lncRNA *THAP9-AS1* Promotes Pancreatic Ductal Adenocarcinoma Growth and Leads to a Poor Clinical Outcome via Sponging miR-484 and Interacting with YAP

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ABSTRACT

Purpose: Long noncoding RNAs (lncRNA) have been observed in various cancer types. Our bioinformatic analysis of existing databases demonstrated overexpression of lncRNA *THAP9-AS1* in pancreatic ductal adenocarcinoma (PDAC). We aimed to investigate the roles and mechanisms of *THAP9-AS1* in PDAC.

Experimental Design: The overexpression of *THAP9-AS1* in samples of patients with pancreatic cancer was characterized and was associated with clinical outcomes. The nonprotein coding property of the *THAP9-AS1* was verified. Various *in vitro* and *in vivo* experiments were performed to investigate the interaction between *THAP9-AS1* and YAP signaling.

Results: We demonstrated that lncRNA *THAP9-AS1* is overexpressed in PDAC in multiple patient sample sets, which is significantly associated with poor outcome of patients with PDAC. *THAP9-AS1* promotes PDAC cells growth both *in vitro* and *in vivo*. *THAP9-AS1* exerts its effects via enhancing YAP signaling. Ectopic YAP expression overcame the effects of *THAP9-AS1* knockdown. Inversely, YAP knockdown diminished the effects of *THAP9-AS1* overexpression. *THAP9-AS1* acts as a competing endogenous RNA for miR-484, leading to YAP upregulation. Moreover, *THAP9-AS1* binds to YAP protein and inhibits the phosphorylation-mediated inactivation of YAP by LATS1. Reciprocally, YAP/TEAD1 complex promotes *THAP9-AS1* transcription to form a feed-forward circuit. Importantly, *THAP9-AS1* level positively correlates with YAP expression in PDAC tissues. YAP overexpression also predicts a poor outcome in patients with PDAC.

Conclusions: Our findings indicate that *THAP9-AS1* plays an important role in PDAC growth via enhancing YAP signaling, which in turn also modulates *THAP9-AS1* transcription. *THAP9-AS1*/YAP axis may serve as a potential biomarker and therapeutic target for PDAC treatment.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is still a highly aggressive cancer with a 5-year overall survival rate of only 3%–5%, which is, at least in part, due to its poor response to existing conventional or targeted treatments (1). Thus, novel and effective therapies are urgently required. Because of the endogenous genetic alterations including the mutations and/or amplification of *KRAS* oncogene and the mutations or losses of *CDKN2A*, *TP53*, and *SMAD4* cancer suppressor genes, PDAC evolves through a series of histopathologic changes, referred to as increasingly dysplastic precursor lesions, or pancreatic intraepithelial neoplasias (PanIN), toward invasive and finally metastatic pancreatic cancer (2–6). The essential roles of these genetic aberrations in PDAC initiation and progression have been well characterized by several genetically engineered mouse models (7). In addition, the critical role of dysregulation of epigenetic modifiers, for example, noncoding RNAs in the development and progression of many human cancer types including PDAC is also increasingly emphasized.

Emerging evidence has regarded long noncoding RNAs (lncRNA) as major regulators of both development and disease. LncRNAs are an important set of endogenous transcripts that are longer than 200 nucleotides but do not encode proteins (8). LncRNAs function in a wide range of biological activities, including cell-cycle regulation, stem cell pluripotency, lineage differentiation, and cancer progression, by regulating gene expression by various mechanisms (9). Studies point out that lncRNAs play vital roles in mRNA transcription and translation, protein abundance and location, as well as chromatin and

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Translational Relevance

Because of its poor prognosis and poor response to existing conventional or targeted treatments, pancreatic ductal adenocarcinoma (PDAC) remains a significant therapeutic challenge. Recent evidence has shown abnormal long noncoding RNAs (lncRNA) expression in a variety of cancers. Our study observed extensive overexpression of lncRNA *THAP9-AS1* in PDAC and demonstrated that *THAP9-AS1* promotes PDAC growth via enhancing YAP signaling. *THAP9-AS1* facilitated YAP expression by sequestrating miR-484. *THAP9-AS1* can also bind YAP to inhibit phosphorylation-mediated inactivation by LATS1. High *THAP9-AS1* and YAP expression are associated with poor prognosis in patients with PDAC. YAP functions as an oncogene in various cancer types. However, targeting Hippo/YAP signaling is currently unsuccessful given the negative regulation of YAP by its upstream kinases. Thus, our findings provide insights into the *THAP9-AS1*/YAP axis as potential therapeutic target against PDAC, implying important translational implications.

protein conformation (10–12). Recent evidence has shown abnormal lncRNA expression in a variety of cancers, and lncRNAs have been reported to play roles of oncogene, tumor suppressor or both, according to their multifaceted functions in a wide range of biological processes, such as proliferation, apoptosis, cell migration, and metastasis (13–15). Recently, although several lncRNAs have been shown to be involved in growth, invasion, metastasis, and stemness of PDAC (13, 16, 17), their specific roles and detailed mechanisms in pancreatic carcinogenesis still remain incompletely revealed.

The annotated potential lncRNA *THAP9-AS1* (THAP9 antisense RNA 1) was previously reported to be lowly expressed in multiple human normal tissues, especially in pancreas (18). Via a bioinformatic analysis of publicly available data, we found that *THAP9-AS1* is overexpressed in PDAC. In this study, we validated and characterized the upregulation of *THAP9-AS1* in PDAC and corroborated the hypothesis that *THAP9-AS1* is a key lncRNA involved in progression of PDAC.

Materials and Methods

Ethical statement

This study was reviewed and approved by the Ethnics Committees of Guangzhou Medical University and Affiliated Cancer Hospital (Guangzhou, Guangdong, China) and Wayne State University (Detroit, MI). The "informed written consent" was obtained from each subject or each subject's guardian. All related procedures were performed with the approval of the internal review and ethics boards of the hospital. The study was conducted in accordance with the Declaration of Helsinki.

Cell culture, transfection, and tissue samples

Pancreatic ductal adenocarcinoma cell lines PANC-1, SW1990, CFPAC-1, and BxPc-3, and HEK293T cells were obtained from ATCC. Cells were cultured in RPMI1640 or DMEM (Gibco) supplemented with 10% FBS. To establish stable transfectants with knockdown or overexpression, cell lines were transfected with psi-LVRU6GP vectors with *THAP9-AS1* shRNAs (target sequence for sh-1#: 5′-AATGGGGAAGCTCTTGGCATG-3′, sh-2#: 5′- GTCTACAACTTCACTCTTTGC-3′, sh-3#: 5′- GCACTTTGGGTGGCTAAAGCA-3′), or with YAP shRNAs (target sequence for sh-1#: 5′- GGAAGCTGCCCGACTCCTTCT-3′, sh-2#: 5′- GCAGGTTGGGAGATGGCAAAG-3′), or with gene overexpression vectors EXP-LV203-*THAP9-AS1* or pEZ-Lv203-YAP using Lipofectamine 3000 following the manufacturer's instructions (Invitrogen). To knockdown TEAD1, the shRNAs (target sequence for sh-1#: 5′-GGTTCTTGCCAGAAGGAAA-3′, sh-2#: 5′-GGATCAGACTGCAAAGGAT-3′, sh-3#: 5′-GCTTGAATCAGTGGACATT-3′, sh-4#: 5′-GCCGATTTGTATACCGAAT-3′) was used for transfection. To increase miR-484 level and inhibit miR-484 function, related cells were transfected with miR-484 mimics and miR-484 inhibitor (miRCURY LNA miRNA inhibitor for miR-484), respectively (Exiqon). The frozen-fresh and paraffin-embedded PDAC and noncancerous tissues were collected from patients at the Affiliated Cancer Hospital of Guangzhou Medical University (Guangzhou, Guangdong, China). The "informed written consent" was obtained from each subject or each subject's guardian. All related procedures were performed with the approval of the internal review and ethics boards of the hospital.

RNA immunoprecipitation assay

RNA immunoprecipitation (RIP) assay was performed to validate the interaction of *THAP9-AS1* with miR-484, and of YAP mRNA 3′UTR with miR-484; HEK293T cells were cotransfected with MS2bs vectors cloned with related DNA sequences (MS2bs, MS2bs-*THAP9-AS1*-WT, MS2bs-*THAP9-AS1*-Mut, MS2bs-YAP-3′UTR-WT, or MS2bs-YAP-3′UTR-Mut) and MS2bp-GFP overexpression vector (Addgene). To validate the interaction between *THAP9-AS1* and YAP protein, HEK293T cells were cotransfected with pReceiver-M11 vector with N-terminal Flag-tag–expressing YAP truncated fragments and EXP-LV203 vector-expressing *THAP9-AS1*, or EXP-LV203 vector-expressing *THAP9-AS1* truncated fragments and pEZ-Lv203-YAP. At 48 hours; related cells were used to perform RIP assay according to the manufacturer's instructions of the EZ-Magna RIP Kit (Millipore), using anti-GFP, anti-Flag or anti-YAP antibodies, or normal rabbit IgG. After extraction of RNAs, miR-484 and *THAP9-AS1* truncated fragments were examined by qRT-PCR.

Xenograft model in athymic mice

The animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of Guangzhou Medical University (Guangzhou, Guangdong, China). Standard animal care and laboratory guidelines were followed according to the IACUC protocol. BxPc-3/sh-Con, BxPc-3/sh-*THAP9-AS1*, BxPc-3/sh-*THAP9-AS1*+Vector, and BxPc-3/sh-*THAP9-AS1*+*YAP* cell lines were injected at 1×10^6 cells in 200 μL, respectively, subcutaneously into the armpit of female Balb/C athymic nude mice to generate xenograft tumors (6 mice/group). The tumor growth was measured every 5 days. The wet weight of tumor was recorded after excision at the experimental endpoint.

qRT-PCR, plasmid constructs, proliferation assay, tumor sphere assay, Western blot, immunofluorescence, transactivation of transcription factors, luciferase reporter assay, ChIP assay, co-immunoprecipitation, *in situ* hybridization, IHC, and primers. These methods used are described in the Supplementary Experimental Procedures.

Statistical analysis

All data were presented as the mean ± SD. The Student *t* test and the χ^2 test were used to compare the differences among different groups. Survival curves were plotted using the Kaplan–Meier method and compared using the log-rank test. Statistical analyses were performed using GraphPad Prism 6. $P < 0.05$ was considered statistically significant.

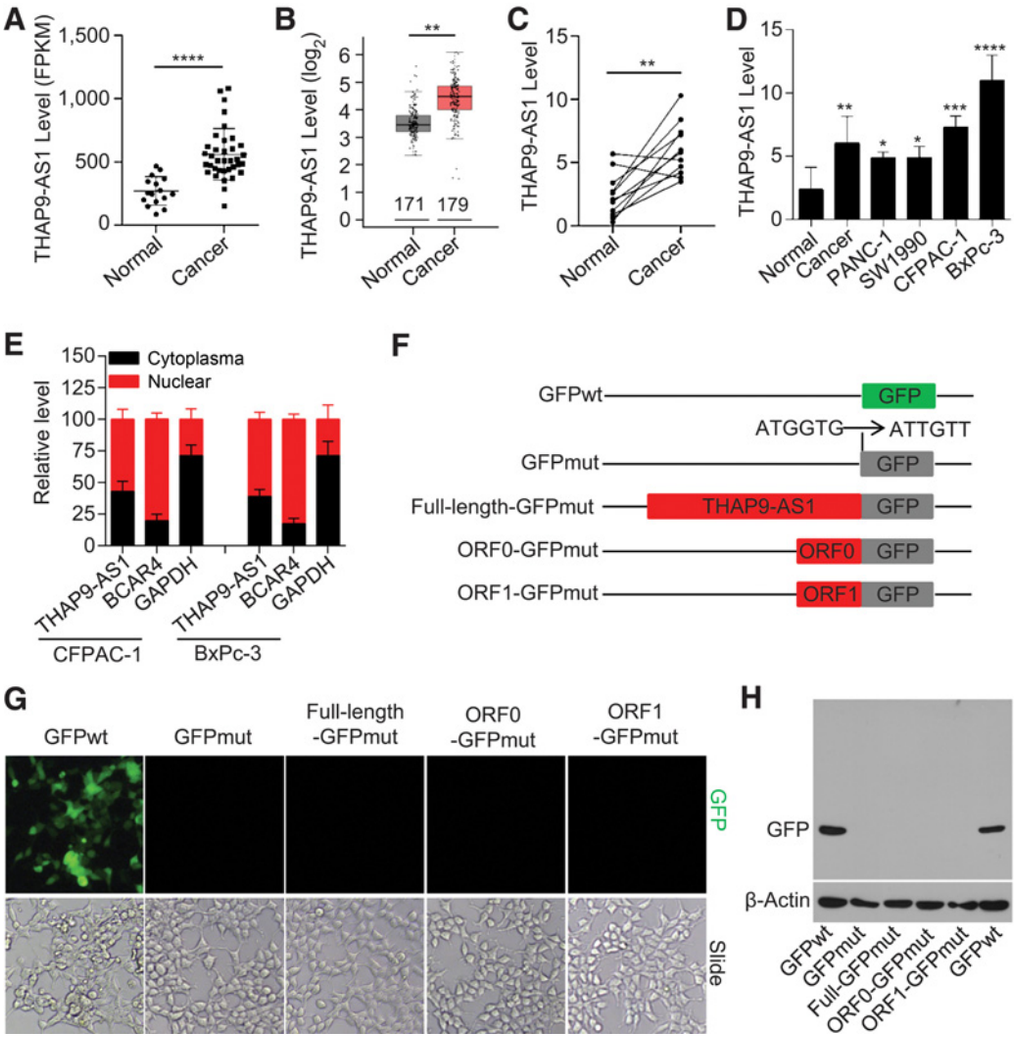

Figure 1.

THAP9-AS1 as an lncRNA is upregulated in PDAC. **A,** *THAP9-AS1* transcript between PDAC tissues and normal pancreatic tissues was analyzed in the publicly accessible samples. **B,** Scatter plots comparing *THAP9-AS1* expression in PDAC samples ($n = 179$) and normal pancreatic tissue samples ($n = 171$). **C,** The relative expression levels of *THAP9-AS1* were detected by qRT-PCR and normalized against an endogenous control (GAPDH) in paired PDAC tissues ($n = 11$). **D,** The expression levels of *THAP9-AS1* in human PDAC cells were detected by qRT-PCR. **E,** Fractionation of PDAC cells followed by qRT-PCR. BCAR4 served as a positive control for nuclear gene expression and GAPDH served as a positive control for cytoplasmic gene expression. **F,** Diagram of the GFP constructs fused to a series of *THAP9-AS1* constructs used for transfection in HEK293T cells. The start codon ATGGTG of the *GFP* (as *GFPwt*) gene is mutated to ATTGTT (as GFPmut). The indicated constructs were transfected into HEK293T cells for 24 hours and the GFP fluorescence was detected (**G**), and the GFP fusion protein levels were examined by Western blot using anti-GFP antibody (**H**). Student *t* test, mean ± SD (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

Results

THAP9-AS1 as an lncRNA is upregulated in PDAC

To explore lncRNAs involved in pancreatic ductal adenocarcinoma (PDAC), a publicly accessible microarray dataset from patients with PDAC was analyzed between PDAC tissues and normal pancreatic tissues (GEO: GSE16515). Among the annotated potential lncRNAs, *THAP9-AS1* levels were found to be increased in PDAC tissues compared with those in normal pancreatic tissues (**Fig. 1A**). Next, online analysis based on TCGA and GTeX data (http://gepia.cancer-pku.cn/) indicates that there are also significantly higher *THAP9-AS1* levels in PDAC tissues compared with normal pancreatic tissues (**Fig. 1B**). Furthermore, the *THAP9-AS1* levels were determined in 11 pairs of PDAC tissues and matched noncancer tissues collected from patients at the Affiliated Cancer Hospital of Guangzhou Medical University (Guangzhou, Guangdong, China). The *THAP9-AS1* levels were also significantly increased in primary PDAC tissues as compared with their matched adjacent normal tissue (**Fig. 1C**). Moreover, *THAP9-AS1* levels in PDAC cell lines were also increased compared with that in the adjacent normal pancreatic tissues (**Fig. 1D**).

Analysis shows that *THAP9-AS1* is composed of five exons, with a transcript of poly (A)-negative (Ensembl: ENSG00000251022). *THAP9-AS1* is located in both cytoplasm and nucleus (**Fig. 1E**). The protein coding potentiality of *THAP9-AS1* was analyzed by online software, that is, PhyloCSF and CPAT. The LNCipedia database based on both softwares failed to predict its coding potentiality

(Supplementary Fig. S1A). Although a Bazzini small ORF is predicted, there is no translation initiation site in the *THAP9-AS1* transcript (Supplementary Fig. S1A). In addition, RegRNA 2.0 online software also predicted two short ORFs, but there is no ribosome-binding site in the *THAP9-AS1* transcript (Supplementary Fig. S1B), supporting that no protein-coding potential of *THAP9-AS1* is discovered. To further experimentally verify the protein-coding ability of *THAP9-AS1* or the activation of ORFs, a series of constructs was generated in which a GFPmut ORF (in which the start codon ATGGTG is mutated to ATTGTT) was fused to the full-length *THAP9-AS1* transcript or predicted ORFs (**Fig. 1F**). No substantial expression of GFP was observed in HEK293T cells transfected with full-length GFPmut or ORFs-GFPmut construct (**Fig. 1G**). Western blot analysis using anti-GFP antibody further confirmed that *THAP9-AS1* is of no protein-coding ability (**Fig. 1H**). These data show that expression of *THAP9-AS1*, as a lncRNA, is upregulated in PDAC cells.

THAP9-AS1 promotes PDAC cell growth

Given that *THAP9-AS1* is overexpressed in PDAC, to determine the roles of *THAP9-AS1* in PDAC cells, loss- and gain-of-function approaches were employed. First, *THAP9-AS1* was knocked down using shRNAs. We found that sh-1# and sh-3# could effectively knockdown *THAP9-AS1* levels in CFPAC-1 and BxPc-3 cells after transient transfection (Supplementary Fig. S2A). Then, the stable *THAP9-AS1*-knockdown CFPAC-1 and BxPc-3 cell lines expressing sh-1# or sh-3# shRNAs were established (**Fig. 2A**). Their proliferation ability was determined by MTS assay. As shown, *THAP9-AS1* knockdown decreased the proliferation of PDAC cells (**Fig. 2B**). The colony-forming assay also showed that *THAP9-AS1* knockdown significantly inhibited colony-forming capacity of PDAC cells (**Fig. 2C**; Supplementary Fig. S2B). In addition, to confirm the role of *THAP9-AS1* in PDAC cells, *THAP9-AS1* was overexpressed in PANC-1 cells that exhibit relatively low level of endogenous *THAP9-AS1* expression (**Fig. 2D**). *THAP9-AS1* overexpression markedly promoted cell proliferation and colony-forming capacity (**Fig. 2D**; Supplementary Fig. S2C). Moreover, *THAP9-AS1* knockdown inhibited tumor sphere formation of PDAC cells represented in sphere number and sphere diameter (**Fig. 2E**; Supplementary Fig. S2D), whereas, ectopic overexpression of *THAP9-AS1* enhanced tumor sphere–forming ability (**Fig. 2F**; Supplementary Fig. S2E). These data show that lncRNA *THAP9-AS1* drives PDAC cells proliferation and growth.

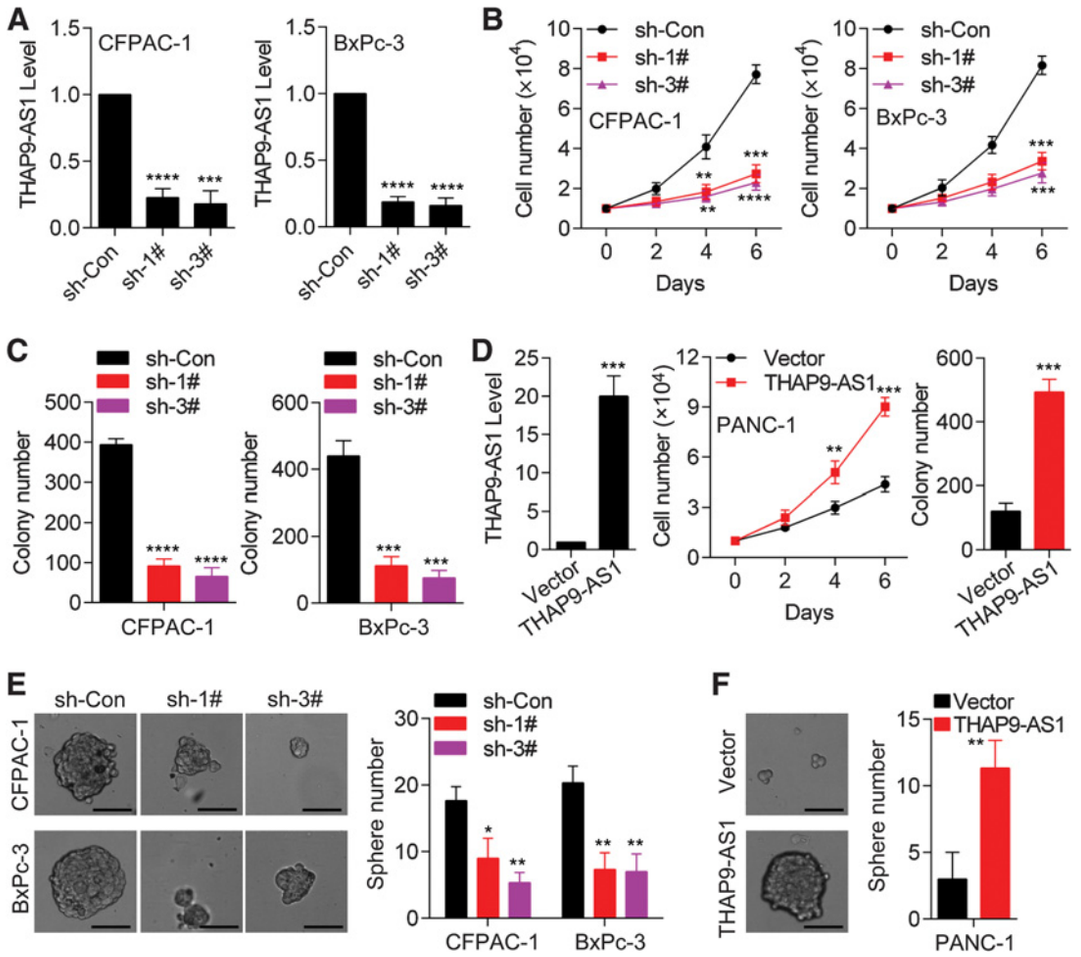

Figure 2.

THAP9-AS1 promotes PDAC cell growth. **A,** *THAP9-AS1* was stably knocked down in PDAC cells. **B** and **C,** The effects of *THAP9-AS1* knockdown on proliferation and colony-forming ability were measured in PDAC cells. **D,** *THAP9-AS1* overexpression enhanced proliferation and colony-forming ability in PDAC cells. **E** and **F,** Representative photo pictomicrographs and quantification of tumor sphere–forming ability of PDAC cells with *THAP9-AS1* knockdown or overexpression. Student *t* test, mean ± SD (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

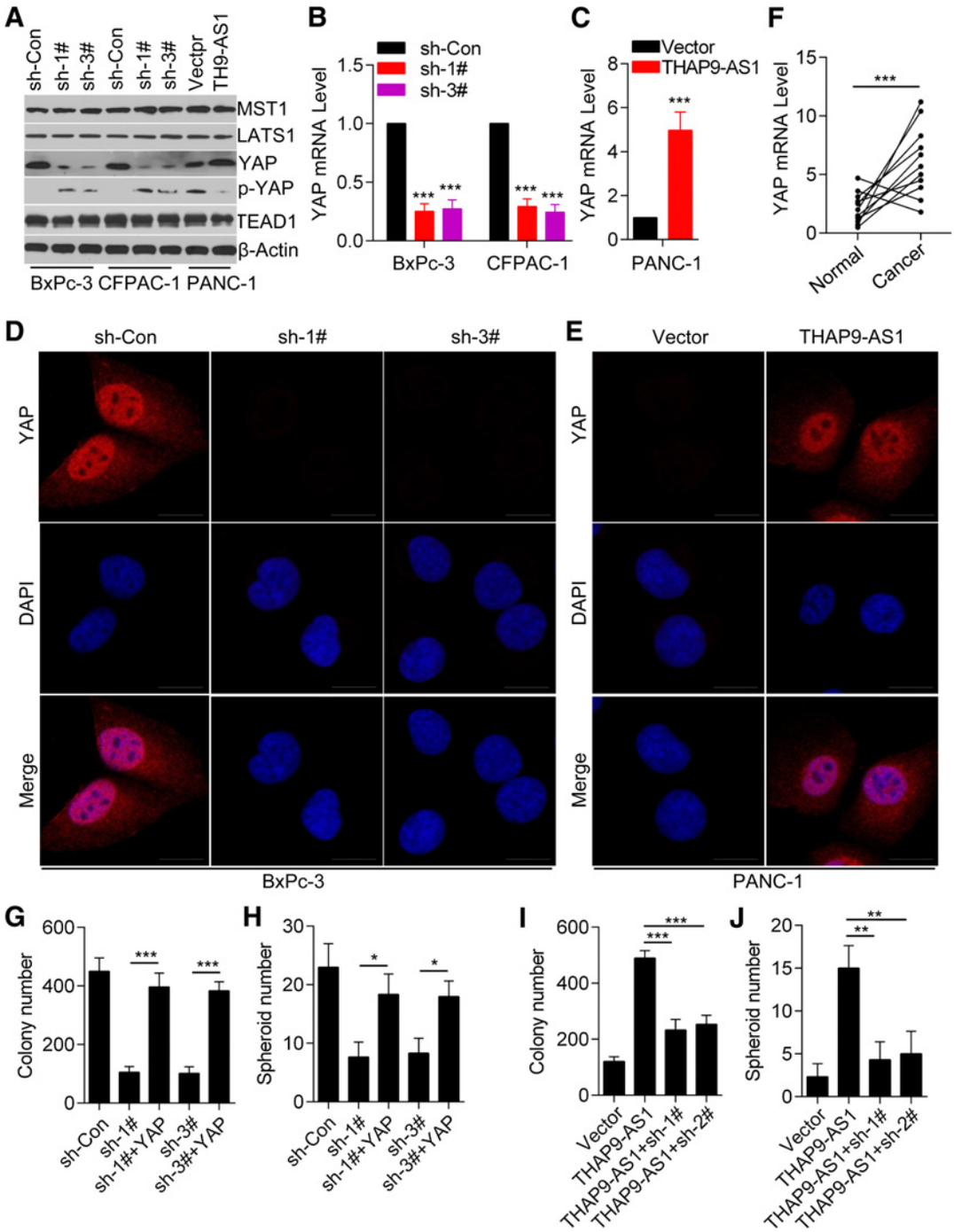

Figure 3.

THAP9-AS1 enables PDAC cell growth via enhancing YAP activation. **A,** The protein levels were detected by Western blot in indicated clones. **B** and **C,** The YAP mRNA levels in indicated cell lines with *THAP9-AS1* knockdown or overexpression were detected by qRT-PCR. **D** and **E,** Immunofluorescence detection of YAP in *THAP9-AS1* knocked down or overexpressed PDAC cells. Scale bar, 20 μm. **F,** The YAP mRNA levels in tissues were detected by qRT-PCR. **G–J,** The colony-forming ability and tumor sphere–forming ability of indicated cell lines were analyzed. Student *t* test, mean ± SD (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

THAP9-AS1 enables PDAC cell growth via enhancing YAP activation

Given the upregulation and oncogenic role of *THAP9-AS1* in PDAC cells, to identify the downstream signaling pathway of *THAP9-AS1*, we examined the transactivation of a series of transcription factors after *THAP9-AS1* overexpression in PANC-1 cells, but there was no significant change observed (Supplementary Fig. S3A). Unexpectedly, we found that the expression of YAP downstream target genes *CTGF* and

CYR61 was significantly increased after *THAP9-AS1* overexpression (Supplementary Fig. S3B), whereas *THAP9-AS1* knockdown decreased the expression of CTGF and CYR61 in PDAC cells (Supplementary Fig. S3C), suggesting that *THAP9-AS1* level was potentially correlated with YAP activation.

To access whether *THAP9-AS1* regulates YAP activity, we examined the protein levels of the genes regulating YAP activity. As shown, *THAP9-AS1* knockdown decreased YAP protein level, but did not influence the protein levels of YAP upstream kinases LATS1 and MST1 (**Fig. 3A**). YAP mRNA level was also decreased after *THAP9-AS1* knockdown (**Fig. 3B**). Phosphorylation at YAP serine 127 by LATS1 is one of the most common posttranslational modifications resulting in cytoplasmic localization of YAP to repress its activity (19). *THAP9-AS1* knockdown increased YAP serine 127 phosphorylation (**Fig. 3A**). Immunofluorescence staining revealed that *THAP9-AS1* knockdown markedly alleviated protein level and nuclear translocation of YAP (**Fig. 3D**; Supplementary Fig. S3D). On the other hand, ectopic overexpression of *THAP9-AS1* positively regulated YAP expression at both protein (**Fig. 3A**) and mRNA levels (**Fig. 3C**), but negatively regulated YAP serine 127 phosphorylation (**Fig. 3A**). Immunofluorescence staining further confirmed that *THAP9-AS1* overexpression enhanced YAP protein level and nuclear translocation (**Fig. 3E**).

To further verify this potential regulation *ex vivo*, we further examined YAP expression in the aforementioned eleven pairs of fresh-frozen primary PDAC tissues and their matched adjacent nontumoral (NT) pancreatic tissues in which *THAP9-AS1* level has been examined. We found that YAP expression was also increased in the primary PDAC tissues as compared with their matched normal tissue (**Fig. 3F**). *THAP9-AS1* level was positively correlated with YAP mRNA level (Supplementary Fig. S3E). Moreover, online analysis based on TCGA and GTeX data indicates that the expression of YAP and YAP target gene *CTGF* and *CYR61* was upregulated in PDAC tissues as compared with the normal pancreatic tissue (Supplementary Fig. S3F). *THAP9-AS1* level was positively correlated with YAP, CTGF, and CYR61 levels (Supplementary Fig. S3G).

To access whether YAP acted as the downstream effector to mediate the role of *THAP9-AS1* in PDAC, we overexpressed YAP in BxPc-3 cells in which *THAP9-AS1* has been stably knocked down (Supplementary Fig. S3H). YAP overexpression significantly overcame the effects of *THAP9-AS1* knockdown on proliferation (**Fig. 3G**; Supplementary Fig. S3I) and tumor sphere formation (**Fig. 3H**; Supplementary Fig. S3J). Inversely, we knocked down YAP expression in PANC-1 cells with *THAP9-AS1* overexpression (Fig. S3H). YAP knockdown abolished the effects of *THAP9-AS1* overexpression on proliferation (**Fig. 3I**; Supplementary Fig. S3K) and tumor sphere formation (**Fig. 3J**; Supplementary Fig. S3L). These data indicated that *THAP9-AS1* exerted its effects in PDAC cells via regulating YAP activity.

THAP9-AS1 increases YAP expression by sponging miR-484

Recent evidences demonstrate that lncRNAs are involved in cancer progression by acting as competing endogenous RNAs (ceRNA; ref. 20). That means lncRNAs act as molecular sponges to regulate the levels of mRNAs by competitively binding their same miRNAs targeting mRNAs. Given that knockdown of *THAP9-AS1* decreased, whereas induction of *THAP9-AS1* increased both YAP mRNA and protein levels, we wonder whether *THAP9-AS1* could act as a miRNA sponge to regulate YAP expression. To test the possibility, we first performed the bioinformatic analysis using the online tool DIANA (21) and miR-484 was predicted to target *THAP9-AS1*. The RNA targeting by miRNAs in animals primarily relies on a "seed region" mapping to positions 2–7 at the miRNA's 5′end. DIANA tools predicted three putative complementary sequences at *THAP9-AS1* for miR-484, including one 10-mer site (**Fig. 4A**), one 7-mer-A1 site, and one 6-mer site (Supplementary Fig. S4A). The RegRNA 2.0 online tool also predicted a 10-mer site at the same position as what DIANA tools predicted, which has suitable RNA secondary structure for miRNA binding (Supplementary Fig. S4B).

The interaction between *THAP9-AS1* and miR-484 was further predicted and the minimum free energy at the binding site was calculated by using RNAhybrid (**Fig. 4B**; ref. 22). Concurrently, the 3′ untranslated region (3′-UTR) of the YAP mRNA contains one 7-mer-m8 site that matches to the miR-484 seed region predicted by online server TargetScan (**Fig. 4A**; Supplementary Fig. S4C).

We next determined whether *THAP9-AS1* potentially interacted with miR-484 and whether miR-484 potentially targeted YAP via the predicted binding motif (**Fig. 4A**). To validate the direct binding between *THAP9-AS1* and miR-484, the *THAP9-AS1* with or without deletion mutations in miR-484 targeting site were cloned into MS2b plasmid to transcribe RNA combined with MS2-binding sequences and then cotransfected with the MS2bp-GFP expression plasmid and miR-484 mimics into HEK-293T cells. Subsequently, we performed RIP assays to pull down miRNAs associated with *THAP9-AS1* via GFP antibody and demonstrated via qRT-PCR analysis that miR-484 associated with *THAP9-AS1* via targeting site, while the nontargeting miR-191–5p used as negative control, was not associated with *THAP9-AS1* (**Fig. 4C**). RIP assays also indicated that miR-484 also associated with YAP 3′-UTR specifically (**Fig. 4D**). To further confirm the association of *THAP9-AS1* with miR-484, based on pMir-reporter plasmid we constructed luciferase reporters containing the 5′-500nt of *THAP9-AS1*, which contains wild-type or deletion-mutated miR-484 targeting site. These reporters were cotransfected with miR-484 mimics into HEK-293T cells. We found that miR-484 mimics reduced the reporter activity of the construct with wild-type *THAP9-AS1* (**Fig. 4E**).

To determine whether YAP is a bona fide target of miR-484, based on pMir-reporter plasmid we also constructed luciferase reporters containing the 500nt of the 3′-UTR of YAP mRNA, which contains wild-type or mutated miR-484 targeting site. These reporters were cotransfected with miR-484 mimics into HEK-293T cells. We found that miR-484 mimics reduced the reporter activity of the construct with wild-type 3′-UTR of YAP mRNA (**Fig. 4F**), suggesting that 3′-UTR of YAP mRNA was inhibited by miR-484. We further performed function gain- and loss-experiments and found that treatment by miR-484 mimics resulted into decrease of endogenous YAP mRNA and protein levels in BxPc-3 and CFPAC-1 cells, whereas miR-484 inhibitor showed the contrary effect in PANC-1 cells (**Fig. 4G**; Supplementary Fig. S4D).

Given that miR-484 efficiently targets both *THAP9-AS1* and YAP, we wonder whether *THAP9-AS1* decoys the miR-484 from YAP to enhance YAP upregulation. We detected YAP expression change upon simultaneous interference of *THAP9-AS1* expression and miR-484 function in PDAC cells. We found that function loss of miR-484 by its inhibitor greatly overcame *THAP9-AS1* knockdown–induced decrease of YAP mRNA and protein levels in BxPc-3 cells (**Fig. 4H**; Supplementary Fig. S4E). On the contrary, induction of miR-484 by mimics markedly reversed *THAP9-AS1* overexpression–mediated increase of YAP mRNA and protein levels in PANC-1 cells (**Fig. 4H**; Supplementary Fig. S4E). Taken together, these data indicated that *THAP9-AS1* acts as a miR-484 sponge and attenuates the inhibitory effect of miR-484 on YAP, thereby resulting into increase of YAP expression in PDAC cells.

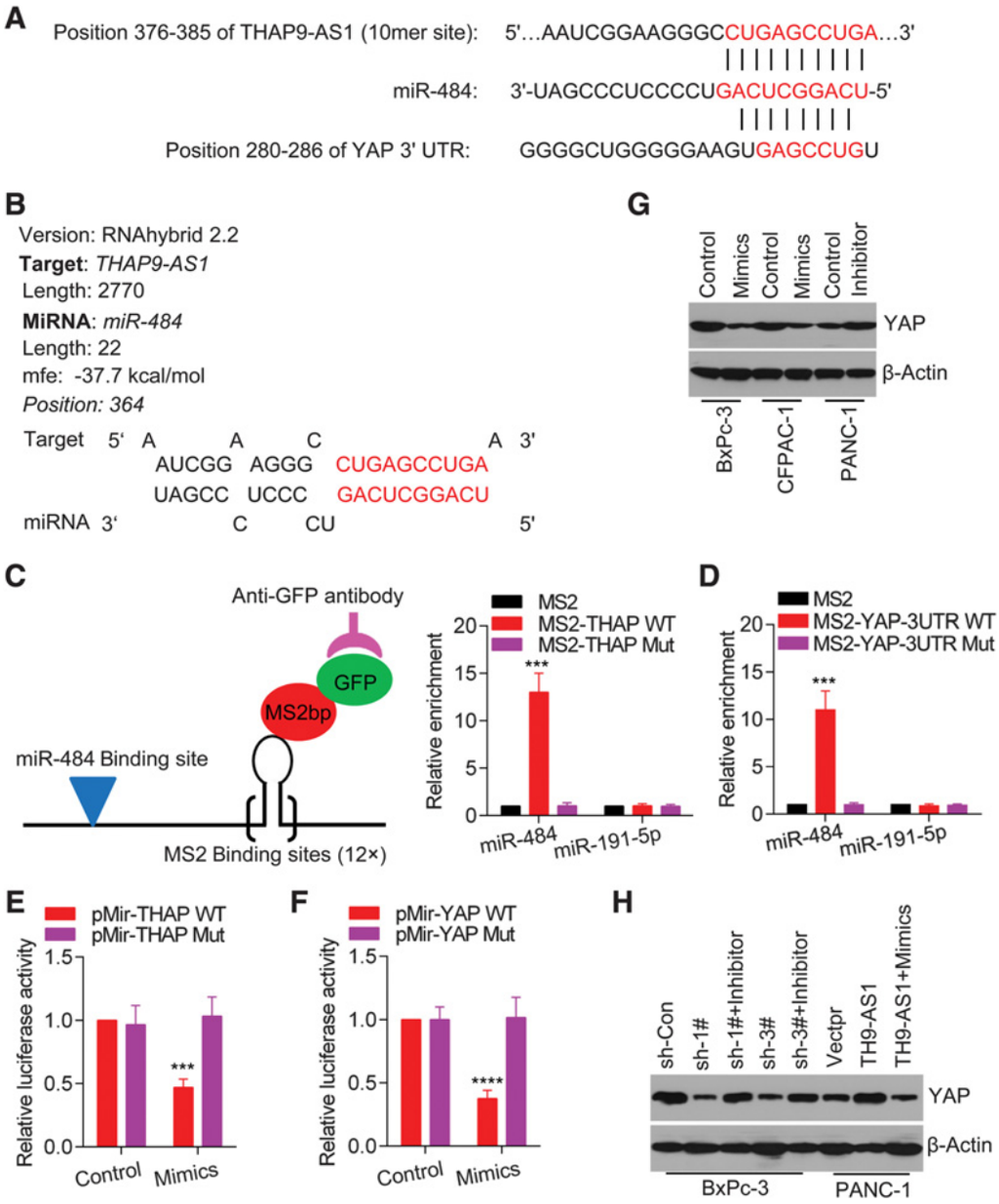

Figure 4.

THAP9-AS1 increases YAP expression by sponging miR-484. **A,** Illustration of the base pairing between *THAP9-AS1* and miR-484 predicted with DIANA tools (http://carolina.imis.athena-innovation.gr/diana_tools/web/index.php), and between miR-484 and YAP predicted by TargetScan (http://www.targetscan.org/vert_72/). **B,** The interaction between *THAP9-AS1* and miR-484 and the minimum free energy (mfe) was predicted with RNAhybrid software (https://bibiserv.cebitec.uni-bielefeld.de/rnahybrid). **C,** MS2-RIP followed by miRNAs qRT-PCR to detect the association between miRNAs and *THAP9-AS1*. **D,** MS2-RIP followed by miRNAs qRT-PCR to detect the association between miRNAs and YAP mRNA 3′-UTR. **E,** Luciferase activity indicated miR-484 targeting *THAP9-AS1*. **F,** Luciferase activity indicated miR-484 targeting YAP. **G** and **H,** Protein levels in indicated cell clones were detected by Western blot. Student *t* test, mean ± SD (***, $P < 0.01$; ****, $P < 0.001$).

THAP9-AS1 interacts with YAP to block LATS1-mediated YAP phosphorylation

Given that *THAP9-AS1* not only increased YAP expression but also its activity, and that lncRNAs can exert their effects through RNA–protein interactions, we asked whether *THAP9-AS1* also modulated YAP activity beyond expression regulation. Here, we used inhibitor of miR-484 to eliminate the expression interference on YAP protein level and to validate the role of *THAP9-AS1* on YAP activity. There were comparable protein levels of LAST1 in selected PDAC cell lines (**Fig. 3A**). Function inhibition of miR-484 with inhibitor endogenously increased levels of total YAP and phosphorylated YAP, which is possibly due to the stable LAST1 level in PANC-1 cells (**Fig. 5A**), while *THAP9-AS1* overexpression reduced YAP phosphorylation, but without influence on total YAP and

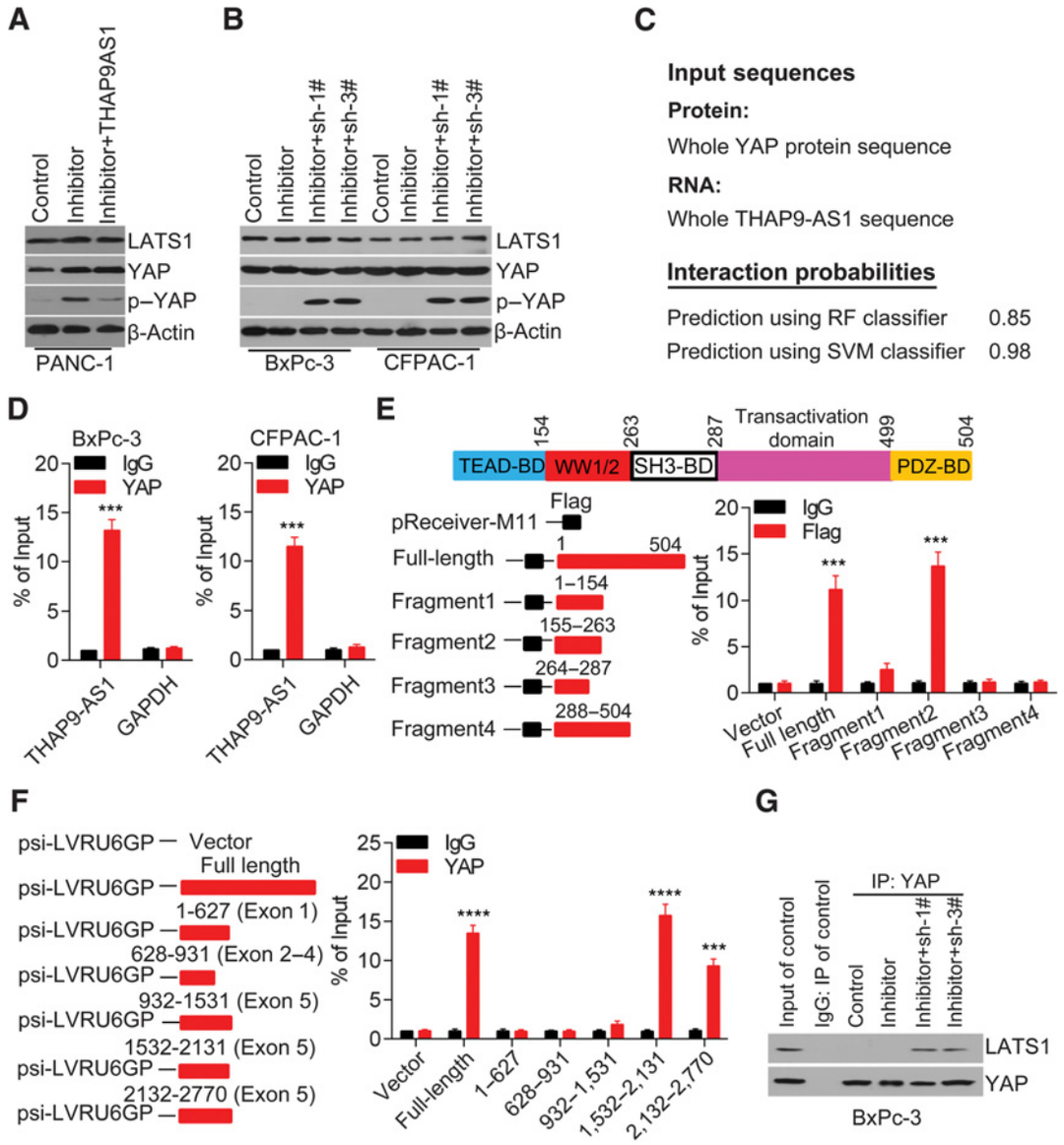

Figure 5.

THAP9-AS1 interacts with YAP to block LATS1-mediated YAP phosphorylation. **A** and **B,** Related protein levels in indicated cell clones were determined by Western blot. **C,** The interaction between THAP9-AS1 and YAP was predicted by online server RPISeq (http://pridb.gdcb.iastate.edu/RPISeq/index.html). **D,** RIP assay indicated the endogenous interaction between THAP9-AS1 and YAP protein in PDAC cells. **E,** Identify domain of YAP protein interacting with THAP9-AS1. The fragments of the YAP protein were illustrated (left); the interaction of YAP protein regions with THAP9-AS1 in HEK293T cells was confirmed by an RIP assay (right). **F,** Analysis regions of THAP9-AS1 interacting with YAP. Schematic diagram of THAP9-AS1 full-length and truncated fragments (left); the interaction of THAP9-AS1 truncated fragments with YAP in HEK293T cells was verified by an RIP assay (right). **G,** The interaction of YAP with LATS1 was verified by a co-IP assay. Student *t* test, mean ± SD (***, $P < 0.01$; ****, $P < 0.001$).

LAST1 levels (**Fig. 5A**). In *THAP9-AS1* highly expressed BxPc-3 and CFPAC-1 cell lines, to exclude the effect of miR-484 upon *THAP9-AS1* knockdown, inhibitor of miR-484 was applied. Transfection with inhibitor of miR-484 showed no significant effect on YAP and YAP phosphorylation, potentially due to the endogenous functional exhaustion of miR-484 by high *THAP9-AS1* level (**Fig. 5B**). However, *THAP9-AS1* knockdown increased YAP phosphorylation, but without influence on total YAP and LAST1 levels (**Fig. 5B**).

To access how *THAP9-AS1* regulates YAP activity, we first predicted that *THAP9-AS1* would physically interact with YAP protein by online tool RPISeq (RNA–protein interaction prediction) analysis via inputting protein and RNA sequences, respectively (**Fig. 5C**). Experimentally, we performed a RIP assay by using YAP antibody to pull down mRNAs interacting with YAP protein. RIP result presented that *THAP9-AS1* but not GAPDH mRNA could interact with YAP in PDAC cells (**Fig. 5D**). First, to identify the domain of YAP interacting with *THAP9-AS1*, we constructed truncated YAP mutants fused with

flag tag. In HEK293T cells cotransfected with YAP truncations and *THAP9-AS1*–overexpressing vectors, RIP assay using flag antibody unraveled that the WW1/2 domain of YAP (residues 155–263) was responsible for its interaction with *THAP9-AS1* (**Fig. 5E**). Second, to predict the region of *THAP9-AS1* responsible for interacting with YAP, a series of *THAP9-AS1* truncations were analyzed and the probabilities to interact with YAP were predicted (Supplementary Fig. S5A). To validate this hypothesis, a series of *THAP9-AS1* truncations were constructed to map its fragment interacting with YAP. The experimental results from RIP assay using YAP antibody solidly confirmed the interaction between YAP and *THAP9-AS1* at two sites within the 3′-region in HEK293T cells cotransfected with truncations of THAP-AS1 and YAP-overexpressing vectors (**Fig. 5F**). Third, the WW1/2 domain of YAP is required to interact with LATS1 to induce YAP phosphorylation and subsequent cytoplasmic retention. Thus, we speculated whether *THAP9-AS1* interaction with YAP blocks LATS1–YAP interaction. We performed coimmunoprecipitation (co-IP) assay and showed that *THAP9-AS1* knockdown facilitated the interaction between YAP and LATS1 upon the presence of miR-484 inhibitor to maintain the high endogenous YAP protein level in BxPc-3 PDAC cells (**Fig. 5G**). These results indicated that *THAP9-AS1* could interact with YAP to block its interaction with LATS1 and then promote its activity.

YAP/TEAD1 complex feedback transactivates *THAP9-AS1*

Given the high expression and role of *THAP9-AS1* in PDAC, and the involvement of regulatory feed-forward loops in tumor (23), we wonder whether *THAP9-AS1*–regulated YAP would have a feedback regulation for *THAP9-AS1* expression. Expectedly, *THAP9-AS1* levels were decreased upon YAP knockdown (**Fig. 6A**), while THAP9-AS1 levels were increased upon ectopic YAP overexpression in PDAC cells (**Fig. 6A**), indicating YAP might positively regulate *THAP9-AS1* transcription. YAP as a transcriptional coactivator without DNA-binding domains usually binds with transcription factors such as TEAD1-4 to modulate target genes expression. We performed bioinformatic analysis (The JASPAR database) to screen potential transcription factors that are located within a 3-kb region upstream of the *THAP9-AS1* transcription start site and identified a TEAD1 binding

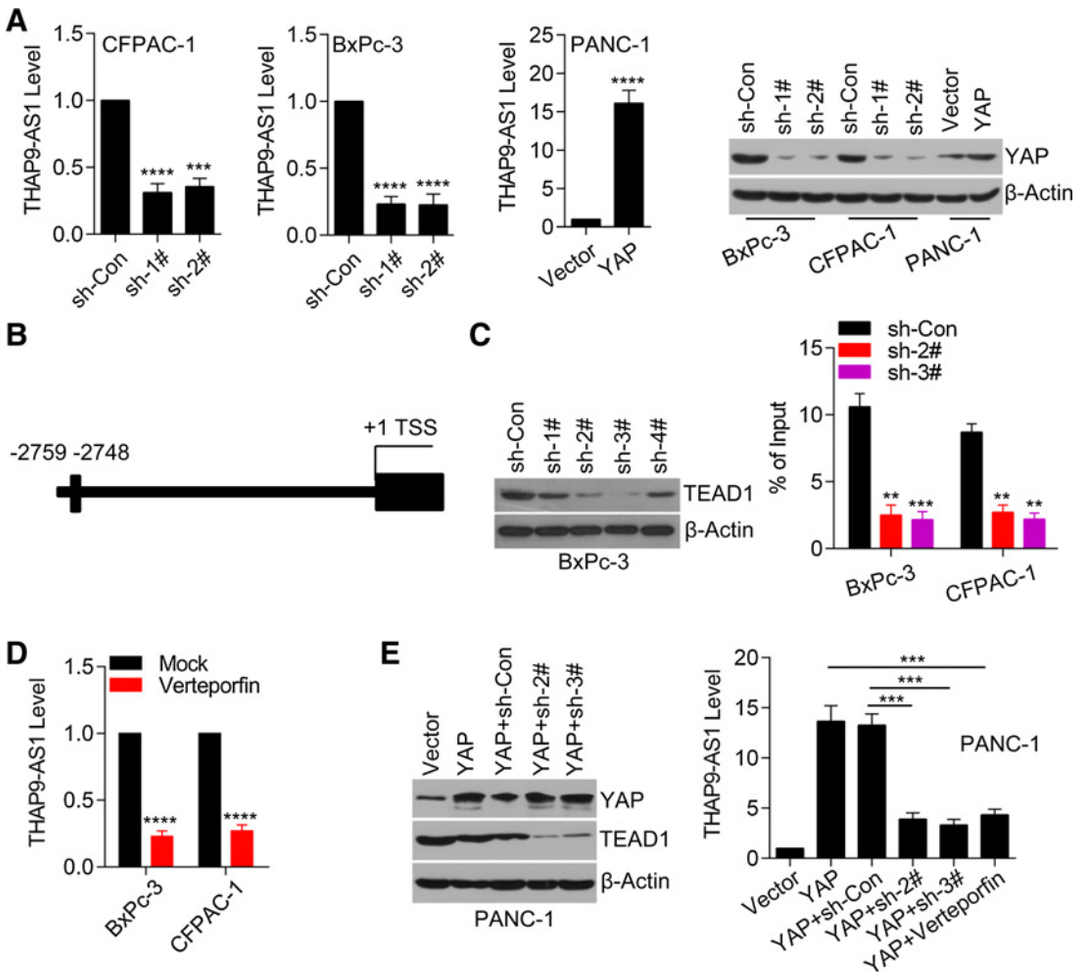

Figure 6.
YAP/TEAD1 complex feedback transactivates THAP9-AS1. **A,** The mRNA level was determined by qRT-PCR, and the protein level was determined by Western blot. **B,** The putative TEAD1 binding site on the potential promoter region of THAP9-AS1 was predicted by online server analysis (http://jaspar.binf.ku.dk). **C,** The enrichment of YAP on THAP9-AS1 promoter was determined with ChIP assay. **D,** The level of THAP9-AS1 transcript was detected by qRT-PCR. **E,** The protein level was determined by Western blot, and the mRNA level was determined by qRT-PCR. Student *t* test, mean ± SD (***, $P < 0.01$; ****, $P < 0.001$).

site at the potential promoter (**Fig. 6B**). Chromatin immunoprecipitation (ChIP) assay indicated the occupancy of YAP on *THAP9-AS1* promoter in PDAC cells (**Fig. 6C**). TEAD1 was consistently expressed in PDAC cells (**Fig. 3A**). Here, we found that TEAD1 knockdown reduced the enrichment of YAP on *THAP9-AS1* promoter (**Fig. 6C**). Also, treatment with verteporfin, a drug selectively disrupts YAP–TEAD interaction, significantly repressed *THAP9-AS1* transcription in BxPc-3 and CFPAC-1 cells (**Fig. 6D**). Moreover, TEAD1 knockdown disrupted the increase of *THAP9-AS1* expression induced by YAP overexpression (**Fig. 6E**). Verteporfin treatment also normalized the increased expression of *THAP9-AS1* induced by YAP overexpression in PANC-1 cells (**Fig. 6E**). These results indicated that YAP bound to *THAP9-AS1* promoter via TEAD1 and promoted *THAP9-AS1* transcription via a positive feedback regulatory loop in PDAC cells.

THAP9-AS1 promotes PDAC growth via YAP *in vivo* and correlates with poor outcome in patients with PDAC

To evaluate the biological and clinical consequences of the function abnormality of *THAP9-AS1* in cancer, we explored the role of *THAP9-AS1* in tumor growth. We implemented subcutaneous injection of BxPc-3 cells with *THAP9-AS1* knockdown and sh-Con cells into nude mice. *THAP9-AS1* knockdown repressed tumor volume growth and tumor weight growth, but restoration of YAP expression rescued the effect of *THAP9-AS1* knockdown on tumor growth *in vivo* (**Fig. 7A**).

To further define the role of *THAP9-AS1* clinically and to further verify its correlation with YAP in clinical samples, we measured *THAP9-AS1* and miR-484 expression via *in situ* hybridization (ISH) in a cohort of PDAC specimens ($N = 57$). A *THAP9-AS1* expression signal was detected in about 82% of the specimens. *THAP9-AS1*–positive samples also exhibited a signal in both cytoplasm and nucleus (**Fig. 7B**). Whereas, a miR-484 expression signal was detected in all the specimens (**Fig. 7B**). Moreover, we performed IHC staining of YAP in PDAC samples, which have been subjected to ISH analysis of *THAP9-AS1*. As expected, the expression of *THAP9-AS1* was positively correlated with YAP protein level (**Fig. 7C**). Importantly, either high *THAP9-AS1* or YAP protein level in PDAC significantly predicts a poor outcome of patients with PDAC (**Fig. 7D**). In addition, online analysis based on TCGA data (http://gepia.cancer-pku.cn/) also showed that THAP9-AS1 and YAP levels were negatively correlated with overall survival of patients with PDAC (**Fig. 7E**). These data indicate that *THAP9-AS1*/YAP axis is strongly correlated with PDAC tumor growth and poor outcome in patients with PDAC.

Discussion

In this study, we for the first time delineated the critical role of the lncRNA *THAP9-AS1* in PDAC. Our findings provided several advanced insights into the underlying mechanisms for PDAC growth: (i) *THAP9-AS1* is overexpressed in PDAC to (ii) promote PDAC growth via enhancing YAP activity; (iii) *THAP9-AS1* acts as a competing endogenous RNA (ceRNA) to upregulate YAP expression; (iv) *THAP9-AS1* interacts with YAP to block LATS1-mediated YAP inactivation; and (v) YAP/TEAD1 complex transcriptionally regulates *THAP9-AS1* expression to form a positive feedback loop in PDAC cells (**Fig. 7F**).

Emerging evidence has shown the important roles of lncRNAs in cancers, especially their effects on cancer growth and malignant transformation. Dysregulation of some lncRNAs have been reported to regulate important cancer biological processes, such as proliferation (24), metabolism (25), metastasis (26) and cancer cells stemness (15). Recent evidence suggests that lncRNAs can act as ceRNAs to regulate miRNAs, subsequently to regulate expression of target genes. For instance, LncRNA-ATB regulated by TGFβ activates the invasion-metastasis cascade through competitively binding miR-200s with ZEB1 and ZEB2 to inducing EMT process in hepatocellular carcinomas (14). HOTAIR promotes gastric cancer progression by sponging miR-331-3p to upregulate HER2 expression (27). Exosome-transmitted lncARSR functions as a sponge of miR-34/miR-449 to induce c-MET and AXL expression to mediate sunitinib resistance in renal cell carcinoma (28). In this study, we found that *THAP9-AS1* shared miR-484 response elements with YAP and facilitated YAP expression by sponging miR-484. YAP was experimentally validated to be a bona fide target of miR-484. Function inhibition of miR-484 effectively rescues the decreased expression of YAP mRNA and protein that are induced by *THAP9-AS1* knockdown in PDAC cells, indicating that *THAP9-AS1* acts as a ceRNA. Recent studies reported the important involvement of miR-484 in cancer progression. Reduced expression of miR-484 in cervical cancer promoted proliferation, invasion, and EMT process, due to upregulation of its targets ZEB1 and Smad2 (29). In colorectal cancer with MSI (microsatellite instability), miR-484 was decreased because of the CpG island methylation. miR-484 suppresses MSI colorectal cancer cell growth via inhibiting CD137L/IL8 axis (30). Our results also extended the regulatory mechanism for miR-484 function.

Our findings that *THAP9-AS1* knockdown increases the phosphorylation of YAP at serine 127 are new. In mammalian cells, the major components of the Hippo signaling pathway contain the transcriptional coactivator YAP, nuclear transcription factors TEAD1-4, and their upstream kinases (MST1/2 and LATS1/2; ref. 31). Beyond transcriptional regulation due to amplification (32), YAP is also tightly regulated posttranscriptionally by kinases-mediated degradation or cytoplasmic sequestration. In response to unfavorable extracellular- or intracellular signal, MST1/2 phosphorylates and activates LATS1/2. Activated LATS1/2 in turn phosphorylates YAP at serine 127, which as one of the most common posttranslational modifications providing the binding site for the 14–3-3 protein and resulting in cytoplasmic location of YAP to repress its activity (19). Otherwise, unphosphorylated YAP translocates into the nucleus and functions as a transcriptional coactivator of TEAD (33). Here, to confirm the function of *THAP9-AS1* on YAP protein, we used inhibitor of miR-484 to exclude the change of total YAP protein level. Our results indicated that *THAP9-AS1* negatively regulated the phosphorylation at YAP serine 127, irrespective of LATS1 level in PDAC cells. Recent evidence indicates that lncRNAs could directly bind to proteins to regulate proteins modification and activity. Bian and colleagues reported that FEZF1-AS1 could bind the PKM2 protein to increase cytoplasmic and nuclear PKM2 protein levels, resulting in increased aerobic glycolysis and STAT3 activation (25). Here, we identified the physical binding between *THAP9-AS1* and YAP protein. This binding prevents YAP from interaction and phosphorylation by LATS1. Our data indicated that *THAP9-AS1* regulated YAP signaling at two aspects: promoting expression and modifying function in PDAC cells. YAP as oncogene is known to be involved in cell proliferation, invasion, EMT, and metastasis. YAP activation has been shown to correlate with poor outcome in several cancer types (34). Evenly, YAP activation mediated the tumor relapse of PDAC with addiction to Kras oncogene (35). Furthermore,

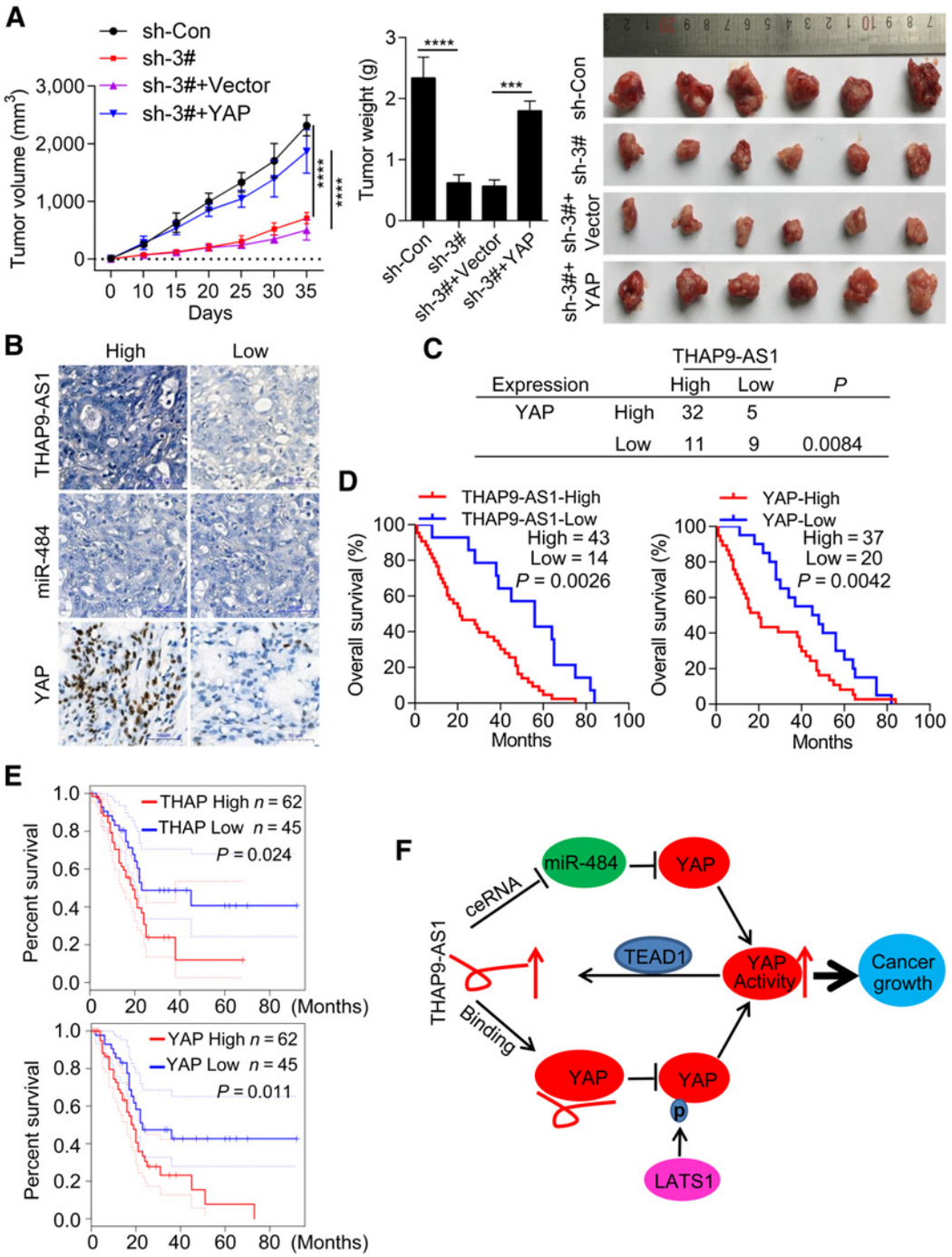

Figure 7.

THAP9-AS1 promotes PDAC growth via YAP *in vivo* and correlates with poor survival in patients with PDAC. **A,** THAP9-AS1 knockdown inhibited BxPc-3–derived tumor growth *in vivo*, but restored YAP expression disturbed the effect of THAP9-AS1 knockdown. **B,** THAP9-AS1 and miR-484 expression in PDAC tissues was examined by ISH assay (blue staining: THAP9-AS1; red staining: nucleus) and YAP expression was examined by IHC assay ($n = 57$). Scale bar, 50 μm. **C,** The correlation between THAP9-AS1 and YAP in PDAC tissues was analyzed. **D,** Kaplan–Meier analysis indicated a correlation between high THAP9-AS1 or YAP expression and poor overall survival in patients with PDAC. **E,** The negative correlation between YAP expression and overall survival was analyzed on the basis of TCGA data in patients with PDAC. **F,** The working model of function and mechanisms of THAP9-AS1 in PDAC.

we showed YAP could transcriptionally regulate *THAP9-AS1* expression via YAP/TEAD1 complex, forming a feed-forward loop to maintain YAP signaling.

In conclusion, our work presented here shows that as lncRNA *THAP9-AS1* is upregulated in PDAC, which is associated with poor clinical outcome. *THAP9-AS1* promotes PDAC growth via YAP signaling. *THAP9-AS1* promotes YAP activity by sponging miR-484 to upregulate YAP expression and by binding YAP protein to enhance its activation. Our findings provide insight into the *THAP9-AS1*/YAP axis as promising therapeutic target against PDAC, implying important translational implications. However, further studies should be performed to develop precise strategies targeting *THAP9-AS1*/YAP signaling.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: N. Li, H. Liu, W. Liu, G. Zheng
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): N. Li, G. Yang, L. Luo, X. Wang, L. Shi
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): N. Li, L. Luo, X. Jia, Z. Long, G. Zheng
Writing, review, and/or revision of the manuscript: N. Li, W. Hu, W. Liu, G. Zheng
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): G. Yang, L. Luo
Study supervision: J. Liu, Z. He, G. Zheng

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