be made about which one is indispensable for survival and fitness. The viability of *T. evansi*, or mammalian rho^0 cells that have been supplemented with uridine and pyruvate, argue against the mitochondrial translational function being essential. Another hint comes from a systematic screen for genetic interactions that convert essential genes to dispensable genes (thus that makes essentiality "bypassable"). It revealed that genes involved in mitochondrial transcription and translation are the most bypassable through their interaction with other bypassable genes [76]. LETM1's gene essentiality may rather derive from the conservation of mitochondrial Ca^{2+} or K^+ homeostasis and highlight the essential nature of mitochondrial Ca^{2+} or K^+ release. As mitochondria have different pathways to release Ca^{2+} and only one for K^+, this may explain the essentiality of the KHE without excluding a key role of the CHE, which may however be involved in other essential functions. The phenotypic rescue by the chemical KHE nigericin in yeast, human cells and trypanosome or monovalent cation exchanger monensin indicates compensation for loss LETM1 by restoring K^+ homeostasis, suggesting that KHE is the primary function of LETM1.

4. LETM1 and structural aspects

4.1. *LETM1 topology N_{out} C_{in}?*

Early studies on the topology of LETM1 included orthologues from yeast, human and mouse and consisted in proteinase K protection assays. These assays mainly used mitochondria incubated in isotonic or hypotonic isolation media to distinguish between intact mitochondria and mitoplasts [44,54,56] or in the presence of detergent [77], which permeabilize the outer or both membranes proportionally to increasing concentrations. Probing western blots with antibodies against the C-terminal part of LETM1 or the C-terminally fused HA [44,54,56] or the HA tag N-terminally inserted after the targeting sequence indicated that full-length LETM1 was protected from proteinase K as long as the inner membrane remained intact. Tamai et al. show partial degradation in mitoplasts, suggesting a region exposed to the IMS [44]. The studies proposed that the C-terminal part faces the matrix and the N-terminal part faces the IMS, and the topology was consistent with the existence of a single TM spanning region.

However, newer methods have been developed to provide high throughput topological information based on mass spectrometry of *in situ* radical-labeled tyrosine residues [78]. Phenoxyl radical labeling can be generated *in situ* by genetically targeted peroxidase (APEX) in any submitochondrial compartment. Using this approach, protein architecture mapping analysis of LETM1 proposed a second possible TM region (TM2) [78]. The suggestion for TM2 has a fundamental implication on that topology, as with two TMs, N and C ends would be facing the same side. The proposed TM2 stretch is relatively short and could be embedded in or cross the IM. The presence of TM2 implicating a matrix-sided N-terminus would radically change our view of the LETM domain, which instead of being fully located in the matrix would be split into a first part facing the IMS and a second part facing the matrix. With the LETM domain being the most evolutionary conserved protein stretch, fundamental changes in its topology would also affect LETM1 orthologues and has consequences on previous findings. Indeed, the finding that PINK1 phosphorylates LETM1 at the residue T192 was supported by the fact that PINK1 distributes in the OM, IMS and IM but not in the matrix, and can phosphorylate T192 because it faces the IMS [79]. This phosphorylation is important since overexpression of phosphorylated LETM1 increases the Ca^{2+} transport activity. Thus, the confirmation of TM2 would question the interaction between LETM1 and PINK1 and its effect on Ca^{2+} transport (Fig. 1). The driving force for crista membrane bending was attributed to the residues D359 and the R382/G383/M384 (RGM) residues. With a single TM, this driving force is applied from the matrix side, with two TMs from the IMS side (Fig. 1), both possibilities implying different mechanistic models. The presence of TM2 also restricts the ribosomal binding portion of the LETM domain, which could be assessed by mutational analysis. So far, mutational analysis on Ca^{2+} transport have not yet identified residues involved in Ca^{2+} binding or sensing, except for E221. A change of the LETM1 topology also affects the orientation of E221, which is on the matrix side with one TM and would shift to the IMS side if TM2 is confirmed. The

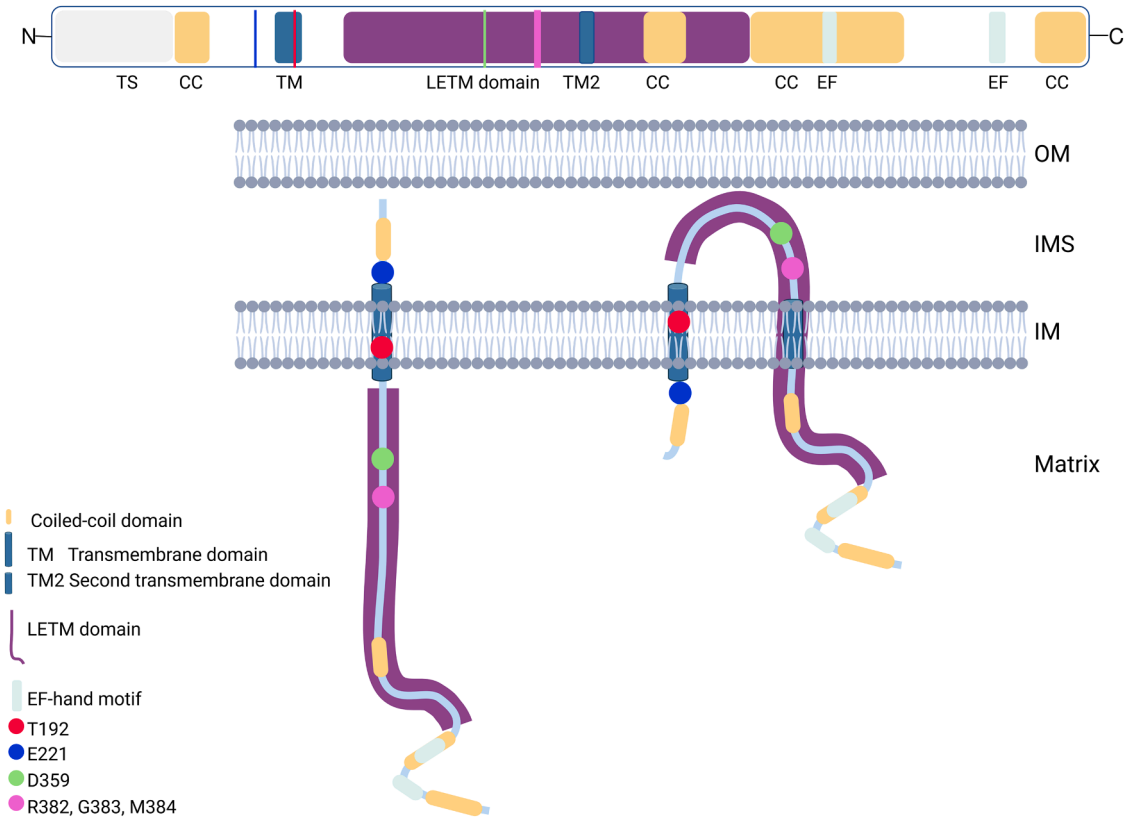

Fig. 1. LETM1 structure, LETM domain topology and implication for residues with roles in Ca^{2+} transport activation, posttranslational modification, and crista membrane bending. Schematic illustration of the LETM1 structure depicts the coiled coil domains (CC), EF hand motifs (EF) transmembrane domains TM and TM2 and relevant residues in the indicated color codes and the proposed topologies.

same applies to the conserved counterpart in Mdm38p, E152, which is essential for KHE [71]. While the orientation of the C-terminus, which carries different numbers of coiled coil segments and the putative EF binding hand motifs, would not be concerned by the presence of TM2, and the number of TMs may not challenge all functional aspects, it will be important to consider the role of the LETM domain orientation for

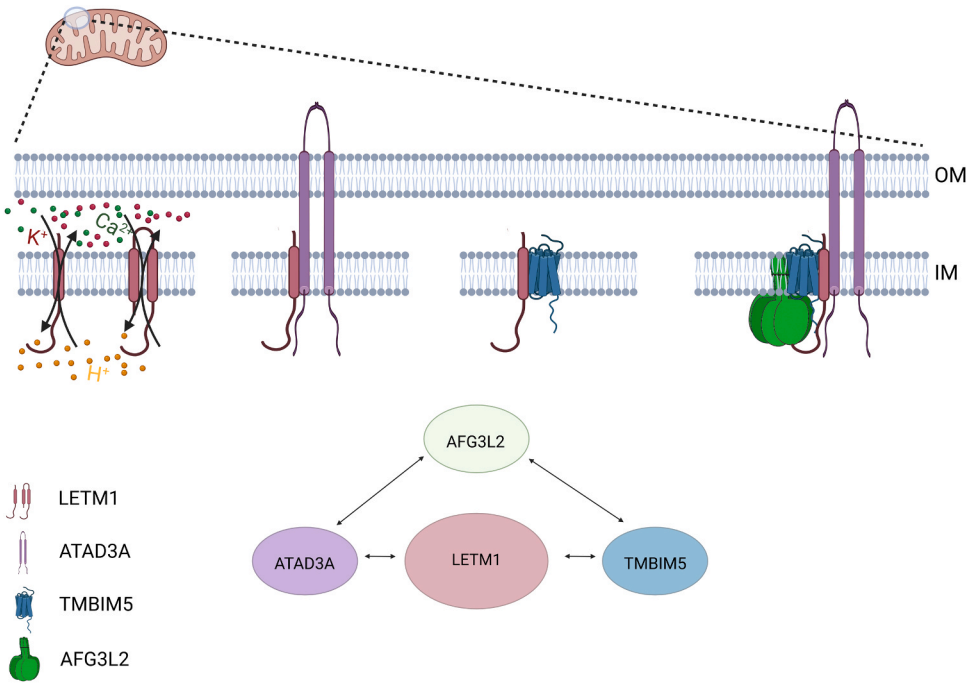

Fig. 2. Interconnections of the novel interactors of LETM1. Schematic representation of monomeric LETM1, the interactors LETM1 and ATAD3A or LETM1 and TMBIM5 or with both and AFG3L2, the common interaction partner of TMBIM5 and ATAD3A.

Ca^{2+} or K^+ transport.

New inspection of the LETM1 topology based on previous proteinase K protection assay does not globally contradict the two-transmembrane theory. In mitoplasts, in which the OM is osmotically detached, proteinase K cleaves all parts of inner membrane proteins that protrude to the IMS. Thus, if the N-terminal side faces the IMS, and is therefore accessible to proteinase K, western blot would show cleaved LETM1 if the C-terminal end of LETM1 is decorated with the antibody, or absence of LETM1 if the N-terminal side is decorated with the antibody. However, data show that full length of LETM1 is resistant to proteinase K treatment. This would rather indicate that N-and C-terminal ends are protected from proteinase K accession by the IM, thus both ends facing the matrix.

To dispel speculations on the topology with its possible and significant interactional and functional implications, future biochemical proteinase K protection assays will need to carefully validate the number of TM.

4.2. LETM1 and interactional aspects

LETM1 appeared as high confident in the interactome of other proteins. For example, Moraes's lab showed recently that human AAA domain-containing protein 3 member A (ATAD3A) associates with several mitochondrial proteins of the inner membrane like AFG3L2 among others, and one of them being LETM1 [80] (Fig. 2). LETM1 interacts through its C-terminus with the C-terminal region of ATAD3A. ATAD3A is a nuclear-encoded mitochondrial membrane protein and member of the ATPase protein family. ATAD3A spans the OM and the IM and its C-terminus locates in the matrix and contains two Walker motifs (A and B) that control the ATP catalytic activity of the ATPase, with the domain A having a AAA+ domain binding function. Mutational analysis excluded the requirement of the Walker motif A for the interaction with LETM1. Mentionable is that ATAD3A also associates with the assembly factor for respiratory complex III, BCS1L, and that previous studies found BCS1L and LETM1 forming a protein complex [44]. Functionally, ATAD3A is involved in many processes like mitochondrial DNA (mtDNA) maintenance through controlling the binding of TFAM to mtDNA, thus affecting nucleoid organization, mitochondrial fragmentation, cholesterol trafficking at contact sites, IM and cristae organization [81]. Mitochondrial defects in nucleoids organization, increased fragmentation and cristae loss are also known for LETM1. While the physiological role of the interaction between LETM1 and ATAD3A is still not clear, data from [80] may connect LETM1 and ATAD3A in the incorporation of proteins into the membrane or in the organization of the cristae. So far, we even do not know whether the function of ATAD3A is impaired in absence of LETM1, or that of LETM1 in absence of ATAD3A, thus whether LETM1 or ATAD3A are indispensable for each other.

A proteomics screening for the LETM1 interactome identified TransMembrane Bax Inhibitor Motif containing protein 5 (TMBIM5) as interaction partner (Fig. 2). Co-IP and reverse co-IP studies confirmed the interaction and functional studies in intact, permeabilized cells and liposomes characterized TMBIM5 as the long-sought mitochondrial Ca^{2+}/H^+ exchanger [40]. The discovery of TMBIM5 a Ca^{2+}/H^+ as an interaction partner of LETM1 raised the question of whether TMBIM5 needs LETM1 for its Ca^{2+} exchanging activity or the other way around, LETM1 requires TMBIM5 for K^+/H^+ exchange. To address these questions, both proteins were depleted one at a time and the K^+ and Ca^{2+} exchange activity of the mitochondria was measured. Results showed that while LETM1 knockdown impedes H^+-dependent K^+ fluxes, the presence or absence of LETM1 does not affect TMBIM5-mediated mitochondrial Na^+-independent Ca^{2+} efflux. However, in the inverse setting, absence of TMBIM5 abolishes Na^+-independent Ca^{2+} efflux and impedes on LETM1-mediated H^+-dependent K^+ fluxes. This points to TMBIM5 interaction with LETM1 or TMBIM5-mediated fluxes being required for the KHE, a point currently under investigation.

In a parallel study on the m-AAA protease AFG3L2, Patron et al. identified TMBIM5 as an interaction partner [41]. The study also confirmed the function of TMBIM5 as mitochondrial CHE exchanger, and based on deletion data proposed that TMBIM5 was necessary to inhibit the proteolytic activity of AFG3L2. Combining the findings of the interaction studies by the Moraes, Langer and Nowikovsky labs may indicate a network linking ATAD3A, LETM1, TMBIM5 and AFG3L2 (see Fig. 2). An additional study by the Methner lab confirmed that TMBIM5 controls LETM1-mediated KHE activity and supports the involvement of TMBIM5 in mitochondrial Ca^{2+} transport, however not as CHE [82].

The interactions between LETM1 and TMBIM5 or ATAD3A and between AFG3L2 and TMBIM5 or ATAD3A (Fig. 2) are remarkable and may indicate common or interdependent functions that would be promising to investigate. The consequences of *ATAD3A* deletion on the KHE function of LETM1 have not yet been examined and TMBIM5 is the first identified LETM1 interactor that is required for LETM1-mediated KHE activity [40,82]. An intriguing point is that yeast has no Ca^{2+} transport pathways and no TMBIM5 counterpart, and that in yeast, LETM1-mediated KHE activity functions independently of other mitochondrial Ca^{2+} transporters. Further studies on LETM1 mapping the residues required for intermolecular interactions and determining the functional, spatial and temporal nature of the interactions in different organisms would provide new insights helping to understand LETM1 function and effects.

4.3. LETM1 and pathogenicity

The first known disease associated with LETM1 is WHS, based on the phenotype–genotype correlation linking seizures and LETM1 haploinsufficiency. A later study confirmed the correlation between seizures and decreased LETM1 expression levels in patients with temporal lobe epilepsy. The same study revealed that seizure susceptibility increases with decreasing LETM1 in an epilepsy rat model in which seizures are induced with pilocarpine, and that knockdown of LETM1 in rats leads to earlier onset, increased frequency and duration of seizures [83]. Other studies using animal model systems support the involvement of LETM1 in neuronal functions. Depletion of the fly LETM1 orthologue CG4589 showed decreased locomotor activity and synaptic neurotransmitter release as well as increased neuronal firing [58,84]. Since then, the number of known pathogenic conditions affecting the brain and involving LETM1 is growing [85]. Screening genes that show decreased synaptic numbers [86] identified LETM1 among 19 other candidates which are associated with axonal degeneration characteristics morphological anomalies. A recent study suggests a link between LETM1-caused mitochondrial Ca^{2+} overload and loss of long-term memory [87]. The well-established role of mitochondrial Ca^{2+} dynamics in modulating Ca^{2+}-dependent neuronal functions explains the focus on investigating the Ca^{2+} role of LETM1 in neuronal firing rather than its role in mitochondrial translation, OXPHOS, or K^+ homeostasis, which could potentially also be involved in or underlie altered neuronal metabolism and excitability.

A study on *LETM1* bi-allelic mutations widens the spectrum of clinical presentations [49]. In addition to neurological disorders, they include optic atrophy, bilateral cataracts, sensorineural deafness, diabetes, myopathy, spastic ataxia and developmental regression. Studies of fibroblasts from patients with LETM1 haploinsufficiency in WHS or bi-allelic LETM1 variants indicate several mitochondrial dysfunctions. In WHS patients hemizygous for LETM1, there is mtDNA nucleoid aggregation and decreased mitochondrial translation. Overlapping dysfunctions are observed in fibroblasts from both WHS and bi-allelic LETM1 variant patients. These include mitochondrial fragmentation, cristae disorganization, matrix swelling, and decreased levels of certain OXPHOS subunits. A significant aspect, demonstrated for the first time in patient cells with pathogenic *LETM1* mutations, is the clustering of most of these mutations in the LETM domain, leading to KHE inhibition. Consistent with the function of LETM1 in maintaining K^+ homeostasis,

mitochondrial morphological phenotypes such as fragmentation and increased volume are restored by the chemical KHE nigericin. These morphological changes are phenocopied by the K^+ ionophore valinomycin and reversed by nigericin in control cells, pointing to deficient K^+ homeostasis being likely a cause of the observed phenotypes.

The effects of nigericin on mtDNA have not yet been investigated in patient cells, and it could be speculated that nucleoids organization is affected by loss of interaction with ATAD3A. Considering the different genetic background of patient cells, but also the pleiotropic effects and the numerous interactors of LETM1, which may be associated with similar neurological and muscular diseases, it would be of great benefit for future rescue studies to exclude phenotypes that are not rescued by the reintroduction of wildtype LETM1.

5. Conclusion

Independent studies over the years have uncovered many aspects of LETM1's role, which while in some important aspects remain controversial, depict a very pleiotropic picture and raise many unanswered questions. For example, what is its core function and how does it affect other functions? Is this function universal or dependent on the organism, perhaps varying across tissues or developmental stages? How does LETM1's interactome modulate its function, or vice versa? Will future consideration of LETM1 in the landscape of its interactors explain the manifold pleiotropic effects of LETM1? Addressing these questions is crucial for better understanding the essentiality of LETM1 and the pathogenic consequences of LETM1 mutations. While we are still far from understanding the full pleiotropy of LETM1, we have many pieces of the puzzle that, once comprehensively assembled, can resolve the current controversy and guide future research efforts.

CRediT authorship contribution statement

Karin Nowikovsky: Writing – original draft, Funding acquisition, Conceptualization. **Sami E. M. Mohammed:** Writing – original draft, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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