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RESEARCH ARTICLE

Prohibitin regulates mTOR pathway via interaction with FKBP8

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Abstract The ability of tumor cells to sustain continuous proliferation is one of the major characteristics of cancer. The activation of oncogenes and the mutation or inactivation of tumor suppressor genes ensure the rapid proliferation of tumor cells. The PI3K–Akt–mTOR axis is one of the most frequently modified signaling pathways whose activation sustains cancer growth. Unsurprisingly, it is also one of the most commonly attempted targets for cancer therapy. FK506 binding protein 8 (FKBP8) is an intrinsic inhibitor of mTOR kinase that also exerts an anti-apoptotic function. We aimed to explain these contradictory aspects of FKBP8 in cancer by identifying a "switch" type regulator. We identified through immunoprecipitation–mass spectrometry-based proteomic analysis that the mitochondrial protein prohibitin 1 (PHB1) specifically interacts with FKBP8. Furthermore, the downregulation of PHB1 inhibited the proliferation of ovarian cancer cells and the mTOR signaling pathway, whereas the FKBP8 level in the mitochondria was substantially reduced. Moreover, concomitant with these changes, the interaction between FKBP8 and mTOR substantially increased in the absence of PHB1. Collectively, our finding highlights PHB1 as a potential regulator of FKBP8 because of its subcellular localization and mTOR regulating role.

Keywords prohibitin 1; FKBP8; mTOR; cell proliferation; cancer

Introduction

The mammalian target of rapamycin (mTOR) is a major intracellular coordinator of growth and nutrient signals in eukaryotic cells. mTOR regulates several fundamental cellular processes, including the metabolism of protein, glucose, nucleotide, fatty acid, and lipid; mitochondrial biogenesis; and autophagy. As a serine–threonine protein kinase, mTOR plays a predominant role in the regulation of protein synthesis by affecting the activity of its target proteins, the translation initiation factor binding protein 4E-BP1, and the ribosomal S6 protein kinase (p70 S6 kinase) [1]. The phosphorylation of either target protein enhances protein synthesis, which results in increased cell proliferation. Therefore, the mTOR pathway is frequently activated in cancer, where the proliferation of cancer cells requires the coordination between oncogenic signaling and metabolic alteration [2]. For this reason, the signaling pathways that regulate mTOR have been intensively studied. Upstream regulating pathways, such as the PI3K–Akt pathway, inhibit tuberous sclerosis complex (TSC1/2), which results in the activation of mTOR; the LKB1–AMPK pathway activates TSC1/2, which leads to the downregulation of Rheb and the inhibition of mTOR. Although mTOR activation via TSC1/2 and Rheb has been widely investigated, little is known about how mTOR is intrinsically inhibited.

FK506 binding protein 8 (FKBP8) is the only intrinsic inhibitor of mTORC1 reported [3]. FKBP8, also known as FKBP38, belongs to the FK506 binding protein family [4]. The protein structure of FKBP8 consists of an N-terminal Glu-rich region, a peptidyl-prolyl cis-transisomerases domain, three quadruple peptide repeats, a calmodulin binding domain, and a C-terminal transmembrane (TM) domain [5,6]. FKBP8 is anchored in the outer mitochondrial membrane through its C-terminal TM domain, which exposes its N-terminal part to the cytoplasm [7,8]. FKBP8 binds to mTOR and inhibits its activity in a similar way to that of the FKBP12–rapamycin complex; thus, it inhibits

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the mTOR-mediated signaling pathway for cell proliferation and growth [3,9]. Later studies have also shown that FKBP8 is an important anti-apoptotic protein; FKBP8 inhibits apoptosis by anchoring anti-apoptotic proteins Bcl-2 and Bcl-XL to the mitochondria through its interaction with them [7,10,11]. Interestingly, FKBP8 has a paradoxical role in cancer with anti-apoptotic and anti-proliferation functions. However, how these anti-apoptotic and mTOR inhibitory functions of FKBP8 were regulated and what their roles are in cancer progression remain to be illustrated. Hence, we identified the interactome of FKBP8 to dissect the regulatory mechanism of FKBP8 in cell proliferation and apoptosis and found that FKBP8 binds to prohibitin 1.

Prohibitin 1 (PHB1/BAP32) and prohibitin 2 (PHB2/BAP37) belong to the stomatin/prohibitin/flotillin/HflKC protein family [12]. PHB1 and PHB2 form about 1 MDa functional ring super complex structure on the inner membrane of the mitochondria [13]. PHB1 was first identified as an anti-proliferative protein [14]; however, this function was later found to be caused by the 3′ untranslated region of PHB1 mRNA and not the protein itself. PHB1 has controversial roles on cell proliferation in different cancer types. PHB1 can inhibit the cell proliferation of estrogen receptor-positive cancer cells, such as positive breast cancer [15] and osteosarcoma cells [16]. In addition, the expression level of PHB1 in nasopharyngeal carcinoma and hepatic carcinoma cells has decreased substantially [17,18]. However, PHB1 has the opposite effect in other cancer types. For instance, PHB1 is overexpressed in gallbladder carcinoma, bladder cancer, ovarian cancer, and prostate cancer [19–23]. The oncogenetic function of PHB1 is associated with the activation of the c-RAF–MEK–ERK and downstream pathway [24], whereas the role of PHB1 in the regulation of mTOR pathway remains to be determined.

In this study, we found that FKBP8 is localized in the mitochondria and binds to PHB1. Upon PHB1 knockdown, FKBP8 is released from the mitochondria and results in increased binding with mTOR, and the silencing of PHB1 leads to the inhibition of the mTOR pathway and the subsequent inhibition of cell proliferation. Moreover, PHB1 expression is upregulated in ovarian cancer tissue compared with normal tissue. Thus, PHB1 has cancer-promoting function.

Materials and methods

Antibodies and reagents

The antibodies used in this study were anti-FKBP8 (#391505, R&D System, USA), anti-phospho-mTOR Ser 2448 (#5536, Cell Signaling Technology, USA), anti-hemagluttinin (HA)–horseradish peroxidase (HRP; #A00169-40, GeneScript, China), anti-phospho-p70S6K Thr 389 (#04-392, Sigma-Aldrich, USA), anti-phosphor-4EBP1 Ser 65 (#9451S, Cell Signaling Technology, USA), anti-GAPDH (#60004, Proteintech, USA), anti-Bcl-2 (#15071, Cell Signaling Technology, USA), anti-Akt (#T308, Cell Signaling Technology, USA), anti-phospho-Akt Ser 473 (#9271T, Cell Signaling Technology, USA), anti-COX IV (#4844, Cell Signaling Technology, USA), anti-mTOR (#2983P, Cell Signaling Technology, USA), and anti-PHB1 (ab70672, Abcam, USA). Lipofectamine 2000 was purchased from Thermo, and MG132 was purchased from APExBIO (USA). For *in vitro* experiments, MG132 (20 mmol/L) was dissolved in dimethyl sulfoxide (DMSO).

Cell culture

HeLa and SK-OV-3 cell lines were purchased from Cell Resource Center, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. Mes-Sa and HEK293 cell lines were purchased from The American Type Culture Collection. HeLa and HEK293 cell were cultured in high-glucose Dulbecco's modified Eagle's medium (Hyclone) supplemented with 10% fetal bovine serum (Gibco), 100 U/mL penicillin, and 100 mg/mL streptomycin. SK-OV-3 and Mes-Sa cell lines were cultured in McCoy's 5A (basal media) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin. All cell lines were tested negative for mycoplasma. Cell culture and all biological experiments were performed at 37 °C in 5% CO_2 conditions in a cell culture incubator. For insulin stimulation, the cells were grown to ~70% confluence on culture dishes under normal condition, then starved with serum-free DMEM for 20 h, and treated with 150 nmol/L insulin diluted in serum-free DMEM-for 30 min. The protein was harvested for the next experiment.

siRNA

RNA interference was performed using siRNAs that target FKBP8 (5′-GAGUGGCUGGACAUUCUGG-3′) and PHB1 (5′-GCGACGACCUUACAGAGCG-3′) and a negative control (5′-UUCUCCGAACGUGUCACGUUU-3′). All the siRNAs were synthesized from Sangon Biotech (Shanghai, China). siRNA (100 nmol/L) transfection was performed using Lipofectamine 2000 (Thermo Fisher Scientific, MA, USA) according to the manufacturer's instructions.

Western blot

Cells were rinsed with ice-cold Dulbecco's phosphate

buffered saline (DPBS) three times and lysed for approximately 30 min. The pyrolysis solution was composed of mammalian cell lysis buffer (MCLB; 50 mmol/L Tris, pH 7.5; 150 mmol/L NaCl; 0.5% NP40), complete EDTA-free protease inhibitor (Roche), 1 mmol/L phenylmethylsulfonyl fluoride (PMSF, Amresco), and phosphatase inhibitor cocktail (Roche, USA). The cell lysate was centrifuged at 13 000 × *g* for 20 min, and then protein concentrations were measured by Quick Start™ Bradford (Bio-Rad, USA). Total protein (25 μg) was separated by 10% sodium dodecyl sulfate (SDS)–polyacrylamide gels using an electrophresis apparatus (BioTanon, shanghai, China) and transferred to nitrocellulose filter membrane (Millipore, USA). The membranes were blocked with 5% skim milk for 2 h and incubated with the indicated primary antibodies overnight at 4 °C. Next, the membranes were washed three times with Tris-buffered saline with Tween (20 mmol/L Tris, pH 7.4; 137 mmol/L NaCl; 0.05% Tween-20) and then incubated with secondary antibodies at room temperature for 40 min. Ultimately, the immunoreactive protein bands were detected by enhanced chemiluminescence using the ChampChemi imaging system (Sage Creation Science).

Immunoprecipitation (IP)

HEK293 cells were co-transfected with HA-tagged PHB1 and enhanced green fluorescent protein (EGFP)-tagged FKBP8 and cultured for 48 h under normal conditions. The cells were rinsed with ice-cold DPBS three times and lysed for approximately 30 min. The cell lysate was centrifuged for 20 min at 13 000 × *g*, and 1 mg protein supernatant was incubated with HA magnetic beads (Thermo Fisher Scientific, MA, USA) overnight at 4 °C. Next day, the precipitated immunocomplexes were washed four times with MCLB and denatured by 5 × SDS loading and then subjected to Western blot analysis described above.

Plasmid construction and transfection

The cDNA encoding FKBP8 and PHB1 were reverse transcribed from HEK293 mRNA and amplified by polymerase chain reaction (PCR). The amplified fragments of FKBP8 and PHB1 were inserted into Pdonr223 vector and then subcloned in pHAGE-C-HA-Puro lentivirus vector. FKBP8 was also subcloned in pHAGE-N-EGFP-Puro lentivirus vector. The mixed construction plasmid and packaging plasmid (TAT + MPMG + Rev + VSV-G) were transfected into HEK293 cells. The cell supernatant was collected and filtered after 48 h, and the virus supernatant was added to HeLa or HEK293 cells in the presence of 10 μg/mL polybrene to generate the corresponding cell lines. Infected cells were maintained in 1 μg/mL puromycin for 4 days and expanded for immunofluorescence experiments.

Cell viability assay

Cell proliferation was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Twenty-four hours after the SK-OV-3 and HeLa cells were transfected with siRNAs (siCTL and siPHB1), the cells were plated into 96-well plate at a density of 3×10^3 cells/well. Then, 20 μL MTT (Sigma-Aldrich, USA) solution was added into the cells and incubated for 4 h at 37 °C with 5% CO_2. Afterward, the media was removed, and 150 μL of DMSO was added. The plates were incubated for 10 min at 37 °C. The absorbance of MTT solution was measured at 490 nm.

Immunofluorescence

HeLa cells were transfected with N-terminal EGFP-tagged FKBP8 construct, and HEK293 cells were transfected with C-terminal HA-tagged FKBP8 construct. The HeLa and HEK293 cells were separately seeded in six-well plates containing glass plates and treated with Mito-tracker red/Mito-tracker green for 45 min. The cells were incubated in complete medium for 1 h, washed with PBS, and fixed with 4% paraformaldehyde at room temperature for 10 min. After that, the cells were permeabilized with 0.2% TritonX-100 for 8 min, washed with PBS for 5 min, blocked in 2% goat serum and 2% BSA/PBS for 1 h, incubated with specific primary antibody and then with Alexa Fluor 488/568-conjugated secondary antibody, and finally stained with 4′,6-diamidino-2-phenylindole (DAPI). The cell slides were observed under a Leica confocal microscope.

Real-time quantitative PCR (qPCR)

Total RNA isolated from HeLa cells using TRIzol (Tiangen Biotech, Beijing, China) was subjected to reverse transcription with the use of a HiScript III RT SuperMix for qPCR (with gDNA wiper) Kit (Vazyme Biotech, Nanjing, China), and the resulting cDNA was subjected to PCR with HieffTM qPCR SYBR Green Master Mix performed on the Light Cycler® 480 qPCR System (Roche, Switzerland). The relative expression level of the target mRNA was analyzed using $2^{-\Delta\Delta CT}$ method. The sense and antisense qPCR primer sequences were 5′-CTGGCCA-AGGTCATCCATGAC-3′ and 5′-CTTGCCCACAGC-CTTGGCAG-3′ for human GAPDH, 5′-GACCACG-TAATGTGCCAGTCA-3′ and 5′-CATCATAGTCCT-CTCCGATGCT-3′ for human PHB1, and 5′-AACCTTC-CAACAAGACGATCC-3′ and 5′-CCCAGCATTTT-CCGGTACAAG-3′ for human FKBP8, respectively.

IP and mass spectrometry (MS)

HEK293 cells were transfected with a pHAGE lentiviral vector that contains the open reading frame of FKBP8 and HA-Flag tag [25]. After 48 h of transfection, 4 × 15 cm^2 plates with approximately 80%–90% confluent cells were washed once with ice-cold DPBS and then harvested in NP-40 lysis buffer (50 mmol/L Tris-HCl, pH 7.5; 0.5% NP-40 substitute; 150 mmol/L NaCl) supplemented with complete Mini EDTA-free protease inhibitor tablets (Roche, USA) and PMSF. Extracts were clarified for 20 min at 13 000 × *g*, and the soluble fraction was incubated with anti-HA–agarose beads (Sigma, USA) overnight at 4 °C. The beads were washed five times with NP-40 lysis buffer and then eluted three times for 30 min each time at room temperature with 500 μg/mL HA peptide (Sigma, USA). The eluted proteins were concentrated with trichloroacetic acid (Sigma, USA), trypsinized, loaded onto stage tips, and analyzed by liquid chromatography–tandem mass spectrometry (LC–MS/MS) in duplicate runs according to previously published protocols [25]. Peptides were identified using Sequest and a target–decoy strategy [25,26].

Duolink *in situ* colocalization assay

Mes-Sa cells were plated onto coverslips in six-well plates and cultured overnight. The cells were fixed and permeabilized as described above. Next, *in situ* colocalization assays were performed using the Duolink *In Situ* Orange Starter Kit Mouse/Rabbit (Sigma-Aldrich) according to the manufacturer's instructions. Briefly, the cells were blocked with blocking buffer for 30 min at 37 °C and incubated in diluted primary antibody (1:200 anti-PHB rabbit antibody, Abcam #70672; 1:100 anti-FKBP8 mouse antibody, R&D Systems #MAB3580) for 1 h at 37 °C. Unbound antibodies were removed by washing the samples three times with wash buffer A. The cells were incubated with secondary antibodies conjugated with proximity ligation assay (PLA) probe for 1 h at 37 °C. The samples were then washed three times with wash buffer A and subjected to ligation reaction for 30 min and amplification reactions for 100 min at 37 °C. Unbound labeled oligonucleotides were removed by washing the samples three times with buffer B before embedding the samples in Prolong Gold antifade mounting medium.

Immunohistochemistry (IHC)

Human ovarian cancer and normal tissue samples were obtained from the Department of Obstetrics and Gynecology, Ruijin Hospital Affiliated to Shanghai Jiao Tong University School of Medicine. The tissue samples were embedded in paraffin and cut into 4 μm-thick sections for IHC. The slides were deparaffinised, steamed in heat-mediated antigen retrieval buffer (10 mmol/L Tris-1 mmol/L EDTA, pH = 9.0) for 15 min, incubated with primary mouse monoclonal antibody against Prohibitin (Abcam #1836, 1:100) overnight at 4 °C, and incubated with HRP-linked secondary antibody (Dako, #K5007) for 30 min at 37 °C using an enhanced labeled polymer system. Finally, all sections were treated with diaminobenzidine. The IHC sections were observed and analyzed according to staining intensity: unstained or weak staining was defined as low expression, and medium-strength staining was defined as high expression.

Statistical analysis

The relative quantification of qPCR data was calculated using $2^{-\Delta\Delta CT}$ method. All data were analyzed using GraphPad Prism software V8 and represented as the mean ± standard deviation (SD) of three independent experiments. Two-sided Student *t*-test was used to analyze the differences between groups. Each experiment was repeated three times. Significance was defined by $P < 0.05$.

Results

FKBP8 is localized in the mitochondria through its C-terminal, and the overexpression of non-mitochondrial-localized FKBP8 inhibits the mTOR pathway

FKBP8 shuttles from the mitochondria to other cellular locations when in stressed condition [27]. Thus, the subcellular localization of FKBP8 may be essential for its function. The localization of FKBP8 on the outer mitochondrial membrane is mediated by a TM sequence at the C terminus of its protein structure. Therefore, we constructed a FKBP8 plasmid with HA tag at the C terminus and another FKBP8 construct with EGFP tag at the N terminus and established stable FKBP8 overexpression cell lines. We found that the FKBP8 protein with HA tag at the C terminus was localized in the mitochondria and also diffused in the cytoplasm (Fig. 1A). The FKBP8 with EGFP tag at the N terminus was localized in the mitochondria (Fig. 1B). Our immunofluorescence result shows that endogenous FKBP8 was predominantly localized in the mitochondria (Fig. 1C). These results indicated that disrupting the C-terminal sequence of FKBP8 through an epitope tag affects the mitochondrial localization of FKBP8. However, disrupting the N-terminal sequence did not have such effect.

The overexpression of C-terminal HA-tagged FKBP8 inhibited the phosphorylation of p70S6K, which suggests the inhibition of mTOR signaling (Fig. 1D). By contrast,

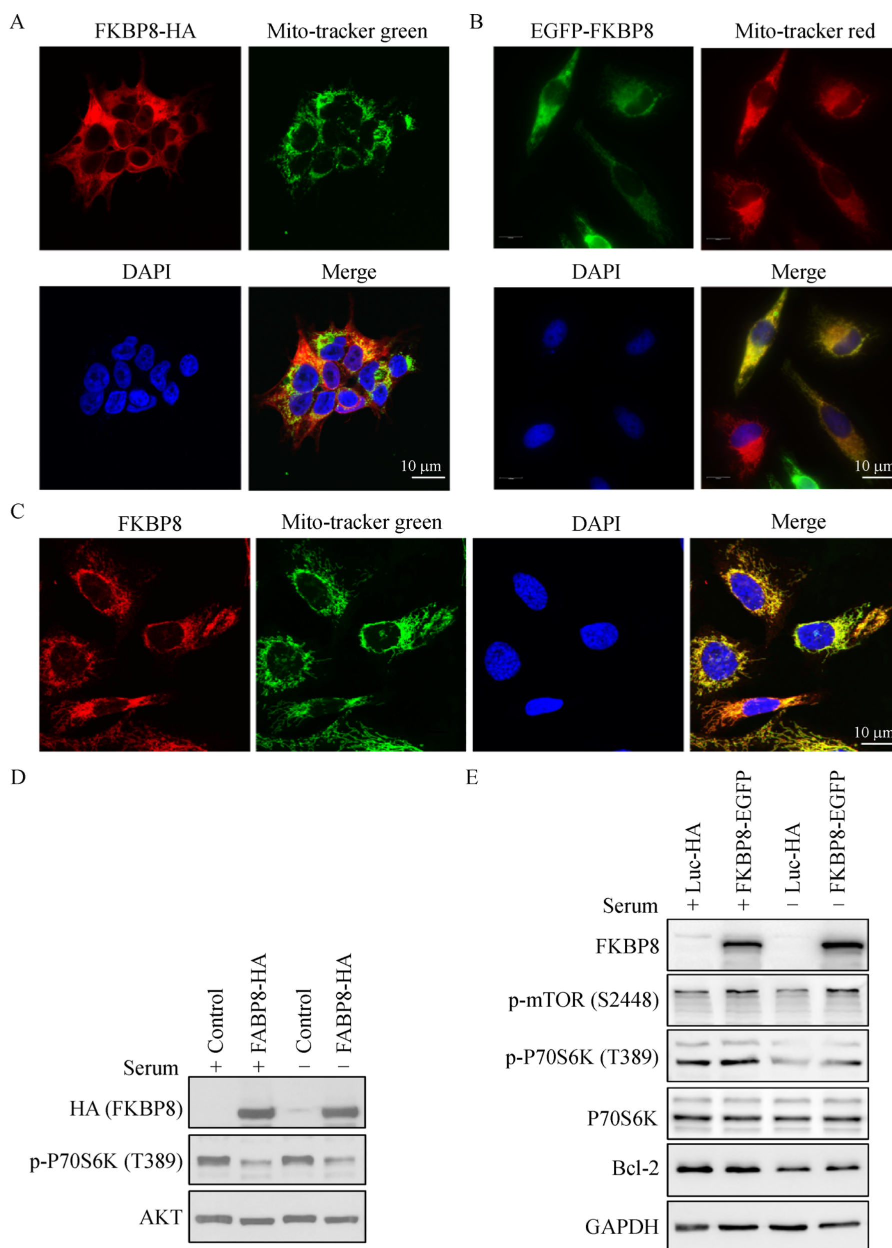

Fig. 1 FKBP8 is localized in the mitochondria through its C terminal and the overexpression of non-mitochondrial-localized FKBP8 inhibits the mTOR pathway. (A) HEK293 cells were stably transfected with C-terminal HA-tagged FKBP8, incubated with 200 nmol/L Mito-tracker green for 45 min, and subjected to immunofluorescence assay with anti-HA (red) antibody. Nuclei were stained with DAPI (blue). Scale bar, 10 μm. (B) Fluorescence analysis of HeLa cells stably transfected with N-terminal EGFP-tagged FKBP8 and stained by Mito-tracker red. Nuclei are stained with DAPI (blue). Scale bar, 10 μm. (C) SK-OV-3 cells were incubated with 200 nmol/L Mito-tracker green for 45 min and subjected to immunofluorescence assay with anti-FKBP8 (red) antibody. Nuclei were stained with DAPI (blue). Scale bar, 10 μm. (D) HEK293 cells were transfected with plasmid expressing C-terminal HA-tagged FKBP8 for 48 h followed by serum starvation overnight. Proteins were extracted and subjected to Western blot analysis. mTOR pathway signaling was assessed by p-P70S6K. AKT was used as loading control. (E) HEK293 cells were transfected with plasmid expressing N-terminal EGFP-tagged FKBP8 for 48 h and subjected to serum starvation overnight. Proteins were extracted and subjected to Western blot analysis. mTOR pathway signaling was assessed by p-mTOR and p-P70S6K. GAPDH was used as loading control.

N-terminal EGFP-tagged FKBP8 slightly increased the phosphorylation of mTOR and p70S6K (Fig. 1E). This result indicated that the localization of FKBP8 affects its function as an inhibitor of the mTOR signaling pathway.

FKBP8 interacts with PHB1

We identified the interactome of FKBP8 by IP–MS-based proteomic analysis to investigate how FKBP8 function was regulated spatially. We determined the FKBP8 interaction spectrum (Fig. 2A) and identified several novel FKBP8-interacting proteins, including the mitochondrial protein PHB1. Tandem IP using HEK293 cells with overexpressed C-terminal HA-tagged PHB1 successfully pulled down the N-terminal EGFP-tagged FKBP8 co-overexpressed in the same cells (Fig. 2B), and IP using HEK293 cells with overexpressed C-terminal HA-tagged FKBP8 successfully pulled down endogenous PHB1 (Fig. 2C). PHB1 is present in multiple compartments of the cell but it is primarily located in the mitochondria. We performed endogenous immunofluorescence staining in Mes-Sa cells and analyzed the results by confocal microscopy to determine the regions where PHB1 and FKBP8 are co-localized. PHB1 and FKBP8 were found to be co-localized predominantly in the mitochondria, although some cytoplasmic staining was apparent (Fig. 2D). Additionally, we used the Duolink *in situ* PLA to measure intracellular PHB1–FKBP8 interaction. The experiment revealed the Duolink signal as a red fluorescent puncta that indicates an *in situ* interaction event (Fig. 2E). Together, these results demonstrate that FKBP8 co-localized and interacted with PHB1 in mitochondria.

PHB1 knockdown results in the downregulation of mTOR and decreased cell proliferation

PHB1 affects the proliferation of various cancer cells. We investigated the role of PHB1 in the regulation of cell proliferation in the ovarian cancer SK-OV-3 cells. The successful knockdown of PHB1 by siRNA remarkably inhibited the proliferation of SK-OV-3 cells compared with that of the control group (Fig. 3A and 3B). We also examined the effect of the absence of PHB1 on the colony forming ability of HeLa cells and found that PHB1 silencing remarkably reduced the number of clones compared with that of the control group (Fig. 3C).

Considering the unique interaction between PHB1 and FKBP8, we wondered whether disrupting the expression of PHB1 would affect the inhibitor role of FKBP8 in the mTOR signaling pathway. PHB1 siRNA-treated and control siRNA-treated SK-OV-3 cells were starved for 20 h and treated with 150 nmol/L insulin for 15 or 30 min to stimulate mTOR activation. We found that the phosphorylation of mTOR substrate protein P70S6K was remarkably reduced after PHB1 was silenced (Fig. 3D). Furthermore, in Mes-Sa cells and chemo-resistant Mes-Sa cell line Mes-Sa-Dx5 cells, PHB1 silencing decreased P70S6K and 4EBP1 phosphorylation, which indicated the inhibition of mTOR signaling. Meanwhile, we observed an increase in AKT phosphorylation at Ser473, which suggested that mTOR inhibition upon PHB1 silencing induced the negative feedback activation of AKT (Fig. 3E). Taken together, PHB1 knockdown inhibited the mTOR signaling pathway, and this inhibition was concurrent with the suppression of cell proliferation.

Knockdown of PHB1 resulted in the release of FKBP8 from mitochondria and increased FKBP8–mTOR interaction

We tested the expression level of FKBP8 after silencing PHB1 in SK-OV-3 cells to investigate how PHB1 silencing led to the inhibition of mTOR signaling pathway and whether this inhibition is via FKBP8. The cells were subjected to serum starvation for 20 h and then stimulated with 150 nmol/L insulin for 30 min. Interestingly, we observed that the level of FKBP8 decreased when PHB1 was silenced for more than 72 h (Fig. 4A). FKBP8 is located in the mitochondria; thus, we suspected that downregulating PHB1 would affect the protein abundance of FKBP8 in the mitochondria. Forty-eight hours after siRNA transfection, cells were collected, then mitochondrial and cytoplasmic proteins were separately extracted, and FKBP8 expression was detected by Western blot. The protein level of FKBP8 in mitochondrial proteins was remarkably reduced (Fig. 4B). PHB1 and PHB2 form a complex structure on the inner membrane of mitochondria [13]; hence, the protein level of PHB2 decreased substantially after silencing PHB1. This finding confirmed that PHB1 and PHB2 are interdependent at the protein level. Furthermore, extended knockdown time resulted in the reduction of total FKBP8 protein level and reduced the protein level of Bcl-2 (Fig. 4C). These results indicated that PHB1 silencing resulted in the release of FKBP8 from the mitochondria, which eventually decreased the overall FKBP8 protein level.

Meanwhile, qPCR analysis showed no considerable change in FKBP8 mRNA levels after PHB1 knockdown (Fig. 4D). The cells were treated with 20 μmol/L MG132 for 8 h after transfection with siPHB1 for 72 h. We found that compared with mitochondrial marker COX IV, FKBP8 protein decreased significantly after PHB1 silencing but was not inhibited by MG132. This result suggests that the degradation of FKBP8 protein level may not be through a proteasome pathway (Fig. 4E). FKBP8 is an endogenous inhibitor of mTOR [3]. We further investigated whether the inhibitory effect of PHB1 on mTOR signaling is mediated by FKBP8. The results showed that PHB1 silencing

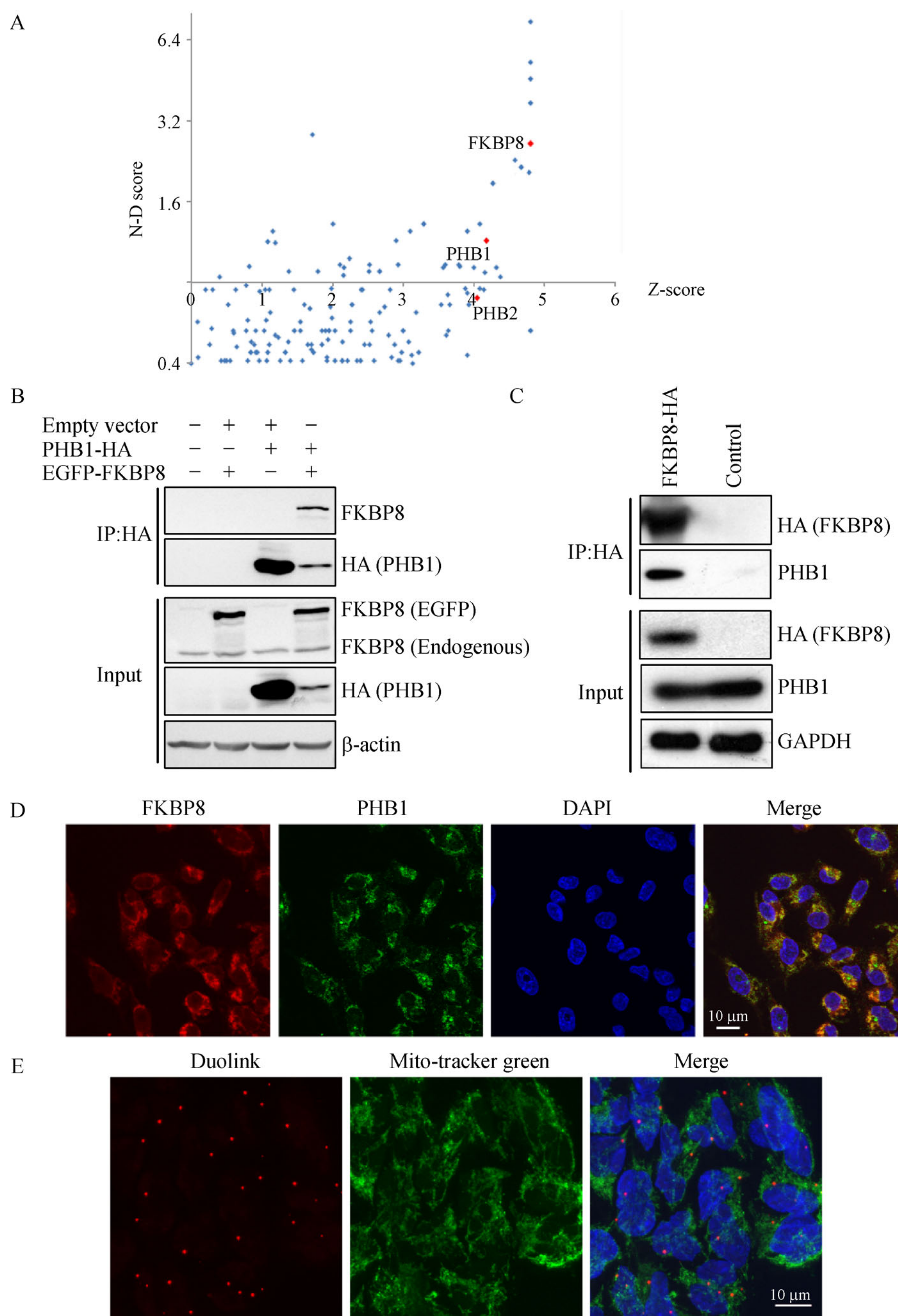

Fig. 2 FKBP8 interacts with PHB1. (A) IP–MS of FKBP8-HA in HEK293 cells. Lysates of PHB-HA-expressing cells were immunoprecipitated with HA resin. Bait complexes were analyzed by LC–MS/MS. High-confidence interacting proteins had normalized weighted D scores $>$ 1 and Z-scores $>$ 4. (B) HEK293 cells were transiently transfected with PHB1-HA and EGFP-FKBP8 plasmids. Proteins were subjected to IP using HA magnetic beads, and co-precipitation FKBP8 (EGFP) was detected by Western blot. Whole cell lysates (input) showed that all plasmids were expressed in the transfected cells. (C) HEK293 cells were transfected with FKBP8-HA plasmid, proteins were subjected to IP using HA magnetic beads, and co-precipitation endogenous PHB1 was detected by Western blot. (D) Immunofluorescent staining of endogenous FKBP8 (red) and PHB1 (green) in Mes-Sa cells. Nuclei were stained with DAPI. Scale bar, 10 μm. (E) *In situ* PLA between FKBP8 and PHB1 was performed with mouse anti-FKBP8 and rabbit anti-PHB1 antibodies using Duolink PLA technology. Each PLA signal (red fluorescent puncta) is indicative of one detected FKBP8–PHB1 interaction event in Mes-Sa cells. Red, PLA signal; blue, DAPI; green, Mito-tracker green. Scale bar, 10 μm.

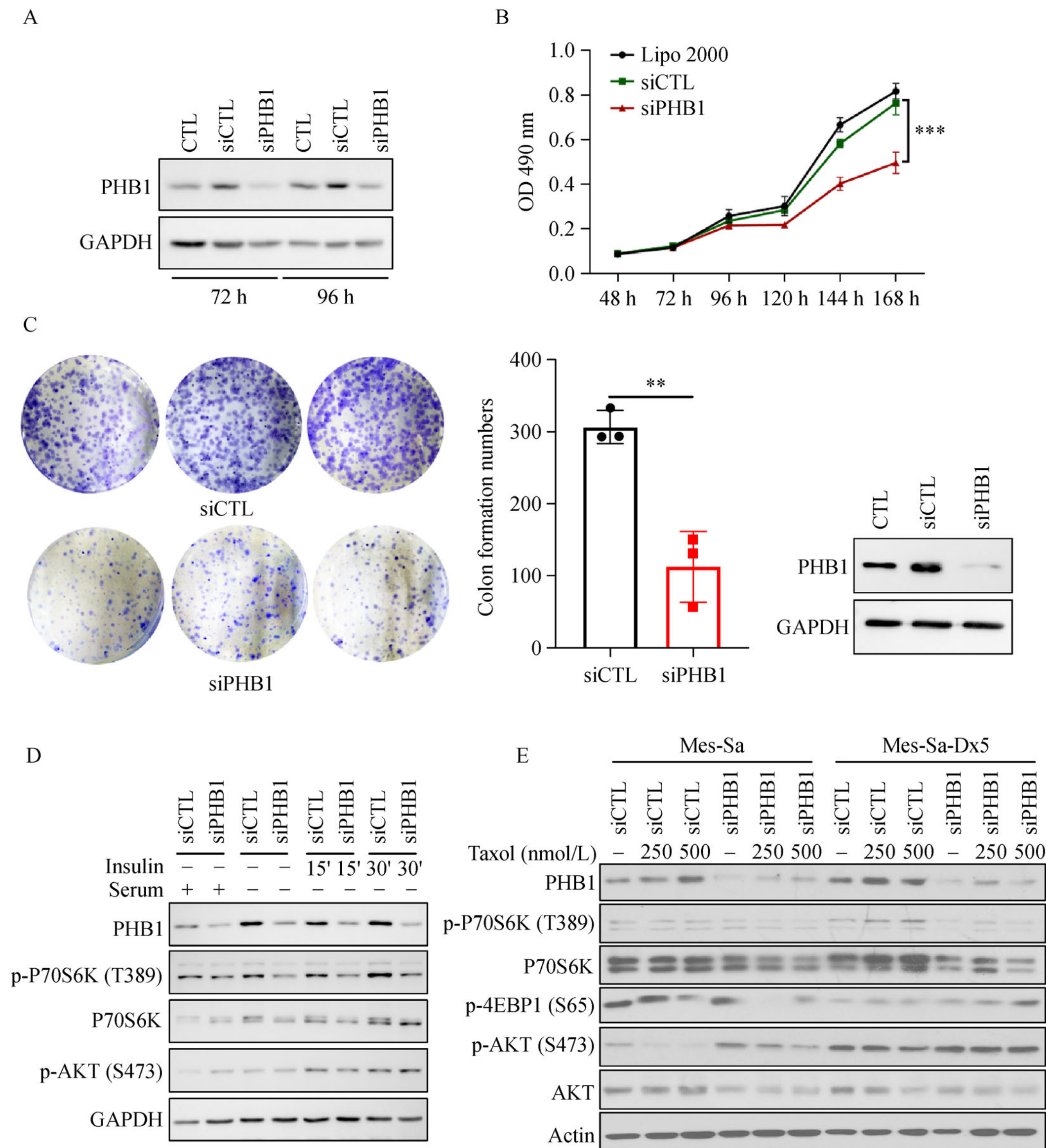

Fig. 3 Silencing of PHB1 decreases cell proliferation and colony formation abilities. (A) SK-OV-3 cells were transfected with siPHB1 for 72 and 96 h, and then proteins were extracted and subjected to Western blot analysis. (B) MTT assay showed that cell proliferation ability was inhibited in SK-OV-3 siPHB1 cells compared with siCTL cells. Data are shown as mean ± SD. $^{***}P < 0.001$. (C) Colony forming assay showed decreased colony formation and colony cell number in HeLa cells transfected with siPHB1, and PHB1 protein level was determined by Western blot. Data are shown as mean ± SD. $^{**}P < 0.01$. (D) HeLa cells were transfected with siPHB1, incubated in the absence of serum for 20 h, and stimulated with 150 nmol/L insulin for 15 or 30 min, and then proteins were extracted and subjected to Western blot analysis. (E) Mes-Sa and Mes-Sa-Dx5 cells were transfected with siPHB1 and treated with 250 or 500 nmol/L paclitaxel for 24 h, and then proteins were extracted and subjected to Western blot analysis.

remarkably enhanced the interaction between FKBP8 and mTOR (Fig. 4F).

PHB1 expression is elevated in ovarian cancer

Given the fact that knocking down PHB1 inhibits mTOR signaling, we investigated whether PHB1 plays a role in ovarian cancer progression. We measured the expression of PHB1 in patients with ovarian cancer by IHC to assess the clinical relevance of PHB1 in ovarian cancer. We collected 64 patient samples, including 13 normal tissues and 51 ovarian cancer tissues, from the gynecology department of Ruijin Hospital. All IHC staining results were scored according to the relative immunostaining intensity of positive tumor cells. We observed that the expression of PHB1 was upregulated in ovarian cancer tissues (Fig. 5A)

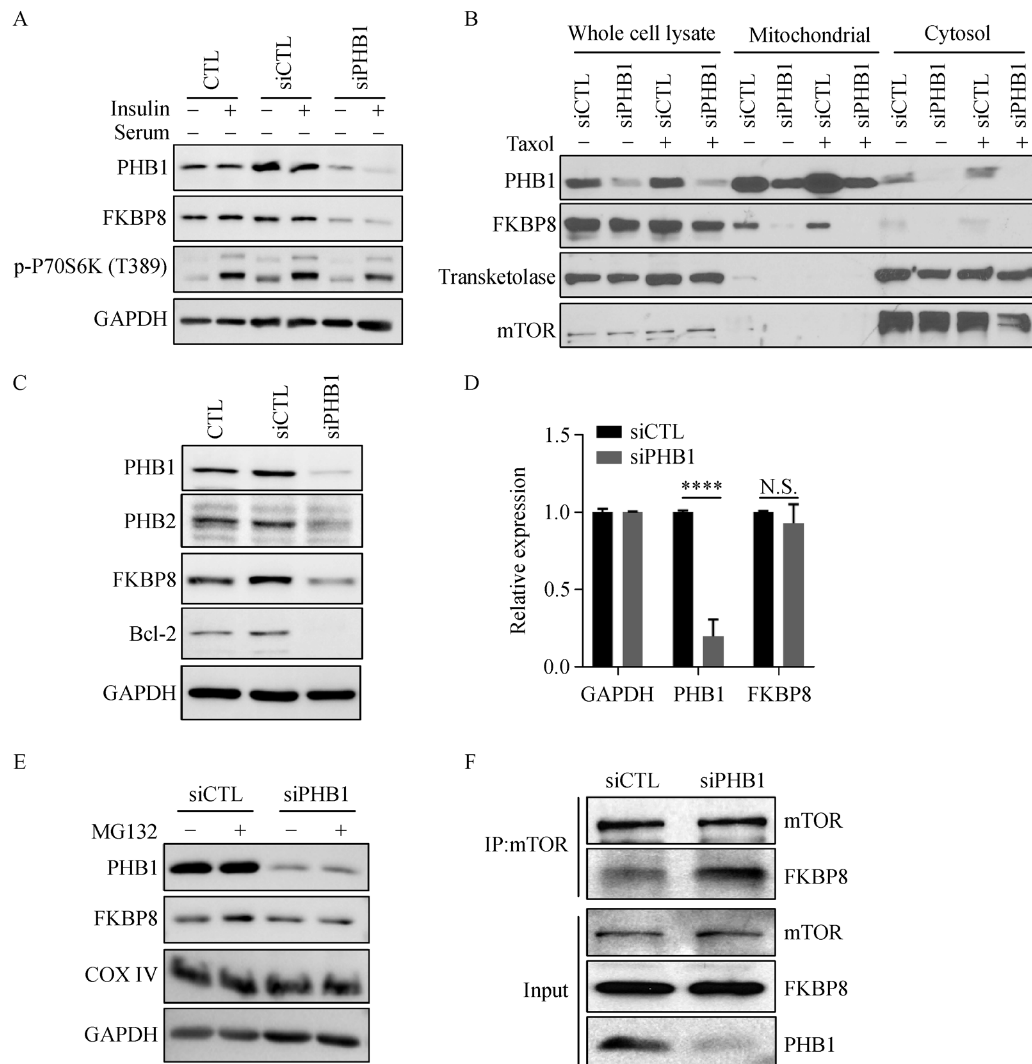

Fig. 4 PHB1 knockdown results in the release of FKBP8 from the mitochondria and increased interaction with mTOR. (A) HeLa cells were transfected with siPHB1, incubated in the absence of serum for 20 h, and stimulated with 150 nmol/L insulin for 30 min, and then proteins were extracted and subjected to Western blot analysis. (B) OVCAR5 cells were transfected with siPHB1 and treated with 250 nmol/L paclitaxel overnight. Mitochondrial fraction and cytosol were isolated, and the amounts of PHB1 and FKBP8 were analyzed by Western blot. Transketolase was used as cytosol loading control. (C) HeLa cells were transfected with siPHB1 or siCTL and cultured under normal condition, and proteins were extracted 72 h post-transfection and subjected to Western blot analysis. (D) qPCR analysis of the mRNA level of FKBP8 in HeLa cells after siPHB1transfection. GAPDH was used as internal reference. Data represent the mean ± SD of three independent experiments; two-tailed unpaired *t*-test, $****P < 0.0001$; N.S., no significance. (E) HeLa cells were transfected with siPHB1 for 72 h and incubated with 20 μmol/L MG132 for 8 h, and then proteins were extracted and subjected to Western blot analysis. (F) Mes-Sa cells were transfected with siPHB1, proteins were subjected to IP using an endogenous mTOR antibody, and co-precipitation FKBP8 was detected by Western blot.

compared with normal tissues (Fig. 5B). High PHB1 expression was present in 43 of 51 (84%) ovarian cancer tissues and in only 2 of 13 (15%) normal tissues (Table 1). The detailed clinical information of 35 of the 51 patients, including histology, staging, and treatment methods, are listed in Table 2. The high expression of PHB1 has no substantial correlation with these characteristics (Table 2). Our IHC results demonstrate that PHB1 is highly expressed in ovarian cancer.

Discussion

The activation of the Akt–mTOR pathway has been shown to participate in cancer development. FKBP8 is the only

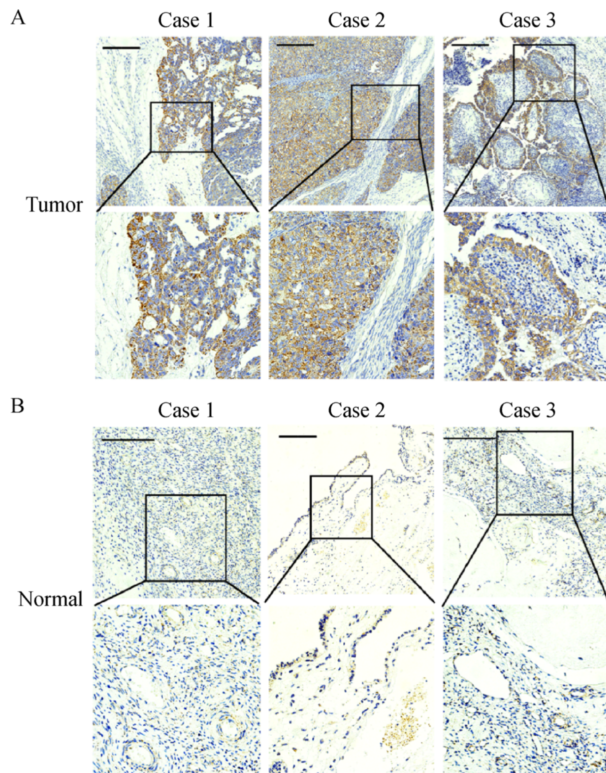

Fig. 5 PHB1 protein expression was detected in ovarian cancer (A) and normal tissues (B) by IHC staining. Boxed areas are enlarged below each image. Scale bars, 100 μm.

Table 1 Correlation analysis of PHB1 expression with ovarian tumor tissues

Tissue type	Total	PHB1-high	PHB1-low	*P* value
Normal	13	2	11	<0.0001
Tumor	51	43	8	

Table 2 Correlation analysis of PHB1 expression with pathology, stage, and treatment of patients with ovarian cancer

Characteristics	Cases, *n*	PHB1-high, *n* (%)	*P* value
Total number	35	29 (82.9%)	
Histology			
Adenocarcinoma	7	6 (85.7%)	0.8226
Serous carcinoma	28	23 (82.1%)	
Treatment			
Non-chemotherapy	5	5 (100%)	0.2719
Chemotherapy	30	24 (80%)	
TNM stage			
I–II	10	9 (90%)	0.6493
III–IV	25	20 (80%)	

reported endogenous inhibitor of mTOR, but its inhibitory role on mTOR is controversial [3,28–30]. We found that C-terminal-tagged FKBP8 is diffusely localized in the cytoplasm and inhibits mTOR signaling, whereas N-terminal-tagged FKBP8 is localized in the mitochondria and has no inhibitory effect on mTOR, which represents the normal physiologic condition. These results suggested that the inhibitory role of FKBP8 on mTOR relies on its subcellular localization.

FKBP8 is a member of the FKBP family with unique characteristics. Previous studies have revealed the paradoxical roles of FKBP8 in cancer. FKBP8 functions as an endogenous inhibitor of mTOR and thus plays a central role in controlling tumor cell growth [3]. Moreover, FKBP8 overexpression prevents tumor cell invasion through the upregulation of anti-invasive syndecan levels and the suppression of pro-invasive MMP9 in melanoma cells [31]. This finding supports the anti-tumor activity of FKBP8 in this cellular context. Nevertheless, FKBP8 exerts its anti-apoptotic effect by recruiting Bcl-2 and Bcl-XL to the mitochondria and protecting Bcl-2 from degradation [10]. FKBP8 also regulates protein degradation by anchoring proteasome to the mitochondrial outer membrane; therefore, it destabilizes PHD2 and increases HIF1 level [32].

We identified the interactome of FKBP8 by performing IP–MS to find out the regulatory factors for these contradictory aspects of FKBP8 in cancer. We identified PHB1 as one of the binding proteins of FKBP8. The results showed that the level of protein and not the level of mRNA of FKBP8 was reduced in cells with PHB1 silencing. Notably, the reduction of FKBP8 was more evident in the mitochondrial fraction than in the whole cell lysates. Hence, PHB1 silencing facilitates the translocation of FKBP8 from the mitochondria to other subcellular compartments and the enhancement of FKBP8 binding to mTOR. Consequently, we found that the extents of the phosphorylation of p70S6K and 4EBP-1 were much lower in PHB1 knockdown cells compared with the control cells. PHB1 is important for maintaining mitochondrial integrity; therefore, PHB1 silencing may result in fragmented and disorganized mitochondria and consequently release FKBP8 from the mitochondria. In our study, no substantial changes in overall mitochondrial contents were noted upon PHB1 silencing as evidenced by the constant expression level of COX IV. This result suggested that the release of FKBP8 from the mitochondria upon PHB1 silencing may not be caused by mitochondrial deconstruction.

PHB1 was first identified as an antiproliferative protein [14]. Early studies believed that the *PHB* gene is a tumor suppressor gene that plays a role in cancer suppression and the occurrence and development of malignant tumors, such as breast and liver cancers. However, recent studies have found that PHB is positively correlated with tumor

progression and metastasis. PHB is highly expressed in various tumor tissues, such as gallbladder, bladder, prostate, and lung cancer tissues. PHB1 is also a potential therapeutic target for drug-resistant tumor cells [33].

The functions of PHB1 in different tissue types is necessary to define considering its multi-intracellular localization and contradictory functions as tumor suppressor gene and oncogene. The present study showed that PHB1 is highly expressed in ovarian cancer, which suggests that PHB1 overexpression may contribute to the tumorigenesis of ovarian tissue. This notion was supported by *in vitro* experiments, which showed that PHB1 silencing considerably reduced the cell proliferation and colony formation of ovarian cancer cell lines. These observations could be explained by the FKBP8-mediated inhibition of mTOR pathway upon PHB1 silencing. Given that PHB1 has multiple functions, the potential involvement of other factors in PHB1-mediated tumorigenic effect cannot be ruled out in this study.

FKBP8 escapes from the mitochondria to the ER upon the induction of mitophage [27]. Another study reported that FKBP8 physically interacts and co-localizes with signal peptide peptidase in the endoplasmic reticulum [30], which facilitates FKBP8 degradation. In our present study, we noticed that FKBP8 escaped from the mitochondria when PHB1 was knocked down. The physiologic relevance of FKBP translocation during mitophagy and PHB1 silencing likely lies in promoting its inhibition effect on mTOR, as well as increasing the cells' susceptibility to apoptosis. Interestingly, PHB1 and FKBP8 are involved in mitochondrial fission/fragmentation and mitophagy under stressed conditions [34,35]. A detailed characterization of their interaction and roles in the regulation of mitophagy needs to be addressed further.

Taken together, we discovered and demonstrated for the first time that PHB1 interacts with FKBP8 and affects its localization and function. Under normal condition, FKBP8 binds to PHB1 and anchors it onto the mitochondrial outer membrane. However, when PHB1 level decreased, FKBP8 escapes from the mitochondria and exerts its inhibitory effect on mTOR kinase. Our present study demonstrated that PHB1 is highly expressed in ovarian cancers and likely promotes tumor progression by regulating mTOR signaling through FKBP8.

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Compliance with ethics guidelines

Jiahui Zhang, Yanan Yin, Jiahui Wang, Jingjing Zhang, Hua Liu, Weiwei Feng, Wen Yang, Bruce Zetter, and Yingjie Xu declare no conflict of interest. All procedures were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the *Helsinki Declaration* of 1975 as revised in 2000. Additional informed consent was obtained from all patients, whose identifying information is included in this article.

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