

Wwox Suppresses Prostate Cancer Cell Growth through Modulation of ErbB2-Mediated Androgen Receptor Signaling

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Abstract

The expression of the *WWOX* tumor suppressor gene is lost or reduced in a large fraction of various cancers, including prostate cancer. We previously reported that *Wwox* overexpression induced apoptosis and suppressed prostate cancer growth *in vitro* and *in vivo*. In this study, pathways through which *Wwox* contributes to control of prostate cancer cell growth have been investigated. We found that *Wwox* interacts with Ap2 γ and prevents it from entering the nucleus to bind the *ERBB2* promoter region to activate transcription of *ERBB2*, a mediator of androgen receptor activity and prostate cancer cell growth at limiting androgen concentration. Ectopic expression of *Wwox* reduced ErbB2 protein expression *in vitro* and expression of *Wwox* protein inversely correlated with expression of ErbB2 protein in prostate cancer tissues. Furthermore, *Wwox* suppressed Ap2 γ /ErbB2-induced prostate cancer cell growth and suppressed prostate-specific antigen secretion through interaction with Ap2 γ and down-modulation of ErbB2, an effect that required functional androgen receptor. (Mol Cancer Res 2007;5(9):957–65)

Introduction

The tumor-suppressor gene, *WWOX* (WW domain containing oxidoreductase), spans one of the most active fragile sites, *FRA16D*, at chromosome 16q23.3-24.1, a region exhibiting loss of heterozygosity in breast, prostate, and other cancers (1-3). *Wwox* protein contains two WW domains in the NH₂-terminal region and a short-chain dehydrogenase/reductase

domain in the central region of the protein; WW domains are involved in protein-protein interactions (4). The *Wwox* short-chain dehydrogenase/reductase domain has amino acid sequence homology to steroid oxidoreductases and the *Wwox* expression level in normal tissues is highest in hormonally regulated tissues such as testis, prostate, and ovary (1, 5), suggesting that *Wwox* may have important functions in hormone-regulated tissues.

We previously showed that *Wwox* expression is reduced or lost in prostate cancer-derived cells and tissues, in part due to hypermethylation in the *WWOX* regulatory region, and that overexpression of exogenous *WWOX* induced apoptosis in prostate cancer cells and suppressed prostate cancer growth *in vitro* and *in vivo* (6). However, the mechanisms through which *Wwox* suppresses prostate cancer growth had not been investigated. Examination of *Wwox* function in breast cancer revealed that *Wwox* down-regulates ErbB2 (Her-2/neu) receptor tyrosine kinase expression and activity through binding and cytoplasmic sequestration of the transcription factor Ap2 γ .¹ The Ap2 transcription factors had been shown to bind the *ERBB2* promoter and activate its transcription in breast cancer cells (7-11). ErbB2, a member of the epidermal growth factor receptor family of receptor tyrosine kinases, is overexpressed in ~30% of human breast cancers, and overexpression is associated with metastatic behavior and poor patient prognosis (12, 13).

Given the role of ErbB2 in breast cancer, ErbB2 has been extensively studied in prostate cancer for the past decade. Cumulative findings suggest that ErbB2 expression is elevated in prostate cancer (14). ErbB2 overexpression in LNCaP prostate cancer-derived cells enhanced androgen receptor (AR) activity and hormone-independent cell growth (12, 15), whereas small interference RNA (siRNA)-mediated ErbB2 knockdown impaired prostate cancer cell growth and AR activity (16). ErbB2 kinase activity is required for optimal transcriptional activity of AR at limiting concentrations of androgen (16, 17). In this study, we explored the effect of *Wwox* restoration on Ap2 γ and ErbB2 signaling in LNCaP (AR positive) and DU145 (AR negative) prostate cancer-derived cells, which express low endogenous *Wwox* levels (6), and the relevance of *Wwox*-mediated effects to prostate cancer cell growth and AR signaling.

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¹D. Iliopoulos, et al. *Wwox* tumor suppressor is a mediator of tamoxifen response, in preparation.

Results and Discussion

Wwox Interacts with Ap2 γ in Prostate Cancer Cells

In this study, the mechanism(s) by which Wwox suppresses the development of prostate cancer has been explored both *in vitro* and *in vivo*. Aqeilan et al. (18) showed interaction of overexpressed Wwox and Ap2 γ in 293 cells, an interaction that involved the Y33 residue in the first WW domain of Wwox, and we had observed that endogenous and exogenous wild-type Wwox, but not WwoxY33R, interacted with Ap2 γ in MCF7 breast cancer cells.¹ The finding that Wwox down-regulated *ERBB2* transcription through interaction and cytoplasmic sequestration of the transcription factor Ap2 γ in breast cancer cells, coupled with reports that the oncoprotein ErbB2 is an important mediator in AR signaling and the development of prostate cancer (12, 14-17, 19), led us to hypothesize that Wwox may similarly mediate ErbB2 signaling in prostate cancer. To test this, we used AR-positive prostate cancer cell LNCaP, a widely used *in vitro* model for studying androgen-dependent prostate cancer and AR signaling events, and prostate cancer cell DU145, where appropriate, as an AR negative control. We first determined if Wwox and Ap2 γ interact in LNCaP cells through coimmunoprecipitation of exogenously expressed proteins. Cells were transiently cotransfected with expression plasmids for Wwox and Ap2 γ and lysates were extracted for coimmunoprecipitation. Wwox and Ap2 γ were coimmunoprecipitated using antisera against Wwox or Ap2 γ , as illustrated in Fig. 1. Ap2 γ and WwoxY33R did not coimmunoprecipitate (not shown), indicating that the Y33 residue is essential for binding, consistent with previous findings (18). Interaction of exogenous Wwox and Ap2 γ was also detected in DU145 cells (not shown).

Wwox Down-Modulates ERBB2 Transcription through Interaction with Ap2 γ

We next determined if restoration of Wwox affects *ERBB2* transcription and if it does so through Ap2 γ binding. LNCaP cells were cotransfected with *ERBB2*-luciferase reporter plasmid (*ERBB2*-LUC) along with indicated plasmids, as shown in Fig. 2A. Cotransfection of *ERBB2*-LUC and *AP2 γ* carrying plasmids led to an ~3.5-fold increase in luciferase activity compared with the empty vector-cotransfected controls. In the presence of *WWOX*-cotransfecting plasmid, Ap2 γ was not able to activate the *ERBB2* reporter, whereas cotransfection with *WWOXY33R* carrying plasmid did not

significantly affect Ap2 γ -enhanced luciferase activity, suggesting that, as in breast cancer-derived cells, Wwox affects *ERBB2* transcription through binding Ap2 γ and preventing it from entering the nucleus to activate *ERBB2*.

Knockdown of Ap2 γ Reduced ErbB2 Protein Expression

If Wwox down-regulates *ERBB2* through mediating Ap2 γ nuclear/cytoplasmic shuttling, then knockdown of Ap2 γ , leading to reduced level of nuclear Ap2 γ , should result in down-regulation of ErbB2. To test this, we used RNA interference to transiently knockdown Ap2 γ expression and observed that Ap2 γ knockdown resulted in reduced ErbB2 protein level compared with cells treated with scrambled siRNA (Fig. 2B), confirming that Ap2 γ is downstream of Wwox signaling in down-modulating *ERBB2* transcription.

Wwox Abrogates Recruitment of Ap2 γ Transcription Factor to ERBB2 Promoter

The transcription factor Ap2 γ is known to bind and activate the *ERBB2* promoter in breast cancer cells (7-11). An Ap2 binding site was identified in the *ERBB2* proximal promoter at 500 bp (9-11) upstream from the transcription start site, enhancing *ERBB2* gene transcription. To directly show that binding of Ap2 γ to the *ERBB2* promoter is affected in the presence of Wwox in LNCaP cells, we did chromatin immunoprecipitation (ChIP) assays to assess the recruitment of Ap2 γ and RNA polymerase II to the *ERBB2* promoter using the primer set amplifying the -500-bp *ERBB2* locus of interest. The -5,300-bp locus of the *ERBB2* distal promoter was amplified as a negative control (11). Normal IgG antibody-precipitated samples served as negative controls. The results are given as percentage of input DNA. Although the IgG-negative controls gave very low signals in every reaction, significant occupancy of Ap2 γ at the -500-bp locus of the *ERBB2* promoter was observed after overexpression of Ap2 γ (Fig. 2C), and this effect was dramatically abrogated in the presence of exogenously expressed Wwox. Coexpression of Wwox led to ~3-fold reduction in Ap2 γ recruitment to the locus of interest, relative to overexpression of Ap2 γ alone. Recruitment of RNA polymerase II to the same region corresponded closely with Ap2 γ binding. The -5,300-bp *ERBB2* locus was not enriched in either the anti-Ap2 γ or the anti-RNA polymerase II immunoprecipitates in the presence or absence of Wwox (Fig. 2D). This confirms the specificity of *in vivo* Ap2 γ recruitment to the -500-bp locus of the *ERBB2* promoter.

Exogenous Wwox Expression Abolishes Ap2 γ Nuclear Localization and Leads to Reduced ErbB2 Expression

We further determined, through immunoblotting, whether ErbB2 protein level was affected by Wwox restoration. Expression of ErbB2 and nuclear Ap2 γ was reduced in cells infected with Ad-*WWOX* compared with noninfected and Ad-GFP-infected controls (Supplementary Fig. S1). To confirm this finding and to confirm that down-regulation of ErbB2 by Wwox is in part through Wwox binding to Ap2 γ , we transfected LNCaP cells with pCMV vector, or the same vector containing cDNA for *WWOX* or *WWOXY33R*. pCMV-*WWOX* but not pCMV-*WWOXY33R* transfection resulted in reduced

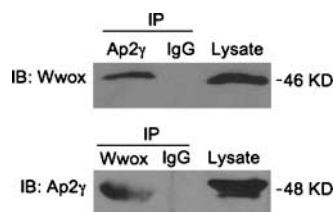


FIGURE 1. Wwox interacts with Ap2 γ in prostate cancer cells. LNCaP cells were transiently cotransfected with *WWOX* (8 μ g) and *AP2 γ* (8 μ g) expression plasmids. Forty-eight hours after transfection, whole-cell lysates were immunoprecipitated with anti-Wwox, anti-IgG, and anti-Ap2 γ sera. The immunoprecipitates were analyzed by immunoblotting with antisera against Wwox and Ap2 γ . IB, immunoblot; IP, immunoprecipitate.

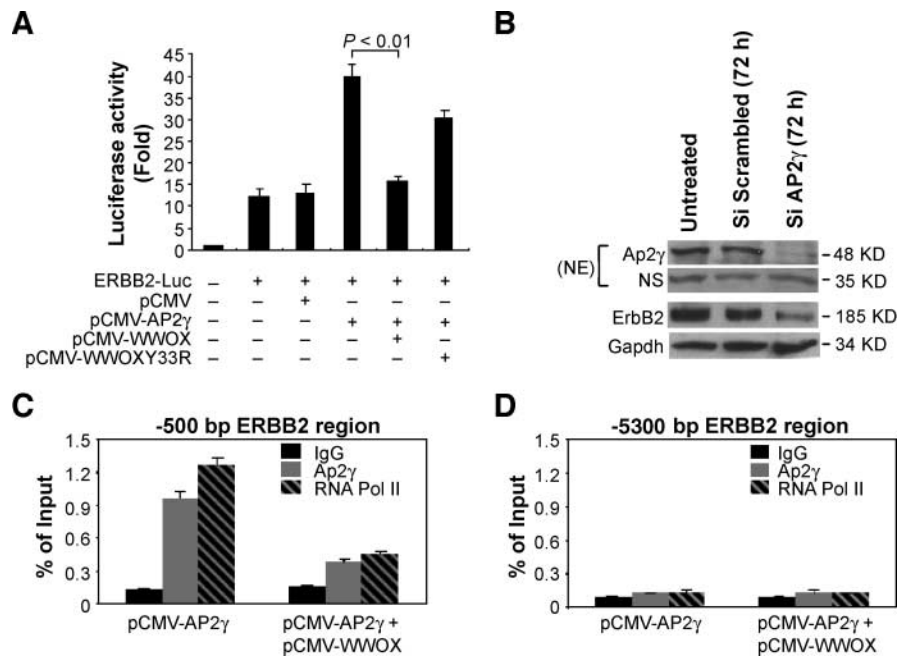


FIGURE 2. Ectopic expression of Wwox down-modulates *ERBB2* transcription through Ap2γ binding. **A.** LNCaP cells in normal growth medium were seeded at a density of 5×10^4 per well in 12-well plates in triplicate and were transiently cotransfected with *ERBB2*-Luc (0.2 μg) and expression plasmid for AP2γ (0.5 μg), *WWOX* (0.5 μg), or *WWOXY33R* (0.5 μg). Empty vector was cotransfected to normalize plasmid concentration and 1 ng of the *Renilla* vector pRL-TK was cotransfected to normalize transfection efficiency. Forty-eight hours after transfection, cells were lysed and luciferase assay was done using Promega Dual-Luciferase Reporter Assay System kit. **B.** LNCaP cells were transfected with AP2γ siRNA or scrambled siRNA control and collected 72 h after transfection. Cell lysates were extracted for immunoblotting. NE, nuclear extract. NS, nonspecific band. Because nuclear extracts were used, levels of a nonspecific protein detected by the Ap2γ antiserum were used to show equivalent loading, as described (33, 34). **C** and **D.** ChIP analyses at *ERBB2* locus. LNCaP cells were transfected with either pCMV-AP2γ or cotransfected with pCMV-AP2γ and pCMV-WWOX. At 48 h posttransfection, ChIP assays were done; antisera used in the ChIP assay are indicated. DNA samples from ChIP preparation were analyzed by real-time PCR analyses using primer sequences to amplify the -500 bp *ERBB2* locus of interest (**C**) with the -5,300 bp locus of the *ERBB2* promoter serving as a negative control (**D**). The value for each immunoprecipitation sample was obtained from the average of triplicate real-time PCR reactions. Columns, mean; bars, SD. Input values were obtained from samples treated identically as the experimental ones except that no immunoprecipitation steps were done. Data shown are representative of three independent experiments that yielded similar results.

ErbB2 expression and decreased nuclear Ap2γ (Fig. 3A), suggesting that interaction of Wwox and Ap2γ correlates with reduced ErbB2 expression and cytoplasmic localization of Ap2γ.

Wwox Knockdown Leads to Increased Nuclear Ap2γ and Elevated ErbB2 Expression

Because ectopic expression of Wwox in Wwox-deficient prostate cancer cells led to reduced nuclear Ap2γ level and suppressed ErbB2 protein expression, we further used RNA interference to address the reciprocal question of whether knockdown of Wwox will lead to elevated nuclear Ap2γ and increased ErbB2 expression. To test this, we used PWR-1E noncancer prostate epithelial cells, which express a relatively abundant level of Wwox compared with the prostate cancer cell lines. We found that, as expected, siRNA knockdown of Wwox led to increased nuclear Ap2γ and enhanced ErbB2 protein expression (Fig. 3B).

Wwox Sequesters Ap2γ in the Cytoplasm

It has been reported that Wwox localizes in the cytoplasm (18, 20-22) and interacts with Ap2γ, which, in turn, causes retention of Ap2γ in the cytoplasm in NIH3T3 and MCF7 cells (18).¹ To extend these findings to prostate cancer cells, we examined the subcellular localization of Ap2γ in the presence

or absence of Wwox in LNCaP cells via immunofluorescence staining. In cells transfected with pCMV-AP2γ alone, Ap2γ localized predominantly in the nucleus (Fig. 3C). In cells cotransfected with plasmids expressing Ap2γ and wild-type Wwox, Ap2γ became cytoplasmic. Cotransfection with plasmids expressing Ap2γ and WwoxY33R mutant did not affect nuclear localization of Ap2γ. These data suggest that binding of Wwox to Ap2γ prevented Ap2γ from entering the nuclei to activate or repress transcription of target genes such as *ERBB2*.

Wwox Suppresses Ap2γ/ErbB2-Induced LNCaP Cell Growth in Low Androgen Environment, Possibly through Mediating AR Signaling

We next investigated the biological effect of the binding of Wwox and Ap2γ. We had previously reported that Wwox restoration suppressed prostate cancer cell growth (6). The ErbB2 receptor tyrosine kinase has been found to be elevated in prostate cancer tissues (14, 23-28), and overexpression of ErbB2 led to androgen-independent cell proliferation through modulation of the AR signal pathway and decreased tumor latency of xenografts in castrated mice (12, 15). We hypothesized that Wwox might suppress prostate cancer cell growth by down-modulating ErbB2/AR signaling. Growth assays were done to determine if binding of Ap2γ and subsequent down-modulation of ErbB2 by Wwox has an effect

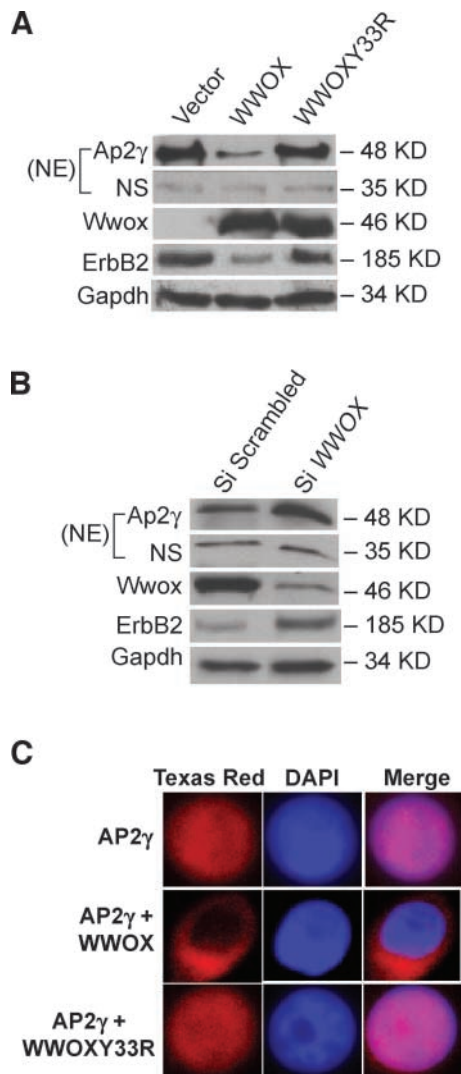


FIGURE 3. Ectopic expression of Wwox suppresses ErbB2 expression through binding Ap2 γ ; knockdown of Wwox using siRNA results in elevated nuclear Ap2 γ level and enhanced ErbB2 protein expression; Wwox binds and sequesters Ap2 γ in the cytoplasm. **A.** LNCaP cells were transfected with either the empty vector or the same plasmid carrying *WWOX* or *WWOXY33R*. At 48 h posttransfection, cells were harvested and proteins were extracted for immunoblotting. **B.** PWR-1E cells were transfected with *WWOX* siRNA or siRNA scrambled control and collected 72 h after transfection. Cell lysates were extracted for immunoblotting. **C.** LNCaP cells cultured on coverslips in 12-well plates were transfected with expression plasmids for *AP2 γ* or cotransfected with *WWOX* or *WWOXY33R* expression plasmids. At 48 h posttransfection, cells were fixed and stained for immunofluorescent visualization of subcellular location. Ap2 γ was sequestered to the cytoplasm in cells cotransfected with expression plasmids for *AP2 γ* and *WWOX*, but not *WWOXY33R*.

on cell growth. Because ErbB2 regulates AR function and prostate cancer cell growth at limiting concentrations of androgen (12, 14-17), we cultured LNCaP cells in phenol red-free medium supplemented with 10% charcoal/dextran-stripped fetal bovine serum (CS-FBS) and 1 nmol/L dihydrotestosterone. Because it is difficult to create an environment where androgen is absolutely absent in the human body, prostate cancer patients undergoing maximal androgen ablation (either surgical or medical castration, plus antiandrogen

therapy) still have low androgen levels (dihydrotestosterone at 1 nmol/L or less) in their serum (15). Thus, 1 nmol/L of dihydrotestosterone in our experiments simulates an androgen-independent environment relevant to the clinical setting. Cells were transfected with the indicated expression plasmids (Fig. 4). We observed that the growth rate for cells transfected with Ap2 γ expression plasmid was ~85% greater than empty vector-transfected controls (Fig. 4A). Cotransfection with *WWOX* expression plasmid reduced the growth rate to below the level of vector controls, whereas growth rates of cells cotransfected with *WWOXY33R* plasmid remained ~50% greater compared with cells cotransfected with wild-type *WWOX* plasmid. Thus, ectopic expression of Ap2 γ , leading to increased expression of ErbB2 (Fig. 4B), resulted in increased LNCaP cell growth in an androgen-independent environment, which was completely suppressed by wild-type Wwox but not mutant WwoxY33R. These data suggest that ErbB2 is downstream of Wwox in a signal pathway that suppresses prostate cancer cell growth, and that binding of Wwox and Ap2 γ is necessary for down-modulation of ErbB2 by Wwox.

To determine if the growth suppression by Wwox through binding Ap2 γ and down-modulating ErbB2 is AR-related, we first examined the expression level of the specific AR downstream target gene, *prostate-specific antigen (PSA)*, whose transcription is strictly regulated by androgen, using immunoblot analysis. Elevated ErbB2 level due to Ap2 γ overexpression resulted in increased PSA protein level in LNCaP cells (Fig. 4B). Cotransfection of *WWOX* plasmid led to reduced ErbB2 expression and subsequent reduction of PSA, whereas cotransfection of *WWOXY33R* plasmid did not cause an apparent difference in expression level of ErbB2 and PSA compared with *AP2 γ* plasmid-transfected cells. Interestingly, AR protein level was not altered by transfection with *AP2 γ* -containing plasmid or cotransfection with *WWOX*-containing plasmids. We next used prostate cancer cell DU145, which does not express endogenous AR, in transfection and growth curve assays under the same conditions. We observed that Ap2 γ overexpression in DU145 cells was much less effective in inducing cell proliferation than in LNCaP cells. The growth rate in *AP2 γ* expression plasmid-transfected cells was ~17% higher than the vector controls (Fig. 4C). Cotransfection with *WWOX* expression plasmid led to ~21% reduction below the vector controls. Growth rate in cells cotransfected with *WWOXY33R* expression plasmid was similar to the vector controls. Furthermore, PSA levels in DU145 cells remained undetectable in cells transfected with either the *AP2 γ* expression plasmid, or cotransfected with *WWOX* or *WWOXY33R* plasmids (not shown). Taken together, the dramatic growth-suppressive effect by Wwox through targeting Ap2 γ /ErbB2 in LNCaP cells is likely through regulating AR signaling.

To mimic the effect of elevated ErbB2 level, induced by Ap2 γ , on prostate cancer cell growth, we transfected LNCaP and DU145 cells with either the pCMV empty vector or the pCMV-*ERBB2* plasmid and did the growth assays again. As expected, ErbB2 overexpression resulted in ~2-fold increase in LNCaP cell growth relative to the empty vector control (Fig. 4D), but had much less effect on AR-negative DU145 cell

growth (Fig. 4E). This supports the proposal that AR signaling is involved in Wwox-mediated growth suppression in prostate cancer cells.

Wwox Suppresses AR Transactivation through Modulation of ErbB2; Reduced PSA Secretion by Wwox Requires Functional AR

Because transfection of either *AP2γ* or cotransfection with *WWOX* expression plasmids did not alter AR protein level in LNCaP cells, but *Ap2γ*/ErbB2–induced PSA expression was reversed by restoration of Wwox, and PSA has been well characterized as an androgen target gene, we were interested in determining if PSA reduction through Wwox-mediated ErbB2 down-regulation occurs through modulation of AR transactivation. We measured AR transactivation activity by transiently cotransfecting LNCaP cells with the pGL3E-probasin plasmid [androgen response element (ARE)-LUC] expressing the luciferase reporter gene driven by a promoter containing an ARE and the indicated expression plasmids (Fig. 5A). At low dihydrotestosterone concentration (1 nmol/L), *Ap2γ* expression increased AR-promoted luciferase activity 13.3-fold relative to the basal level, and this effect was suppressed in the presence of Wwox, but not WwoxY33R, suggesting that Wwox suppressed *Ap2γ*/ErbB2–induced AR transactivation through binding and cytoplasmic sequestration of *Ap2γ*. We extended the determination of AR transactiva-

tional activity from the artificial promoter to the endogenous androgen regulated gene, *PSA*. The level of PSA secreted in cell supernatants was highest in cells transfected with *Ap2γ*, in the presence of dihydrotestosterone (1 nmol/L), whereas cotransfection with *WWOX* but not *WWOXY33R* expression plasmid significantly abrogated this effect.

To confirm that AR signaling is required for down-regulation of *Ap2γ*/ErbB2–induced PSA secretion by Wwox, AR-negative DU145 cells were transfected with *ARE-LUC* and cotransfected with the indicated plasmids (Fig. 5B), and luciferase assay was done. In the absence of AR, *Ap2γ*/ErbB2 had no effect on ARE-LUC activity. Cotransfection of *AR* and *AP2γ* expression plasmids induced ARE-LUC activity 4.6-fold in the absence of dihydrotestosterone, compared with transfection with *AR* expression plasmid alone. The combination of *Ap2γ*, *AR*, and dihydrotestosterone led to a 16.2-fold increase in ARE-LUC activity compared with a 4.3-fold increase with *AR* and dihydrotestosterone. This effect was significantly compromised in the presence of wild-type Wwox, but not WwoxY33R. Accordingly, the level of PSA in the cell supernatant was undetectable in the absence of AR after transfection of *Ap2γ* expression plasmid and was highest in the presence of *Ap2γ*, *AR*, and dihydrotestosterone. Cotransfection of *WWOX* but not *WWOXY33R* expression plasmid abolished the effect of *Ap2γ*/ErbB2 on PSA secretion. Thus, Wwox suppressed AR transactivation

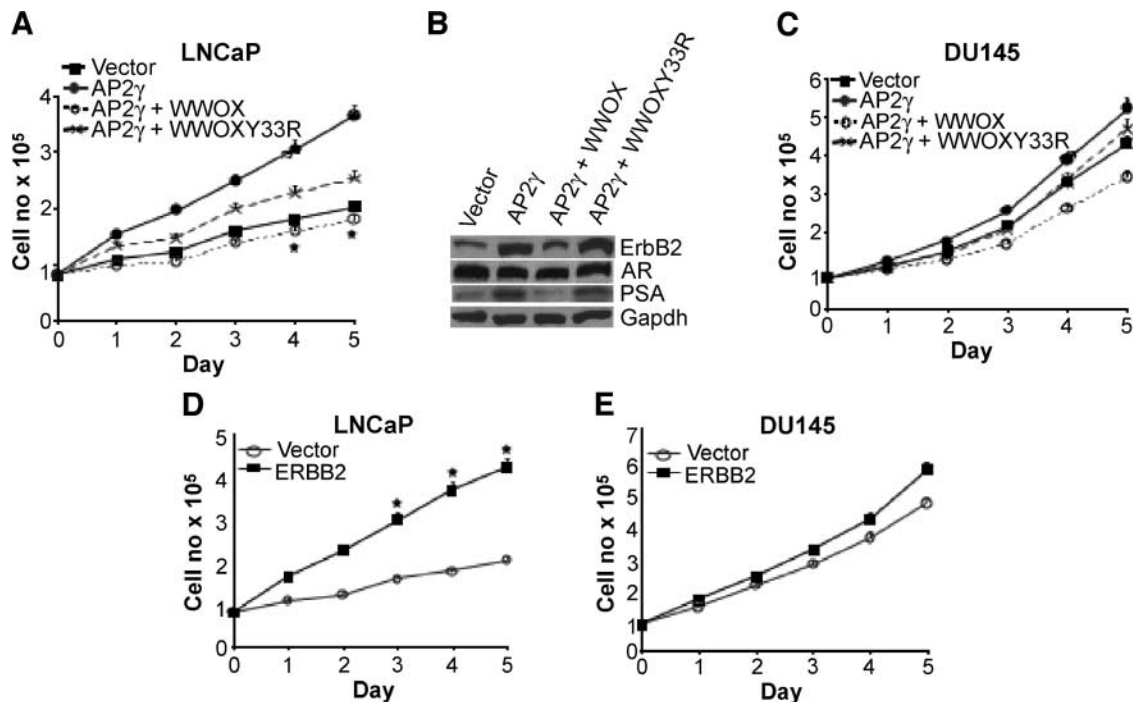


FIGURE 4. Wwox suppresses *Ap2γ*/ErbB2–induced LNCaP cell growth in an AR-dependent manner. **A.** LNCaP cells cultured in phenol red–free medium supplemented with 10% CS-FBS and 1 nmol/L dihydrotestosterone were transfected with the indicated plasmids, and cells were counted every 24 h for 5 d. *, $P < 0.05$, statistically significant growth suppression in cells cotransfected with Wwox relative to *Ap2γ* transfection alone. **B.** LNCaP cells cultured under the same conditions were transfected with the indicated plasmids. At 48 h posttransfection, cells were harvested and lysates were extracted for immunoblotting. Gapdh, glyceraldehyde-3-phosphate dehydrogenase. **C.** Growth curves were measured under the same conditions in DU145 cells transfected with the same expression plasmids. LNCaP (**D**) and DU145 (**E**) cells cultured in phenol red–free medium supplemented with 10% CS-FBS and 1 nmol/L dihydrotestosterone were transfected with either the pCMV empty vector or the same plasmid carrying *ERBB2* cDNA and growth analyses were done. *, $P < 0.05$, statistically significant difference relative to the controls.

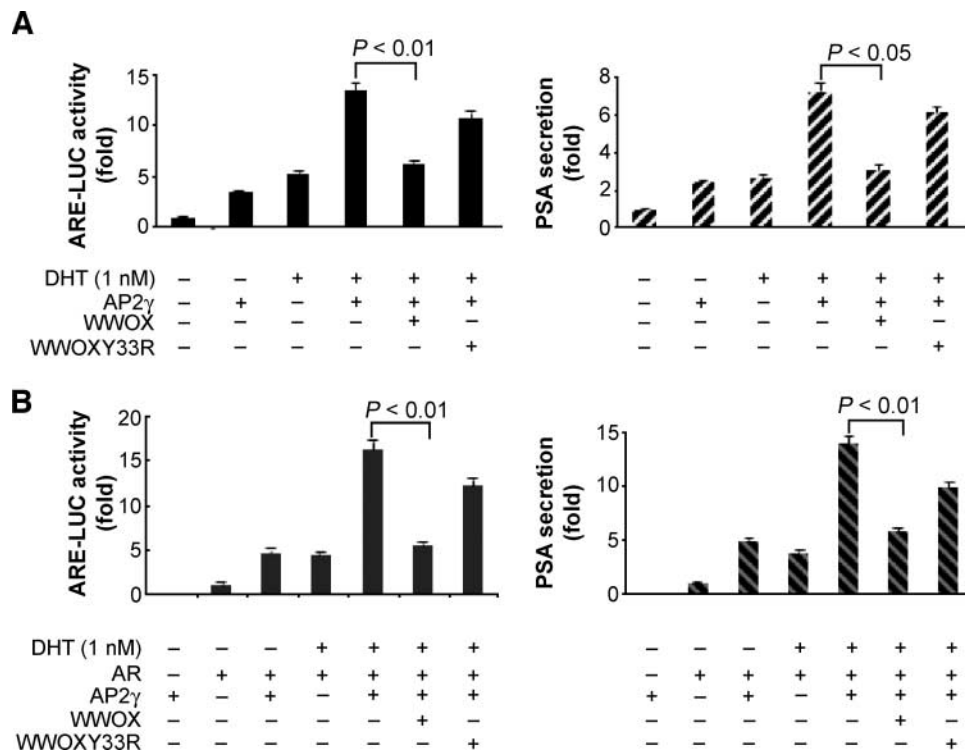


FIGURE 5. Wwox suppresses AR transactivation through regulation of Ap2 γ /ErbB2 signaling. **A.** LNCaP cells in phenol red-free medium supplemented with 10% CS-FBS were seeded at a density of 5×10^4 per well in 12-well plates in triplicate and were transiently cotransfected with *ARE-LUC* (0.2 μ g) and *AP2 γ* (0.5 μ g), *WWOX* (0.5 μ g), or *WWOXY33R* (0.5 μ g) expression plasmids. At 24 h posttransfection, medium was changed and 1 nmol/L dihydrotestosterone was added. After another 24 h, cells were lysed and luciferase assay was done using Promega Dual-Luciferase Reporter Assay System kit, and cell supernatant medium was collected for ELISA assay (Anogen) following manufacturer's instructions. **B.** DU145 cells cultured under the same conditions were transiently cotransfected with *ARE-LUC* (0.2 μ g) and *AR* (0.5 μ g), *AP2 γ* (0.5 μ g), *WWOX* (0.5 μ g), or *WWOXY33R* (0.5 μ g) expression plasmids. At 24 h posttransfection, medium was changed and 1 nmol/L dihydrotestosterone was added. Luciferase assay for ARE-LUC activity and ELISA assay for secreted PSA level were done.

through down-modulation of Ap2 γ /ErbB2, and subsequent reduction of PSA by Wwox required AR signaling.

Wwox Expression Is Inversely Correlated with Expression of ErbB2 and Gleason Scores

To provide *in vivo* confirmation for the importance of Wwox in modulating the ErbB2 signal pathway in prostate cancers, Wwox and ErbB2 protein expression was evaluated in 52 primary human prostate cancers on a tissue microarray by immunohistochemical analysis to assess the correlation between Wwox and ErbB2 protein expression. Of the 52 tumor cases, Wwox expression was high only in 10 (19.2%) but low to negative in 42 (80.8%) cases (Table 1), consistent with our previous findings (6). Reports of ErbB2 expression in prostate cancer have not been consistent (14), possibly due to methodologic differences in tissue preparation and antisera used. More recent studies have shown experimental evidence that ErbB2 oncoprotein is overexpressed in 20% to 60% of prostate carcinomas and is correlated with higher Gleason grade, more advanced stage of disease, and shortened survival (14, 23-28). In our study of the 52 prostate cancer tissue samples, ErbB2 expression was high in 14 cases (27%), and low to negative in 38 cases (73%); 23 of the 52 cases expressed Wwox, or ErbB2 or both, and were used to assess the correlation between expression levels of Wwox tumor-suppressor

protein and ErbB2 oncoprotein. There was a statistically significant inverse correlation between Wwox and ErbB2 expression, as determined by nonparametric Spearman's correlations (two-tailed, $P < 0.01$, $r = -0.737$; Table 1). Furthermore, higher Wwox expression tended to be associated with lower Gleason score, whereas higher ErbB2 expression

Table 1. Association of Wwox and ErbB2 Expression and Gleason Scores in 23 of the 52 Prostate Cancer Patient Tissue Samples that Express Wwox or ErbB2 or Both

Gleason Score	Wwox Expression		ErbB2 Expression		Total No. Samples
	High	Low	High	Low	
6	8	1	2	7	9
7	1	2	2	1	3
8	1	7	7	1	8
9	0	3	3	0	3
Wwox					
Spearman's ρ	ErbB2		r^*	-0.737 [†]	
			P (two-tailed)	<0.01	
			No. samples	23	

*Correlation coefficient.

[†]Correlation is significant at the 0.01 level (two-tailed).

was associated with higher Gleason score. Representative images from the same TMA cores showing high expression of Wwox while ErbB2 expression is reduced, and vice versa, are shown in Supplementary Fig. S2.

Twenty frozen prostate cancer tissue samples were obtained to confirm the correlation between levels of Wwox and ErbB2 protein expression. Western blot and densitometry analysis confirmed a statistically significant inverse correlation of Wwox and ErbB2 protein expression ($r = -0.554$, $P < 0.01$, as determined by nonparametric Spearman's correlations test; Fig. 6A and B).

Frozen prostate cancer tissues were homogenized and lysed, and protein was extracted for coimmunoprecipitation analysis to confirm the interaction between endogenous Wwox and Ap2 γ proteins *in vivo*. We observed coimmunoprecipitation of Wwox and Ap2 γ , as shown in Fig. 6C, thus confirming the binding of Wwox and Ap2 γ *in vivo*.

In summary, we have shown that restoration of Wwox suppressed ErbB2-induced AR transactivational activity and prostate cancer cell growth through binding and cytoplasmic sequestration of Ap2 γ *in vitro* and *in vivo*. An unsolved clinical problem in prostate cancer is its progression from an androgen-dependent to an androgen-independent state after androgen ablation therapy. The latter state is characterized by functional AR signaling, as indicated by the expression of AR and the increase in circulating levels of the androgen-dependent protein PSA, despite antiandrogen therapy, suggesting that the AR pathway is reactivated by ligand(s) other than androgen, or in a ligand-independent fashion (12, 29). Overexpression of ErbB2 has been reported to activate AR signaling at limiting concentration of androgen and to induce hormone-independent prostate cancer growth (12, 14-16). In line with this, the data reported here showed that Ap2 γ /ErbB2 activates AR transactivational activity at low level of androgen, which may

account for the increased growth rate induced by ErbB2, and elevated PSA level in LNCaP cells. More importantly, overexpression of Wwox reversed the effect of ErbB2 on prostate cancer cell growth and AR activity. Therefore, restoration of Wwox or the Wwox signal pathway may provide new alternatives to battle hormone-refractory prostate cancer.

Materials and Methods

Cell Culture

LNCaP, DU145, and PWR-1E cell lines were obtained from the American Type Culture Collection. LNCaP and DU145 cells were cultured in RPMI 1640 supplemented with 10% FBS. For androgen ablation, cells were grown in phenol red-free RPMI 1640 supplemented with 10% CS-FBS (Gemini Bio-Products) for 48 h before treatment with dihydrotestosterone (a gift from Dr. Ching-Shih Chen, College of Pharmacy at The Ohio State University, Columbus, OH). PWR-1E cells were maintained in keratinocyte-SFM (Invitrogen Corp.).

Plasmids and Transfection

The construction of pCMV-*WWOX* (wild-type *WWOX*), pCMV-*WWOXY33R* (mutant *WWOX*), and pCMV-*AP2 γ* expression vectors was described previously (18). pCMV-SPORT6-*ERBB2* plasmid was purchased from the American Type Culture Collection. *ERBB2*-pGL3-basic vector was kindly provided by Dr. Helen C. Hurst (University of London, London, England). pGL3E/probasin firefly luciferase reporter plasmid (*ARE-LUC* plasmid) was a gift from Dr. Ralph W. deVere White (University of California, Davis, Davis, CA). pCMV-*hAR* was provided by Dr. James Dalton (The Ohio State University, Columbus, OH). All transfections were done using LipofectAMINE 2000 (Invitrogen) following manufacturer's instructions.

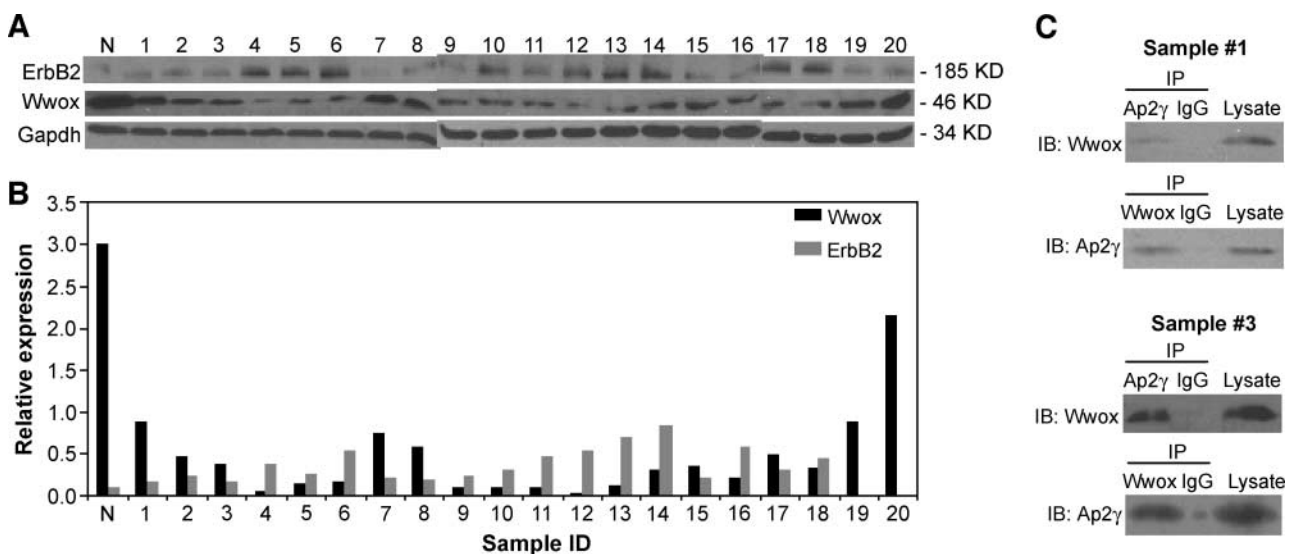


FIGURE 6. *In vivo* expression of Wwox and ErbB2 are inversely correlated; Wwox and Ap2 γ physically interact *in vivo*. Western blot analysis (A) followed by densitometry analysis (B) for 20 frozen prostate cancer tissue samples. N, PWR-1E cell lysates, a noncancer control. Spearman's nonparametric analysis showed a statistically significant inverse correlation between Wwox and ErbB2 expression ($r = -0.554$, $P < 0.01$). C, Examples of coimmunoprecipitation experiments for detection of Wwox and Ap2 γ interaction in prostate cancer tissues. Wwox and Ap2 γ coimmunoprecipitated.

Protein Expression Assays

Immunoblot analysis was done using both cell and homogenized tissue lysates. Protein extraction, quantification, and immunoblot analyses were carried out as described (30). Nuclear extracts were prepared as described (31). The following primary antisera and dilutions were used for immunoblotting: Rabbit polyclonal anti-Wwox, 1:20,000 (32), and mouse monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase, 1:2,500, were from Calbiochem. Mouse monoclonal anti-Ap2 γ (6E4/4), 1:200; rabbit polyclonal anti-ErbB2 (C-18), 1:200; rabbit polyclonal anti-AR (N-20), 1:500; and goat polyclonal anti-PSA (C-19), 1:1,000, were all from Santa Cruz Biotechnology. The amount of secreted PSA protein in supernatant medium was determined using the Human PSA ELISA Kit (Anogen).

Coimmunoprecipitation

Protein-protein interactions were detected by coimmunoprecipitation followed by immunoblot analysis. Briefly, cells or homogenized tissues were lysed in buffer containing 30 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 10% glycerol, and 1% NP40 supplemented with proteinase and phosphatase inhibitors. Lysates were incubated on ice for 30 min and centrifuged at 13,000 rpm for 15 min at 4°C. Supernatants (600 μ g) were precleared with normal mouse and rabbit IgG for 30 min at 4°C on an orbital shaker. Immunoprecipitations were carried out with anti-Wwox and anti-Ap2 γ ; 20 μ L of Ultralink immobilized protein A/G beads (Pierce) were added and incubated on a rocker for 4 h to overnight. Pellets were collected by centrifugation at 2,500 rpm and washed four times with the above buffer. Pellets were then resuspended in 2.5 \times SDS-PAGE sample buffer, and heated to 95°C for 5 min for immunoblotting.

Luciferase Assays

Transient cotransfection of LNCaP cells with 0.2 μ g of the firefly luciferase reporter plasmid and 1 ng of the *Renilla* vector pRL-TK (Promega), together with the indicated expression vector combinations were done using LipofectAMINE 2000 reagent. Cells were collected 48 h after transfection and luciferase activity was quantified and normalized to *Renilla* activity using a luciferase assay kit (Promega), with the aid of a luminometer. Results are shown as fold induction of the luciferase activity compared with control cells.

RNA Interference

AP2 γ and WWOX siRNA and scrambled control siRNAs were purchased from Ambion, Inc. Sequences for AP2 γ siRNA were sense 5'-GGUACUGAAGCUUUUUUUGtt-3' and antisense 5'-CAAUAAAGCUUCAGUACt-3'. Sequences for WWOX siRNA were sense 5'-GGACGGCUGGGUUUACU-ACtt-3' and antisense 5'-GUAGUAAACCCAGCCGUCct-3'. Cells (2×10^5) were seeded in a six-well plate and cultured in normal growth medium. Twenty-four hours later, cells were transfected with AP2 γ and WWOX siRNAs and scrambled control siRNAs using siPORT NeoFX lipid-based transfection agent (Ambion). Seventy-two hours later, cells were harvested and lysates were extracted for immunoblotting.

ChIP Assays

LNCaP cells (7.5×10^6 /150-mm dish) were transfected with pCMV-AP2 γ vector, or cotransfected with pCMV-AP2 γ and pCMV-WWOX vectors for 48 h. ChIP assays were done using a ChIP assay kit (Upstate Cell Signaling Solutions) following the manufacturer's instructions. Sonication of chromatin was done as follows using a Sonic Dismembrator Model 100 (Fisher Scientific): 20-s pulse followed by 40-s rest on ice to eliminate warming, repeating once for each output power (60%, 70%, and 80%).

Antisera used for immunoprecipitation were as follows: anti-Ap2 γ (H-77, Santa Cruz Biotechnology), control rabbit IgG (Santa Cruz Biotechnology), and anti-phosphorylated RNA polymerase II (phosphorylated S5; Abcam, Inc.). One microliter of each DNA sample from ChIP preparations was analyzed by real-time PCR using SYBR Green PCR Master Mix (Bio-Rad) under conditions recommended by the manufacturer and an iCycler iQ Multicolor real-time PCR detection system (Bio-Rad). All reactions were run in triplicate. Primer sequences amplifying the -500-bp locus of interest and the -5,300-bp locus (negative control) of the ErbB2 promoter were as described by Begon et al. (11). Serial dilutions of input DNAs were used in PCRs to establish a standard curve for each individual pair of primers. The results are given as a percentage of input. All ChIP experiments were repeated at least thrice and consistent results were obtained.

Immunofluorescence

LNCaP cells were placed on coverslips in 12-well plates at an initial density of 3.5×10^4 per well; 24 h later, cells were transfected with pCMV-AP2 γ (0.5 μ g) or cotransfected with pCMV-WWOX (0.5 μ g) or pCMV-WWOX-Y33R (0.5 μ g), and pCMV-AP2 γ (0.5 μ g). Forty-eight hours later, cells were fixed in 3.7% PBS-buffered formaldehyde for 10 min, blocked with 3% bovine serum albumin, and incubated with primary antisera for Ap2 γ (1:200) and Wwox (1:5,000) for 1 h in 1% bovine serum albumin in PBS, and with secondary antisera under the same conditions. Secondary antisera used were anti-mouse Texas red-conjugated IgG (Molecular Probes). The coverslips were mounted with Vectashield hardset mounting medium with 4',6-diamidino-2-phenylindole (Vector Laboratories). Cells were visualized using a Zeiss LCM 510 microscope.

Cell Growth Assay

In the first assay, LNCaP and DU145 cells were seeded at 5×10^4 per well in 12-well plates in triplicate and cultured in phenol red-free medium supplemented with 10% CS-FBS and 1 nmol/L dihydrotestosterone for 24 h. Cells were transfected with either the empty vector (0.5 μ g), or plasmids containing AP2 γ (0.5 μ g), AP2 γ and WWOX (0.5 μ g), or AP2 γ and WWOXY33R (0.5 μ g). In the second assay, cells were cultured under the same conditions and transfected with either the empty vector or plasmid containing ERBB2 (0.5 μ g). Medium was changed 6 h later. Cells were then counted every 24 h for 5 days using the Vicell counter (Beckman Coulter).

Immunohistochemical Analysis

A prostate tissue microarray with cores from 52 prostate cancers was stained for Wwox and ErbB2 expression by

immunohistochemistry as described (6), using rabbit polyclonal anti-Wwox (1:5,000; ref. 32) and rabbit polyclonal anti-ErbB2 sera (1:200; Dako North America, Inc.). Individual prostate cancer cores were scored by a pathologist for high or low Wwox and ErbB2 immunoreactivity.

Statistics

In vivo tissue microarray and Western blot quantification data were analyzed using nonparametric Spearman's correlation method. All other statistical analyses were done using one-way ANOVA, where appropriate. Statistical significance was set at $P < 0.05$.

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