



PIH1D1 interacts with mTOR complex 1 and enhances ribosome RNA transcription



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ABSTRACT

PIH1D1 is the defining component of the R2TP complex. Recently, R2TP has been reported to stabilize mTOR (mammalian target of rapamycin), an important regulator of cell growth and protein synthesis. Two complexes of mTOR, mTORC1 and mTORC2, have been identified. We demonstrate that immunoprecipitation (IP) of PIH1D1 results in the co-IP of Raptor (mTORC1 specific), but not Rictor (mTORC2 specific), and that knockdown of PIH1D1 decreases mTORC1 assembly, S6 kinase phosphorylation (indicator of mTORC1 activity), and rRNA transcription without affecting mTORC2 in human breast cancer MCF-7 cells. In addition, we provide evidence that PIH1D1 is overexpressed in various breast cancer cell lines. These findings collectively suggest that PIH1D1 may have an important role in mTORC1 regulation in breast cancers.

Structured summary of protein interactions:

mTOR physically interacts with **PIH1D1** and **Raptor** by anti tag coimmunoprecipitation (View interaction)

Rictor physically interacts with **mTOR** and **Tel2** by anti bait coimmunoprecipitation (View interaction)

Raptor physically interacts with **Tel2**, **PIH1D1** and **mTOR** by anti bait coimmunoprecipitation (View interaction)

PIH1D1 physically interacts with **mTOR** and **Raptor** by anti bait coimmunoprecipitation (View interaction)

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1. Introduction

Mammalian target of rapamycin (mTOR) is a member of the phosphatidylinositol 3-kinase-related protein kinase (PIKK) family, which also includes ATM (ataxia telangiectasia mutated), ATR (ATM- and Rad3-related), DNA-dependent protein kinase catalytic subunit (DNA-PKcs), suppressor with morphological effect on genitalia 1 (SMG-1), and TRRAP (transformation/transcription domain-associated protein) [1]. mTOR functions within two distinct multi-subunit complexes, mTOR complex 1 and mTOR complex 2 (mTORC1 and mTORC2, respectively) [2]. mTORC1 consists of mTOR, mLST8 (also known as GβL) and Raptor. mTORC2 also contains mTOR and mLST8 but, instead of Raptor, it contains Rictor.

mTORC1 controls cell growth and protein synthesis by S6 kinase (S6K), via its ability to phosphorylate multiple substrates including S6 and eIF4B, whereas mTORC2 regulates cell survival by phosphorylating downstream effector Akt. Recently, Tel2 (telomere maintenance 2) has been identified to interact with all known mammalian PIKKs and essentially regulate their abundance [3].

The R2TP complex, identified in yeast by systematic proteomic and genomic approaches, is a multi-subunit Hsp90 interacting complex formed by Rvb1, Rvb2, Tah1, and Pih1 [4–7]. It has been reported that PIKK family including mTOR is stabilized by Tel2–R2TP complex [8,9]. We have reported that Monad and its binding proteins, R2TP complex, regulate apoptosis [10–16]. Among the components of R2TP complex, Pih1 (PIH1D1 in human) directly binds to Tel2 [9] and shown to be unstable [6,7,15], raising the possibility that PIH1D1 may be a critical regulator of R2TP complex. We have reported that PIH1D1 possesses cell protective function against apoptosis in human osteosarcoma U2OS cells [13], however, the mechanism has not been fully explored. Rvb1 and Rvb2

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(pontin and reptin in human) have been reported to be overexpressed in several cancers including hepatocellular carcinoma, colon and bladder cancer, and melanoma; they are potential targets for cancer therapy [17–21]. The role of PIH1D1 in cancer has not been clarified.

Ribosome biogenesis is likely to be a critical process dysregulated in cancer. mTORC1 regulates the synthesis of ribosomal components, including transcription and processing of ribosome RNA (rRNA) and expression of ribosomal proteins [22]. RNA polymerase (Pol) I transcribes a single 47S rRNA precursor, which is processed into 18S, 5.8S and 28S rRNA. Mayer et al., showed that the mTOR–S6K pathway activates the regulatory element tripartite motif-containing protein-24, which promotes its interaction with Pol I and the expression of rRNA [23]. By contrast, the mTOR–S6K axis has also been shown to control rRNA expression by promoting the interaction of upstream binding factor with SL1, an event that facilitates Pol I activation [24]. Since PIH1D1 is reported to be involved in rRNA transcription [25], the role of PIH1D1 in mTOR-regulated rRNA transcription is worth investigating.

In this study, we provide evidence that PIH1D1 is overexpressed in various breast cancer cells and interacts with mTORC1, but not mTORC2 in human breast cancer MCF-7 cells. We also present a data showing that PIH1D1 positively regulates mTORC1-dependent rRNA transcription.

2. Materials and methods

2.1. Reagents and antibodies

Anti-PIH1D1 antibody was from Santa Cruz Biotechnology. Anti-mTOR, anti-GβL, anti-Raptor (24C12), anti-Rictor (D16H9), anti-p70 S6K, anti-Phospho-S6K (Thr389), anti-Akt, or anti-Phospho-Akt (Ser-473) antibody was from Cell Signaling Technology. Anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase, 23040091) antibody was from Chemicon. Anti-Tel2 antibody was generous gift from Dr. de Lange and Dr. Takai (The Rockefeller University). Anti-RPAP3 antibody and anti-Flag antibody (M2) were from Sigma. Rapamycin was from Calbiochem. Flag-mTOR vector was generous gift from Dr. Mizushima (Tokyo University) and described previously [26].

2.2. Cell Culture

Breast cancer cell lines (MDA-MB-231, SKBr, BT549, MDA-MB-231, T47, and MCF-7) were obtained from the American Type Culture Collection. Breast cancer cell lines and HEK293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum containing 100 µg/ml streptomycin, 100 IU/ml penicillin and 1 µl/ml amphotericin B. Normal human mammary epithelial cell line was obtained from Lonza and cultured according to manufacturer's instructions.

2.3. Transfection and immunoblotting

HEK293 cells were seeded onto 60-mm Petri dishes and grown for 24 h. Indicated plasmids were transfected with Lipofectamine 2000 according to manufacturer's instructions (Invitrogen). After 4 h-transfection, the cells were returned to growth medium and incubated for 48 h. Cells were lysed in extraction buffer (0.3% CHAPS, 120 mM NaCl, 5 mM EDTA, 10% glycerol, and 20 mM Tris, pH 7.4) including protease inhibitor cocktail (Roche). Total protein was mixed with Laemmli denaturing buffer, separated by SDS-PAGE and transferred to PVDF membranes (Millipore Corporation). Immunoblotting was carried out as described previously [27].

2.4. Immunoprecipitation (IP)

IP was carried out as described previously [28]. Briefly, equal protein concentrations of lysates were incubated with A/G agarose beads (Pierce) precoated with indicated antibody or normal mouse or rabbit IgG (Santa Cruz). Associated proteins were recovered by boiling in Laemmli buffer.

2.5. Knockdown experiments

Tel2 or PIH1D1-specific siRNA was purchased from Qiagen and targeted the following sequences: 5'-CAGGGCACGGGCTCTCA GAAA-3' or 5'-CCTACGACGTAGCTGTCAATT-3' (target-1); 5'-CCCGC TGCAGATCAACTCTCA-3' (target-2) and transfected with Lipofectamine RNAiMAX according to the manufacturer's instructions (Invitrogen). AllStars Negative Control siRNA (Qiagen) was used as a control.

2.6. Lentiviral transduction and establishment of stable cell line

The siRNA-resistant PIH1D1 was cloned into pENTR/D TOPO and subcloned into pLenti6.3/V5-DEST using Gateway System (Invitrogen). This vector or pLenti3.3/TR was cotransfected into 293FT cells with ViraPower packaging mix (Invitrogen) to generate the lentivirus according to the manufacturer's protocol. MCF-7 cells were transduced with the lentivirus and stable cell lines were generated by selecting with geneticin (pLenti3.3/TR) or blasticidin (pLenti6.3/V5-DEST), respectively. Silent mutations (indicated by bold/italic letters) that were introduced are 5'-CCCCTGCAGAT AAATTCCA-3'. Site-directed mutagenesis was performed using the QuikChange II Site-directed Mutagenesis Kit (Agilent Technologies) and all mutations were confirmed by sequencing.

2.7. Quantitative RT-PCR for pre-rRNA synthesis

Quantitative RT-PCR (RT-qPCR) was performed as previously described [28]. The relative expression of pre-rRNA was normalized to β-actin using the comparative CT method. Specific primers for pre-rRNA (forward: 5'-GAACGG TGGTGTGTCGTTTC-3'; reverse: 5'-GCGTCTCGTCTCGTCTACT-3') and β-actin (forward: 5'-ATCGTC-CACCGCAAATGCTTCTA-3'; reverse: 5'-AGCCATGCCAATCTCATC TTGTT-3') were used for PCR amplification [29].

2.8. Statistical analysis

Data are expressed as mean ± S.E.M. Statistical differences between groups were determined using Tukey test after ANOVA.

3. Results

3.1. Expression of PIH1D1 in breast cancer cell lines

We analyzed PIH1D1 expression using anti-PIH1D1 antibody and found that PIH1D1 is highly expressed in various human breast cancer cell lines (Fig. 1). On the other hand, PIH1D1 expression was low in normal human mammary epithelial cells (HMEC) and human breast cancer BT549 cells (Fig. 1). RPAP3 expression was not different between normal and cancer cells (Fig. 1).

3.2. PIH1D1 interacts with mTORC1

Previous studies have demonstrated that mTOR interaction with Raptor and Rictor is disrupted when cells are lysed with 1% Triton X-100-containing buffer, but not with 0.3% CHAPS-containing buffer [30]. When we transfected HEK293 cells with Flag-mTOR

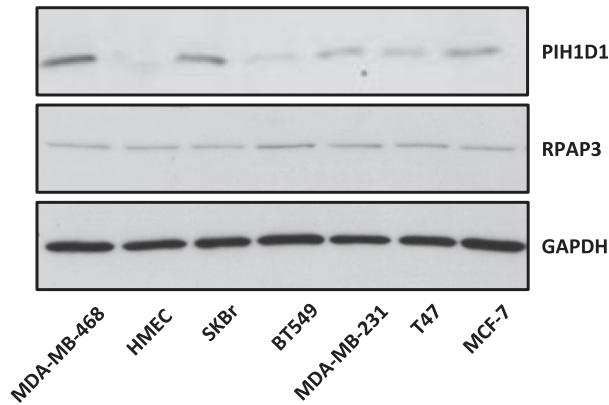


Fig. 1. PIH1D1 expression in breast cancer cell lines. Lysates from breast cancer cell lines were subjected to SDS-PAGE followed by immunoblotting with anti-PIH1D1, RPAP3, or GAPDH antibody. A given immunoblot is a representative of three independent experiments.

vector, PIH1D1–mTOR interaction was observed in CHAPS-containing buffer but was disrupted in Triton X-100-containing buffer (Fig. 2A). We thus used the CHAPS-containing buffer in the IP experiments. The endogenous interaction between mTOR and PIH1D1 in human breast cancer MCF-7 cells was also detected by IP using an antibody against PIH1D1, suggesting that their interaction is physiologically relevant (Fig. 2B). We also investigated the endogenous interaction between PIH1D1 and either Raptor (specific for mTORC1) or Rictor (specific for mTORC2) and found that PIH1D1 interacted with Raptor, but not Rictor in MCF-7 cells (Fig. 2B and C). As reported previously [3], Tel2 was present both in mTORC1 and mTORC2 (Fig. 2C).

3.3. PIH1D1 regulates mTORC1 activity

mTORC1 can phosphorylate S6K, whereas mTORC2 phosphorylates AKT [2]. We analyzed whether PIH1D1 regulates the activity of mTORC1 and mTORC2. Small interfering RNA (siRNA)-mediated knockdown of PIH1D1 was very effective and reduced PIH1D1 expression to almost undetectable levels in MCF-7 cells (Fig. 3A). In these cells, mTOR expression levels were not changed. Knockdown of PIH1D1 suppressed the phosphorylation of S6K without affecting the amount of S6K. To rule out off-target effects of siRNA, we used a rescue strategy. We established doxycycline (DOX)-regulatable MCF-7 cells overexpressing PIH1D1 and introduced silence mutations in the target sequence of siRNA and made the mRNA insensitive to siRNA. As shown in Fig. 3C, induced overexpression of PIH1D1 by DOX rescued inhibitory effect of PIH1D1 siRNA on the phosphorylation of S6K without affecting the amount of S6K. These results, together with the data that PIH1D1 interacts with mTORC1, suggest that PIH1D1 positively regulates mTORC1 activity. Next, we analyzed the involvement of PIH1D1 in mTORC2 regulation. The phosphorylation of Akt at Ser-473, which is a well known indicator of mTORC2 activity was not changed in cells treated with PIH1D1 siRNA (Fig. 3A), suggesting that PIH1D1 is not important for mTORC2 activity. In contrast, in Tel2-knockdown cells, the phosphorylation of S6K and Akt was inhibited (Fig. 3B), as reported previously [3]. Also, mTOR expression levels were decreased in Tel2-knockdown cells (Fig. 3B), consistent with previous report [3].

3.4. PIH1D1 is important for mTORC1 assembly

Decreased mTORC1 activity was clearly observed even when mTOR protein levels were not changed following knockdown of PIH1D1 (Fig. 3A). It has been reported that Tel2–R2TP complex

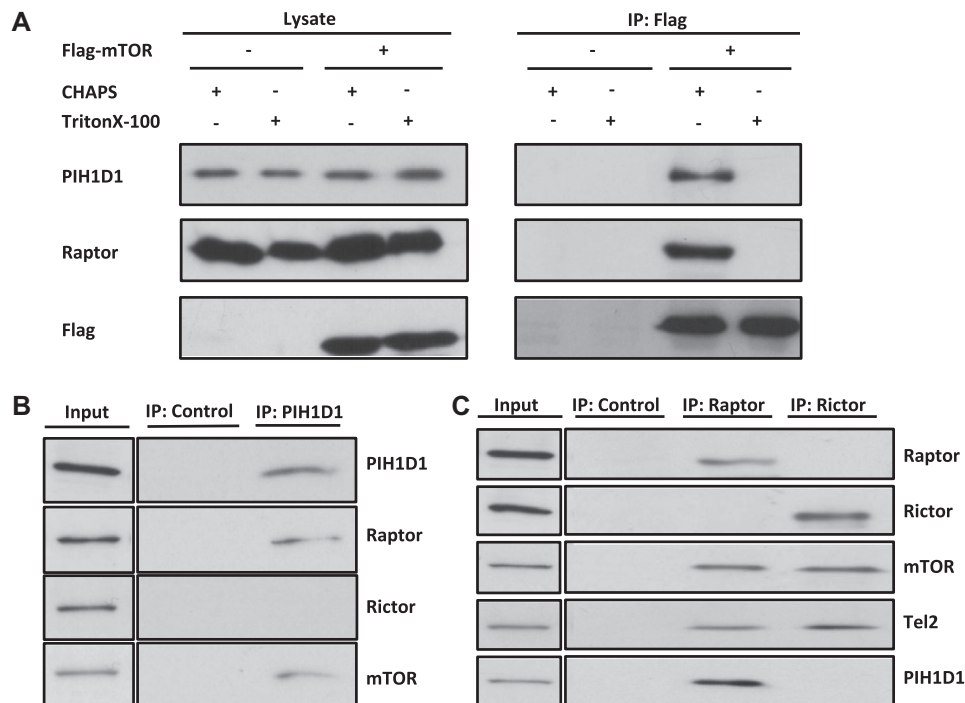


Fig. 2. PIH1D1 interacts with mTORC1. (A) HEK293 cells were transfected with an empty vector or Flag-mTOR. After 48 h, cells were harvested and lysed in a buffer containing either 0.3% CHAPS or 1% Triton X-100. IP was then performed with anti-Flag antibody. (B and C) MCF-7 cells were lysed and IP was performed with anti-PIH1D1 antibodies or normal mouse IgG (B) or anti-Raptor, anti-Rictor antibodies, or normal rabbit IgG (C) to detect endogenous interactions. A given immunoblot is a representative of three independent experiments.

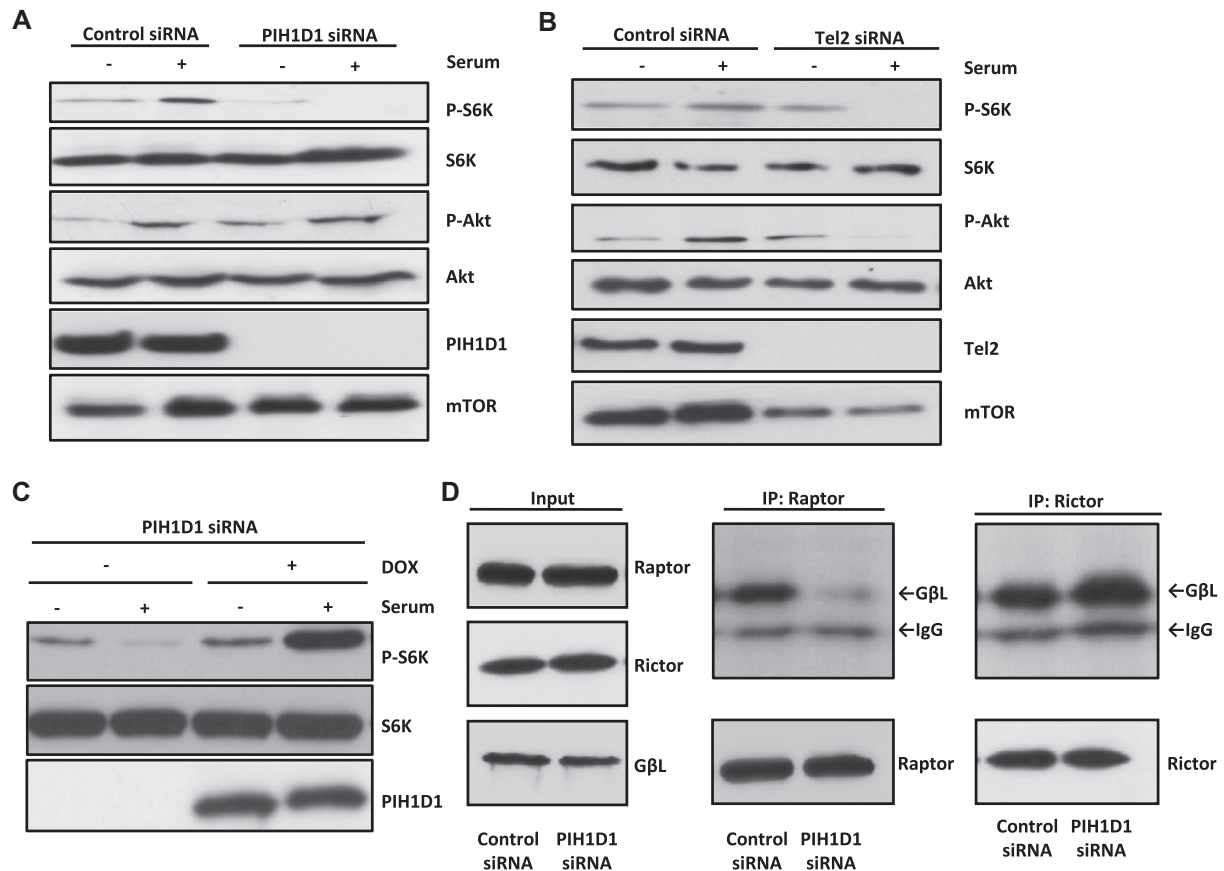


Fig. 3. PIH1D1 potentiates mTORC1 activity. MCF-7 cells were transfected with PIH1D1 (A) or Tel2 (B) siRNA in serum free conditions and after 48 h, the phosphorylation of S6K and Akt was examined by immunoblotting following 2 h serum treatment. (C) MCF-7 cells overexpressing DOX-regulatable and siRNA-resistant PIH1D1 were transfected with PIH1D1 siRNA. The phosphorylation of S6K was analyzed by immunoblotting at 48 h after the treatment with PIH1D1 siRNA. (D) MCF-7 cells were transfected for 48 h with PIH1D1 siRNA and the assembly of mTORC1 (the interaction of GβL and Raptor) or mTORC2 (the interaction of GβL and Rictor) was determined by immunoblotting. A given immunoblot is a representative of three independent experiments.

stabilizes mTOR [8,9], and we confirmed that Tel2 indeed stabilized mTOR (Fig. 3B), these observations prompted us to hypothesize that PIH1D1 might have functions other than the stabilization of mTOR protein to regulate mTORC1 activity. One possibility was that PIH1D1 facilitates mTOR complex formation. We tested this possibility by immunoprecipitating the mTOR complex. Knockdown of PIH1D1 did not affect the levels of GβL and Raptor in MCF-7 cells, but decreased the amount of GβL in the Raptor immunoprecipitates, while the amount of GβL in the Rictor immunoprecipitates was not changed (Fig. 3D). This suggests that mTORC1, but not mTORC2, is partially disassembled in PIH1D1-depleted cells.

3.5. PIH1D1 regulates rRNA transcription

It has been reported that PIH1 is a positive regulator in the transcription of rRNA genes [25]. mTORC1 is a major regulator of ribosomal biogenesis and protein synthesis. The lack of S6K phosphorylation in PIH1D1-silenced cells prompted us to examine whether PIH1D1 silencing can inhibit rRNA transcription. Three of the rRNAs, 5.8S, 18S and 28S, are transcribed as a single precursor, 47S pre-rRNA, by Pol I in the nucleolus, which is then processed through several steps to generate the mature rRNAs. We measured the level of the pre-rRNA in MCF-7 cells, and found that knockdown of PIH1D1 decreases rRNA level (Fig. 4A). When rapamycin (20 nM) was added, no further inhibitory effect of PIH1D1 silencing on rRNA level was observed (Fig. 4A), suggesting that the effect of PIH1D1 is mTOR-dependent.

4. Discussion

Increased protein synthesis and cell growth are critical for tumorigenesis. Tumor cells are generally larger than normal cells and contain more and bigger nucleoli, a phenotype indicative of increased ribosome synthesis [31,32]. mTORC1 signaling plays an essential role in cancer [2], and gain of function of this pathway has been demonstrated in various cancers. Multiple components of this pathway are either oncogenes or tumor suppressors. mTORC1 coordinates the synthesis of both ribosomal proteins and rRNA to modulate ribosome biogenesis [22–24]. These observations thus indicate that control of ribosome biogenesis through mTORC1 pathway is critical for tumorigenesis.

It has been reported that PIKK family including mTOR is stabilized by Tel2–R2TP complex [8,9]. However, the precise mechanism has not been explored. We first questioned which mTOR complex contains PIH1D1. Importantly, PIH1D1 was detected in Raptor immunoprecipitates, but not in Rictor immunoprecipitates, suggesting that PIH1D1 specifically targets mTORC1. As reported previously [3], Tel2 was detected in both complexes. We also detected Raptor and mTOR, but not Rictor in PIH1D1 immunoprecipitates. These results indicate that PIH1D1 is mTORC1-specific protein. We also demonstrated that PIH1D1 regulates mTORC1, but not mTORC2 activity. It has been reported that mTORC2 activity is negatively modulated by feedback inhibition of mTORC1 [2]. We confirmed that by rapamycin treatment, which inhibits mTORC1 activity, Akt phosphorylation in MCF-7 is up-regulated

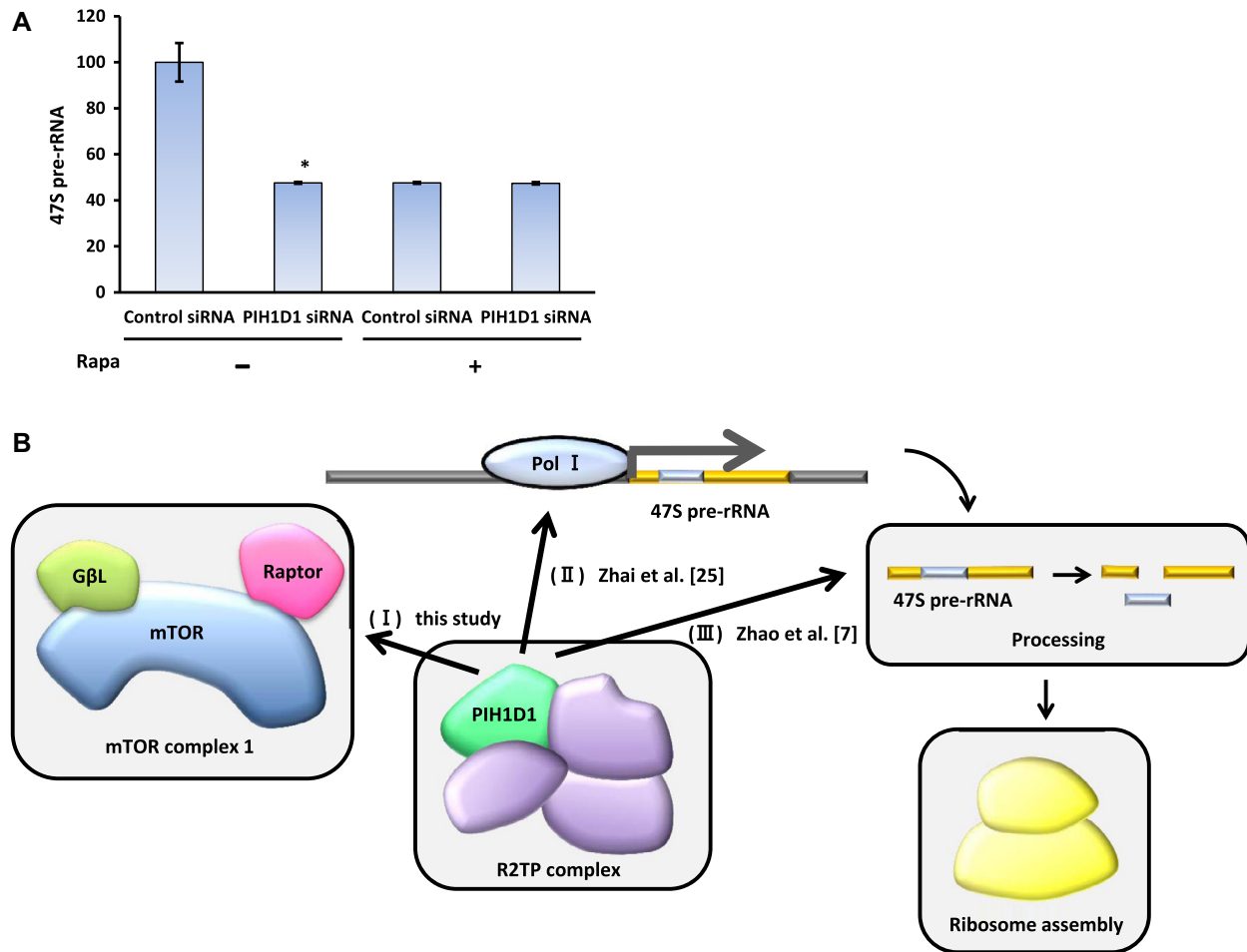


Fig. 4. (A) Knockdown of PIH1D1 decreases pre-rRNA levels. MCF-7 cells were transfected with PIH1D1 siRNA in the presence or absence of rapamycin (Rapa, 20 nM), and pre-rRNA levels were determined after 48 h by RT-qPCR. Values are means \pm S.E.M. from four independent experiments. Data were normalized based on β -actin mRNA copy numbers. * $P < 0.01$ vs control in the absence of rapamycin. (B) Possible targets of PIH1D1. PIH1D1 could modulate ribosome biogenesis at multiple steps, by affecting mTORC1 (I), rRNA transcription (II), or rRNA processing (III). See text for details.

(data not shown), as reported previously [2]. Although the mechanism by which negative feedback effect on mTORC2 is canceled in PIH1D1-depleted cells is unclear, it is interesting to note that similar phenomena have been recently reported [33]. Further investigation is needed for the elucidation of the mechanism by which PIH1D1 selectively influences on mTORC1. mTOR controls ribosome biogenesis by at least two mechanisms: by promoting the translation of mRNAs for ribosomal proteins and by affecting rRNA synthesis. We showed that pre-rRNA transcription is positively regulated by PIH1D1, and this is consistent with previous report by Zhai et al., [25]. They focused on silencing nucleolar remodeling complex (NoRC) and found that PIH1D1 is involved in the derepression of NoRC-silenced rRNA genes [25]. Since pre-rRNA transcription is enhanced by mTOR [34], based on our new findings, we propose that the effect of PIH1D1 on pre-rRNA transcription could be mTOR-dependent (Fig. 4B). Regarding the effect of PIH1D1 on rRNA transcription, it is possible that both mTOR-dependent and -independent mechanisms exist. It has been also reported that pre-rRNA processing is regulated by R2TP complex [7]. Interestingly, mTOR, PIH1D1, and another component of R2TP complex, Rvb1 (pontin in human) are localized in nucleoli [34–36].

PIH1D1 is known to be an unstable protein [7], and we have reported that PIH1D1 is stabilized by another component of R2TP complex, Tah1 (RPAP3 in human) [15]. In this study, we showed

that PIH1D1 is overexpressed in breast cancers. However, overexpression of RPAP3 was not observed in breast cancer cells, suggesting that another mechanism exists by which PIH1D1 is stabilized. Using comprehensive meta-analysis of The Cancer Genome Atlas data sets, Kim et al. found that the expression of many R2TP complex mRNA was significantly higher in breast carcinomas when compared to their normal tissue controls [37]. They hypothesized that elevated expression of R2TP complex promotes mTORC1 signaling and metabolic process that are necessary for tumor cell growth. mTORC1 is an important regulators of not only ribosome biogenesis and metabolic process, but also cell survival. Choi et al., has recently reported that inhibition of S6K enhances glucose deprivation-induced cell death in MCF-7 cells [38]. In this study, knockdown of PIH1D1 in MCF-7 cells did not affect both Akt, well-known regulator of cell survival (Fig. 3A) and cell survival rate estimated by MTT assay (data not shown). The role of R2TP complex and PIH1D1 in cell survival awaits further investigation.

Clearly, the data presented here along with the recent studies describing the role of Tel2–R2TP complex on mTORC1 [39–41] demonstrate that mTORC1 is a direct target for PIH1D1. Considering that mTORC1 has been proposed as a promising target for cancer treatment [2], understanding PIH1D1–mTORC1 axis in breast cancer may lead to the development of additional targeted agents to suppress this axis, and result in improved treatments for breast cancer.

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