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Yin Yang 1 Regulates the Transcriptional Activity of Androgen Receptor

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Abstract

The multifunctional protein Yin Yang 1 (YY1) plays an important role in epigenetic regulation of gene expression. YY1 is highly expressed in various types of cancers, including prostate cancer. Currently, the mechanism underlying the functional role of YY1 in prostate tumorigenesis remains unclear. In this report, we investigated the functional interplay between YY1 and androgen receptor (AR), and the effect of YY1 on AR-mediated transcription. We found that YY1 physically interacts with AR both in a cell-free system and in cultured cells. YY1 is required for the optimal transcriptional activity of AR in promoting the transcription of the prostate specific antigen (PSA) promoter. However, ectopic YY1 expression in LNCaP cells did not further enhance the reporter driven by the PSA promoter, suggesting an optimal level of YY1 is already established in prostate tumor cells. Consistently, YY1 depletion in LNCaP cells reduced endogenous PSA levels, but overexpressed YY1 did not significantly increase PSA expression. We also observed that YY1-AR interaction is essential to YY1-mediated transcription activity of AR and YY1 is a necessary component in the complex binding to the androgen response element (ARE). Thus, our study demonstrates that YY1 interacts with AR and regulates its transcriptional activity.

Keywords

androgen receptor; Yin Yang 1; prostate cancer; PSA promoter; transcription

INTRODUCTION

As a transcription factor, YY1 (Yin Yang 1) either represses or activates gene expression, depending on the recruited cofactors. Many YY1-regulated genes play crucial roles in cell proliferation and differentiation (Shi *et al.*, 1997; Thomas and Seto, 1999), while YY1 gene is also regulated by other factors, such as prohibitin and NF-kappaB (Joshi *et al.*, 2007; Wang *et al.*, 2007). In addition, the function of YY1 is modulated by different

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posttranslational modifications (Deng *et al.*, 2007; Rylski *et al.*, 2008; Yao *et al.*, 2001). Several studies, including ours, demonstrated that YY1 is a negative regulator of p53 (Bain and Sinclair, 2005; Gronroos *et al.*, 2004; Santiago *et al.*, 2007; Sui *et al.*, 2004). YY1 also recruits p300, HDAC1 and Ezh2 to mediate histone modifications (Caretti *et al.*, 2004; Yang *et al.*, 1996; Yao *et al.*, 2001) indicating a pivotal role of YY1 in genomic imprinting and chromatin remodeling.

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YY1 is overexpressed in different human cancers, including prostate cancer (PCa) (Baritaki *et al.*, 2007; Begon *et al.*, 2005; de Nigris *et al.*, 2006; Erkeland *et al.*, 2003; Seligson *et al.*, 2005). Recent studies suggest that YY1 plays a role in PCa development and progression. YY1 is increased in the prostatic intraepithelial neoplasia stage (Seligson *et al.*, 2005). Importantly, YY1 is essential to histone methylation mediated by Ezh2 (Caretti *et al.*, 2004; Wilkinson *et al.*, 2006) that is an oncogene in prostate tumorigenesis (Bracken *et al.*, 2003). Moreover, p53 mutations are frequently observed in the late stage of PCa, but not the primary tumors. Therefore, YY1 may antagonize the tumor suppression surveillance of p53 during malignant transformation of prostate cells.

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Androgenic hormones are necessary for normal prostate development and also play critical roles in stimulating the proliferation and progression of PCa cells through androgen receptor (AR) (Heinlein and Chang, 2004). As a ligand-regulated transcription factor, AR shares common features with other members of the nuclear receptors. AR protein contains two activation domains (AF-1 and AF-2), a DNA binding domain (DBD) and a ligand-binding domain (LBD) (Dehm and Tindall, 2005). The binding of androgens changes the conformation of AR and composition of the AR-containing complex, which leads to its translocation from cytoplasm to nucleus. Nuclear AR associates with androgen response elements (AREs) in the promoters and enhancer regions of its target genes, and stimulates their expression (Dehm and Tindall, 2005).

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Due to the crucial role of AR in PCa progression, androgen deprivation is used as a therapeutic approach for patients with advanced PCa (Sharifi and Farrar, 2006). Several studies indicate that this treatment exerts selective pressure on androgen signaling pathways (Craft *et al.*, 1999; Taplin *et al.*, 1999). This leads to elevated AR levels (Gregory *et al.*, 2001; Koivisto *et al.*, 1997), increased AR sensitivity to androgens or other steroid hormones (from gain-of-function mutations) (Gaddipati *et al.*, 1994; Veldscholte *et al.*, 1992) and enhanced AR nuclear localization (Lapouge *et al.*, 2007). These adaptive changes sustain the function of AR in stimulating cell proliferation and preventing apoptosis, and consequently cause recurrent cancers that are apparently androgen-independent.

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Since YY1 has great potential in regulating cancer development and is increasingly expressed in early stages of PCa, we investigated whether there is a functional interplay between YY1 and AR. In this study, we focused on the role of YY1 in mediating the transcriptional activity of AR.

MATERIALS AND METHODS

Antibodies and DNA vectors

All antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) unless otherwise indicated. Plasmids expressing AR proteins, including pcDNA3/Flag-AR, GST-AR(1–556), GST-AR(557–919) and pHis×6-AR(557–919), were generated in our laboratory. Plasmids expressing HA-YY1, GST-YY1 wild type (wt) and mutants, and YY1 chimera 17 (Chi17, with the second zinc finger of YY1 replaced by a zinc finger from growth factor independence-1) were described previously (Galvin and Shi, 1997; Sui *et al.*, 2004). The reporter plasmid, PSA-Fluc, with the 5.8-kilo base pair (bp) PSA promoter driving Firefly luciferase (Fluc) was kindly provided by Dr. Weber (Gioeli *et al.*, 2002). The PSA promoter with its putative YY1 binding site mutated from “AAGATGGTC” to “AAACTAGTC” was generated by PCR and confirmed by DNA sequencing analysis.

Gaussia luciferase (Gluc) (Tannous *et al.*, 2005) is a secreted protein and its activity can be measured in collected culture medium. To generate PSA-Gluc, we subcloned either the PSA promoter or its mutated form into the HindIII site of pGluc-Basic (New England Biolabs). The Lentiviral vector, pLu-Puro, was used to deliver U6/siRNAs expression cassettes. U6/scrambled and U6/*yy1* siRNAs were described previously (Sui *et al.*, 2004). The construction of U6/*AR* siRNA followed our previously published protocol (Sui and Shi, 2005; Sui *et al.*, 2002), with a target sequence as “GAGGCACCTCTCTCAAGAGTT”.

Cell culture and transfection

293T cells were maintained in DMEM containing 10% fetal bovine serum (FBS). LNCaP cells were cultured in RPMI1640 medium with 10% FBS and transfected at 80% confluence in 12-well plates with Lipofectamine 2000 (Invitrogen). After 6 h of transfection, the medium was replaced with phenol-red-free RPMI 1640 containing 10% charcoal stripped serum (CSS). If needed, R1881 was added in the medium to a final concentration of 10 nM at 24 h post transfection. Cell lysates or medium were collected at 48 h post transfection for further analyses.

Lentiviral production and infection

Lentivirus production followed a previous report (Rubinson *et al.*, 2003) by transfecting 293T cells with a Lentiviral plasmid and three packaging plasmids (pMDLg/pRRE, pRSV-REV and pVSV-G). To infect cells, concentrated Lentivirus was added to the medium with 8 µg/ml polybrene and incubated for 6 h before changing back to normal medium.

Reporter assay

We used three reporter constructs: PSA-Gluc, PSA-Fluc and 3×ARE-Fluc. The detailed construction procedure of the 3×ARE-Fluc containing three concatemeric repeats of the ARE-I (Rao *et al.*, 2003) from the PSA promoter and one PSA proximal promoter is described in the supplementary material. The Actin-SEAP plasmid with β-actin promoter-driven secreted alkaline phosphatase (SEAP) was used as a transfection control and each condition was tested in triplicate and repeated over 3 times. For cells transfected with PSA-Gluc, 50 µl of medium, collected at 48 h post transfection, was mixed with 100 µl of

substrate solution containing 0.5 µg/ml of coelenterazine (CTZ), 200 mM NaCl, 50 mM Tris•HCl and 0.01% Triton X-100, pH 8.7. Light emission was measured at 480 nm and normalized to the SEAP expression. The assays with Fluc activity were performed as described before (Deng *et al.*, 2007).

Protein interaction studies

The *in vitro* protein binding studies and immunoprecipitation experiments for transfected or endogenous proteins have been described previously (Deng *et al.*, 2007).

Electrophoretic mobility shift assay (EMSA)

The EMSA followed a previously published protocol (Cleutjens *et al.*, 1997). We incubated nuclear protein extract with the ARE-I probe labeled by ³²P-dATP (Perkin Elmer) and used a control double-stranded probe generated by annealing the following two oligonucleotides: 5'-TATCAGAAGGACGTATACGTATTACAACCT-3' and 5'-GATCAGTTGTAATACGTATACGTCCTTCT-3'. To determine the role of YY1 in the AR complex, nuclear extract was individually pretreated by YY1 antibodies (13G10, Cell Signaling and H-10) and normal antibodies before adding the probe.

Quantitative PCR (qPCR) analysis

This was conducted using Taqman® Gene Expression Assay kit (Applied Biosystems). Briefly, RNA extraction and reverse transcription were carried out according to the manufacturer's instruction. mRNA levels of PSA and GAPDH were determined by unlabeled PCR primers and the FAM™ dye-labeled TaqMan MGB probes (Hs03063374 for PSA and Hs99999905 for GAPDH) using the ABI7000 Real-time PCR system. The samples were analyzed in triplicates and repeated in 3 individual experiments. Comparative Ct method was used to calculate relative levels of PSA mRNA normalized to GAPDH.

Statistical analysis

All data in reporter assay and qPCR are presented as mean ± SD. Comparisons between two groups on a single parameter were conducted using Student's *t*-test. Statistical analyses were performed using SigmaStat (Systat Software Inc). The criterion for statistical significance was set at $p < 0.05$.

Results

YY1 directly interacts with AR

We conducted immunoprecipitation experiment in 293T cells cotransfected with pcDNA3/Flag-AR and pcDNA3/HA-YY1. When the cell lysates were immunoprecipitated with an AR antibody (N-20) and analyzed by Western blot using an HA antibody (F-7), we detected HA-YY1 brought down by the AR antibody in the sample with both plasmids transfected (lane 4, Figure 1A). HA-YY1 was not detected if Flag-AR was absent in transfection or a control rabbit IgG replaced the AR antibody (lanes 3 and 2, Figure 1A). In the reciprocal immunoprecipitation experiment, when HA antibody (F-7) was used to bring down HA-YY1, Flag-AR could be pulled down when both HA-YY1 and Flag-AR were transfected

(lane 4, Figure 1B). However, no such significant amount of Flag-AR could be brought down when the control antibody was used or when Flag-AR was replaced by an empty vector (lanes 2 and 3, Figure 1B). We further examined whether endogenous YY1 and AR interact in prostate cells. We immunoprecipitated AR from LNCaP cell lysates using AR antibody N-20 and C-19, recognizing the N- and C-terminal of AR, respectively. YY1 could be coimmunoprecipitated by both AR antibodies, but not the control IgG (lanes 3, 4 vs. 2, Figure 1C). AR N-20 antibody could pull down more YY1 than AR C-19, proportional to the AR amounts brought down by them (lower panel). In the reciprocal experiment, both YY1 antibodies (C-20 and H-414) brought down significant amounts of endogenous AR compared to the control rabbit IgG (lanes 3, 4 vs. 2, Figure 1D). Again, the amounts of AR brought down by these antibodies were proportional to the levels of immunoprecipitated YY1 (lower panel).

We then asked whether the YY1-AR association occurred through direct protein-protein interaction. Since the full length AR was poorly expressed in bacteria, we produced and purified recombinant AR N-terminal (1–555, containing AF-1 domain), AR C-terminal (556–919, containing DBD, LBD and AF-2 domains), and full length YY1, fused with either 6-histidine tag (His \times 6) or GST, from *E. coli* Rosetta cells. First, we incubated His \times 6-YY1 with GST-AR-N-terminal(1–555), GST-AR-C-terminal(556–919) and GST alone, followed by the addition and incubation of glutathione-conjugated agarose (Sigma). GST-AR(556–919), but not GST or GST-AR(1–555), brought down His \times 6-YY1 (lanes 4 vs. 2 and 3, Figure 2A), which showed a similar intensity to that brought down by GST-p53 (lane 5), as we previously demonstrated (Sui *et al.*, 2004). We also performed a reciprocal binding assay by incubating His \times 6-AR(556–919) with GST-YY1 and GST. We observed that GST-YY1, but not GST alone, could bring down His \times 6-AR(556–919) (lanes 3 vs. 2, Figure 2B). We mapped the interacting domain of AR on YY1 protein using three GST-YY1 mutants, and observed that YY1(198–414) and YY1(331–414), but not YY1(1–224), interacted with His \times 6-AR(556–919) (lanes 5, 6 vs. 4, Figure 2B), suggesting that AR binding site is located at the C-terminal of YY1. In this experiment, GST-p53 (lane 7) served as a positive control, since p53 directly interacts with AR (Shenk *et al.*, 2001).

YY1 enhances the transcriptional activity of AR

After observing direct YY1-AR interaction, we asked whether YY1 has any regulatory effect on AR-regulated gene expression. We determined the response of the PSA promoter to different YY1 concentrations with or without AR. We transfected 293T cells with PSA-Fluc (300 ng), pcDNA3/Flag-AR (300 ng) and increasing amounts (75, 150 and 300 ng) of pcDNA3/HA-YY1, and cultured the cells with or without the synthetic androgen R1881. While the activation of AR to the PSA promoter was greatly stimulated by R1881 (#8 vs. #2, Figure 3A), HA-YY1 alone also slightly enhanced the PSA promoter regardless of the R1881 presence (#3 vs. #1, and #9 vs. #7). When Flag-AR was cotransfected, an initial amount (75 ng) of HA-YY1 displayed increased transcription in the absence of R1881 compared to HA-YY1 or Flag-AR alone (#4 vs. #3 and #4 vs. #2), but this increase was markedly enhanced by R1881 (#10 vs. #9 and #10 vs. #8), exhibiting a synergistic effect of HA-YY1 and Flag-AR. Strikingly, further YY1 increases (150 and 300 ng) inversely affected this synergistic activation (#5, #6 vs. #4, and #11, #12 vs. #10). Western blot

analysis indicated that YY1 increases did not apparently alter AR levels (right, Figure 3A). We repeated this reporter assay with lower concentrations (50 and 75 ng) of YY1, but did not observe any significant difference between these two conditions (data not shown).

To determine how YY1 affects endogenous AR, we studied the effects of altered YY1 expression on transcriptional activity of AR in LNCaP cells that highly express both YY1 and AR proteins. We cotransfected LNCaP cells with 80 ng of PSA-Gluc and three different amounts (50, 100 and 200 ng) of HA-YY1. HA-YY1 increases did not significantly change the PSA promoter activity with or without R1881 (#2, #3, #4 vs. #1 and #6, #7, #8 vs. #5, Figure 3B). A representative Western blot analysis of endogenous AR and transfected HA-YY1 is on the right. The results suggested that highly expressed endogenous YY1 in LNCaP cells is sufficient for the optimal activity of AR; hence, ectopically introduced YY1 would not further enhance its activity. Instead, when YY1 increased from 100 to 200 ng, we observed a slight decrease in luciferase activity driven by the PSA promoter, which is consistent with the effects of increased amounts of YY1 in 293T cells (Figure 3A).

To determine whether YY1 is required for AR-mediated transcription, we cotransfected LNCaP cells with U6/scrambled siRNA or U6/*yy1* siRNA and 80 ng of PSA-Gluc, with or without R1881. In the presence of R1881, YY1 depletion (38% of its original level, right in Figure 3C) decreased Gluc activity by 50% (#4 vs. #3, $p < 0.01$). Without R1881, the reporter expressed basal levels of Gluc despite of YY1 expression (#2 vs. #1).

YY1 knockdown leads to decreased endogenous PSA expression

Since YY1 is required for AR-mediated expression (Figure 3), we asked whether altered YY1 expression changes the endogenous PSA level. We infected LNCaP cells with Lentivirus generated from an empty pSL2 vector or pSL2-YY1 that employed the CMV promoter to drive *yy1* cDNA (upper panel, Figure 4A) and then cultured the cells with or without R1881. Although endogenous PSA expression could be increased by ectopically expressed YY1 in the absence of R1881 (1.8-fold, lanes 3 vs. 1, Figure 4B), there was no obvious change of PSA levels in the presence of R1881 (lanes 4 vs. 2, Figure 4B). In addition, YY1 increase did not change the expression of endogenous AR. This result is consistent with our observation in the reporter assay (Figure 3B).

We further studied how YY1 knockdown affects endogenous PSA expression. LNCaP cells were individually infected with lentiviruses carrying U6/scrambled, U6/*yy1* and U6/*AR* siRNAs (lower panel, Figure 4A) and then cultured with or without R1881 for 3 days, followed by Western blot analysis. The two middle panels of Figure 4C indicate that the endogenous YY1 and AR were correspondingly knocked down. Without R1881, PSA expression was low when the scrambled siRNA was introduced, but markedly decreased when either YY1 or AR was knocked down (lanes 3, 5 vs. 1, top panel of Figure 4C). Consistently, when R1881 was added, both YY1 and AR knockdown also reduced the expression of endogenous PSA compared to the scrambled siRNA control (lanes 4, 6 vs. 2, Figure 4C).

To quantitatively analyze the effects of YY1 depletion on PSA expression, we determined the levels of PSA mRNA in LNCaP cells expressing the scrambled siRNA and *yy1* siRNA,

respectively. As shown in Figure 4D, the expression of PSA mRNA dropped to 55.6% in the YY1 depleted cells compared to the cells expressing scrambled siRNA.

YY1-AR, but not YY1-DNA, interaction is essential to YY1-enhanced AR activity

Since YY1 directly binds to AR, we asked whether YY1-AR interaction determines YY1-regulated transcriptional activity of AR. As we mapped the AR binding domain to the C-terminal of YY1 (Figure 2A), we tested the interaction of AR with YY1 chimera 17 (Chi17) that has the second zinc finger of YY1 replaced by a zinc finger from growth factor independence-1 (Figure 5A) (Galvin and Shi, 1997). In the *in vitro* protein binding experiments, while wt His×6-YY1 interacted with GST-AR-C-terminal, His×6-Chi17 lost this interaction (lanes 8 vs. 4, Figure 5B). Reciprocally, GST-YY1, but not GST-Chi17, brought down His×6-AR(556–919) (lanes 3 vs. 4, Figure 5C), with GST-PIASy as a positive control (lane 5), as it interacts with AR (Gross *et al.*, 2004). We asked if the loss of interaction between YY1-Chi17 and AR could result from a destructive distortion of YY1 conformation after the substitution of its second zinc finger. We therefore tested the binding affinity of YY1-Chi17 with Hdm2 and PIASy, which have been identified by us to interact with the spacer region and Zinc finger domain of YY1, respectively (Deng *et al.*, 2007; Sui *et al.*, 2004). As shown in Figures 5D and 5E, GST-wt YY1 and GST-YY1-Chi17 exhibited comparable affinity to His×6-Hdm2 and His×6-PIASy, suggesting YY1-Chi17 likely retains a conformation similar to wt YY1. The result in Figure 5E also indicated that, although PIASy binds to the zinc finger region of YY1, the zinc finger 2 is not essential to this interaction.

To determine whether YY1-AR interaction is necessary to YY1-mediated transcription activity of AR, we individually transfected pcDNA3/HA-YY1 and pcDNA3/HA-YY1(Chi17) with both PSA-Fluc and pcDNA3/Flag-AR in 293T cells and tested Fluc activity in cell lysates. While HA-YY1 could synergistically stimulate PSA promoter transcription (#3 vs. #1 and #2, Figure 6A), HA-YY1(Chi17) completely lost this function (#3 vs. #4). Comparable expression of HA-YY1 and HA-YY1(Chi17) was demonstrated by Western blots with GAPDH as a control (right, Figure 6A).

Since YY1 is a transcription factor, we asked whether YY1 could potentially bind to the PSA promoter and whether YY1-DNA interaction could affect AR-mediated PSA transcription. Using an algorithm to search for binding elements of transcriptional factors (Heinemeyer *et al.*, 1998), we identified a potential YY1 binding site (A⁻²⁰⁴⁰AGATGGTC⁻²⁰³²) in the PSA promoter. The nucleotides are numbered relative to the transcription start site of PSA mRNA and the underlined “ATGG” is reversely complementary to the core sequence (“CCAT”) of YY1 binding element (Shi *et al.*, 1997). To study whether this potential YY1 binding site determined YY1-mediated PSA transcription, we disrupted this site by mutating “A⁻²⁰⁴⁰AGATGGTC⁻²⁰³²” to “A⁻²⁰⁴⁰AACTAGTC⁻²⁰³²”, which deleted the YY1 binding elements and introduced a SpeI site (ACTAGT) used in DNA subcloning. Hence, we generated a reporter construct with an YY1-binding site mutated PSA promoter driving Gluc cDNA (designated as “m-PSA-Gluc”, Figure 6B).

To determine whether YY1-DNA interaction is required for PSA promoter expression, we individually transfected PSA-Gluc and m-PSA-Gluc reporter plasmids into LNCaP cells with or without endogenous YY1 knockdown. As shown in Figure 6C, wt and m-PSA promoters showed no significant differences in driving Gluc expression, either with or without R1881. Similar results were obtained in 293T cells (data not shown). We also tested the effect of YY1 on an artificial promoter, 3×ARE, which contains three concatemeric repeats of the PSA promoter ARE-I and lacks an YY1 binding element (Figure 6D). In this reporter assay using 3×ARE-Fluc, YY1 knockdown decreased the AR-mediated transcription by 35% ($p < 0.01$) in the presence of R1881 (#4 vs. #3, Figure 6E), similar to the YY1's effects on AR-mediated transcription of the endogenous PSA promoter (compare Figure 6E with Figure 3C).

YY1 is required for the AR-ARE-complex

We used EMSA to determine whether YY1 regulates AR-ARE association. We first analyzed the ^{32}P -labeled synthetic probe containing ARE-I of the PSA promoter without or with the incubation of LNCaP cell nuclear extract and detected a slowly migrated band when the nuclear proteins was added (lines 1 vs. 2, Figure 7A). This band likely corresponds to an AR-ARE-containing complex, since its intensity increased when the cells were cultured in R1881-containing medium (lanes 3 vs. 2), which enhances AR-ARE association and/or promotes AR expression, and decreased when the unlabeled probe was added to compete with the labeled probe (lanes 4 vs. 3). To further validate the identity of this band, we individually incubated the ^{32}P -labeled the ARE-I-containing probe and a control probe consisting of a scrambled sequence with the LNCaP cell nuclear extracts. As shown in Figure 7A (lanes 5 vs. 6), the ARE-I probe exhibited a specific band with the same migration as lane 2, but the control showed mostly nonspecific binding. To confirm the presence of AR in this band, we incubated the labeled ARE-I-containing probe with the nuclear protein extracts of LNCaP cells infected by lentiviruses expressing the scrambled siRNA or the AR siRNA that efficiently knocked down endogenous AR (data not shown). The depletion of AR markedly reduced the intensity of the detected band (lanes 7 vs. 8, Figure 7A). Therefore, these gel-shift experiments unequivocally indicate that the slowly migrate band, as pointed at the left of Figure 7A, is the AR-ARE-I-containing complex.

To determine the presence of YY1 in this AR-ARE-I-containing complex, we pretreated the LNCaP nuclear extracts with YY1 antibodies (13G10 generated from rabbit and H-10 from mouse) prior to the analysis of EMSA. Both YY1 antibodies markedly decreased the intensity of the AR-ARE-I-containing complex, compared to the samples treated with the control antibodies (lanes 2 vs. 1 and 4 vs. 3, Figure 7B). These results indicate that YY1 is an essential component of the AR-ARE-I-complex, since the association of YY1 and YY1 antibody disrupted the complex formation.

Discussion

YY1 regulates different epigenetic processes by mediating gene expression and protein modifications. The multifunctional properties of YY1 may explain its elevated expression in different cancers, including PCa. Our current study provided unequivocal evidence of the

functional YY1-AR interaction and unveiled another regulatory activity of YY1: acting on AR-mediated transcription through direct protein-protein interaction. Our data suggest that YY1 plays an important role in prostate cell differentiation and PCa development.

We and others demonstrated that YY1 enhances Mdm2-mediated p53 ubiquitination/ degradation, inhibits p300-mediated p53 acetylation and antagonizes p53's transcriptional activity (Gronroos *et al.*, 2004; Sui *et al.*, 2004). In the current studies, we revealed that YY1 promotes AR-mediated transcription without significantly altering its expression and stability. Therefore, YY1 exhibits differential regulatory functions to different proteins. Since YY1 interacts with various transcriptional cofactors, it is possible that the binding of YY1 provides an additional interface to AR-mediated gene expression and determines the components in the AR-containing complex.

Ample evidence indicates that YY1 regulates gene expression through recruiting various chromatin remodeling proteins to target promoters. Depending on the recruited cofactors, YY1 can either activate or inhibit gene expression. In the present study, we observed that a medium increase of YY1 in 293T cells could markedly enhance AR-mediated transcription of the PSA promoter. However, further increases in transfected YY1 did not add to, and actually slightly decreased, AR-mediated transcription (Figure 3A). Similarly, in LNCaP cells, although YY1 knockdown significantly reduced PSA promoter transcription in the presence of R1881, ectopically expressed YY1 did not cause any increased transcription (Figure 3B). We believe that the already high levels of YY1 in prostate cancer cells, such as LNCaP, contribute to the elevated activity of AR. Conversely, instead of promoting AR's function, the further increase in YY1 inversely affects the activity of AR due to the "squenching effect" resulted from an excessive titration of a transcription factor, as described previously by Gill et al (Gill and Ptashne, 1988). Consequently, this leads to compromised performance of AR, as shown in the cotransfection experiments in 293T cells (Figure 3A). Based on this observation, we proposed that the regulation of AR by YY1 follows a model schematically depicted in Figure 8. In a condition with an optimal increase of YY1, it is recruited by AR and activates gene expression. In PCa cells that exhibit elevated YY1 expression (Seligson *et al.*, 2005), we propose that this recruitment leads to stimulated expression of AR-targeted genes and consequently promotes proliferation of PCa cells. When YY1 is either depleted by siRNA or robustly overexpressed, the expression of AR-targeted genes will be significantly reduced by the absence of YY1 or attenuated due to the squenching effect caused by excessive YY1, respectively.

The manner of YY1-regulated transcriptional activity of AR is reminiscent to a reported correlation between YY1 expression and outcomes of prostate cancer patients (Seligson *et al.*, 2005). In that study, YY1 expression was predominantly elevated in the prostatic intraepithelial neoplasia stage and in tumors from intermediate to high morphologic grade. However, increased YY1 expression in prostate cancer tissues was inversely associated with a higher risk to develop recurrent disease. Therefore, although YY1 could be used as a diagnostic and prognostic marker, its functional role may be either proliferative or antiproliferative, depending on the stages of prostate cancer development and progression.

Androgen receptors are critical for the proliferation and survival of prostate cancer cells. Current therapeutic approaches in PCa to inhibit AR function include preventing ligand synthesis and using androgen antagonists that bind to AR (Hirawat *et al.*, 2003). Although these treatments are initially effective, they will eventually fail due to restored AR activity despite the presence of therapeutics. Therefore, understanding the molecular mechanism of AR regulation may provide fundamental support to the therapeutic treatment of PCa. In this study, we demonstrated that YY1 physically interacts with the C-terminal of AR and is essential to the AR-mediated transcription of the PSA promoter. These results imply that YY1 may be an alternative therapeutic target to inhibit AR's activity and PCa development. In addition, the regulation of YY1 to AR's transcriptional activity depends on the YY1-AR interaction but not on the YY1-DNA association, suggesting that YY1 may either mediate the posttranslational modification of AR or act as a cofactor, recruiting other proteins to facilitate AR-mediated transcription. Further investigation is required to delineate whether altered YY1 expression will affect the posttranslational modification of AR and/or the association of AR with other critical transcription cofactors.

We have demonstrated that the YY1-AR interaction is important to AR-mediated transcription of the PSA promoter. Whether the direct binding of YY1 to the PSA promoter affects PSA expression is still unclear. Our results indicate that the transcription of the PSA promoter and the artificial 3×ARE-I promoter without any YY1 binding site exhibited similar effects to altered YY1 expression (Figures 3C and 6D). Thus, it is unlikely that YY1-DNA association is critical to PSA promoter transcription. However, since increased YY1 expression inversely affected AR-mediated transcription, it is also possible that excess YY1 in the nucleus may enhance the YY1-DNA association, in turn reducing transcription of the PSA promoter.

Both AR and YY1 are proteins with multiple functions and their regulatory roles in prostate cancer have been indicated by many previous reports. Our current study revealed the functional interplay between these two proteins in PCa cells. We demonstrated that, as a novel AR-interacting protein, YY1 is an essential component of the ARE-complex and crucial in maintaining the AR-mediated transcription. Future study is needed to delineate the mechanism underlying YY1-regulated function of AR.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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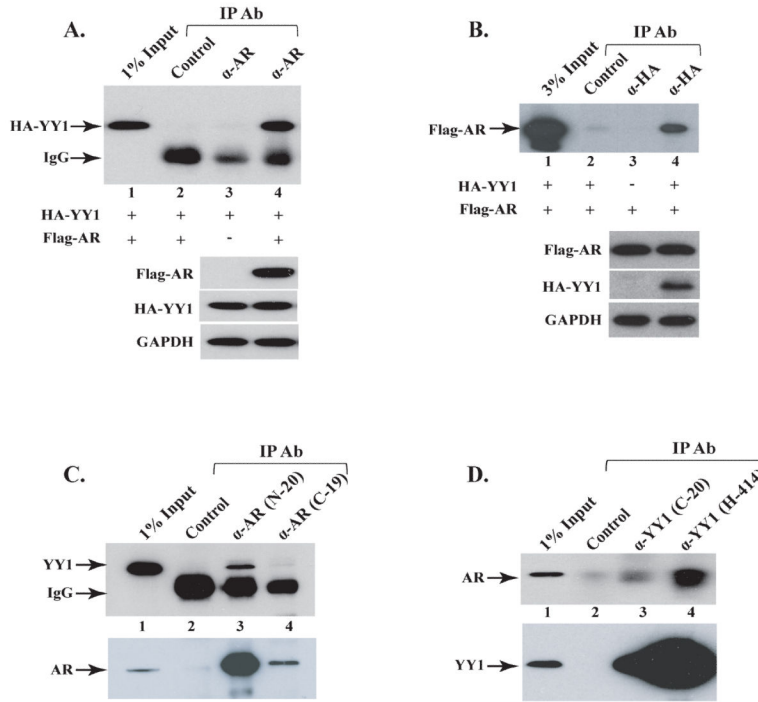


Figure 1. Interaction of YY1 and AR in cells

A and B. Interaction of transfected YY1 and AR in 293T cells. One µg of pcDNA3/HA-YY1 or an empty vector and 1 µg of pcDNA3/Flag-AR or the empty vector were cotransfected into 293T cells. One day post transfection, cells were cultured in medium containing 10 nM R1881 for 24 h, followed by cell lysis. In co-immunoprecipitation (CoIP) experiments, AR polyclonal antibody (N-20), HA antibody, or control antibodies were incubated with cell lysates. Precipitated samples were applied to Western blot analysis using monoclonal antibodies against HA (A) or Flag (B). HA-YY1 and Flag-AR are indicated at left. Lower panels: Western blots of the protein inputs in CoIP. C and D. Interaction of endogenous YY1 and AR in LNCaP cells. LNCaP cells were treated with 10 nM R1881 for 24 h and 500 µg of cell lysates were used in reciprocal CoIP analysis using indicated antibodies (3 µg/each). Antibodies used in Western blot studies (upper and lower panels) are indicated at left.

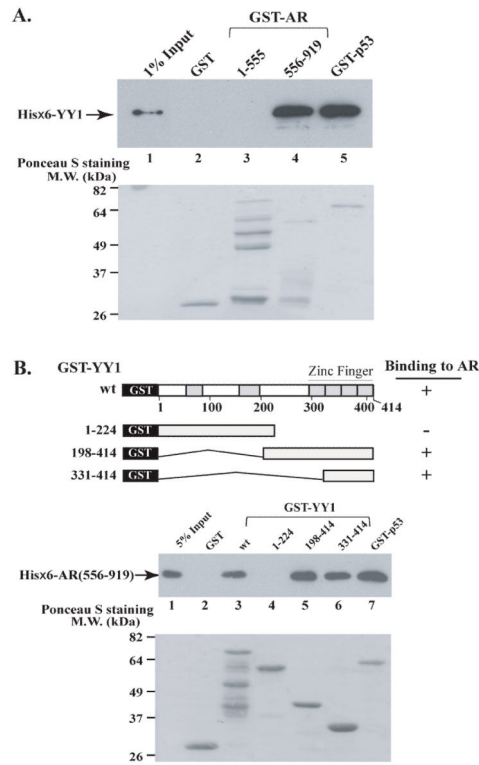


Figure 2. *In vitro* protein binding studies to determine YY1 and AR interaction domains
A. Identification of YY1 binding domain on AR. Purified GST-AR(1–555), GST-AR(556–919) and GST-p53 proteins (3 μg each) were individually incubated with His×6-YY1 (1.2 μg). Samples brought down by glutathione agarose beads were analyzed by Western blot using YY1 (H-10) antibody. Lower panel: Ponceau S staining of transferred membrane to show input of GST fusion proteins. **B. Identification of AR binding domain on YY1.** Top panel: Diagram of GST-YY1 fusion proteins. Purified GST-YY1 proteins (3 μg each) and GST-p53 were individually incubated with purified His-AR(556–919) (1.2 μg each). Samples brought down by glutathione agarose beads were analyzed by Western blot using AR (N-20) antibody. Lower panel: Ponceau S staining of the transferred membrane to demonstrate input of the GST fusion proteins.

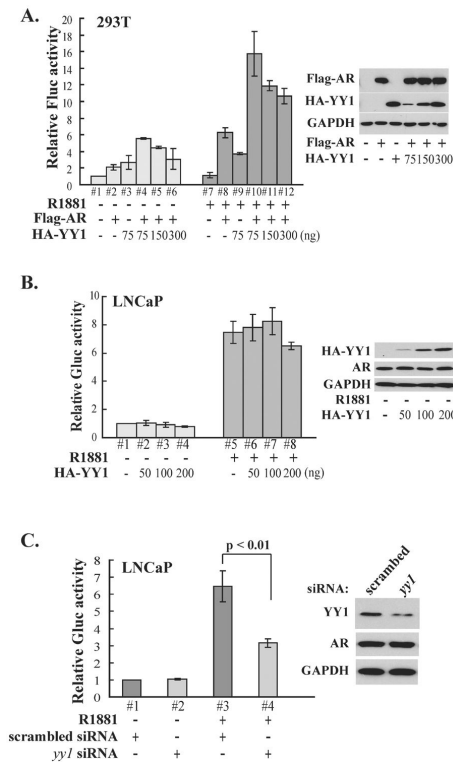


Figure 3. Studies of YY1 expression on the activity of AR-mediated PSA promoter

A. Effects of YY1 increase on PSA promoter activity with transfected AR in 293T cells. pcDNA3/HA-YY1 (75, 150 and 300 ng), pcDNA3/Flag-AR (300 ng), PSA-Fluc reporter (300 ng) and Actin-SEAP (100 ng) were cotransfected into 293T cells cultured in 12-well plates with or without 10 nM R1881. Each transfection was in triplicate and total DNA was compensated to the same amount with an empty vector, if necessary. Fluc in cell lysates was measured at 48 h after transfection and then normalized against SEAP activity in the same sample. Right panel: representative Western blot analysis of Flag-AR, HA-YY1 and GAPDH. B. Effects of YY1 increase on the PSA promoter in LNCaP cells. Differing amounts of pcDNA3/HA-YY1 (75, 150 and 300 ng), PSA-Gluc reporter (80 ng) and Actin-SEAP (100 ng) were cotransfected into LNCaP cells cultured in the absence or presence of R1881 in triplicates with an empty vector to compensate for each transfection if necessary. Gluc in the medium was measured at 48 h after transfection and then normalized against SEAP activity in the same sample. Right panel: representative Western blot analysis of HA-YY1, endogenous AR and GAPDH. C. Effects of YY1 depletion on the PSA promoter in LNCaP cells. LNCaP cells were cotransfected with PSA-Fluc reporter (500 ng), Actin-SEAP (100 ng) and the plasmid expressing the scrambled siRNA or *yy1* siRNA (300 ng), as labeled, in triplicates. After cells were cultured in medium with or without 10 nM R1881 for 48 h, Fluc activity in the cell lysates was measured and normalized to SEAP activity in the same sample. Expression of YY1 and AR was analyzed by Western blot and shown at right.

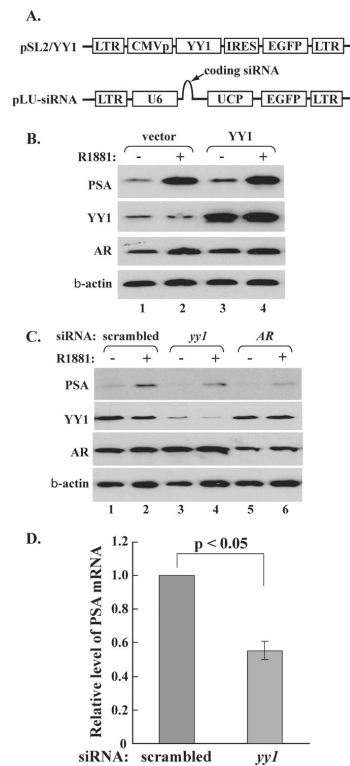


Figure 4. Effect of YY1 on expression of endogenous PSA in LNCaP cells

A. Schematic diagrams of lentiviral vectors used to express YY1 and siRNAs in LNCaP cells. Sequences between the two long terminal repeats (LTRs) in the Lentiviral vectors are integrated into the genome of the infected cells. CMVp: CMV promoter; IRES: internal ribosomal entry site; UCP: ubiquitin C promoter. EGFP: enhanced green fluorescent protein.

B. Effect of overexpressed YY1 on endogenous PSA. LNCaP cells were infected with Lentivirus generated from empty pSL2 vector or pSL2/YY1. Infected cells were first cultured in Phenol-red free RPMI medium with 10% CSS for 48 h, followed by another 24 h of culture with or without 10 nM R1881. Cell lysates were then analyzed by Western blot using antibodies against PSA (BiosPacific Inc., Emeryville, CA), YY1 (H-10), AR (N-20) and β -actin (MAB1501, Chemicon International Inc.). **C and D.** Effect of YY1 depletion on endogenous PSA. LNCaP cells were infected by Lentivirus generated from pLu-U6/scrambled siRNA, pLu-U6/*yy1* and pLu-U6/*AR*. Infected cells were treated as described above and Western blot was used to detect expression of PSA, YY1, AR and β -actin (C). The samples infected by the scrambled siRNA and *yy1* siRNA were also analyzed by Real-time qPCR for PSA expression with GAPDH as a control (D).

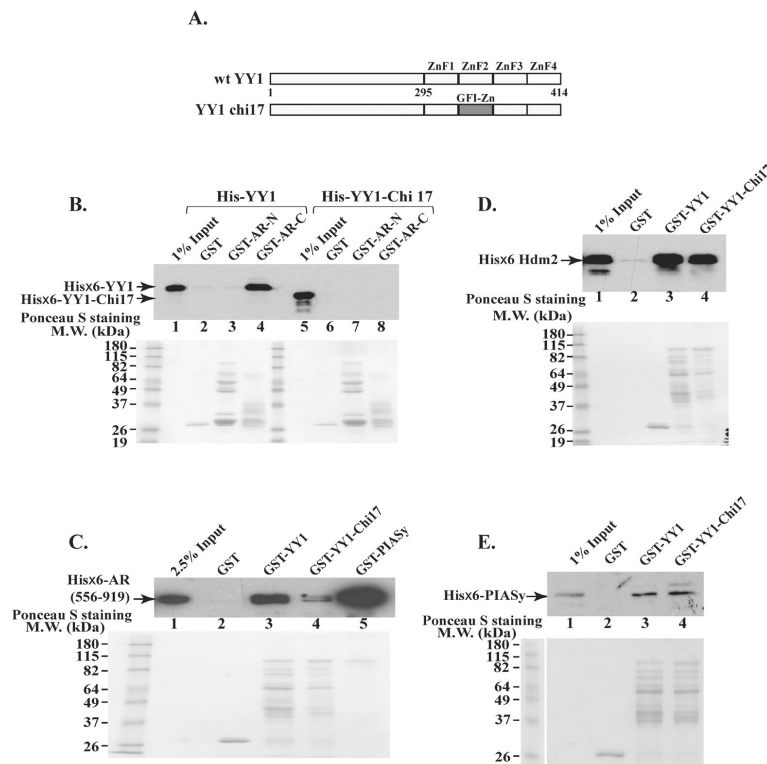


Figure 5. Zinc finger 2 of YY1 is essential to YY1-AR interaction

A. Schematic diagram of domain structures of wild type YY1 and YY1 Chi17. The four zinc fingers of YY1 (ZnF1 to ZnF4) are labeled on the top. GFI: growth factor independence-1.

B and C. Substitution of zinc finger 2 of YY1 disrupts YY1-AR interaction. YY1 Chi17 mutant has the second zinc finger of YY1 replaced by a zinc finger of GFI-1 (Galvin and Shi, 1997). In “B”, equal amount (3 μ g) of GST, GST-AR-N (i.e. AR 1–555) and GST-AR-C (i.e. AR 556–919) were individually incubated with 1.5 μ g of His \times 6-YY1 or His \times 6-YY1 Chi17 for 4 h at 4 $^{\circ}$ C. Samples brought down by glutathione agarose were analyzed by Western blot using YY1 (H-10) antibody. Lower panel: Ponceau S staining of transferred membrane to demonstrate input of GST fusion proteins. In “C”, GST, GST-YY1, GST-YY1-Chi17 and GST-PIASy (3 μ g each) were individually incubated with 1.5 μ g of His \times 6-AR(556–919) under the same conditions described above. Samples pulled down by glutathione agarose were applied to Western blot to detect His \times 6-AR(556–919) using AR antibody (C-20). Lower panel: protein inputs. D and E. YY1 Chi17 retains its interaction with Hdm2 and PIASy. GST, GST-YY1 and GST-YY1-Chi17 (3 μ g each) were individually incubated with 1.5 μ g of His \times 6-Hdm2 or His \times 6-PIASy, respectively. Samples brought down by glutathione agarose were analyzed by Western blot using Hdm2 antibody (Smp14) or PIASy antibody (I-19). Lower panels: protein inputs stained by Ponceau S.

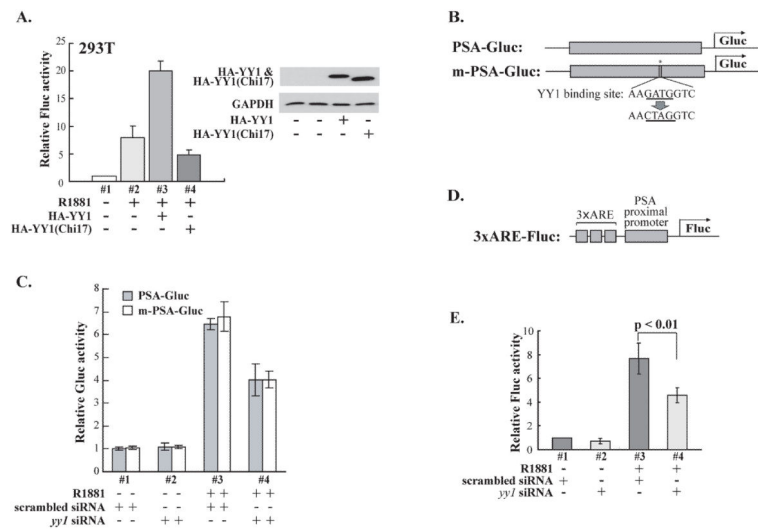


Figure 6. YY1-AR interaction is essential to YY1-enhanced AR transcriptional activity

A. YY1-Chi17 does not activate AR-mediated transcription of the PSA promoter. 100 ng of pcDNA3/HA-YY1 or pcDNA3/HA-YY1-Chi17 were cotransfected with 300 ng of pcDNA3/Flag-AR, 300 ng of PSA-Fluc and 100 ng of Actin-SEAP into 293T cells in 12-well plates cultured in medium with or without 10 nM R1881, as described above. Fluc activity was normalized against SEAP activity (left panel); expression of HA-YY1, HA-YY1-Chi17 and GAPDH is shown on the right panel. B. Schematic diagram of reporter constructs of PSA-Gluc and m-PSA-Gluc with mutated putative YY1 binding site. Mutation of the putative YY1 binding site is indicated. C. YY1 could stimulate the expression of the PSA promoter with mutated putative YY1 binding site. LNCaP cells were cotransfected by 300 ng of siRNA plasmid carrying either the scrambled or *yy1* siRNA, 80 ng of PSA-Gluc or m-PSA-Gluc, and 100 ng of Actin-SEAP, in triplicates. Transfected cells were cultured in medium without or with 10 nM R1881; normalized Gluc activity in each condition is presented. D. Schematic diagram of the 3×ARE-Fluc reporter construct. See “Materials and Methods” for detail. E. Effects of YY1 depletion on 3×ARE promoter in LNCaP cells. LNCaP cells were cotransfected with 3×ARE-Fluc reporter (500 ng), Actin-SEAP (100 ng) and plasmid (300 ng) expressing scrambled siRNA or *yy1* siRNA, as labeled.

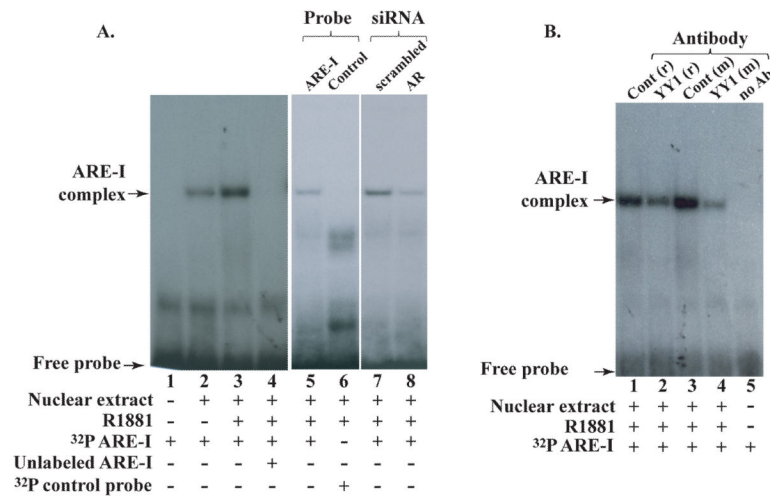


Figure 7. YY1 is essential to the formation of ARE complex

A. Validation of the AR-ARE complex by EMSA. Lanes 1: ³²P-labeled ARE-I containing probe incubated with BSA. Lanes 2–4: labeled probe was incubated with nuclear protein extracts from LNCaP cells cultured without or with R1881, as indicated. Sample in lane 4 was co-incubated with unlabeled probe. Lanes 5 and 6: nuclear protein extracts from R1881-treated LNCaP cells were incubated with ³²P-labeled DNA probes containing either the ARE-I sequence or a scrambled sequence, as indicated on top. Lanes 7 and 8: the extracts from R1881-treated LNCaP cells infected by lentivirus expressing either a scrambled siRNA or the AR siRNA (indicated on top) were incubated with the labeled ARE-I-containing probe. Samples were applied to a 5% non-denaturing polyacrylamide gel for EMSA. **B.** YY1 antibodies attenuate the formation of AR-ARE complex. Nuclear protein extracts in lanes 1–4 were pretreated with different antibodies (indicated on top) before the incubation with labeled probe. Lane 5: labeled probe incubated with BSA. Cont: control; r: rabbit; m: mouse; YY1 (r): YY1 antibody 13G10; YY1 (m): YY1 antibody H-10. no Ab: no antibody added.

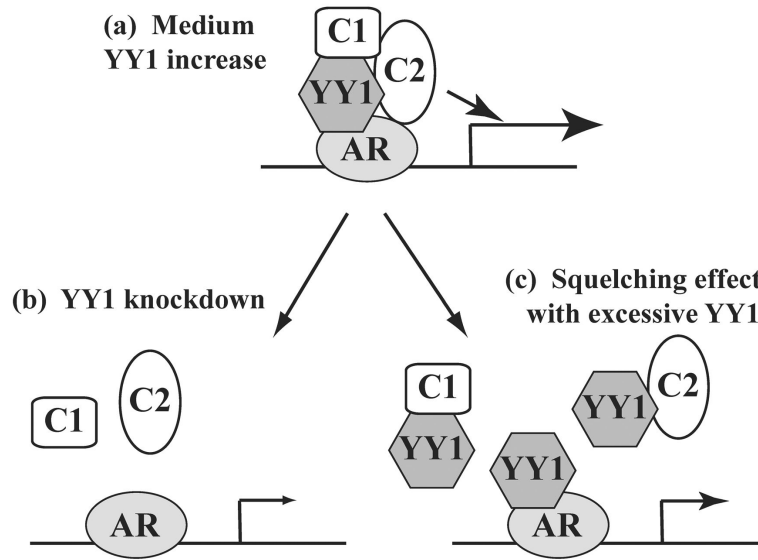


Figure 8. Schematic model for the effects of altered YY1 expression on AR-mediated gene expression
 At the condition of YY1 increase (a), such as in prostate cancer, YY1 binds to AR and recruits cofactors to facilitate the expression of AR-targeted genes. When AR was depleted by siRNA (b), the cofactors will not be recruited and therefore the AR-mediated gene transcription is decreased. When YY1 is robustly overexpressed (c), excessive YY1 will individually interact with AR and the cofactors, which interferes with the formation of the optimized transcriptional complex. This so-called “squelching effect” (Gill and Ptashne, 1988) will inversely affect the expression of AR-targeted genes. The sizes of the arrows on AR-targeted promoter represent the strength of transcription. C1 and C2: cofactors recruited by YY1.