Androgen attenuates the inactivating phospho–Ser-127 modification of yes-associated protein 1 (YAP1) and promotes YAP1 nuclear abundance and activity

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Abstract

The transcriptional coactivator yes-associated protein 1 (YAP1) regulates cell proliferation, cell–cell interactions, organ size, and tumorigenesis. Posttranscriptional modifications and nuclear translocation of YAP1 are crucial for its nuclear activity. The objective of this study was to elucidate the mechanism by which the steroid hormone androgen regulates YAP1 nuclear entry and functions in several human prostate cancer cell lines. We demonstrate that androgen exposure suppresses the inactivating posttranslational modification phospho–Ser-127 in YAP1, coinciding with increased YAP1 nuclear accumulation and activity. Pharmacological and genetic experiments revealed that intact androgen receptor signaling is necessary for androgen's inactivating effect on phospho–Ser-127 levels and increased YAP1 nuclear entry. We also found that androgen exposure antagonizes Ser/Thr kinase 4 (STK4/MST1) signaling, stimulates the activity of protein phosphatase 2A (PP2A), and thereby attenuates the phospho–Ser-127 modification and promotes YAP nuclear localization. Results from quantitative RT-PCR and CRISPR/Cas9-aided gene knockout experiments indicated that androgen differentially regulates YAP1-dependent gene expression. Furthermore, an unbiased computational analysis of the prostate cancer TCGA data revealed that *YAP1* and *AR* transcript levels correlate with each other in prostate cancer tissues. These findings indicate that androgen regulates YAP1 nuclear localization and its transcriptional activity through the AR–STK4/MST1–PP2A axis, which may have important implications for human diseases such as prostate cancer.

Introduction

YAP1 (yes-associated protein 1) and its paralog WWTR1 (WW domain-containing protein) are transcriptional coactivators (1,2). YAP1 is a well-characterized nuclear effector of the Hippo pathway in mammals (2-4). The STK4/MST1, STK3/MST2, and LATS1/2 protein kinases are core components of the Hippo pathway (5). YAP1 was initially identified from the protein complexes of the Src family kinases (6). YAP1 regulates diverse cellular activities, including cell proliferation, cell survival, cell differentiation, stem cell maintenance, cell-cell interaction, organ size, and tumorigenesis (2). Nuclear localization of YAP1 is critical for its transcriptional-dependent biological functions, even though a majority of YAP1 proteins are present in the cytoplasm (7,8). YAP1 exerts its transcriptional-dependent biological activity by interacting with transcription factors. The family of the TEAD transcription factors is a critical mediator of the YAP1-dependent gene transcription. Mounting evidence indicates that interaction between the YAP1 and TEAD proteins are mutual because YAP1 functions as a coactivator for TEAD-dependent gene expression (3,8,9).

Although the exact mechanisms are mostly unknown, YAP1 shuttles between the cytoplasm and nucleus. Protein-protein interactions (8,10) or nuclear import or export signaling are possible mechanisms that control the translocations of YAP1 between the cytoplasm and nucleus (11-14). Posttranslational modification through phosphorylation is one of the best-characterized mechanisms that modulate the nuclear-cytoplasmic translocation of YAP1 (15). The MST1/2 and LATS1/2 kinase cascade phosphorylate Ser127 (S127) and inactivate YAP1 (3,7,16). In addition to the covalent modification, a mechanical force could also increase YAP1 nuclear import (14,17). For example, the PDLIM5/7 family of PDZ and LIM domain-containing proteins increased YAP1 nuclear import through integrin-mediated mechanotransduction (18). Regardless, TEAD or 14-3-3 protein binding mediates YAP1 nuclear localization (11,19,20). Our published study suggests that androgen hormone signaling regulates the nuclear-cytoplasmic translocation of YAP1 (7), but the mechanism is unknown.

In this study, we have demonstrated that androgen exposure can suppress the inhibitory phospho-S127 on YAP1, which causes increases in YAP1 protein levels and nuclear abundance. We showed that androgen receptor (AR) signaling was critical for the regulation of YAP1 by androgen. We also showed that androgen exposure antagonized the Hippo/STK4 signaling and promoted PP2A activity. Our data further demonstrate that androgen signaling regulates the YAP1-dependent gene expression. Thus, our study uncovers a new mechanism of YAP1 regulation that involves androgen hormone signaling.

Results

Expression of YAP1 protein and transcript varies in prostate cancer cell models

To identify suitable cell lines that allow studying the effects of androgen on YAP1, we analyzed the levels of YAP1, WWTR1, and AR in the established prostate cancer cell models. We found that the levels of YAP1, WWTR1, and AR protein varied among the cell lines tested (Figure 1A, B). Surprisingly, LNCaP and its androgen insensitive LN-81 and C4-2 sublines showed no detectable WWTR1 protein. Also, the 22Rv1 cell line was YAP1-deficient compared with LNCaP and C4-2. The ARCaP and PC3 cell lines expressed YAP1, but they were AR-negative. Similarly, the expression of YAP1 transcripts also varied in the select cell lines (Figure 1C). Thus, LNCaP is the ideal cell model to study the mechanism of YAP1 regulation by androgen because it harbors an intact AR and YAP1 signaling pathway (7).

Androgen attenuates the inhibitory phospho-S127 modification on YAP1

To determine whether androgen signaling posttranscriptionally regulates phospho-S127, a potent inhibitor of YAP1 nuclear translocation (3,7,16), we examined the levels of phospho-S127 and the total amount of the YAP1 protein in LNCaP and its hormone-independent C4-2 subline. Both cell lines were exposed to dihydrotestosterone (DHT) at varying times (0, 4, 16, or 24h). We also examined the levels of phospho-S397 on YAP1 as a control. Androgen significantly inhibited phospho-S127 in LNCaP cells, but without affecting phospho-S397 (Figure 2A, B), suggesting that androgen specifically regulates phospho-S127 on YAP1. Notably, the inhibition of phospho-S127 by androgen correlated with increases in the total amount of YAP1 protein (13,21,22), which overlapped with AR activity (Figure 2A). Surprisingly, androgen did not significantly alter the levels of the phospho-S127 and total amount of YAP1 protein in C4-2 cells compared with that of LNCaP (23) under the same experimental conditions (Figure 2C, D).

To further confirm the specificity of the effects of androgen on YAP1, we analyzed the levels of phospho-S127 and total YAP1 in the PC3 cell line, which is AR-negative (24), and the MCF7 cell line, which is AR-positive and responsive to androgen (25,26). Treatment of these cells with DHT did not alter the phospho-S127 in PC3 cells (Figure 2E). Surprisingly, MCF7 cells did not show detectable YAP1 protein, though expressing its paralog WWTR1 protein (Figure 2F). Androgen did not alter the levels of the WWTR1 protein in both PC3 and MCF7 cells. Taken together, the effects of androgen on phospho-S127 are specific.

Androgen attenuates phospho-S127 through AR and Hippo/SKT4 signaling

To demonstrate whether the AR activity is critical for the inhibition of phospho-S127 by androgen, we performed a series of experiments. First, we evaluated the levels of phospho-S127 and total YAP1 protein in LNCaP cells after treatment with a vehicle, DHT, and DHT plus ENZ, a direct pharmacological inhibitor of the AR (Figure 3A, B). Compared with DHT, ENZ exposure reversed the inhibitory effects of androgen on phospho-S127, accompanied by a reduction in the total YAP1 protein, which likely occurred due to the induction of phospho-S127 (Figure 3A, lane 2 vs. lane 3). Second, we also examined the levels of phospho-S127 and total YAP1 protein in LNCaP with or without AR knockdown by siRNA (Figure 3C, D). The results showed that androgen was unable to reduce phospho-S127 in LNCaP cells with AR silencing compared with the

scrambled siRNA control (lane 4 vs. lane 5), which overlapped with a diminished total amount of YAP1 protein. The treatment of cells with ENZ had the opposite effect (Figure 3C, lane 6 vs. lane 5). Nevertheless, AR knockdown reduced the total YAP1 protein relative to the control siRNA (Figure 3C, D).

We also wanted to know if androgen antagonizes STK3/4 signaling in a way that suppresses phospho-S127, given that the STK3/4 kinase mediates phospho-S127 on YAP1 (16). To test this possibility, we assessed the levels of phospho-S127 and total YAP1 protein in LNCaP cells with LATS1/2 and STK3/4 knockdown. In comparison with the vehicle, androgen exposure slightly decreased phospho-S127 in LATS1/2 knockdown, but without altering the levels of phospho-S127 in STK3/4 knockdown cells (Figure 3E, F).

Androgen antagonizes the Hippo/STK4 signaling through the Ser/Thr phosphatases

Previously, we reported that controlled, ectopic expression of MST1/STK4 in the engineered C4-2/HA-MST1/STK4 cell line prevented YAP1 nuclear localization (7). We utilized this cell line to demonstrate if androgen antagonizes Hippo/STK4 signaling in a way that inhibits phospho-S127. In addressing this idea, we analyzed the levels of phospho-S127 and total YAP1 protein in C4-2/HA-STK4 cells that were exposed to doxycycline (Dox) along with the vehicle, or DHT. Dox was used to induce ectopic MST1/STK4 expression. Compared with vehicle control, androgen exposure significantly reduced phospho-S127, which resulted in increases in total YAP1 protein (Figure 4A, B).

The Ser/Thr phosphatases PP1/PP2A are known to inactivate the STK4/3 signaling (27-29). Okadaic acid (OKA), a potent inhibitor of the PP1/PP2A phosphatases, activates STK4/MST1 (30). Thus, we proposed that androgen positively regulates PP2A, which, in turn, attenuates Hippo/STK4 signaling. To test this possibility, we assessed the levels of phospho-S127 and total YAP1 protein in LNCaP cells after treatment with DMSO (mock) control and OKA. Compared with DMSO treatment, OKA reduced the mobility of phospho-S127 and YAP1 protein in the reduced SDS-PAGE (Figure 4C, D). We suggest that a reduction in the mobility of YAP1 protein accounts for its persistent multiple phosphorylation sites that increased the molecular weight of the YAP1 protein. This is likely due to the inactivation of the Ser/Thr phosphatases by OKA (30). However, compared with the vehicle, androgen slightly accelerated the YAP1 mobility, most likely by activating the Ser/The phosphatases, which, in turn, decreased the phospho-modifications on YAP1 (29,31). Furthermore, immunofluorescence imaging showed that androgen exposure enhanced YAP1 nuclear abundance in Dox-treated C4-2/HA-STK4 cells in comparison with vehicle treatment (Figure 4E, F). Hence, androgen may activate the Ser/Thr phosphatases to attenuate the Hippo/STK4 activity.

Androgen promotes YAP1 nuclear localization through AR and PP2A

To examine whether AR activity is necessary for the androgen-induced nuclear accumulation of YAP1, we conducted a co-immunofluorescence analysis of native AR and YAP1 proteins in LNCaP cells, followed by treatment with vehicle, DHT, or DHT plus ENZ (Figure 5A). Compared with the mock treatment, androgen exposure significantly increased the co-localization of YAP1 and AR protein in the cell nuclei. ENZ, however, reversed the effects of androgen on YAP1 and AR nuclear abundance (Figure 5B). Consistently, co-immunoprecipitation and western blot experiments demonstrated that androgen enhanced the interaction of YAP1 with AR and the catalytic C subunit of the PP22A, particularly in the nuclear fraction, under the same experimental conditions (Figure 5C). These findings indicate that AR, in concert with PP2A, mediates the reduction of phospho-S127 and induction of YAP1 nuclear localization by androgen.

Androgen promotes YAP1-dependent gene expression

The CCN1-encoded CYR61, the CCN2-encoded CTGF, and the ANKRD1 genes are well-characterized YAP1 targets (32,33). To test

whether androgen regulates the transacting functions of YAP1, we assessed the levels of CYR61, CTGF, and ANKRD1 transcripts, including YAP1 in the YAP1-WT (wild type) and YAP1-KO (knockout) LNCaP cell lines after treatment with the vehicle, DHT, or DHT plus ENZ (Figure 6A-E). Quantitative PCR demonstrated that androgen signaling differentially modulated the expression of CYR61, CTGF, and ANKRD1 transcripts in YAP1-WT cells. Regulation of these genes by androgen was YAP1-dependent because the silencing of YAP1 by CRISPR/Cas9 gene KO technology completely abolished the expression of CYR61, CTGF, and ANKRD1, including YAP1.

AR and YAP1 activity correlates in prostate tumor tissues

To verify the physiological significance of our observations in cultures, we conducted a computational analysis of the TCGA (The Cancer Genome Atlas) prostate cancer data sets, which is accessible via the www.cbioportal.org website. The results revealed that the expression of AR and YAP1 transcripts were significantly positively correlated (Pearson correlation = 0.45, p = 1.57e-14) in 493 prostate cancer patients (Figure 6F). Overall, our data indicate that the regulation of YAP1 activity by androgen is physiologically and clinically relevant.

Discussion

Here, we have demonstrated that androgen attenuates the phospho-S127 modification to promote YAP1 nuclear localization and activity. We have also shown that the AR-Hippo/STK4-PP2A axis mediates the effects of androgen on YAP1. Our findings suggest a new mechanism of YAP1 regulation that involves androgen hormone signaling.

Our data have revealed that androgen signaling suppressed phospho-S127 on YAP1 in an AR-dependent manner because the disruption of AR activity by genetic and pharmacological methods attenuated the inhibition of phospho-S127 modification by androgen. Also, inhibition of phospho-S127 resulted in increases in the total YAP protein and nuclear abundance. Our current and published (7) studies demonstrated that androgen promoted protein-protein interaction between YAP1 and AR in the androgen-sensitive LNCaP cell line. Unlike LNCaP cells, the YAP1 and AR interaction occurred independently of androgen exposure in the androgen-independent C4-2 cell model that mainly expresses nuclear AR and YAP1 proteins (7). Based on these observations, we suggest that AR modulates YAP1 activity by at least three mechanisms. First, the AR may function as a chaperone for YAP1 through the protein binding, which increases the stability of YAP1. Second, the AR binding to the YAP1 may prevent the phosphorylation of S127 by the kinases. Finally, the AR may serve as a cargo protein to import YAP1 into the nucleus via protein-protein interaction. Therefore we suggest that protein-protein interaction provides critical mechanistic insights into the regulation of YAP1 by androgen in the cell.

In addition, we have shown that androgen may antagonize Hippo/STK4 signaling to attenuate phospho-S127 and enhance YAP nuclear abundance, possibly by activating the Ser/Thr phosphatase PP1/PP2A. Our findings are consistent with the literature that PP2A was shown to inactivate the Hippo/STK4 activity (29,31). In a published study, we demonstrated that androgen enhanced the protein complex formation between AR and the full-length MST1 (MST1-FL) (34). We also found that the MST1-FL protein localized to the cell nuclei, where the MST1-FL was devoid of phospho-Thr183 in the activation loop, a critical side of the MST1 activity (34). It is possible that the AR binding restricts the ability of the kinases to phosphorylate S127 on YAP1. In addition, androgen might antagonize STT4/MST1 by enhancing phospho-Thr120 modification (34). Phospho-Thr120 was shown to reduce phospho-Thr183, which, in turn, results in the inactivation of STK4/MST1 activity (35).

Moreover, our co-IP and western blot experiments demonstrated that in addition to the AR, androgen enhanced the protein-complex formation between the catalytic, C subunit of PP2A and YAP1 in cell nuclei. This observation

suggests that androgen maintains nuclear YAP1 abundance by promoting the protein-protein interaction between YAP1 and PP2A, possibly via an AR-dependent manner because androgen also augmented YAP-AR interaction under the same experimental conditions. Nevertheless, the precise mechanism of how androgen regulates the nuclear YAP-PP2A interaction and whether it is biologically functional warrants further investigation, which is not the subject of the current study. Our finding is consistent with the literature that YAP1 interacts with PP2A in cell nuclei (36,37).

Furthermore, our proteomic analysis of the YAP1 proteome indicated that 14-3-3 was part of the YAP1 proteome (not shown). Our finding suggests the possibility that androgen also regulates phospho-S127 through the 14-3-3 protein binding. This notion is consistent with the literature that the 14-3-3 protein was shown to bind the phosphorylated serine residues, including phospho-S127 on YAP1 (38). Also, the 14-3-3 binding caused the ubiquitination and proteasomal degradation of YAP1 (39). The LATS and CK1δ/ε protein kinase signaling cascade phosphorylates and leads to the ubiquitination and proteasomal degradation of YAP1 through SCF (beta-TRCP) E3 ubiquitin ligase (22). LATS could reduce the stability and transcriptional activity of YAP1 via the Amot130–AIP4 complex in serum-starved conditions (40).

Nevertheless, we were unable to demonstrate androgen-regulated YAP1 ubiquitination, although we made several attempts (not shown). Evidence suggests that other kinases and also the phosphorylation sites on YAP1 could also regulate YAP1 nuclear localization (3,21,22,41,42). However, it remains unknown whether androgen influences the activity of other kinases, such as Src family kinases (6), to modulate YAP nuclear import (22,43). Our work also revealed that androgen hormone signaling regulates the YAP1-dependent gene expression. Therefore, further research is necessary to precisely determine the mechanism of how androgen signaling modulates the stability, nuclear localization, and transcriptional activity of YAP1 in future studies.

Based on our observations, we have provided a model (Figure 6G), in which androgen modulates the Hippo/STK4-AR-PP2A axis to attenuate phospho-S127 and to promote YAP1 nuclear abundance and activity. Our findings are biologically crucial because androgen regulates YAP1-dependent gene expression by possibly enhancing the interaction of YAP1 with AR and the PP2A protein phosphatase. Our results are also are physiologically and clinically significant because the YAP1 and AR transcripts correlate in the subset of prostate cancer tissues. Overall, our study uncovers a new mechanism of YAP1 regulation that is mediated by the AR-Hippo/MST1-PP2A axis. Our findings have important implications for human diseases, given that the Hippo-YAP pathway regulates a range of cellular events, including cell differentiation, stem cell biology, and immune responses.

Experimental procedures

Cell culture

LNCaP, LNCaP-81, C4-2, C4-2B, 22Rv1, PC3, and ARCaP cells were grown in RPMI 1640 cell culture medium at 37°C in 5% CO_2 incubator. 5% fetal bovine serum (FBS) and 1% penicillin and streptomycin antibiotics were added to the cell culture medium. LNCaP, C4-2, C4-2B, 22Rv1, and PC3 cell lines were purchased from American Type Culture Collection (ATCC).

YAP1-knockout cell line

LNCaP cells (3 x 10^5 cells per well in six-well plate) were seeded overnight before transfection. Cells were co-transfected with 1-3 μg human YAP1 HDR plasmid (Cruz Biotechnology, Inc (SCBT, sc-400040-HDR) and human YAP1 CRISPR/Cas9 KO (knockout) plasmid (SCBT, sc-400040) or control CRISPR/Cas9 plasmid (SCBT, sc-418922). Plasmids were transfected using lipofectamine 3000 reagents according to the manufacturer's instructions ThermoFisher Scientific (TFS, L3000001). After 72 hours of transfection, cells were exposed to an increasing dose of puromycin (2-10 μg/mL) to select puromycin-resistant clones. Individual clones

were transferred to new tissue culture plates and grown in a medium supplemented with puromycin (2 μg/mL). Western blot and quantitative-PCR analysis were performed to verify the loss of YAP1 expression in selected clones. All protocols and procedures were conducted according to the manufacturer's instructions (SCBT).

RNA isolation and quantitative polymerase chain reaction

Total RNA was isolated at 80% confluency using TRIzol RNA isolation reagent according to the manufacturer's instruction (TFS). GoTaq 1-Step RT-qPCR System (Promega, A6020) was used to carry out quantitative qPCR according to the manufacturer's instruction. The gene expression was determined using a 2-ΔCt method (44) normalized to the 18S ribosomal RNA control in qPCR results. The gene-specific primers used in qPCR are listed in Table 1.

Protein analysis

Total proteins were extracted from cells using ice-cold lysis buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 0.5% NP-40, 1 mM EDTA, and 1X protease inhibitors and phosphatase inhibitors (Calbiochem). Cytoplasmic and nuclear extracts were prepared according to the published protocol (45). Cells were washed with an ice-cold Phosphate Buffer Saline (PBS) three times before protein isolation. Co-immunoprecipitation (co-IP) was performed with anti-YAP1 at 4°C as described (7). The YAP1 immune complexes were captured with Protein A-Sepharose conjugate (GE Healthcare) and washed with a lysis buffer three times. Bound proteins were eluted and analyzed by 8% SDS–PAGE and western blotting. Membranes were blocked with PBS containing 0.1% Tween-20 and 5% (w/v) skim milk, followed by incubation with the protein-specific antibody to YAP1 (Cell Signaling Technology (CST, 8418 and 12395S, 1:1000 or SCBT (sc-376830, 1:100), phospho-S127-YAP1 (CST, 4911, 1:1000), phospho-S397-YAP1 (CST, 13619, 1:1000), STK4/MST1 (Abnova, H00006789-M01, 1:2000), AR (EMD Millipore, 06-680, 1:1000), HA-Tag (CST, 3724, 1:1000), PSA (CST, 332475P, 1:1000), β-Actin (Sigma-Aldrich, A2228, 1:3000), GAPDH (CST, 5174, 1:2000), α-Tubulin (CST, 2144, 1:2000), PP2A C subunit (CST, 2028S, 1:1000, or TOPI (CST, 79971, 1:1000). Protein signals were detected using a Luminata Forte Western HRP substrate (Millipore Sigma, WBLUFO500) and Bio-Rad ChemiDoc MP Imaging System.

Immunofluorescence and microscopy

Immunofluorescence imaging was conducted according to a published protocol (7). Briefly, LNCaP cells seeded in a chamber slide were androgen-starved overnight, followed by treatment with EtOH (ethanol), 10 nM dihydrotestosterone (DHT), or DHT plus enzalutamide (ENZ) in 5% CSS-fed condition for 16-18h. Cells were fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.3% Triton-X-100 for 10 min, and blocked with blocking buffer (2% BSA and 0.2% Triton-X-100 prepared in PBS) for 1h at room temperature. Cells were incubated with the primary YAP1 mouse monoclonal (G-6) antibody (SCBT, sc-376830, 1:50 dilution), AR rabbit polyclonal antibody (Millipore Sigma, 06-680, 1:400), or HA-Tag rabbit monoclonal (C29F4) antibody (CST, 3724, 1:100) at 4 °C overnight. Cells were incubated with the secondary antibody conjugated to Alexa Fluor 488 (Invitrogen, anti-mouse A32723, or anti-rabbit A32731) or conjugated to Alexa Fluor 647 (Invitrogen, anti-mouse A32728 or anti-rabbit A32733) for 1h at room temperature. Antibody dilutions were prepared in a blocking buffer. Cells were washed with PBS, 3x, after each procedure. Slides were mounted using Prolong Gold-Antifade reagent containing DAPI (CST, 89615). YAP1, AR, or HA-STK4 signals were captured using confocal microscopy (Zeis SP5-X, USA) at 40X magnification.

RNAi and plasmids

Scramble (control), or AR, STK4/3, or LATS1/2 siRNA were purchased from Dharmacon/Fisher Scientific (Pittsburgh, PA). Construction of tetracycline or doxycycline-inducible and HA-tagged STK4/MST1 expression plasmid was described previously (45). Briefly, PCR-amplified HA-Tag STK4/MST1-WT cDNA was

inserted into the BamH1, and MluI enzyme sites in the pRetro-X-Pur vector (Clontech Laboratories, Inc.), and the resulting plasmid was designated as pRXTP-HA-STK4/MST1. The gene-specific siRNA were transfected using DharmaFect-2 or Lipofectamine RNAi MAX Reagent (TFS 13778150) in Opti-MEM I reduced serum medium (TFS, 31985070) according to the manufacturer's instructions.

Data Mining

Prostate cancer gene expression data was mined from The Cancer Genome Atlas (TCGA) Pan-Cancer (PANCAN) dataset for patients with prostate adenocarcinoma (PRAD) (46). Batch normalized Illumina RNAseq V2 data from 493 patients was accessed via the cBioPortal website (https://www.cbioportal.org) (47,48). Co-expression and correlation analysis of AR and YAP1 mRNA levels was performed using cBioPortal co-expression tools (Pearson correlation = 0.45, p = 1.57e-14).

Data availibiltiy

All data described in the manuscript are contained within the manuscript.

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Author contributions

BC conceptualized the study and designed the experiments. BC and MAM conducted experiments and analyzed the data; CSM performed the TCGA data mining and analysis and edited the manuscript. SAK provided reagents and resources and edited the manuscript. BC wrote the manuscript.

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Table 1. Primer set used in quantitative polymerase chain reactions (qPCR). Fv: Forward; Rv: Reverse.

Gene ID	Primer Set (5'→ 3')	
CCN1	Fv: GGCAAGAAATGCAGCAAGAC	Rv: CAGTACTTGGGCCGGTATTT
CCN2	Fv: CATTCTCCAGCCATCAAGAGAC	Rv: CCACAAGCTGTCCAGTCTAATC
ANKRD1	Fv: CAAGACTCCTTCAGCCAACA	Rv: GGAGCAGAGATGTGGGAATG
YAP1	Fv: TGCGTAGCCAGTTACCAACACTG	Rv: TCGAGAGTGATAGGTGCCACTG
18S rRNA	Fv: GCTTAATTTGACTCAACACGGGA	Rv: AGCTATCAATCTGTCAATCCTGTC

Figures

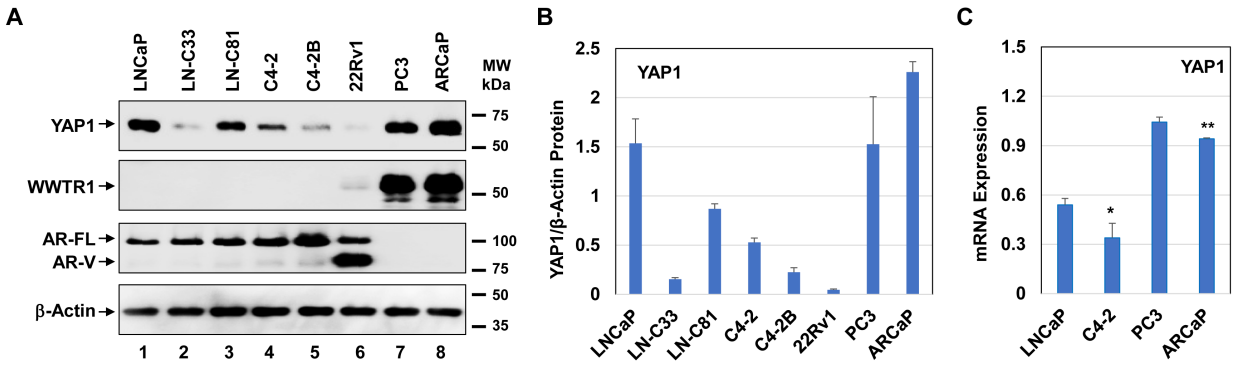

Figure 1. Expression of YAP1, WWTR1, and AR in prostate cancer cell models. (A) Total proteins were isolated from cells at 80% confluence in steady-state growth conditions and analyzed by western blotting for YAP1, WWTR1, or AR protein abundance. β-actin was used as a loading control in immunoblots. Membranes were blotted with the protein-specific antibody. (B) Quantification of YAP1 protein was normalized to the β-actin. *ImageJ* software was used to quantify YAP1 and β-actin protein blots. (C) Expression of YAP1 transcripts in the select cell lines. Total RNA was isolated from cells at 80% confluence in steady-state conditions 24h after cell seeding and analyzed by quantitative RT-PCR for the abundance of YAP1 transcripts, *,**$p<0.01$. YAP1 mRNA expression was normalized to 18S rRNA transcript. The data (±SD) are from three independent experiments.

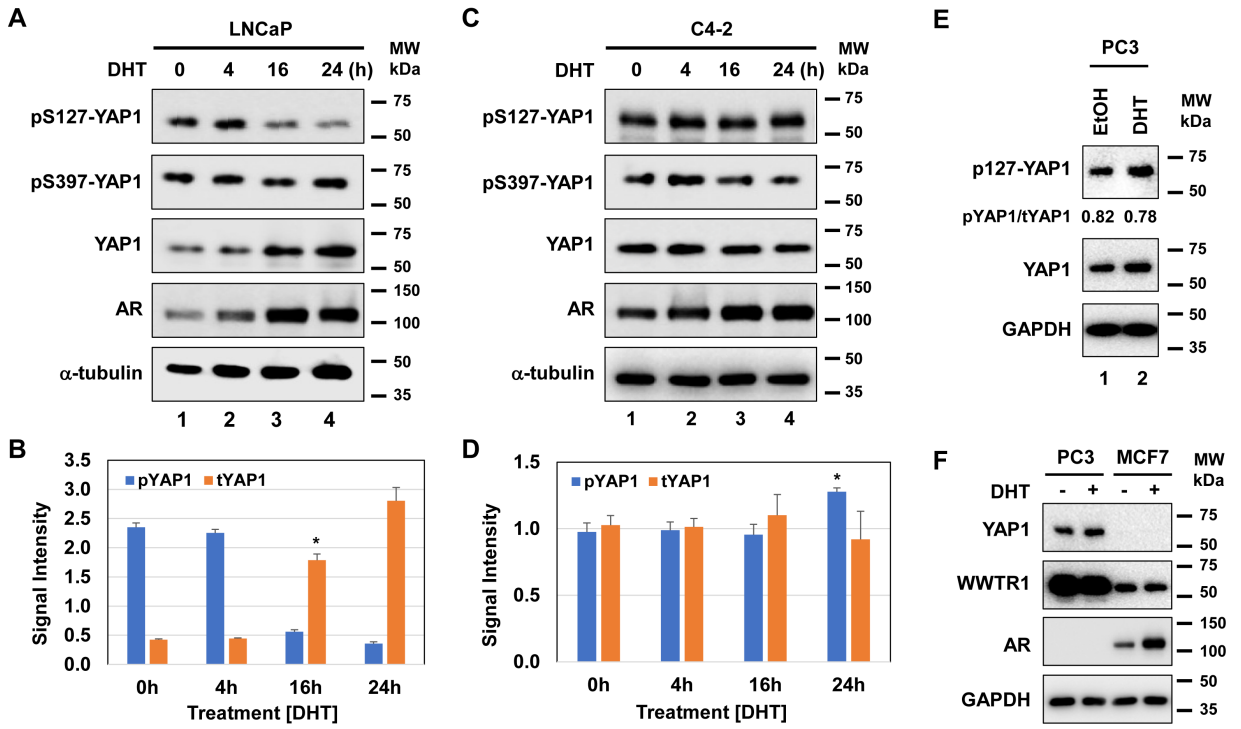

Figure 2. Steroid hormone androgen regulates phospho-S127 and YAP1 protein levels in cultures. (A-D) Western blot and quantification of phospho-S127 and total YAP1 proteins in androgen-dependent LNCaP, *$p<0.01$, and androgen-independent C4-2, *$p>0.05$ cells, respectively. Cells were exposed to dihydrotestosterone (DHT, 10 nM) at varying times. The data in panel B and C were normalized to α-tubulin. The phospho-S127 blot was included as a control. (E, F) Western blot analysis of YAP1 and WWTR1 proteins in the PC3 prostate cancer and MCF7 breast cancer cell lines that were also treated with vehicle (ethanol) or DHT overnight (16-18h). DHT treatment was conducted in 5% CSS–fed growth conditions. *ImageJ* software was used to quantify the intensity of phospho-S127, total YAP, and α-tubulin protein bands. YAP1 and AR blots were included as a positive control in panel F. The α-tubulin or GAPDH protein blot was incorporated as a loading control. Membranes were blotted with the protein-specific antibody. The data (±SD) are the representation of two independent experiments.

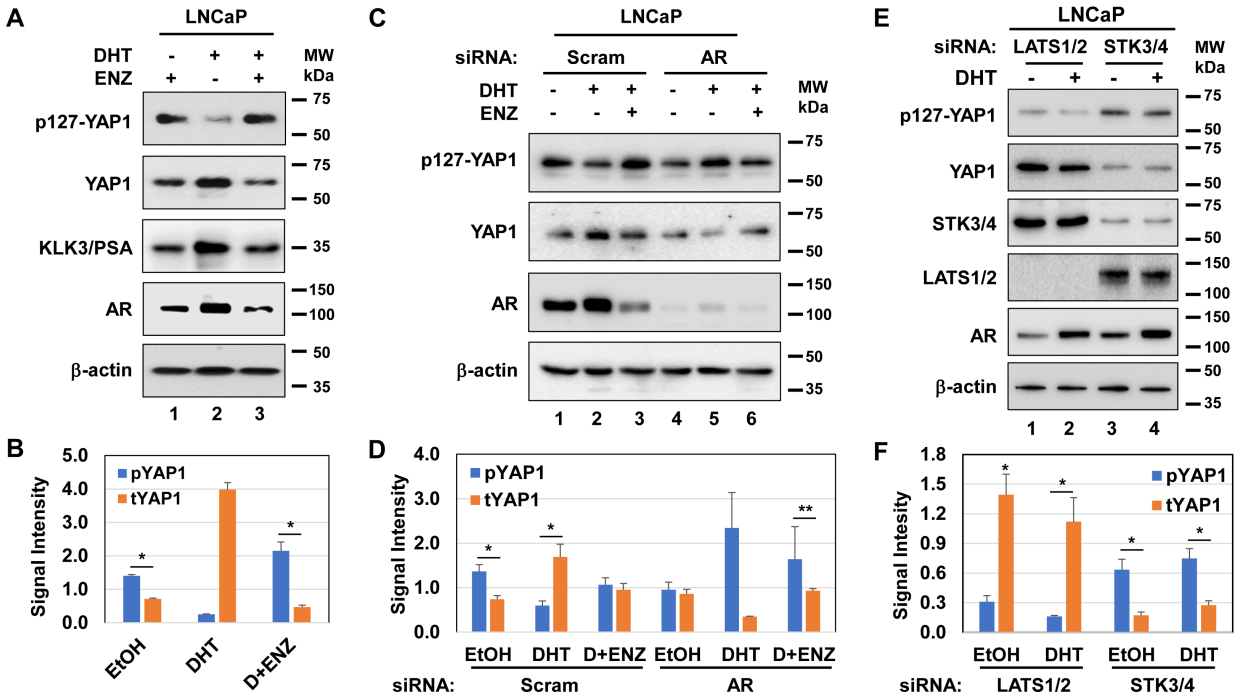

Figure 3. AR and STK4/3 signaling is necessary for the inhibition of phospho-S127 by androgen. (A, B) Western blot and quantification of phospho-S127 and total YAP1 proteins, respectively, **,**p<0.01*. KLK3/PSA blot was included as a positive control to assess the activity of DHT. (C,D) Western blot and quantification of phospho-S127 and total YAP1 protein in LNCaP cells after transient transfection with the pool of scrambled (Scram) or the AR gene-specific siRNA for 36h, followed by treatment with or without DHT and ENZ overnight in 5% CSS-fed conditions. (E, F) Western blot and quantification of phospho-S127 and total YAP1 in LNCaP cells transfected with Scram siRNA or the LATS1/2 and STK4/3 (MST1/2) gene-specific siRNA for 36h, followed by treatment with or without DHT overnight in 5% CSS-fed growth conditions. β-actin was used as a loading control in immunoblots. Membranes were blotted with the protein-specific antibody. *ImageJ* software was used to quantify the intensity of the phospho-S127 and the total YAP1 signal. The data (±SD) are the representation of three independent experiments.

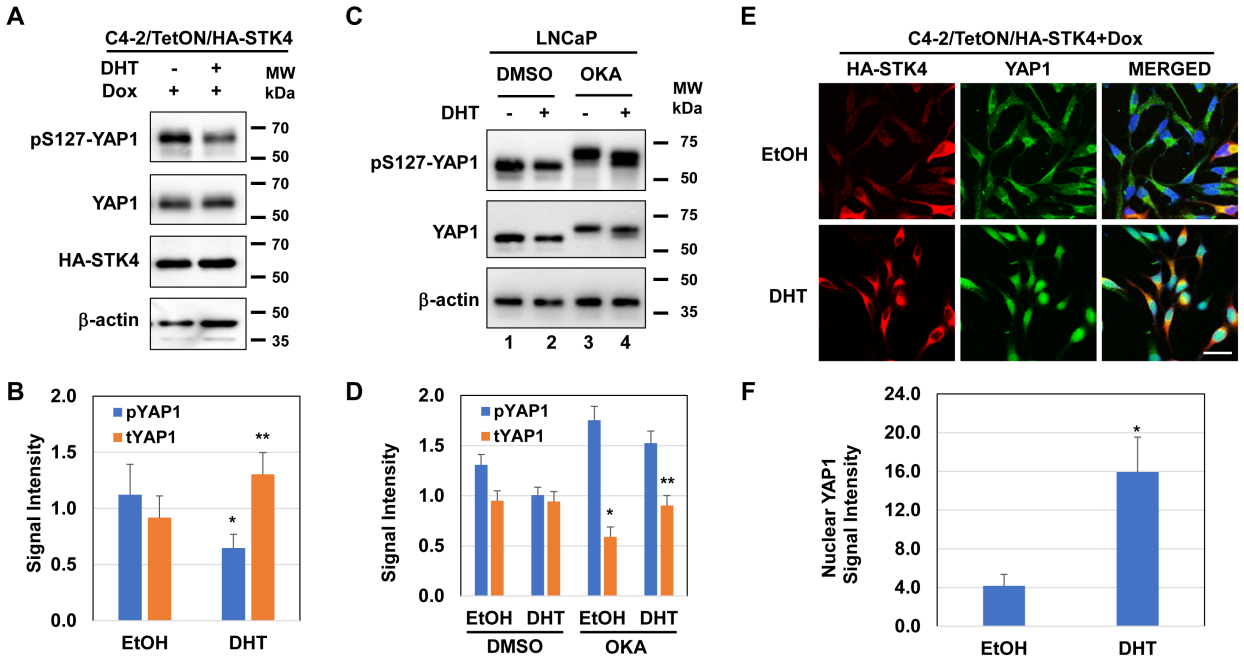

Figure 4. Androgen negatively regulates the Hippo/STK4 signaling. (A, B) Western blot and quantification of phospho-S127 and total YAP1 proteins in C4-2/HA-STK4/MST1 cells that express tetracycline or doxycycline (Dox) inducible HA-STK4 protein (45), respectively. (C, D) Western blot and quantification of phospho-S127 and total YAP1 protein in LNCaP cells that were exposed to DMSO (vehicle) or OKA (okadaic acid) followed by treatment with or without DHT overnight in 5% CSS-fed growth condition. β-actin was used as a loading control in immunoblots. **(E)** Immunofluorescence analysis of ectopic HA-STK4 (red), and native YAP1 (green) proteins and nuclei (DAPI, blue) in Dox-treated C4-2/HA-STK4 cells. Scale bar: 20 μm. Micrographs are the representation of multiple images. **(F)** Quantification of nuclear YAP1 protein by *ImageJ* from multiple images and nuclear YAP1 signals were normalized to the total number of cells subjected to image quantification. The ectopic expression of HA-STK4/MST1 protein was assessed using an HA-tag antibody. Cells were treated with EtOH or DHT (10 nM) and Dox (1 μg/ml) in serum-depleted conditions overnight before analysis, **,**p<0.05*.

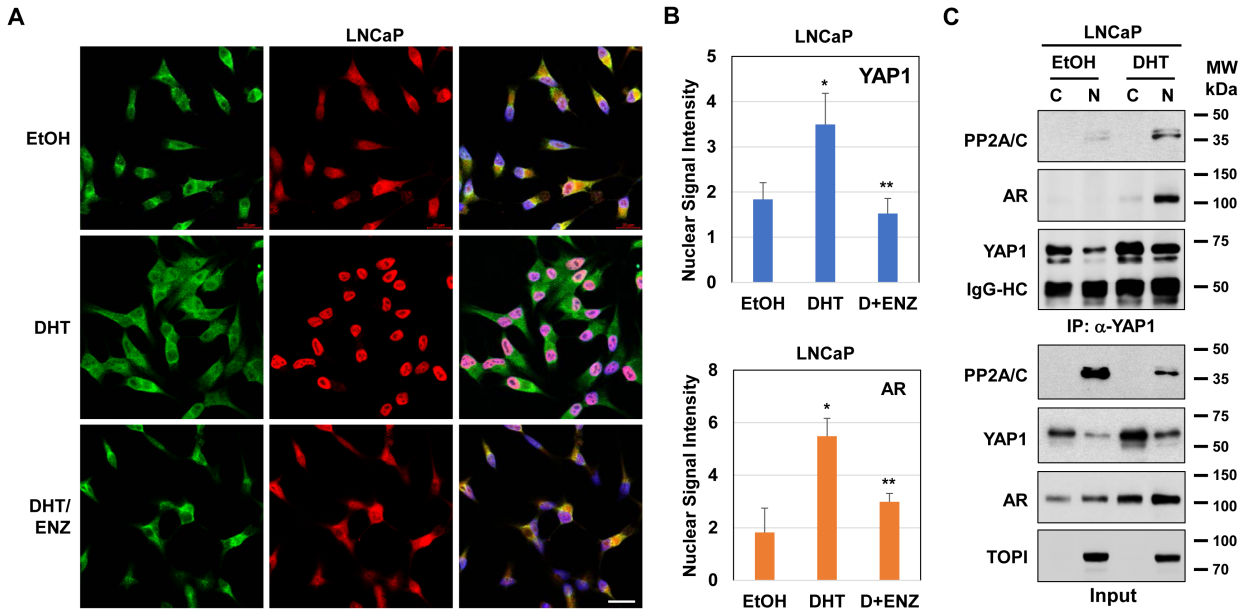

Figure 5. Androgen promotes YAP1 nuclear localization. (A, B) Co-immunofluorescence imaging of YAP1 and AR proteins in LNCaP cells. Cells were treated with or without DHT (10 nM) and enzalutamide (ENZ, 20 μM) overnight in 5% CSS-fed growth conditions. Images of AR (Alexa 647, red), YAP1 (Alexa 488, green), and nuclei (DAPI, blue) were acquired using confocal microscopy. Scale bar: 20 μm. The micrographs are the representation of multiple images. (B) *ImageJ* software was used to quantify the intensity of the nuclear AR and YAP1 protein from multiple images. The data (±SD) normalized to the total cell number from multiple images of three independent experiments, *,**$p<0.0001$. (C) Co-immunoprecipitation and western blot analysis of the cytoplasmic and nuclear YAP1, PP2A C, and AR proteins in LNCaP cells after treatment with or without DHT overnight in 5% CSS-fed growth condition. Topoisomerase I (TOPI) was used as a nuclear marker. Blots are the representation of three independent experiments.

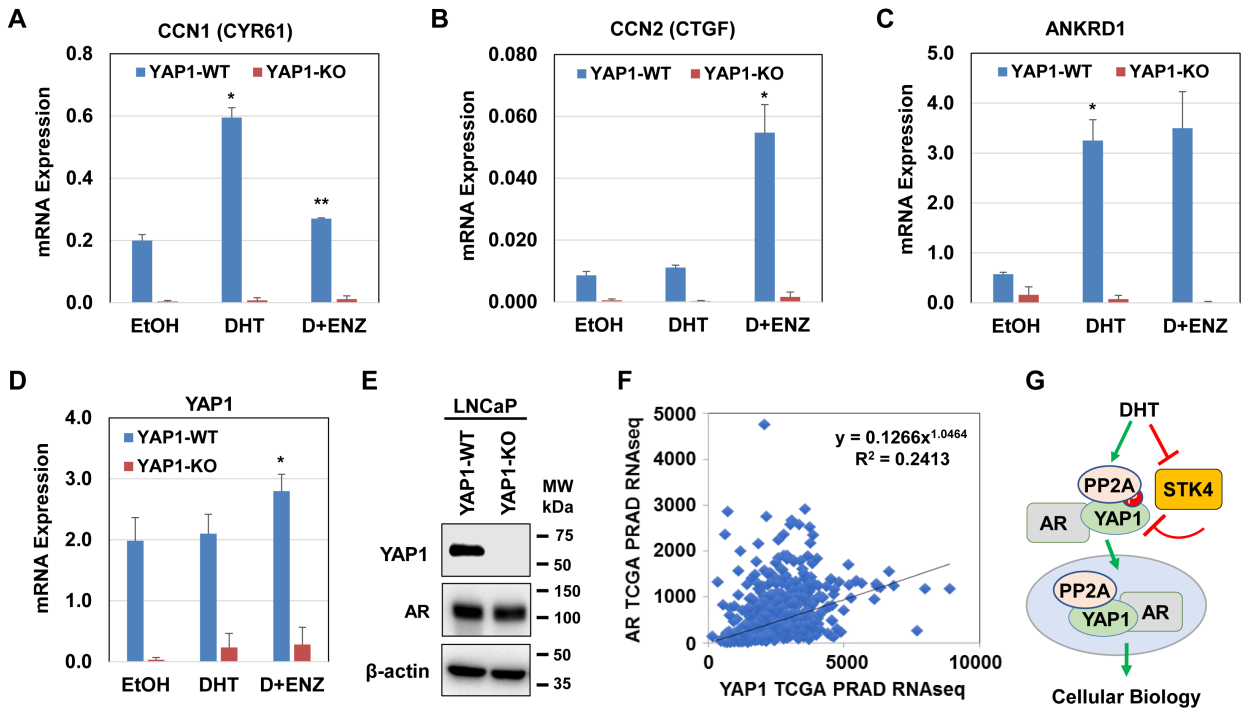

Figure 6. Androgen signaling regulates the transcriptional activity of YAP1. (A-C) Quantitative RT-PCR analysis of YAP1 and the YAP1 target gene expression in YAP1-WT and YAP1-KO LNCaP cell models after treatment with EtOH, DHT, and DHT plus ENZ overnight in 5% CSS-fed growth condition. (D-E) Quantitative RT-PCR and western blot analysis YAP1 transcripts and protein expression in YAP1-WT and YAP1-KO LNCaP cell models, respectively. The data (±SD) are from three independent experiments, *,**$p<0.01$. (F) Co-expression and correlation analysis of AR and YAP1 mRNA levels in The Cancer Genome Atlas (TCGA) Pan-Cancer (PANCAN) prostate adenocarcinoma dataset (PRAD) from 493 patients (46). Pearson correlation = 0.45, p = 1.57e-14). The data was accessed using the cBioPortal website, https://www.cbioportal.org. (G) A model illustrates the regulation of YAP1 nuclear localization by androgen. In this model, androgen antagonizes the Hippo/STK4/MST1 in a way that attenuates phospho-YAP and induces the YAP1 nuclear localization. Androgen also enhanced YAP1 nuclear localization by promoting protein-protein interaction between YAP1, PP2A, and AR, leading to cellular biology.