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Original Article

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# Inhibition of the AKT/mTOR pathway negatively regulates PTEN expression via miRNAs

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## Abstract

PI3K/AKT/mTOR pathway plays important roles in cancer development, and the negative role of PTEN in the PI3K/AKT/mTOR pathway is well known, but whether PTEN can be inversely regulated by PI3K/AKT/mTOR has rarely been reported. Here we aim to investigate the potential regulatory relationship between PTEN and Akt/mTOR inhibition in MEFs. AKT1$^{E17K}$ and TSC2$^{-/-}$ MEFs were treated with the AKT inhibitor MK2206 and the mTOR inhibitors rapamycin and Torin2. Our results reveal that inhibition of AKT or mTOR suppresses PTEN expression in AKT1$^{E17K}$ and TSC2$^{-/-}$ MEFs, but the transcription, subcellular localization, eIF4E-dependent translational initiation or lysosome- and proteasome-mediated degradation of PTEN change little, as shown by the real time PCR, nucleus cytoplasm separation assay and immunofluorescence analysis. Moreover, mTOR suppression leads to augmentation of mouse PTEN-3′UTR-binding miRNAs, including miR-23a-3p, miR-23b-3p, miR-25-3p and miR-26a-5p, as shown by the dual luciferase reporter assay and miRNA array analysis, and miRNA inhibitors collaborately rescue the decline of PTEN level. Collectively, our findings confirm that inhibition of mTOR suppresses PTEN expression by upregulating miRNAs, provide a novel explanation for the limited efficacy of mTOR inhibitors in the treatment of mTOR activation-related tumors, and indicate that dual inhibition of mTOR and miRNA is a promising therapeutic strategy to overcome the resistance of mTOR-related cancer treatment.

**Key words** miRNA, PI3K/AKT/mTOR, PTEN

## Introduction

Phosphatase and tensin homologue (PTEN) is a classical tumor suppressor that has been identified to be frequently disrupted in human sporadic tumors. In addition to inducing tumorigenesis after loss-of-function mutation, PTEN also governs a series of cellular processes under physiological conditions, including cell survival, proliferation, senescence, motility and polarity [1]. Phosphoinositide 3-kinase (PI3K) promotes the phosphorylation of phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) into phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P3), which activates the downstream AKT pathway. PTEN antagonizes the function of PI3K by dephosphorylating PIP3 back to PIP2, hence inactivating AKT [2]. PTEN carries out most of its biological functions by intervening in the PI3K/AKT pathway as a lipid phosphatase. As the downstream molecule of PI3K, AKT directly phosphorylates tuberous sclerosis protein 2 (TSC2) and then disrupts the TSC1/2 complex, which inhibits the activity of RAS-related small GTPase-activating protein enriched in the brain (Rheb) [3,4]. Mammalian target protein of rapamycin (mTOR), a serine/threonine kinase, has been demonstrated to participate in many biological processes, such as nutrient metabolism, cell cycle progression and autophagy [5,6]. Consistent with TSC2 deficiency, both PTEN deficiency and AKT activating-mutation (AKT1$^{E17K}$) lead to mTOR consecutive activation via negative regulation of Rheb [7,8]. However, the roles they play on

AKT are different: PTEN deletion and AKT1$^{E17K}$ mutation lead to phosphorylation of AKT1 on Ser473, whereas TSC2 knockout results in inhibition of AKT1 phosphorylation.

Given the important role played by PTEN under physiological conditions, the expression and activity of PTEN are tightly regulated through several mechanisms, including epigenetic silencing, transcriptional repression, posttranscriptional regulation by non-coding RNAs, inhibition of translation initiation [9], posttranslational modifications [10] and aberrant subcellular localization [11]. Furthermore, the interaction between PTEN and other cellular proteins may also affect its function, activity and abundance, either positively or negatively [12]. Although the regulation of PTEN has been investigated widely and the inhibitory activity of PTEN on the PI3K/AKT/mTOR pathway is well established, whether PTEN can be inversely regulated by PI3K/AKT/mTOR has rarely been reported.

In this study, to gain more insight into the regulatory relationship between PTEN and the PI3K/AKT/mTOR signaling pathway, PTEN protein levels were examined in AKT1$^{E17K}$ and TSC2$^{-/-}$ mouse embryonic fibroblasts (MEFs) after treatment with AKT or mTOR inhibitors. The results confirmed that the regulatory role of the PI3K/AKT/mTOR pathway on PTEN is dependent on mTOR, and inhibition of either AKT or mTOR effectively suppresses PTEN expression.

## Materials and Methods

### Cell culture and transfection

AKT1$^{E17K}$, TSC2$^{-/-}$ and control MEFs were isolated from the pregnant mice by the collagen digestion method and preserved in our lab. All cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, USA) containing 10% fetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin (Thermo Fisher Scientific, Waltham, USA) at 37°C, 5% $CO_2$, and 95% humidity in 12-well plates (Corning, New York, USA), untile the cell confluence reached 70%–80%. Before transfection, the supernatant was removed, and the MEFs were washed with phosphate-buffered saline (PBS) and cultured in fresh serum-free DMEM without antibiotics. After dilution, the complex of the plasmids and Lipofectamine 3000 (Thermo Fisher Scientific) was gently added into the well, and then the transfection mixture was discarded and replaced by fresh full DMEM 4–6 h later. The transfected cells were cultured in an incubator for another 24-48 h before further assays.

### Western blot analysis

Cells were harvested and lysed for protein expression analysis. Equal amounts of total protein from each group were isolated by 12.5% SDS-PAGE and transferred to nitrocellulose filter (NC) membranes (Millipore, Billerica, USA). After being blocked with 5% nonfat milk (BD, Franklin Lakes, USA) for 1 h at room temperature, the membranes were incubated with a specific primary antibody overnight at 4°C. After three times wash with TBST, the blots were incubated with IRDye 680RD goat anti-mouse or IRDye 800RD goat anti-rabbit secondary antibodies (LI-COR, Nebraska, USA) for 1 h at room temperature. Then, the fluorescence on the membrane was detected with the ODYSSEY Clx Capture system (LI-COR) and imaged. Finally, the band intensity was analysed with ImageJ software. The details of the primary antibodies were as follows: PTEN (9559, 1:1000; CST, Shanghai, China), 4E-BP1 (9644, 1:1000; CST), P-4E-BP1$^{T37/46}$ (2855, 1:1000; CST), S6 (2217, 1:1000; CST), P-S6$^{Ser235/236}$ (4858, 1:1000; CST), AKT1 (2938, 1:1000; CST), P-AKT1$^{S473}$ (9018, 1:1000), TSC2 (4308, 1:1000; CST), Lamin A/C (4777, 1:1000; CST), GAPDH (97166, 1:1000; CST), eIF4E (2067, 1:1000; CST), P62 (8025, 1:1000; CST), β-catenin (9587S, 1:1000; CST), and β-actin (SC-47778, 1:3000; Santa Cruz, Shanghai, China).

### miRNA and mRNA microarray data analysis

Total small RNA samples of TSC2$^{-/-}$ MEFs after treatment with 10 nM rapamycin (Sigma, St. Louis, USA) were prepared and analysed with a microarray (02_M12.0_090121; PTM BIO, Beijng, China). The normalized microarray data were processed with ArrayPro (Array Nonlinear Dynamics). Moreover, total mRNA samples of TSC2$^{+/+}$, TSC2$^{-/-}$ and TSC2$^{-/-}$ MEFs treated with rapamycin were prepared and measured using an Affymetrix mouse genome 4302.0 array (Affymetrix, PTM BIO, Beijng, China). A fold change greater than 2.0 and a $P$ value less than 0.05 were considered statistically significant.

### Total RNA isolation, miRNA isolation and expression analysis

Total RNA was extracted after AKT1$^{E17K}$, TSC2$^{-/-}$ and control MEFs were treated with 5 μM MK2206 (Selleck, Houston, USA), 10 nM rapamycin and 50 nM Torin2 (MedChemExpress, Princeton, USA), respectively, using the TRIzol reagent (Invitrogen, Carlsbad, USA). The concentration and purity of total RNA were measured using a Nanodrop spectrophotometer (Thermo Fisher Scientific). Then, 1 μg total RNA was reverse transcribed to cDNA using the first strand cDNA synthesis kit (11141; Yi Sheng, Beijing, China). Total small RNA was obtained with a miRNA isolation kit (AM1561, Invitrogen) according to the manufacturer's instructions. Moreover, a miRNA first strand cDNA synthesis kit (B532451; Sangon Biotech, Shanghai, China) was used to reverse transcribe miRNA into cDNA. Real-time polymerase chain reaction (PCR) was conducted using 2 × qPCR Master Mix (Abm, Richmond, Canada) following the manufacturer's protocol. The PCR conditions were: 95°C for 20 s, followed by 40 cycle of amplification at 95°C for 1 s, 60°C for 10 s, and 72°C for 30 s. *Actin* and *U6* were used as normalized controls for detecting mRNA and miRNA expression, respectively. The relative expression level was calculated by the $2^{-\Delta\Delta Ct}$ method. The primers used for PCR are listed in Table 1.

### RNA transfection

The siRNAs of mouse 4E-BP1 were designed and synthesized by TsingKe Biotechnology (Beijing, China). The shRNA sequences of mouse Eif4e are as follows: Sh-1, 5′-CCGGCCTTCGATTGATCTC TAAGTTCTCGAGAACTTAGAGATCAATCGAAGGTTTTTG-3′; Sh-2, 5′-CCGGCCGAAGATAGTGATTGGTTATCTCGAGATAACCAAT CACTATCTTCGGTTTTTG-3′; Sh-3, 5′-CCGGGCAAGCAAACCTTC GATTGATCTCGAGATCAATCGAAGGTTTGCTTGCTTTTTG-3′. The siRNAs were transfected into AKT1$^{E17K}$ or TSC2$^{-/-}$ MEFs to knockdown 4E-BP1 with Lipofectamine™ RNAiMAX (Invitrogen) transfection reagent according to the manufacture instructions. The Eif4e shRNA were synthesized, annealed and inserted into the PLKO.1 vector to construct lentivirus plasmids and then transfected into AKT1$^{E17K}$ or TSC2$^{-/-}$ MEFs to knockdown eIF4E according to the protocol of Lipofectamine™ 3000 Reagent (Invitrogen). Mimics of NC (miR1N0000001-1-5), miR-23a-3p (miR10000532-1-5), miR-

**Table 1. The sequences of primers used for real time PCR in this study**

| Primer | Sequence |
|---|---|
| PTEN-F | 5′-TGGATTCGACTTAGACTTGACCT-3′ |
| PTEN-R | 5′-GCGGTGTCATAATGTCTCTCAG-3′ |
| Actin-F | 5′-AGAGGGAAATCGTGCGTGAC-3′ |
| Actin-R | 5′-CAATAGTGATGACCTGGCCGT-3′ |
| mmu-miR-26b-5p-F | 5′-TTCAAGTAATTCAGGATAGGT-3′ |
| mmu-miR-721-F | 5′-CAGTGCAATTAAAAGGGGGAA-3′ |
| mmu-miR-301a-3p-F | 5′-CAGTGCAATAGTATTGTCAAAGC-3′ |
| mmu-miR-301b-3p-F | 5′-CAGTGCAATGGTATTGTCAAAGC-3′ |
| mmu-miR-130a-3p-F | 5′-CAGTGCAATGTTAAAAGGGCAT-3′ |
| mmu-miR-130b-3p-F | 5′-CAGTGCAATGATGAAAGGGCAT-3′ |
| mmu-miR-130c-F | 5′-CAGTGCAATGTTCCAAGGTGTG-3′ |
| mmu-miR-6341-F | 5′-CAGUGCAAUGAUAUUGUCACUAU-3′ |
| mmu-miR-6389-F | 5′-CAGUGCAAUGUUAAACUUUGC-3′ |
| mmu-miR-132-3p-F | 5′-TAACAGTCTACAGCCATGGTCG-3′ |
| mmu-miR-212-3p-F | 5′-TAACAGTCTCCAGTCACGGCCA-3′ |
| mmu-miR-17-5p-F | 5′-CAAAGTGCTTACAGTGCAGGTAG-3′ |
| mmu-miR-26a-5p-F | 5′-TCGGCAGGTTCAAGTAATCCAG-3′ |
| mmu-miR-23a-3p-F | 5′-TCGGCAGGATCACATTGCCAG-3′ |
| mmu-miR-23b-3p-F | 5′-TCGGCAGGATCACATTGCCAG-3′ |
| mmu-miR-25-3p-F | 5′-TCGGCAGGCATTGCACTTGTCT-3′ |
| U6-F | 5′-ctcgcttcggcagcaca-3′ |
| U6-R | 5′-aacgcttcacgaatttgcgt-3′ |

The common reverse primer for miR is provided by the first strand cDNA synthesis kit.

23b-3p (miR10000125-1-5), miR-25-3p (miR10000652-1-5), miR-26a-5p (miR10000533-1-5), and inhibitors of NC (miR2N0000001-1-5), miR-23a-3p (miR20000532-1-5), miR-23b-3p (miR20000125-1-5), miR-25-3p (miR20000652-1-5), miR-26a-5p (miR20000533-1-5) were purchased from RiboBio (Guangzhou, China) and transfected into AKT1$^{E17K}$ or TSC2$^{-/-}$ MEFs with Lipofectamine™ RNAiMAX transfection reagent respectively.

### Nucleus and cytoplasm separation

Nuclear protein and cytoplasmic protein were separated using a nucleus-cytoplasm separation kit (sc-003; Invent, Beijing, China) after AKT1$^{E17K}$ and TSC2$^{-/-}$ MEFs were treated with 10 nM rapamycin for 24 h.

### Immunofluorescence microscopy

After treatment with 10 nM rapamycin for 24 h, AKT1$^{E17K}$ and TSC2$^{-/-}$ MEFs grown on slides (Corning, New York, USA) were washed with PBS and then fixed with 4% paraformaldehyde (Servicebio, Wuhan, China) for 30 min at room temperature. After incubation with 0.1% Triton X-100 (Solarbio, Beijing, China), the cells were blocked with 3% BSA (Thermo Fisher Scientific) and further incubated with a rabbit monoclonal anti-PTEN antibody (9559, 1:500; CST) in 3% BSA blocking buffer overnight at 4°C. The slides were then incubated with a Cy3-conjugated goat anti-rabbit secondary antibody (1:300; Servicebio) for 1 h at room temperature. Finally, the cell nuclei were stained with DAPI-anti-fluorescence quenching-fluoromount (ab104139; Abcam, Cambridge, England), and the fluorescence images were examined and captured under a confocal microscope (Leica, Weztlar, Germany).

### Dual-luciferase reporter assay

The 3′-untranslated region (3′UTR) of mouse PTEN was amplified from C57B6 L mouse genome DNA, and the whole fragment was divided into four segments since it was too long. The four separate fragments were cloned into the pmirGLO dual-luciferase reporter plasmid (Promega, Madison, USA) to construct four plasmids named pmirGLO-PTEN-3′UTR-F1, F2, F3, and F4 using primers shown in Table 2. The dual-luciferase reporter plasmids and pmirGLO control plasmid were transfected into TSC2$^{-/-}$ MEFs using Lipofectamine 3000 transfection reagent, respectively. The transfection mixture was removed and replaced by fresh full DMEM containing 10 nM rapamycin after 4-6 h of transfection. Then, the cells were harvested and lysed 24 h later. The lysates were added to black 96-well plates to measure Renilla luciferase activity using the Dual Luciferase Reporter Gene Assay Kit (11402ES60; Yisheng, Beijing, China). Renilla luciferase activity was regarded as a normalized control, and all data are shown with the luciferase/Renilla ratio.

### Statistical analysis

All data are presented as the mean ± standard deviation from at least three independent experiments. One-tailed Student's *t* test or analysis of variance (ANOVA) was performed with GraphPad Prism 8.0 Software (GraphPad, La Jolla, USA). *P* values less than 0.05 were considered statistically significant.

## Results

### Inhibition of AKT or mTOR reduces PTEN expression in AKT1$^{E17K}$ MEFs

mTOR is the downstream molecule of AKT, and consecutively activated AKT leads to hyperactive mTOR. To investigate the effect of aberrant AKT activation on PTEN protein level, western blot analysis was performed to examine PTEN and key signaling molecules of the Akt/mTOR pathway in AKT1$^{E17K}$ MEFs. Consecutive mTOR activation upon AKT1 E17K mutation was confirmed by increased phosphorylation of 4E-BP1 and S6, as previously described [13]. However, PTEN protein level was not affected by aberrant AKT activation (Figure 1A). To further ascertain whether inhibition of AKT or mTOR affects PTEN expression, western blot analysis was performed after AKT1$^{E17K}$ MEFs were treated with 5 μM Akt inhibitor MK2206, 50 nM mTOR inhibitor Torin2 and 10 nM mTOR inhibitor rapamycin for 24 h respectively. The results showed that PTEN expression was markedly reduced upon treatment with Akt or mTOR inhibitors in AKT1$^{E17K}$ MEFs (Figure 1B–D). Intriguingly, the

**Table 2. The primers of PTEN-3′UTR construction**

| Primer | Sequence |
|---|---|
| F1 | 5′-tgCTCGAGtgacaccactgactctgatccag-3′ |
| R1 | 5′-ggGTCGACCGATAGTAGTTGTACTCTTGC-3′ |
| F2 | 5′-tgCTCGAGgtggtagagttgggattagggc-3′ |
| R2 | 5′-gaGTCGACGAGGCATTATCCTGTACACGTC-3′ |
| F3 | 5′-tgCTCGAGaccccgattcagcctcttcaga-3′ |
| R3 | 5′-tgGTCGACCCCCAAGGACATGAGAATTGTG-3′ |
| F4 | 5′-gaCTCGAGgtgaagatggcaggatagtgtc-3′ |
| R4 | 5′-gtGTCGACCTAGTCTTATGTCCATTGGTAGCC-3′ |

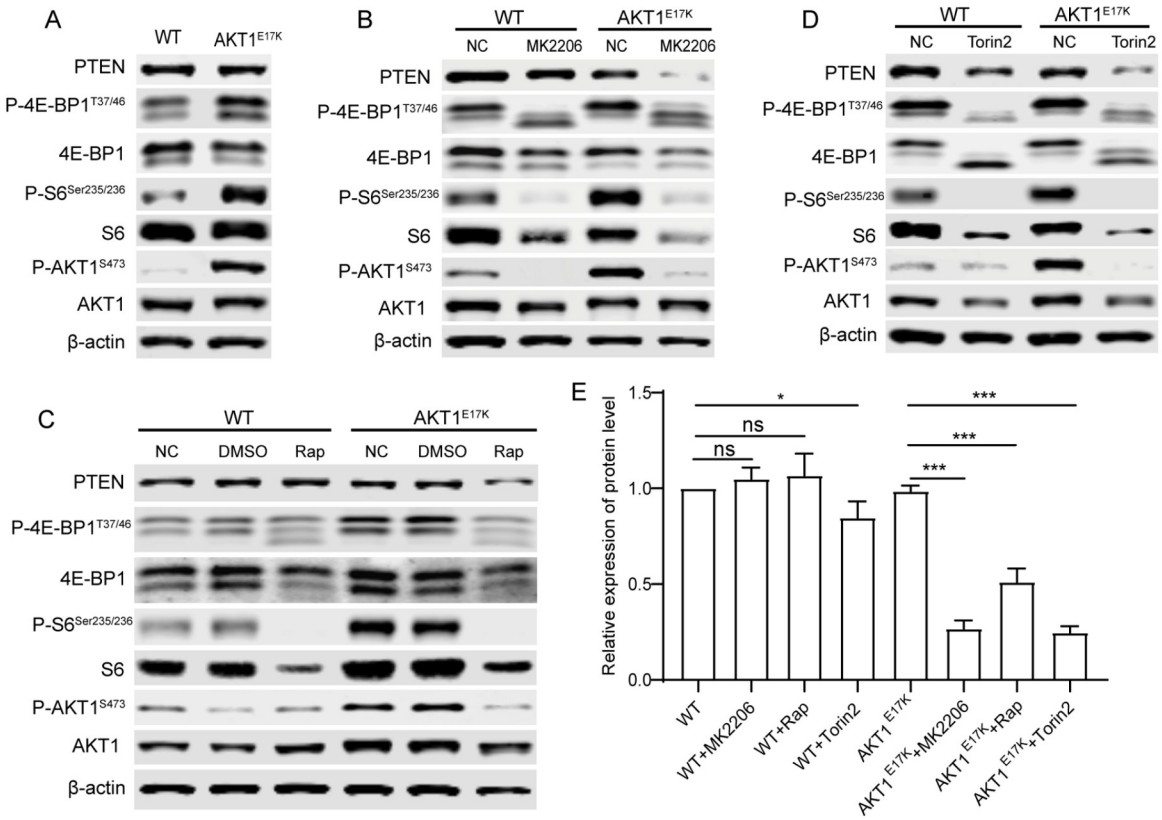


**Figure 1. Inhibition of AKT or mTOR downregulates PTEN expression in AKT1$^{E17K}$ MEFs** (A) The protein level of PTEN in AKT1$^{E17K}$ and control MEFs. (B) The PTEN level in AKT1$^{E17K}$ MEFs after treatment with 5 μM MK2206 for 24 h. (C) The PTEN level in AKT1$^{E17K}$ MEFs after treatment with 10 nM rapamycin for 24 h. DMSO was used as a vehicle control. (D) The PTEN level in AKT1$^{E17K}$ MEFs after treatment with 50 nM Torin2 for 24 h. (E) Quantification of the relative protein level of PTEN. Data are expressed as the mean ± SD ($n = 3$). *$P < 0.05$, ***$P < 0.001$. ns, not significant.

PTEN protein level remained almost the same in WT MEFs treated with MK2206 or rapamycin but was downregulated by Torin2 treatment. These data indicated that inhibition of AKT or mTOR reduces the expression of PTEN in MEFs with aberrant AKT1 activation.

## Inhibition of mTOR downregulates PTEN level in TSC2$^{-/-}$ MEFs

Similar to the AKT1$^{E17K}$ mutation, *TSC2* knockout also leads to consecutive mTOR activation. Thus, whether PTEN expression changes after *TSC2* knockout was also investigated. Western blot analysis results revealed that *TSC2* knockout did not affect the expression of PTEN (Figure 2A). Consistent with AKT1$^{E17K}$ MEFs, the PTEN protein level was reduced in TSC2$^{-/-}$ MEFs after treatment with rapamycin or Torin2 (Figure 2B,C). Due to the inhibited AKT phosphorylation under the condition of *TSC2* knockout, MK2206 was not used to treat TSC2$^{-/-}$ MEFs.

In summary, inhibition of AKT or mTOR remarkably suppresses PTEN expression in mTOR-activated MEFs, and this finding suggests the potential feedback regulation of PTEN expression by the Akt/mTOR pathway.

## Inhibition of AKT or mTOR does not alter the mRNA level of PTEN

To explore the potential regulatory relationship between AKT/mTOR inhibition and PTEN expression, real-time PCR was performed to analyse PTEN mRNA level. Upon AKT or mTOR inhibition, the mRNA level of PTEN only changed slightly (Figure 2E,F). Furthermore, the transcription factors (TFs) that are able to bind with the mouse *PTEN* promoter were predicted online (http://jaspar.genereg.net/), and the expression level of the identified TFs was examined according to the mRNA microarray data of TSC2$^{-/-}$ MEFs. The results showed that their expressions were not altered with statistical significances (Table 3). Taken together, suppression of PTEN upon AKT or mTOR inhibition was not achieved through transcriptional regulation.

## Inhibition of AKT or mTOR does not affect the subcellular localization of PTEN

PTEN localizes to the cytoplasm and nucleus, and increasing evidence suggests the importance of nuclear PTEN in controlling chromosome stability and cell cycle progression [14,15]. Extra stimulation, which mediates neddylation or phosphorylation of PTEN, has been proposed to promote PTEN translocation between the cytoplasm and nucleus [11,16]. Furthermore, the degradation approach of nuclear PTEN and cytoplasmic PTEN is not totally the same. To elucidate whether the downregulated PTEN level is related to the alteration of PTEN subcellular localization, nucleus cytoplasm separation assay was performed after AKT1$^{E17K}$ and TSC2$^{-/-}$ MEFs were treated with rapamycin. The results showed that PTEN expression was decreased in both the cytoplasm and nucleus

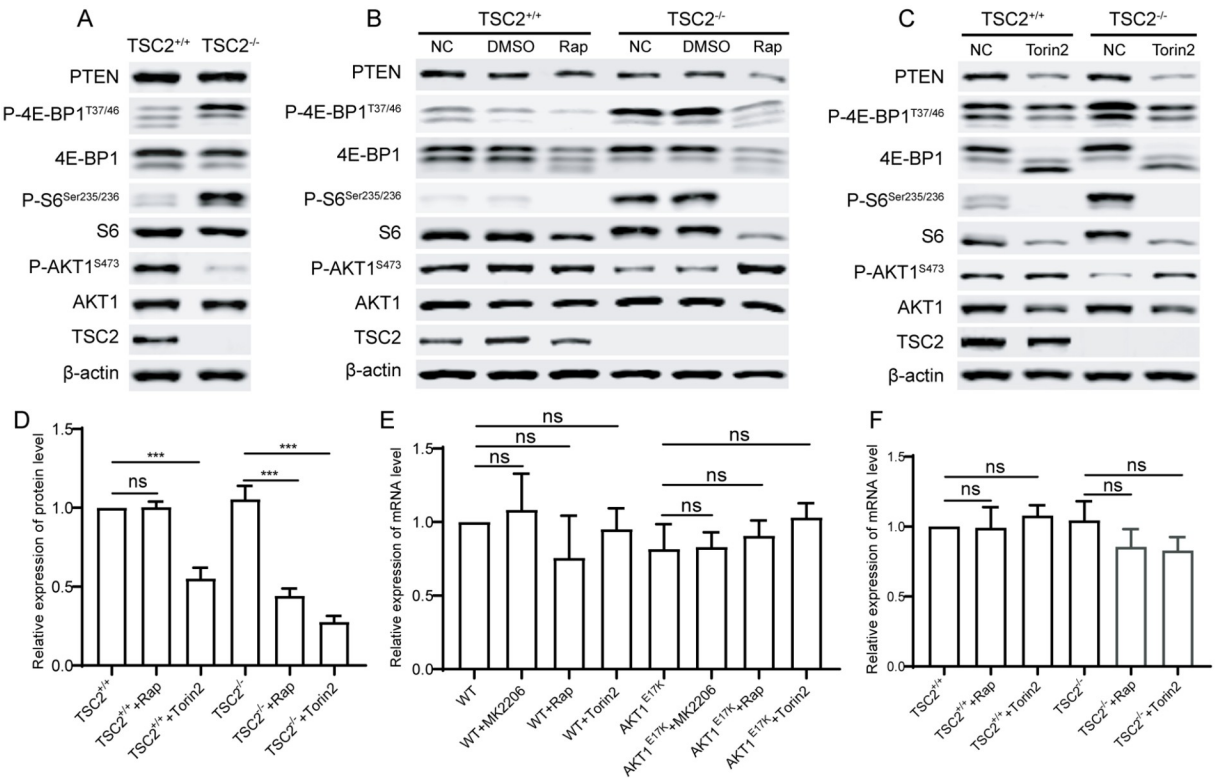


**Figure 2. Inhibition of mTOR reduces PTEN levels in TSC2$^{-/-}$ MEFs** (A) The protein level of PTEN in TSC2$^{-/-}$ and TSC2$^{+/+}$ MEFs. (B) The protein level of PTEN in TSC2$^{-/-}$ MEFs after treatment with 10 nM rapamycin for 24 h. (C) The protein level of PTEN in TSC2$^{-/-}$ MEFs after treatment with 50 nM Torin2 for 24 h. (D) Quantification of the relative protein level of PTEN. Data are expressed as the mean ± SD ($n$ = 3). (E) Relative mRNA levels of PTEN in AKT1$^{E17K}$ and control MEFs after treatment with 5 μM MK2206, 10 nM rapamycin and 50 nM Torin2 for 24 h. (F) Relative mRNA level of PTEN in TSC2$^{-/-}$ and TSC2$^{+/+}$ MEFs after treatment with 10 nM rapamycin and 50 nM Torin2 for 24 h, respectively. ***$P$ < 0.001. ns, not significant.

**Table 3. The mRNA level of predicted transcription factors of *PTEN***

| Gene | Tsc2$^{-/-}$ vs Tsc2$^{+/+}$ | | Tsc2$^{-/-}$R vs Tsc2$^{-/-}$ | |
|---|---|---|---|---|
| | Fold change | Description | Fold change | Description |
| C/EBPβ | 1.61277 | Up | −1.02346 | Down |
| YY1 | −1.15627 | Down | 1.09839 | Up |
| C/EBPα | −1.00968 | Down | −1.25871 | Down |
| myogenin | −1.00658 | Down | 1.06402 | Up |
| HOXA5 | −1.09352 | Down | 1.06891 | Up |
| Jun D | −1.02044 | Down | 1.2457 | Up |
| SP1 | 1.04168 | Up | 1.01331 | Up |
| RelA | −1.05628 | Down | −1.04292 | Down |
| Hes1 | 1.25847 | Up | −1.45703 | Down |

The mRNA abundance of transcription factors that predicted to regulate PTEN in Tsc2$^{+/+}$ MEFs, Tsc2$^{-/-}$ MEFs, and Tsc2$^{-/-}$ MEFs treated with rapamycin (R) was measured using Affymetrix mouse genome 4302.0 array.

upon rapamycin treatment (Figure 3A,B). To further confirm the accurate subcellular localization alteration of PTEN, immunofluorescence microscopy was performed, and the results showed that PTEN can be clearly seen in the cell-cell junctions, cytoplasm and nucleus of AKT1$^{E17K}$ and TSC2$^{-/-}$ MEFs, but the fluorescence intensity became weaker in both the cytoplasm and nucleus after rapamycin or Torin2 treatment (Figure 3C–E). Collectively, inhibition of mTOR decreased the abundance of PTEN in both the cytoplasm and nucleus, but the subcellular localizations of PTEN were not altered.

## Intervening with eIF4E-dependent translational initiation is not able to rescue the decrease in PTEN level caused by mTOR inhibition

Since transcriptional regulation cannot explain the decreased PTEN level, more efforts should be made to search for other mechanisms, such as translational regulation. Among the initiation, elongation, termination, and ribosome recycling of eukaryotic translation

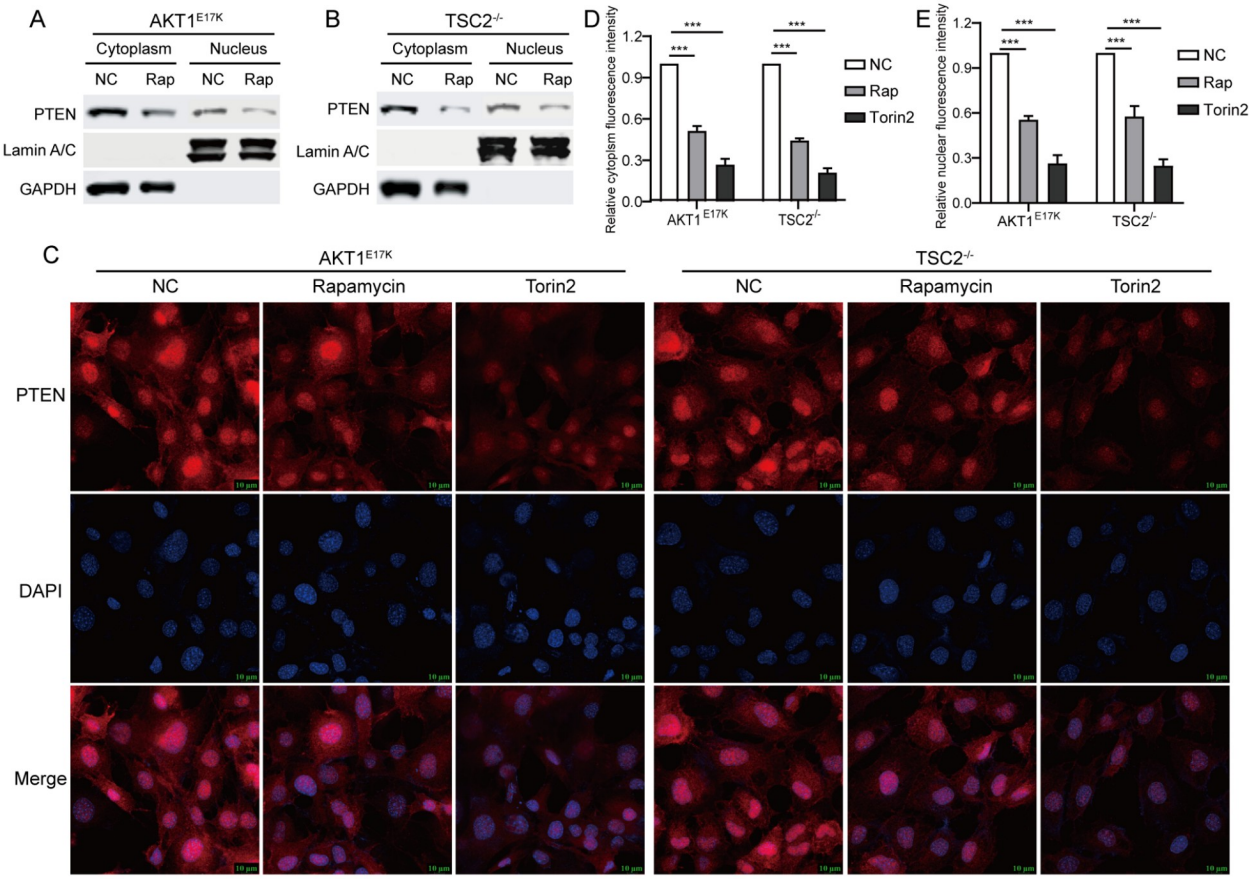


**Figure 3. Inhibition of mTOR does not affect the subcellular localization of PTEN** (A) The protein level of PTEN in the cytoplasm and nucleus of AKT1$^{E17K}$ MEFs after treatment with 10 nM rapamycin for 24 h. (B) The protein level of PTEN in the cytoplasm and nucleus of TSC2$^{-/-}$ MEFs after treatment with 10 nM rapamycin for 24 h. Lamin A/C and GAPDH are markers of nuclear and cytoplasmic proteins, respectively. (C) Immunofluorescence microscopy was used to visualize the subcellular localization of PTEN in AKT1$^{E17K}$ and TSC2$^{-/-}$ MEFs after treatment with 10 nM rapamycin or 50 nM Torin2 for 24 h. Cy3-labelled PTEN and DAPI were used to stain the nuclei. (D) Quantification of the fluorescence intensity in the cytoplasm. (E) Quantification of the fluorescence intensity in the nucleus. ***$P < 0.001$.

processes, most regulation is exerted at the initiation stage [17]. Eukaryotic translation initiation factor 4E (eIF4E) is a subunit of the eIF4F translation initiation complex, which plays a complicated role in the translational initiation of eukaryotes. 4E-BP1, a member of the eIF4E-binding proteins (4E-BPs), is a substrate of mTORC1 and can be phosphorylated upon mTOR activation [18]. The phosphorylation state of 4E-BP1 determines its interaction with eIF4E. Hypophosphorylated 4E-BP1 strongly binds with eIF4E to prevent eIF4F complex assembly, while hyperphosphorylated 4E-BP1 stimulates its release from the eIF4E/4E-BP complex and allows eIF4E to constitute the translation initiation complex with eIF4G, thereby increasing the translation initiation rates [19]. 4E-BP1 in AKT1$^{E17K}$ and TSC2$^{-/-}$ MEFs was hyperphosphorylated, while inhibition of mTOR suppressed 4E-BP1 phosphorylation (Figures 1 and 2).

To determine whether inhibition of mTOR reduces PTEN protein synthesis by interfering with 4E-BP1/eIF4E-dependent translational initiation, siRNA was applied to knockdown 4E-BP1 or eIF4E in AKT1$^{E17K}$ and TSC2$^{-/-}$ MEFs, and PTEN protein level was subsequently detected after the siRNA-transfected MEFs were treated with rapamycin. The results showed that knockdown of 4E-BP1 or eIF4E could not rescue the decreased PTEN level under mTOR inhibition (Figure 4A–D). These data indicated that inhibition of mTOR reduces PTEN expression not through interfering with 4E-BP1/eIF4E-dependent protein synthesis.

## Reduced PTEN expression upon mTOR inhibition is not caused by proteasome- or lysosome-mediated degradation

Post-translation modifications, including phosphorylation, acetylation, oxidation and ubiquitylation, also play indispensable roles in regulating the function and expression of PTEN. The former three modifications principally regulate the activity of PTEN, while ubiquitylation mainly modulates protein abundance. Since we have excluded the possible involvement of transcriptional regulation and translational initiation in decreased PTEN level upon mTOR inhibition, other mechanisms that mediate the abundance changes in proteins, such as degradation, should be taken into consideration. Ubiquitinated degradation of PTEN has been reported in numerous human cancers [20,21]. In addition to ubiquitylation mediating proteasome degradation, the lysosome degradation mechanism should also be considered. Our results showed that the protein abundance of PTEN changed little after AKT1$^{E17K}$ and TSC2$^{-/-}$ MEFs were treated with rapamycin or Torin2 combined with proteasome inhibitor MG132 or lysosome degradation inhibitor chloroquine and $NH_4Cl$, respectively (Figure 5A–D). In addition, to exclude the off-target effects of MG132, chloroquine and

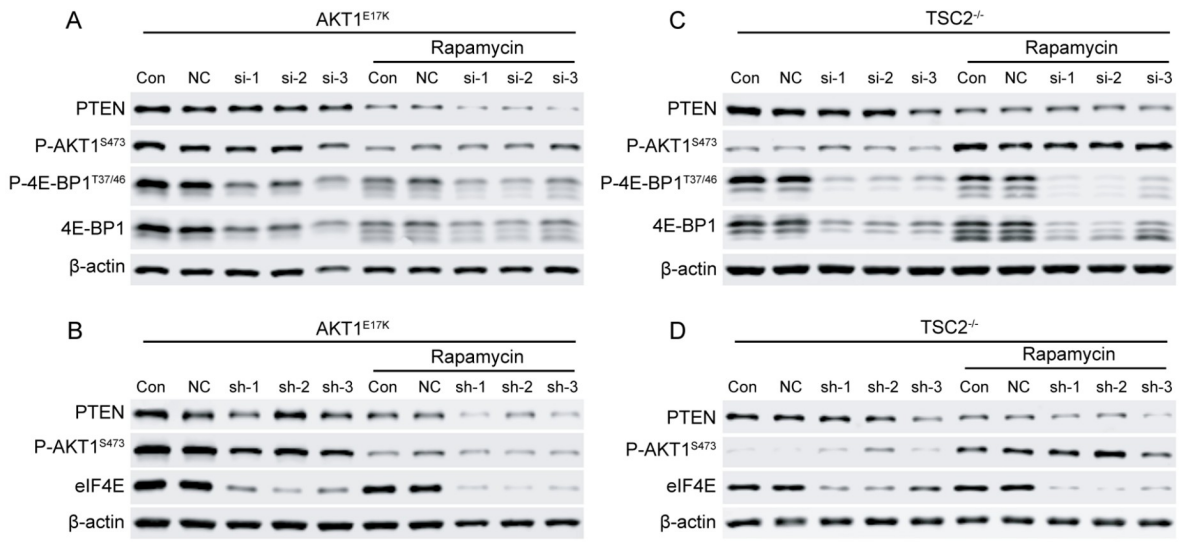


**Figure 4. Intervening with 4E-BP1/eIF4E-dependent translation initiation is not able to rescue the decreased PTEN mediated by mTOR inhibition** (A,C) The protein level of PTEN in AKT1$^{E17K}$ and TSC2$^{-/-}$ MEFs after treatment with 10 nM rapamycin for 24 h after 4E-BP1 knockdown. (B,D) The protein level of PTEN in AKT1$^{E17K}$ and TSC2$^{-/-}$ MEFs after treatment with 10 nM rapamycin for 24 h after eIF4E knockdown.

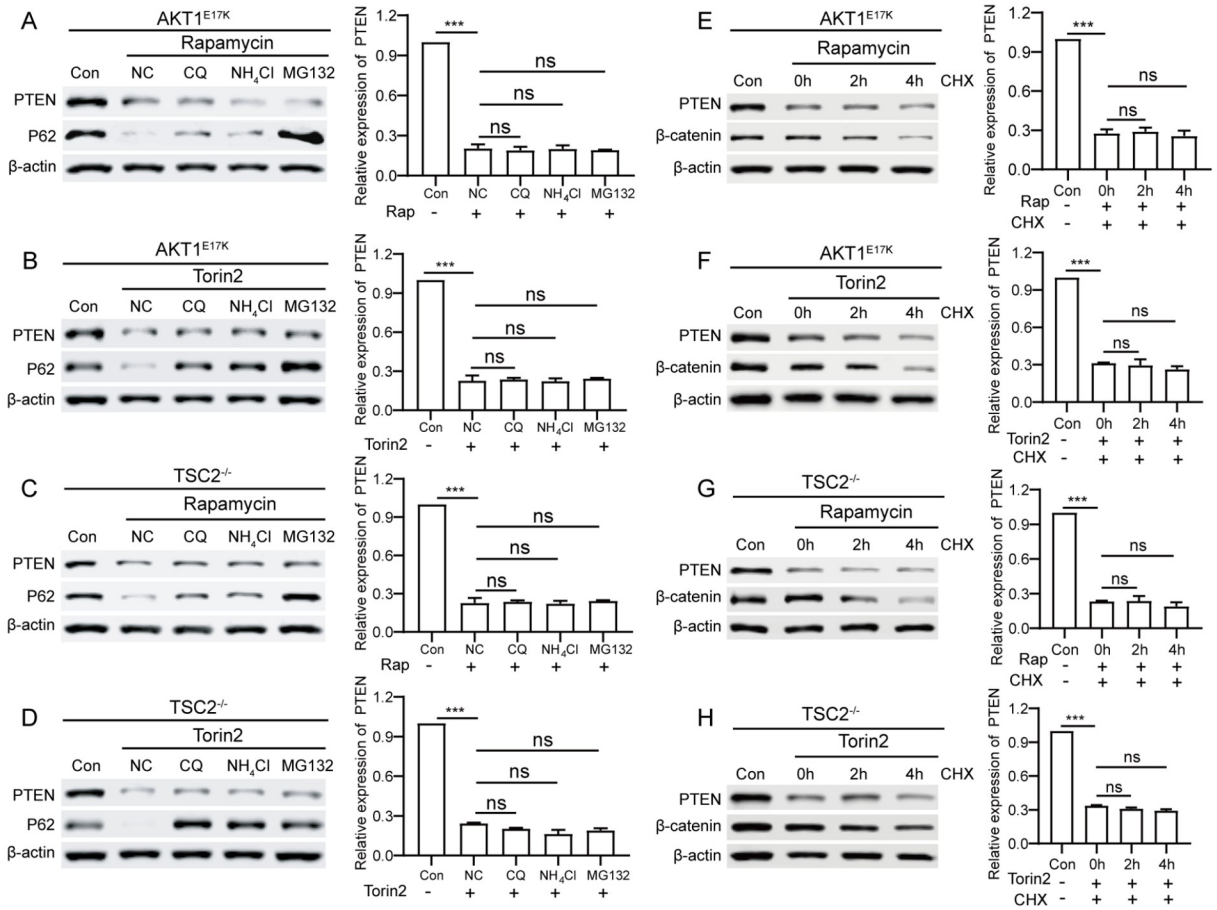


**Figure 5. Suppression of proteasome or lysosome degradation cannot rescue the downregulated PTEN level** (A,C) The protein level of PTEN in AKT1$^{E17K}$ and TSC2$^{-/-}$ MEFs after treatment with 10 nM rapamycin combined with 10 μM chloroquine (CQ), 5 mM $NH_4Cl$ and 5 μM MG132 for 24 h. P62 was used as a positive control. (B,D) The protein level of PTEN in AKT1$^{E17K}$ and TSC2$^{-/-}$ MEFs after treatment with 50 nM Torin2 combined with 10 μM CQ, 5 mM $NH_4Cl$ and 5 μM MG132 for 24 h. (E,G) The protein level of PTEN in AKT1$^{E17K}$ and TSC2$^{-/-}$ MEFs pretreated with 100 μg/mL CHX for different time and then incubated with 10 nM rapamycin for 24 h. β-Catenin was used as a positive control. (F,H) The protein level of PTEN in AKT1$^{E17K}$ and TSC2$^{-/-}$ MEFs pretreated with 100 μg/mL CHX for different time and then incubated with 50 nM Torin2 for 24 h. ***$P<0.001$. ns, not significant.

$NH_4Cl$, cycloheximide (CHX)-pretreated $AKT1^{E17K}$ and $TSC2^{-/-}$ MEFs were incubated with mTOR inhibitors to determine the stability of PTEN. The results showed that mTOR inhibitors did not promote PTEN degradation (Figure 5E–H).

Ubiquitination and deubiquitination are dynamically balanced under physiological conditions. Accumulating evidence has revealed that disruption of this balance is involved in numerous human diseases [22]. To further evaluate the expression of E3 ubiquitin ligase and deubiquitinase (DUB), which have been reported to regulate PTEN polyubiquitylation degradation [20,21,23–26], mRNA microarray data were analysed. The results showed that the expressions of the E3 ligases and DUB (Table 4) were only slightly altered with no statistical significances. These data suggest that rapamycin or Torin2 decreases PTEN protein level not through regulating protein degradation.

### miRNAs play an indispensable role in PTEN expression under mTOR inhibition

MicroRNAs (miRNAs) are a class of endogenous single-stranded small noncoding RNAs that negatively modulate the expression of target genes through binding with the seeding sequence on the 3′ UTR [27]. As one of the most crucial post-transcription regulators, miRNAs have been demonstrated to play important roles in various physiological and pathological processes. Numerous miRNAs have been reported to downregulate PTEN expression to promote tumorigenesis or other disorders [28]. Additionally, Ogórek *et al.* [29] proposed that TSC2 regulates microRNA biogenesis via mTORC1.

To confirm whether the inhibition of mTOR-decreased PTEN level is related to miRNAs, four mouse PTEN-3′UTR dual-luciferase reporter plasmids were transfected into $TSC2^{-/-}$ MEFs, and a dual-luciferase reporter assay was performed. The luciferase activities of PTEN-3′UTR-F1 and F2 were decreased after rapamycin treatment, while the luciferase activities of PTEN-3′UTR-F3 and F4 changed little (Figure 6A,B). In addition, the online software TargetScan (http://www.targetscan.org) was used to predict the potential miRNA-binding sites on the mouse PTEN-3′UTR, and the predicted binding sites mainly locate on the F1 and F2 regions. And then, microRNA array was performed to verify the prediction after $TSC2^{-/-}$ MEFs were treated with rapamycin. Among 56 differential miRNAs, 4 predicted miRNAs that have the potential to bind with PTEN-3′UTR-F1 and F2 regions were found to be upregulated (Figure 6C). Next, real-time PCR was performed to confirm the expressions of miRNAs predicted to be able to bind with PTEN-3′UTR-F1 and F2. The results showed that among the 16 detected miRNAs, only miR-23a-3p, miR-23b-3p, miR-25-3p and miR-26a-5p were obviously upregulated (Figure 6D,E). Surprisingly, similar results were also obtained in $AKT1^{E17K}$ MEFs (Figure 6F). To demonstrate the direct negative role of the 4 miRNAs in regulating PTEN expression, miRNA mimics were cotransfected with PTEN-3′UTR-F1 and F2 dual-luciferase reporter plasmids into $TSC2^{-/-}$ MEFs. The results showed that luciferase activity was decreased (Figure 7A,B). In addition, the PTEN protein level was also found to be decreased after miRNA mimics were transfected into $AKT1^{E17K}$ or $TSC2^{-/-}$ MEFs (Figure 7C,D). To further verify that inhibition of mTOR suppresses PTEN expression by upregulating these 4 miRNAs, the miR-23a-3p, miR-23b-3p, miR-25-3p and miR-26a-5p inhibitors were transfected into rapamycin-treated $AKT1^{E17K}$ or $TSC2^{-/-}$ MEFs. The results showed that each miRNA inhibitor partially rescued the decreased PTEN level in both $AKT1^{E17K}$ and $TSC2^{-/-}$ MEFs (Figure 7E,F). In addition, cotransfection with all these 4 inhibitors seemed more effective than cotransfection with each inhibitor alone (Figure 7G,H). These data indicated that inhibition of mTOR suppresses PTEN expression through upregulating the levels of miRNAs.

## Discussion

Our current findings demonstrated that MK2206 not only inhibited the phosphorylation of AKT1 at Ser473 but also inhibited mTOR phosphorylation and PTEN expression in $AKT1^{E17K}$ MEFs. Both the mTOR allosteric inhibitor rapamycin and the kinase inhibitor Torin2 effectively decreased PTEN level in $AKT1^{E17K}$ and $TSC2^{-/-}$ MEFs. Additionally, the reduced PTEN level was also observed in control MEFs treated with Torin2 (Figures 1D and 2C), although the degree of reduction in control MEFs was relatively low compared with that in $AKT1^{E17K}$ and $TSC2^{-/-}$ MEFs. Overall, inhibition of mTOR suppresses PTEN expression. However, the phenomenon was not clearly observed in control MEFs after MK2206 or rapamycin treatment. Torin2 inhibits mTOR by competitively binding to the ATP-binding sites of mTOR and completely blocks mTOR kinase activity, whereas rapamycin inhibits mTOR via allosteric regulation and only blocks certain mTOR activities [30,31]. Similar to rapamycin, MK2206 is a selective allosteric inhibitor of AKT which acts through binding to the pleckstrin homology domain to lock

**Table 4. The mRNA level of reported E3ubiquitin ligases and deubiquitinase of *PTEN***

| Type | Gene | $Tsc2^{-/-}$ vs $Tsc2^{+/+}$ | | $Tsc2^{-/-}$R vs $Tsc2^{+/+}$ | |
|---|---|---|---|---|---|
| | | Fold change | Description | Fold change | Description |
| E3 ligase | Nedd4-1 | −1.41008 | Down | 1.37135 | Up |
| | WWP1 | 1.01048 | Up | 1.03184 | Up |
| | WWP2 | 1.27317 | Up | −1.15902 | Down |
| | FBXO22 | 1.11451 | Up | −1.14743 | Down |
| | XIAP | −1.11014 | Down | −1.00557 | Down |
| | TRIM27 | 1.18987 | Up | −1.29726 | Down |
| | RNF146 | 1.08809 | Up | −1.04308 | Down |
| DUB | OTUD3 | 1.04892 | Up | −1.34057 | Down |
| | USP13 | 1.67728 | Up | −1.47363 | Down |

The mRNA abundance of E3ubiquitin ligases and deubiquitinase once reported to regulate PTEN in $Tsc2^{+/+}$, $Tsc2^{-/-}$ MEFs, and $Tsc2^{-/-}$ MEFs treated with rapamycin (R) was measured using Affymetrix mouse genome 4302.0 array.

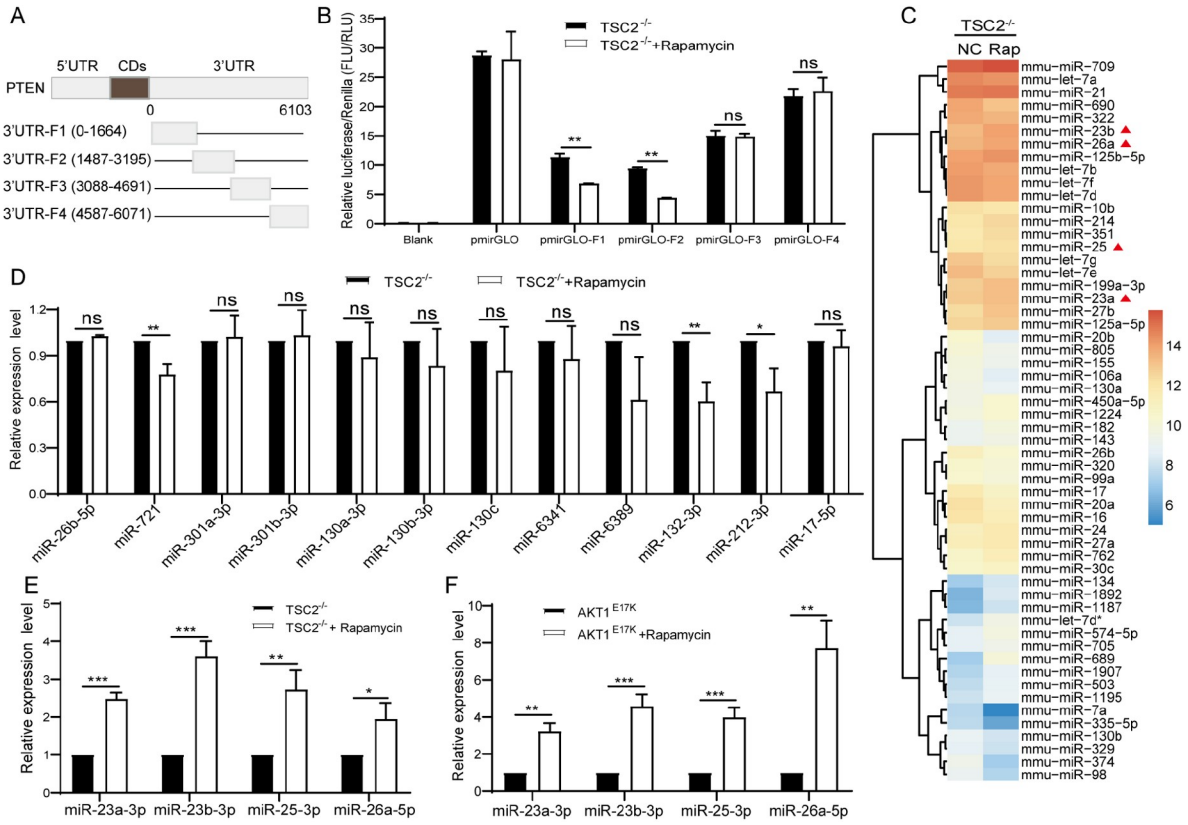


**Figure 6. Inhibition of mTOR upregulates the expressions of miR-23a-3p, miR-23b-3p, miR-25-3p and miR-26a-5p** (A) Construction of four mouse PTEN-3′UTR dual-luciferase reporter plasmids. (B) A dual-luciferase reporter assay confirmed the potential miRNA binding sites in the mouse PTEN-3′UTR. (C) Heatmap of differentially expressed miRNAs in TSC2$^{-/-}$ MEFs after treatment with 10 nM rapamycin. Red triangles indicate upregulated miR-23a-3p, miR-23b-3p, miR-25-3p and miR-26a-5p. (D,E) Real-time PCR analysis of miRNA expression in TSC2$^{-/-}$ MEFs after treatment with 10 nM rapamycin for 24 h. (F) Real-time PCR confirmed the 4 upregulated miRNAs in AKT1$^{E17K}$ MEFs after rapamycin treatment. *$P$ < 0.05, **$P$ < 0.01, ***$P$ < 0.001. ns, not significant.

AKT in an inactive conformation [32]. The different mechanism of action could potentially explain the difference in PTEN protein expression in WT MEFs upon treatment with different inhibitors.

mTOR forms two distinct signaling complexes with different subunits, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) [33]. mTORC1 integrates nutrients and the microenvironment to promote anabolic metabolism and inhibit catabolic metabolism through downstream S6K and 4E-BP1 [13], while mTORC2 phosphorylates Ser-473 on AKT to regulate cytoskeletal rearrangements and cellular survival [34]. Intriguingly, inhibition of mTOR increased the phosphorylation level of AKT1 in TSC2$^{-/-}$ MEFs in our study. Given the positive role of mTORC2 on Akt Ser-473, Akt1 phosphorylation should theoretically be decreased after mTOR inhibition. However, considering that PTEN negatively regulates Akt phosphorylation, the increased Akt phosphorylation could be explained by the reduction in PTEN protein expression. Unfortunately, the severely attenuated phosphorylation of AKT1 on Ser473 in *TSC2*-knockout MEFs has been a challenge for decades, and our present discovery still cannot explain the phenomenon. Furthermore, it is still not clear why PTEN expression is not correspondingly increased upon mTOR activation but decreased upon mTOR inhibition. Given the role of AKT/mTOR signaling in cell survival and nutrient metabolism under physiological conditions [35], the homeostatic regulation of cells to prevent damage or death led by mTOR pathway overinhibition might explain the downregulation of PTEN expression upon mTOR inhibition. Similar to the activation of PI3K/AKT/mTOR signaling is also attenuated by feedback inhibition through a series of AKT- or mTOR-dependent mechanisms [36,37].

Our results demonstrated that inhibition of mTOR does not regulate PTEN expression at the transcription level, but whether epigenetic modifications, such as aberrant promoter methylation and histone acetylation, are involved in this process synergistically is still unknown. Surprisingly, in contrast to previous findings [9], our data suggested that mTOR inhibition has little influence on eIF4E/4E-BP1-mediated translation initiation of *PTEN*. However, whether mTOR inhibition decreases PTEN expression through other stages of PTEN translation requires further investigation. A possible explanation for the discrepancy between our results and previous findings [9] could be that our experiments were performed in MEFs with a simple genetic background, whereas their experiments were conducted in cancer cells with numerous genetic alterations that could potentially affect the results. In addition, even though our results confirmed that mTOR inhibition-mediated PTEN downregulation is not achieved through proteasome or lysosome degradation approaches, other degradation mechanisms for PTEN expression cannot be totally ruled out, since the cellular regulatory network is intricate. Finally, four miRNAs were found to be remarkably upregulated in TSC2$^{-/-}$ and AKT1$^{E17K}$ MEFs after rapamycin treatment, and the miRNA inhibitors rescued the

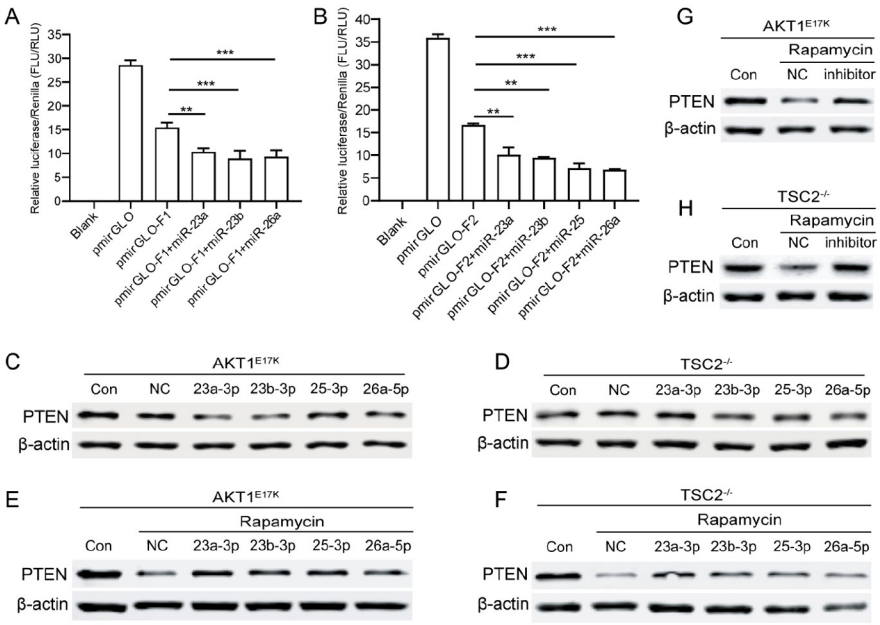


**Figure 7. Inhibition of mTOR suppresses PTEN expression by upregulating miRNAs** (A,B) Dual-luciferase reporter assay after miR-23a-3p, miR-23b-3p, miR-25-3p and miR-26a-5p mimics were cotransfected with PTEN-3′UTR-F1 and F2. (C,D) The protein level of PTEN in AKT1$^{E17K}$ and TSC2$^{-/-}$ MEFs after miR-23a-3p, miR-23b-3p, miR-25-3p and miR-26a-5p mimics were transfected. (E,F) The protein level of PTEN in AKT1$^{E17K}$ and TSC2$^{-/-}$ MEFs after the 4 miRNA inhibitors were transfected followed by treatment with rapamycin. (G,H) PTEN protein level in AKT1$^{E17K}$ and TSC2$^{-/-}$ MEFs after cotransfection with the 4 miRNA inhibitors followed by rapamycin treatment. $^{**}P < 0.01$, $^{***}P < 0.001$.

decreased PTEN level synergistically. These findings provide some creative guides for PTEN regulation, but how mTOR inhibition upregulates the four miRNAs is still unknown. Collectively, although our present discovery sheds new light on the relationship between AKT/mTOR and PTEN, the existing limitations of our project should not be ignored.

Given the importance of PI3K/AKT/mTOR in numerous biological processes, the dysregulation of the pathway is closely related to many diseases, such as metabolic syndrome and cancers. Emerging evidence has revealed that mTOR is abnormally activated in a plethora of human cancers [38,39]. Thus, tumor therapy targeting mTOR seems promising and has attracted the attention of numerous scholars. For instance, the mTOR allosteric inhibitors rapamycin, everolimus and temsirolimus have been approved by the FDA for the treatment of breast cancer [40], kidney renal cell carcinoma [41] and endometrial cancer [42], respectively. Although mTOR inhibitors have been applied for clinical therapy for decades, the therapeutic efficacy was not confirmed in all patients [43]. For instance, Kwiatkowski *et al.* [44] recently declared the failure of a phase II clinical trial of everolimus in pan-cancer patients with mTOR pathway alterations. The decreased PTEN level in AKT1$^{E17K}$ and TSC2$^{-/-}$ MEFs after treatment with rapamycin or Torin2 from our data may provide a rational explanation for mTOR activation-related tumors resistant to mTOR inhibitors to a certain degree.

In summary, this study confirmed the regulatory role of the AKT/mTOR pathway on PTEN and demonstrated the nonnegligible role of miRNAs in regulating PTEN expression. Taking the PTEN level into consideration may provide a theoretical basis and research value for identifying potential therapeutic targets for PI3K/AKT/mTOR pathway activation-related tumors. Because of the popular investigation of noncoding RNAs in cancers, mTOR/miRNA dual inhibition may be a novel strategy to overcome therapeutic resistance in the future.

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## Conflict of Interest

The authors declare that they have no conflict of interest.

## References

1. Hollander MC, Blumenthal GM, Dennis PA. PTEN loss in the continuum of common cancers, rare syndromes and mouse models. *Nat Rev Cancer* 2011, 11: 289–301
2. Lee YR, Chen M, Pandolfi PP. The functions and regulation of the PTEN tumour suppressor: new modes and prospects. *Nat Rev Mol Cell Biol* 2018, 19: 547–562
3. Inoki K, Li Y, Zhu T, Wu J, Guan KL. TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. *Nat Cell Biol* 2002, 4: 648–657
4. Zhang H, Cicchetti G, Onda H, Koon HB, Asrican K, Bajraszewski N, Vazquez F, *et al.* Loss of Tsc1/Tsc2 activates mTOR and disrupts PI3K-Akt signaling through downregulation of PDGFR. *J Clin Invest* 2003, 112: 1223–1233
5. Shaw RJ, Cantley LC. Ras, PI(3)K and mTOR signalling controls tumour

cell growth. *Nature* 2006, 441: 424–430
6. Wang H, Liu Y, Wang D, Xu Y, Dong R, Yang Y, Lv Q, *et al.* The upstream pathway of mTOR-mediated autophagy in liver diseases. *Cells* 2019, 8: 1597
7. Carpten JD, Faber AL, Horn C, Donoho GP, Briggs SL, Robbins CM, Hostetter G, *et al.* A transforming mutation in the pleckstrin homology domain of AKT1 in cancer. *Nature* 2007, 448: 439–444
8. Inoki K, Li Y, Xu T, Guan KL. Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling. *Genes Dev* 2003, 17: 1829–1834
9. Mukherjee R, Vanaja KG, Boyer JA, Gadal S, Solomon H, Chandarlapaty S, Levchenko A, *et al.* Regulation of PTEN translation by PI3K signaling maintains pathway homeostasis. *Mol Cell* 2021, 81: 708–723.e5
10. Kotelevets L, Trifault B, Chastre E, Scott MGH. Posttranslational regulation and conformational plasticity of PTEN. *Cold Spring Harb Perspect Med* 2020, 10: a036095
11. Xie P, Peng Z, Chen Y, Li H, Du M, Tan Y, Zhang X, *et al.* Neddylation of PTEN regulates its nuclear import and promotes tumor development. *Cell Res* 2021, 31: 291–311
12. Smith SL, Pitt AR, Spickett CM. Approaches to investigating the protein interactome of PTEN. *J Proteome Res* 2021, 20: 60–77
13. Ben-Sahra I, Howell JJ, Asara JM, Manning BD. Stimulation of de novo pyrimidine synthesis by growth signaling through mTOR and S6K1. *Science* 2013, 339: 1323–1328
14. Ho J, Cruise ES, Dowling RJO, Stambolic V. PTEN nuclear functions. *Cold Spring Harb Perspect Med* 2020, 10: a036079
15. Misra S, Ghosh G, Chowdhury SG, Karmakar P. Non-canonical function of nuclear PTEN and its implication on tumorigenesis. *DNA Repair* 2021, 107: 103197
16. Chen JH, Zhang P, Chen WD, Li DD, Wu XQ, Deng R, Jiao L, *et al.* ATM-mediated PTEN phosphorylation promotes PTEN nuclear translocation and autophagy in response to DNA-damaging agents in cancer cells. *Autophagy* 2015, 11: 239–252
17. Sonenberg N, Hinnebusch AG. Regulation of translation initiation in eukaryotes: mechanisms and biological targets. *Cell* 2009, 136: 731–745
18. Böhm R, Imseng S, Jakob RP, Hall MN, Maier T, Hiller S. The dynamic mechanism of 4E-BP1 recognition and phosphorylation by mTORC1. *Mol Cell* 2021, 81: 2403–2416.e5
19. Peter D, Igreja C, Weber R, Wohlbold L, Weiler C, Ebertsch L, Weichenrieder O, *et al.* Molecular architecture of 4E-BP translational inhibitors bound to eIF4E. *Mol Cell* 2015, 57: 1074–1087
20. Ge MK, Zhang N, Xia L, Zhang C, Dong SS, Li ZM, Ji Y, *et al.* FBXO22 degrades nuclear PTEN to promote tumorigenesis. *Nat Commun* 2020, 11: 1720
21. Chuang HY, Hsu LY, Pan CM, Pikatan NW, Yadav VK, Fong IH, Chen CH, *et al.* The E3 ubiquitin ligase NEDD4-1 mediates temozolomide-resistant glioblastoma through PTEN attenuation and redox imbalance in Nrf2–HO-1 axis. *Int J Mol Sci* 2021, 22: 10247
22. Nicklas S, Hillje AL, Okawa S, Rudolph IM, Collmann FM, van Wuellen T, del Sol A, *et al.* A complex of the ubiquitin ligase TRIM32 and the deubiquitinase USP7 balances the level of c-Myc ubiquitination and thereby determines neural stem cell fate specification. *Cell Death Differ* 2019, 26: 728–740
23. Lee YR, Yehia L, Kishikawa T, Ni Y, Leach B, Zhang J, Panch N, *et al.* WWP1 gain-of-function inactivation of PTEN in cancer predisposition. *N Engl J Med* 2020, 382: 2103–2116
24. Ma L, Yao N, Chen P, Zhuang Z. TRIM27 promotes the development of esophagus cancer via regulating PTEN/AKT signaling pathway. *Cancer Cell Int* 2019, 19: 283
25. Yuan L, Lv Y, Li H, Gao H, Song S, Zhang Y, Xing G, *et al.* Deubiquitylase OTUD3 regulates PTEN stability and suppresses tumorigenesis. *Nat Cell Biol* 2015, 17: 1169–1181
26. Zhang J, Zhang P, Wei Y, Piao H, Wang W, Maddika S, Wang M, *et al.* Deubiquitylation and stabilization of PTEN by USP13. *Nat Cell Biol* 2013, 15: 1486–1494
27. O'Brien J, Hayder H, Zayed Y, Peng C. Overview of MicroRNA biogenesis, mechanisms of actions, and circulation. *Front Endocrinol* 2018, 9: 402
28. Li N, Miao Y, Shan Y, Liu B, Li Y, Zhao L, Jia L. MiR-106b and miR-93 regulate cell progression by suppression of PTEN via PI3K/Akt pathway in breast cancer. *Cell Death Dis* 2017, 8: e2796
29. Ogórek B, Lam HC, Khabibullin D, Liu HJ, Nijmeh J, Triboulet R, Kwiatkowski DJ, *et al.* TSC2 regulates microRNA biogenesis via mTORC1 and GSK3β. *Hum Mol Genet* 2018, 27: 1654–1663
30. Ballou LM, Lin RZ. Rapamycin and mTOR kinase inhibitors. *J Chem Biol* 2008, 1: 27–36
31. Liu Q, Xu C, Kirubakaran S, Zhang X, Hur W, Liu Y, Kwiatkowski NP, *et al.* Characterization of Torin2, an ATP-competitive inhibitor of mTOR, ATM, and ATR. *Cancer Res* 2013, 73: 2574–2586
32. Jansen VM, Mayer IA, Arteaga CL. Is there a future for AKT inhibitors in the treatment of cancer? *Clin Cancer Res* 2016, 22: 2599–2601
33. Xu J, Pham CG, Albanese SK, Dong Y, Oyama T, Lee CH, Rodrik-Outmezguine V, *et al.* Mechanistically distinct cancer-associated mTOR activation clusters predict sensitivity to rapamycin. *J Clin Invest* 2016, 126: 3526–3540
34. Sarbassov DD, Guertin DA, Ali SM, Sabatini DM. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* 2005, 307: 1098–1101
35. Saxton RA, Sabatini DM. mTOR signaling in growth, metabolism, and disease. *Cell* 2017, 168: 960–976
36. Rodrik-Outmezguine VS, Chandarlapaty S, Pagano NC, Poulikakos PI, Scaltriti M, Moskatel E, Baselga J, *et al.* mTOR kinase inhibition causes feedback-dependent biphasic regulation of AKT signaling. *Cancer Discovery* 2011, 1: 248–259
37. Hopkins BD, Pauli C, Du X, Wang DG, Li X, Wu D, Amadiume SC, *et al.* Suppression of insulin feedback enhances the efficacy of PI3K inhibitors. *Nature* 2018, 560: 499–503
38. Murugan AK. mTOR: role in cancer, metastasis and drug resistance. *Semin Cancer Biol* 2019, 59: 92–111
39. Mossmann D, Park S, Hall MN. mTOR signalling and cellular metabolism are mutual determinants in cancer. *Nat Rev Cancer* 2018, 18: 744–757
40. Ballhausen A, Wheler JJ, Karp DD, Piha-Paul SA, Fu S, Pant S, Tsimberidou AM, *et al.* Phase I study of everolimus, letrozole, and trastuzumab in patients with hormone receptor—positive metastatic breast cancer or other solid tumors. *Clin Cancer Res* 2021, 27: 1247–1255
41. Voss MH, Chen D, Reising A, Marker M, Shi J, Xu J, Ostrovnaya I, *et al.* PTEN expression, not mutation status in *TSC1*, *TSC2*, or *mTOR*, correlates with the outcome on everolimus in patients with renal cell carcinoma treated on the randomized RECORD-3 trial. *Clin Cancer Res* 2019, 25: 506–514
42. Venkata PP, Chen Y, Alejo S, He Y, Palacios BE, Loeffel I, Liu J, *et al.* KDM1A inhibition augments the efficacy of rapamycin for the treatment of endometrial cancer. *Cancer Lett* 2022, 524: 219–231
43. Sun SY. mTOR-targeted cancer therapy: great target but disappointing clinical outcomes, why? *Front Med* 2021, 15: 221–231
44. Adib E, Klonowska K, Giannikou K, Do KT, Pruitt-Thompson S, Bhushan K, Milstein MI, *et al.* Phase II clinical trial of everolimus in a pan-cancer cohort of patients with mTOR pathway alterations. *Clin Cancer Res* 2021, 27: 3845–3853