P4HA2-induced prolyl hydroxylation suppresses YAP1-mediated prostate cancer cell migration, invasion, and metastasis

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Running title: The role of prolyl hydroxylation of YAP1 in prostate cancer

Key words: YAP1, prolyl hydroxylation, P4A2, prostate cancer, cell migration, invasion, metastasis

Conflict of interest: C. J. Logothetis reports receiving commercial research grants from Bayer, Sanofi, Janssen, Astellas Pharma, Pfizer; and honoraria from Bayer, Janssen, Sanofi, Astellas Pharma. No potential conflicts of interest were disclosed by the other authors.

ABSTRACT

Yes-associated protein 1 (YAP1), a key player in the Hippo pathway, has been shown to play a critical role in tumor progression. However, the role of YAP1 in prostate cancer cell invasion, migration, and metastasis is not well defined. Through functional, transcriptomic, epigenomic, and proteomic analyses, we showed that prolyl hydroxylation of YAP1 plays a critical role in the suppression of cell migration, invasion, and metastasis in prostate cancer. Knockdown (KD) or knockout (KO) of *YAP1* led to an increase in cell migration, invasion, and metastasis in prostate cancer cells. Microarray analysis showed that the EMT pathway was activated in *Yap1*-KD cells. ChIP-seq analysis showed that Yap1 target genes are enriched in pathways regulating cell migration. Mass spectrometry analysis identified P4H prolyl hydroxylase in the YAP1 complex and YAP1 was hydroxylated at multiple proline residues. Proline-to-alanine mutations of YAP1 isoform 3 identified proline 174 as a critical residue, and its hydroxylation suppressed cell migration, invasion, and metastasis. KO of *P4ha2* led to an increase in cell migration and invasion, which was reversed upon *Yap1* KD. Our study identified a novel regulatory mechanism of YAP1 by which P4HA2-dependent prolyl hydroxylation of YAP1 determine its transcriptional activities and its function in prostate cancer metastasis.

INTRODUCTION

Yes-associated protein 1 (YAP1), a key transcriptional coactivator in the Hippo pathway, has been shown to be an important driver promoting cancer development and progression in multiple cancer types (1). Although YAP1 plays an oncogenic role in various cancer types, multiple studies also support a tumor-suppressive function for YAP1 in cancers of various origins, such as head and neck cancers (2), breast cancer (3-5), hematological cancers (6), and colorectal cancer (7, 8). Thus, the functions of YAP1 are likely context dependent (9). YAP1 was shown to be overexpressed in prostate adenocarcinoma and is associated with cell proliferation and invasiveness in castration-resistant prostate cancer (10-12). However, YAP1 was found to be downregulated in the highly aggressive NEPC subset (12). Importantly, despite the low frequency of *YAP1* amplification observed in prostate cancer, *YAP1* homozygous deletion and mutation were also observed in a small subset of prostate cancers (1% or 23 out 5883: 4 primary tumors, and 19 metastatic tumors) as shown in cBioPortal (13) (**Suppl. Fig. 1A**). On the cellular level, YAP1 has been shown to promote prostate cancer cell proliferation through cell autonomous and non-autonomous mechanisms (11, 12, 14, 15), but its role in prostate cancer metastasis is not clearly defined.

In addition to regulating gene expression, post-translational modification of YAP1, such as phosphorylation and ubiquitination, has also been shown to regulate YAP1 cellular localization, stability, and activities (16, 17). Interestingly, we found that Yap1 proteins in prostate cancer cells are modified by proline hydroxylation. Proline hydroxylation is an important post-translational modification that modulates protein folding and stability in mammalian cells (18, 19). Proline hydroxylation is induced by prolyl hydroxylases, such as prolyl hydroxylase domain proteins (PHD) and collagen prolyl 4-hydroxylase (P4H). Whether

YAP1 is subjected to proline hydroxylation was previously unknown, as were the effects of such modification on YAP1 function. In this study, we identified a surprising role for YAP1 in the suppression of cell migration, invasion, and metastasis in prostate cancer. We found that YAP1 interacts with the P4H complex and is hydroxylated at multiple proline residues. The status of proline hydroxylation of YAP1 determines its oncogenic activity in prostate cancer cell migration, invasion, and metastasis.

RESULTS

YAP1 suppresses prostate cancer cell migration, invasion, and metastasis

Previously, we showed that Yap1 was highly expressed in primary tumors from the Pten/Smad4 prostate conditional knockout (KO) mouse model that developed distant metastasis (12). Given the highly metastatic nature of Pten/Smad4-deficient cancer cells (referred to as PS cells hereafter) (20), we first examined the effect of *Yap1* knockdown (KD) on cell migration, invasion, and metastasis in these cells. Western blot analyses confirmed the efficient KD of *Yap1* with two independent shRNAs (**Fig. 1A**). Surprisingly, *Yap1* KD dramatically enhanced cell migration and invasion (**Fig. 1B-C**). To validate this finding, we examined the effect of *Yap1* KO using the CRISPR/Cas9 system on the migration and invasion of PS cells. Western blot analysis confirmed the complete loss of Yap1 expression in the KO cells (**Fig. 1D**). Consistent with the findings from the shRNA KD experiments, *Yap1* KO led to a significant increase in cell migration and invasion (**Fig. 1E & Suppl. Fig. 1B**). Of note, *YAP1* KD or KO did not have a significant effect on cell proliferation as measured by the total number of cells at the end of the assays (**data not shown**), suggesting that the reduced cell migration and invasion were not due to a reduction in cell proliferation. Furthermore, re-expression of human *YAP1* in *Yap1*-KO cells

suppressed cell migration and invasion compared to the green fluorescent protein (GFP) control (**Fig. 1F-G & Suppl. Fig. 1C**). To demonstrate that these effects are not limited to PS cells, shRNAs were used to knock down *YAP1* in C4-2b cells, and similar effects on cell migration and invasion were observed (**Fig. 1H-J**). Moreover, *Yap1* KD in PS cells promoted metastasis, as shown by the enhanced colonization of cancer cells in the lung in a tail vein injection model (**Fig. 1K**). Taken together, our data suggest that YAP1 suppresses cell migration, invasion, and metastasis in prostate cancer cells.

Yap1 interacts with the prolyl 4-hydroxylase complex, and its proline residues are hydroxylated

To understand the mechanisms by which Yap1 suppresses cell migration, invasion, and metastasis, we performed microarray analysis of RNA isolated from PS cells in which *Yap1* was knocked down by doxycycline-inducible shRNA and the control cells. Gene set enrichment analysis (GSEA) (21) identified epithelial to mesenchymal transition (EMT) as the top pathway activated in *Yap1*-KD cells (**Fig. 2A**), consistent with our observed effects of Yap1 on cell migration, invasion, and metastasis. We then performed qPCR analyses and confirmed that the expression of several genes involved in EMT, including *Postn*, *Cdh11*, *Acta2*, *Rgs4*, and *Mgp*, were upregulated upon *Yap1* KD (**Suppl. Fig. 2A**).

Since YAP1 has been shown to act as a transcriptional coactivator that interacts with the TEAD transcription factor family, we sought to determine whether *Postn*, *Cdh11*, *Acta2*, *Rgs4*, and *Mgp* are direct target genes of YAP1. We performed ChIP-seq of endogenous Yap1 in PS cells and found that Yap1 binds mostly to the distant intergenic region and to other introns (**Fig. 2B**), which is consistent with previous reports (22). We then performed pathway analyses of the top 2000 Yap1 binding sites using Gene Ontology (GO) and the Kyoto Encyclopedia of Genes

and Genomes (KEGG). As expected, “Hippo signaling pathway” was identified in the KEGG pathway analysis (**Suppl. Fig. 2B**). In addition, multiple pathways related to cell migration were identified as top pathways, including “positive regulation of locomotion,” “positive regulation of cellular component movement,” “positive regulation of cell mobility,” “positive regulation of cell migration,” “focal adhesion,” and “axon guidance” (**Fig. 2C & Suppl. Fig. 2B**). Known motif analysis or de novo motif analysis using Homer (23) identified TEAD1 and AP1 motifs (**Suppl. Fig. 2C & data not shown**), which is consistent with the known physical and functional interaction between Yap1, TEAD, and AP1 (22). We then examined whether *Postn*, *Cdh11*, *Acta2*, *Rgs4*, and *Mgp* were direct target genes of YAP1. Indeed, YAP1 binds to the distant intergenic region upstream or downstream of *Postn*, *Cdh11*, *Acta2*, *Rgs4*, and *Mgp* (**Suppl. Fig. 2D & data not shown**). Additionally, we confirmed the binding of Yap1 to its known target genes, including *Cxcl5* and *Ccnd1* (**Suppl. Fig. 2E**).

Since the functions of Yap1 are regulated through its interacting partners, as well as by post-translational modifications (16, 17), we performed immunoprecipitation (IP) of Yap1 in PS cells followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis to identify Yap1-interacting proteins and novel post-translational modifications (**Fig. 2D**). As expected, we identified multiple proteins previously shown to interact with Yap1, such as Amot, Amotl1, Amotl2, Lats1, and 14-3-3 family proteins (**Fig. 2D**). Interestingly, proteins of the collagen prolyl 4-hydroxylase (P4H) complex, including P4ha1, P4ha2, and P4hb, were among the top Yap1-interacting proteins identified with mass spectrometry analysis (**Fig. 2D**). Collagen P4H, an α2β2 tetrameric α-ketoglutarate (α-KG)-dependent dioxygenase, catalyzes 4-hydroxylation of proline (18, 19). The P4H α subunit (P4HA) is responsible for both peptide binding and catalytic activity. Because P4ha2 was the most abundant protein of the P4H complex

that was pulled down in our IP-MS experiment, we focused on its interaction with Yap1. We performed co-immunoprecipitation (co-IP) of overexpressed Flag-YAP1 and P4HA2 in 293T cells to examine their interaction. As expected, YAP1 efficiently pull down P4HA2 in 293T cells (**Fig. 2E**). Also, endogenous Yap1 was found to interact with P4ha2 in PS cells (**Fig. 2F**).

Give the proline hydroxylase activity of the P4H complex (18, 19), we examined whether proline residues in Yap1 were hydroxylated. We identified nine proline residues (proline 60, 70, 105, 157, 159, 347, 353, 395, 398) of mouse Yap1 isoform 1 (NP_001164618) that were hydroxylated in PS cells (**Fig. 2G, Suppl. Fig. 3A & data not shown)**. Eight of these proline residues (proline 60, 70, 105, 157, 159, 353, 395, 398) were evolutionarily conserved between mouse and human (**Suppl. Fig. 3B**), suggesting that they might play a role in the regulation of Yap1 functions.

Hydroxylation at proline 174 of Yap1 plays a critical role in suppressing cell migration, invasion and metastasis but is dispensable for cell proliferation

Given that proline hydroxylation plays a critical role in the regulation of protein functions (18, 19), we decided to examine whether proline hydroxylation modulates YAP1 functions. We first generated hydroxylation-defective mutants of human YAP1 protein isoform 3 by mutating proline to alanine (YAP Mut1-3: P75/85/120A; Mut4-5: P172/174A; Mut6-9: P348/352/394/397A) (**Fig. 3A**). These YAP1 mutants were overexpressed in *Yap1*-KO PS cells to avoid the possible interference of the endogenous wild type (WT) YAP1. We first confirmed the protein expression levels of YAP1 WT and mutants were similar in western blot analysis (**Fig. 3B**). We then determined whether the proline-to-alanine mutations affect the proliferation of PS cells. We did not observe any significant difference in cell proliferation as shown by foci-

forming assay (**Suppl. Fig. 4A**) or *in vivo* growth (**Suppl. Fig. 4B)** between YAP1 WT and mutants.

Given that *Yap1* KD led to an increase in cell migration, invasion, and metastasis, we compared the effects of overexpression of YAP1 WT and mutants on these processes. We found that there is an increase in cell migration and invasion in YAP1 Mut4-5–overexpressing cells compared to YAP1 WT-overexpressing cells (**Fig. 3C-D**), suggesting that mutations of proline 172 and 174 to alanine abolished the activity of WT YAP1 in suppressing cell migration and invasion. Furthermore, we determined the effect of proline hydroxylation mutants on metastasis *in vivo* using tail vein injection. Consistent with the *in vitro* findings, PS cells with YAP1 Mut4-5 overexpression increased the colonization of cancer cells in the lung compared to WT, Mut1-3, and Mut6-9 (**Fig. 3E & Suppl. Fig. 4C**).

To determine whether prolyl hydroxylation controls the transcriptional activities of YAP1, we examined the expression of YAP1 target genes *Ccnd1*, *Mcm6*, *Cxcl1*, and *Cxcl5* in *Yap1*-KO cells that overexpressed GFP, YAP1 WT, YAP1 Mut4-5 or the constitutively active S127A mutant (24). Both YAP1 Mut4-5 and the constitutively active S127A mutant dramatically increased the expression of these three genes compared to the YAP1 WT (**Fig. 3F**), suggesting that the non-hydroxylated YAP1 is more transcriptionally active than the hydroxylated YAP1.

To further pinpoint which proline residue of YAP1 is critical for its function in suppressing cell migration and invasion, we generated site-specific proline-to-alanine mutants of YAP1 (P172A and P174A mutants). We overexpressed GFP control, YAP1 WT, and YAP1 mutants (YAP1 Mut4-5: P172/174A; Mut4: P172A; Mut5: P174A) in *Yap1*-KO cells (**Fig. 3G**) and examined their effects on cell migration and invasion. We found that both YAP1 Mut4-5 and YAP1 Mut5, but not the Mut4, dramatically increased cell migration and invasion compared to

the GFP control (**Fig. 3H-I)**, suggesting proline 174 is the critical hydroxylation site that regulates YAP1 activity in cell migration and invasion. Importantly, overexpression of Mut4-5 and Mut5 similarly increased the cell migration of PC3 and TRAMPC2 cells (**Suppl. Fig. 4D**). Taken together, our data suggest that hydroxylation at proline 174 of YAP1 plays a critical role in the regulation of cell migration, invasion, and metastasis.

Loss of P4ha2 promotes cell migration and invasion through Yap1

Given the observed hydroxylation of Yap1 in PS cells (**Fig. 2G**) and the effect of hydroxylation-defective YAP1 mutants on migration and invasion (**Fig. 3C-I**), the ability of Yap1 to suppress cell migration and invasion appeared to be regulated by the P4H complex. To examine the effect of *P4ha2* KO on cell migration and invasion, we first generated *P4ha2* KO in PS cells (**Fig. 4A**). We found that *P4ha2* KO significantly increased cell migration and invasion compared to WT cells (**Fig. 4B-C**). The increase in cell migration and invasion was not due to an increase in cell proliferation, as there was no difference in cell numbers between *P4ha2*-KO cells and *P4ha2* WT cells (**data not shown**). Given that P4ha2 may have multiple substrates, we examined whether the increase in cell migration and invasion in *P4ha2*-KO cells was mediated through Yap1. We found that KD of *Yap1* in *P4ha2*-WT cells increased cell migration and invasion as expected (**Fig. 4D-E & Suppl. Fig. 4E**). However, KD of *Yap1* in *P4ha2*-KO cells abolished the effect of *P4ha2* KO on cell migration and invasion (**Fig. 4D-E & Suppl. Fig. 4E**). Taken together, our data indicate that P4ha2 suppresses cell migration and invasion through proline hydroxylation of Yap1.

DISCUSSION

We have shown that prolyl hydroxylation is a novel post-translational modification of YAP1 , which regulates YAP1's function in cell migration, invasion, and metastasis. We showed that the proline 174 hydroxylation-defective mutant of YAP1 is more transcriptionally active than the WT and is comparable to the constitutively active S127A mutant. Interestingly, proline 174 is within the first WW domain of YAP1, which has been shown to be crucial for the transcriptional co-activation and oncogenic activities of YAP1 through its interaction with transcription factors that contain PPxY motifs (25). Thus, our findings suggest that prolyl hydroxylation at P174 of Yap1 may impair its interaction with transcription factors that are critical for its transcriptional activities, such as c-JUN (22), resulting in a decrease in transcriptional activation of its target genes. Alternatively, YAP hydroxylation may be required for the efficient recruitment of the transcription corepressor complexes, such as NuRD (26). YAP1 is known to be regulated by phosphorylation at multiple serine residues (27). Our studies shed light on a new aspect of YAP1 regulation, which may also be involved in many YAP1-mediated cellular activities yet to be identified.

In contrary to previous findings that KD of *YAP1* in LNCaP-C4-2 cells impaired cell migration and invasion, we demonstrated that KD or KO of *Yap1* in mouse (PS and TRAMPC2) and human (C4-2b and PC3) prostate cancer cells led to enhanced cell migration, invasion, and metastasis. The discrepancy between our study and the previous one is not clear. In our studies, we used *YAP1* KD by shRNA, KO by CRISPR/Cas9, and re-expression of *YAP1* in *Yap1*-KO cells in multiple cell lines to study YAP1's effect on cell migration, invasion, and metastasis. These findings were further supported by microarray analysis showing YAP1 suppressed EMT and by ChIP-seq analysis showing that Yap1 target genes were strongly associated with pathways regulating cell migration. Thus, our data strongly suggest that YAP1 suppresses cell

migration, invasion, and metastasis in this subset of prostate cancer cells. However, we cannot rule out the possibility that YAP1 may have distinct roles in different cell lines, which may be due to diverse regulation of YAP1 in different cellular contexts.

The P4H α subunit (P4HA) has three isoforms (P4HA1-3) in mammalian cells. We confirmed the physical interaction between P4HA2 and YAP1 and demonstrated that the function of P4HA2 in the suppression of cell migration and invasion is in large part mediated through YAP1. Since both P4ha1 and P4ha2 were identified as Yap1-interacting proteins in our MS analysis, it is possible that P4ha1 also suppresses cell migration, invasion, and metastasis through prolyl hydroxylation of Yap1. However, P4HA1 was previously shown to promote prostate cancer progression (28), and further studies are needed to clarify the role of P4HA1 in the regulation of YAP1 functions and cell migration, invasion, and metastasis. Due to the lack of an antibody that can specifically recognize the hydroxylated proline 174 of YAP1, we cannot assess the clinical relevance of prolyl hydroxylation of YAP1 in tumor samples from prostate cancer patients. The development of such antibodies is warranted, and would allow us to examine the association of prolyl hydroxylation of YAP1 and P4HA expression in prostate cancer specimens.

In summary, our findings support a model in which P4HA2-mediated prolyl hydroxylation serves as a molecular switch that controls the activities of YAP1 in cell migration, invasion, and metastasis (**Fig. 4F**).

MATERIALS AND METHODS

Cell lines

293T, TRAMPC2, RM-1, C4-2b, and PC3 were obtained from ATCC, Manassas, VA, USA. 293FT was obtained from Thermo Fisher Scientific, Waltham, MA. Castration-resistant Pten/Smad4-deficient (*Pten*$^{pc-/-}$*Smad4*$^{pc-/-}$) prostate cell line has been described previously (20).

Lentivirus-mediated shRNA knockdown, CRISPR/Cas9 genome editing, and open reading frame overexpression

All lentiviral shRNA clones targeting Yap1, P4ha2, and nontargeting shRNA control were obtained from Sigma Aldrich in the pLKO vector (29). The TRC clone IDs for the shRNA used in this study are as follows: non-targeting control 202 (SHC202); mouse Yap1 sh#432 (TRCN0000238432); mouse Yap1 sh#434 (TRCN0000238434); human YAP1 sh#265 (TRCN0000107265); human YAP1 sh#325 (TRCN0000300325). Inducible Yap1 for targeting mouse Yap1 using TRCN0000238432 has been described previously (12). sgRNAs for Yap1 and P4ha2 were cloned into lentiCRISPR v2 plasmid (Addgene #52961) as described (30) using the following the sgRNAs: *Yap1:* CACCGAGTCGGTCTCCGAGTCCCCG; *P4ha2*#1, GGCAGCTGATCTGCTGGTCA; *P4ha2*#2, CCGCAGCTGGGCCAGCAAGA. pENTR-3xFLAG-YAP1-S127A (31) (human YAP1 isoform 3) was a gift from William Pu (Addgene plasmid#42239; http://n2t.net/addgene:42239; RRID:Addgene_42239). The described *Yap1* proline-to-alanine mutants were generated by Gibson Assembly Master Mix (NEB) using the pENTR-3xFLAG-YAP1 as a template. YAP1 WT and mutant open reading frames (ORFs) and human P4HA2 ORF (clone ID: OHS5894-202498515) were cloned into lentiviral plasmid pHAGE-EF1a-IRES-GFP (32) and pcDNA-DEST40 using Gateway LR Clonase II Enzyme mix (Thermo Fisher Scientific). Lentivirus (shRNA, ORF, CRISPR/cas9) was generated to transduce cancer cells as described (12).

Western blotting, immunoprecipitation, and liquid chromatography-tandem mass spectrometry (LC-MS/MS)

For Western blot analysis, cells were lysed on ice using RIPA buffer (Boston BioProducts) supplemented with protease and phosphatase inhibitors (Roche). For immunoprecipitation, cells were lysed on ice using Pierce IP Lysis Buffer (Thermo Fisher Scientific). To identify Yap1-interacting proteins and post-translational modifications, the immunoprecipitates from control IgG and anti-YAP1 antibody were subjected to SDS-PAGE, followed by silver staining, and liquid chromatography-tandem mass spectrometry (LC-MS/MS) as described (33). The antibodies used in this study were listed in the supplementary materials and methods.

Foci-formation, transwell migration, and invasion assay

For foci-formation assay, 1×10^3 cells were seeded in 6-well plates and cultured for 5 to 7 days before they were fixed and stained with crystal violet. Cell migration and invasion assays were carried out with Falcon Permeable Support for 24-well Plate with 8.0 µm Transparent PET Membrane (Corning) and BioCoat Matrigel Invasion Chambers with 8.0 µm PET Membrane (Corning). Briefly, 5×10^4 cells in 500 µL serum-free media were seeded in the inserts and 750 µL media containing 10% FBS was added to the well as the chemoattractant. Migrated and invaded cells were fixed and stained with crystal violet after 16 hours. Quantification of migrated and invaded cells was performed using ImageJ.

Subcutaneous implantation, tail vein injection, and bioluminescence imaging

For subcutaneous implantation, $1x10^6$ cells were injected into the flank of male nude mice. For tail vein injection, $1x10^5$ cells labeled with luciferase were injected into nude mice, followed by bioluminescence imaging using D-luciferin (Gold Biotechnology). The relative luciferase signal was quantified using Living Image (Perkin Elmer).

mRNA expression analysis and microarray

RNAeasy Kit (Qiagen) was used to isolate RNA from cells. iScript cDNA Synthesis Kit (Bio-Rad) was used to generate cDNA for quantitative PCR (qPCR) analysis using PerfeCTa reg SYBR reg Green SuperMix Reaction Mixes (QuantaBio). The primers used for qPCR analysis are listed in the supplementary materials and methods. For microarray analysis, Pten/Smad4-deficient cells infected with inducible Yap1 or control shRNA targeting luciferase were treated with doxycycline for 48 hours before total RNA was isolated for microarray analysis with Mouse Genome 430 2.0 Array (Thermo Fisher Scientific). The raw data have been submitted to the NCBI Gene Expression Omnibus (GEO). Differentially expressed genes were identified by Transcriptome Analysis Console (TAC) Software. GSEA was performed using GenePattern as described (34).

Chromatin-immunoprecipitation sequencing (ChIP-seq)

ChIP-seq was performed in Pten/Smad4-deficient cells as described (35) using anti-Yap1 antibody (Novus, NB110-58358). Libraries were prepared using NEBNext Ultra DNA Library Kit (E7370). Sequencing was performed using an Illumina HiSeq 2500 instrument. Reads were aligned to a reference genome (UCSC mm9). Homer package was used to perform ChIP-seq data analysis as described (23). Top 2000 peaks were selected for GO and KEGG analysis. The raw data has been submitted to the NCBI Gene Expression Omnibus (GEO).

Statistical analysis

Data are presented as mean ± SD unless indicated otherwise. Student's t test assuming two-tailed distributions was used to calculate statistical significance between groups. **** $P < 0.0001$, *** $P < 0.001$; ** $P < 0.005$; * $P < 0.05$.

ACKNOWLEDGEMENTS

We thank Ronald DePinho and Haoqiang Ying for helpful discussions and critical reviews and Sarah E. Townsend for editing the manuscript. G.W. is supported by the Prostate Cancer Moon Shot, Institutional Research Grant (IRG), and Prostate Cancer Research Program at the University of Texas MD Anderson Cancer Center. G.W. is also supported by the University of Texas STARs Award, the National Institutes of Health (NIH) R00 CA194289 and Prostate Cancer SPORE (P50 CA140388). Supported by the NIH/NCI under award number P30CA016672 and used the Research Animal Support Facility - Houston (RASF-H), Flow Cytometry and Cellular Imaging Core Facility, and Functional Genomics Core.

COMPETING INTERESTS

C. J. Logothetis reports receiving commercial research grants from Bayer, Sanofi, Janssen, Astellas Pharma, Pfizer; and honoraria from Bayer, Janssen, Sanofi, Astellas Pharma. No potential conflicts of interest were disclosed by the other authors.

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FIGURE LEGENDS

Figure 1. YAP1 suppresses cell migration, invasion, and metastasis in prostate cancer. (**A**) WB analysis of Yap1 expression in PS cells transduced with control shRNA (shCon) and *Yap1* shRNAs. (**B-C**) Cell migration and invasion assay using PS cells transduced with control shRNA and *Yap1* shRNAs. (**D**) WB analysis of YAP1 expression in *Yap1*-WT and -KO PS cells. (**E**) Cell migration assay using *Yap1*-WT PS cells and *Yap1*-KO cells. (**F**) WB analysis of YAP1 re-expression in *Yap1*-KO PS cells. (**G**) Cell migration assay using *Yap1*-KO cells with GFP overexpression and YAP1 overexpression. (**H**) WB analysis of YAP1 in C4-2b cells transduced with control shRNA and *YAP1* shRNAs. (**I-J**) Cell migration and invasion assay using C4-2b cells transduced with control shRNA and *YAP1* shRNAs. (**K**) Luciferase imaging in mice injected with PS cells transduced with control shRNA and *Yap1* shRNAs through the tail vein.

Figure 2. Microarray, ChIP-seq, and immunoprecipitation-mass spectrometry analyses. (**A**) GSEA analysis of microarray data from PS cells transduced with doxycycline-inducible Yap1 shRNA identified EMT as the top pathway activated in *Yap1*-KD cells. (**B-C**) ChIP-seq analysis identified Yap1 binding sites and Yap1-regulated pathways. (**D**) Immunoprecipitation-mass spectrometry analysis identified known Yap1-interacting proteins and novel Yap1-interacting proteins. (**E**) Exogenous YAP1 interacts with exogenous P4HA2 when overexpressed in 293T cells by transfection of the indicated plasmids for co-immunoprecipitation experiments. (**F**) Endogenous Yap1 interacts with endogenous P4ha2 in PS cells. (**F**) Multiple prolyl hydroxylation sites were identified in peptides of Yap1 isoform 3 from the LC- MS/MS analysis.

Figure 3. Prolyl hydroxylation of Yap1 suppressed cell migration, invasion, and metastases. (**A**) Scheme showing the strategy to generate prolyl hydroxylation–defective YAP1 mutants by mutating proline to alanine (PA): Mut1-3 (P75/85/120A), Mut4-5 (P172/174A), and Mut6-9

(P348/352/394/397). (**B**) Expression of YAP1 WT and PA mutants in Yap1-KO PS cells. (**C-D**) Cell migration and invasion assay in *Yap1*-KO PS cells with overexpression of YAP1 WT and PA mutants. (**E**) Tail vein injection of *Yap1*-KO cells with overexpression of Yap1 WT and PA mutants. (**F**) qPCR analysis of Yap1 target genes in *Yap1*-KO PS cells with overexpression of Yap1 WT, Mut4-5, and the constitutively active S127A mutant. (**G**) WB analysis of YAP1 in *Yap1*-KO PS cells with overexpression of GFP, YAP1 WT, Mut4-5, Mut4, and Mut5. (**H-I**) Cell migration and invasion assay using *Yap1*-KO PS cells with overexpression of GFP, YAP1 WT, Mut4-5, Mut4, and Mut5.

Figure 4. P4ha2 suppresses cell migration and invasion through Yap1. (**A**) WB analysis of P4ha2 in *P4ha2*-WT and *P4ha2*-KO PS cells. (**B-C**) Cell migration and invasion using *P4ha2*-WT and *P4ha2*-KO PS cells. (**D**) WB analysis of Yap1 in *P4ha2*-WT and *P4ha2*-KO PS cells transduced with Yap1 shRNAs. (**E**) Cell invasion assay in *P4ha2*-WT and *P4ha2*-KO PS cells transduced with shYap1#434. (**F**) A model for hydroxylation-dependent YAP1 function in cell migration, invasion, and metastasis (Created with BioRender.com). Left: P4HA2-mediated hydroxylation of YAP1 may impair its interactions with transcription factors such as JUN or enhance the recruitment of corepressor complex NuRD, which results in a decrease in the expression of genes involved in cell migration, invasion, and metastasis. Right: In the absence of P4HA2, non-hydroxylated YAP1 may efficiently interact with transcription factors such as JUN to activate genes involved in cell migration, invasion, and metastasis.

Figure 1

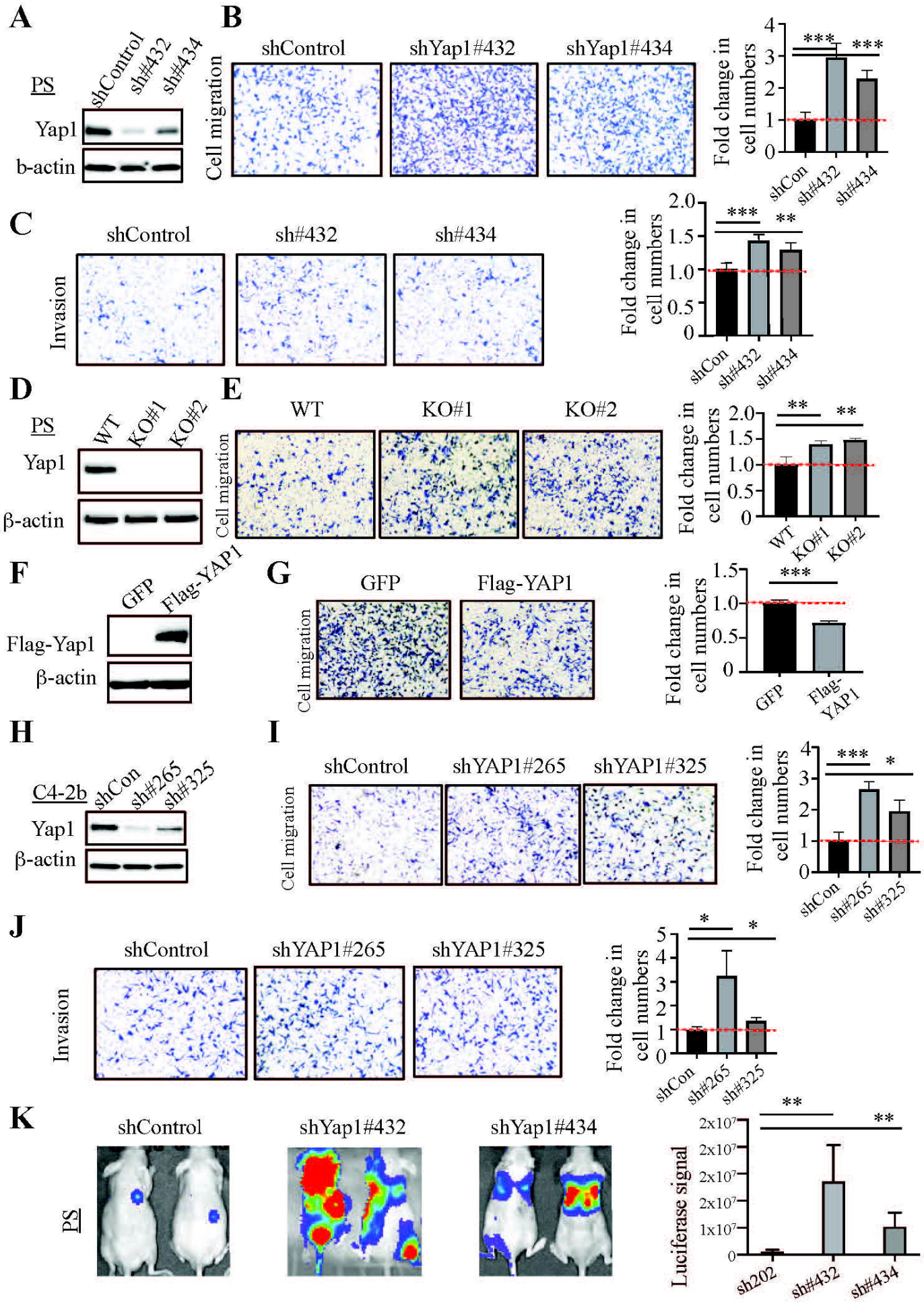
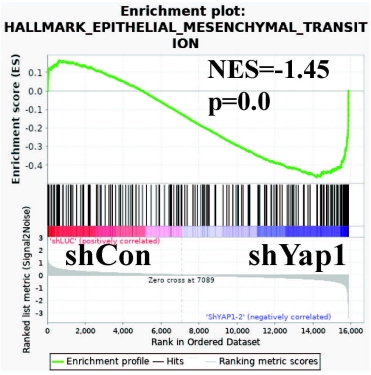

A
PS
shControl
sh#432
sh#434
Yap1
b-actin
B
shControl
shYap1#432
shYap1#434
Cell migration
Fold change in cell numbers
shCon
sh#432
sh#434
C
shControl
sh#432
sh#434
Invasion
D
PS
WT
KO#1
KO#2
Yap1
β-actin
E
WT
KO#1
KO#2
Cell migration
F
GFP
Flag-YAP1
Flag-Yap1
β-actin
G
GFP
Flag-YAP1
H
C4-2b
shCon
sh#265
sh#325
Yap1
β-actin
I
shControl
shYAP1#265
shYAP1#325
J
K
shControl
shYap1#432
shYap1#434
PS
Luciferase signal
sh202

Figure 2

A



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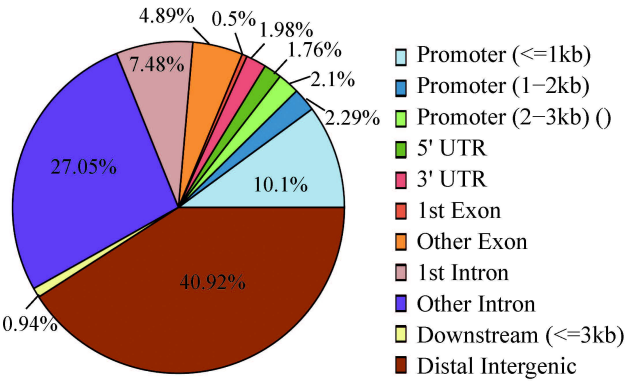

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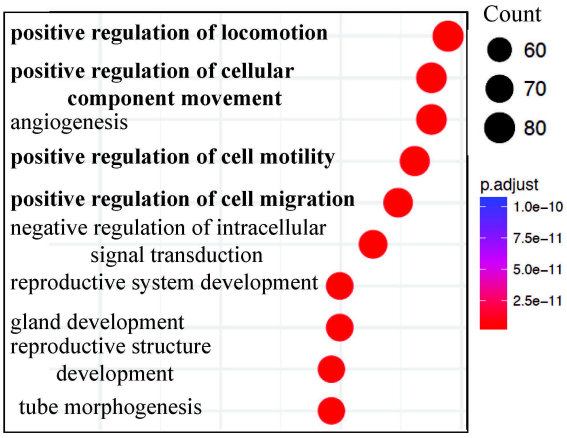

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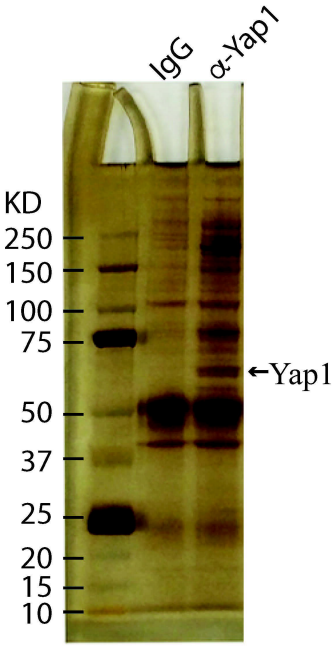
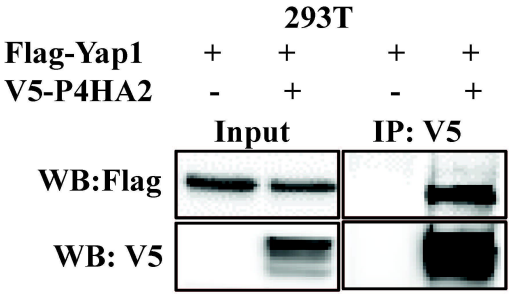


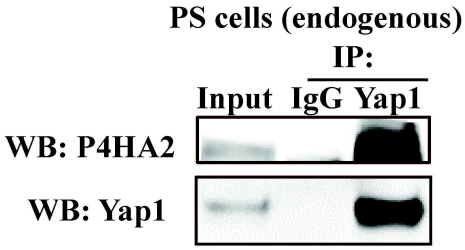
Pulldown peptide #

	IgG.	IP: Yap1	
Yap1	0	133	Known Yap1 interactors
Amot	2	75	
Amotl2	0	58	
Amotl1	0	27	
Lats1	0	11	
Ywhag	1	24	
Ywhab	0	20	
Ywhah	0	19	
Ywhaz	0	19	
Ywhae	2	14	
P4ha2	1	53	P4H complex
P4hb	4	31	
P4ha1	0	13	

E

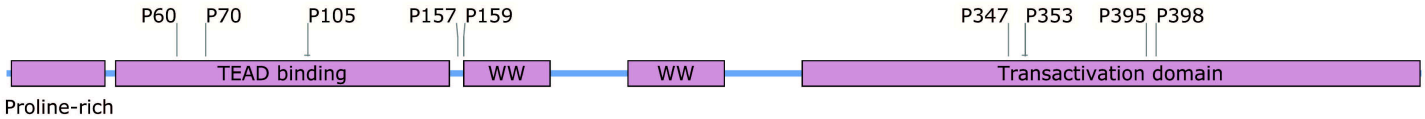


F



G

Yap1 mouse isoform 1 (NP_001164618)



Peptides identified from LC-MS/MS

Proline 60: GDSETDLEALFNAVMN**p**K
Proline 70: TANV**p**QTVPMRLR
Proline 105: QASTDAGTAGALT**p**QHVR
Proline 157/159: QSSFEIPDDV**p**L**p**AGWEMAK
Proline 347/353: SQLPTLEQDGGT**p**NAVSS**p**GMSQELR
Proline 395: DESTDSGLSMSSYSI**p**R
Proline 398: T**p**DDFLNSVDEMDTGDTISQSTLPSQQSR

Figure 3

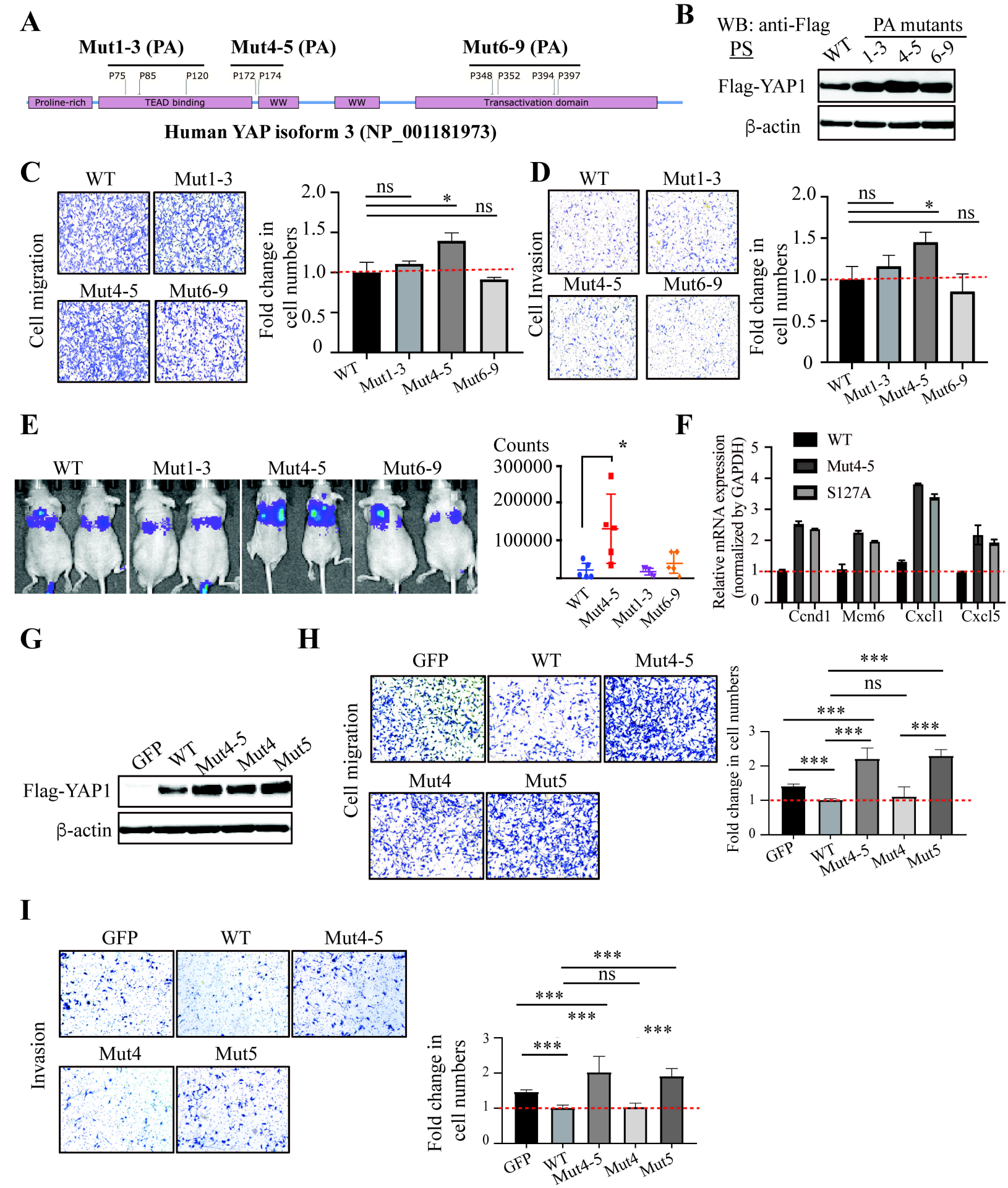
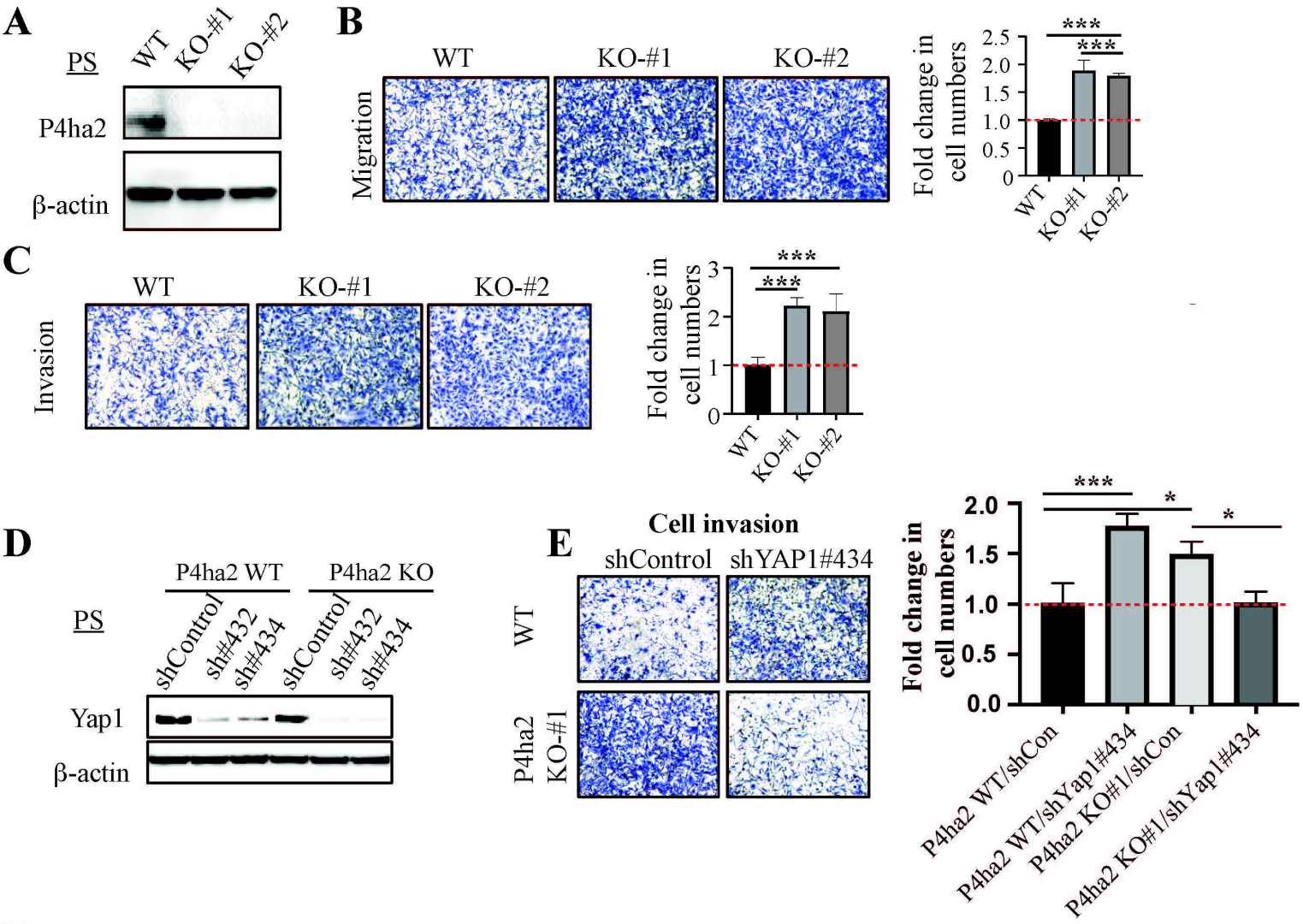
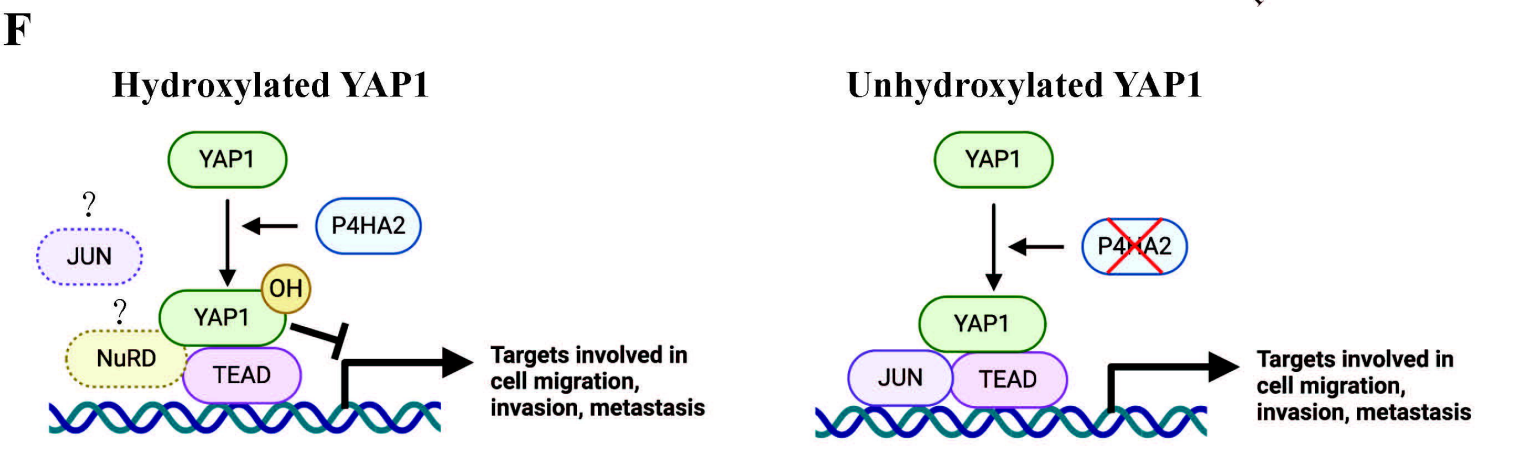

A
Mut1-3 (PA)
Mut4-5 (PA)
Mut6-9 (PA)
P75 P85 P120
P172 P174
P348 P352 P394 P397
Proline-rich
TEAD binding
WW
WW
Transactivation domain
Human YAP isoform 3 (NP_001181973)
B
WB: anti-Flag
PA mutants
PS
WT
1-3
4-5
6-9
Flag-YAP1
β-actin
C
WT
Mut1-3
Mut4-5
Mut6-9
Cell migration
Fold change in cell numbers
ns
*
ns
D
Cell Invasion
E
Counts
F
Relative mRNA expression (normalized by GAPDH)
WT
Mut4-5
S127A
Ccnd1
Mcm6
Cxcl1
Cxcl5
G
GFP
Mut4
Mut5
H
I
Invasion
***

Figure 4


A
PS
WT
KO-#1
KO-#2
P4ha2
β-actin
B
WT
KO-#1
KO-#2
Migration
Fold change in cell numbers
2.5
2.0
1.5
1.0
0.5
0
***
***
WT
KO-#1
KO-#2
C
WT
KO-#1
KO-#2
Invasion
Fold change in cell numbers
3
2
1
0
***
***
WT
KO-#1
KO-#2
D
P4ha2 WT
P4ha2 KO
PS
shControl
sh#432
sh#434
shControl
sh#432
sh#434
Yap1
β-actin
E
Cell invasion
shControl
shYAP1#434
WT
P4ha2 KO-#1
Fold change in cell numbers
2.0
1.5
1.0
0.5
0.0
***
*
*
P4ha2 WT/shCon
P4ha2 WT/shYap1#434
P4ha2 KO#1/shCon
P4ha2 KO#1/shYap1#434


F
Hydroxylated YAP1
YAP1
P4HA2
?
JUN
OH
?
YAP1
NuRD
TEAD
Targets involved in cell migration, invasion, metastasis
Unhydroxylated YAP1
YAP1
P4HA2
YAP1
JUN
TEAD
Targets involved in cell migration, invasion, metastasis