LETM1 is required for mitochondrial homeostasis and cellular viability (Review)

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Abstract. Leucine zipper/EF-hand-containing transmembrane protein 1 (LETM1) has been identified as the gene responsible for Wolf-Hirschhorn syndrome (WHS), which is characterized by intellectual disability, epilepsy, growth delay and craniofacial dysgenesis. LETM1 is a mitochondrial inner membrane protein that encodes a homolog of the yeast protein Mdm38, which is involved in mitochondrial morphology. In the present review, the importance of LETM1 in WHS and its role within the mitochondrion was explored. LETM1 governs the mitochondrion ion channel and is involved in mitochondrial respiration. Recent studies have reported that LETM1 acts as a mitochondrial Ca2+/H+ antiporter. LETM1 has also been identified as a K+/H+ exchanger, and serves a role in Mg2+ homeostasis. The function of LETM1 in mitochondria regulation is regulated by its binding partners, carboxyl-terminal modulator protein and mitochondrial ribosomal protein L36. Therefore, we describe the remarkable role of LETM1 in mitochondrial network physiology and its function in mitochondrial-mediated cell death. In the context of these findings, we suggest that the participation of LETM1 in tumorigenesis through the alteration of cancer metabolism should be investigated. This review provides a comprehensive description of LETM1 function, which is required for mitochondrial homeostasis and cellular viability.

1. Introduction

Wolf-Hirschhorn syndrome (WHS) patients display pleiotropic phenotypes including growth delay, intellectual disability, congenital hypotonia, distinct facial appearance, congenital heart defects, midline defects and seizures (1,2). The deletion of either of two critical regions (WHSCR and WHSCR-2) within chromosome band 4p16.3 has been proposed to be necessary for minimal clinical manifestation of WHS (3,4). A gene within WHSCR-2, leucine zipper/EF-hand-containing transmembrane protein 1 (LETM1), which is deleted in almost all patients with the full phenotype, and was therefore suggested as an excellent candidate gene for the seizure development (2,3,5). LETM1 was proposed to contribute to the neuromuscular features of WHS patients (6). Multiple genes are responsible for the characteristics of WHS disorders; thus, mouse models have been developed to complement ongoing patient-based studies (7). Phenotypes of a genetically modified mouse model for FGFR3, MAEA, Sax2/Nkx1-1 and CTBP1 showed skeletal malformations, hematopoietic dysgenesis, post-natal growth defects, and later growth defects, respectively (7). The most severe pathogenic phenotype of WHS is epilepsy, which has been shown in mouse lines carrying TACC3 and FAM53A mutations (7,8). LETM1 is also accepted to be tightly linked to the epilepsy pathogenesis in WHS. In Drosophila melanogaster model, neuronal-specific knockdown of LETM1 resulted in the impairment of locomotor behavior in the fly and reduced synaptic neurotransmitter release. These results revealed the function of LETM1 in epilepsy: One of the severe pathogenic phenotypes of WHS (2,9). Decreased LETM1 levels in the neo-cortices of temporal lobe epilepsy patients and in the hippocampi and adjacent cortices of rats

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following the onset of seizures has suggested that reduction of LETM1 contributes to the seizure phenotype. Knockdown of LETM1 leads to reduced MT-CYB expression and induction of susceptibility to seizures, which suggested that dysfunctional LETM1 together with mitochondrial swelling and disturbed MT-CYB expression is important for the deteriorated behavioral phenotype of epilepsy (10). However, Nigericin, a K+/H+ ionophore, fails to prevent epileptic seizures, and does not reverse lentivirus-shLETM1-mediated facilitation of epilepsy, indicating that further studies are required to determine the detailed mechanism of LETM1 function and delineate the involvement of LETM1 in seizure pathogenesis (10). Therefore a detailed understanding of LETM1 function will provide great advantages for WHS patients.

2. LETM1 functions in mitochondria

LETM1 structure and localization. LETM1 is a protein with a molecular mass of 70 kDa; it has also been identified as a 83-kDa endogenous protein in HeLa cells, suggesting that it is synthesized as a cytosolic precursor with a presequence (11). LETM1, like its yeast counterpart Mdm38 (12), is a mitochondrial inner membrane protein (1,9,13); it comprises a C-terminal domain bearing two EF-hand domains (578-590 and 676-698) and an N-terminal domain bearing a protein kinase C phosphorylation site (Fig. 1A) (6,14). LETM1 is an integral mitochondrial inner membrane protein facing toward the matrix (Fig. 1B, right). LETM1 is co-localized with HSP60, a mitochondria matrix protein (11). Partial digestion of LETM1 under hypotonic conditions has suggested that LETM1 contains a small and/or protease-resistant region exposed to the inner membrane space (11). The N-terminus of LETM1 is located in the inter-membrane space, connected to the inner membrane by a transmembrane domain containing three conserved proline residues (206-228), and the C-terminus extends to the mitochondrial matrix (6,15) [Fig. 1B, left (1)]. However, a recent study (16) revealed that LETM1 contains another transmembrane domain, from residues 413 to 421. This discovery has led to controversy regarding LETM1 N-terminal localization, which was strongly suggested to face the matrix [Fig. 1B, left (2)] (15).

LETM2, a homolog of LETM1, has already been identified (11,17). The LETM2 gene is located at human chromosome 8 and was also found in rat through a homolog search using human LETM1 cDNA (17). LETM2 is a mitochondrial inner membrane protein that shares some features with LETM1 (e.g., they are both mitochondrial inner membrane proteins with a leucine-zipper motif); however, it has a function distinct from that of LETM1, as demonstrated by its failure to suppress mitochondrial swelling caused by LETM1 knockdown (11). LETM1 has been detected in nearly all rat tissues, whereas LETM2 is expressed specifically in the testis, with a molecular size of 45 kDa, during the developmental stages from spermatocyte to spermatozoon. These expression environments indicate its probable function in inner and cristae membrane reorganization in the mitochondria during spermatogenesis (11).

LETM1 regulates mitochondrial ion-channels. As indicated in Fig. 2, LETM1 was recently reported to function as a mitochondrial ion channel regulator. The presence of two putative Ca2+ binding sites on LETM1 suggests that impaired mitochondrial Ca2+ homeostasis due to lack of LETM1 could explain the seizures observed in some WHS patients (1). An RNAi screen was conducted to identify the genes that control mitochondrial Ca2+ transport in Drosophila S2 cells which stably express mitochondria-targeted ratiometric pericam. CG4589, the Drosophila homolog of the human gene LETM1, was identified as a gene strongly affecting (Ca2+)m and (H+)m responses (18). LETM1 catalyzes the 1:1 electrogenic exchange of Ca2+ for H+ which is driven by the negative potential of mitochondria and by protons leaving the mitochondrial matrix (18,19). The mitochondrial calcium uniporter (mCU) drives rapid and massive Ca2+entry, but only at the high cytosolic Ca2+ concentrations (>10 µM) that are reached in microdomains near Ca2+ release channels on the endoplasmic reticulum (ER) (20). The new Ca2+/H+ antiporter operates at low cytosolic Ca2+ concentrations (<100 nM) and is limited by the pH gradient generated by the mitochondrial electron transport chain (21). LETM1 shares a role with mCU in catalyzing Ca2+ uptake into mitochondria; this role can be inhibited by ruthenium red. Thus, unlike mCU, which is critical only for Ca2+ uptake, LETM1 catalyzes Ca2+ uptake into mitochondria in exchange for H+, implying that proton efflux from mitochondria can drive LETM1-dependent Ca2+ entry into mitochondria. mCU conveys rapid Ca2+ transients from the cytosol to the matrix but exposes mitochondria to Ca2+ overload and alterations in ER Ca2+ handling. LETM1 is bidirectional and can extrude Ca2+ along with the Na+/Ca2+ exchanger during large Ca2+ loads, allowing mitochondria to bear small cytosolic Ca2+ elevations without risking Ca2+ overload (21). Knockdown and overexpression of LETM1 in cells has been shown to alter [Ca2+]m and [pH]m responses in a pattern consistent with Ca2+/H+ exchange (18). Reconstitution of the purified protein in liposomes confirmed that LETM1 mediates Ca2+/H+ exchange. This transport is electrogenic and therefore blocked by ruthenium red. Functional data in LETM1-depleted HeLa cells have indicated that the new antiporter can mediate both Ca2+ uptake and Ca2+ extrusion from mitochondria; however, these observations remain to be confirmed by simultaneous Ca2+ and pH measurements during physiological stimulation (18). LETM1 and UCP2/3 independently contribute two molecularly distinct pathways for mitochondrial Ca2+ uptake in endothelial cells. Knockdown of LETM1 resulted in highly depleted sequestration of entering Ca2+, but had no effect on Ca2+ uptake at ER-mitochondrial junctions, suggesting that LETM1 and UCP2/3 contribute Ca2+ uptake from different sources and at different Ca2+ concentrations (22). However, it has been proposed that LETM1 works as a Ca2+/H+ antiporter in mitochondria, but also that LETM1 functions as a mitochondrial K’/H’ exchanger (12). Measurements in isolated mitochondria labeled with K+ and H+ fluorescent dyes confirmed the presence of a K’/H’ exchanger in the inner mitochondrial membrane of yeast (12). Mutants in the yeast LETM1 homolog Mdm38, which lacks the two Ca2+ binding sites present in LETM1, cause loss of mitochondrial K’/H’ exchange activity, resulting in highly abundant K’ within the mitochondrial matrix and low membrane potential (∆Ψm), followed by water influx and organelle swelling (23,24). LETM1 expression restores Mdm38 and potassium acetate indicating
Figure 1. LETM1 structure and localization. (A) Schematic representation of LETM1 and LETM2. Specific domains and sites are symbolized by respective colors as indicated. (B) Sub-mitochondrial localization of LETM1. OM, Outer Membrane; IM, inner membrane; LETM1, leucine zipper/EF-hand-containing transmembrane protein 1.

Figure 2. Regulation of ion-channels in mitochondria by LETM1. MCU drives massive calcium entry with high cytosolic Ca\(^{2+}\) concentrations (>10 µM) in microdomains near Ca\(^{2+}\) release channels on ER but LETM1 functions as Ca\(^{2+}\)/H\(^+\) antiporter, at low cytosolic Ca\(^{2+}\) concentrations (>100 nM). The pH gradient generated by the ETC limits its action. It can also extrude Ca\(^{2+}\) along with the NCLX during Ca\(^{2+}\) overload. Furthermore, it also functions as the K\(^{+}\)/H\(^+\) exchanger as well as a Mg\(^{2+}\) transporter. ETC, electron transport chain; MCU, Mitochondrial Ca\(^{2+}\) uniporter; NCLX, Na\(^{+}\)-Ca\(^{2+}\) exchanger; VDAC, voltage dependent anion channel; SR, sarcoplasmic Reticulum; ER, endoplasmic reticulum; LETM1, leucine zipper/EF-hand-containing transmembrane protein 1.
a defect in $K^+/H^+$ exchange activity-induced swelling (23). Furthermore, Mdm38 depletion resulted in an early loss of $K^+/H^+$ exchange-mediated swelling capacity of mitochondria, loss of $\Delta \Psi m$ and mitophagy during the extensive interaction of mitochondria with vacuoles (24,25). These effects strongly support the notion that $K^+/H^+$ exchange activity is the primary cause of morphological changes in mitochondria, which in turn trigger the process of mitophagy (24). This evidence of mitophagy induction via Mdm38 depletion feasibly suggests that LETM1 regulates mitophagy and cell viability.

Mitochondrial Mg$^{2+}$ transport processes may play an important role in cellular Mg$^{2+}$ homeostasis and the regulation of cell and mitochondrial functions (26). MRS2 encodes a mitochondrial membrane protein, that is an essential component of the major electrophoteric Mg$^{2+}$ influx system in mitochondria (26). Mutant alleles of MRS2 and its over-expression increase intra-mitochondrial Mg$^{2+}$ concentrations. Two yeast homolog genes of LETM1, MRS7 and YOL027, are multicopy suppressors of MRS2D (disrupting the open reading frames of MRS2 and defective in mitochondrial Mg$^{2+}$ influx, respectively), whereas Yol027p overexpression may improve Mg$^{2+}$ influx in MRS2D cells (23,26). Furthermore, Mdm38 mutation suppresses the growth deficiency caused by the mutation in the MRS2 gene (27,28). Together, these findings suggest LETM1 acts as an Mg$^{2+}$ transporter in the mitochondrion.

**LETM1 and its partners cooperate to influence mitochondria morphology, respiration and biogenesis.** Yeast Mdm38, a homolog of LETM1 was identified in a comprehensive genome-wide screen for mutants with mitochondrial morphology defects (29). Within LETM1-knockdown cells, mitochondrial swelling and the disorganization of cristae structures were observed using electron microscopy (11). LETM1 knockdown caused mitochondria to become dot-like structures and lose their tubular networks to a significantly greater extent than fragmented mitochondria observed in OPA1-knockdown cells (11,30). Images of mitochondria lacking LETM1 were reminiscent of observations following overexpression of pro-fission proteins such as Fis1 (31) or knockdown of pro-fusion proteins stf1g such as OPA1 (32). In both cases, inhibition of the dynamin-related protein Drp1-dependent fission machinery, by silencing or using a dominant negative, caused the Drp1K38A mutant to recover its mitochondrial morphology (33,34). Nevertheless, Drp1 inhibition in LETM1 knockdown cells, did not recover the fragmented phenotype, indicating that blockage of the fission machinery is not induced by reduction of LETM1 and that lack of LETM1 causes Drp1-independent mitochondrial fragmentation (30). Moreover, following the silencing of both Drp1 and LETM1, mitochondria remained partly swollen, suggesting that mitochondrial membrane fusion is unaffected by downregulation of LETM1 or co-silencing of Drp1 (11). These results suggest that LETM1 is not directly implicated in mitochondrial membrane fission and fusion. Thus, neither lack nor excess of LETM1 is beneficial to cells, and fragmented mitochondria and swollen cristae were observed in LETM1-overexpressed HeLa cells (14).

LETM1 downregulation caused reduced numbers, and morphological changes in cristae, leading to a substantially lower membrane potential ($\Delta \Psi m$), which is consistent with reports that isolated mitochondria from yeast Mdm38 mutants exhibited low membrane potential (23,35); however, no significant changes in $\Delta \Psi m$ were observed between controls and LETM1-transduced cells (14). LETM1 knockdown caused the disassembly of three different proton pumps complex I (NADH dehydrogenase), III (cytochrome b complex), and IV (cytochrome c oxidase) (36), suggesting that LETM1 regulates the biogenesis of respiratory chains. Low membrane potential appears to be a consequence of the failure to form super-complexes: Complexes I, III and IV of the respiratory chains (11).

Therefore, LETM1 is critical for respiratory chain biogenesis, being physically associated with mitochondrial ribosomes to mediate membrane insertion of several proteins of nuclear and mitochondrial origin, and facilitating their transport across the inner membrane (24,35). A ribosome-associated site has been identified on the LETM1 ribosome binding domain (RBD); it displays a matrix-exposed 14-3-3-like fold, which is critical for respiratory chain assembly through the regulation of Cox1 and Cytb translation (37). Mitochondrial ribosomal protein L36 (MRPL36) has been reported to be associated with the inner-membrane (38). The addition of puromycin was used to access possible interactions between LETM1 and MRPL36; LETM1 associated with MRPL36 both in vivo and in vitro. MRPL36 siRNA significantly recovered LETM1-mediated ATP reduction, suggesting that LETM1 may regulate the mitochondrial translation system and reduce mitochondrial biogenesis through association with MRPL36 (14). The inhibition of mitochondrial biogenesis by LETM1 offers a possible explanation of WHS phenotypes, especially neuromuscular defects and seizures, which likely reflect oxidative phosphorylation defects and thus resembles classical mitochondrial encephalomyopathies such as mitochondrial encephalomyopathy, lactic acidosis, and stroke like episodes (35).

Cells lacking mitochondrial DNA lose active respiratory chains, but maintain mitochondrial tubular networks, indicating that mitochondrial swelling caused by LETM1 knockdown is not caused by the disassembly of respiratory chains (11). Human AAA-ATPase BCSIL, which is a mitochondria inner membrane protein, is responsible for human disorders and the assembly of respiratory chains (39-41). BCSIL interacts specifically with LETM1, stimulating the formation of the LETM1 major complex; thus, BCSIL and LETM1 function in different process to maintain mitochondria and form tubular network structures (11).

**Role of LETM1 in mitochondrial quality control.** The term mitophagy was first introduced by Dr J.J. Lemasters for the selective autophagic degradation of damaged and dysfunctional mitochondria (42). An accumulation of studies suggest that mitochondrial dysfunction and morphological changes, which are interrelated factors, are responsible for the induction of mitophagy (24,43,44). Mitochondrial fission produces two subsets of daughter mitochondria with either increased or decreased membrane potential. The depolarized daughter mitochondrion, which is incapable of fusing into the polarized network, is removed through the process of mitophagy (44). Loss of $K^+/H^+$ activity and subsequent
Mitochondrial morphology is also strongly associated with energy metabolism, as mitochondria with increased respiration levels appear in morphologically interconnected networks with enlarged cristae compartments, whereas those with low respiration and therefore high glycolysis levels are fragmented, with smaller inter-cristae space (62). LETM1 has been demonstrated to cause such fragmented morphology (24,45) along with altered energy metabolism leading to tumorigenesis (14). Taken together, these findings demonstrate that the role of LETM1 in mitochondrial volume and morphological homeostasis is critical to determining cell viability and correlation with tumorigenesis.

LETM1 contributes to cancerous metabolic alteration. In 1931, Otto Warburg was awarded the Nobel Prize for oncology for his breakthrough hypothesis and research on cancerous metabolism (63). He observed that cancer cells are predominantly dependent on energy produced by glycolysis, rather than by pyruvate oxidation in mitochondria (57). Although the exact mechanisms responsible for this metabolic alteration remain to be elucidated, mitochondrial respiratory defects, due in part to mitochondrial DNA mutations/deletions and hypoxia, are thought to be important contributing factors. Mitochondrial respiration deficiency and an increase in ATP
production via glycolysis, leading to activation of the PKB/Akt survival pathway through NADH-mediated inactivation of PTEN, is a novel mechanism contributing to altered cancerous metabolism (64). Consistent with these results, Piao and colleagues demonstrated that LETM1 overexpression (45); led to increased ATP production via glycolysis and increased lactate production, also causing PTEN inactivation and PKB activation, suggesting that LETM1-induced mitochondrial dysfunction resulted in PKB activation which increased the ratio of NADH/NADPH to inactivate PTEN enzyme activity. -Similar levels of LETM1 expression in cancerous tissue and in overexpressed HeLa cells, along with the detection of LETM1 in two bands from six patients who had undergone surgery for malignant cancer, strongly suggest that LETM1 overexpression is a feature of altered cancerous metabolism accompanied by metastasis (14). In contrast, AMPK activation and consequent inhibition of G1/S cell cycle progression, as well as decreased PKB and mTOR phosphorylation by LETM1 overexpression can alter lung cancer cell growth (65). Using liquid chromatography tandem mass spectrometry and a power law global error model for reliable bio-signature mapping of hepatocellular carcinoma, LETM1 was found to have increased expression level, and its potential translocation to the tumor nuclear fraction was inferred, especially to the peripheral nuclear region and outer nucleus as confirmed by western blot, immunohistochemical, and immunofluorescent analyses (66). Collectively, these finding underscore the putative role of LETM1 in altered cancerous metabolism and indicate the need for further study to explore the roles of LETM1 in different tumors, and establish it as an effective therapeutic target in cancer-treatment.

Association of LETM1 with mitochondrial ribosomes and mitochondrial ATP regulation. Mutations in mitochondrial DNA have been shown to play a key role in tumorigenesis within various organs, as these mutations lead to the malfunction or/malfunctioning of the mitochondrial respiratory chain, compelling cells to depend on glycolysis to fulfill their ATP demand (67,68). The biogenesis of respiratory chain complexes requires the synthesis of proteins encoded by the mitochondrial genome and their subsequent insertion into the inner membrane. Mdm38 has been found to be associated with newly synthesized mitochondrial proteins via the ribosome, and is specifically required for efficient membrane insertion of cytochrome b and Atp6, and polytopic membrane proteins of complexes III and V, respectively (35). The LETM1 ortholog Mdm38 plays a role in respiratory chain function at the cellular level, as demonstrated by growth defects and reduced Δψ observed in Mdm38Δ mitochondria. LETM1 partially rescues growth defects in Mdm38Δ cells, suggesting that both proteins fulfill similar cellular functions (23).

An LETM1 RBD has been shown to be important to respiratory chain assembly through regulation of Cox1 and Cytb translation; this matrix-exposed domain displays a 14-3-3-like fold (37). LETM1 overexpression has been reported to decrease mitochondrial ATP production, oxygen consumption, and MRPL36 depletion through the use of siRNA-MRPL36 to significantly revert the LETM1-mediated ATP decrease, suggesting a functional association between LETM1 and mitochondrial ribosomes (14). Thus, the association of the LETM1 family with mitochondrial ribosomes and its consequent role in the mitochondrial translation system and biogenesis highlights the putative role of LETM1 in tumorigenesis.

Figure 3. Schematic representation of proposed LETM1 function. Schematic diagram depicting the proposed physiological as well as pathophysiological roles of LETM1. It shows the functions of LETM1 in the mitochondria and the consequences of loss in these functions. LETM1, leucine zipper/EF-hand-containing transmembrane protein 1.
4. Conclusion

LETM1 has been cloned in an attempt to identify the genes deleted in WHS (6), and was found to be responsible for seizure development (4,10). Since this discovery, LETM1 has been shown to play prominent roles in mitochondrial K+ and Ca2+ homeostasis, volume, and morphology maintenance, and respiratory chain biogenesis, and to be indispensable in maintaining organelle function and subsequent tumorigenesis. Mitophagy has been reported as the selective degradation of depolarized mitochondria, as a result of lost K+/H+ exchange due to Mdm38 depletion (24); however, this process must be further explored to determine the contributing roles of the PINK/PARKIN pathway and LETM1. Studies of the possible interactions between LETM1 and mitochondrial ribosomes and its functional relevance as a translocase in mitochondrial protein synthesis and export machinery may help to establish this protein as a potential therapeutic target for various diseases such as WHS, epilepsy, cancer, and other pathophysiological conditions (Fig. 3).

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Availability of data and materials

All data used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

YL conceived the present study. QT analyzed the LETM1 literature and made the substantial contribution in the finalization of this manuscript. LP assessed the experimental data. RS and SP contributed to data interpretation and writing the discussion. JIP contributed to data interpretation and discussion, particularly regarding the mitochondria function of LETM1. JoP contributed to designing the study and was involved in data interpretation and writing the discussion. JoP also approved the final manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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