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Ersuo Jin
Soochow University Laboratory of Cancer Molecular Genetics, Suzhou Medical College of Soochow University

Shengjie Wang
Department of Basic Medicine, Kangda College of Nanjing Medical University

Donglai Chen
Department of Thoracic Surgery, Zhongshan Hospital, Fudan University

Yuanyuan Zeng
Department of Respiratory Medicine, The First Affiliated Hospital of Soochow University, Suzhou Medical College of Soochow University

Hong-Tao Zhang (htzhang@suda.edu.cn)
Soochow University Laboratory of Cancer Molecular Genetics, Suzhou Medical College of Soochow University

https://orcid.org/0000-0003-4182-421X

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Prolyl 4-hydroxylase alpha-2 directly activates mTOR kinase

Ersuo Jin\textsuperscript{1,2}, Shengjie Wang\textsuperscript{3}, Donglai Chen\textsuperscript{4}, Yuanyuan Zeng\textsuperscript{5} & Hong-Tao Zhang\textsuperscript{1,2,6*}

\textsuperscript{1}Soochow University Laboratory of Cancer Molecular Genetics, Suzhou Medical College of Soochow University; Suzhou 215123, China.
\textsuperscript{2}Department of Genetics, School of Biology and Basic Medical Sciences, Suzhou Medical College of Soochow University; Suzhou 215123, China.
\textsuperscript{3}Department of Basic Medicine, Kangda College of Nanjing Medical University; Lianyungang 222000, China.
\textsuperscript{4}Department of Thoracic Surgery, Zhongshan Hospital, Fudan University; Shanghai 200032, China.
\textsuperscript{5}Department of Respiratory Medicine, The First Affiliated Hospital of Soochow University, Suzhou Medical College of Soochow University; Suzhou 215006, China.
\textsuperscript{6}Suzhou Key Laboratory for Molecular Cancer Genetics; Suzhou 215123, China.

*Corresponding author: htzhang@suda.edu.cn (H.-T.Z.)

Abstract

Mammalian target of rapamycin (mTOR) kinase functions as a central regulator of cell growth and metabolism, and its complexes mTORC1 and mTORC2 phosphorylate distinct substrates\textsuperscript{1-3}. Dysregulation of mTOR signaling is commonly implicated in human diseases, including cancer\textsuperscript{4,5}. Despite three decades of active research in mTOR, much remains to be determined\textsuperscript{1}. In fact, how mTOR kinase is directly activated and recognize distinct substrates are still unknown. Here, we demonstrate that prolyl 4-hydroxylase alpha-2 (P4HA2) can hydroxylate mTOR protein and thereby activating mTOR signal. By performing co-immunoprecipitation, GST pull-down assays combined with mass spectrometry-based proteomic analysis in 293T cells, we found that P4HA2 binds directly to mTOR and hydroxylates one highly conserved proline 2341 (P2341) within a kinase domain of mTOR. Moreover, we discover that the hydroxylation of P2341 strengthens mTOR stability and allows mTOR to accurately recognize its substrates such as S6K and AKT. Our study reveals an undiscovered hydroxylation-regulatory mechanism by which P4HA2 directly activates mTOR kinase, providing insights for therapeutically targeting mTOR kinase-driven cancers.
Target of rapamycin (TOR), an evolutionarily conserved serine/threonine protein kinase that integrates a variety of stimuli including growth factors, nutrients, oxygen availability and stress, functions as a central regulator of cell growth and metabolism by forming two distinct complexes: TORC1 and TORC2. Mammalian TORC1 (mTORC1) and mTORC2 exert their function through controlling several important kinases, such as S6 kinase (S6K) and AKT. Dysregulation of mTOR signaling is commonly implicated in human diseases that include diabetes, neurodegeneration and cancer. Of note, hypoxia partially inhibits mTOR kinase activity by rapidly causing hypo-phosphorylation of mTOR and its targets including S6K and AKT. Moreover, hypoxia-induced inhibition of mTOR is dominant over mTOR activation via multiple environmental cues and occurs independently of HIF-1α. Using human embryonic kidney (HEK) 293T cells treated with insulin, we also found that the hypoxia-mediated dephosphorylation of S6K and AKT was rapidly reversed after 1 h of reoxygenation (Fig. 1A), supporting that mTOR activity is regulated by molecular oxygen (O2). In addition, α-ketoglutarate (α-KG)-activated prolyl hydroxylases indirectly induce mTORC1 activity by promoting GTP loading of RAGB. These findings indicate that O2 and α-KG are involved in mTOR kinase activity. However, it is still a puzzle for how mTOR is directly activated in cell growth and metabolism.

To identify candidate mTOR-interacting molecules, we performed an anti-Flag immunoprecipitation (IP) followed by mass spectrometry (MS)-based proteomic analysis in 293T cells overexpressing Flag-tagged mTOR. Among 634 potential mTOR-interacted proteins (Supplementary Table 1A), prolyl 4-hydroxylase subunit alpha-2 (P4HA2) was paid more attention (Fig. 1B) in view of the fact that a prolyl hydroxylase may bind to its cofactors, Fe2+ and α-KG, its substrate and oxygen to transiently yield a prolyl hydroxylation reaction. Endogenous P4HA2 was co-immunoprecipitated with Flag-tagged mTOR in 293T cells (Extended Data Fig. 1A). These results suggest that mTOR interacts with P4HA2, and vice versa (Supplementary Table 1B and Extended Data Fig. 1B). Using co-IP experiments in 293T cells co-
transfected with Flag-mTOR and HA-P4HA2, we confirmed the interaction of P4HA2 with mTOR (Fig. 1C). More co-IP experiments showed that a small percentage of P4HA2 and P4HB, α- and β-subunits of P4H, interacted with mTOR in 293T cells (Extended Data Fig. 1C, D), reflecting the dynamic nature of enzyme-substrate interactions. Glutathione S-transferase pull-down assays indicated a direct interaction of P4HA2 with mTOR (Extended Data Fig. 1E). In support of this, immunofluorescence staining analyses showed that P4HA2 and mTOR were co-expressed in the cytoplasm (Extended Data Fig. 1F). Furthermore, N-terminally truncated mTOR (mTORAN) containing kinase domain (residues 1376-2549) was specially found to bind with P4HA2 (Extended Data Fig. 1G).

Based on the evidence that dysregulation of mTOR signaling and upregulation of P4HA2 in human cancers (Extended Data Fig. 1H), we subsequently focused on lung cancer to investigate whether and how P4HA2 affects mTOR function. Interestingly, short hairpin RNA (shRNA)-mediated knockdown of P4HA2 not only diminished mTOR expression but also inhibited S6K389 and AKT473 phosphorylation in A549 and H1299 cells (Fig. 1D and Extended Data Fig. 1I), suggesting that P4HA2 positively regulates mTOR signaling. This is in consistency with the findings in glioma, where P4HA2 enzymatic inhibitor ethyl-3,4-dihydroxybenzoate (EDHB) or P4HA2 knockdown led to a reduction in phosphorylation levels of AKT9. In fact, P4HA1 and P4HA2 are two major isoforms of P4HA, which individually form α2β2 tetramers with P4HB and thereby contributing to P4HA enzyme activity in most cell types10,11. However, P4HA1 knockdown failed to restrain mTOR signaling in A549 cells (Extended Data Fig. 1J). These findings suggest an important role of P4HA2 in mTOR signaling activation.

Given that P4HA is metabolically regulated by α-KG during collagen hydroxylation and associates with mTOR kinase activity, we hypothesized that P4HA2 activates mTOR via a hydroxylation regulation. To test this, we firstly monitored the changes in total mTOR abundance, and p-S6K389 and p-AKT473 levels in A549 and H1299 cells transfected with an increasing dose of P4HA2. Results of this experiments showed that the amount of mTOR, p-S6K389 and p-AKT473 elicited an increasingly dynamic
response to P4HA2. (Fig. 2A and Extended Data Fig. 2A). When compared with cells overexpressing wild-type P4HA2, the cells with hydroxylation-deficient mutants of P4HA2 \(^\text{13}\) presented lower levels of mTOR, p-S6K\(^{389}\) and p-AKT\(^{473}\) (Fig. 2B and Extended Data Fig. 2B). Using an assay of mTOR in vitro kinase activity \(^\text{14}\), we found that P4HA2 overexpression and knockdown increased and reduced mTOR-catalyzed phosphorylation of S6K\(^{389}\) and AKT\(^{473}\), respectively (Fig. 2C), further demonstrating the promotive effect of P4HA2 on mTOR activation. EDHB, an α-KG mimic, was used as a P4HA2 inhibitor \(^\text{15}\) to treat P4HA2-overexpressing cells, resulting in attenuated levels of p-S6K\(^{389}\) and p-AKT\(^{473}\) (Fig. 2D and Extended Data Fig. 2C). These results imply a hydroxylation role of P4HA2 for regulating mTOR signaling.

Inspired by our aforementioned findings, we speculate that mTOR can be hydroxylated by P4HA2. Thus, using 293T cells overexpressing Flag-mTOR, Flag-mTOR/HA-P4HA2, and Flag-mTOR/HA-P4HA2-overexpressing cells treated with EDHB, we observed that the hydroxylation levels of total protein were elevated in Flag-mTOR/HA-P4HA2-overexpressing cells and this increment was abolished by EDHB treatment (Extended Data Fig. 2D). Next, we conducted an anti-Flag IP to capture and enrich mTOR protein, and the immunoprecipitates were subjected to MS analysis. Intriguingly, we identified a mTOR peptide sequence \(^{[2123\text{SLAVSMVGYILGLGDRH} \#\text{PSNLMLDR}_{2348}]}\) carrying P2341 hydroxylation modification, which exhibits +16 Da mass shifts at the \(y^8\) ion of the fragmentation spectrum (Fig. 2E, middle). Therefore, MS analysis discovered P4HA2-catalyzed hydroxylation of mTOR kinase domain at proline 2341, which positioned within H-P-S motif. This is supported by the notion that P4HA2-induced hydroxylation sites are not limited to the \(X_{(aa)}-P-G\) triplets \(^\text{16-18}\). Using the program ConSurf \(^\text{19}\), we calculated that P2341 is highly conserved in mTOR kinase domain (Fig. 2F), suggesting a crucial role of P2341 in controlling mTOR functions. Moreover, P2341A (proline 2341 to alanine) mutant of mTOR significantly suppressed not only P4HA2-induced proline hydroxylation of mTOR but also the interaction
between mTOR and P4HA2 (Fig. 2G), indicating that P4HA2 directly and specifically hydroxylates mTOR on P2341.

The hydroxylation of proline residues in collagen strengthens the folding and stability of collagen \(^{20,21}\). Qi et al also linked increased Argonaute 2 (Ago2) stability to hydroxylation of Ago2 at proline 700 \(^{22}\). Coincidentally, in this study, the hydroxylated P2341 (HYP2341) of mTOR may be negatively charged at physiological pH (~6.50) and evoke electrostatic forces because pH 6.30 is the isoelectric point for proline and pH 5.83 for HYP. Therefore, HYP2341 can electrostatically interact with positively charged arginine 2378 (R2378), and this interplay is supported by using Cryo-EM structure analyses in mTORC1 and mTORC2 (Fig. 3A). These data encouraged us to explore whether HYP2341 regulates mTOR stability by interacting with R2378. As expected, mTOR\(^{P2341A}\) led to destabilization of mTOR in protein biosynthesis inhibitor cycloheximide (CHX)-treated 293T cells overexpressing P4HA2 (Fig. 3B), but this effect was blocked by the proteasome inhibitor MG132 (Fig. 3C). R2378A (arginine 2378 to alanine) mutant of mTOR (mTOR\(^{R2378A}\)) resulted in the same effects (Fig. 3D, E). Moreover, the stabilization of endogenous mTOR by P4HA2 overexpression (Fig. 3F) was further enhanced by MG132 (Fig. 3G), suggesting that non-hydroxylated mTOR could be degraded via a ubiquitin-mediated proteasome-dependent mechanism. Thus, we examined the ubiquitylation of mTOR in the presence or absence of P4HA2 and P4HB. Co-IP experiments revealed that P4HA2 or P4HB overexpression inhibited mTOR polyubiquitylation in 293T cells, whereas P4HA2 or P4HB knockdown had the opposite effect (Fig. 3H). Collectively, these results provide a notion that the hydroxylation of P2341 by P4HA2 is required for mTOR stability.

Considering the previous findings that mTOR and suppressor of morphogenesis in genitalia 1 (SMG1) belong to the phosphatidylinositol kinase-related kinase family \(^{23,24}\) and mTOR-CDK2 superposition reveals a coincidence of mTOR H2340 and CDK2 K129 side chains \(^{25}\), we focused on insights into the structural position of mTOR P2341 site and found that HYP2341 is just located in the catalytic pocket.
This is evidenced by the mTOR-SMG1-CDK2 superposition (Extended Data Fig. 3A-E), suggesting that hydroxylation of P2341 is a critical event causing mTOR activation.

Next, we determined the role of mTOR P2341 hydroxylation in mTOR substrate phosphorylation and cell growth. Upon insulin stimulation, 293T cells overexpressing a hydroxylation-deficient mutant of mTOR (mTORP2341A) exhibited significantly reduced activation of mTOR, showing lower levels of p-S6K389 and p-AKT473 (Fig. 4A). Even if P4HA2 was overexpressed in 293T cells, the incorporation of mTORR2378A mutant partially decreased mTOR kinase activity (Extended Data Fig. 3F). Interestingly, we did not find any cancer-associated mTOR mutations at P2341 and R2378 (Supplementary Table 2A) using the cBio Cancer Genomics Portal (http://cbioportal.org) 26, inspiring us to investigate the significance of mTOR P2341 hydroxylation modification for cancer progression. Overexpression of mTORP2341A in A549 cells caused a significant reduction in phosphorylation of mTOR-activated substrates S6K or AKT (Fig. 4B), and thereby attenuating tumor cell growth (Fig. 4C, D).

Hydroxyproline residues have a key role in stabilizing collagen 20,21. The pyrrolidine ring of HYP makes conformationally contacts with the pyrrolidine ring in the triple-helical structure of collagen (Extended Data Fig. 4A). Therefore, it is likely that HYP2341 contributes to substrate recognition of mTOR. Overlapping the structure of mTORC1 or mTORC2 catalytic pocket with SMG1, we predicted that like collagen, mTOR recognizes substrates in the catalytic pocket composed of HYP-proline contact structure (Fig. 4E, F and Extended Data Fig. 4B-G). Thus, we tested whether the contact is required for the recognition of substrate and affects insulin-stimulated mTOR kinase activity. As a result, S6KP393A and AKTP470A mutants abolished mTORC1-catalyzed S6K T389 phosphorylation and mTORC2-catalyzed AKT S473 phosphorylation, respectively. (Fig. 4G, H). No cancer-associated mutations were found at S6K P393 and AKT P470 (Supplementary Table 2B, C), indicating their significance in substrate recognition of mTOR.

Taken together, our experiments discover a hydroxylation regulatory mechanism that activates and
stabilizes mTOR kinase, and provide insights for therapeutically targeting mTOR kinase-driven cancers.

Methods

Cell culture

Human embryonic kidney (HEK) 293T cell line, and lung cancer cell lines A549 and H1299 were obtained from the Cell Bank of Chinese Academy of Sciences. All the cell lines were authenticated with STR profiling and checked free of mycoplasma contamination by PCR. HEK 293T cells were cultured in Dulbecco’s Modified Eagle medium with high glucose (DMEM, Thermo Scientific, 21013024) and 10% fetal bovine serum (FBS). A549 and H1299 cells were cultured in RPMI-1640 (Corning, 10-040-CV) with 10% FBS. Hypoxia treatment was made by culturing cells in a hypoxic chamber containing 1% oxygen (Coy Laboratory Products). Cells were serum-starved in DMEM for 24 h followed by stimulation with 200 nM insulin (Beyotime, P3376) for evaluating mTOR activation levels.

Antibodies and reagents

The antibodies used were as follows: phosphor (p)-AKT\(^{473}\) (Cell Signaling Technology (CST), 4060S), AKT (CST, 4691S), mTOR (CST, 2972S), RAPTOR (CST, 2280S), RICTOR (CST, 9476S), IgG (CST, 3900), HA-tag (CST, 3724S), DYKDDDDK (Flag)-tag (CST, 14793S), HIF-1α (Santa Cruz, sc-13515), p-S6K\(^{389}\) (Proteintech, 28735-1-AP), S6K (Proteintech, 14485-1-AP), P4HA2 (Proteintech, 13759-1-AP), P4HA1 (Beyotime, AG2783), P4HB (Beyotime, AG2786), β-actin (ABclonal, AC004), Myc-tag (ABclonal, AE070), hydroxyproline (Biorbyt, orb184229), horseradish peroxidase (HRP)-conjugated secondary antibodies rabbit (Beyotime, A0208) or mouse (Beyotime, A0216). The reagents used were as follows: MG132 (Beyotime, S1748), cycloheximide (CHX, Beyotime, SC0353), ethyl-3,4-dihydroxybenzoate (EDHB, TCI, D0571).

Construction of vectors and transfections

The full-length coding sequence (CDS) of mTOR (GenBank Accession number: NM_001386500.1), P4HA2 (NM_001017974.2), S6K (NM_001272042.2), and AKT (NM_001014431.2) were amplified and
then subcloned into pcDNA3.1-Flag/HA vectors with endonucleases NheI/HindIII (New England Biolabs, NEB; R0131L/R0104L) to generate pcDNA3.1-mTOR-Flag, pcDNA3.1-P4HA2-HA, pcDNA3.1-S6K-HA, and pcDNA3.1-AKT-Flag transient expression vectors. The mutant constructs containing mTORP2341A or R2378A, P4HA2H430S, H501S or H519S, S6KP393A, and AKT-P470A were generated using a PCR-based site-directed mutagenesis and ClonExpress Ultra One Step Cloning Kit (C115-01, Vazyme). The pcDNA3.1-mTOR-Flag was used as DNA template to yield the constructs containing N-terminally truncated mTOR (mTORΔN, residues 1376-2549). All plasmids were validated with Sanger sequencing (Tsingke). Next, HEK 293T, A549, or H1299 cells were transiently transfected with above-constructed vectors using Lipofectamine 3000 (Thermo Fischer, L3000015) (1 μg DNA : 2 μl Lipofectamine).

**Establishment of P4HA2-silenced stable cell lines**

Two shRNAs targeting P4HA2 (sh-P4HA2-1, CCGGGCCGAATTCTTCACCTCTATTCTCGAGAAATCAGAGGTAAGAATTCGGCTTTTG; sh-P4HA2-2, CCGGGCAGTCTCTGAAAGAGTACATCTCAGATGTACTCTTTCAGAGACTGCTTTTTTG) (Tsingke) and negative control shRNA (sh-NC, CCGGTTCGACAGCTTGCTTTCATCGAGAAACGTGACACGTTCGAGAATTTTTG) were subcloned into a lentiviral vector pLKO.1-puro (GENEWIZ) with endonucleases AgeI/EcoRI (NEB, R055L/R0101L) to create pLKO.1-sh-P4HA2-1/2 and pLKO.1-sh-NC. Subsequently, the aforementioned constructs were respectively co-transfected with packaging plasmids psPAX2 and pMD2.G (RIBOBIO) into HEK 293T cells using Lipofectamine 3000. Packaged lentiviruses were collected and used to infect the selected cells for 72 h. Finally, P4HA2-silenced stable cells were selected with 0.2 μg/ml puromycin (Solarbio, P8230).

**RNA interference**

Two short interfering RNAs (siRNAs) targeting P4HA1 were designed and synthesized (GenePharma). The siRNA sequences of siRNA were as follows: si-P4HA1-1, GGAAACCGGUAAUCCUAAU; si-P4HA1-2, GAUAAAGUCUCUGUUCUAG. A scrambled siRNA served as negative control (si-NC).
Cells were transiently transfected with 100 pmol of siRNAs using Lipofectamine 3000. At 48 h post-transfection, cells were harvested for further experiments.

**Immunoblot analysis**

Immunoblot analysis was conducted as previously described by us\(^\text{27}\). Briefly, cells were collected and lysed in ice-cold RIPA buffer (Thermo Fisher Scientific, 89900) supplemented with protease inhibitor cocktail (Sangon Biotech, C600387) and phosphatase inhibitor cocktail (Sangon Biotech, C500017). Lysates were cleared by centrifugation at 14,000 g for 15 min at 4°C and total protein was quantitated using BCA Protein Quantification Kit (Yeasen Biotechnology, 20201ES76). Equal amounts of whole cell lysates were subjected to SDS-PAGE gel electrophoresis and transferred onto nitrocellulose membrane (Merck, HATF00010) followed by immunoblot with indicated antibodies. Eventually, proteins were detected by ECL chemiluminescence kit (Fdbio science, FD8020).

**Co-immunoprecipitation (Co-IP)**

Co-IP was performed as described previously\(^\text{27}\) with some modification. Briefly, cells were grown on 10-cm dishes and lysed with ice-cold IP buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% NP-40, 10% glycerol) for 30 min. Cell lysates were incubated with magnetic beads carrying antibodies against Flag or HA (Bimake, B26102 or B26202), or incubated with the appropriate antibodies coupled to protein A/G agarose beads (Thermo Fisher Scientific, 88802) for 3 h at 4°C. Subsequently, the immunoprecipitated beads were washed with IP buffer. Immunoprecipitates and input were subjected to immunoblot or MS-based analysis.

**Protein silver-staining assay**

The anti-Flag or anti-HA Co-IP products from HEK 293T cells transfected with Flag-mTOR or HA-P4HA2 were resolved on SDS-PAGE gel and then identified with Silver Staining Kit (CWBIO, CW2012S). The protocol was performed as previously described\(^\text{27}\).

**Mass spectrometry assay**
The protein mixture was reduced with 10 mM DTT for 30 min, alkylated with 55 mM iodoacetamide (IAM) for 45 minutes, and digested with trypsin overnight. The resulting tryptic peptides were purified using a C18 column and analyzed on an Orbitrap Elite hybrid mass spectrometer (Thermo Fisher Scientific) coupled with the Dionex liquid chromatography (LC). MS/MS spectra were collected for the selected precursor ion within a 0.02 Da mass isolation window. All spectral data were searched against the UniProt protein database (https://www.uniprot.org) using the Proteome Discoverer 1.4 (Thermo Scientific).

**Immunofluorescence staining**

Cells were seeded on coverslips in a 24-well culture plate. Twenty-four hours later, cells were washed with PBS buffer, fixed with 4% paraformaldehyde for 30 min, permeabilized with 0.2% Triton X-100, and then blocked with 5% BSA for 1 h. Subsequently, cells were incubated with rabbit anti-P4HA2 (Proteintech, 13759-1-AP) and mouse anti-mTOR (Santa Cruz, sc-51764) antibodies (1:500 dilution for both) at 4°C overnight. The following day, cells were washed with PBST buffer and incubated with FITC-conjugated anti-rabbit secondary antibody (Beyotime, A0562) and Cy3-conjugated anti-mouse secondary antibody (Beyotime, A5021) for 2 h in dark. Nuclei were counterstained with 4’,6-diamidino-2-phenylindole (DAPI). Finally, protein localization was observed and photographed using a confocal laser scanning microscope (Leica SP8), and images were digitally merged for co-localization examination.

**Expression and purification of GST fusion proteins**

P4HA2, S6K, and AKT CDSs were subcloned into pGEX-4T-2 vector with N-terminal GST-tag using endonucleases BamHI/NotI (NEB, R0136L/R0189L) to yield pGEX-GST-P4HA2, pGEX-GST-S6K, and pGEX-GST-AKT expression vectors. *E. coli* strain BL21 cells were transformed with above-constructed vectors or pGEX-4T-2 (empty vector) and cultured at 37°C overnight, and then induced with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 16°C with vigorous shaking for 12 h. Cells were collected and sonicated on ice, and incubated with glutathione-Sepharose beads (Sangon Biotech, C600327). Purified GST-P4HA2 or GST proteins were used for GST pull-down analysis, while purified
GST-S6K and GST-AKT were prepared for assay of mTOR \textit{in vitro} kinase activity.

**GST pull-down**

Purified GST-P4HA2 or GST proteins from \textit{E. coli} strain BL21 cells were mixed with lysates of HEK 293T cells overexpressing Flag-mTOR. The mixture was incubated with rotating at 4°C overnight. After washing for three times, bound proteins were separated on SDS-PAGE gel and visualized with Coomassie Blue staining and immunoblot.

**Assay of mTOR \textit{in vitro} kinase activity**

Assay of mTOR \textit{in vitro} kinase activity was done as previously described \textsuperscript{14} with some modification. Insulin (200 nM)-stimulated HEK 293T cells were lysed with the buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1× protease inhibitor cocktail, 1.0 mM 2-mercaptoethanol, 1.0 % Triton, 10 % Glycerol and 1.0 mM MgCl\textsubscript{2}). The lysates were immunoprecipitated with anti-RAPTOR and anti-RICTOR antibodies (0.4 μg for both). RAPTOR or RICTOR immunoprecipitates were washed in kinase buffer (25 mM HEPES pH 7.4, 20 mM KCl, and 10 mM MgCl\textsubscript{2}). Then, the complex mTORC1 (RAPTOR) or mTORC2 (RICTOR) were incubated with mTOR kinase buffer (25 mM HEPES, 50 mM KCl, 10 mM MgCl\textsubscript{2}, and 500 μM ATP) with bacterially purified inactive 50 ng GST-S6K (for mTORC1 complex) or 50 ng GST-AKT (for mTORC2 complex) substrate at 37°C. After 30 min, the kinase-catalyzed reactions were stopped. Finally, the reaction mixture was subjected to immunoblot analysis with antibodies against p-S6K\textsuperscript{389} or p-AKT\textsuperscript{473}. The mTOR kinase activities were assessed by the ratio of phosphorylated substrate to total substrate in each reaction.

**Identification of prolyl hydroxylation site of mTOR**

HEK 293T cells were transfected with Flag-mTOR, Flag-mTOR/HA-P4HA2 for 24 h, respectively. Flag-mTOR/HA-P4HA2-overexpressing cells were treated with EDHB (200 μM) for 24 h. Forty-eight hours later, the whole cell lysates from above-treated cells were collected and incubated with anti-Flag M2 Affinity Gel (Sigma-Aldrich, A2220) at 4°C for 5 h. Then the Flag beads were washed with cold lysis
buffer and treated with 8 M Urea, followed by incubation with 50 mM DTT solution for 30 min, IAM solution at room temperature for 45 min in dark. Next, 50 mM DTT solution was added to stop alkylation. Eluates containing mTOR protein were prepared for protease digestion (trypsin) and desalted with C18 Ziptip (Millipore, ZTC18S096). The pooled supernatants were vacuum concentrated to dryness and subjected to mass spectrometry analysis with Orbitrap Fusion Lumos Tribrid (Thermo Scientific). The prolyl hydroxylated peptide spectrum (15.99491@Pro) matches for mTOR were obtained after Proteome Discoverer 2.0 database (Thermo Scientific) search.

**Colony formation and CCK8 assays**

Cells were seeded into 6-well plates (1.5×10^3 cells/well) and left for 8-10 days until formation of visible colonies. Colonies were washed with PBS buffer, fixed with methanol for 30 min, and then stained with 0.1% crystal violet in 20% ethanol overnight. After staining, the plates were washed and air-dried. Eventually, the colony numbers were counted. Five independent experiments were performed for colony formation analysis.

For Cell Counting Kit-8 (CCK8) assay, cells (2.0×10^3) were cultured in 96-well plates for 1 day, 2 days, 3 days and 4 days, respectively. Then, CCK8 solution (Beyotime, C0038) was added into each well in dark. Optical density (OD) values were determined by measuring the absorbance at 450 nm and 630 nm.

**Structural superimposition**

Human SMG1 kinase complex bound to an UPF1 phosphorylation site was applied as the structure model. Superimposition of the substrate binding grooves of SMG1 with an UPF1 substrate peptide (PDB ID:6Z3R) and mTORC1 (PDB: 5H64) or mTORC2 (PDB: 5ZCS) was visualized using the PyMol software (Schrodinger LLC). The mTOR phosphorylation substrates were superimposed with UPF1 substrate peptide for mimicking the binding of mTOR to its substrate peptides.

**Statistical analysis**

The difference between two groups was compared by paired or unpaired Student’s t-test. Data were
expressed as mean ± SD. *P* values less than 0.05 was considered statistically significant. Statistical analyses were performed using GraphPad Prism 7.01 software (GraphPad).
References


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**Author contributions** E.J. and H.-T.Z. contributed to study concept and design. E.J. and S.W. performed all experiments. Y.Z. provided technical support. E.J., D.C., and H.-T.Z. analyzed the data, wrote the manuscript, and contributed to interpretation of data and critical revision of the manuscript. H.-T.Z. supervised the study.

**Competing interests** The authors declare no competing interests.

**Data and materials availability** All data are available in the main text or the supplementary materials.
Fig. 1. P4HA2 interacts with mTOR. (A) Serum-starved 293T cells were incubated in normoxia or hypoxia for 30 min, and then stimulated with 200 nM insulin for 45 min and reoxygenated for 1 h as indicated. Whole cell lysates (WCL) were immunoblotted for mTOR, total or phosphorylated S6K, AKT and HIF-1α. β-actin served as internal control. (B) WCL from 293T cells transfected with Flag-mTOR were immunoprecipitated with anti-Flag antibody, and bound proteins were eluted and subjected to silver staining and MS-based proteomic analysis. (C) WCL of 293T cells co-expressing Flag-mTOR and/or HA-P4HA2 were subjected to co-IP using anti-Flag or anti-HA antibodies. (D) Immunoblot analysis of mTOR, total or phosphorylated S6K, and AKT in WCL from A549 cells transfected with sh-P4HA2.
Fig. 2. P4HA2 hydroxylates mTOR kinase domain at proline 2341 and activates mTOR signaling. (A) Immunoblot analysis of mTOR, total or phosphorylated S6K, and AKT in A549 cells transfected with an increasing dose of P4HA2. (B) A549 cells were transfected with wild-type P4HA2 and hydroxylation-deficient P4HA2 mutants for 48 h, and WCL were immunoblotted for mTOR, total or phosphorylated S6K and AKT. (C) Insulin (200 nM)-stimulated 293T cells with P4HA2 overexpression and sh-P4HA2 were immunoprecipitated with anti-RAPTOR or anti-RICTOR antibodies, and the immunoprecipitates were subjected to an assay of mTOR in vitro kinase activity by using recombinant S6K (50 ng) or AKT (50 ng) as mTORC1 and mTORC2 substrates. (D) P4HA2-overexpressing A549 cells were treated with
EDHB (200 μM) for 24 h, and the immunoblot analysis was conducted as above. (E) 293T cells were transfected with Flag-mTOR, Flag-mTOR/HA-P4HA2 for 24 h, respectively. Flag-mTOR/HA-P4HA2-overexpressing cells were treated with EDHB (200 μM) for 24 h. WCL were harvested, and anti-Flag IP was performed to enrich mTOR protein followed by MS analysis. The fragmentation spectrum of mTOR peptide \([2123]\text{SLAVMSMVGYILGLGDRHP(OH)SNLMLDR}_{2348}\) carrying P2341 hydroxylation modification is shown with annotated y-ions (blue) and b-ions (red). The y-ion series displays +16 Da mass shifts at the y\(^8\) ion (middle panel). P(OH) represents hydroxylated-proline. (F) Amino acid conservation of P2341 among different species was analyzed using the program ConSurf \(^{19}\). (G) 293T cells were co-transfected with HA-P4HA2 and wild-type Flag-mTOR or proline 2341 to alanine (P2341A) mutants of mTOR, and treated with EDHB as indicated. WCL were used for anti-Flag IP experiments and then immunoblotted with antibodies against Flag, HA, and hydroxylation (Hy)-OH.
Fig. 3. P4HA2-catalyzed hydroxylation is required for mTOR stability. (A) Crystallographic structure of mTORC1 (PDB:6BCU) and mTORC2 (PDB:5ZCS) showing the positions of P2341 of mTOR-catalyzed substrate binding site and R2378. HYP2341, hydroxylated P2341. (B-E) 293T cells co-transfected with Flag-mTORWT or Flag-mTORP2341A or R2378A and HA-P4HA2 were treated with CHX (50 μM) or MG132 (25 mM) for the indicated times, and immunoblotted with antibodies against Flag, HA and β-actin as loading control. (F and G) 293T cells overexpressing P4HA2 and empty vector were treated as above. Endogenous mTOR and P4HA2 expression were detected by immunoblot. (H) 293T cells were co-transfected with Flag-mTOR, Myc-Ubiquitin (Ub), HA-P4HA2 and HA-P4HB, or sh-P4HA2 and sh-P4HB and then treated with MG132 (25 mM) for 8 h as indicated. WCL were immunoprecipitated with anti-Flag antibodies and immunoblotted with the indicated antibodies.
Fig. 4. Hydroxylation of mTOR P2341 affects substrate phosphorylation and recognition of mTOR.

(A) Serum-starved 293T cells with Flag-mTOR WT or Flag-mTOR P2341A were stimulated with insulin (200 nM) for the indicated times. The WCL were immunoblotted with antibodies against Flag, total or phosphorylated S6K and AKT. (B) Immunoblot analysis of mTOR, total or phosphorylated S6K and AKT in A549 cells with Flag-mTOR WT or Flag-mTOR P2341A. (C and D) Colony formation and CCK8 assays were performed in the above-mentioned A549 cells (B). Data are shown as the mean ± SD (n=5). ***P < 0.001 by unpaired Student’s t-test. (E and F) Superimposition of the substrate binding grooves of SMG1 (PDB: 6Z3R) and mTORC1 (PDB: 5H64) or mTORC2 (PDB: 5ZCS) was visualized using the PyMol software. S6K1 substrate peptide containing P393 near T389 or AKT substrate peptide containing P470...
near S473 were overlaid into the catalytic pockets of mTORC1 or mTORC2, respectively. This overlay yields the contact of mTOR P2341 with P393 embodied in S6K1 substrate peptide, or the contact of mTOR P2341 with P470 embodied in AKT substrate peptide. (G and H) 293T cells transfected with HA-S6K<sup>WT</sup> and HA-S6K<sup>P393A</sup>, or Flag-AKT<sup>WT</sup> and Flag-AKT<sup>P470A</sup> were serum-starved for 24 h and stimulated with insulin (200 nM). The WCL were immunoblotted with antibodies against mTOR, HA or Flag, phosphorylated S6K<sup>389</sup> and AKT<sup>473</sup>.
Extended Data Figures

Extended Data Fig. 1. P4HA2 directly interacts with mTOR. (A) Mass spectrometry (MS)-based proteomic analysis of mTOR and P4HA2 interaction (upper panel). Whole cell lysates (WCL) of 293T cells transfected with Flag-mTOR were immunoprecipitated with anti-Flag antibody, and endogenous P4HA2 was detected by immunoblot (lower panel). (B) WCL from 293T cells transfected with HA-P4HA2 were immunoprecipitated with anti-HA antibody, and bound proteins were eluted and subjected to silver staining and MS-based proteomic analysis (left panel). MS analysis of P4HA2 and mTOR interaction (upper right panel). WCL of 293T cells transfected with HA-P4HA2 were immunoprecipitated with anti-HA antibody, and endogenous mTOR was detected with immunoblot (lower right panel). (C) Co-IP analysis of mTOR with P4HA2 or P4HB using anti-mTOR antibody in 293T cells. (D) Co-IP was performed in 293T overexpressing HA-P4HB. Endogenous mTOR or P4HA2 were immunoprecipitated
with anti-HA antibody. Binding of mTOR or P4HA2 to P4HB was determined by immunoblot. (E) Recombinant Glutathione S-transferase (GST)-P4HA2 pulls down Flag-mTOR in a cell-free system. (F) 293T cells were co-incubated with a rabbit anti-P4HA2 antibody and a mouse anti-mTOR antibody and then stained with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (green, for P4HA2) and Cy3-conjugated anti-mouse IgG (red, for mTOR). Cell nuclei were counterstained and visualized with DAPI. Scale bar: 10 μm. (G) N-terminally truncated mTOR ((mTORΔN)) was shown in schematic diagram of mTOR protein as indicated (left panel). WCL of 293T cells transfected with Flag-mTOR or Flag-mTORΔN and HA-P4HA2 were subjected to co-IP using anti-Flag antibody and immunoblotted with indicated antibodies (right panel). (H) Analysis of P4HA2 mRNA expression in pan-cancer tissues and normal tissues using TCGA database. ns, not significant; *P < 0.05, **P < 0.001 and ***P < 0.001 by paired Student’s t-test. (I) Immunoblot analysis of mTOR, total or phosphorylated S6K, and AKT in WCL from H1299 cells transfected with sh-P4HA2. (J) WCL of A549 cells transfected with sh-P4HA1 were immunoblotted with antibodies against mTOR, total or phosphorylated S6K and AKT.
Extended Data Fig. 2. P4HA2 activates mTOR via a hydroxylation regulation. (A) Immunoblot analysis of mTOR, total or phosphorylated S6K, and AKT in H1299 cells transfected with an increasing dose of P4HA2. (B) H1299 cells were transfected with wild-type P4HA2 and hydroxylation-deficient P4HA2 mutants for 48 h, and WCL were immunoblotted for mTOR, total or phosphorylated S6K and AKT. (C) P4HA2-overexpressing H1299 cells were treated with EDHB (200 μM) for 24 h, and the immunoblot analysis was conducted as above. (D) 293T cells were co-transfected with Flag-mTOR and HA-P4HA2, and treated with EDHB as indicated. WCL were immunoblotted with antibodies against hydroxylation (Hy)-OH, mTOR, HA and β-actin as loading control.
Extended Data Fig. 3. Hydroxylated P2341 electrostatically interacts with R2378 and thereby causing mTOR activation. (A) Comparison among the amino acid sequences within catalytic pockets of mTOR, SMG1 and CDK2. Positions of P2341/R2378, L2338/R2374 and P130/S188 were marked in the corresponding sequences as indicated. (B-E) Superimposition of mTOR (PDB:5H64) with SMG1 (PDB:6Z3R) and CDK2 (PDB:3QZW) showing potentially different contribution of P2341/R2378 to substrate recognition of mTOR when compared with those of L2338/R2374 and P130/S188 to substrate recognition of SMG1 and CDK2. (F) Serum-starved 293T cells co-transfected with Flag-mTORWT or Flag-mTORR2378A and HA-P4HA2 were stimulated with insulin (200 nM) for the indicated times. The WCL were immunoblotted with antibodies against Flag, HA, total or phosphorylated S6K and AKT.
Extended Data Fig. 4. The contact of hydroxylated P2341 with prolines of mTOR substrates contributes to substrate recognition of mTOR. (A) Structure of a collagen triple-helix fragment composed of (Pro-Hyp-Gly)n strands (PDB: 1CAG). (B-E) Superimposition of the substrate binding grooves of SMG1 (PDB: 6Z3R) and mTORC1 (PDB: 5H64) was visualized using the PyMol software, and 4E-BP1 substrate peptides containing P38 near T37, P47 near T46, P66 near S65 or P71 near T70 were overlaid into the catalytic pockets of mTORC1. This overlay yields the contact of mTOR P2341 with the prolines (P38, P47, P66, and P71) embodied in 4E-BP1 substrate peptides. (F and G) Superimposition of the substrate binding grooves of SMG1 (PDB: 6Z3R) and mTORC2 (PDB: 5ZCS) was visualized using the PyMol software, and PKC substrate peptides containing P635 near T631 or P639 near T638 were overlaid into the catalytic pockets of mTORC2. This overlay yields the contact of mTOR P2341 with the prolines (P635 and P639) embodied in PKC substrate peptide.
Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTable1.xlsx
- SupplementaryTable2.xlsx
- SupplementaryTable3.xlsx