



## HEATR1, a novel interactor of Pontin/Reptin, stabilizes Pontin/Reptin and promotes cell proliferation of oral squamous cell carcinoma



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### ABSTRACT

Pontin and Reptin are closely related proteins belonging to the AAA+ (ATPases Associated with various cellular Activities) family. They form a hetero-oligomeric complex, Pontin/Reptin, which is involved in protein stability and assembly of the protein complexes as a molecular chaperone. Overexpression of Pontin and Reptin in tumor cells has been reported and is implicated in the development of various cancers. However, the molecular mechanism of Pontin/Reptin function in oral squamous cell carcinoma (OSCC) development remains unclear. Here, we identify HEAT repeat-containing protein 1 (HEATR1) as a novel binding factor of Pontin/Reptin. Functionally, HEATR1 stabilizes Pontin/Reptin and positively regulates OSCC cell proliferation by activating mTOR and pre-rRNA synthesis. We also find that HEATR1 expression is markedly upregulated in tumor region of OSCC tissue. Hence, we propose that HEATR1 is involved in the regulation of mTOR and ribosome biogenesis as a potential protein stabilizer of Pontin/Reptin in OSCC.

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### 1. Introduction

OSCC is the most frequent head and neck squamous cell carcinoma and shows a high rate of local invasiveness and metastasis to neck lymph nodes [1]. Despite progress in the treatment and diagnostic techniques of this disease, the overall survival rates remain low [2,3]. Therefore, a search for more promising therapeutic targets and understanding the mechanism of OSCC development would be beneficial for increasing the efficacy of the treatment.

Pontin (also known as RUVBL1, TIP49, TIP49a, and Rvb1) and Reptin (also known as RUVBL2, TIP48, TIP49b, Rvb2) are well-

conserved ATPases of AAA + superfamily [4,5]. They form a Pontin/Reptin complex and participate in INO80 and TIP60 complexes, which are involved in chromatin remodeling [6]. As a part of R2TP (Pontin, Reptin, PIH1D1, and RPAP3)-TTT (TELO2, TTI1 and TTI2) complex, which is a chaperone for quaternary structure formation, Pontin/Reptin is involved in stabilization and proper assembly of various protein complexes, such as phosphatidylinositol 3-kinase (PI3K)-related protein kinase (PIKK) family proteins: mammalian target of rapamycin (mTOR), ATM, ATR, DNA-PK and SMG-1 [7–10]. Further, Pontin/Reptin is a component of PAQosome (Particle for Arrangement of Quaternary structure), which is a complex of R2TP and prefoldin-like (R2TP/PFDL), providing a platform for the assembly and maturation of various multiple protein complexes [11].

It has been reported that Pontin/Reptin is involved in cancer progression through the regulation of transcriptional oncogenic factors: MYC, E2F1,  $\beta$ -catenin, and p53, DNA damage response, and

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ribosome biogenesis [6]. Overexpression of Pontin and Reptin have been identified in various types of cancers, such as hepatocellular carcinoma, colorectal, breast cancers, and OSCC [12,13]. We recently revealed that Pontin and Reptin, as the components of R2TP, contribute to malignant characteristics of OSCC, such as cell proliferation and migration [14]. Although evidence is accumulating in support of the functional roles of Pontin/Reptin in oncogenesis, further investigation would be needed to understand its underlying mechanism.

Here, we identify HEAT repeat-containing 1 (HEATR1) as a novel interactor of Pontin/Reptin in human OSCC-derived cells, such as HSC-3, HSC-4, and SAS, and investigate its functional role in OSCC. We reveal that HEATR1 contributes to the protein stabilization of Pontin/Reptin and positively regulates OSCC cell proliferation by activating mTOR and ribosome biogenesis.

## 2. Materials and methods

### 2.1. Cell culture

Human OSCC cell lines, HSC-3, HSC-4, and SAS were obtained from RIKEN Cell Bank (Tsukuba, Japan) and they were cultured in Dulbecco's modified Eagle's medium, D-MEM containing 4.5 g/L glucose supplemented with 10% Fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37 °C humidified atmosphere of 5% CO<sub>2</sub>.

### 2.2. Western blot analysis

Western blot analysis was performed as we previously described [14]. Cells were lysed with RIPA buffer. Protein concentrations were measured by using Protein Assay Bicinchoninate kit (Nacalai Tesque). The proteins were separated by SDS-PAGE and transferred onto PVDF membrane (PALL). The membranes were incubated with primary antibodies: monoclonal or polyclonal antibodies against Pontin (06–1299, MilliporeSigma), Reptin (10195-1-AP, Proteintech), HEATR1 (sc-390445, Santa Cruz Biotechnology), mTOR (#2972S, Cell Signaling Technology), p-mTOR (S2448) (#2971, Cell Signaling Technology), PIH1D1 (NBP1-92269, NOVUS Biologicals), RPAP3 (23741-1-AP, Proteintech), and  $\beta$ -Tubulin (T5326, MilliporeSigma). Then, the membranes were incubated with HRP-conjugated secondary antibodies, and the signals were detected by using CCD imager (GE Healthcare). The experiments were performed three times independently, and the densitometry of the protein band was analyzed using NIH imageJ (National Institutes of Health, USA).

### 2.3. Co-immunoprecipitation (co-IP)

Cells were washed with PBS and lysed in CHAPS buffer (40 mM HEPES pH 7.5, 120 mM NaCl, 1 mM EDTA, 0.3% CHAPS, 10 mM glycerol phosphate) containing one tablet EDTA-free protease inhibitors (Roche) per 10 ml. Rabbit polyclonal *anti*-Reptin (NBP1-40354, NOVUS Biologicals) and *anti*-HEATR1 (ab241610, Abcam) were used for co-IP. Normal rabbit IgG (#2729, Cell Signaling Technology) was used as a negative control. Antibody was conjugated with SureBeads Protein G Magnetic Beads (Bio-Rad), and incubated with cell lysate for 2 h at 4 °C. The beads were washed with CHAPS buffer, and proteins were eluted in Laemmli sample buffer.

### 2.4. Immunofluorescence staining

Cells grown on glass coverslips in 24-well plate were fixed using 4% paraformaldehyde for 15 min and permeabilized with 1% TritonX-100 in PBS for 10 min. The cells were blocked with 1% BSA

in PBS for 1 h at room temperature and incubated with primary antibodies in PBS containing 1% BSA. After overnight incubation at 4 °C, the cells were treated with goat anti-rabbit or anti-mouse conjugated with Alexa Fluor 488 or 594 (Thermo Fisher Scientific) and DAPI in PBS containing 1% BSA for 1 h at room temperature. The images were acquired by Zeiss Axio Imager (ZEISS). *Anti*-Pontin (SAB4200194, Sigma-Aldrich), *anti*-Reptin (A302-536A, BETHYL Laboratories), and *anti*-HEATR1 (sc-390445, Santa Cruz Biotechnology) were used for primary antibody.

### 2.5. Mass spectrometry

The isolated proteins co-IP with *anti*-Reptin antibody were mixed with Laemmli buffer and boiled for 10 min, and then were co-polymerized with acrylamide. The gels were reduced with 10 mM dithiothreitol and carbamoylmethylated with 55 mM iodoacetamide and subjected to in-gel trypsin digestion. Trypsin digests were finally dissolved in 0.3% formic acid and analyzed in duplicate by a nanoflow LC (Eksigent nanoLC with ekspert cHiPLC, Sciex) coupled with a tandem mass spectrometer (TripleTOF 5600+, Sciex).

### 2.6. RNA interference

OSCC cells were seeded in 6-well plates at a concentration of  $2 \times 10^5$  cells/well. The siRNA transfections were carried out using Lipofectamine RNAiMAX (Thermo Fisher Scientific) according to the manufacture's manual. The following siRNA oligonucleotides were used: siPontin (5'-GACAGAAAUCACAGACAAATT-3'), siReptin (5'-GACGCUAUGGGCUCCAGATT-3'), siHEATR1 (5'-GAUGUUGUUUGUCGGCUATT-3'), and siRNA Universal Negative Control #1 (Sigma-Aldrich) for a negative control, siCtrl.

### 2.7. Cell proliferation assay

Cell proliferation assay was performed using Cell Counting Kit-8 (DOJINDO). Cells were seeded in 96-well plates at a density of  $1 \times 10^3$  cells/well. 10  $\mu$ l of CCK-8 solution was added to each well and incubated at 37 °C of 5% CO<sub>2</sub> for 2 h. The absorbance at 450 nm was measured every 24 h for 72 h using a plate reader.

### 2.8. Immunohistochemistry

This study was approved by the Medical Ethics Committee of Niigata university medical and dental hospital (Approval No. 2019–0080). Informed consent was obtained from all patients. The formalin-fixed paraffin-embedded tongue cancerous and adjacent tumor tissues were obtained from Niigata university medical and dental hospital. Tissue sections were deparaffinized and dehydrated. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide in methanol. Antigen retrieval was achieved by 10 mM citric sodium buffer (pH 6.0). The sections were blocked and incubated with primary antibodies and then reacted with EnVision Plus (Dako) and developed with 3,3-diaminobenzidine. After counterstaining with hematoxylin, the slides were dehydrated and mounted. *Anti*-HEATR1 (sc-390445, Santa Cruz Biotechnology) and *anti*-Ki67 (M7240, Dako) were used for the primary antibody.

### 2.9. Real-time PCR

Cell lysis, total RNA isolation, reverse-transcription of total RNA to cDNA, and PCR amplification were performed using PureLink™ RNA Mini Kit (Thermo Fisher Scientific), RQ1 RNase-Free DNase (Promega) and Fast Advanced Cells-to-Ct Kit (Thermo Fisher Scientific), according to the manufacture's instruction. PCR reactions

were analyzed using QuantStudio 1 Real-time PCR System (Thermo Fisher Scientific). Specific primers were obtained from Thermo Fisher Scientific. For 47S pre-rRNA (Forward: 5'-CCTCCCCAGGCGTCCCTCG-3'; Reverse: 5'-GGCAGCGCTACCA-TAACGGA-3') [15],  $\beta$ -actin (Forward: 5'-ATCGTCCACCG-CAAATGCTTCTA-3'; Reverse: 5'-AGCACTGCCAATCTCATCTTGT-3'), Pontin (Hs00186558\_m1), Reptin (Hs01090542\_m1), HEATR1 (Hs00985319\_m1) and hu GAPDH (4326317E) were used for PCR amplification. The results were analyzed using  $2^{-\Delta\Delta CT}$  method and expressed as fold change to siCtrl.

### 2.10. Protein stability assay

To examine Pontin, Reptin and HEATR1 protein stability in the cells, 5  $\mu$ M MG132 (Sigma-Aldrich) or 0.1% Dimethyl sulfoxide as a control was added to siRNA-treated HSC-4 cell for 4 h. Equal amount of proteins were loaded onto SDS-PAGE for Western blot analysis.

### 2.11. Statistical analysis

Statistical analysis was performed using Statcel (OMS, Saitama, Japan). Data were presented at mean  $\pm$  S.D. of three independent experiments and analyzed using ANOVA and Tukey's test.  $p < 0.05$  was considered statistically significant.

## 3. Results

### 3.1. HEATR1 is a novel binding factor of Pontin/Reptin in OSCC

To search for a novel binding protein of the Pontin/Reptin, we performed co-IP assay with an anti-Reptin antibody using HSC-4 cell lysate, and subsequently analyzed the isolated proteins by tandem mass spectrometry analysis. As expected, we identified the components of the R2TP-TTT chaperone complex, Reptin, Pontin, PIH1D1, RPAP3, TELO2, TTI1, and TTI2, as well as its target protein, mTOR (Fig. 1A). Additionally, we found HEATR1 as a potential novel interactor of Reptin (Fig. 1A). We verified the interaction between HEATR1 and Pontin/Reptin in OSCC-derived cell lines, HSC-3, HSC-4, and SAS, by Western blotting (Fig. 1B). Immunofluorescence staining showed that HEATR1 was colocalized with Pontin and Reptin in the nucleoli in HSC-3, HSC-4, and SAS cells (Fig. 1C). These results suggest that HEATR1 and Pontin/Reptin are associated in the nucleolus in OSCC cells.

### 3.2. HEATR1 is required for protein stability of Pontin/Reptin

To investigate the functional relationship among Pontin, Reptin, and HEATR1, we performed siRNA knockdown of Pontin, Reptin, or HEATR1 in OSCC cells, HSC-3, HSC-4, and SAS, and the protein levels were determined by Western blotting (Fig. 2A and Fig. S1). Consistent with a previous study in hepatocellular carcinoma [16], either Pontin or Reptin knockdown significantly reduced both Pontin and Reptin levels in OSCC cell lines, suggesting that Pontin and Reptin coregulate the protein levels each other in OSCC. Intriguingly, HEATR1 knockdown decreased not only HEATR1 protein levels but also Pontin and Reptin in HSC-3, HSC-4, and SAS (Fig. 2A). However, HEATR1 protein levels were not affected by Pontin or Reptin depletion. This result raised a possibility that HEATR1 could regulate mRNA and/or protein levels of Pontin and Reptin. The knockdown of Pontin, Reptin, or HEATR1 led to a decreased mRNA levels to only each targeted gene in HSC-4 cells but not affected others (Fig. 2B). We next examined the effect of HEATR1 knockdown on the protein stability of Pontin and Reptin by using a proteasome inhibitor MG132. We found that the decreased

protein levels of Pontin and Reptin led by HEATR1 knockdown were recovered in the presence of MG132, indicating that HEATR1 is required for the protein stability of Pontin and Reptin (Fig. 2C).

### 3.3. Pontin, Reptin and HEATR1 promote OSCC cell proliferation by upregulating mTOR phosphorylation and pre-rRNA synthesis

We compared protein levels of Pontin, Reptin, and HEATR1 in HSC-3, HSC-4, and SAS cells. The levels of Pontin, Reptin, and HEATR1 were significantly higher in HSC-4 and SAS compared with HSC-3 (Fig. 3A). Also, the cell proliferation rate of HSC-4 and SAS were higher than HSC-3 (Fig. 3B), suggesting a correlation between the expression levels of Pontin, Reptin, and HEATR1 and OSCC cell proliferation activity. The depletion effect of Pontin, Reptin, or HEATR1 on the OSCC cell proliferation rate was determined. Consistent with our recent study [14], Pontin or Reptin knockdown significantly decreased the proliferation rate of HSC-3, HSC-4, and SAS (Fig. 3C). Similar trends were also observed in HEATR1 knockdown cells (Fig. 3D). These results indicated that Pontin, Reptin, and HEATR1 positively regulate the cell proliferation of OSCC cells.

It has been shown that Pontin/Reptin are involved in tumor development through activating mTOR signaling, and also increased phosphorylated mTOR ( $p$ -mTOR) is known to be associated with poor prognosis in OSCC patients [17–19]. Thus, we analyzed the involvement of Pontin, Reptin, or HEATR1 in mTOR regulation. The total mTOR protein levels were unchanged, whereas  $p$ -mTOR (S2448) levels were significantly decreased by depleting Pontin, Reptin, or HEATR1 (Fig. 3E). Additionally, 47S pre-rRNA levels, which are positively regulated by mTOR pathway [20], were also decreased in Pontin, Reptin, or HEATR1 knocked down HSC-4 cells (Fig. 3F), suggesting that Pontin/Reptin and HEATR1 are involved in the activation of mTOR signaling and ribosome biogenesis.

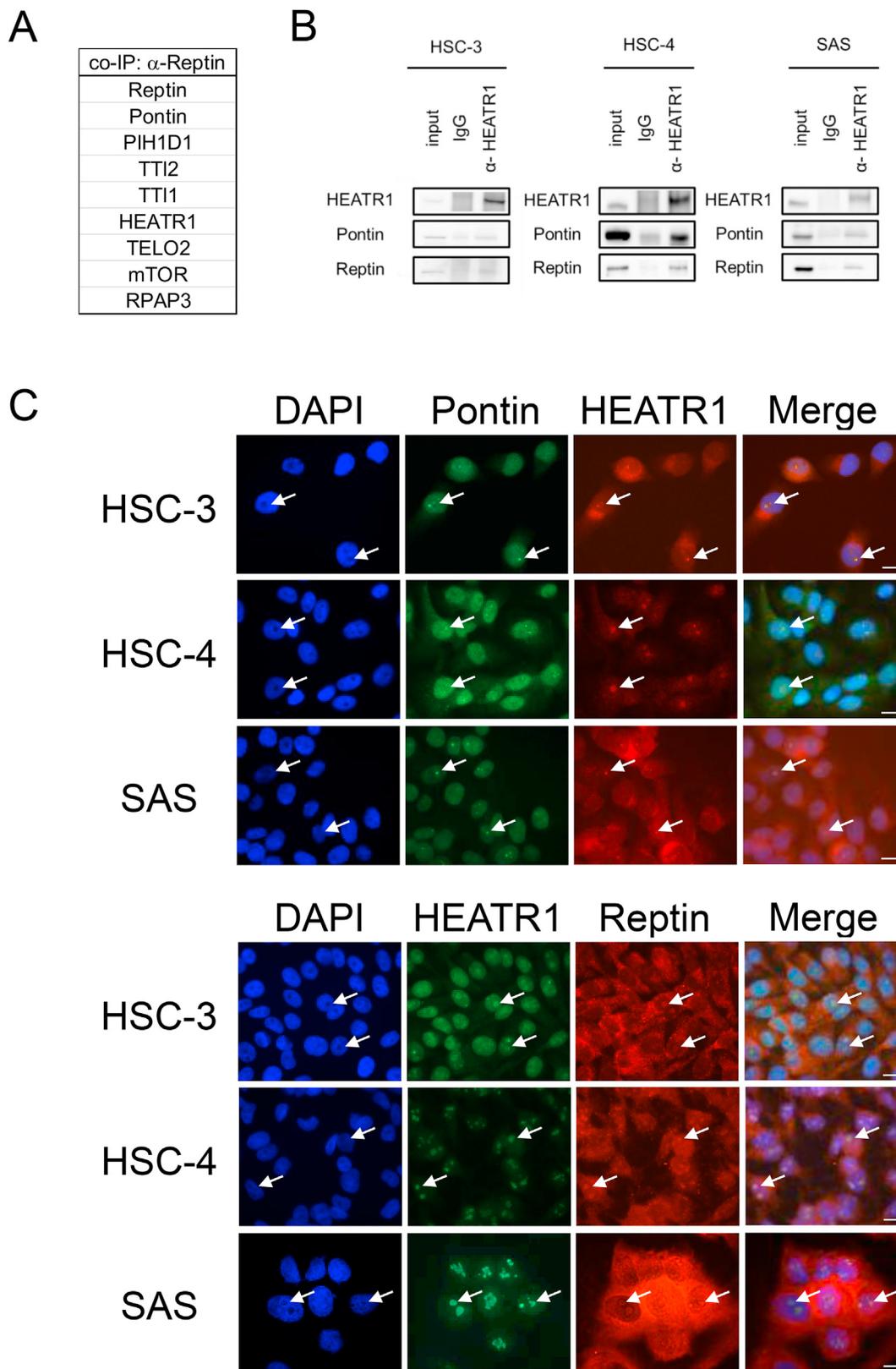
### 3.4. HEATR1 is upregulated in the tumor region of OSCC tissue

Recently, we have shown that Pontin and Reptin are strongly expressed in the cells with high proliferative activity in OSCC as well as normal oral epithelia [14]. We, thus, determined the expression of HEATR1 in OSCC clinical specimens. Both HEATR1 and Ki-67 expression in the tumor region of OSCC were remarkably elevated compared with the normal tissue (Fig. 4A). The positive staining of HEATR1 was detected in the nucleoli of tumor cells (Fig. 4B), which is consistent with the localization in OSCC cell lines (Fig. 1C).

## 4. Discussion

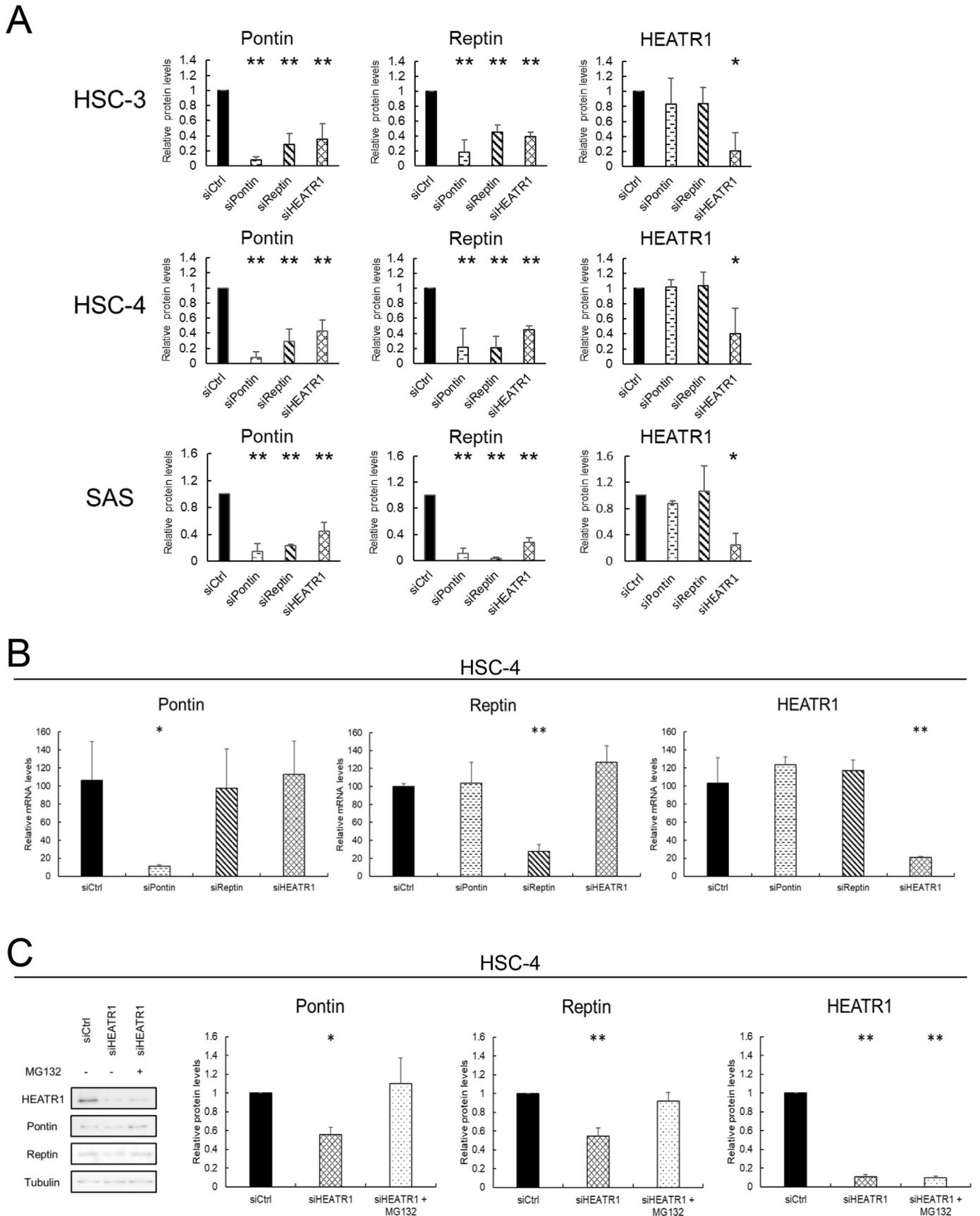
In this study, we identified HEATR1 as a novel interactor of Pontin/Reptin in OSCC (Fig. 1A and B). Since Pontin/Reptin has been shown to contribute to the stabilization of the target proteins, we analyzed whether Pontin/Reptin is required for the protein stability of HEATR1, and found that, contrary to our expectation, Pontin and Reptin are not required for the HEATR1 stability, but HEATR1 stabilizes Pontin and Reptin proteins (Fig. 2). Functionally, HEATR1, together with Pontin/Reptin, promotes OSCC cell proliferation by activating mTOR and pre-rRNA synthesis (Fig. 3). Besides, we observed that HEATR1 is markedly upregulated and accumulated in the nucleoli of the OSCC tumor cells (Fig. 4).

It has been shown that Pontin/Reptin is a part of R2TP, which interacts with PIKK family proteins through TELO2 [7,21]. Although it would not be surprising that we identified several known interactors of Pontin/Reptin, such as Pontin, Reptin, PIH1D1 and RPAP3 (components of R2TP), TELO2, TTI1, and TTI2 (components of TTT



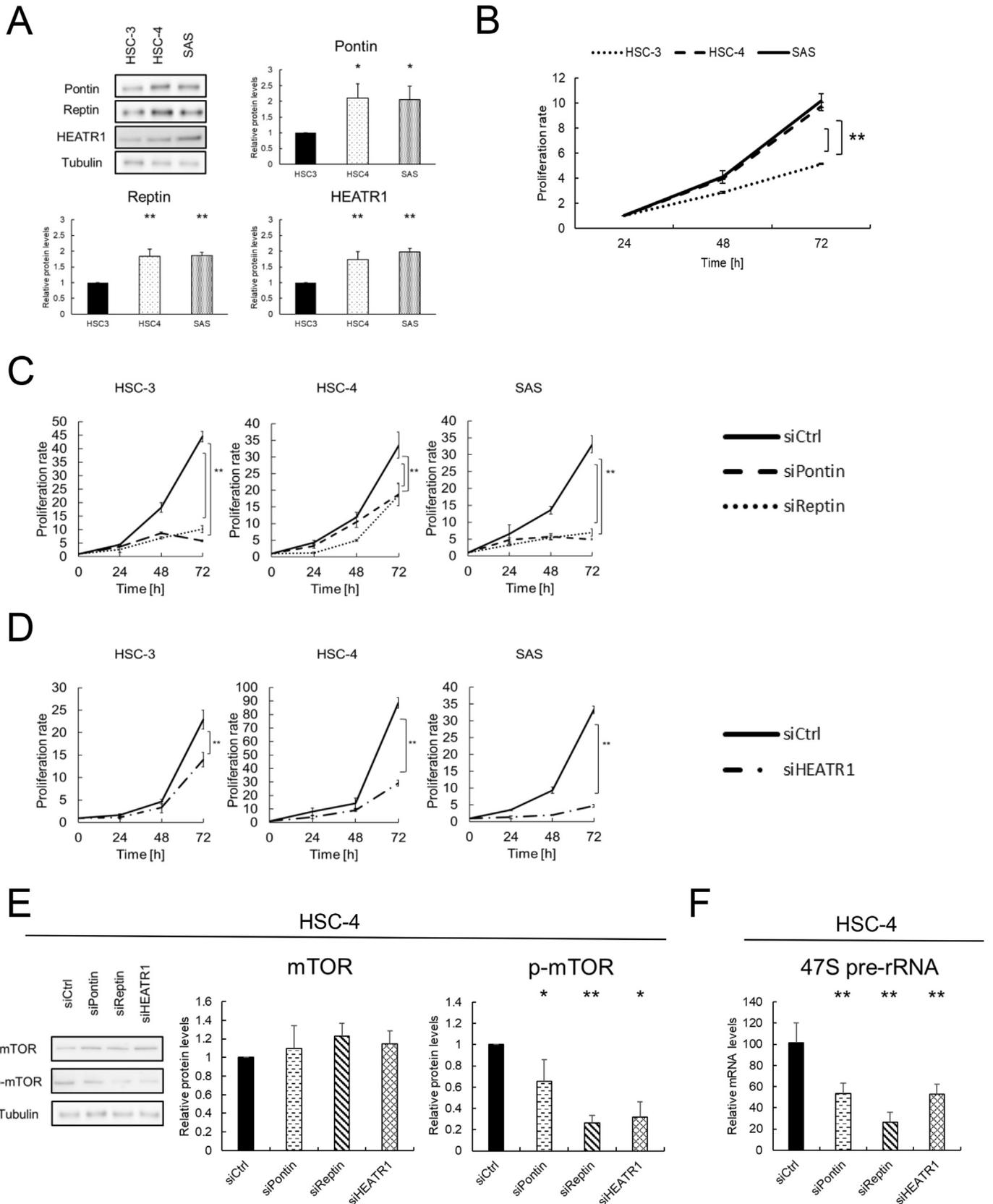
**Fig. 1. HEATR1 interacts with Pontin/Reptin in OSCC cells.**

(A) Summary of Reptin-associated proteins. Co-IP with *anti*-Reptin antibody was performed using HSC-4 cells, and the isolated proteins were identified by mass spectrometry (Supplementary Table S1). (B) Western blotting of Pontin and Reptin for co-IPed proteins with control IgG or *anti*-HEATR1 antibody. (C) Immunofluorescence staining of Pontin, Reptin, and HEATR1 in HSC-3, HSC-4, and SAS. Cells were counterstained with DAPI. The arrows show colocalization of Pontin or Reptin and HEATR1 in nucleoli. Scale bar, 50  $\mu$ m.



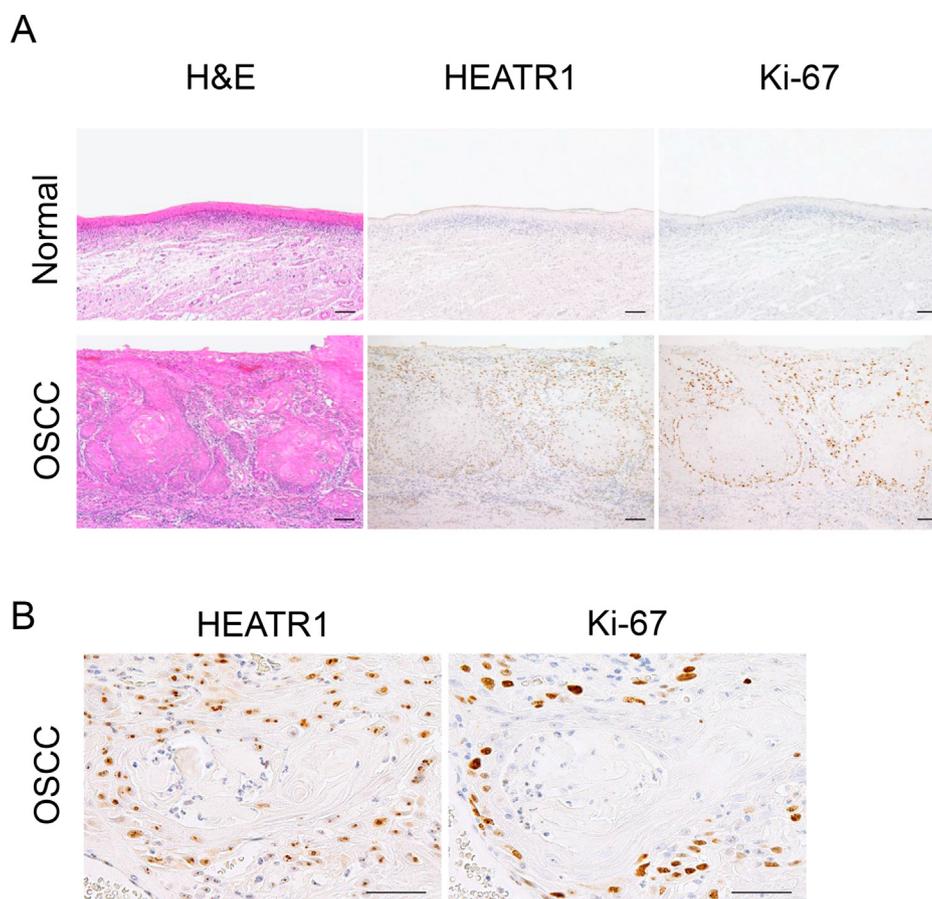
**Fig. 2. HEATR1 is required for protein stability of Pontin and Reptin, but not vice versa.**

HSC-3, HSC-4, and SAS cells were transfected with siRNA targeting Pontin (siPontin), Reptin (siReptin), HEATR1 (siHEATR1), or a control siRNA (siCtrl). (A) Western blotting analyses were performed (Supplementary Fig. S1). Relative protein levels of Pontin, Reptin, and HEATR1 in HSC-3, HSC-4, and SAS cells were shown. Data were expressed as mean  $\pm$  S.D. ( $n = 3$ ). \* $p < 0.05$  and \*\* $p < 0.01$ , versus siCtrl. (B) Relative mRNA levels of Pontin, Reptin, and HEATR1 in HSC-4 cells. Data were expressed as mean  $\pm$  S.D. ( $n = 3$ ). \* $p < 0.05$  and \*\* $p < 0.01$ , versus siCtrl. (C) Western blot analyses of HEATR1, Pontin, Reptin, and  $\gamma$ -Tubulin (loading control) in HEATR1 knocked-down HSC-4 cells with or without MG132 treatment. Relative protein levels of Pontin, Reptin, and HEATR1 are shown in the graphs. Data were expressed as mean  $\pm$  S.D. ( $n = 3$ ). \* $p < 0.05$  and \*\* $p < 0.01$ , versus siCtrl.



**Fig. 3. Pontin, Reptin and HEATR1 promote OSCC cell proliferation by upregulating mTOR phosphorylation and pre-rRNA synthesis.**

(A) Western blot analysis of Pontin, Reptin, HEATR1, and  $\gamma$ -Tubulin (loading control). The graphs show the relative protein levels of Pontin, Reptin, and HEATR1 in HSC-3, HSC-4, and SAS cells, calculated by densitometry of the blots. Data were expressed as mean  $\pm$  S.D. ( $n = 3$ ). \* $p < 0.05$  and \*\* $p < 0.01$ , versus HSC-3. (B) Relative cell proliferation rates of HSC-3, HSC-4, and SAS. Data were expressed as mean  $\pm$  S.D. ( $n = 3$ ). \*\* $p < 0.01$ , versus HSC-3. (C) Relative cell proliferation rates of HSC-3, HSC-4, and SAS, transfected with siCtrl, siPontin, or siReptin. Data were expressed as mean  $\pm$  S.D. ( $n = 3$ ). \*\* $p < 0.01$ , versus siCtrl. (D) Relative cell proliferation rates of HSC-3, HSC-4, and SAS, transfected with siCtrl or siHEATR1. Data were expressed as mean  $\pm$  S.D. ( $n = 3$ ). \*\* $p < 0.01$ , versus siCtrl. (E) Western blot analysis of mTOR, p-mTOR, and  $\gamma$ -Tubulin (loading control) in HSC-4. The graphs show the relative protein levels of mTOR and p-mTOR. Data were expressed as mean  $\pm$  S.D. ( $n = 3$ ). \* $p < 0.05$  and \*\* $p < 0.01$ , versus siCtrl. (F) Relative expression levels of 47S pre-rRNA analyzed by real-time PCR in HSC-4. Data were expressed as mean  $\pm$  S.D. ( $n = 3$ ). \*\* $p < 0.01$ , versus siCtrl.



**Fig. 4.** HEATR1 is upregulated in tumor region of OSCC tissue. (A) H&E and immunohistochemical staining of HEATR1 and Ki-67 in normal oral epithelial and tumor region of OSCC tissue. Scale bar represents 100  $\mu\text{m}$ . (B) Enlarged image of the tumor region of the OSCC tissue in Fig. 4A. Scale bar represents 50  $\mu\text{m}$ .

complex), as well as mTOR, we found HEATR1 as a novel interactor of Pontin/Reptin in OSCC. HEATR1 is an ortholog of yeast U3 small nucleolar RNA-associated protein 10 (UTP 10), involving the transcription and processing of pre-ribosomal RNA [22]. HEATR1 and UTP10 have a conserved HEAT repeat motif at their C-terminal end [23]. HEAT repeats are composed of tandem repeat of two alpha-helices linked by a short loop and function as a flexible scaffold for the assembly of other protein components [24]. Intriguingly, in this study, we identified three HEAT repeat family proteins, TELO2, mTOR and HEATR1, as the Pontin/Reptin interactors, suggesting that HEAT repeat could be a recognition motif for Pontin/Reptin. Recently, Pontin/Reptin has been shown to directly interact with N-terminal HEAT repeat region of mTOR [25], implying that the interaction between Pontin/Reptin and HEATR1 is also possibly mediated by HEAT repeat. Further, it has been shown that Pontin/Reptin interacts with mTOR and activates the mTOR complex under energy-rich conditions and promotes the transcription of rRNA, as well as ribosome biogenesis [25–27]. In this context, we propose that HEATR1 is involved in the regulation of mTOR and ribosome biogenesis as a potential protein stabilizer of Pontin/Reptin in OSCC.

There have been several reports on the association of HEATR1 with cancer development. HEATR1 is highly expressed in gastric and non-small cell lung cancers, and depletion of HEATR1 significantly suppressed cell proliferation and colony formation and increased cell apoptosis [28,29]. Also, HEATR1 knockdown significantly decreased xenograft tumor growth in mice [28,29]. In U2OS cells, HEATR1 depletion also showed the inhibition of proliferation through p53-dependent cell cycle arrest [30]. Taken together with

our results, HEATR1 shows tumor-promoting effect by positively regulating cell proliferation. However, it has been reported that HEATR1 negatively regulates the development of pancreatic cancer, and the loss of HEATR1 gives rise to the resistance to chemotherapy [31,32]. These contradicting data may be due to differences in the expression levels and functional roles of HEATR1 in different tissues and cancers.

In head and neck squamous cell carcinoma, including OSCC, mTOR pathway is frequently upregulated and an aberration in this pathway leads to malignant characteristics, such as cell survival, invasion, and metastasis [33]. Hence, we discovered HEATR1 as a novel interactor of Pontin/Reptin and regulates mTOR activity and ribosome biogenesis. It is necessary to further clarify the HEATR1 protein interaction network and comprehensively reveal the functional role of HEATR1 in the mechanism of OSCC progression, including the mTOR pathway.

#### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbrc.2021.04.021>.

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