

Original Article

mTOR-related linc-PMB promotes mitochondrial biogenesis via stabilizing SIRT1 mRNA through binding to the HuR protein

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Abstract

Mitochondrial dysfunction is implicated in numerous disorders, including type 2 diabetes, Alzheimer's disease and cancer. Long non-coding RNAs (lncRNAs) are emerging as pivotal regulators of cellular energy metabolism, yet their roles remain largely unclear. In this study, we identify an lncRNA named linc-PMB, which is associated with mTOR and promotes mitochondrial biogenesis, through microarray analysis. We demonstrate that the knockdown of *linc-PMB* results in significantly impaired mitochondrial respiration and biogenesis, along with altered expressions of related genes. Conversely, overexpression of linc-PMB markedly increases mitochondrial function. We further reveal that linc-PMB interacts with the RNA-binding protein HuR, promoting the stabilization of SIRT1 mRNA and a substantial increase in SIRT1 expression, which in turn activates the PGC-1 α /mtTFA pathway and mitochondrial biogenesis. Collectively, our findings reveal a novel regulatory pathway in which linc-PMB, through its interaction with HuR, modulates the SIRT1/PGC-1 α /mtTFA axis to maintain mitochondrial biogenesis and function.

Key words HuR, long non-coding RNA, mitochondrial biogenesis, mTOR, SIRT1

Introduction

Long non-coding RNAs (lncRNAs), defined as non-protein-coding transcripts longer than 200 nucleotides, constitute a significant portion of the mammalian transcriptome. They play regulatory roles in transcription through various mechanisms, including acting as signals, decoys, guides, and scaffolds, while also governing multiple aspects of post-transcriptional processes such as pre-mRNA processing and splicing, transport, translation, and degradation [1–3].

lncRNAs can extensively influence cellular functions by responding to various stimuli and interacting with key metabolic components and regulatory factors within signaling pathways [4]. Recent studies have revealed that lncRNAs engage in crosstalk with crucial signaling networks, such as Akt [5], WNT [6], Notch [7], and TGF β [8], thereby modulating numerous cellular pathways and

biological processes [2].

Mammalian target of rapamycin (mTOR) is an evolutionarily conserved serine/threonine kinase that consists of two distinct signaling complexes: mTORC1 and mTORC2. mTOR integrates signals from upstream pathways, including insulin, growth factors, amino acids, oxygen, and energy levels. Therefore, the mTOR pathway serves as a central coordinator of mammalian metabolism and physiology, playing key roles in the function of various tissues, including the liver, muscle, adipose tissue, and brain [9–11]. Rapamycin inhibits mTORC1 activity by interacting with its intracellular receptor FKBP12 [12]. Dysfunction of mTOR signaling has been implicated in many human diseases, such as cancer, diabetes, and neuronal disorders [9,13,14]. In recent years, several groups have reported that the inhibition of mTORC1, in addition to reducing protein synthesis, profoundly affects gene transcription

induced by specific transcription factors, including STAT3, SREBP, PPAR γ , PPAR α , HIF1 α , YY1-PGC1 α , and TFEB [15,16].

lncRNAs represent an emerging dimension of the modulatory architecture of the mTOR signaling network. Recent evidence has indicated that crosstalk between lncRNAs and the mTOR pathway occurs in many types of cancer [17,18]. Several key components of the mTOR pathway are potentially targeted by lncRNAs, such as H19 [19], FLJ11812 [20], and SNHG6 [21]. Conversely, the expressions of certain lncRNAs, including IRT1 [22] and TP53COR1 [23], are induced by mTOR treatment.

To further investigate the roles of lncRNAs in the mTOR signaling pathway, we analyzed global lncRNA expression changes via a microarray in hepatocellular carcinoma cells (HepG2) treated with rapamycin. We identified eight lncRNAs whose expressions significantly changed. Among these, one lncRNA named linc-PMB (promoting mitochondrial biogenesis) was selected for further study. We discovered that this lncRNA markedly affected mitochondrial biogenesis by enhancing the stability of SIRT1 mRNA through binding to the HuR protein.

Materials and Methods

Cell culture

HepG2 cells were obtained from the Stem Cell Bank, Chinese Academy of Sciences (Shanghai, China), and cultured in DMEM medium (Gibco, Carlsbad, USA) supplemented with 10% FBS and 1% antibiotic/antimycotic solution. The cells were incubated in a CO₂ incubator (Thermo Fisher Scientific, Irwindale, USA) maintained at 37°C with humidified air and 5% CO₂. For rapamycin treatment, 70%–80% confluent HepG2 cells were cultured in serum-free medium supplemented with vehicle (0.1% DMSO) or rapamycin (20 nM; Selleck Chemicals, Houston, USA) for 8 h.

Microarray analysis

lncRNA microarray analysis was performed on three paired samples from the rapamycin treatment group and the vehicle control group. The RNA was amplified and reverse transcribed into fluorescent (Cy5- and Cy3-dCTP)-labeled cDNA and then hybridized to the Agilent human lncRNA + mRNA array V4.0 that contains probes interrogating 77,000 lncRNAs (4 × 180K; CapitalBio Corp, Beijing, China). After hybridization and washing, the processed slides were scanned, and the raw data were extracted as pair files via Feature Extraction software. Gene expression differences and *P* values were then calculated via GeneSpring GX software. The thresholds for up- and down-regulated genes were set at a fold change ≥ 2.0 and a *P* value < 0.05 . Hierarchical clustering of the lncRNA and mRNA profiles was performed via Cluster 3.0. Quantitative real-time PCR (qRT-PCR) was conducted to confirm the reliability of the microarray analysis, with primers for each target gene listed in [Supplementary Table S1](#).

RNA extraction and qRT-PCR analysis

Total RNA was isolated from cells grown under the indicated conditions via TRIzol reagent (Invitrogen, Carlsbad, USA), and reverse transcription was performed via the PrimeScript™ RT Reagent Kit (Takara Bio, Dalian, China). The resulting cDNA was quantified by qRT-PCR using SYBR® Premix Ex Taq™ II (Tli RNaseH Plus; Takara Bio). mRNA transcript levels were measured via the Applied Biosystems StepOne™ Real-Time PCR System v2.2 software. β -Actin was chosen as the endogenous control, and data

analysis was performed via the 2^{- $\Delta\Delta$ CT} method. The primers used for each target gene are listed in [Supplementary Table S1](#).

Western blot analysis

The cells were washed in cold phosphate buffered saline (PBS) and lysed in RIPA buffer (Beyotime, Shanghai, China) containing PMSF (Beyotime). After lysis for 30 min, insoluble material was removed by centrifugation at 13,000 g for 15 min at 4°C. The protein content was determined with a BCA Protein Concentration Determination Kit (Beyotime). Equal amounts of protein were treated with 1× SDS-PAGE sample buffer and heated at 100°C for 5 min. The samples were separated by 10% SDS-PAGE and transferred to PVDF membranes. The membranes were blocked for 2 h at room temperature with 5% non-fat dry milk in TBS containing 0.1% (v/v) Tween-20 and incubated overnight with primary antibody at 4°C. The membranes were then incubated with a suitable secondary antibody conjugated with horseradish peroxidase for 1 h at room temperature, and hybridization was visualized via Meilunbio® FGSuper Sensitive ECL Luminescence Reagent (Meilunbio, Dalian, China).

The primary antibodies used included those against mTOR (1:1000, #2972), phospho-S2448 mTOR (1:1000, #2971), p70 S6 kinase (1:1000, #9202), phospho-T389 p70 S6 kinase (1:1000, #9205), 4E-BP1 (1:1000, #9452), phospho-T37/46 4E-BP1 (1:1000, #9459), PGC-1 α (3G6) (1:1000, #2178) (Cell Signaling Technology, Massachusetts, USA), SIRT1 (1:1000, #ab189494), NRF1 (1:1000, #ab55744), mtTFA (1:1000, #ab176558) (Abcam, Cambridge, UK), COX1 (C-20) (1:1000), COX3 (N-20) (1:500), ND1 (C-18) (1:1000), ND4 (A-16)-R (1:1000), cytochrome b (H-300) (1:500) (Santa Cruz Biotechnology, Santa Cruz, USA), and β -actin (1:1000, #AF0003; Beyotime). The secondary antibodies used were HRP-labeled goat anti-rabbit IgG (H + L) (1:2000, #A0208) and HRP-labeled goat anti-mouse IgG (H + L) (1:2000, #A0216) (Beyotime).

siRNA transfection

The cells were transfected with the negative control or linc-PMB Smart Silencer using a riboFECT™ CP Transfection Kit from RiboBio (Guangzhou, China). The linc-PMB Smart Silencer, synthesized by RiboBio, is composed of a mixture containing three siRNAs and three ASOs. Briefly, the negative control or linc-PMB Smart Silencer was diluted to 200 nM with riboFECT™ CP Buffer, mixed with riboFECT™ CP Reagent and incubated for 10 min at room temperature to form complexes. The complexes were then added to adherent cells via normal culture medium supplemented with 10% FBS but lacking antibiotics.

Cell proliferation assay

A total of 1000 cells were seeded into each well of 96-well plates. Cell viability was assessed using a Cell Counting Kit-8 (CCK-8; MCE, Monmouth Junction, USA) at the indicated time points by incubating the plates at 37°C for 2 h, followed by absorbance measurement at 450 nm.

Rapid amplification of cDNA ends (RACE) assay

The 5' and 3' RACE methods were adopted to determine the full length and identify the transcriptional initiation and termination sites of linc-PMB with a FirstChoice® RLM-RACE Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. The sequences of the gene-specific PCR primers used for 5' RACE analysis were 5'-TGTTTCCGCCTTCTGATCCT-3', and those used for

3' RACE analysis were 5'-AGGGAAGTCAACAAACGCCATCTGT-3'. The sequences of the primers for the full-length linc-PMB were as follows: forward: 5'-TCACAGGTCATAGCATCTAC-3', reverse: 5'-CCAGCTGGGAGGAATTTCTG-3'.

Prediction of the protein-coding potential of linc-PMB

The protein-coding potential of linc-PMB sequence was evaluated by two well-established algorithms: the Coding Potential Assessment Tool (CPAT) [24,25] and ORF finder software from the National Centre for Biotechnology Information (NCBI) [26]. For CPAT (<http://lilab.research.bcm.edu/>), HOTAIR served as non-coding RNA control, and β -actin and GAPDH served as coding RNA controls. For ORF finder software (<https://www.ncbi.nlm.nih.gov/orffinder/>), the putative ORFs were then searched against the UniProtKB/Swiss-Prot database (<https://www.uniprot.org/>) to check whether there are matched proteins.

RNA fluorescence *in situ* hybridization (RNA-FISH)

RNA-FISH for linc-PMB was performed via an RNA-FISH Kit (RiboBio, Guangzhou, China) according to the manufacturer's instructions. The linc-PMB FISH Probe Mix was synthesized by RiboBio. Briefly, HepG2 cells were seeded on slides at the bottom of a 24-well plate and incubated until the degree of cell fusion reached 60%–70% before the experiment. After cell fixation and permeabilization, the cells were treated with pre-hybridization buffer at 37°C for 30 min and then hybridized in hybridization buffer containing 300 nM FISH Probe Mix at 37°C overnight. After several times wash with washing buffer at 42°C and PBS at room temperature, the cells were stained with DAPI. Finally, images were captured under an immunofluorescence microscope (Nikon, Tokyo, Japan).

Immunofluorescence (IF) staining

For IF staining, HepG2 cells were seeded on slides at the bottom of a 24-well plate and incubated. After cell fixation and permeabilization, the cells were blocked with 5% BSA for 1 h at room temperature and incubated with an anti-HuR antibody (1:250; Abcam) at 4°C overnight. The cells were subsequently incubated with an Alexa Fluor 488-labeled anti-rabbit secondary antibody (Beyotime) for 1 h at room temperature. Finally, the cells were stained with DAPI for 20 min, followed by image acquisition via a confocal microscope (Leica, Wetzlar, Germany).

Determination of mitochondrial respiration

A Seahorse XF96 Flux Analyzer (Seahorse Biosciences, Billerica, USA) was used to determine the metabolic profiles of the HepG2 cells. A total of 5000 cells/well were seeded into Seahorse XF96 microplates and incubated at 37°C overnight, and then the oxygen consumption rate (OCR) was measured. Additional measurements were performed after the injection of three compounds: oligomycin A (1 μ M; Sigma-Aldrich, St Louis, USA), carbonyl cyanide 4-trifluoromethoxy-phenylhydrazone (FCCP, 100 nM; Sigma-Aldrich), and rotenone (1 μ M; Sigma-Aldrich). The results were normalized to the protein concentration.

Mitochondrial mass and DNA copy number measurement

The cells were stained with 300 nM MitoTracker (Invitrogen) for 30 min in a cell culture incubator. The cells were then collected by

trypsinization and subjected to flow cytometry analysis (BD Biosciences, San Jose, USA).

Total DNA was extracted from cells via proteinase K digestion and ethanol precipitation. A total of 0.2 μ g of total DNA was used for qRT-PCR as described above. Mitochondrial DNA was quantified via the cytochrome c oxidase I (MT-COI) gene, and nuclear DNA was quantified via the 18S gene.

RNA pulldown assay

RNA pulldown assay was performed via a Pierce™ Magnetic RNA-Protein Pull Down Kit (Thermo Fisher Scientific). First, full-length linc-PMB or linc-PMB-antisense was synthesized via *in vitro* transcription with RiboMAX™ Large Scale RNA Production Systems T7 (Promega, Madison, USA) according to the manufacturer's instructions. The 3' terminus of linc-PMB or linc-PMB-antisense was subsequently attached to a single desthiobiotinylated nucleotide. HepG2 cells were lysed in IP lysis buffer and centrifuged at 15,000 g for 30 min, after which the supernatant was collected. The desthiobiotinylated linc-PMB or linc-PMB-antisense and cell lysates (0.8–1 mg per sample) were incubated at 4°C overnight. The RNA-protein complexes were further isolated with streptavidin magnetic beads. The recruited proteins were subject to SDS-PAGE and Coomassie Blue Staining (Beyotime). The gels containing all the retrieved proteins were cut and identified via LC-MS/MS analysis by PTM Bio (Hangzhou, China).

RNA immunoprecipitation (RIP) assay

RIP assay was performed via the Magna RIP™ RNA-binding Protein Immunoprecipitation Kit (Millipore, Bedford, USA) according to the manufacturer's instructions. Briefly, 2×10^7 cells were lysed in 0.1 mL of lysis buffer containing protease inhibitors and RNase inhibitor. The lysates were immunoprecipitated with anti-HuR (Abcam) and IgG antibodies. Finally, the RNA was extracted, purified, and subjected to qRT-PCR analysis.

Vector construction and cell transfection

For the establishment of linc-PMB-overexpressing HepG2 cells, the human *linc-PMB* sequence was amplified and cloned and inserted into the *Sal*I and *Xba*I sites of the lentivirus expression vector pCDH to generate pCDH-linc-PMB. HEK293T cells were transfected with pCDH-linc-PMB via the LentiStarter 2.0 Kit (System Biosciences, Palo Alto, USA) according to the manufacturer's instructions. The virus particles were collected 48 h after transfection. HepG2 cells were infected with recombinant lentivirus, incubated at 37°C for 72 h, and assayed for reporter expression.

Actinomycin D assay

After transfection for 48 h, actinomycin D (ActD, 4 μ g/mL; Sigma-Aldrich) was added to the culture medium, followed by incubation for 0 h, 1 h or 2 h. SIRT1 mRNA stability in the ActD treatment group was analyzed by qRT-PCR.

Statistical analysis

Data are presented as the mean $\pm$ standard deviation (SD). The statistical significance of differences was determined by Student's *t* test or one-way ANOVA with Bonferroni's post hoc comparison where appropriate. All cell-based *in vitro* experiments were repeated at least 3 times. *P* values less than 0.05 were considered to indicate statistical significance.

Results

Aberrant expression of lncRNAs in HepG2 cells treated with rapamycin

To identify lncRNAs that are potentially involved in the mTOR signaling pathway, microarray analysis was performed to reveal the difference between rapamycin and vehicle (0.1% DMSO) treatments in HepG2 cells. After treatment with 20 nM rapamycin for 8 h, mTOR phosphorylation was inhibited (Figure 1A). Concurrently, the levels of phosphorylation of the mTORC1 substrates S6K1 and 4E-BP1 were also reduced (Figure 1A). The microarray chip contained probes targeting 77,000 lncRNAs. Hierarchical clustering was used to represent the differential lncRNA expression profiles (Figure 1B). In total, 8 lncRNAs were identified as significantly differentially expressed (fold change ≥ 2.0 , $P < 0.05$) in rapamycin-treated HepG2 cells compared with their vehicle-treated counterparts (Figure 1C and Supplementary Table S2). To further validate these differentially expressed lncRNAs, qRT-PCR assay was performed, confirming four of these lncRNAs as observed in the microarray analysis (Figure 1D).

linc-PMB expression is associated with mTOR phosphorylation

To further determine whether these aberrantly expressed lncRNAs are associated with mTOR phosphorylation, insulin (100 nM) was used to activate the PI3K/mTOR pathway, followed by the detection of mTORC1 phosphorylation (Figure 1E). The expression levels of three of the four aforementioned lncRNAs significantly decreased after a 2-h treatment with insulin (100 nM) but increased with the addition of rapamycin (Figure 1F). One of these three lncRNAs, linc-PMB, was demonstrated to promote mitochondrial biogenesis in subsequent experiments. Further observations revealed that treatment with rapamycin (20 nM) could counteract the down-regulation caused by knockdown with the lncRNA Smart Silencer (Figure 1G). Interestingly, the silencing of linc-PMB led to a reduction in mTOR phosphorylation (Supplementary Figure S1A) and inhibited cell proliferation (Supplementary Figure S1B). Conversely, the overexpression of linc-PMB increased the level of mTOR phosphorylation (Supplementary Figure S1C). Additionally, linc-PMB had no effect on AMPK phosphorylation (Supplementary Figure S1D,E).

linc-PMB is an intergenic long non-coding RNA located on chromosome 8p 11.22 in the human genome. Its 1529 bp full-length sequence was obtained through 5' and 3' RACEs. This sequence revealed an additional 240 bp in the middle, a 90 bp deletion at the 5' end and a 272 bp deletion at the 3' end compared with the predicted sequence (Supplementary Figure S2A–C). The protein-coding potential of linc-PMB was assessed via two well-established algorithms: the CPAT and ORF finder software [24–26]. CPAT assigned a protein coding potential score of 0.0041 to linc-PMB, indicating that it is a non-coding RNA (Supplementary Figure S2D). The ORF finder software identified eleven putative open reading frames (ORFs) over 75 bp in length using ATG as the start codon, none of which exceeded 200 bp in length (Supplementary Figure S2E). These putative ORFs were searched against the UniProtKB/Swiss-Prot database, but no matching reference proteins or non-redundant protein sequences were found. Furthermore, cellular fractionation assay indicated that linc-PMB is predominantly localized in the nucleus (Supplementary Figure S2F), and RNA-FISH clearly revealed strong expression around the nuclear region

(Supplementary Figure S2G). Therefore, it is inferred that linc-PMB is highly expressed in the nuclear membrane.

Taken together, these findings suggest that linc-PMB, as a lncRNA, is closely associated with mTOR phosphorylation in HepG2 cells.

linc-PMB silencing impairs mitochondrial respiration capacity and mitochondrial biogenesis

To directly examine the physiological role of linc-PMB in energy metabolism, mitochondrial function in HepG2 cells was assessed via a Seahorse Flux Analyzer, which measures the oxygen consumption rate (OCR), an indicator of oxidative phosphorylation (OXPHOS) [27]. linc-PMB expression was reduced by approximately 70% using lncRNA Smart Silencer, which consists of a mixture of three siRNAs and three ASOs, acting as a more effective lncRNA inhibitor (Figure 2A). The mitochondrial respiration capacity was evaluated 48 h post-transfection, and mitochondrial oxidative respiration was significantly inhibited as a result of linc-PMB knockdown (Figure 2B). Non-mitochondrial respiration, basal respiration, and ATP production were markedly decreased, while the proton leakage was slightly reduced (Figure 2C–F). These data strongly suggest that mitochondrial function is compromised in cells with linc-PMB knockdown.

The underlying mechanism of impaired mitochondrial function in linc-PMB-silenced cells was further explored. SIRT1 and its substrate, PGC-1 α , are known to regulate aspects of energy metabolism through the mitochondria [28]. mTOR can modulate mitochondrial oxidative function through a YY1–PGC-1 α transcriptional complex [29]. Our experiments revealed significant decreases in both SIRT1 and PGC-1 α levels, which corresponded to reduced expressions of NRF1 and mtTFA (Figure 3A). Moreover, the mitochondrial DNA content (Figure 3B) and mitochondrial mass (Figure 3C) were markedly reduced with Smart Silencer treatment. Furthermore, the expressions of mitochondrial proteins, including MT-CO1, MT-CO3, MT-ND1, MT-ND4, and MT-CYB, were also downregulated (Figure 3D,E).

linc-PMB overexpression promotes mitochondrial respiration capacity and mitochondrial biogenesis

Lentivirus-mediated overexpression of linc-PMB in HepG2 cells was successfully established (Figure 4A). Our findings revealed that overexpression of linc-PMB dramatically promoted mitochondrial respiration, as evidenced by increased non-mitochondrial respiration, proton leakage, basal respiration, and ATP production (Figure 4B–F). Notably, linc-PMB overexpression led to substantial increases in the protein levels of SIRT1, PGC-1 α , NRF1, and mtTFA (Figure 5A). Consequently, both the mitochondrial DNA content (Figure 5B) and the mitochondrial mass (Figure 5C) were significantly increased with linc-PMB overexpression. The expressions of the mitochondrial coding genes mentioned above increased with the overexpression of linc-PMB (Figure 5D,E).

Additionally, when HepG2 cells were subjected to linc-PMB overexpression following the 12-h inhibition of linc-PMB, both the expression (Supplementary Figure S3A) and function of linc-PMB were restored in comparison to those in cells treated with only the linc-PMB Smart Silencer. Consequently, not only was the mitochondrial DNA content increased (Supplementary Figure S3B), but the mitochondrial mass was also partially restored (Supplementary Figure S3C). Furthermore, the downregulation of mitochondrial

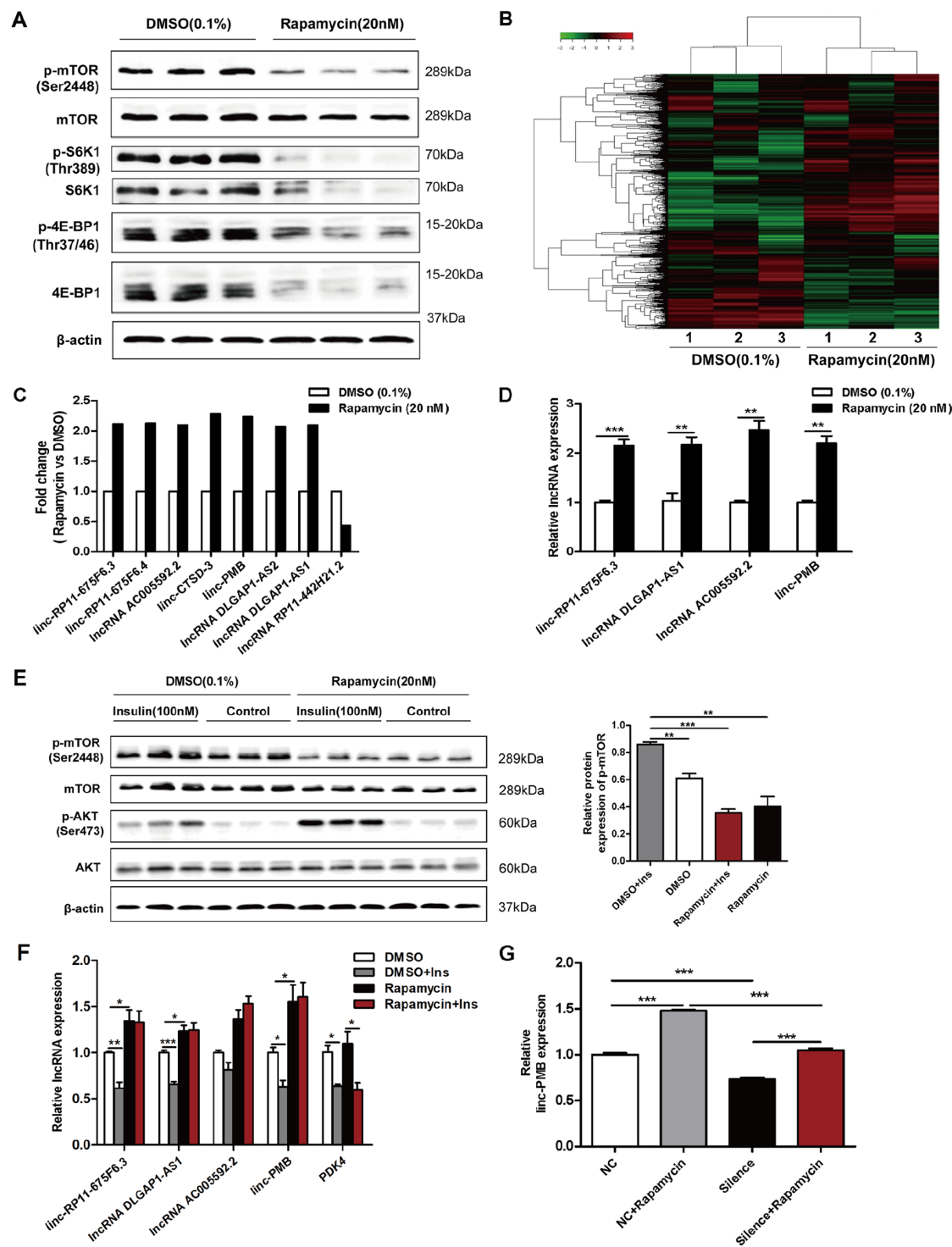


Figure 1. Identification of an lincRNA associated with mTOR phosphorylation in HepG2 cells (A) Western blot analysis of HepG2 cells treated with or without rapamycin (20 nM) for 8 h. (B) Hierarchical clustering image of lincRNAs differentially regulated in rapamycin-treated cells compared with vehicle-treated cells. Each row represents an lincRNA. Red: higher expression levels. Green: lower expression levels. (C) Differential expression (fold change ≥ 2) of 8 lincRNAs in rapamycin-treated cells versus vehicle-treated cells, as determined by microarray analysis. (D) Validation of the differential expression of 4 lincRNAs in rapamycin-treated cells versus vehicle-treated cells via a microarray assay and qRT-PCR. (E) Western blot analysis of HepG2 cells treated with insulin (100 nM) for 20 min followed by 8 h of rapamycin (20 nM) treatment. The relative protein expression of mTORC1 phosphorylation (p-mTOR) was quantified via Image J software (right panel). (F) Gene expression in HepG2 cells treated with or without insulin (100 nM) for 2 h after 8 h of rapamycin (20 nM) or vehicle treatment. PDK4 served as a positive control. (G) Expression levels of linc-PMB in control HepG2 cells and in linc-PMB-knockdown HepG2 cells treated with negative control (NC) or Smart Silencer (Silence) for 24 h, followed by rapamycin (20 nM) treatment for an additional 24 h. Data are presented as the mean $\pm$ SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

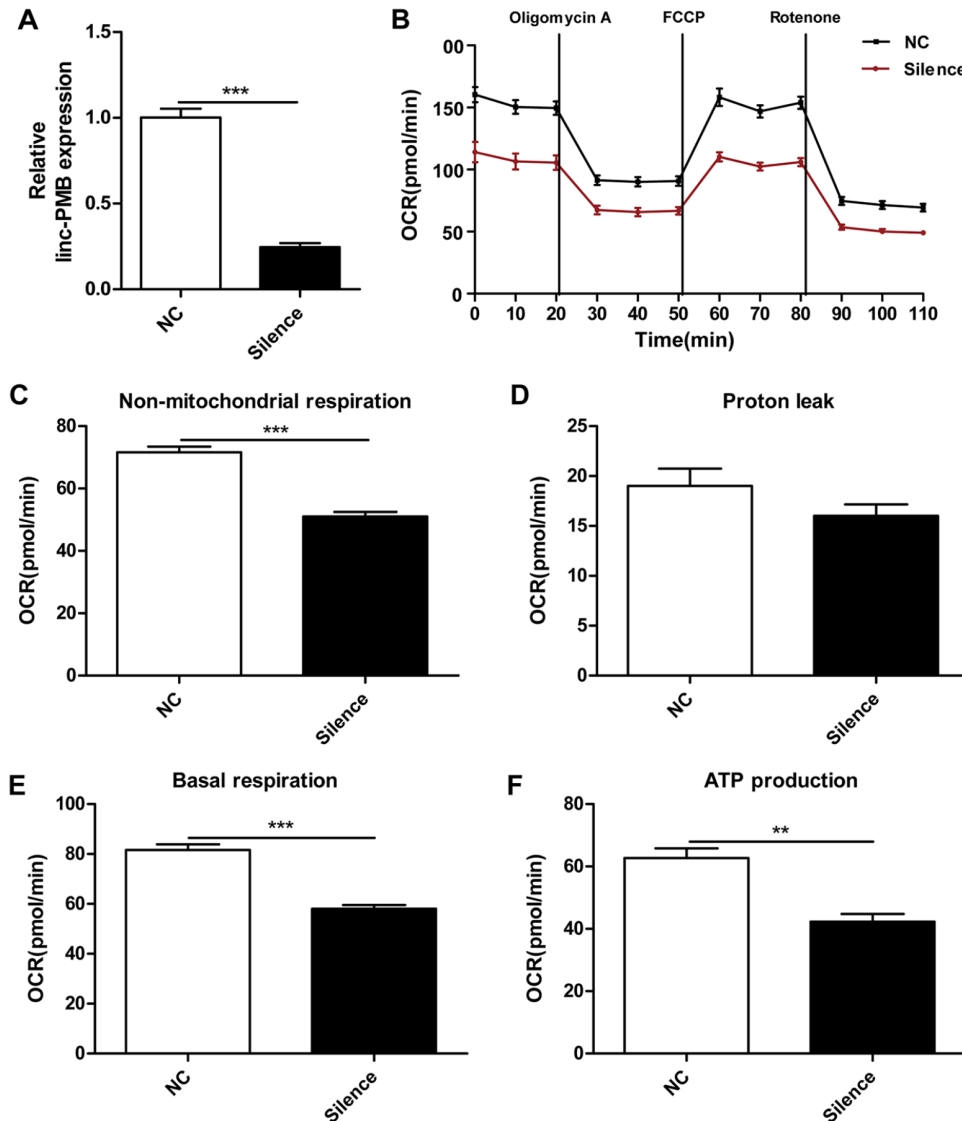


Figure 2. linc-PMB knockdown inhibits mitochondrial respiration capacity in HepG2 cells (A) Expression levels of linc-PMB in control HepG2 cells and in linc-PMB-knockdown HepG2 cells treated with a negative control (NC) or Smart Silencer (Silence) for 24 h. (B) Seahorse XF96 Flux analysis of control HepG2 cells and linc-PMB-knockdown HepG2 cells treated with negative control (NC) or Smart Silencer (Silence) for 48 h. (C) Non-mitochondrial respiration in control HepG2 cells and linc-PMB-knockdown HepG2 cells treated with negative control (NC) or Smart Silencer (Silence) for 48 h. (D) Proton leak in control HepG2 cells and linc-PMB-knockdown HepG2 cells treated with negative control (NC) or Smart Silencer (Silence) for 48 h. (E) Basal respiration in control HepG2 cells and linc-PMB-knockdown HepG2 cells treated with negative control (NC) or Smart Silencer (Silence) for 48 h. (F) ATP production in control HepG2 cells and linc-PMB-knockdown HepG2 cells treated with negative control (NC) or Smart Silencer (Silence) for 48 h. Data are presented as the mean $\pm$ SD of three independent experiments. ** $P < 0.01$, *** $P < 0.001$.

coding genes induced by linc-PMB knockdown was reversed due to the overexpression treatment (Supplementary Figure S3D,E). All of these functional changes were consistent with the recovery of the expression levels of proteins, including that of SIRT1, PGC-1 α , NRF1, and mtTFA (Supplementary Figure S3E).

Together, these findings indicate that linc-PMB promotes mitochondrial respiration capacity and mitochondrial biogenesis.

linc-PMB interacts with HuR to stabilize SIRT1 mRNA

To elucidate the molecular mechanism by which linc-PMB promotes mitochondrial biogenesis, RNA pulldown assay was performed to capture proteins potentially interacting with linc-

PMB. Two independent pull-down experiments identified six proteins through tandem mass spectrometry analysis (Supplementary Table S3). Among these candidates, the RNA binding protein ELAV-like protein 1 (HuR) was selected for further investigation. To confirm the interaction between HuR and linc-PMB, RIP assay was performed, revealing significant enrichment of linc-PMB in anti-HuR precipitates (Figure 6A). Moreover, we performed simultaneous FISH for linc-PMB and IF staining for HuR in HepG2 cells, and the results revealed colocalization between linc-PMB and HuR (Figure 6B).

HuR, a member of the embryonic lethal abnormal-vision (ELAV) family of mRNA-binding proteins, contains three RNA-recognition

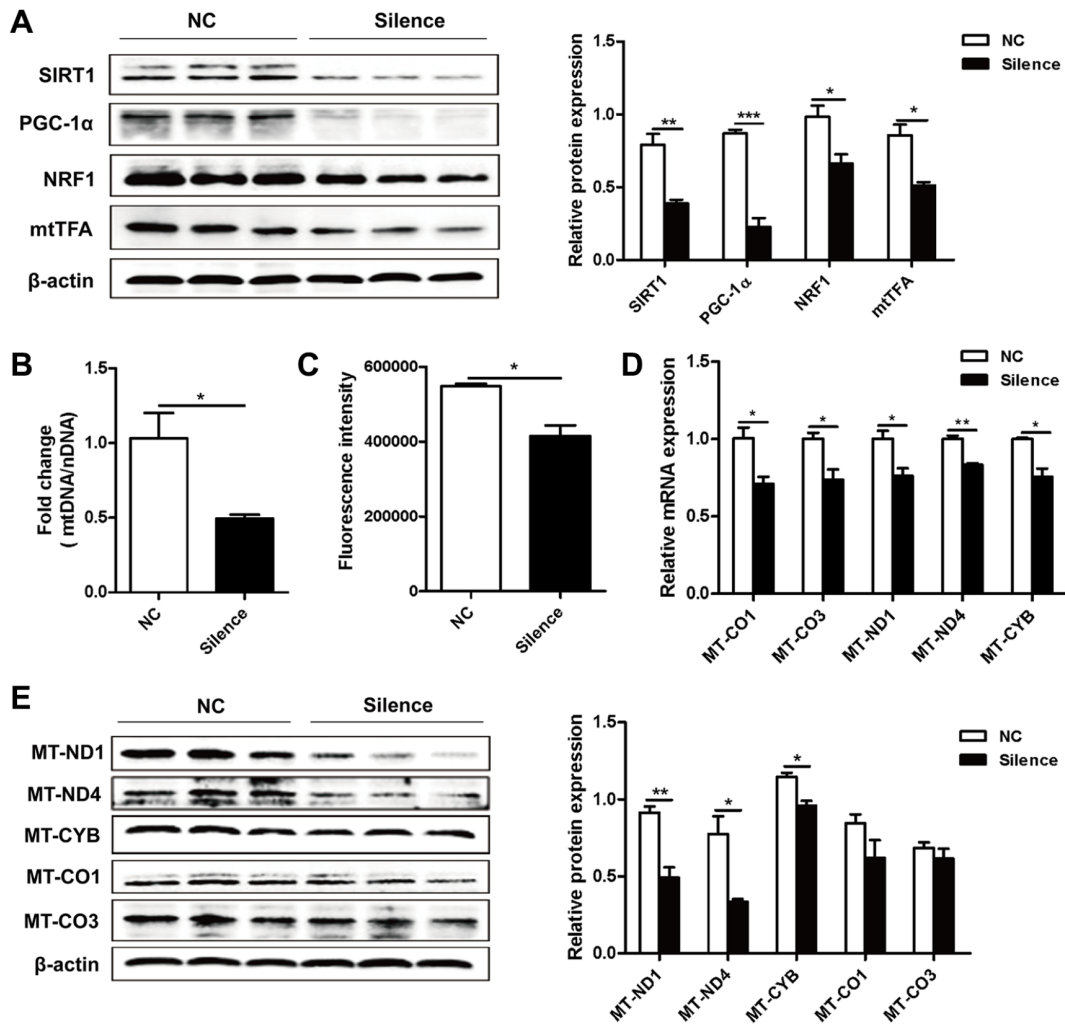


Figure 3. linc-PMB knockdown inhibits mitochondrial biogenesis in HepG2 cells (A) SIRT1, PGC-1 α , NRF1, and mtTFA were detected via western blot analysis in control HepG2 cells and in linc-PMB-knockdown HepG2 cells treated with a negative control (NC) or Smart Silencer (Silence) for 72 h. The relative protein expression was quantified via Image J software (right panel). (B) mtDNA content in control HepG2 cells and in linc-PMB-knockdown HepG2 cells treated with negative control (NC) or Smart Silencer (Silence) for 72 h. COX I and 18S were amplified via qRT-PCR in total DNA extracts to calculate the mtDNA/nDNA ratio. (C) Mitochondrial mass in control HepG2 cells and in linc-PMB-knockdown HepG2 cells treated with negative control (NC) or Smart Silencer (Silence) for 72 h was determined via MitoTracker (300 nM) staining and flow cytometry analysis. (D) Expressions of mitochondrial-encoded genes in control HepG2 cells and in linc-PMB-knockdown HepG2 cells treated with negative control (NC) or Smart Silencer (Silence) for 72 h. (E) Expressions of mitochondrial-encoded proteins were measured by western blot analysis in control HepG2 cells and in linc-PMB-knockdown HepG2 cells treated with negative control (NC) or Smart Silencer (Silence) for 72 h. The relative protein expression was quantified via Image J software (right panel). Data are presented as the mean $\pm$ SD of three independent experiments. * P < 0.05, ** P < 0.01, *** P < 0.001.

motifs that bind to the 3'UTR of the SIRT1 mRNA with high affinity, thereby maintaining the steady-state level of SIRT1 mRNA [30,31]. To assess the impact of linc-PMB on SIRT1 mRNA stability, the half-life ($t_{1/2}$) of SIRT1 mRNA was determined following treatment with actinomycin D to inhibit de novo transcription, and SIRT1 mRNA level was measured via qRT-PCR. The half-life of SIRT1 mRNA was reduced to 1 h with linc-PMB knockdown compared with 2 h in the negative control (NC) group (Figure 6C). Conversely, the half-life of SIRT1 mRNA was longer in the linc-PMB-overexpressing group than in the control group (Figure 6D). These results suggest that the binding of linc-PMB to HuR promotes the stabilization of SIRT1 mRNA, leading to substantial upregulation of SIRT1 level in HepG2 cells.

Discussion

Despite the rapid advancement of RNA sequencing, which has led to the identification of tens of thousands of lncRNAs, the functions of the majority of these lncRNAs are still largely unknown [32,33]. Several studies have reported that the mTOR pathway positively regulates lncRNA expression [17,18]. To uncover novel lncRNAs and their functions, we utilized rapamycin to inhibit the mTOR signaling pathway in the HepG2 cell line, which was then subjected to lncRNA profiling analysis via a human lncRNA microarray. This approach was expected to identify lncRNAs associated with the mTOR network. Indeed, eight lncRNAs were found to be significantly differentially expressed (fold change > 2.0) in HepG2 cells treated with rapamycin. The expressions of four of these

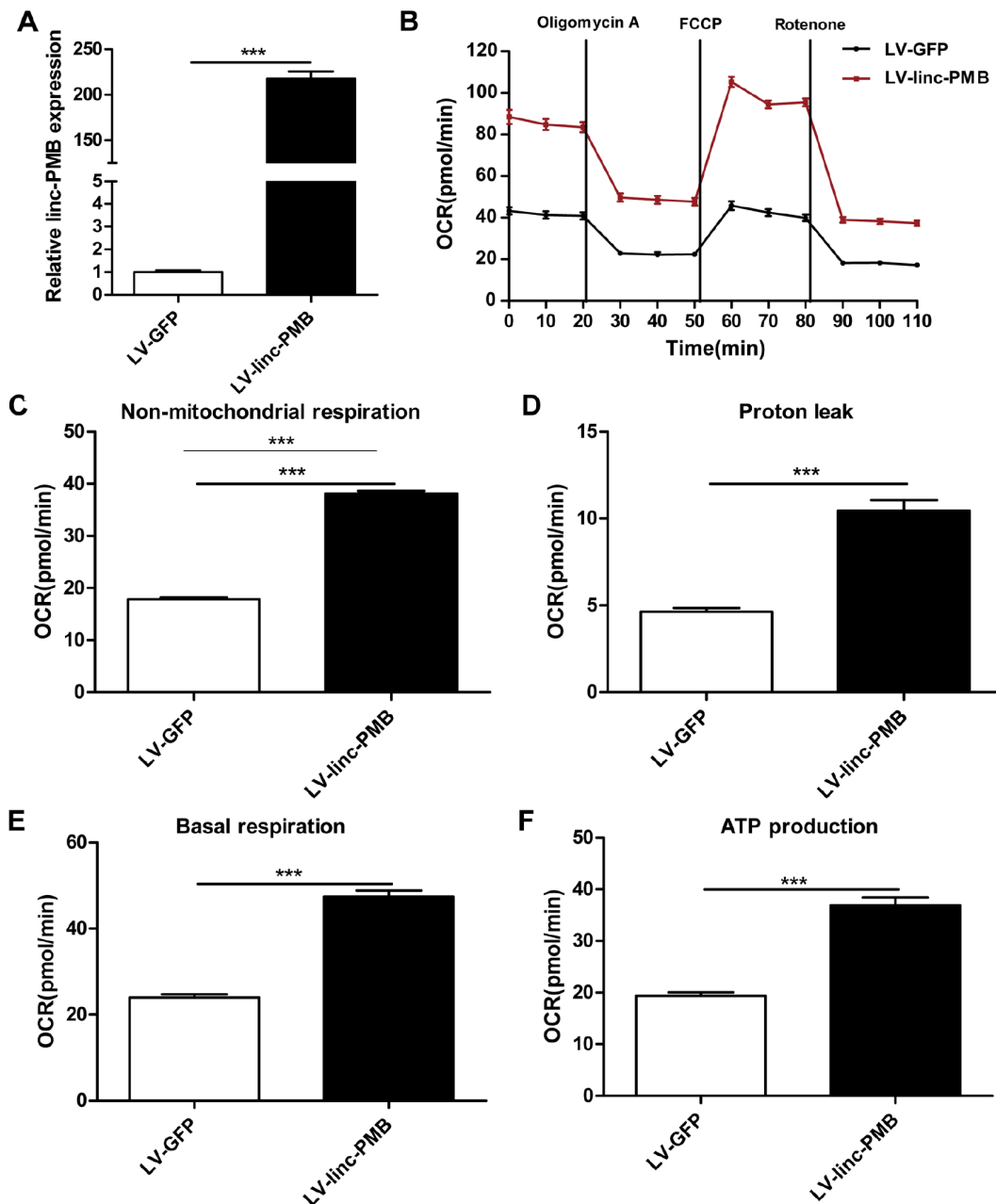


Figure 4. linc-PMB overexpression enhances mitochondrial respiration capacity in HepG2 cells (A) Establishment of linc-PMB-overexpressing HepG2 cells. LV-GFP: overexpression of empty vector; LV-linc-PMB: overexpression of linc-PMB. (B) Seahorse XF96 Flux analysis of LV-GFP HepG2 cells and LV-linc-PMB HepG2 cells. (C) Non-mitochondrial respiration of LV-GFP HepG2 cells and LV-linc-PMB HepG2 cells. (D) Proton leak of LV-GFP HepG2 cells and LV-linc-PMB HepG2 cells. (E) Basal respiration of LV-GFP HepG2 cells and LV-linc-PMB HepG2 cells. (F) ATP production by LV-GFP HepG2 cells and LV-linc-PMB HepG2 cells. Data are presented as the mean $\pm$ SD of three independent experiments. *** $P < 0.001$.

lncRNAs were confirmed via qRT-PCR. Notably, when mTOR was activated by insulin treatment, three of the four lncRNAs were downregulated compared with those with rapamycin treatment. These results suggest that mTOR can induce the transcription of lncRNA genes, although the underlying mechanisms remain to be elucidated. Among these three lncRNAs, linc-PMB was identified as a positive regulator of mTORC1 phosphorylation. The reciprocal interaction between linc-PMB and mTOR implies that linc-PMB is involved in the regulation of the mTOR signaling network.

In addition to its established role in mRNA translation regulation,

numerous studies have shown that mTOR also modulates mitochondrial functions, such as respiration, the tricarboxylic acid (TCA) cycle, and biogenesis [34]. In mammals, mTOR balances energy consumption by regulating mRNA translation and enhancing mitochondrial energy production through the synthesis of nucleus-encoded proteins related to mitochondrial function, including mtTFA, mitochondrial ribosomal proteins, and complexes I and V [35]. The mTOR inhibitor rapamycin has been shown to reduce the gene expression of key mitochondrial transcriptional regulators, such as PGC-1 α , ERR α , and NRFs, leading to decreased

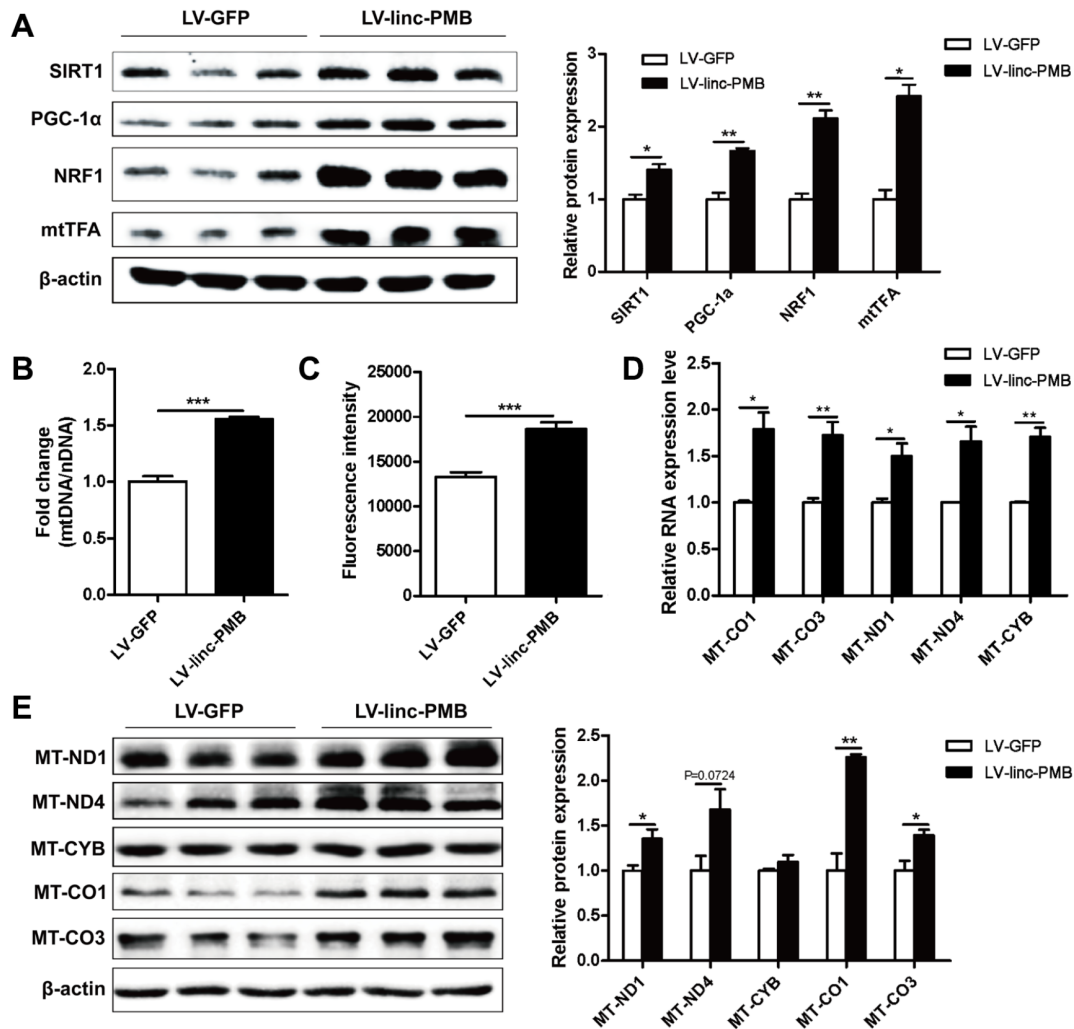


Figure 5. linc-PMB overexpression enhances mitochondrial biogenesis in HepG2 cells (A) Western blot analysis of SIRT1, PGC-1 α , NRF1, and mtTFA in LV-GFP HepG2 cells and LV-linc-PMB HepG2 cells. The relative protein expression was quantified via Image J software (right panel). (B) mtDNA content in LV-GFP HepG2 cells and LV-linc-PMB HepG2 cells. COX I and 18S were amplified via qRT-PCR in total DNA extracts to calculate the mtDNA/nDNA ratio. (C) Mitochondrial mass in LV-GFP HepG2 cells and LV-linc-PMB HepG2 cells was determined by MitoTracker (300 nM) staining and flow cytometry analysis. (D) Expressions of mitochondrial-encoded genes in LV-GFP HepG2 cells and LV-linc-PMB HepG2 cells. (E) Western blot analysis of mitochondrial-encoded proteins in LV-GFP HepG2 cells and LV-linc-PMB HepG2 cells. The relative protein expression was quantified via Image J software (right panel). Data are presented as the mean $\pm$ SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

mitochondrial gene expression and oxygen consumption [29].

In this study, we observed that linc-PMB expression was upregulated in response to mTORC1 inhibition by rapamycin. Knockdown of linc-PMB in HepG2 cells markedly decreased the expressions of PGC-1 α , NRF1, and mtTFA, resulting in reduced mitochondrial biogenesis and impaired mitochondrial respiration. Conversely, overexpression of linc-PMB enhanced mitochondrial biogenesis and mitochondrial respiration by increasing the expressions of PGC-1 α , NRF1 and mtTFA. These findings suggest a counteracting effect of linc-PMB on mTOR inhibition. Generally, lncRNAs are less abundant than mRNAs [36]. Although the elevated transcription of linc-PMB did not fully reverse the effects of rapamycin treatment, it likely mitigated the severe reduction in mitochondrial biogenesis and respiration. These results imply that linc-PMB plays an important role in maintaining the physiological function of mitochondria, especially when negative signals are present.

PGC-1 α stimulates the expressions of NRF1 and mtTFA, thereby promoting mitochondrial biogenesis, and its activity is modulated by various key factors, with AMPK and SIRT1 being particularly crucial [37]. However, in this study, the phosphorylation of AMPK was unaffected by changes in linc-PMB expression (Figure S1D, E). SIRT1 regulates the activity of PGC-1 α through deacetylation [28,38]. The expression of SIRT1 is significantly influenced by linc-PMB, suggesting that mitochondrial biogenesis and respiration are affected by linc-PMB via the SIRT1/PGC-1 α /mtTFA axis.

To elucidate the mechanism by which lncRNAs influence SIRT1 expression, RNA pulldown combined with LC-MS/MS was used to identify interacting proteins, leading to the identification of six proteins. Among them, the RNA-binding protein HuR is known to bind to the 3'UTR of SIRT1 mRNA, which encodes a protein involved in longevity and stress response, thereby stabilizing the mRNA and increasing SIRT1 expression level [30]. The regulation of

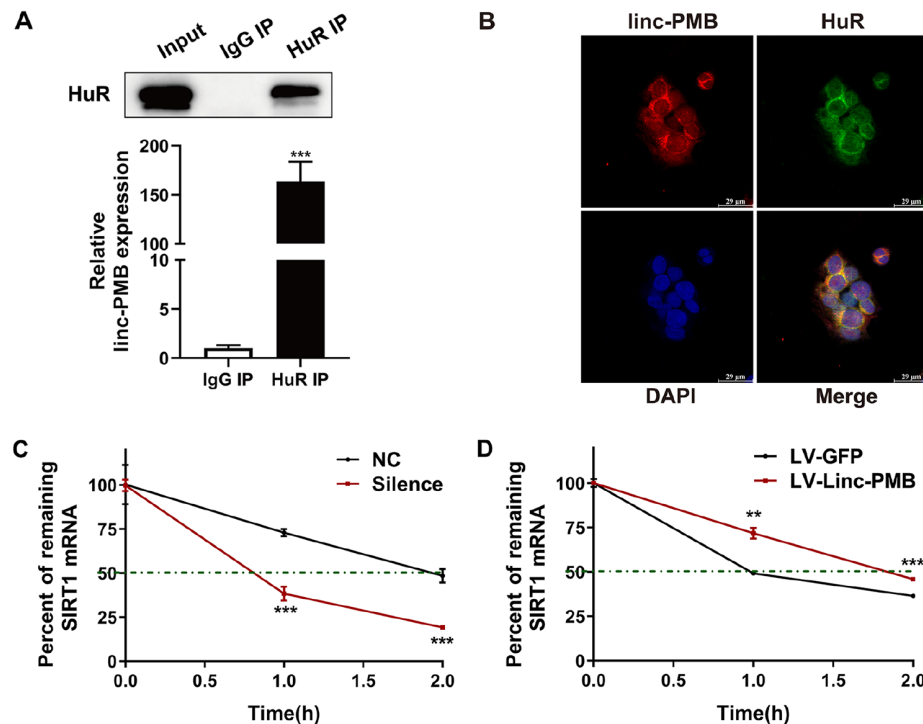


Figure 6. linc-PMB stabilizes SIRT1 mRNA by binding to HuR (A) The interaction of linc-PMB with HuR was verified via RNA RIP assay. The products obtained from HuR-RIP (HuR IP) and IgG-RIP (IgG IP) were determined via qRT-PCR (lower panel); the binding efficiency of the HuR antibody was validated via western blot analysis (upper panel). IgG IP was used as a negative control. (B) Intracellular localization of the linc-PMB and HuR proteins in HepG2 cells was visualized via RNA-FISH and IF staining, respectively. DAPI: 4',6-diamidino-2-phenylindole. (C) The half-life of SIRT1 after treatment with 4 μ g/mL actinomycin D for the indicated time intervals in linc-PMB-knockdown HepG2 cells. (D) The half-life of SIRT1 after treatment with 4 μ g/mL actinomycin D for the indicated time intervals in linc-PMB-overexpressing HepG2 cells. Data are presented as the mean $\pm$ SD of three independent experiments. * P < 0.05, ** P < 0.01, *** P < 0.001.

SIRT1 transcript abundance occurs at both the transcriptional and post-transcriptional levels, with HuR being a key post-transcriptional regulator [39]. Here, our findings suggest that lncRNAs may mediate specific interactions between HuR and the 3'-UTRs of its target mRNAs; specifically, linc-PMB may facilitate the binding of HuR to the SIRT1 mRNA, thereby promoting its stabilization.

Mitochondrial dysfunction has been strongly linked to a variety of age-related conditions, including type 2 diabetes, Alzheimer's disease, and cancer [40,41]. Our study reveals a mechanism by which lncRNA-linc-PMB promotes mitochondrial biogenesis and suggests that a previously unknown pathway potentially connects mTORC1 to mitochondrial function.

Supplementary Data

Supplementary data is available at *Acta Biochimica et Biophysica Sinica* online.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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