LncRNA coordinates Hippo and mTORC1 pathway activation in cancer

Shugeng Zhang1,2, Shuhang Liang1,2, Dehai Wu1, Hongrui Guo1, Kun Ma1 and Lianxin Liu1✉

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INTRODUCTION
Cancer is the consequence of dysregulated signalling pathways. Dissecting the molecular mechanisms of cancer signalling pathways lays the foundation for developing targeted cancer therapies. YAP1 and TAZ are transcription factors/cofactors that regulate signature gene expression to control development and homoeostasis [1–4]. YAP1 has been demonstrated to facilitate the proliferation, invasion and epithelial–mesenchymal transition of various types of cancer cells. As a consequence, hyperactivation of YAP1 and expression of YAP1 target genes promote cancer initiation, progression and drug resistance [5–7]. The YAP1 pathway is regulated by upstream Hippo tumour suppressors, including MST1/2 and LATS1/2. Recent research has shown that a variety of molecular mechanisms regulate the activity of YAP/TAZ [8–11]. Upon nutrient stress, low energy triggers AMPK activation, which leads to YAP1 phosphorylation and degradation [12–14]. In cancer cells, the negative regulatory role of AMPK could be uncoupled by unknown mechanisms, leading to hyperactivation of the YAP1 pathway.

The mammalian target of rapamycin (mTOR) signalling pathway has important role in regulating cell growth, metabolism and survival. mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) are two of the major complexes [15]. mTORC1 contains the catalytic mTOR subunit and regulatory subunits, including Raptor [16]. Reports have proposed that Raptor modulates the enzymatic activity of mTOR and the recruitment of substrates, such as S6K and 4E-BP1 [17, 18]. mTORC1 plays essential roles in promoting cell growth and proliferation by regulating protein biosynthesis, autophagy, lipid biogenesis, mitochondrial metabolism and other pathways. The activity of mTORC1 is regulated by the integration of intracellular and extracellular stimuli, such as growth factors, nutrition and energy stress [19].

Recent advances in genome-wide transcriptome profiling have indicated the vast transcription of IncRNAs [20–22]. The upregulation of IncRNAs in cancer suggests the potential functional role of IncRNAs in modulating cellular activities, such as antiapoptosis, cancer metabolism reprogramming, antigen presentation, transcription regulation, mitochondrial metabolism and drug resistance [23–25]. Recently, IncRNAs have been demonstrated to play important roles in modulating cancer signalling pathways through RNA–protein interactions [11, 26–30]. Hence, IncRNAs may act as key signalling mediators that regulate cancer signalling cascades, which leads to malignancies. Here, we report that in breast cancer and cholangiocarcinoma, the YAP1 pathway and mTORC1 pathway are coactivated. Mechanistically, the expression of the IncRNA HPR is upregulated in breast cancer and cholangiocarcinoma compared with normal tissues. HPR is directly involved in YAP1 activation and associated with both the HEAT and WD40 domains of Raptor, and it regulates the enzymatic activity of mTORC1. Knocking down or genetically depleting HPR increases the phosphorylation of Raptor at Ser792, which is repressed in the presence of exogenous HPR. Knockdown or knockout of HPR in breast cancer and cholangiocarcinoma cells leads to a reduction in tumour growth. Compared with HPR WT cells, HPR-overexpressing cells exhibited an accumulation of YAP1 in the nucleus and significantly blocked the downregulation of mTORC1 signalling induced by energy stress. Hence, our research work demonstrates a lncRNA directed molecular mechanism for coactivation of the Hippo and mTORC1 pathways in cancer.

RESULT
Coactivation of the Hippo and mTORC1 pathways in cancer
Both the Hippo and mTORC1 pathways are significantly upregulated in cancer [31, 32], hence, we performed a correlation analysis.
of the mRNA expression of Hippo and mTORC1 pathway-related genes in the TCGA database. The findings showed that the expression of Hippo and mTORC1 pathway genes is significantly correlated in breast and colon cancer (Fig. 1a–d). Using a tumour tissue microarray, we validated the status of the key factors in the Hippo and mTORC1 pathways using phosphorylated YAP1 and p70S6K. Our data indicated that YAP1 and phosphorylated 70S6K (Thr389) exhibited high positive rates compared with paired adjacent normal tissues in breast cancer, cholangiocarcinoma and colon cancer (Fig. 2a, b, d, e and Supplement Figs. 1A, 1B). Furthermore, the status of nuclear YAP1 overlapped with the positive staining of phospho-70S6K (Fig. 2c, f and Supplement Fig. 1C). Therefore, our data suggested that the Hippo and mTORC1 pathways are coactivated in cancer.

**LncRNA HPR is involved in YAP1 activation**

To identify cancer-relevant lncRNAs that might be involved in the YAP1 signalling pathway, a previous study transfected the human Lincode siRNA library into MCF-7 cells that were engineered with a TEA domain transcription factor TEAD-driven luciferase reporter and subsequently determined the relative YAP1 activity [11]. More than 40 IncRNAs were potentially required for YAP1-dependent transcription. However, how these candidates regulate the YAP1 activation is still unclear. To further investigate whether these candidates were involved in YAP1 activation, we targeted IncRNAs to MCF10A, MDA-MB-231 and HS578T cells using locked nucleic acids (LNAs) together with the TEAD reporter 8xGTIIC-luciferase. Consistent with other studies, YAP1-dependent transcription activation was high in MDA-MB-231 and HS578T breast cancer cells compared with the MCF10A normal cells (Supplemental Fig. 2). Interestingly, knockdown of lncRNA-FLG14107 dramatically downregulated YAP1 activation in breast cancer cells but not normal cells, thus, we renamed this lncRNA hippo pathway-related lncRNA (HPR) (Fig. 3a). However, YAP activation upregulated in the HPR-overexpressing MCF10A cells (Fig. 3b). Furthermore, the expression level of YAP1 substrates was also downregulated in the HPR-knockdown breast cancer cell lines (Fig. 3b). To further confirm that HPR was associated with the YAP1 signalling pathway, we used CRISPR-Cas9 technology to generate two HPR-knockout MDA-MB-231 cell clones (Fig. 3d), qRT-PCR data
Fig. 2  Coactivation of the Hippo and mTORC1 pathways in cancer. a, b Immunohistochemical staining using antibodies against YAP1 (a) and phospho-p70S6K1 (b) in human breast cancer tissues. Upper panel: representative images (scale bars, 100 µm); lower panel: statistical analysis of immunohistochemical staining (**P < 0.001). The results are the mean ± s.e.m. of n = 3 independent experiments. P values were determined by one-way ANOVA. c Pearson’s correlation analysis comparing staining density between YAP1 and phospho-p70S6K1, n = 20. d, e Immunohistochemical staining using antibodies against YAP1 (d) and phospho-p70S6K1 (e) in human cholangiocarcinoma tissues. Upper panel: representative images (scale bars, 400 µm); lower panel: statistical analysis of immunohistochemical staining (**P < 0.001). The results are the mean ± s.e.m. of n = 3 independent experiments. P values were determined by one-way ANOVA. f Pearson’s correlation analysis comparing staining density between YAP1 and phospho-p70S6K1, n = 20.
**Fig. 3**  *HPR* regulates Hippo pathway activation. 

(a) Reporter assay detection of the activation of YAP1 in MDA-MB-231, HS578T and MCF10A cells transfected with the LNA of *HPR* (*P* < 0.05, **P** < 0.01). The results are the mean ± s.e.m. of *n* = 3 independent experiments. *P* values were determined by one-way ANOVA. 

(b) Reporter assay detection of the activation of YAP1 in MCF10A cells transfected with *HPR* cDNA (*P* < 0.05, **P** < 0.01). The results are the mean ± s.e.m. of *n* = 3 independent experiments. *P* values were determined by one-way ANOVA. 

(c) qPCR detection of *HPR* and substrates of YAP1 expression (**P** < 0.01). The results are the mean ± s.e.m. of *n* = 3 independent experiments. *P* values were determined by one-way ANOVA. 

(d) CRISPR-Cas9 knockout of *HPR* in MDA-MB-231 cells. 

(e) qPCR detection of *HPR* expression in the MDA-MB-231 cells (**P** < 0.01). The results are the mean ± s.e.m. of *n* = 3 independent experiments. *P* values were determined by one-way ANOVA. 

(f) Reporter assay detection of the activation of YAP1 (*P* < 0.05, **P** < 0.01). The results are the mean ± s.e.m. of *n* = 3 independent experiments. *P* values were determined by one-way ANOVA. 

(g), (h) Immunoblotting detection using the indicated antibodies in *HPR*-knockout MDA-MB-231 cells. Three independent experiments were performed and yielded similar results. 

(i) Immunofluorescence detection using indicated antibodies in the *HPR*-knockout MDA-MB-231 cells. 

(j) Immunoblotting detection using the indicated antibodies in *HPR*-knockout MDA-MB-231 cells. Three independent experiments were performed and yielded similar results.
showed that HPR was not expressed in these two clones (Fig. 3e). We next tested whether HPR is required for YAP1 activation in this specific signalling context, and found that HPR depletion significantly impaired TEAD luciferase activity (Fig. 3f). The phosphorylation of LATS1 (Thr1079) and YAP1 (Ser127) was all increased following HPR knockout (Fig. 3g). Furthermore, we expressed full-length HPR in HPR KO cells for rescue experiments. We found that the expression of HPR decreased the phosphorylation of LATS1 (Thr1079) and YAP1 (Ser127) (Fig. 3h). At the same time, the expression of HPR restored YAP1 nuclear localisation (Fig. 3i, j). Thus, we found that HPR was associated with the Hippo pathway and regulated YAP1 activation.

LncRNA HPR is associated with cancer

We assumed that HPR may be expressed at lower levels in normal cells than in cancer cells, thus, to identify the mechanisms underlying the above discrepancies, we performed RNA in situ hybridisation on breast cancer tissue microarrays using RNA fluorescence in situ hybridization (FISH) technology to test the HPR expression level and examine the potential correlation of HPR with cancer. Our data indicated that YAP1 and phosphorylated 70S6K (Thr389) exhibit high positive correlations compared with paired adjacent normal tissues in breast cancer, cholangiocarcinoma and colon cancer (Fig. 4a–c). We also examined the expression of HPR in a panel of breast cancer cell lines and identified a higher...
expression of HPR in breast cancer cell lines than in normal cell lines (Fig. 4d). To detect whether HPR was highly expressed in other cancer types, we also examined the expression of HPR in cholangiocarcinoma cell lines and found higher expression of HPR in cholangiocarcinoma cell lines than in normal bile duct cells (Fig. 4e). We also employed the RNA FISH assay to analyse HPR expression in normal and cancer tissues from multiple organs and observed increased HPR expression in many types of human cancer tissues, including colorectal, lung and oesophageal tissues, compared with normal tissues (Table 1). Taken together, these results demonstrated the strong correlation of HPR expression with cancer progression and the relevance of elevated HPR expression to human cancer development and progression. We examined the subcellular localisation of HPR by RNA FISH and found that the HPR transcript was localised in the cytoplasm and nucleus (Fig. 4F).

**HPR interacts with Raptor**

To identify how HPR regulates YAP1 activation, we performed RNA pulldown experiments using MDA-MB-231 cell lysates to identify whether HPR binds Hippo-YAP1 pathway proteins (Fig. 5a). Interestingly, HPR binds Raptor, S6K and 4E-BP1 but does not bind any Hippo-YAP1 pathway proteins, suggesting that it may mediate mTORC1 pathway activation. HPR binding to Raptor was confirmed by an in vivo RIP assay and an in vitro pulldown assay (Fig. 5b–d). Further study revealed that we find out HPR associates with both the HEAT and WD40 domains of Raptor, and regulates the enzymatic activity of mTORC1 (Fig. 5e).

**HPR regulates YAP1 and mTORC1 activation under cellular energy stress conditions**

Tumour cell growth is an energy-consuming process and must be coordinated with cellular energy status. Given the key function of the mTORC1 pathway in tumour cell growth regulation, we investigated whether mTORC1 is regulated by HPR under energy stress. We tested mTORC1 activation in the HPR-knockdown breast cancer cells and found that HPR depletion significantly decreased the phosphorylation of 70S6K (Thr389) but not mTOR (Ser2448) (Fig. 6a). Besides, the phosphorylation of 70S6K (Thr389) and the phosphorylation of RP56 (Ser235/236) were also drastically decreased in cholangiocarcinoma (Fig. 6b). This result suggests that HPR does not regulate mTORC1 activation through direct regulation of the level of phosphorylated mTOR. To further verify the coordinated connection between the Hippo and mTORC1 pathways, we tested the activities of YAP1 when treating the HPR-overexpressing cell line with rapamycin (Supplement Fig. 4). The results revealed that HPR affects both Hippo and mTORC1 signalling activities in two independent ways. Recent studies have shown that mTORC1 activation can be down-regulated under energy stress through direct AMPK directly phosphorylation of Raptor at site 792. Interestingly, we found that deletion of HPR significantly increased the level of phosphorylated Raptor (Ser792) under glucose starvation (Fig. 6c). Similarly, overexpression of HPR blocked AMPK phosphorylation of Raptor under glucose starvation (Fig. 6d). Interestingly, two recent studies found that AMPK phosphorylated YAP1 under glucose starvation and then inhibited YAP1 nuclear localisation. These data suggest that HPR may block AMPK phosphorylation of YAP1 and Raptor and then promote tumour cell growth under energy stress.

**DISCUSSION**

The development of effective preventative and therapeutic strategies for cancer relies on a comprehensive understanding of the molecular mechanisms of the disease. Our findings demonstrate that IncRNAs coregulate both the YAP1 and mTORC1 pathways in cancer. Through large-scale screening of siRNAs targeting human long noncoding RNAs, we identified that one IncRNA, named HPR, can regulate YAP1 activation. Interestingly,
HPR can also promote mTOR-dependent phosphorylation of S6K and 4E-BP1 by directly binding to Raptor, an important component of the mTORC1 complex. We also revealed that HPR blocks S792 phosphorylation of Raptor by AMPK under energy stress. The activation of the YAP and mTOR pathways is correlated in human cancer tissues. These findings suggest that HPR is important for co-regulating mTOR and YAP activation in cancer.

Cellular growth, proliferation and survival are critical processes that must be finely regulated to preserve the structure and function of different organs. The mechanistic targets of the rapamycin (mTOR) and Hippo pathways have recently emerged as the major signalling transduction cascades regulating organ size and cellular survival. The mTOR pathway promotes protein synthesis, cellular growth and survival. Although the Hippo pathway exerts opposite effects by inhibiting the cellular growth and proliferation and inducing cell death. These findings imply that tight coordination of these two pathways is important for the regulation of organ size and cellular integrity and strongly suggest the existence of multiple crosstalk mechanisms between the mTOR and Hippo signalling cascades. Although a recent study showed that YAP1 mediates crosstalk between the Hippo and PI3K-TOR pathways by suppressing PTEN via mIR-29 [33], some PTEN-deficient cancer cells and tumour tissues also showed coactivation of the YAP and mTOR pathways. We also found that the knockdown of HPR increased T1029 phosphorylation of LATS. We first considered that mTORC1 may regulate LATS activation, because both pathways are regulated by HRP. In HRP-overexpressing CCLP1 cells, mTORC1 activity, as indicated by the decreased p70S6K1 T389 phosphorylation by the rapamycin treatment (Supplementary Figure 4), suggesting that HPR did not regulate mTORC1 activation through direct regulation of the level of phosphorylated mTOR. On the other hand, Hippo pathway activation was not affected by rapamycin-treated HPR-overexpressing CCLP1 cells (Supplementary Figure 4), indicating that mTORC1-independent pathways are involved in the regulation of LATS kinase activity in response to the manipulation of HPR expression. Therefore, the regulatory mechanisms that involve direct crosstalk between the Hippo and mTOR pathways remain unknown.

We believe that the molecular mechanisms presented in our study have broad biological implications. In conditions where nutrients are scarce, AMPK acts as a metabolic checkpoint that inhibits cellular growth. AMPK regulates cell growth by suppressing mTORC1 pathway via phosphorylated TSC2 and RAPTOR. Interestingly, two recent studies found that AMPK directly phosphorylated YAP1 and retained the cytoplasmic localisation of YAP1 under glucose starvation conditions [12, 13]. As nutrient concentrations in tumours are different from those in normal tissues, cancer cells in vivo may have metabolic dependencies that are not shared by normal cells. In particular, tumour glucose concentrations are frequently three- to tenfold lower than those in non-transformed tissues. These data suggest that some factors can coregulate...
mTORC1 and YAP1 activation and promote tumour growth under low glucose concentrations. Therefore, our study reveals that the lncRNA HPR, which shows high tumour expression, may play a role in tumour growth by co-regulating mTORC1 and YAP1 activation.

METHODS

Clinical samples
The primary tumour and some paired normal tissues were obtained from individuals with breast, cholangiocarcinoma and colon cancer diagnosed at the First Affiliated Hospital of Harbin Medical University. The protocol was approved by the First Affiliated Hospital of Harbin Medical University Research Ethics Committee. All tissue samples were collected in compliance with the informed consent policy.

Animal studies
All animal experiments were performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee of the First Affiliated Hospital of Harbin Medical University. Luciferase-labelled QBC-939 and CCLP1 cells (5 × 10⁶) were suspended in 150 μL PBS to be subcutaneously injected into the flanks of mice. Tumour growth was examined by bioluminescent imaging every week using an IVIS Spectrum Xenogen Imaging System (Caliper Life Sciences) at the Small Animal Imaging Facility of the First Affiliated Hospital of Harbin Medical University.

Cell culture, transfection and treatments

The human normal biliary cell line HIBEpiC was obtained from ScienCell Research Laboratories (Carlsbad, CA, USA). The HuCCT1 cell line was kindly provided by the Cancer Cell Repository of the Tohoku University in Japan. The CCLP1 and KMBC cell lines were purchased from BeNa Culture Collection (Beijing, China). The RBE, QBC-939 and HCCC9810 cell lines were purchased from Shanghai Bioleaf Biotech Corporation (Shanghai, China). The RBE, HCCC9810, QBC-939, CCLP1 and HuCCT1 were authenticated using short tandem repeat (STR) analysis. The human breast cancer cell lines MDA-MB-231, HS578T and the human embryonic kidney cell line HEK293T were purchased from American Type Culture Collection (ATCC) and maintained in Dulbecco’s modified essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in 5% CO₂.
Fig. 7  HPR inhibits the growth of cancer. a Clonogenicity assay detection of the growth of HPR-knockout MDA-MB-231 cells. b Clonogenicity assay detection of the growth of HPR-knockdown QBC-939 cells and overexpressed CCLP1 cells. c In vivo analyses of tumour growth in mice that were subcutaneously injected with HPR-knockdown QBC-939 cells. d In vivo analyses of tumour growth in mice that were subcutaneously injected with HPR-overexpressing CCLP1 cells. e Graphic illustration of the functional role of HPR coordinates the activation of the Hippo and mTORC1 pathways in cancer.
His6-RNC, His6-HEAT and His6-WD40 proteins were expressed in pairs of sgRNAs (oligonucleotide sequences and primers) to generate experiments. For cell transfection, LNA transfections were performed (25 mM), rapamycin (10 nM) and Torin1 (10 nM) for 4 h in various conditions. His6-HEAT and His6-WD40) were constructed in the pET-DEST42 vectors for wild-type hRPP was constructed by subcloning the gene sequences into pBabe (Addgene). The 8xGTCI Luciferase vector was obtained from Addgene (Plasmid #34615). The following recombinant proteins were used in this study, plasmids for expression were designed (10) using four pairs of sgRNAs (oligonucleotide sequences and primers) to generate stable knockout cell lines of MDA-MB-231 cell lines (Gene Editing/Cellular Model Core Facility, MD Anderson Cancer Center). Full-length human 56K1 protein was purchased from Abcam (ab167933) and full-length human 4E-BP1 protein was purchased from Sigma (SRPS162-50UG). Recombinant His6-HEAT, His6-RNC and His6-WD40 proteins were expressed in Escherichia coli strain BL21-CodonPlus (DE3)-RIPL (Agilent Technologies) and purified. Antigen was visualized with 4% PFA at 4 °C for 10 min at room temperature. The samples were blocked in 10% normal goat serum, and PBS with 0.25% Triton X-100 for 1 h at room temperature. Primary antibodies targeting YAP1 were incubated overnight at 4 °C in a blocking solution. After extensive washing in PBS-0.25% Triton X-100, nuclei were stained with DAPI (2 μg/ml; Life Technologies) for 10 min at room temperature. Slides were mounted with ProLong Gold anti-fade reagent (Life Technologies) and imaged with a confocal microscope (Zeiss).

**RNA fluorescence in situ hybridisation**

RNA FISH was performed using LNA FISH technology according to the manufacturer’s instructions (Exiqon) with minor modifications, which have been described previously [34]. The probe is listed in the Oligonucleotide Sequences and Primers section.

**RNA pulldown and mass spectrometry analysis**

RNA pulldown followed by a mass spectrometry analysis, and in vitro RNA-protein binding assay, and an in vitro RNA pulldown assays were performed as described previously [34]. In brief, the RNA-capture beads were incubated with recombinant proteins (1 μg): His-p70S6K and His-4E-BP1; and His-RNC, His-HEAT and His-WD40 in binding buffer [50 mM Tris-HCl pH 7.9, 10% glycerol, 100 mM KCl, 5 mM MgCl2, 10 mM β-ME 0.1% NP-40] for 1 h at 30 °C.

**Immunohistochemistry and immunofluorescence**

For immunohistochemistry, tumour tissues were fixed in neutral buffered formalin overnight and embedded in paraffin. Five-micrometre sections were baked for 30 min at 60 °C, and then deparaffinized. Antigen was retrieved at 98 °C for 20 min in 10 mM citrate buffer pH 6. Endogenous peroxidase activity was quenched by incubating the sections in 3% hydrogen peroxide solution for 10 min. All incubations were performed at room temperature unless otherwise stated. After blocking in 5% BSA/0.05% Tween-20, primary antibodies were applied (anti-rabbit YAP, 1:200; anti-rabbit phospho-p70S6 kinase (Thr389), 1:200 and anti-rabbit phospho 4E-BP1 (Ser65) (Cell Signaling) were applied overnight at 4 °C. Subsequently, the sections were incubated with labelled polymer-HRP (Dako; K4065) for 30 min. For all staining, counterstaining with DAB served as the internal reference gene. The results are
reported as the mean ± standard error of the mean of at least three independent experiments. Each exact $n$ values is indicated in the corresponding figure legend. Comparisons were performed using two-tailed paired Student’s t test or two-way analysis of variance (n.s. $p > 0.05$, $p < 0.05$, **$p < 0.01$ and ***$p < 0.001$) as indicated in the individual figures. Pearson chi-square test or Fisher’s exact test was implemented for statistical analyses of the associations between markers and clinical parameters, as indicated in the individual figures.

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