Association of LETM1 and MRPL36 Contributes to the Regulation of Mitochondrial ATP Production and Necrotic Cell Death

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Abstract

Leucine zipper/EF hand–containing transmembrane-1 (LETM1) is a mitochondrial inner membrane protein that was first identified in Wolf-Hirschhorn syndrome, and was deleted in nearly all patients with the syndrome. LETM1 encodes for the human homologue of yeast Mdm38p, which is a mitochondria-shaping protein of unclear function. Here, we describe LETM1-mediated regulation of mitochondrial ATP production and biogenesis. We show that LETM1 overexpression can induce necrotic cell death in HeLa cells, in which LETM1 reduces mitochondrial biogenesis and ATP production. LETM1 acts as an anchor protein and associates with mitochondrial ribosome protein L36. Adenovirus-mediated overexpression of LETM1 reduced mitochondrial mass and expression of many mitochondrial proteins. LETM1-mediated inhibition of mitochondrial biogenesis enhanced glycolytic ATP supply and activated protein kinase B activity and cell survival signaling. The expression levels of LETM1 were significantly increased in multiple human cancer tissues compared with normals. These data suggest that LETM1 serves as an anchor protein for complex formation with the mitochondrial ribosome protein L36. Adenovirus-mediated overexpression of LETM1 reduced mitochondrial mass and expression of many mitochondrial proteins. LETM1-mediated inhibition of mitochondrial biogenesis enhanced glycolytic ATP supply and activated protein kinase B activity and cell survival signaling. The expression levels of LETM1 were significantly increased in multiple human cancer tissues compared with normals. These data suggest that LETM1 serves as an anchor protein for complex formation with the mitochondrial ribosome and regulates mitochondrial biogenesis. The increased expression of LETM1 in human cancer suggests that dysregulation of LETM1 is a key feature of tumorigenesis. [Cancer Res 2009;69(8):3397–404]

Introduction

Mitochondria play essential and diverse roles in the physiology of eukaryotic cells. Mitochondria provide energy for cells, participate in numerous metabolic reactions, and play a central role in apoptosis (1). Impairments of mitochondrial function have been implicated in a wide variety of human diseases, including cancers and age-related disorders (2). In oncology, the Warburg effect is the observation that most cancer cells predominantly produce energy by glycolysis followed by lactic acid fermentation in the cytoplasm, rather than by oxidation of pyruvate in mitochondria like most normal cells (3). It is now recognized that the Warburg effect represents a prominent metabolic characteristic of malignant cells. Although the exact mechanisms responsible for this metabolic alteration remain to be elucidated, malfunction of mitochondrial respiration or "respiration injury" due, in part, to mitochondrial DNA (mtDNA) mutations/deletions is thought to be an important contributing factor (4–7). Recent studies revealed that cancer cells of various tissue origins exhibit frequent mutations in their mtDNA (8–10). Because mtDNA encodes for 13 protein components of the mitochondrial respiratory chain, it is likely that certain mtDNA mutations may cause malfunction of the respiratory chain, forcing the cells to increase glycolysis to maintain their ATP supply.

The phosphoinositide-3 kinase/protein kinase B (PKB) pathway promotes cell survival and proliferation through a series of downstream events by enhancing nutrient uptake and energy metabolism (11), stimulating aerobic glycolysis (12), and suppressing apoptosis via phosphorylation of Bad (13). The phosphoinositide-3 kinase/PKB pathway in cancer has been shown to play a key role in cell survival, growth, cell cycle entry, and cell migration—all of which are key characteristics of cancer cells (14, 15). The PKB survival pathway is negatively regulated by phosphatase and tensin homologue deleted on chromosome 10 (PTEN), which limits the half-life of phosphoinositide-3 kinase protein PtdIns[3,4,5]P3 in cells (16). Mitochondrial dysfunction enhances glycolysis and increases cellular NADH levels caused by defective consumption of mitochondrial NADH (17). As a consequence, NADPH levels decrease, caused by increased flux of glucose through glycolysis at the expense of the pentose phosphate pathway (17). These effects lead to oxidation of the tumor suppressor PTEN, resulting in up-regulation of PKB activity (18).

In this current study, we have provided clear evidence that leucine zipper/EF hand–containing transmembrane-1 (LETM1) causes necrotic cell death in a long-term overexpression system. Inhibition of mitochondrial biogenesis and ATP production seems to be the main cause of LETM1-mediated necrotic cell death in HeLa cells. In addition, LETM1 acts as an anchor protein and associates with mitochondrial ribosome protein L36 (MRPL36; an inner mitochondrial membrane–associated protein). LETM1 also enhanced glycolysis-mediated ATP supply, together with activation of the PKB survival pathway due to mitochondria dysfunction. Furthermore, the expression levels of LETM1 were significantly increased in multiple human cancer tissues compared with normal tissues. Taken together, these results show that LETM1 regulation of mitochondrial biogenesis is an important feature of human cancer.
Materials and Methods

Antibodies and reagents. Anti-Myc 9E10 and anti-HA 12CA5 monoclonal antibodies were produced from hybridomas. All commercial antibodies were purchased from the following: anti-LETM1 (Abnova); anti-AIF, anti-COX IV, anti-PAR, and anti-VDAC (Cell Signaling); anti-GFP (Santa Cruz); anti-PARP and anti-Hsp60 (BD Biosciences); and anti-SDHA, anti-MTCO I, and anti-ND 6 antibodies (Abcam).

Construction of expression vectors. Myc, His, or DsRed COOH-terminal–tagged full-length LETM1 (pcDNA3.1/Myc-LETM1, pET28a-LETM1 and pDsRed2-N1-LETM1) were prepared by amplifying the HeLa cells’ cDNA library with corresponding primers. HA, GST, and GFP COOH-terminal–tagged full-length MRPL36 (pcDNA3.1/Zeo-CHA-MRPL36, pGEX4T.1-MRPL36, and pEGFP-N1-MRPL36) were prepared by the same strategies. Adenoviral expression vector for wild-type LETM1, GFP, and Lac Z were prepared by using Adenoviral Expression Kit (Invitrogen). All constructs were confirmed by automated DNA sequencing. Sequences of the mutagenic oligonucleotides are available upon request.

Cell culture, adenovirus infection, and transfection. HeLa cells were maintained in DMEM supplemented with 10% fetal bovine serum, 2 mmol/L of glutamine, 100 units/mL of penicillin, and 100 μg/mL of streptomycin (Life Technologies). HeLa cells were infected with the appropriate adenovirus for the indicated time when cells reached 80% confluence. The medium was changed 6 h postinfection, and was not changed in the time course experiment. For transfection, these cells were cotransfected with HA-MRPL36 and Myc-LETM1 by using LipofectAMINE (Invitrogen).

Immunoprecipitation analysis. HeLa cells were cotransfected with HA-MRPL36 and Myc-LETM1 for 24 h, then placed on ice and extracted with lysis buffer containing 50 mmol/L of Tris-HCl (pH 7.5), 1% v/v Nonidet NP-40, 120 mmol/L of NaCl, 25 mmol/L of sodium fluoride, 40 mmol/L of β-glycerol phosphate, 0.1 mmol/L of sodium orthovanadate, 1 mmol/L of phenylmethylsulfonyl fluoride, 1 mmol/L of benzamidine, and 2 mmol/L of microcystin-LR. HA-MRP36 or Myc-LETM1 protein was immunoprecipitated from 50 μg of cell-free extracts. The immune complexes were washed once with lysis buffer containing 0.5 mol/L of NaCl followed by lysis buffer and finally with PBS.

Flow cytometry analysis. Mitochondrial mass per cell was measured by flow cytometry (FACS) using MitoTracker Green FM (Molecular Probes). HeLa cells were collected by trypsinization, suspended in 0.5 mL of PBS, stained with 100 mmol/L flow cytometry MitoTracker Green FM for 30 min at room temperature in the dark and analyzed by FACS Calibur (BD Bioscience). Both annexin V and propidium iodide labeling for necrotic cell death detection was performed by using annexin V/propidium iodide staining kit (BD Bioscience).

Confocal imaging analysis. HeLa cells were grown on glass coverslips until they were 50% to 70% confluent and then transfected with pEGFP-N1-MRP36, pDsRed-LETM1, or pDsRed-Mito constructs (Clontech). After 24 h, the cells were fixed in 4% paraformaldehyde at room temperature for 10 min and mounted with Fluoromount-G (Vector Laboratories) and visualized using an OLYMPUS confocal microscope.

ATP measurement from the subcellular compartments. Firefly luciferase from pGL3-base vector was subcloned into pcDNA3.1-HA vector. For the mitochondrial luciferase (Luc-m), it was fused downstream of the sequence encoding the mitochondrial targeting signal of subunit 8 of cytochrome c oxidase (COX 8). The COX 8 targeting sequence has been used successfully in other mitochondrial targeting experiments (19), and a similar COX 8–luciferase construct was shown to be targeted appropriately to the mitochondrial matrix. Light emission was measured in a luminometer at 5-s intervals until the maximum value of luminescence was reached. To normalize for the variability of luciferase expression in Figure 1.

LETM1-induced necrotic cell death in HeLa cells. A, HeLa cells were infected with GFP (Ad-GFP), LETM1-adenovirus (Ad-LETM1), or without adenovirus (Control), or treated with 10 μmol/L of CCCP, a mitochondrial uncoupler, for the indicated times. Cells were stained with propidium iodide and measured by FACS. Results are representative of three independent experiments. B, HeLa cells were harvested from the 6-d experiment. Results are representative of three independent experiments. C, HeLa cells were infected with Lac Z (Ad-Lac Z) or LETM1-adenovirus and grown for 4 d. Cells were labeled annexin V and propidium iodide (PI) and analyzed by FACS. Results are a representative of three independent experiments. D, HeLa cells harvested from the 6-d experiment. Lysates were analyzed by immunoblotting using anti-PARP or anti-PAR antibodies. Results are representative of three independent experiments.
transfected cells, the relative luminescence values in each cell compartment were expressed as a ratio to the total potential luminescence measured on equal aliquots of the same lysed cells with a luciferase assay kit (Promega) in the presence of excess ATP (final concentration, 0.5 mmol/L).

**Measurement of oxygen consumption.** HeLa cells were infected with Lac Z or LETM1-adenovirus for 2 days. Cells (2 \times 10^5) were divided into aliquots in a BD Oxygen Biosensor System plate (BD Biosciences) in triplicate. Plates were read on a SAFIRE multimode microplate spectrophotometer at 10-min intervals for 120 min.

**Expression of GST or His fusion protein in bacteria and GST pull-down assay.** GST-MRPL36 or His-LETM1 were isolated from BL21DE3 cells as previously described (20). For GST pull-down assays, 2 μg of GST-MRPL36 and His-LETM1 were incubated for 4 h at 4°C. Bound LETM1 was detected by anti-His antibody. Binding was compared with that of 10% of the added lysates. GST-MRPL36 and His-LETM1 were also determined by Coomassie blue staining solution.

**Immunohistochemistry.** A human cancer tissue array slide was purchased from Bio Max. Histostain-Plus kits (Zymed Laboratories) were used for the immunohistochemistry of tissue array. Paraffin sections were analyzed with anti-LETM1 antibody as previously described (20). Pictures were taken with a microscope.

**Statistical analysis.** Data are expressed as the mean ± SD from at least three separate experiments performed in triplicate. The differences between groups were analyzed using Student’s t test. P < 0.05 (*) was considered significant and P < 0.01 (**) was highly significant compared with corresponding control values. Statistical analyses were carried out using SPSS software ver. 13.0 (SPSS, Inc.).

**Results**

LETM1-induced cell death is necrotic, not apoptotic. Using siRNA knockdown, it has been reported that reduction of LETM1 expression causes swollen mitochondria (21, 22) and decreased cristae compared with control cells (22). On the other hand, down-regulation of LETM1 caused “necrosis-like” death, without activation of caspases and was not inhibited by overexpression of Bcl-2 (23). In order to investigate the effect of LETM1 on cell viability, adenovirus-mediated overexpression was used. LETM1-adenovirus–transduced HeLa cells underwent cell death starting 3 days after LETM1 overexpression (Fig. 1A). At 6 days post-infection, 42% of cells were in sub-G1 phase compared with controls (Fig. 1B). To identify the type of cell death, annexin V and propidium iodide stainings were performed. As shown in Fig. 1C, most of the LETM1-overexpressed cells were stained by propidium iodide indicating that LETM1-induced cell death was necrotic in nature. To further confirm LETM1-induced necrotic cell death, the activation of poly(ADP-ribose) polymerase (PARP) was analyzed by immunoblotting. PARP activation, indicated by

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**Figure 2.** Effects of LETM1 on mitochondrial biogenesis. A, HeLa cells were infected with Lac Z (Ad-Lac Z), LETM1-adenovirus (Ad-LETM1), or without adenovirus (Control) for 48 h. Mitochondria were stained with MitoTracker Green FM dye. Mitochondrial mass was measured by FACS. Results are representative of three independent experiments. Representative confocal images of each condition (bottom). Bars, 20 μm. B, the mean of fluorescence intensity per cell (Y-axis). Typically, a minimum of 10,000 cells per sample were measured in each experiment. Statistical differences of fluorescence intensity were determined by comparing the values for control cells. Columns, mean of three independent experiments; bars, SD; ***, P < 0.01 using Student’s t test. C, HeLa cells were infected with GFP (Ad-GFP), LETM1-adenovirus (Ad-LETM1), or without adenovirus (Control) for 48 h. Mitochondrial proteins were analyzed by immunoblotting with the indicated antibodies (mitochondrial-encoding respiration chain proteins: MT-CO I, a complex IV subunit; ND6, a complex I subunit; nuclear-encoding respiration chain proteins: COX IV, a complex IV subunit; SDHA, a complex II subunit and cytochrome c; and another mitochondrial protein: AIF, HSP 60, and VDAC). Results are representative of three independent experiments.

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LETM1 Induces Necrotic Cell Death

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PAR activation was observed in LETM1-overexpressing cells (Fig. 1D). Apoptosis-inducing factor (AIF) translocation and caspase activation in this process were also evaluated using AIF-antibody and treatment of cells with z-VAD (a pan-caspase inhibitor), because AIF translocation to the nucleus (24) and caspase activation (25) is a typical signature for apoptotic cell death. LETM1 overexpression did not induce the nuclear translocation of AIF when compared with staurosporine, an apoptosis-inducing agent (Supplementary Fig. S1A). Furthermore, LETM1-induced cell death was not blocked by the treatment of cells with z-VAD (Supplementary Fig. S1B), suggesting that LETM1-mediated cell death is not dependent on apoptotic signaling. As shown in Supplementary Fig. S1C, infection of cells with adenovirus Ad-TIM50 and Ad-LETM1-DN did not induce a similar degree of cell death as Ad-LETM1-WT, indicating that LETM1-induced cell death was not simply a result of overexpressing mitochondria protein.

Figure 3. Changes in ATP production and oxygen consumption due to LETM1 expression. HeLa cells were transfected with pcDNA3.1/Zeoc-CHA-Luc (A) or pcDNA3.1/Zeoc-CHA-COX8-Luc (B) for 24 h, then infected with Lac Z and LETM1-adenovirus and cultured for 48 h. HeLa cells were measured for luciferase activity. Columns, averages of the ratio between cytoplasmic and mitochondrial luminescence per total potential luminescence (i.e., the maximal luminescence that can be obtained with excess exogenous ATP in lysed transfected cells); bars, SD (n = 3). Expression of each protein was monitored (top blots). C, columns, averages of the relative ratio between cytoplasmic and mitochondrial luminescence in three independent experiments; bars, SD (n = 3); *, P < 0.05; **, P < 0.01 using Student's t test. D, HeLa cells were infected for 48 h. Equal volumes of packed cells were separated into aliquots in wells of a 96-well BD Oxygen Biosensor plate. The fluorescence in each well was recorded with a SAFIRE multimode microplate spectrophotometer. Points, mean of three independent experiments; bars, SD.

Figure 4. Effects of LETM1 on lactate production and PKB signaling. A, HeLa cells were infected with GFP (Ad-GFP), LETM1-adenovirus (Ad-LETM1), or without adenovirus (Control) for 48 h. The culture medium was collected and pH measured. Columns, mean of three independent experiments; bars, SD; *, P < 0.05 using Student's t test. B, total lysates were analyzed with the indicated antibodies. Results are representative of three independent experiments.
Inhibition of mitochondrial biogenesis by LETM1 in HeLa cells. To further investigate the molecular mechanisms leading to the induction of necrotic cell death by LETM1, we monitored changes in mitochondrial mass in LETM1-overexpressed cells. Mitochondrial mass was decreased in LETM1-overexpressed cells compared with controls (Fig. 2A and B). To further evaluate this effect, the expression levels of mitochondrial proteins were analyzed. The protein levels of all these proteins except Hsp60 were significantly decreased in LETM1-overexpressed cells (Fig. 2C), suggesting that LETM1 overexpression might be involved in regulating mitochondrial biogenesis.

Regulation of ATP production and oxygen consumption by LETM1. Causes of cellular necrosis include prolonged exposure to injury, infection, cancer, infarction, toxins, and inflammation. The majority of causes involve ATP deprivation within cells (26). Therefore, mitochondrial ATP production and oxygen uptake were monitored in LETM1-overexpressing cells. By using two targeted luciferase expression vectors (27), total amounts of cytosolic or mitochondrial ATP were measured. ATP amounts were notably decreased in LETM1-infected HeLa cells compared with control and Lac Z–infected cells (Fig. 3A and B), indicating that LETM1 inhibited the production of mitochondrial ATP production, suggestive of necrotic cell death. However, the ratio of cytoplasmic to mitochondrial ATP was increased in LETM1-infected cells (Fig. 3C), indicating that cells were forced to enhance glycolysis to maintain their ATP supply. Furthermore, mitochondrial oxygen consumption was significantly inhibited by LETM1 expression (Fig. 3D). Taken together, results suggest that LETM1 induced necrotic cell death by inhibiting mitochondrial biogenesis as well as mitochondrial ATP production.

LETM1 expression enhances lactate production and activates PKB signaling. Based on our previous observations that LETM1 led to mitochondrial malfunction and an induction of glycolysis in cytoplasm to maintain their ATP supply in these cells (Fig. 3C), the pH of the culture medium was monitored. As expected, the pH of the culture medium was significantly decreased (Fig. 4A), indicating that up-regulation of the glycolytic pathway in the cytoplasm lead to increased lactate production. It has been reported that respiratory deficiency could increase intracellular NADH levels and cause inactivation of PTEN through a redox modification mechanism, leading to PKB activation (18). Therefore, the possible involvement of PKB signaling was tested by immunoblot analysis. Interestingly, PKB signaling, including GSK3β phosphorylation, was increased whereas PTEN phosphorylation was decreased in LETM1-overexpressed cells (Fig. 4B; Supplementary Fig. S2). Results suggest that overexpression of LETM1 led to the inhibition of mitochondrial biogenesis, forcing cells to increase glycolysis and NADH levels, which can effectively compete with NADPH and compromises the ability of NADPH/thioredoxin to keep PTEN protein in the reduced state (18), leading to inactivation of PTEN and activation of PKB. In addition, tumor suppressor gene, p53 did not seem to be involved in this process (Supplementary Fig. S3).

LETM1 associates with mitochondrial ribosomal protein L36 (MRPL36) in vivo. Previously, it had been shown that Mdm38 (yeast homologues of LETM1) plays a critical role in the biogenesis

Figure 5. Interaction between LETM1 and MRPL36. A, HeLa cells were cotransfected with pEGFP-N1-MRPL36 and pDsRed-Mito (top) or pEGFP-N1-MRPL36 and pDsRed-N1-LETM1 (bottom) for 24 h. Then cells were fixed in 4% paraformaldehyde and imaged with confocal microscopy. Bars, 20 μm. B, HeLa cells were transfected with Myc-LETM1 and HA-MRPL36, immunoprecipitated with anti-Myc antibody and probed for associated proteins with anti-HA antibody. C, HeLa cells were transfected with Myc-LETM1 and HA-MRPL36 cultured for 24 h, immunoprecipitated with anti-HA antibody, and probed for associated proteins with anti-Myc antibody. Results are representative of three independent experiments. D, GST-MRPL36 or His-LETM1 was isolated from BL21DE3 cells. GST-MRPL36 and His-LETM1 were incubated for 4 h at 4°C. Bound LETM1 was detected (top). Inputs from each protein were also detected (bottom). Similar results were obtained in three separate experiments.
of the respiratory chain by coupling ribosomal function to protein transport across the inner mitochondrial membrane (28). Furthermore, mitochondrial ribosome proteins were shown to be bound to the inner membrane (29). Additionally, it has been reported that only MRPL36 remained associated with the inner membrane (30) following the addition of puromycin, an antibiotic that inhibits protein synthesis (31) and induces the release of nascent chains from ribosome membrane complex (32). Therefore, the possible interaction between LETM1 and MRPL36 was assessed. Confocal microscopic analysis of cells revealed that Red-LETM1 and GFP-MRPL36 were perfectly merged in mitochondria (Fig. 5A), indicating that LETM1 colocalizes with MRPL36. This observation was further extended by biochemical analysis of immunoprecipitates which showed that LETM1 physically associated with MRPL36 in vivo (Fig. 5B and C). Importantly, GST pull-down assays indicated that LETM1 bound MRPL36 in vitro, whereas LETM1 displayed negligible binding with beads alone (Fig. 5D), suggesting that these proteins directly interact in vivo. Taken together, results suggested that LETM1 may serve as a membrane anchor for the mitochondrial ribosomes. The importance of this interaction was confirmed by using siRNA-MRPL36 (Supplementary Fig. S4), suggesting that MRPL36 is required for LETM1 function.

**LETM1 is overexpressed in various human cancers.** Cancer cells of various tissue origin exhibit frequent mutations in their mtDNA (8–10). Because mtDNA encodes for 13 protein components of the mitochondrial respiratory chain, it is likely that certain mtDNA mutations may cause a malfunction of the respiratory chain, forcing the cells to increase glycolysis to maintain their ATP supply (33). Therefore, the expression level of LETM1 in various human cancers was measured using the multiorgan human tissue array with normal and cancer tissues. Immunohistochemical analysis of tissue array with anti-LETM1 antibody revealed that the expression levels of LETM1 were markedly increased in different human cancers compared with normal tissue from breast, colon, esophagus, lung, ovary, rectum, stomach, and uterine cervix (Fig. 6A). To further evaluate these findings, total cell lysates from normal and cancerous tissues from three patients (P1–3 for liver cancer; P4–6 for colon cancer) who underwent surgery for malignant cancer were analyzed by Western blotting with anti-LETM1 antibody. As shown in Fig. 6B, liver or colon cancer tissues expressed LETM1 at a significantly higher level than the normal tissues in all samples. Interestingly, LETM1 migrated as a doublet in cancerous tissue only, suggesting that increased expression

![Figure 6. Overexpression of LETM1 in cancer tissues. A, human multiple tissue arrays were performed as described under Materials and Methods. Bars, 100 μm. B, total cell lysates from normal (N) and cancerous (C) tissues from three patients (P1–3 for liver cancer; P4–6 for colon cancer) with malignant cancer were analyzed for protein levels of LETM1 (top). Control for equal loading was checked with antiactin antibody. C, schematic illustration of the working model for the actions of LETM1 in cancer. Long-term expression of LETM1 inhibited mitochondrial biogenesis, leading to mitochondrial dysfunction via MRPL36 interaction. Consequently, these events result in the reduction of mitochondrial mass and inhibition of mitochondrial ATP production, leading to an increase in glycolysis and lactate production. Therefore, the ratio of NADH/NADPH is changed and consequent PTEN inactivation occurs by compromising the ability of NADPH/thioredoxin, resulting in PKB activation. All these changes contribute to cancerous metabolic alteration.](http://www.aacrjournals.org/cancerres/article-pdf/69/8/3402/320297/CancerRes69_08_3402.pdf)
and posttranslational modification of LETM1 occurs in cancerous tissues.

Discussion

Apoptosis is considered to be a programmed and controlled mode of cell death, whereas necrosis has long been described as uncontrolled and accidental cell death resulting from extremely harsh conditions (26). In this study, we unexpectedly found that long-term overexpression of LETM1 triggered cell death in a time-dependent manner (Fig. 1A). The mode of cell death was found to be necrotic cell death (Fig. 1C) because it was independent of AIF nuclear translocation and caspase activation (Supplementary Fig. S1A and B). However, Dimmer and colleagues found that down-regulation of LETM1 also caused necrotic cell death (25). It is currently unclear how both gain and loss of LETM1 causes a similar phenotype in cells.

One of the critical features of necrosis is early rupture of the plasma membrane, which is considered to be a result of ATP depletion because a reduction in the function of the ATP-dependent ion pumps on the plasma membrane due to energy depletion might disturb intracellular homeostasis (26). Interestingly, we found that LETM1 overexpression lead to necrotic cell death via deprivation of intracellular ATP levels in cells. These effects can be partially recovered by providing extra glucose (Supplementary Fig. S6), indicating that LETM1-mediated cell death is mediated by energy deprivation. Adenovirus-mediated overexpression of LETM1 caused a significant reduction of total mitochondrial mass (Fig. 2A and B) due to inhibition of mitochondrial biogenesis (Fig. 2C). This led to a decrease in the mitochondrial oxygen consumption (Fig. 3D) as well as in the amount of ATP per cell (Fig. 3A and B), suggesting that LETM1-induced necrotic cell death is an ATP-dependent process. However, we cannot exclude the possibility that specific decrease per mitochondrion in ATP production could have occurred due to the lack of specific mitochondrial proteins per mitochondrion.

LETM1 is a mitochondrial protein that was first identified as a responsible gene for Wolf-Hirschhorn syndrome with core characteristics, including mental retardation, epilepsy, growth delay, and craniofacial dysgenesis, and was deleted in nearly all patients with the syndrome (22, 34). LETM1 is an inserting mitochondrial inner membrane protein with its COOH-terminal large domain part facing the matrix (23). Recently, LETM1 was shown to associate with an AAA-ATPase BCSIL in the inner membrane (21), forming a protein complex of approximately 550 kDa (22, 23). Thus, it is important to elucidate what proteins bind to the COOH-terminal large domain of LETM1 for increased insight into LETM1 function, as well as to better understand the pathogenesis of Wolf-Hirschhorn syndrome.

In mammalian cells, mitochondrial biogenesis involves a complex interplay between the mitochondrial and nuclear genetic systems (35). Nuclear-encoding mitochondrial proteins are recognized and imported into mitochondria by translocases such as TOM and TIM23 complexes (36). However, within the mitochondria, proteins are synthesized in the mitochondrial ribosomes and inserted into the inner membrane by insertion machinery, such as Oxa 1 complex (37). Mitochondrial ribosome protein complex is localized in the matrix and is bound to the inner membrane (32). Several tethering factors coordinate cotranslational membrane insertion in mitochondria (30). MRPL36 is shown to localize at the inner membrane (30) following the treatment of cells with puromycin, which is a mitochondrial protein synthesis inhibitor (31), and disrupts mitochondrial ribosome-membrane complex formation (32). Yeast Mdm38 is a human homologue of LETM1, which can interact with mitochondrial ribosome (28). Indeed, we found that LETM1, as a mitochondrial inner membrane protein, associates with MRPL36 (Fig. 5), probably via the COOH-terminal large domain of LETM1 in the matrix. Furthermore, ablation of MRPL36 by siRNA confirmed that MRPL36 significantly reverses the phenotype of ATP reduction, induced by LETM1 (Supplementary Fig. S4), suggesting that association of LETM1 and MRPL36 is required for LETM1 function. Analysis of the carbonate-mediated extraction of MRPL36 in the absence of LETM1 revealed (Supplementary Fig. S5) that LETM1 acts as a real anchor protein for the ribosome complex formation. These data indicate that LETM1 may regulate the mitochondrial translation system and reduce mitochondrial biogenesis through association with MRPL36.

A curious but common property of invasive cancers is altered glucose metabolism. Under aerobic conditions, glycolysis is inhibited and normal mammalian cells rely mainly on mitochondrial oxidative phosphorylation for their energy supply (38). However, cancer cells display a significant increase in glycolysis and lactate production even in the presence of oxygen (38). mtDNA mutations/deletions might explain this phenotype (6, 7). Recent studies revealed that cancer cells of various tissue origins exhibit frequent mutations in their mtDNA (8–10). Supporting previous data, LETM1 overexpression led to increased ATP production via glycolysis (Fig. 3C) and to decreased pH of cell culture medium (Fig. 4A), indicating that LETM1-induced mitochondrial dysfunction led to increased glycolysis and lactate production. Both mitochondrial dysfunction and an increase in ATP production via glycolysis can modulate the ratio of NADH/NADPH (18). NADPH is an important molecule for maintaining thioredoxin enzyme activity (39). The increased NADH effectively competes with NADPH and compromises the ability of NADPH/thioredoxin to keep PTEN protein in the reduced state, leading to inactivation of PTEN and activation of PKB (18). Immunoblot analysis of LETM1-expressed cells revealed that LETM1 overexpression caused PTEN inactivation and PKB activation (Fig. 4B; Supplementary Fig. S2), suggesting that LETM1-induced mitochondrial dysfunction resulted in PKB activation which increased the ratio of NADH/NADPH to inactivate PTEN enzyme activity. This phenotype is similar to the metabolic alterations seen in cancer.

One of the most recognized reasons for altered tumor metabolism is the unique physiologic stresses that exist within the tumor (40). Hypoxia is perhaps the most pervasive of these stresses. Therefore, the activity of the hypoxia-inducible factor 1 (HIF1) transcription factor seems to be important factor for tumor cells to adapt in this microenvironment (41). Both of Warburg's original observation—increased aerobic glycolysis and decreased mitochondrial metabolism—can therefore be attributed to the activation of HIF1 in tumor tissue (40). However, this does not exclude a possible role for other oncogenic changes in altering the metabolic profile in the tumor, e.g., the p53 tumor suppressor can also reduce glycolysis when activated (42). Therefore, it will, of course, be interesting to know the possible involvement of HIF1 in the LETM1-mediated metabolic shift of cells. However, p53 does not seem to be involved in our study (Supplementary Fig. S3).

Interestingly, the expression levels of LETM1 were increased significantly in different cancer tissues examined (Fig. 6A). The expression level of LETM1 in cancerous tissue seemed to be at a similar level which was observed in the overexpressed HeLa cells

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(Supplementary Fig. S7), indicating that our results are relevant to the pathophysiological condition. Furthermore, LETM1 was detected in two bands from six patients who underwent surgery for malignant cancer (Fig. 6B), suggesting that LETM1 overexpression is a feature of human metastasis.

In summary, the following working model is proposed (Fig. 6C). Long-term overexpression of LETM1 primarily causes a shift in cell metabolism to glycolysis (glycolysis-dependent ATP production). The effects of LETM1 on cell survival are secondary to its effects on metabolism and the inactivation of PTEN. The reason why LETM1 caused necrotic cell death in culture is likely due to the depletion of intracellular ATP rather than the induction of apoptosis. This might be different from the in vivo situation in which LETM1-overexpressing cells can survive when glucose is supplied from other tissues in vivo. Overexpression of LETM1 may not lead to enhanced cell survival but rather metabolic alterations, eventually providing a survival advantage to cells via NADH-mediated inactivation of PTEN.

All these changes may contribute to cancerous metabolic alteration. Further studies on transgenic mice overexpressing LETM1 will provide new insights into the physiologic roles of LETM1 in vivo.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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