Characterization of the mitochondrial protein LETM1, which maintains the mitochondrial tubular shapes and interacts with the AAA-ATPase BCS1L

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Summary

LETM1 is located in the chromosomal region that is deleted in patients suffering Wolf-Hirschhorn syndrome; it encodes a homolog of the yeast protein Mdm38 that is involved in mitochondrial morphology. Here, we describe the LETM1-mediated regulation of the mitochondrial volume and its interaction with the mitochondrial AAA-ATPase BCS1L that is responsible for three different human disorders. LETM1 is a mitochondrial inner-membrane protein with a large domain extruding to the matrix. The LETM1 homolog LETM2 is a mitochondrial protein that is expressed preferentially in testis and sperm. LETM1 downregulation caused mitochondrial swelling and cristae disorganization, but seemed to have little effect on membrane fusion and fission. Formation of the respiratory-chain complex was impaired by LETM1 knockdown. Cells lacking mitochondrial DNA lost active respiratory chains but maintained mitochondrial tubular networks, indicating that mitochondrial swelling caused by LETM1 knockdown is not caused by the disassembly of the respiratory chains. LETM1 was co-precipitated with BCS1L and formation of the LETM1 complex depended on BCS1L levels, suggesting that BCS1L stimulates the assembly of the LETM1 complex. BCS1L knockdown caused disassembly of the respiratory chains as well as LETM1 downregulation and induced distinct changes in mitochondrial morphology.

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Key words: BCS1L, LETM1, LETM2, Mitochondrial morphology, Respiratory chains

Introduction

The leucine zipper EF-hand-containing transmembrane protein 1 (LETM1) gene was originally identified as one of the genes that are chromosomally deleted in patients with Wolf-Hirschhorn syndrome (WHS) (Endele et al., 1999). WHS is a congenital complex malformation syndrome characterized by growth delay, mental retardation, seizures, hypotonia and characteristic facial features, and is caused by the partial deletion of the distal short arm of chromosome 4 (Zollino et al., 2003). LETM1 protein is conserved between yeast and humans and is located in the mitochondria (Schlickum et al., 2004). The MDM38 gene, which encodes a yeast homolog of LETM1, was first identified by a mutation that caused enlarged mitochondria (Dimmer et al., 2002). The expression of LETM1 suppresses the growth defect of mdm38-deletion mutants on a non-fermentable carbon source, indicating that yeast Mdm38p is a functional counterpart of human LETM1 (Nowikovsky et al., 2004). The mdm38 mutation suppresses the growth deficiency caused by mutation in the MRS2 gene that encodes a mitochondrial membrane protein related to bacterial Mg2+- transporters (Waldherr et al., 1993; Gregan et al., 2001). Moreover, mitochondria isolated from the mdm38 mutant exhibit little potassium-acetate-induced swelling, suggesting that Mdm38p regulates mitochondrial K+/H+ exchange (Nowikovsky et al., 2004). However, Mdm38p binds to mitochondrial ribosomes, and a MDM38-gene deletion gives rise to impaired transport of mitochondrial proteins from the matrix, suggesting that Mdm38p has a critical role in protein export from the matrix (Frazier et al., 2006).

BCS1L is an AAA-ATPase that has a single hydrophobic segment, homologous to yeast Bcs1p. Mutations in the BCS1L gene cause three different human disorders: complex III deficiency, GRACILE syndrome and Björnstad syndrome. Complex III deficiencies are manifest in heterogeneous clinical presentations, such as neonatal proximal tubulopathy, hepatic involvement, and encephalopathy (de Lonlay et al., 2001). GRACILE syndrome is characterized by profound multisystem organ failure, including fetal growth retardation, lactic acidosis, aminoaciduria, cholestasis and abnormalities in iron metabolism (Visapää et al., 2002). Intriguingly, no defects in enzyme activity or oxygen consumption of the respiratory chains are found in patients with GRACILE syndrome. Björnstad syndrome is an autosomal recessive – but not lethal – disorder that is associated with sensorineural hearing loss and pili torti (Hinson et al., 2007). These various clinical presentations are consequences of different mutations in a single gene, BCS1L, suggesting that BCS1L functions in multiple cellular events through different downstream targets. In yeast, the bcs1 mutant was first identified as a respiratory-deficient mutant without spectroscopically...
discernible cytochrome b (Nobrega et al., 1992). Most subunits of the respiratory chains, which comprise five protein complexes (complex I to complex V), are encoded in the nucleus; only 13 subunits are encoded in mitochondrial DNA. The formation of stable complexes is coordinated by the insertion of subunits into the inner membrane, which is facilitated by several factors, including Oxa1p and chaperones (Tzagoloff, 1995; Yi and Dalbey, 2005). Yeast Bcs1p functions in loading the Rieske iron-sulfur (Fe-S) protein onto the subcomplex of complex III in an ATP-dependent manner (Cruciat et al., 1999). Despite the genetic relationship between BCS1L and human disorders with various characteristic features, the understanding of the molecular mechanisms of BCS1L is still insufficient to obtain an overview of its physiological roles.

Mitochondria are dynamic organelles that continuously divide and fuse, resulting in the maintenance of tubular network structures (Yaffe, 1999; Gripparic and van der Bliek, 2001; Mozdly and Shaw, 2003). Extensive studies have led to the identification of several factors that directly regulate mitochondrial membrane fission and fusion (Yaffe, 1999; Jensen et al., 2000; Karbowskis and Youle, 2003; Westermann, 2003; Zhang and Chan, 2007). Two of these factors, Drp1 (DNM1L) and OPA1, are dynamin-related proteins that control membrane fission and fusion, respectively (Frank et al., 2001; Olichon et al., 2003; Lee et al., 2004; Ishihara et al., 2006). Here, in HeLa cells, we demonstrated that LETM1 knockdown caused mitochondrial swelling and a reduction in the number of cristae structures. Interestingly, LETM1 downregulation had little influence on membrane fission and fusion machinery governed by Drp1 and OPA1. Moreover, LETM1 knockdown caused the disassembly of complexes I, III and IV of the respiratory chains, leading to decreased membrane potential. BCS1L interacted with LETM1, and its downregulation caused a deficiency in the formation of complexes I, III and IV (as in the LETM1 knockdown), suggesting that LETM1 and BCS1L cooperate in the biogenesis of respiratory chains. In addition, BCS1L has a distinct role in mitochondrial morphology. These findings provide crucial insight into the molecular mechanisms underlying human disorders caused by the loss of LETM1 and BCS1L.

Results

**LETM1 is a mitochondrial inner-membrane protein**

It has been reported in a previous study that LETM1 is located in the mitochondria (Schlickum et al., 2004). To confirm that LETM1 is, indeed, a mitochondrial protein, an antibody against human LETM1 was prepared by immunizing rabbits by using a recombinant LETM1 protein as antigen. The anti-LETM1 antibody specifically recognized a single band with a molecular mass of 70 kDa in HeLa cell lysate (Fig. 1A, lane 1). When LETM1 was exogenously expressed, the same band was highlighted (lane 2), indicating that the 70-kDa band corresponds to LETM1 protein. Immunofluorescence microscopic analysis using the antibody against LETM1 showed that LETM1 was detected in intracellular tubular networks and colocalized with the mitochondrial protein Hsp60 (Fig. 1B). Furthermore, subcellular fractionation analysis indicated that the 70-kDa band of LETM1 (solid arrowhead) was recovered in the mitochondrial fraction, but not in the microsomal or cytosolic fraction (Fig. 1C). Thus, LETM1 is a mitochondrial protein with a molecular mass of 70 kDa.

By exogenously expressing LETM1 a slowly migrating band with a molecular mass of 83 kDa was detected in addition to the 70-kDa band (Fig. 1A, lane 2). When LETM1 was expressed that had been C-terminally tagged with three hemagglutinin (HA) molecules (LETM1-3HA), two larger bands were also found (lane 3). On the basis of the deduced amino acid sequences, the molecular mass of LETM1 was predicted to be ~83,353. In subcellular fractionation, the 83-kDa band was detected only in the cytosolic fraction (Fig. 1C, gray arrowhead). These results suggest that LETM1 is synthesized as a cytosolic precursor with a presequence.

To examine the submitochondrial localization of LETM1, the mitochondrial fraction was treated with trypsin under either isotonic (mitochondria) or hypotonic (mitoplasts) conditions (Fig. 1D). An outer membrane protein, Tom20, was digested under both conditions, whereas the matrix protein Hsp60 was resistant to

![Fig. 1. LETM1 is a mitochondrial inner-membrane protein.](image-url)
trypsin treatment even under the hypotonic condition. An inner-membrane protein, Tim17, was degraded only under the hypotonic condition. LETM1 was resistant to trypsin treatment under the isotonic condition, but was partially digested under the hypotonic condition (Fig. 1D, lane 7). All samples were degraded in the presence of Triton X-100 (Fig. 1D, lanes 4, 8), indicating that trypsin digests these proteins when the membrane integrity was lost. Together with the finding that LETM1 is resistant to alkaline extraction (data not shown), these results indicate that LETM1 is a mitochondrial inner-membrane protein. In addition, partial digestion of LETM1 under the hypotonic condition suggested LETM1 has a small and/or protease-resistant region exposed to the intermembrane space.

**LETM1 downregulation causes mitochondrial swelling**

To examine the roles of LETM1 in mitochondrial morphology, LETM1 gene expression was knocked down in RNA interference (RNAi) experiments. LETM1 was reduced by more than 90% after a single transfection with siRNA specifically targeting LETM1 (Fig. 2A). Because mitochondrial structures are fragile, cell fixation often induced mitochondrial fragmentation and subsequent aggregation, which is not easy to distinguish from swelling. To avoid technical problems, we used a HeLa cell line that stably expressed the mitochondria-targeted fluorescent protein Su9-DsRed, to visualize the morphology without requiring cell fixation (Taguchi et al., 2007). Mitochondria usually formed tubular network structures in control cells (Fig. 2Ba). With LETM1 knockdown, mitochondria became dot-like structures and lost their tubular networks (Fig. 2Bb). More than 80% of cells exhibited such dot-like structures after transfection with LETM1 siRNA (Fig. 2C). The tubular networks were partially restored by the introduction of LETM1 cDNA (Fig. 2Bc), but not by the introduction of the cDNA that encodes mitofilin, a mitochondrial inner-membrane protein (data not shown), which indicates that the dot-like structures of mitochondria are caused by LETM1 downregulation. Thus, LETM1 is crucial in the maintenance of mitochondrial tubular networks.

OPA1 regulates mitochondrial membrane fusion and its downregulation causes mitochondrial fragmentation (Olichon et al., 2003; Lee et al., 2004; Ishihara et al., 2006). The dot-like structures caused by LETM1 downregulation (Fig. 2Bb) were significantly larger than the fragmented mitochondria in the OPA1-knockdown cells (Fig. 2De), suggesting that mitochondria in the LETM1-knockdown cells either become swollen or form large aggregates. Simultaneous RNAi for LETM1 and OPA1 induced obvious mitochondrial fragmentation, similar to a phenocopy caused by OPA1 knockdown (Fig. 2De,f), suggesting that the dot-like structures observed in the LETM1-knockdown cells are swollen mitochondria, not aggregates, and that mitochondrial fission occurs normally regardless of the loss of LETM1 function. Drp1 is a dynamin-related protein that is required for mitochondrial fission; its knockdown results in elongated mitochondria (Fig. 2Da) (Frank et al., 2001; Taguchi et al., 2007). When Drp1 and LETM1 were simultaneously knocked down (both by more than 90%; supplementary material Fig. S1, lane 4), more than 60% of cells contained filamentous mitochondria that were partly swollen (Fig. 2Db), suggesting that mitochondrial membrane fusion is unaffected by downregulation of LETM1. Similar results were obtained when using a dominant-negative Drp1 mutant. The GTPase-deficient mutant Drp1(K38A) has been shown to induce mitochondrial elongation (Fig. 2Dc) (Smirnova et al., 1998; Ishihara et al., 2003). When the EGFP-tagged Drp1(K38A) mutant was introduced into LETM1-knockdown cells, most mitochondria were elongated with many lumps on the filaments (Fig. 2Dd). These results suggest that LETM1 is not directly implicated in mitochondrial membrane fission and fusion.

To further examine whether LETM1 knockdown causes mitochondrial swelling, mitochondria were immunostained with antibodies against mitofilin and mHsp70. If the dot-like structures were swollen mitochondria, mitofilin should be observed in the outline of these structures whereas mHsp70 should be observed inside these structures. In control HeLa cells, both proteins were colocalized in the tubular networks. By contrast, mitofilin staining was observed at the outline of the large dot-like structures in LETM1-knockdown cells, whereas the interior of the large dots was immunostained with antibody against mHsp70 (Fig. 2Ef; arrowhead), indicating that mitochondria are swollen in LETM1-knockdown cells. To further investigate these mitochondrial structures, LETM1-knockdown cells were analyzed using conventional electron microscopy. Mitochondria were larger and had fewer cristae in LETM1-knockdown cells compared with control cells (Fig. 2Fa,b). The few remaining cristae were not straight and were often twisted (Fig. 2Fc). Thus, LETM1 downregulation causes the disorganization of cristae structures, as well as mitochondrial swelling. In addition, the apoptotic release of cytochrome c was significantly stimulated by LETM1 knockdown (data not shown), supporting the theory that cristae formation is impaired by inactivation of LETM1.

Because LETM1 knockdown affected mitochondrial morphology, we examined the effects of LETM1 overproduction on mitochondrial morphology by introducing a LETM1-GFP fusion protein into HeLa cells stably expressing Su9-DsRed. In those cells that overexpress LETM1-GFP, mitochondria were clearly fragmented but were not swollen (Fig. 2Ga; superimposed). Thus, changes in LETM1 expression cause the loss of mitochondrial tubular networks.

**LETM2 is a mitochondrial protein that is expressed specifically in testis and sperm**

During cloning of the LETM1 cDNA, we found a rat chromosomal region that was homologous to human LETM1 cDNA. Similarly, a specific region in human chromosome 8 is reported to be homologous to the crucial WHS region that contains the LETM1 gene (Stec et al., 2001). Because the putative proteins encoded in the rat chromosomal region did not contain an ortholog of human LETM1, we attempted to clone a cDNA sequence that is homologous to the LETM1 gene. Using rat total RNA, we amplified a PCR product that contained a coding region for a 459 amino acid polypeptide with a single hydrophobic segment (Fig. 3A).

The protein encoded by the cDNA exhibited 32% overall similarity to human LETM1 protein; higher similarity (53.6%) was found for the region between the hydrophobic segment and a putative leucine-zipper domain. The gene in human chromosome 8 has already been named LETM2, although the entire protein is still unidentified (Stec et al., 2001). Therefore, we named our cloned gene product rat LETM2 to avoid confusion in the nomenclature. We also cloned a cDNA for a splicing variant of LETM2 that lacked a 48 amino acid internal domain with a hydrophobic segment (Fig. 3A); this variant was named LETM2S.

To examine the expression of LETM2 in rat tissues, northern blots were carried out using a DNA probe specifically targeting the LETM2 gene (Fig. 3B). A 2.4-kb mRNA fragment of LETM2 was detected only in the testis. Immunoblotting was performed to confirm the
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Fig. 2. LETM1 downregulation causes mitochondrial swelling. (A) Total-cell lysates from HeLa cells transfected once or twice with siRNA for either GFP (mock) or LETM1 were subjected to immunoblotting with antibodies to LETM1 and GAPDH as a loading control. (B) HeLa cells that stably expressed mitochondrion-targeted Su9-DsRed were transfected with siRNA targeting either GFP (a, control) or LETM1 (b). Then, the expression plasmid, which coexpressed LETM1 and nucleus-targeted GFP, was introduced into the cells transfected with the LETM1-targeting siRNA (c). Live images were obtained by confocal microscopy. Typical images were superimposed. Scale bar, 10 μm. (C) Cells transfected with siRNA (as described for B) were analyzed by immunofluorescence microscopy. Data represent the mean ± s.e. of three independent experiments; 100-200 individual cells were counted. (D) HeLa cells expressing Su9-DsRed were transfected with siRNAs targeting DRP1 (a), DRP1 and LETM1 (b), OPA1 (c), OPA1 and LETM1 (d), or LETM1 (panel d). After siRNA transfection, the plasmid expressing Drp1(K38A)-EGFP was transfected (c,d). Live images were collected by confocal microscopy. Arrowheads indicate large dot structures on the filaments, asterisks indicate cells expressing Drp1-EGFP. Scale bar, 10 μm. Data represent the mean ± s.e. of three independent experiments; 100-200 individual cells were counted. (E) HeLa cells transfected with siRNA targeting GFP (a-d) or LETM1 (e-h) were fixed, permeabilized and immunostained using antibodies against the inner membrane protein mitofilin and the matrix protein mtHsp70. Scale bar, 950 nm. (G) Cells stably expressing Su9-DsRed were transfected with plasmid expressing LETM1-EGFP and analyzed by confocal microscopy. Inset, sigh-magnification image. Arrowhead indicates the cell overexpressing LETM1-EGFP. Scale bar, 10 μm.

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tissue distribution of LETM2, which – with a molecular size of 45 kDa – was found only in the testis. LETM1, however, was detected in all tissues tested (Fig. 3C). In all cultured cell lines tested, LETM1 was expressed ubiquitously, but LETM2 was undetectable (Fig. 3D). Thus, LETM2 is expressed specifically in testis and LETM1 is a ubiquitous protein. Unfortunately, we did not detect a band...
corresponding to LETM2S in any tissues tested, suggesting that LETM2S expression is lower than that of the full-length protein.

None of the cultured cell lines tested by us expressed LETM2. Therefore, LETM2 was exogenously expressed in HeLa cells to determine the intracellular localization. A 45-kDa band was detected in HeLa cells transfected with the \textit{LETM2}-expression plasmid, but not in cells with the vector, and was the same molecular size as that found in rat testis (Fig. 3E). In addition to the 45-kDa band, a 50-kDa band was observed when exogenously expressing LETM2, suggesting that LETM2 is synthesized as a precursor. LETM2S was expressed as a 39-kDa band when the plasmid carrying \textit{LETM2S} cDNA was introduced. Immunofluorescence microscopy showed that LETM2 colocalized with cytochrome c (Fig. 3F). In subcellular fractionation, LETM2 was recovered mainly in the mitochondrial fraction (Fig. 1C). Similar results were obtained for the intracellular distribution of LETM2S (data not shown). Thus, LETM2 and LETM2S that were exogenously expressed in HeLa cells both target the mitochondria.

To determine the cellular localization of endogenous LETM2, immunohistochemical analysis of frozen sections of rat testis was conducted using anti-LETM2 antibody. In the seminiferous tubules, immunostaining of LETM2 was detected in spermatocytes and elongating spermatids (Fig. 4Aa,e). No signal was obtained by immunostaining using pre-immune serum. Thus, LETM2 is expressed specifically in cells that undergo spermatogenesis and spermiogenesis. LETM2 was also found in middle pieces of purified spermatozoa (Fig. 4B). Immunoelectron microscopy revealed that LETM2 was observed in the mitochondria of spermatozoa (Fig. 4C). Together with the mitochondrial localization of LETM2 in HeLa cells, these results indicate that endogenous LETM2 is a mitochondrial protein that is expressed in the developmental stages from spermatocyte to spermatozoon.

**Fig. 3.** LETM2 is a mitochondrial protein that is expressed preferentially in testis and sperm. (A) Scheme of LETM1, LETM2, and LETM2S. Red, blue, orange and green boxes represent putative presequences, hydrophobic segments, leucine-zipper motifs and EF-hand motifs, respectively. (B) Poly(A) RNA from adult rat tissues was hybridized using a radioactive probe recognizing LETM2. The blot was also hybridized with a control probe recognizing β-actin. (C) Total-cell lysates from various rat tissues were subjected to immunoblotting using antibodies against LETM2, LETM1 and GAPDH. (D) Total cell lysates from the indicated cell lines were analyzed by immunoblotting using antibodies against LETM2, LETM1 and Hsp60. (E) Total-cell lysates from HeLa cells transfected with vector (lane 1), or with plasmid expressing LETM2 (lane 2) or LETM2S (lane 3), and rat testis homogenates (lane 4) were subjected to immunoblotting using anti-LETM2 antibody. Black and gray arrowheads indicate LETM2 and LETM2S, respectively. (F) HeLa cells transfected with LETM2 (a-c) or LETM2S expression plasmid (d-f) were fixed, permeabilized and immunostained with antibodies against LETM2 and cytochrome c. Images were collected using confocal microscopy. Scale bar, 10 μm.
LETM1 is crucial for the assembly of supercomplexes of the respiratory chains

LETM1 downregulation caused a reduction in the number of, and morphological changes in, cristae. We therefore examined the effects of LETM1 knockdown on the mitochondrial membrane potential by using Mitotracker Red CMXROS, a membrane-potential-dependent mitochondrial vital dye (Fig. 5A). In control cells, staining with Mitotracker was clearly observed at concentrations ranging from 5-20 nM. By contrast, LETM1-knockdown cells were substantially stained with Mitotracker, only at a concentration of 20 nM. Treatment with 10 nM Mitotracker resulted in faint staining of mitochondria when LETM1 was repressed. Similar results were obtained using TMRM, another membrane-potential-dependent mitochondrial dye (supplementary material Fig. S2). Thus, the membrane potential in LETM1-siRNA-transfected cells is substantially lower than that in control cells, which is consistent with previous reports that isolated mitochondria from yeast mdm38 mutants that exhibit low membrane potential (Nowikovsky et al., 2004; Frazier et al., 2006).

Because LETM1-knockdown cells have abnormal cristae and low membrane potential, we examined the amount and assembly of protein complexes of the respiratory chains (complexes I-V). Immunoblotting showed that LETM1 knockdown did not significantly affect the amounts of the tested subunits and of Tom40 and Tim17, which are outer and inner membrane proteins, respectively (Fig. 5B). The assembly of the protein complexes was analyzed using Blue-Native gel electrophoresis (Schägger and von Jagow, 1991), followed by immunoblotting. In mammals, the respiratory chains contains giant complexes called supercomplexes, including three proton pumps: Complex I (NADH dehydrogenase), III (cytochrome bc1 complex), and IV (cytochrome c oxidase) (Schägger and Pfeiffer, 2000). LETM1 knockdown obviously reduced the formation of the supercomplexes (Fig. 5C, arrowhead). Complexes I and IV failed to form, and the assembly of complex III was significantly decreased. By contrast, the assembly of complex II (succinate dehydrogenase) and complex V (ATP synthase) – which are not proton pumps – was unaffected. Furthermore, the Tom complex and the Tim complex (translocases for mitochondrial proteins in the outer and inner membranes, respectively) formed normally even when LETM1 was downregulated. On Blue-Native gel electrophoresis, LETM1 formed two complexes: a major (~300 kDa) and a minor (500-600 kDa) complex. However, neither LETM1 complex co-migrated with any complexes of the respiratory chains, suggesting that LETM1 is not stably associated with the respiratory chains. Thus, LETM1 is crucial for the formation of supercomplexes, including three different proton pumps, and low membrane potential appears to be a consequence of the failure to form the complexes of the respiratory chains.

The levels of the respiratory chain subunits tested were essentially normal, but the formation of complexes I, III and IV was clearly defective in LETM1-knockdown cells. Therefore, we examined the protein stability and membrane association of the subunits. Unfortunately, there were no differences in sensitivity to trypsin treatment between control and LETM1-knockdown cells for the subunits tested. The association with the inner membrane was thus analyzed carrying out alkaline extraction using buffers with pH ranges between 11.5 and 12.5 (Fig. 5D). As expected, complex IV subunit IV, a protein with a single transmembrane domain, was resistant to alkaline extraction, as was the integral membrane protein Tim17. By contrast, the assembly of complex III has no significant hydrophobic segment, it was resistant to alkaline extraction using buffer at pH 11.5. Extraction with buffer at pH 12.0 resulted in the recovery of half of the core1 protein in the supernatant fraction. Although the core1 protein of complex III is not stably associated with the respiratory chains, this is likely because of the failure to form the complexes of the respiratory chains.

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LETM1 is a nuclear-encoded mitochondrial matrix protein. A, axoneme; ODF, outer dense fiber. Scale bar, 0.1 μm.
buffer at pH 12.5, even in \textit{LETM1}-knockdown cells. The tested subunits of complex I and II were not affected by alkaline extraction (data not shown). Thus, \textit{LETM1} knockdown facilitates the tight association of at least core1 protein with the inner membrane, suggesting that this tight association interrupts the formation of complex III.

Mitochondrial swelling is not caused by defects in the assembly of the respiratory chains \textit{LETM1} knockdown induced abnormal mitochondrial structures and failure in the formation of respiratory-chain complexes. To address the possibility that the incomplete assembly of respiratory chains caused mitochondrial swelling, we used $\rho^0$ HeLa cells.
These cells lack mitochondrial DNA that encodes 13 subunits of the respiratory chains, resulting in a deficiency in oxidative phosphorylation (Hayashi et al., 1992). In ρ0 HeLa cells, the 39-kDa subunit protein of complex I, which is nucleus encoded, was dramatically reduced, but the amounts of the other subunits tested and of LETM1 were essentially unaffected (Fig. 6A). As expected, complexes I, III, and IV completely disappeared, and small complexes of complex V were found in ρ0 HeLa cells (Fig. 6B). Although complex II does not contain mitochondria-encoded subunits, the amount of the complex was reduced. By contrast, LETM1 and Tom complexes were unaffected by the loss of mitochondrial DNA. Fluorescence microscopy of ρ0 HeLa cells transfected with the plasmid carrying Su9-DsRed were analyzed by confocal microscopy. ρ0 HeLa cells maintain mitochondrial tubules, which are not expanded. Scale bar, 10 μm. (D) The ρ0 HeLa cells were fixed, and thin sections were visualized by electron microscopy. Arrowheads indicate mitochondria. Scale bar, 950 nm.

The AAA-ATPase BCS1L interacts with LETM1 and stimulates the formation of the LETM1 complex

In yeast, the AAA-ATPase Bcs1p has a role in the assembly of Rieske Fe-S protein into precomplexes of complex III in an ATP-dependent manner (Crucliat et al., 1999). In addition, mutations in the BCS1L gene cause three different human disorders (de Lonlay et al., 2001; Visapää et al., 2002; Hinson et al., 2007). Because BCS1L and LETM1 are mitochondrial inner-membrane proteins that are responsible for both human disorders and the assembly of respiratory chains, we examined the interaction of LETM1 with BCS1L. After transfection of plasmids that express C-terminal three-FLAG-tagged BCS1L (BCS1L-3FLAG) or the three-FLAG-tagged version of the inner-membrane protein AIF (AIF-3FLAG), immunoprecipitation using anti-FLAG antibody was carried out. Endogenous LETM1 was co-precipitated with BCS1L-3FLAG, but not AIF-3FLAG (Fig. 7A). By contrast, other inner membrane proteins, including the 39-kDa subunit of complex I, complex III core 1 protein and Tim44, were not co-precipitated with BCS1L. Thus, BCS1L interacts specifically with LETM1. Furthermore, the interaction with LETM1 was abolished by a BCS1L point mutation in which proline was substituted for arginine at position 155 (R155P; Fig. 7B); one of four mutations found in patients with
complex-III deficiencies (de Lonlay et al., 2001). The ATPase-deficient mutant K236A (lysine substituted for argenine at position 236) of BCS1L was still able to pull down LETM1, suggesting that ATP hydrolysis is not required for the interaction between BCS1L and LETM1. Immunofluorescence microscopy showed that BCS1L-3FLAG was localized to the mitochondria, as was endogenous BCS1L (Fig. 7Ca,v). All BCS1L mutants were also targeted to the mitochondria (Fig. 7, panels d-u), although the expression of BCS1L(R155P)-3FLAG induced mitochondrial fragmentation and aggregation, indicating that these mutations have little influence on the mitochondrial localization of BCS1L.

LETM1 formed the major (~300 kDa) and minor (500-600 kDa) complexes (Fig. 5C). The overexpression of BCS1L-3FLAG stimulated the formation of the major complex, but the amount of LETM1 was essentially unaffected (Fig. 7D, lanes 4 and 8). Conversely, BCS1L knockdown caused decreases (~50%) in both the amount of LETM1 and the formation of LETM1 major complex (Fig. 7D, lanes 1 and 4). Pulse chase experiment also indicated the
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Fig. 8. BCS1L downregulation causes supercomplex disassembly and abnormal mitochondrial morphology. (A) Total cell lysates from cells transfected with siRNA targeting GFP (control) or BCS1L were immunoblotted with antibodies to the indicated proteins. BCS1L knockdown caused reduction in the supercomplexes of the respiratory chains. (B) Mitochondrial fractions from cells transfected with siRNA targeting GFP (control) or BCS1L were solubilized with digitonin, subjected to Blue-Native gel electrophoresis, and immunoblotted using antibodies against the indicated proteins. Arrowhead indicates supercomplexes of Complex I, III, and IV. Asterisk indicates a nonspecific band. (C) HeLa cells that stably expressed Su9-DsRed were transfected with siRNA for GFP (panel a), BCS1L (panels b,c,d), LETM1 (panel e), or BCS1L and LETM1 (panel f), and further transfected with plasmid co-expressing BCS1L and nucleus-targeted GFP (panel b). BCS1L knockdown induced abnormal mitochondrial morphology. Live images were acquired by confocal microscopy. Typical images are shown in insets at high magnification. Scale bar, 10 μm. (D) Cells transfected with BCS1L siRNA were analyzed by immunofluorescence microscopy. Data represent the mean ± s.e. of three independent experiments; 100-200 individual cells were counted. (E) Model of the functions of BCS1L and LETM1 in mitochondrial biogenesis. Blue arrows indicate functions reported here; gray arrow indicates the BCS1L function reported previously (Cruciat et al., 1999; de Lonlay et al., 2001).

instability of LETM1 in BCS1L-knockdown cells (supplementary material Fig. S3). Thus, the formation of the LETM1 major complex depends on the BCS1L level. Although BCS1L formed a 500-600-kDa complex on Blue-Native gel electrophoresis, LETM1 knockdown had little effect on the formation of the BCS1L complex (Fig. 5C), suggesting that in the native gel most BCS1L is not stably associated with LETM1 as a protein complex. These results indicate that BCS1L stimulates the assembly of the LETM1 complex.

BCS1L downregulation causes the disassembly of the supercomplexes and abnormal mitochondrial morphology

As expected from studies in yeast, BCS1L downregulation dramatically affected the assembly of the respiratory chains. Immunoblotting showed that the Rieske Fe-S protein of complex III and the complex-II 70-kDa subunit incorporated into complex II was not affected, although the total amount was reduced by BCS1L downregulation. Rieske Fe-S protein was hardly detected in any complexes corresponding to Complex III upon BCS1L knockdown (Fig. 8B). In the yeast bcs1 deletion mutants, non-assembled Rieske Fe-S protein is detected as monomer (Cruciat et al., 1999). Monomeric forms of the Rieske Fe-S protein, however, were undetectable in the BCS1L-knockdown cells. In addition, BCS1L downregulation did not influence amounts of the Rieske Fe-S protein recovered in the digitonin-insoluble fraction (supplementary material Fig. S4), suggesting that formation of aggregates including the Rieske Fe-S protein is not stimulated by BCS1L inactivation. Compared with the LETM1-knockdown cells (Fig. 5C), the decrease in the Rieske Fe-S protein of the supercomplexes was more severe in the BCS1L-
knockdown cells (Fig. 8B). Thus, BCS1L downregulation causes the disassembly of the supercomplexes as does LETM1 knockdown, but the effects on the formation of the individual complexes differ. In particular, LETM1 might be responsible for the steps after loading of the Rieske Fe-S protein before formation of the supercomplexes.

The effects of BCS1L knockdown on mitochondrial morphology were analyzed using a HeLa cell line that stably expressed Su9-DsRed. When BCS1L was knocked down, more than 70% of mitochondria lost their network structures but did not swell, resulting in the formation of short, lumpy filaments with few branches (Fig. 8Cc,D). In addition, ring structures were frequently observed (Fig. 8Cd, inset). These mitochondrial morphologies were clearly different from those caused by LETM1 knockdown. Exogenous expression of BCS1L partially suppressed the morphological defects in the BCS1L-knockdown cells (Fig. 8Cb). Thus, BCS1L has a distinct role in the maintenance of mitochondrial morphology that is separate from the LETM1 function. Furthermore, the simultaneous knockdown of LETM1 and BCS1L resulted in a phenotype with both features, i.e. short filaments associated with large dots at their edges (Fig. 8Cf), supporting that BCS1L and LETM1 function in different processes in the formation of tubular network structures, although BCS1L helps LETM1 in the formation of protein complexes.

Discussion
In this study, we showed that LETM1 is a mitochondrial inner-membrane protein with a pre-sequence, which is consistent with recent reports (Dimmer et al., 2007; Hasegawa and van der Bliek, 2007). LETM1 downregulation caused mitochondrial swelling and the loss of tubular networks. The simultaneous knockdown of LETM1 and OPA1 showed that membrane fission is a dominant event, rather than swelling, even when most LETM1 protein had disappeared. Moreover, elongated mitochondria were maintained upon the downregulation of both LETM1 and Drp1, indicating that membrane fusion occurs independently of the loss of LETM1 function. These results strongly suggest that LETM1 is not involved directly in mitochondrial membrane fusion and fission, and imply that the disappearance of the tubular networks in LETM1-knockdown cells is caused by the expansion of the tubular structures. Recent study reported that the silencing together of Drp1 and LETM1 caused the loss of tubular networks, similar to the phenotype caused by LETM1 knockdown (Dimmer et al., 2007), which is inconsistent with our findings. Under our conditions, Drp1 and LETM1 virtually disappeared (supplementary material Fig. S1) and more than 60% of cells had elongated mitochondria with large elongated mitochondria that had dilated tubules in part (Fig. 2D). The differences may be explained by the extent to which Drp1 was knocked down.

LETM1 downregulation induced a reduction in the number of cristae structures and the disassembly of respiratory chains. In particular, complexes I, III and IV failed to form supercomplexes. MDM38 gene deletion also causes defects in the formation of complexes III and IV, together with the association with mitochondrial ribosomes, it is proposed that Mdm38 functions in protein export from the matrix (Frazier et al., 2006). However, knockdown of OXA1L (the human ortholog of yeast Oxa1p), which is a central component of the protein export machinery, induced the severe disassembly of complex V and a moderate reduction in complex I (Stiburek et al., 2007), suggesting that LETM1 does not function in an OXA1L-mediated pathway of the export machinery. The p0 HeLa cells completely lost several respiratory chain complexes, but the mitochondrial tubular shapes were maintained, indicating that mitochondrial swelling caused by LETM1 knockdown is not caused by the failure of respiratory-chain assembly. Furthermore, we found that the disassembly of the respiratory chains proceeded slowly, rather than changes in mitochondrial morphology, with increasing LETM1 inactivation (supplementary material Fig. S5). When transfected once with the LETM1 siRNA, half of the cells had swollen mitochondria but the supercomplexes were normally assembled. In addition, the mitochondrial morphology is altered in the cells, with a 60% reduction in the amount of LETM1, but respiratory chain activity is unaffected (Dimmer et al., 2007). Thus, it is that the disassembly of the respiratory chains is likely to be a secondary effect of changes in mitochondrial morphology, especially in cristae structure. However, we cannot rule out that LETM1 is implicated directly in the assembly of respiratory chain because prolonged respiratory-chain stability may conceal the early effects of LETM1 downregulation.

Although mitochondrial swelling was induced by RNAi targeting LETM1, the molecular mechanisms of how LETM1 regulates mitochondrial volume still remains unclear. The addition of nigericin, an electroneutral ionophore, cancels mitochondrial swelling caused by the loss of yeast Mdm38 function in vivo and in vitro (Nowikovsky et al., 2004; Nowikovsky et al., 2007). In HeLa cells, treatment with nigericin also prevented mitochondrial swelling caused by LETM1 knockdown (supplementary material Fig. S6) (Dimmer et al., 2007). However, the addition of nigericin to mitochondria produced short filaments even in wild-type HeLa cells (supplementary material Fig. S6), suggesting that nigericin works on multiple aspects of mitochondrial morphology in HeLa cells. Furthermore, the simultaneous knockdown of DRP1 and LETM1 caused mitochondria to become elongated and partially swollen (Fig. 2D). Such mitochondrial morphology cannot be simply explained by increasing osmotic pressure in the matrix caused by impaired K+/H+ -exchange activity. Our findings indicate that LETM1 is required for the maintenance of the tubular shape, cristae organization and assembly of the respiratory chain, leading to the possibility that LETM1 functions as a structural protein to maintain normal cristae structure. For example, lamins and lamin-associated proteins act as structural proteins in the nucleus and have scaffolding functions in the regulation of high-order chromatin (Vlcek and Foisner, 2007). LETM1 might fold the inner membranes by interacting with other factors and might scaffold the respiratory chains. As a consequence of LETM1 downregulation, the unfolding of cristae might induce the expansion of the tubular structures.

LETM2 is a mitochondrial protein that is expressed preferentially in spermatocytes and spermatozoa. On the basis of the deduced amino acid sequences, LETM2 is predicted to have a single hydrophobic segment. Analyses of alkaline extraction and protease protection indicated that, similar to LETM1, LETM2 is an inner-membrane protein (our unpublished data). Although LETM2 and LETM1 share common features (e.g. being a mitochondrial inner membrane protein, containing a leucine-zipper motif), mitochondrial swelling caused by LETM1 knockdown was not suppressed by the expression of LETM2 (our unpublished data), indicating that LETM2 cannot be replaced by LETM1. This is consistent with the observation that LETM1 is also expressed in testis (Fig. 3C). Why mitochondrial functions in testis and sperm require LETM2 still remains unclear. Mitochondrial morphology changes dramatically in rat germ cells; the mitochondria become rounded with the diluted
Characterization of LETM1 and BCS1L

intra-crystalline spaces and inner space that contains a dense matrix, and are known as condensed mitochondria (De Martino et al., 1979). LETM2 might contribute to the reorganization of the inner and cristae membranes during spermatogenesis.

BCS1L was involved in the assembly of three respiratory-chain complexes (I, III and IV), as well as the LETM1 (major) complex. The clinical characterization of WHS patients strongly suggests a correlation between LETM1 and epileptic seizures (Rauch et al., 2001; South et al., 2007). Certain patients with GRACILE syndrome also exhibit neurological symptoms such as seizure (Visiàppà et al., 2002). These common features strongly suggest a functional relationship between BCS1L and LETM1. Indeed, LETM1 co-purified with BCS1L, and the interaction was abolished by the R155P mutation in BCS1L. Furthermore, the formation of the LETM1 complex depended on the level of BCS1L, whereas the amount of BCS1L was less affected by LETM1 knockdown, indicating that BCS1L stimulates the assembly of the LETM1 complex. Because LETM1 has been reported to interact with itself in vitro (Hasegawa and van der Bliek, 2007), BCS1L might induce the homo-oligomerization of LETM1. However, BCS1L downregulation caused changes in mitochondrial morphology that were different from those in LETM1-knockdown cells. In addition, the simultaneous knockdown of LETM1 and BCS1L produced distinct mitochondrial morphology comprising both features, which suggests that BCS1L contributes to the maintenance of mitochondrial tubular networks separately from an LETM1-dependent pathway.

We hypothesize a working model of the function of BCS1L and LETM1 in mitochondrial biogenesis (Fig. 8E). Because BCS1L is an AAA-ATPase, the following three functions are downstream targets: (1) respiratory chain assembly, (2) mitochondrial morphology maintenance and, (3) LETM1 complex formation. BCS1L functions directly in the formation of mitochondrial tubular networks, in addition to the assembly of the supercomplexes. LETM1 has a distinct role in maintenance of mitochondrial volume and shapes, which helps – in concert with BCS1L – to achieve the efficient assembly of the respiratory chains. Further investigation of LETM1-associated proteins is necessary to elucidate the physiological relations between the roles in mitochondrial biogenesis and the clinical presentation of human disorders.

Materials and Methods

Cell culture and DNA transfection

HeLa cells and a Su9-DsRed-expressing cell line were maintained at 37°C in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS) and 4.5 mg/ml glucose. The p3HeLa cells (EBB) (Hayashi et al., 1992) were cultured in RPMI160 supplemented with 10% FBS, 4.5 mg/ml glucose, 0.1 mg/ml pyruvate, and 50 µg/ml uridine. DNA transfection was carried out using FuGENE6 (Roche Diagnostics) according to the manufacturer’s instructions. 

cDNA cloning and plasmid construction of human LETM1, rat LETM2 and human BCS1L

A 2.2-kb DNA fragment containing the LETM1 coding region was amplified using a human EST clone (IMAGE 4126510; Invitrogen) as template and primers with the sequences 5'-CCGGCCGCCGAATTCATGGCGTCC-3' and 5'-CCAGGCAGTGT-CTCGAGGCTCTTCACCT-3'. The fragment was then cloned into pEGFP-N1 (Clontech) for the expression of the LETM1-GFP fusion protein and pEI-3HA in which the inserted gene was expressed as a three-tandem HA-tagged protein by the EF1 promoter. LETM1 cDNA was also cloned into pGNCef1, a vector containing humanized Romilio green fluorescent protein (hrGFP, Stratagene) with three tandem SV40 nuclear-localization signals downstream of the internal ribosomal entry site (IRES) sequence, to co-express both hrGFP and LETM1. A 1.4-kb cDNA containing an LETM2 open reading frame was obtained by amplification using total RNA from rat testis, using the primers 5'-AAATGCGCTTCTACAGTAAAATCTA-3' and 5'-TTAGGCTCTTTTGAGAATGCTTGCTG-3', and cloned into pBluescript II (Stratagene). After DNA sequence analysis, the coding regions for LETM2 and LETM2S were subcloned to yield constructs pEF1-LETM2 and pEF1-LETM2S, respectively. The nucleotide sequence data were deposited in the nucleotide sequence databases with the following accession numbers: rat LETM2, AB296367; rat LETM2S, AB296368. A 1.3-kb RT-PCR product encoding human BCS1L was obtained using DNA from HeLa cells and cloned into p3FLAG-CMV14 (Sigma-Aldrich).

Preparation of antibodies

GST-LETM1 or GST-LETM2S-fusion protein was expressed in Escherichia coli BL21 (DE3) cells carrying pET41-LETM1 or pET41-LETM2S, and purified by SDS gel electrophoresis, electrophoretically eluted from the gels, and used to immunize rabbits to generate antibodies. The antisera were affinity-purified by incubation with nitrocellulose membrane strips to which the antigen bound and were eluted with 0.1 M glycine (pH 2.0).

Immunoblotting and immunofluorescence microscopy

Immunoblotting was performed as described previously (Oka et al., 2004). Immunofluorescence microscopy was carried out essentially as described previously (Ungar et al., 2002), with the following modification: cells grown on glass coverslips were fixed at room temperature for 25 minutes with 4% paraformaldehyde, permeabilized by incubation with 0.1% Triton TX-100 for 10 minutes, and incubated with primary antibodies. After incubation with Alexa-conjugated secondary antibodies (Invitrogen), images were acquired using a confocal microscope Radiance 2000 (Bio Rad).

Subcellular localization

Subcellular fractionation was performed as described previously (Horie et al., 2002). To determine the intramitochondrial localization, the isolated mitochondria fraction was treated at 4°C for 30 minutes with 0, 50 or 150 µg/ml trypsin in the presence or absence of 1% Triton X-100 under isotonic and hypotonic conditions. After termination by the addition of 10% TCA, the precipitates were analyzed using SDS-PAGE and immunoblotting.

siRNA transfection

The following siRNA duplexes were transfected into HeLa cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions: LETM1 (sense, 5'-CCAGCAUAUGAGAUAUCUGCUU-3'; antisense, 5'-AAAGCGCAUGAU-UUCCUCAUUGCUG-G-3'), BCS1L (sense, 5'-GAUUGGAGCUGAAGGAGUATT-3'; antisense, 5'-UAUUCUCUUAUGCUGACUUCCT-3'), OPA1 (Ishihara et al., 2006), and Drp1 (Tague et al., 2007). After incubation for 48 hours, the cells were harvested and used for immunoblotting. For Blue-Native gel electrophoresis and morphological analysis cells were transfected with siRNA at least twice.

Immunoprecipitation

Cells were harvested, solubilized at 4°C for 60 minutes with 1% digitonin in buffer A (20 mM HEPESS-KOH, pH 7.4, 150 mM NaCl, and 10% glycerol), and centrifuged to remove insoluble materials. The resultant supernatant was incubated at 4°C for 120 minutes with anti-FLAG (M2) antibody, washed twice with 0.25% digitonin in buffer A, and rinsed with buffer B. The immunoprecipitates were analyzed by SDS-PAGE and immunoblotting.

Blue-Native gel electrophoresis

Blue-Native gel electrophoresis was carried out essentially as described previously (Schägger and von Jagow, 1991). Isolated mitochondria (1 mg/ml) were solubilized at 4°C for 60 minutes with 1% digitonin in buffer A, centrifuged at 100,000 g for 15 minutes, and subjected to electrophoresis on 3-12% gradient gels with 0.02% SERVA Blue G. After incubating at 60°C for 15 minutes with denaturing buffer (20 mM Tris-HCl, pH 6.8, 1% SDS, and 100 mM β-mercaptoethanol), the proteins were electrically transferred to PVDF membranes and analyzed by immunoblotting.

Northern blots

Northern blotting was carried out using multiple tissue blots (Clontech) as described previously (Oka et al., 2001). A 506-bp probe was prepared from LETM2 cDNA (between +875 and +1380 bp, numbering from the first letter of the initiation codon) and used for radioactive labeling.

Electron microscopy

Electron microscopy was carried out as described previously (Eura et al., 2003).

Immunohistochemistry and immunoelectron microscopy

Immunohistochemistry of rat testes and immunoelectron microscopy of spermatocytes were performed as reported previously (Doiguchi et al., 2002).

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