The metastasis suppressor NDRG1 directly regulates androgen receptor signaling in prostate cancer

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N-myc-downregulated gene 1 (NDRG1) has potent anti-cancer effects and inhibits cell growth, survival, metastasis, and angiogenesis. Previous studies suggested that NDRG1 is linked to the androgen signaling network, but this mechanistic relationship is unclear. Considering the crucial role of the androgen receptor (AR) in prostate cancer (PCa) progression, here we examined for the first time the effect of NDRG1 on AR expression, activation, and downstream signaling in LNCaP, 22Rv1, and C4-2B PCa cell types. We demonstrate that NDRG1 effectively promotes interaction of AR with the chaperone HSP90, which in turn stabilizes the AR while decreasing its androgen-mediated activation. The expression of NDRG1 suppressed: (1) AR activation, as measured by p-AR[Ser213] and p-AR[Ser81]; (2) expression of a major AR transcriptional target, prostate-specific antigen (PSA); and (3) AR transcriptional activity, probably *via* inhibiting the c-Jun-AR interaction by reducing c-Jun phosphorylation (p-c-Jun[Ser63]). NDRG1 was also demonstrated to inhibit multiple key molecules involved in androgen-dependent and -independent signaling (namely EGFR, HER2, HER3, PI3K, STAT3, and NF-κB), which promote the development of castration-resistant prostate cancer. We also identified the cysteine-rich secretory protein/antigen 5/pathogenesis related-1 (CAP) domain of NDRG1 as vital for inhibition of AR activity. Examining NDRG1 and p-NDRG1 in PCa patient specimens revealed a significant negative correlation between NDRG1 and PSA levels in prostatectomy patients that went on to develop metastasis. These results highlight a vital role for NDRG1 in androgen signaling and its potential as a key therapeutic target and biomarker in PCa.

Despite recent improvements in the early detection and treatment of prostate cancer (PCa), it remains one of the most diagnosed cancers among men globally, with 1.3 million new cases each year, contributing to 7.1% of total cancer deaths in 2018 (1, 2). Androgen deprivation therapy (ADT) remains the gold standard for PCa treatment, leading to reduced testosterone levels through castration or drug treatments that block or lower circulating androgen levels (3). However, approximately 20% of men develop resistance to ADT, leading to the development of castration-resistant prostate cancer (CRPC) (4). Despite low circulating androgen levels, the androgen receptor (AR) remains a significant driver of CRPC (5, 6) and therefore, is an important target in developing novel PCa therapies.

The normal development and growth of the prostate gland are dependent mainly on the AR, with this protein also playing a major role in driving PCa development and progression (5). Androgens, such as testosterone, are the main hormones driving the development of the normal prostate in activating genomic and nongenomic signaling pathways of the AR (7). Mutation and deregulation of the AR, including loss of function and gain of function and/or its downstream signaling pathways, have played a major role in the development of androgen resistance (5). It is estimated that 10 to 30% of patients with advanced PCa possess AR mutations, especially those treated with ADT (8). Mutations in the ligand-binding domain region of the AR, especially the F876L mutation, were found to induce resistance of PCa to the antiandrogen, Enzalutamide, leading to constitutive AR activation in the presence of this clinically used drug (9).

The signaling pathway of the AR in PCa can be divided into androgen-dependent and -independent mechanisms (4, 6, 10, 11). The classical model of androgen-dependent signaling involves hormone-binding, including testosterone, to the inactive AR, leading to conformational changes, nuclear translocation, and subsequent activation of target genes (7). The androgen-independent signaling pathways of AR have been demonstrated to play major roles in androgen resistance (12). The epidermal growth factor receptor (EGFR) family (*i.e.*, EGFR, HER2, HER3) was demonstrated to be key contributor to androgen-independent AR activation and thus, is associated with PCa metastasis and poor response to therapy (13–15). Activation of the EGFR family by epidermal growth factor (EGF) stimulates the downstream phosphatidylinositol-3-kinase (PI3K)/protein kinase B (AKT) and mitogen-activated protein kinase (MAPK) signaling pathways, which promotes

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increased AR transactivation, transcriptional activity, and CRPC development (16–18).

Studies over the past 20 years have identified a novel molecular player in PCa progression and metastasis, namely the metastasis suppressor, N-myc downstream-regulated gene 1 (NDRG1) (19–23). The expression of NDRG1 is often reduced in cancer compared with normal tissue (19), with higher levels being correlated to better patient survival in prostate, pancreatic, and breast cancers (19, 24–26). Examining PCa, our laboratory and others have demonstrated that NDRG1 expression markedly suppresses the oncogenic phenotype of these cells, including inhibition of proliferation, cell migration, and invasion, while promoting differentiation (19, 21, 23, 27–35). Phosphorylation of NDRG1 (p-NDRG1) at Ser330 and Thr346 by serum- and glucocorticoid-regulated kinase 1 has been reported to be essential for NDRG1 to inhibit downstream oncogenic signaling (36). Further, NDRG1 phosphorylation at both these latter sites is potently increased by pharmacological agents that show marked antitumor and antimetastatic activity (37, 38).

Relevant to its crucial role as a metastasis suppressor, NDRG1 was demonstrated by our laboratory to inhibit the first step in the metastatic process, namely the epithelial-to-mesenchymal transition (EMT) in PCa (33). This activity occurred *via* the ability of NDRG1 to inhibit oncogenic TGF-β signaling, leading to increased expression of the key adherens junction proteins, E-cadherin and β-catenin, on the plasma membrane (33). NDRG1 inhibits multiple critical oncogenic signaling networks that influence the activity of androgens and promote the development of CRPC. These include the EGFR, PI3K/AKT, nuclear factor kappa light chain enhancer of activated B cells (NF-κB), signal transducer and activator of transcription 3 (STAT3), and mitogen-activated protein kinase (MAPK) signaling pathways (30, 39–43).

NDRG1 has been demonstrated to be an androgen-regulated gene, and its interactome was linked to the androgen network through its interactions with heat shock protein 90 (HSP90) and β-catenin (44, 45). Hence, we hypothesized that NDRG1 could play an essential role in the activity of both androgen-dependent and -independent AR signaling in PCa. Notably, the association between NDRG1 expression and the AR has never been characterized, and this was the principal aim of the current study.

Considering the emerging role of NDRG1 in PC progression, we assessed the effect of NDRG1 expression in well-characterized androgen-dependent and -independent PCa cell types. Herein, we demonstrate for the first time that NDRG1 attenuates both androgen-dependent and -independent AR signaling pathways in PCa. This response was demonstrated to occur through a mechanism involving stabilization of the HSP90-AR complex, preventing ligand-mediated AR activation and attenuation of downstream AR signaling and prostate-specific antigen (PSA) levels. Significantly, NDRG1 inhibited EGF-mediated androgen-independent AR signaling *via* its effects on c-Jun, as well as the EGFR, PI3K, STAT3, and NF-κB signaling pathways. Baseline tumor NDRG1 expression was also negatively correlated with blood PSA levels in patients who developed metastatic PCa. This study highlights the clinical importance of NDRG1 expression in PCa and its utility as a biomarker for more aggressive forms of PCa, such as CRPC. This investigation also demonstrates that NDRG1 is a promising therapeutic target for treating metastatic and resistant PCa.

Results

### NDRG1 inhibits androgen-dependent and -independent activation of AR in prostate cancer

To investigate the effect of NDRG1 on AR expression and activation, studies initially utilized the well-characterized LNCaP PCa cell type. These cells were stably transfected to overexpress NDRG1 (designated as "NDRG1" in all figures) and were compared with their respective empty vector-transfected controls (VC; Fig. 1A). LNCaP cells are androgen-dependent and responsive to testosterone in terms of growth and PSA expression (46). Further, this cell type expresses the AR mutant T877A, which is transactivated by lower levels of androgens and other ligands (47, 48).

These NDRG1 overexpression studies are relevant for not only dissecting molecular mechanism, but also for considering the effects of NDRG1-inducing chemotherapeutics that markedly upregulate this protein and have entered clinical trials for cancer treatment (49, 50). These transfected cell types were examined after incubation for 24 h/37 °C with control media alone (Con; Fig. 1) or after incubation with either testosterone (10 nM) for 24 h/37 °C or EGF (100 μg/ml) added in the last 10 min of the 24 h/37 °C incubation (*i.e.*, (+) T or (+) E; Fig. 1). These conditions of testosterone and EGF treatment were used in all experiments throughout this investigation. Testosterone and EGF are major activators of androgen-dependent and -independent AR signaling, respectively (51, 52), and the antioncogenic effect of NDRG1 overexpression was important to understand on these pathways.

The western blot in Figure 1A demonstrates that NDRG1 overexpression in LNCaP cells unexpectedly significantly increased total AR levels relative to the VC under control conditions, but also in the presence of testosterone or EGF. Total AR levels were also significantly upregulated after incubation with testosterone or EGF in VC and NDRG1 overexpressing cells relative to incubation in control medium alone (Fig. 1A).

The phosphorylation of AR at Ser213 (*i.e.*, p-AR^{Ser213}) and Ser81 (*i.e.*, p-AR^{Ser81}) was also examined since these sites are phosphorylated in response to testosterone and are critical for AR activity (53). Examining p-AR^{Ser213} levels under control conditions, there was no significant change after NDRG1 overexpression *versus* the VC, while NDRG1 significantly reduced p-AR^{Ser81} levels under these conditions (Fig. 1A). Upon incubation of VC cells with testosterone or EGF, there was a significant increase in phosphorylation of p-AR^{Ser213} and p-AR^{Ser81} in response to both ligands relative to control-treated VC cells (Fig. 1A), indicating AR activation. In contrast, NDRG1 overexpression significantly decreased p-AR^{Ser213} and p-AR^{Ser81} levels relative to the respective VC
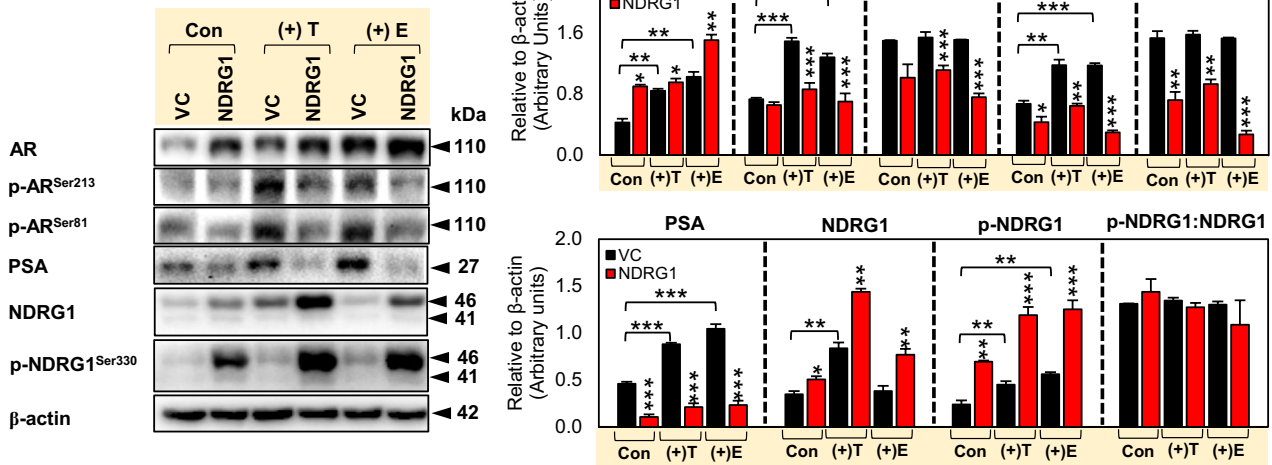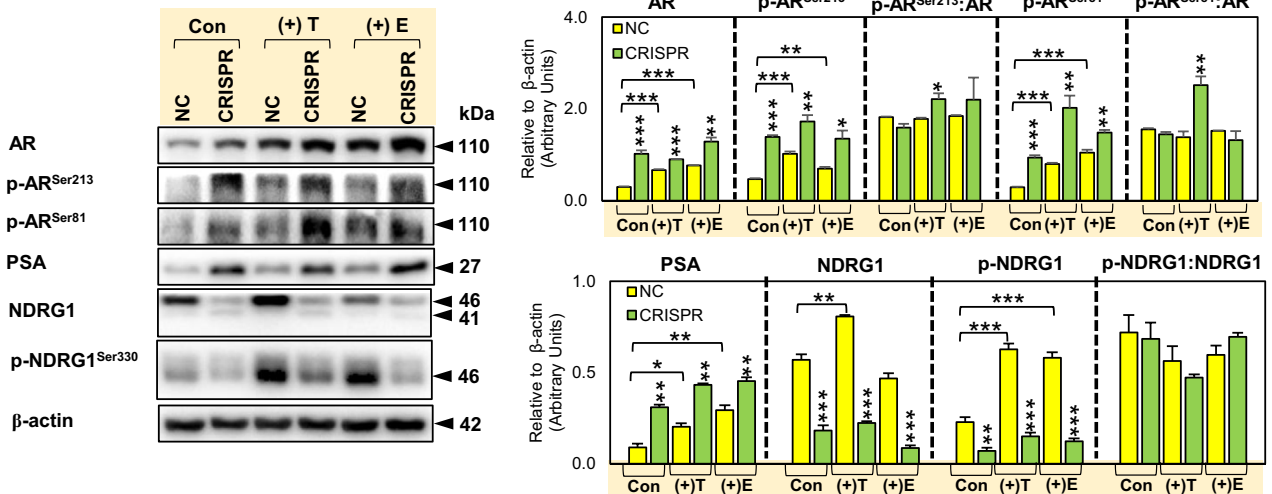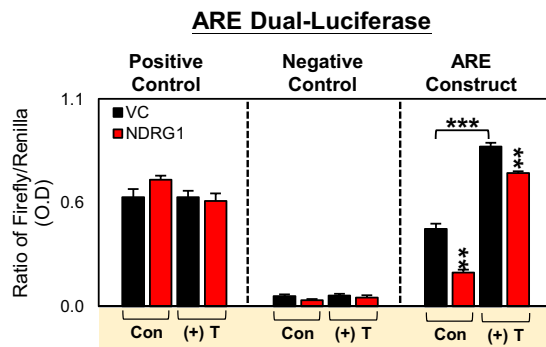
Figure 1. NDRG1 expression downregulates AR activity in LNCaP cells. *A*, LNCaP cells transfected with the vector control (VC) or an NDRG1 expression vector (NDRG1); and (*B*) LNCaP cells with CRISPR *NDRG1* silencing compared with their relevant negative control (NC) cells. These cells were incubated with either control medium (Con) for 24 h/37 °C; or this medium containing either testosterone (+T; 10 nM) for 24 h/37 °C or EGF (+E; 100 μg/ml), which was added in the last 10 min of the 24 h/37 °C incubation. Lysates were prepared and then assessed for protein levels of AR, p-AR^{Ser213}, p-AR^{Ser81}, PSA, NDRG1, and p-NDRG1^{Ser330} *via* western blot. *C*, AR activity in LNCaP cells was determined using an ARE luciferase construct in the presence or absence of testosterone (10 nM) for 24 h/37 °C. Firefly (550 nm) and Renilla (488 nm) luminescence was measured and the results normalized to Renilla luminescence. Results are mean ± S.E.M (n = 3). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ denote statistical significance compared with the relevant VC group, or as otherwise indicated in the graphs.

cells in response to testosterone or EGF. Hence, irrespective of the increased total AR levels, NDRG1 overexpression inhibited AR phosphorylation (p-AR^{Ser213} and p-AR^{Ser81}), indicating AR activation was suppressed. This conclusion was confirmed by examining the ratios of p-AR^{Ser213} and p-AR^{Ser81} to total AR, wherein testosterone- or EGF-treated cells, NDRG1 overexpression resulted in a significant decrease in these ratios *versus* the respective VC (Fig. 1*A*).

Examining a key downstream transcriptional target of AR, namely PSA (54), its expression was markedly and significantly downregulated by NDRG1 overexpression *versus* the VC under all conditions (Fig. 1*A*). These results indicate a pronounced inhibitory effect of NDRG1 expression on downstream AR signaling.

Assessing NDRG1 expression, two bands were generally observed in LNCaP cells upon western blotting, namely a minor band at ~41- and a major band at ~46-kDa (49) (Fig. 1*A*). However, in some experiments, the 41-kDa NDRG1 band was at low levels and difficult to detect. Treatment of LNCaP cells with testosterone significantly increased NDRG1 levels in both the VC and the overexpression clone relative to the respective untreated controls, while EGF did not significantly affect NDRG1 expression (Fig. 1*A*). Under control conditions, overexpression of NDRG1 also significantly increased NDRG1 phosphorylation at Ser330 (*i.e.*, p-NDRG1^{Ser330}; Fig. 1*A*) *versus* the VC, which was reported to be related to its antioncogenic activity (36, 37). Treatment of LNCaP cells with testosterone or EGF led to a pronounced and significant increase in p-NDRG1 levels in the VC and NDRG1 overexpression clone relative to that observed under control conditions. There was no alteration in the p-NDRG1:NDRG1 ratio under all conditions (Fig. 1*A*), suggesting that the increase in phosphorylation observed after testosterone or EGF was due to its increased protein levels.

Considering the effects of NDRG1 overexpression above, we then stably silenced *NDRG1* in LNCaP cells *via* CRISPR-Cas9 gene editing (designated as "CRISPR" in all figures) and compared these cells with the relative nonspecific negative control cells (NC; Fig. 1*B*). Most of the effects due to NDRG1 overexpression in LNCaP cells observed in Figure 1*A* were reversed when *NDRG1* was silenced by CRISPR in this cell type (Fig. 1*B*). In fact, p-AR (*i.e.*, p-AR^{Ser213} and p-AR^{Ser81}) and PSA levels were significantly upregulated in *NDRG1* silenced cells under all conditions (Fig. 1*B*).

However, silencing of *NDRG1* surprisingly increased total AR under all treatment conditions (Fig. 1*B*), which was the same effect observed upon NDRG1 overexpression (Fig. 1*A*). This paradoxical observation is difficult to reconcile, but may suggest other responses to NDRG1 expression that alter total AR levels. To investigate this further, we examined the expression of CHIP, a functional E3 ubiquitin ligase that interacts with HSP70 and HSP90, and promotes proteasomal degradation of AR (55, 56). We examined the expression of CHIP protein levels in LNCaP cells either overexpressing NDRG1 (Fig. S1*A*) or with *NDRG1* silencing (CRISPR; Fig. S1*B*) under control conditions and in response to testosterone or EGF. Upon NDRG1 overexpression, CHIP levels

were markedly and significantly decreased under all conditions (Fig. S1*A*). Notably, the same effect was observed in response to *NDRG1* silencing, with CHIP levels again being significantly decreased under all conditions (Fig. S1*B*). The reduced CHIP levels under all conditions suggest that there is decreased degradation of the AR protein and would explain the increased AR levels in response to both overexpression and silencing of NDRG1. These data underline the complex, multieffector responses elicited by NDRG1 (57, 58).

The response of LNCaP cells to alterations in NDRG1 expression above in Figure 1 prompted further studies comparing their response to two androgen-independent PCa cell types, namely 22Rv1 cells (59) (Fig. S2*A*) and C4-2B PCa cells (60) (Fig. S2*B*). In contrast to both LNCaP (Fig. 1*A*) and C4-2B PCa cells (Fig. S2*B*) where the AR was detected as one 110 kDa band, examining 22Rv1 cells, AR was observed as two bands at 80 and 110 kDa (Fig. S2*A*). This observation is due to an 80 kDa AR splice variant in the 22Rv1 cell type (61, 62). Of note, *NDRG1* silencing had less robust effects on increasing p-AR^{Ser213} and p-AR^{Ser81} levels in C4-2B cells and particularly 22Rv1 cells (Fig. S2, *A* and *B*) relative to that observed in LNCaP cells (Fig. 1*B*).

In the 22Rv1 cell-type, *NDRG1* silencing had little effect on p-AR^{Ser213} levels while significantly upregulating p-AR^{Ser81} under control conditions or after incubation with testosterone (Fig. S2*A*). For C4-2B cells, p-AR^{Ser213} levels were significantly upregulated by *NDRG1* silencing under control conditions and after incubation with testosterone, while p-AR^{Ser81} was increased by silencing *NDRG1* only after incubation with testosterone (Fig. S2*B*). The differences in response to NDRG1 expression could be related to the fact that LNCaP represents an androgen-dependent PCa cell type (46–48), while both 22Rv1 and C4-2B represent androgen-independent cells (59, 60).

Despite the generally less pronounced effect of *NDRG1* silencing on AR activation in the 22Rv1 and C4-2B cells (Fig. S2, *A* and *B*) relative to LNCaP cells (Fig. 1*B*), upregulation of its key target, PSA, was observed, particularly after incubation with testosterone and EGF, as demonstrated using LNCaP cells (Fig. 1*B*). Silencing of *NDRG1* in 22Rv1 and C4-2B cells also resulted in a marked and significant decrease in p-NDRG1^{Ser330} levels and the p-NDRG1/NDRG1 ratio *versus* the respective Con-siRNA-treated controls (Fig. S2, *A* and *B*).

In summary, the studies described in Figure 1*A* indicate that NDRG1 overexpression in LNCaP cells inhibits AR activation (p-AR^{Ser213} and p-AR^{Ser81}) by androgen-dependent (testosterone) and androgen-independent (EGF) pathways, preventing downstream activation of its major transcriptional target, PSA. These alterations are supported by *NDRG1* silencing in androgen-dependent LNCaP cells (Fig. 1*B*), as well as androgen-independent 22Rv1 and C4-2B cells (Fig. S2).

NDRG1 overexpression decreases AR transcriptional activity

To determine if the decreased AR phosphorylation in response to NDRG1 overexpression in LNCaP cells (Fig. 1*A*) corresponded to its reduced activity, we examined the effect of

NDRG1 expression on the transcriptional activity of AR using an androgen response element (ARE) luciferase reporter construct (Fig. 1C). LNCaP cells transfected withthe positive control (constitutively expressing firefly luciferase construct), negative control (noninducible firefly luciferase reporter) or the inducible AR-responsive luciferase reporter construct were incubated in the presence or absence of testosterone (10 nM) for 24 h/37 °C.

In the presence of control medium only, NDRG1 overexpression resulted in a significant decrease in transcriptional activity from the ARE construct *versus* the VC (Fig. 1C). Incubation of LNCaP cells with testosterone significantly increased luciferase activity of the ARE construct in both the VC and NDRG1 overexpressing LNCaP cells. However, cells overexpressing NDRG1 had significantly lower luciferase activity in the presence of testosterone than the VC (Fig. 1C). Collectively, these results demonstrated that NDRG1 overexpression decreases AR transcriptional activity.

NDRG1 expression prevents testosterone-mediated activation of the AR and its nuclear localization

Once activated, AR dissociates from HSP90 and becomes localized within the nucleus, where it can exert its transcriptional effects (10, 63). In the studies in Figure 1A, we demonstrated that NDRG1 overexpression in LNCaP cells inhibits AR phosphorylation (p-AR^{Ser213} and p-AR^{Ser81}) in the presence of its activating ligands such as testosterone and EGF. To determine whether NDRG1 also prevents nuclear localization of AR, we examined active p-AR^{Ser81} levels in both the nuclear and cytoplasmic fractions of LNCaP VC and NDRG1 overexpressing cells (Fig. 2A). The levels of p-AR^{Ser81} were very low in the cytoplasmic fraction and more pronounced in the nuclear fraction, with appropriate fractionation into these compartments being demonstrated by the classical cytoplasmic and nuclear markers, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and histone deacetylase (HDAC), respectively (Fig. 2A). Overexpression of NDRG1 only slightly, but significantly, increased p-AR^{Ser81} levels in the nuclear fraction of control-treated LNCaP cells (Fig. 2A). Importantly, testosterone treatment of LNCaP VC cells caused a pronounced and significant upregulation of nuclear p-AR^{Ser81} *versus* control-treated VC cells, with this increase being markedly and significantly suppressed by NDRG1 overexpression (Fig. 2A). Of note, NDRG1 was predominantly expressed in the cytoplasm rather than the nucleus (Fig. 2A), as previously demonstrated (29, 38, 64).

Overall, these results in Figure 2A suggested that NDRG1 overexpression decreased the robust testosterone-mediated nuclear translocation of active AR in LNCaP PCa cells.

NDRG1 inhibits AR activity *via* upregulation of c-Jun and decreased c-Jun phosphorylation (p-c-Jun^{Ser63})

An important regulator of AR activity is c-Jun, which can directly mediate AR transcriptional activity by either acting as a coactivator or corepressor (65–67), or inhibit AR transcriptional activity indirectly by targeting other downstream

proteins (66). To investigate if NDRG1 expression regulates c-Jun, studies examined the effect of NDRG1 overexpression in LNCaP cells under control conditions, which markedly and significantly increased total c-Jun expression *versus* the VC (Fig. 2B). In contrast, upon incubation with testosterone or EGF, the stimulatory effect of NDRG1 on total c-Jun relative to the respective controls was less marked (Fig. 2B).

Considering these effects of NDRG1 on total c-Jun expression, the activating phosphorylation of c-Jun at Ser63 was then examined as this stabilizes the c-Jun-AR interaction and promotes AR transcriptional activity (66, 68). In fact, p-c-Jun^{Ser63} expression in patient samples has been correlated with significantly shorter relapse-free survival (69). Examining the effect of NDRG1 overexpression under control conditions, there was no significant alteration on p-c-Jun^{Ser63} levels relative to the VC (Fig. 2B). In the presence of testosterone, a slight but significant decrease in p-c-Jun^{Ser63} levels was observed after NDRG1 overexpression *versus* the respective VC (Fig. 2B). In contrast, in the presence of EGF, there was a pronounced and significant increase in p-c-Jun^{Ser63} levels in VC cells relative to when these cells were incubated with control medium. Overexpression of NDRG1 in the presence of EGF potently and significantly decreased p-c-Jun^{Ser63} levels *versus* the respective VC (Fig. 2B). Assessing the ratio of p-c-Jun^{Ser63} to total c-Jun revealed that NDRG1 overexpression significantly decreased c-Jun phosphorylation relative to the VC under all conditions (Fig. 2B), demonstrating the antioncogenic activity of this metastasis suppressor.

Another key molecule that interacts with and influences AR activation is HSP90 (70). In the absence of androgens, HSP90 binds directly to the AR, leading to its stabilization that inhibits activation (10). Upon binding of androgens such as testosterone to the AR, a conformational change occurs, leading to dissociation of AR from HSP90, enabling AR dimerization, phosphorylation, and activation (10). The overexpression of NDRG1 significantly upregulated HSP90 in the presence of testosterone relative to the respective VC (Fig. 2B). Incubation of cells with EGF increased HSP90 levels to an almost equal extent in both the VC and NDRG1 overexpressing cells relative to that observed under control conditions (Fig. 2B)

Considering that NDRG1 overexpression in LNCaP cells increased total c-Jun (Fig. 2B), the silencing of *NDRG1* using CRISPR in this cell type did not significantly alter total c-Jun expression *versus* the NC under control conditions or in the presence of testosterone (Fig. 2C). In contrast, in the presence of EGF, *NDRG1* silencing slightly, but significantly, decreased total c-Jun expression *versus* the respective NC (Fig. 2C). Similar to the effects of NDRG1 overexpression (Fig. 2B), *NDRG1* silencing had little effect on p-c-Jun^{Ser63} levels under control conditions or upon testosterone treatment (Fig. 2C). Only upon incubation with EGF did *NDRG1* silencing significantly increase p-c-Jun^{Ser63} levels *versus* the respective NC (Fig. 2C), which was appropriately opposite to the suppressive effect observed with NDRG1 overexpression (Fig. 2B). Examination of the p-c-Jun: c-Jun ratio again indicated that the only marked effect of *NDRG1* silencing was observed in the
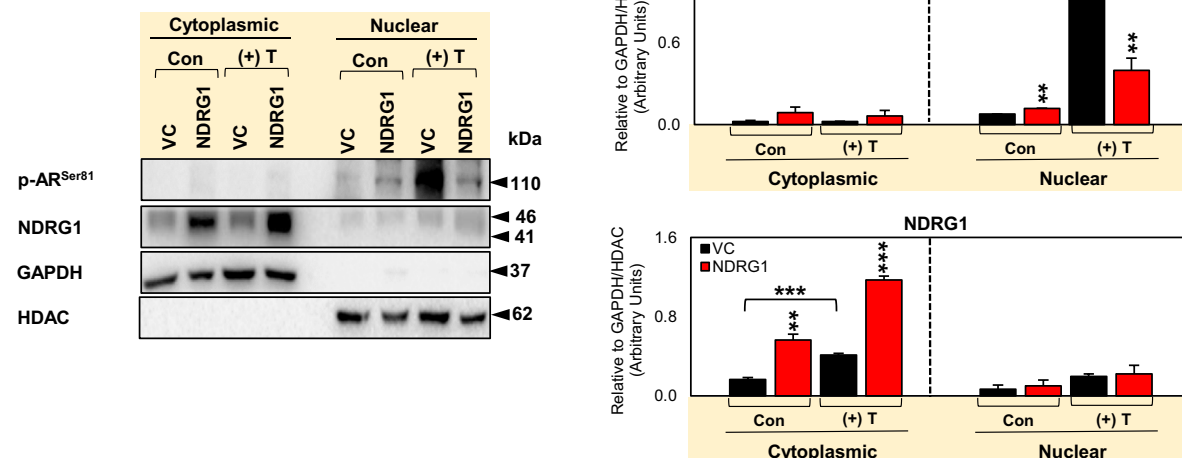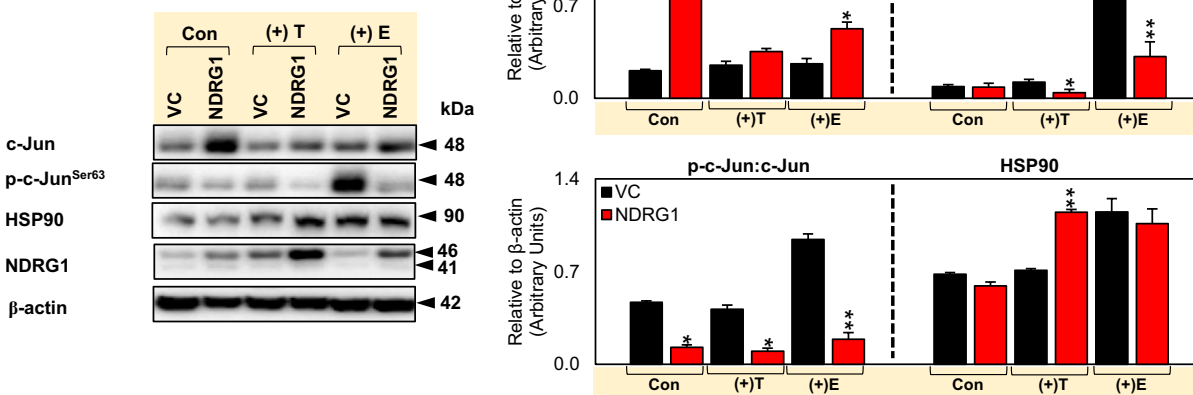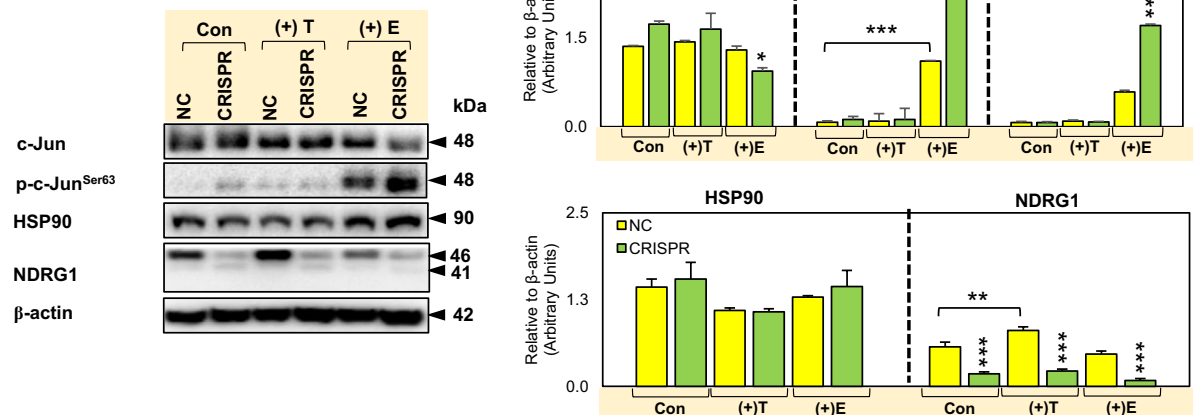
Figure 2. NDRG1 expression prevents nuclear localization of AR and promotes c-Jun and HSP90 expression. *A*, LNCaP cells overexpressing NDRG1 (NDRG1) or the relevant vector control (VC) cells were incubated with testosterone (10 nM) for 24 h/37 °C, and the localization of p-AR[Ser81] and NDRG1 assessed *via* western blot of cytoplasmic and nuclear fractions. GAPDH and HDAC were used as positive controls for cytoplasmic and nuclear localization, respectively. *B*, LNCaP cells transfected with the vector control (VC) or NDRG1 expression vector, and (*C*) LNCaP cells with CRISPR *NDRG1* silencing or their negative control (NC) transfected counterparts, were incubated with control medium, testosterone (+T; 10 nM) for 24 h/37 °C or EGF (+E; 100 μg/ml), which was added in the last 10 min of the 24 h/37 °C incubation, and assessed for protein levels of c-Jun, p-c-Jun[Ser63] and HSP90 *via* western blot. The NDRG1 blots in (*B*) and (*C*) are from Figure 1, *A* and *B*, respectively, and were included as a reference due to the same set of lysates being used to assess these additional proteins. β-actin was used as the loading control. Densitometry results are mean ± S.E.M (n = 3). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ denote statistical significance compared to the relevant VC group, or as otherwise indicated in the graphs.

presence of EGF, where a marked and significant increase in the p-c-Jun: c-Jun ratio was observed (Fig. 2C). Silencing of *NDRG1* did not significantly affect HSP90 expression under all conditions.

Collectively, these results in Figure 2 suggest the NDRG1-mediated inhibition of AR transcriptional activity (Fig. 1C) occurs *via* upregulation of c-Jun and decreased c-Jun phosphorylation (p-c-Jun^{Ser63}), particularly in the presence of EGF. In the presence of testosterone, NDRG1 overexpression increased HSP90, which may account for the reduced AR activation under this condition. These data encouraged further studies below to investigate the role of c-Jun and HSP90 in the antioncogenic activity of NDRG1.

NDRG1 promotes the association between the AR and HSP90, while inhibiting the AR-c-Jun interaction in the presence of testosterone

Considering the results above regarding the effect of NDRG1 on c-Jun and HSP90 expression, further studies assessed if NDRG1 affects c-Jun and HSP90 association with AR. These latter interactions were important to examine, as HSP90 and other chaperone proteins have been demonstrated to stabilize AR in its native state in the absence of ligands (70). Further, c-Jun was found to directly bind AR to promote its transcriptional activity (66). In these studies, coimmunoprecipitation (Co-IP) was performed by pulling down AR in either control or NDRG1 overexpressing LNCaP cells in the presence or absence of testosterone (Fig. 3).

Co-IP results from immunoprecipitating the AR and then probing for HSP90 by western analysis suggested an association between the AR and HSP90 (Fig. 3A). Notably, the AR/HSP90 interaction was significantly greater in NDRG1 overexpressing cells in the absence or presence of testosterone, suggesting that NDRG1 promotes the association between AR and HSP90 under both conditions (Fig. 3A). To determine if NDRG1 overexpression affects the association of c-Jun with the AR, we also examined the AR immunoprecipitate for c-Jun expression in the absence or presence of testosterone. As shown in Figure 3A, there was a strong interaction between AR and c-Jun. While NDRG1 did not alter this interaction under control conditions, there was a significant decrease in the association of AR to c-Jun in NDRG1 overexpressing cells upon incubation with testosterone (Fig. 3A). This finding suggests that NDRG1 inhibits the interaction between c-Jun and AR in the presence of testosterone.

The interaction between AR and HSP90 was further confirmed in LNCaP cells with CRISPR silenced *NDRG1*, which demonstrated a significantly reduced association between AR and HSP90 in the absence and particularly the presence of testosterone (Fig. 3B). Considering the known ability of NDRG1 to bind directly to proteins to affect its antioncogenic activity (40, 44), studies then assessed if NDRG1 itself can associate with HSP90 and the AR. Coimmunoprecipitates generated using an NDRG1 antibody from VC and NDRG1 overexpressing LNCaP cells were then probed for HSP90 and AR (Fig. 3C). These results demonstrate that

NDRG1 also interacts with HSP90, with a marked and significant increase in HSP90 levels being present in NDRG1 immunoprecipitates in cells overexpressing this metastasis suppressor *versus* the VC. On the other hand, while the AR was identified in NDRG1 immunoprecipitates, overexpression of NDRG1 did not increase AR levels in cells treated under control conditions, there being no significant difference relative to the VC (Fig. 3C). Furthermore, incubation of LNCaP cells with testosterone led to a significant decrease in the association between AR and NDRG1 upon NDRG1 overexpression, suggesting a role for testosterone in modulating the interaction. Overall, these results in Figure 3C suggest that NDRG1 associates with the HSP90-AR complex.

Taken together, these results in Figures 1–3 demonstrate that NDRG1 associates with the HSP90/AR complex, which may stabilize the AR in its native state and prevent its testosterone-mediated activation and nuclear localization. NDRG1 also inhibits the c-Jun-AR complex, further reducing AR transcriptional activation and downstream signaling.

The CAP region of the NDRG1 protein is essential for its inhibitory effects on the AR

As demonstrated above, NDRG1 expression can potently inhibit androgen-dependent and -independent AR activation. However, the mechanism by which NDRG1 affects these multiple signaling pathways remains elusive, with NDRG1 having no known function as a transcription factor or enzyme (71). To elucidate what region of the NDRG1 protein is involved in its inhibitory effects on AR activation, we utilized four different deletion mutants of *NDRG1* that target key regions and motifs of this protein (72). These include (1) a C-terminal deletion (ΔC); (2) a deletion of the unique three repeats of 10-amino acids at the protein's C-terminal (Δ3xR); (3) deletion of the CAP region (ΔCAP) that is located within the α/β hydrolase fold of NDRG1; and (4) deletion of a potential nuclear localization helix-turn-helix motif at the N-terminal (ΔHTH) (72) (Fig. 4A). Each mutant *NDRG1* construct was transiently transfected into LNCaP cells and compared with a wild-type NDRG1 (WT-NDRG1; Fig. 4A) transfectant, as well as the relevant VC (Fig. 4B). These transfected cell types were then assessed for AR expression and phosphorylation, as well as PSA levels.

As shown in Figure 4B, transfection of LNCaP cells with all deletion mutants and WT-NDRG1 led to similar significantly upregulated NDRG1 levels compared with VC cells. Total AR levels were not significantly affected by the ΔC and Δ3xR NDRG1 mutants *versus* the VC, but significantly upregulated by the ΔCAP and ΔHTH NDRG1 mutants, as well as by WT-NDRG1 (Fig. 4B). Examining levels of p-AR^{Ser213}, the ΔC and Δ3xR NDRG1 mutants had no significant effect *versus* the VC, while the ΔCAP and ΔHTH NDRG1 mutants significantly increased p-AR^{Ser213} levels (Fig. 4B). Only WT-NDRG1 significantly decreased p-AR^{Ser213} levels *versus* the VC. In contrast to their effects on p-AR^{Ser213}, all deletion mutants except ΔCAP significantly decreased p-AR^{Ser81} levels *versus* the VC (Fig. 4B).
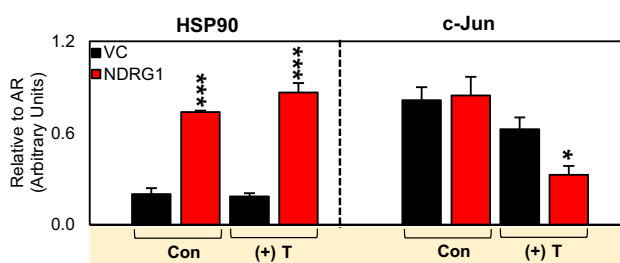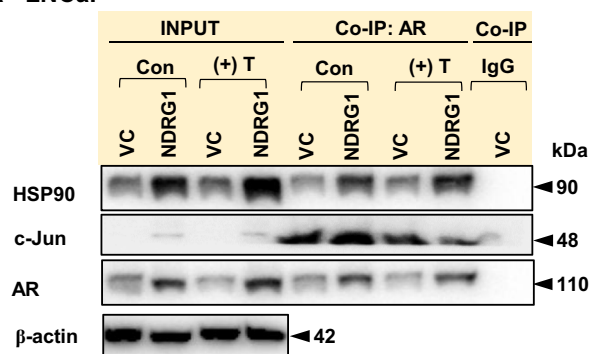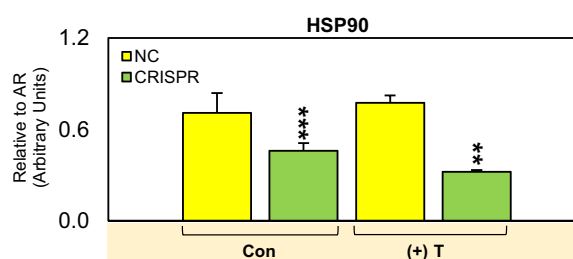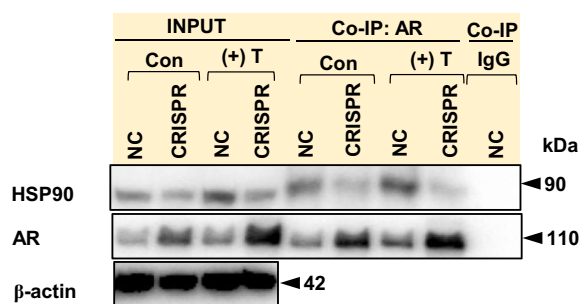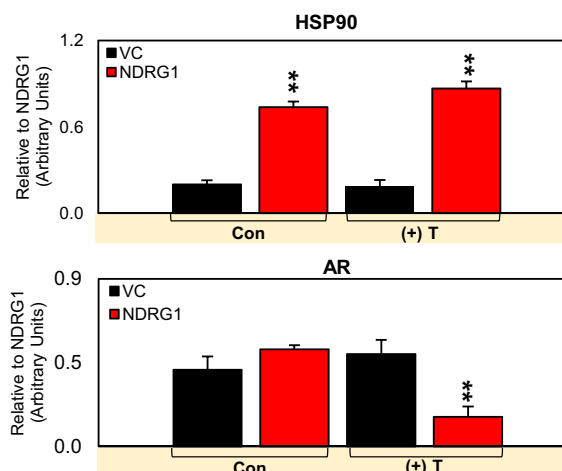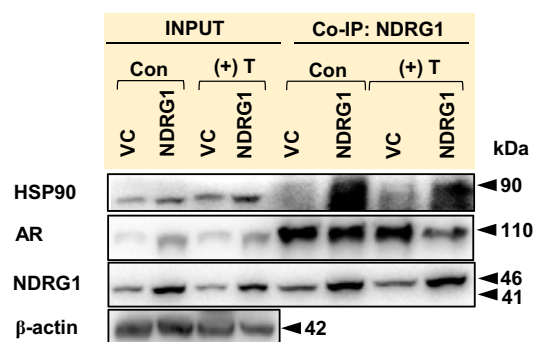
Figure 3. NDRG1 expression promotes the AR association with HSP90, while inhibiting AR binding to c-Jun. LNCaP cells were transfected with either: (*A*) an NDRG1 expression vector (NDRG1) or the empty vector control (VC); or (*B*) CRISPR-Cas9 using three different guide RNAs targeting *NDRG1* (CRISPR) or the empty vector (NC). These cells were incubated with control medium (Con) or this medium containing testosterone (+T; 10 nM) for 24 h/37 °C and the association between the AR, HSP90, and c-Jun assessed *via* coimmunoprecipitation (Co-IP) of the AR. An IgG isotype control antibody was used as a negative control. *C*, LNCaP cells overexpressing NDRG1 or their relevant empty vector control (VC) cells were incubated with Con medium or this medium containing testosterone (+T; 10 nM) for 24 h/37 °C. The association between HSP90 and NDRG1, as well as between AR and NDRG1, was then examined *via* Co-IP of the NDRG1 protein.

Examining PSA expression, which is a major downstream target of AR (54), revealed that the ΔC and Δ3xR NDRG1 mutants significantly decreased PSA levels, demonstrating similar efficacy to that of WT-NDRG1 (Fig. 4*B*). However, the ΔCAP and ΔHTH NDRG1 mutants had no significant effect on PSA compared with the VC (Fig. 4*B*), indicating a loss of its inhibitory activity. Collectively, these results suggest that the HTH and CAP sites of the NDRG1 protein play important roles in its antioncogenic activity. Deletion of the HTH or CAP sites prevented the ability of NDRG1 to downregulate p-AR^{Ser231} and PSA levels. However, only deletion of the CAP site prevented the ability of NDRG1 to downregulate these and

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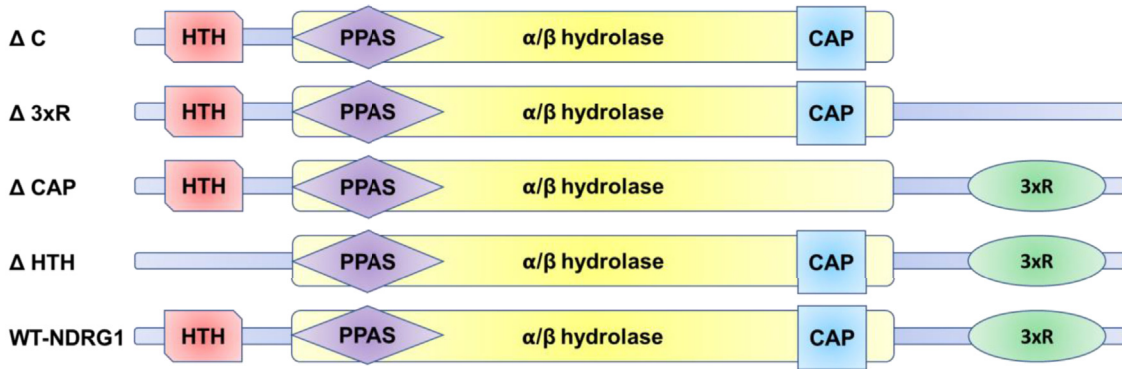
B LNCaP
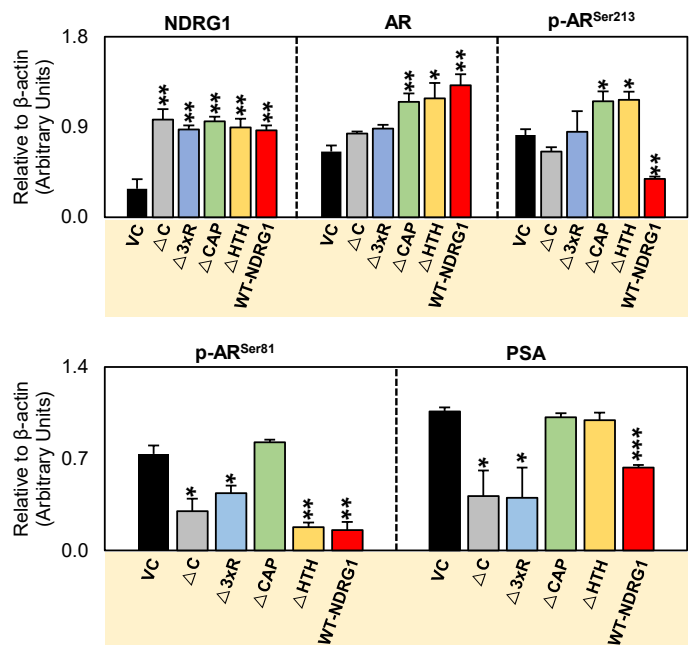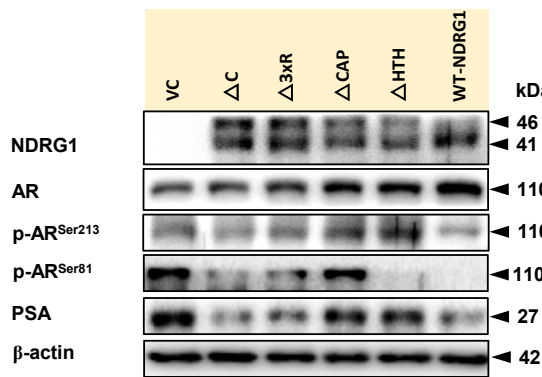
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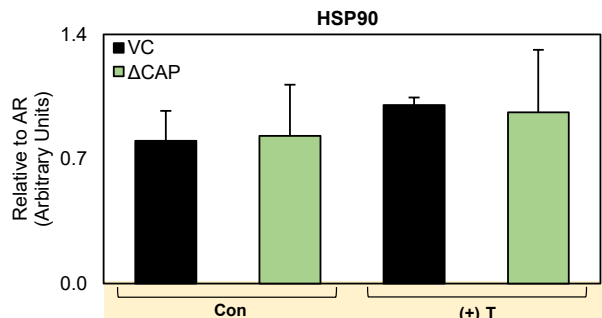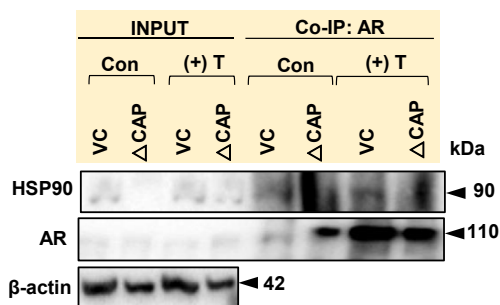
Figure 4. The CAP region of NDRG1 is vital for its comprehensive inhibitory activity on the AR. *A*, schematic diagram of the NDRG1 deletion constructs used in the current study. *B*, LNCaP cells were transiently transfected with the NDRG1 deletion mutants (*i.e.*, ΔC, Δ3xR, ΔCAP, and ΔHTH) or WT-NDRG1 for 72 h/37 °C and then assessed for protein levels of NDRG1, AR, p-AR^{Ser213}, p-AR^{Ser81}, and PSA *via* western blot. Densitometry results are mean ± S.E.M (n = 3). *C*, LNCaP cells were transiently transfected with either the vector control (VC) or the ΔCAP NDRG1 mutant and the association between HSP90 and AR was assessed *via* Co-IP of the AR. Densitometry results are mean ± S.E.M (n = 3). *p < 0.05, **p < 0.01, and ***p < 0.001 denote statistical significance compared with the relevant VC group, or as otherwise indicated on the graphs.

also p-AR^{Ser81} (Fig. 4*B*). Of note, p-AR^{Ser213} was significantly increased by the ΔHTH NDRG1 mutant when compared with the VC, which may counteract any inhibitory effects on PSA expression due to the decreased p-AR^{Ser81} levels. This hypothesis is suggested as p-AR^{Ser213} can also promote AR transcriptional activation (53). As such, only the ΔCAP construct was examined further as its deletion comprehensively prevented the decrease of p-AR^{Ser213}, p-AR^{Ser81}, and PSA that was observed with the WT-NDRG1 construct (Fig. 4*B*).

To further test the role of the CAP NDRG1 region in AR activation and downstream signaling, studies were performed using LNCaP CRISPR cells (Fig. S3), where endogenous NDRG1 expression was markedly decreased (Fig. 1*B*). These cells were transfected with the VC, ΔCAP NDRG1 mutant, or WT-NDRG1 and assessed for the expression of AR, p-AR, and PSA in response to testosterone (Fig. S3). Due to the transfection with ΔCAP NDRG1 and WT-NDRG1, these clones had markedly higher levels of NDRG1 expression when compared with VC cells (Fig. S3). Examining total AR, transfection with ΔCAP NDRG1 and WT-NDRG1 resulted in a significant increase in total AR expression *versus* the VC. This result was consistent with the observations after transfection with the WT-NDRG1 construct in Figure 4*B*. However, while WT-NDRG1 significantly decreased p-AR^{Ser213}, p-AR^{Ser81}, and PSA, ΔCAP NDRG1 had no significant effect on their levels (Fig. S3).

Considering the ΔCAP NDRG1 mutant could not inhibit AR phosphorylation (Fig. 4*B*), we further investigated if this deletion also affected the binding of AR to HSP90 (Fig. 4*C*). Notably, in Figure 3*A* we demonstrated that WT NDRG1 overexpression increased the association of AR with HSP90 in LNCaP cells. However, performing the same study with overexpression of the ΔCAP NDRG1 mutant demonstrated no significant change in the AR-HSP90 interaction relative to the VC (Fig. 4*C*). Hence, the CAP region of NDRG1 plays an important role in the ability of this protein to facilitate the AR-HSP90 association.

Overall, these results in Figure 4 and Fig. S3 indicate that the CAP region of NDRG1 is involved in inhibiting AR activation in LNCaP PCa cells.

NDRG1 overexpression inhibits EGF-mediated activation of EGFR, HER2, and HER3

As demonstrated in Figure 1 and by others (10, 17), AR signaling can also be activated by androgen-independent pathways *via* ligands such as EGF. Thus, we examined the effect of NDRG1 overexpression on the EGF family of receptor tyrosine kinases (ErbBs), including EGFR, HER2, and HER3 (Fig. 5*A*). These proteins are directly activated by EGF, leading to downstream signaling that promotes AR activation, PCa progression, and metastasis (11, 73–75).

As shown in Figure 5*A*, relative to the respective VC, total EGFR protein and p-EGFR^{Tyr1086} levels were not significantly affected by NDRG1 overexpression in LNCaP cells under control conditions or upon incubation with testosterone. In contrast, total EGFR levels were markedly decreased in the VC and NDRG1 overexpression clones upon incubation with EGF relative to incubation with control media alone (Fig. 5*A*). This downregulation of EGFR was accompanied by a pronounced and significant increase in the activating EGFR phosphorylation at Tyr1086 in response to EGF. In the presence of EGF, NDRG1 overexpression significantly decreased p-EGFR^{Tyr1086} levels and also the p-EGFR^{Tyr1086}/EGFR ratio *versus* the respective VC (Fig. 5*A*). These latter results suggested a potent decrease of EGFR activation by NDRG1 overexpression in the presence of EGF.

Examining total HER2 and HER3 expression in LNCaP cells, NDRG1 overexpression significantly reduced their total levels under all conditions *versus* the respective VC cells (Fig. 5*A*). Again, only EGF treatment resulted in an increase in the activating phosphorylation of HER2 at Tyr1221/1222 (*i.e.*, p-HER2$^{Tyr1221/1222}$) and HER3 at Tyr1289 (*i.e.*, p-HER3^{Tyr1289}) in both the VC and NDRG1 overexpressing cell type *versus* the respective control treatments. In the presence of EGF, and upon NDRG1 overexpression, there was a significant decrease in p-HER2$^{Tyr1221/1222}$, p-HER3^{Tyr1289}, the HER2$^{Tyr1221/1222}$: HER2 ratio, and p-HER3^{Tyr1289}: HER3 ratio *versus* the relative VC cells (Fig. 5*A*).

Interestingly, *NDRG1* silencing in LNCaP cells (Fig. 5*B*) reversed many of the most pronounced effects observed after NDRG1 overexpression (Fig. 5*A*) on EGFR, HER2, and HER3 levels in LNCaP cells. For example, when *NDRG1* was silenced in this cell type (Fig. 5*B*), significantly higher p-EGFR^{Tyr1086}, p-HER2$^{Tyr1221/122}$, and p-HER3^{Tyr1289} levels were observed in response to EGF in *NDRG1* silenced (CRISPR) cells relative to the NC cells. Total EGFR levels were significantly reduced in control and testosterone-treated cells, while total HER2 and HER3 levels were not significantly affected by *NDRG1* silencing.

Somewhat similar effects of *NDRG1* silencing were also observed in the two androgen-independent cell-types, namely 22Rv1 (Fig. S4*A*) and C4-2B cells (Fig. S4*B*), where *NDRG1* silencing significantly increased p-HER2$^{Tyr1221/1222}$ levels in the presence of EGF, resulting in a significant increase in the p-HER2: HER2 ratio. In these androgen-independent cell-types, and in contrast to androgen-dependent LNCaP cells (Fig. 5*B*), *NDRG1* silencing did not significantly affect either total EGFR protein or p-EGFR^{Tyr1086} levels (Fig. S4, *A* and *B*). Notably, the response of both 22Rv1 and C4-2B to *NDRG1* silencing was very similar under all conditions examined.

Overall, Figure 5 demonstrates that NDRG1 overexpression can inhibit the EGF-mediated activation of ErbB family members. However, *NDRG1* silencing demonstrated differences in terms of which ErbB members were affected depending on the cell type, and this may be related to their state of androgen dependence (Figs. 5 and S4).

NDRG1 expression inhibits PI3K/AKT signaling in prostate cancer cells

A vital pathway activated by ErbB proteins in response to EGF is the PI3K/AKT pathway, which has emerged as a
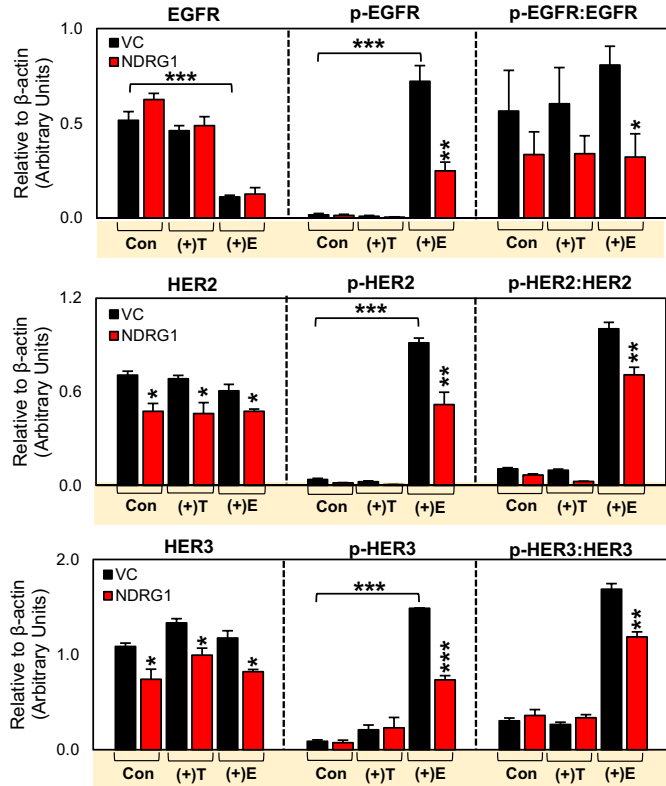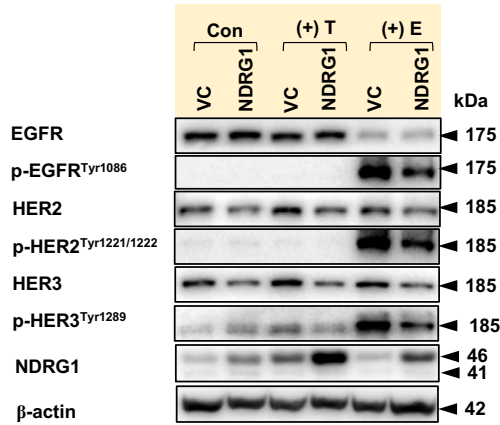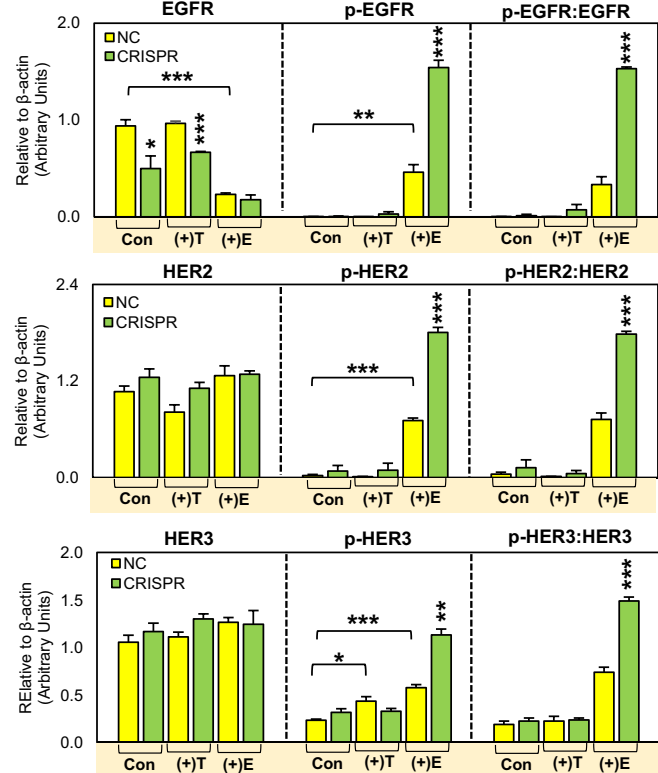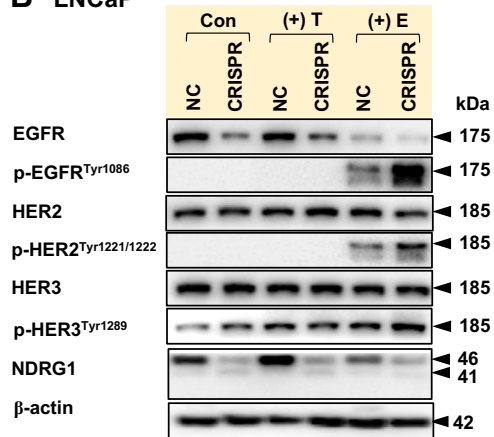
Figure 5. NDRG1 inhibits oncogenic expression and/or activation of EGFR, HER2, and HER3. LNCaP cells were transfected with either: (*A*) an NDRG1 expression vector (NDRG1) or the empty vector control (VC); or (*B*) CRISPR-Cas9 using three different guide RNAs targeting *NDRG1* (CRISPR) or the empty vector (NC). These cells were incubated with control medium (Con), or this medium containing testosterone (+T; 10 nM) for 24 h/37 °C; or EGF (+E; 100 μg/ml), which was added in the last 10 min of the 24 h/37 °C incubation. The following were then assessed using western blot, namely EGFR, p-EGFR^{Tyr1086}, HER2, p-HER2$^{Tyr1221/1222}$, HER3 and p-HER3^{Tyr1289}. The NDRG1 blot in (*B*) is from Figure 1*B* and was included as a reference, as the same set of lysates was used to assess these additional proteins. β-actin was used as the loading control. Densitometry results are mean ± S.E.M (n = 3). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ denote statistical significance compared with the relevant VC, or as otherwise indicated in the graphs.

primary driver of resistance in various cancers, including PCa, where it promotes AR activation (75–77). Hence, considering the results in Figure 5, we next assessed the effect of NDRG1 overexpression on PI3K/AKT levels, activation, and its downstream signaling in LNCaP cells under control conditions and in the presence of testosterone or EGF (Fig. 6A).

Studies first examined the effect of NDRG1 overexpression on the expression and phosphorylation of the PI3K p85 regulatory subunit that plays a key role in PI3K activity (78, 79). Overexpression of NDRG1 in control cells or those treated with EGF had no effect on total PI3K p85 levels *versus* the

respective VC, whereas, in the presence of testosterone, NDRG1 overexpression significantly decreased PI3K p85 levels *versus* the testosterone-treated VC (Fig. 6A). Incubation with EGF significantly decreased PI3K p85 to an almost equal extent in the VC and after NDRG1 overexpression. Examining the activating phosphorylation of PI3K p85 (*i.e.*, p-PI3K^{Tyr458}), it was notable that under all incubation conditions, NDRG1 overexpression significantly decreased its levels relative to the respective VC (Fig. 6A). This latter effect resulted in a marked and significant decrease in the p-PI3K p85: PI3K p85 ratio after NDRG1 overexpression upon incubation with control,
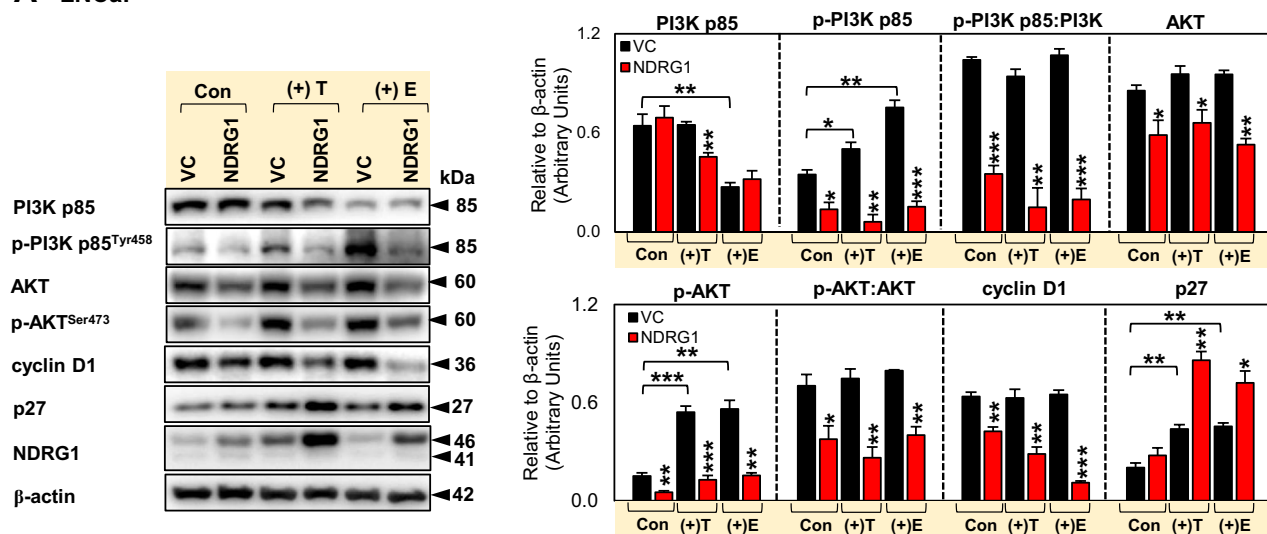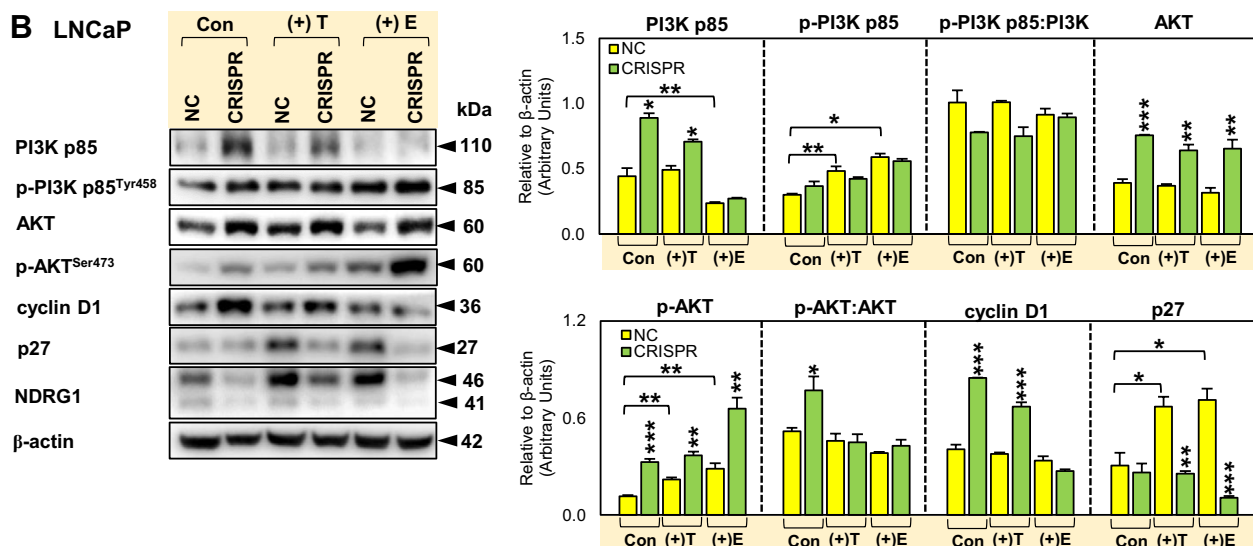
Figure 6. NDRG1 expression inhibits p-PI3K p85, AKT, and cyclin D1 expression, while increasing p27 levels in LNCaP cells. LNCaP cells transfected with either: (*A*) an NDRG1 expression vector (NDRG1) or the empty vector control (VC); or (*B*) CRISPR-Cas9 using three different guide RNAs targeting *NDRG1* (CRISPR) or the empty vector (NC), were incubated with control medium (Con) or this medium containing testosterone (+T; 10 nM) for 24 h/37 °C; or EGF (+E; 100 µg/ml), which was added in the last 10 min of the 24 h/37 °C incubation. The following were then assessed using western blot, namely PI3K p85, p-PI3K p85^{Tyr458}, AKT, p-AKT^{Ser473}, cyclin D1 and p27. The NDRG1 blot in (*A*) is from Figure 1A and was included as a reference, as the same set of lysates was used to assess these additional proteins. β-actin was used as the loading control. Densitometry results are mean ± S.E.M (n = 3). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ denote statistical significance compared with the relevant VC, or as otherwise indicated in the graphs.

testosterone, or EGF. Further, NDRG1 overexpression significantly decreased expression of a major downstream target of PI3K, namely AKT (80), and inhibited the activating phosphorylation of AKT (*i.e.*, p-AKT^{Ser473}) under all incubation conditions (Fig. 6A). In fact, the ratio of p-AKT to total AKT was significantly decreased under all conditions, demonstrating that NDRG1 overexpression inhibited AKT phosphorylation.

Key downstream targets of the PI3K/AKT signaling pathway were also examined, namely cyclin D1 (81), which promotes cell cycle progression, and the cyclin-dependent kinase inhibitor, p27 (82). NDRG1 overexpression led to significant downregulation of cyclin D1 under all conditions *versus* the VC, particularly in the presence of EGF (Fig. 6A). Overexpression of NDRG1 resulted in significant upregulation of the tumor suppressor, p27, relative to the VC after testosterone or EGF treatment, but not under control conditions (Fig. 6A).

These regulatory effects of NDRG1 expression on downstream effectors were generally further confirmed upon silencing *NDRG1* in LNCaP cells, which essentially had an opposite effect to NDRG1 overexpression for AKT, cyclin D1, and p27 (Fig. 6B). Under most incubation conditions, silencing *NDRG1 via* CRISPR enhanced AKT, p-AKT, and cyclin D1 levels. In contrast, *NDRG1* silencing decreased the expression of the tumor suppressor, p27, in these cells after incubation with testosterone or EGF *versus* the respective NC (Fig. 6B). The effects of *NDRG1* silencing on PI3K p85 were variable, with increased total PI3K p85 in control and testosterone-treated cells, while having no effect in EGF-treated cells (Fig. 6B). The levels of p-PI3K p85 and the ratio of p-PI3K p85: PI3K were not significantly affected by *NDRG1* silencing under all incubation conditions relative to the respective NC cells.

Taken together, these results in Figure 6, *A* and *B* demonstrate that NDRG1 inhibits downstream PI3K/AKT signaling under all conditions, which can promote AR activation (75–77).

NDRG1 inhibits JAK-STAT3 signaling in PCa cells

Besides the ErbB family of RTKs, other key oncogenic proteins can activate the AR in an androgen-independent manner and contribute to PCa progression and CRPC (83). These proteins include the Janus kinase (JAK) and signal transducer and activator of transcription (STAT3) (83). Hence, we next assessed the effect of NDRG1 overexpression on JAK2, Tyk2, and STAT3 expression in LNCaP cells (Fig. 7A).

Overexpression of NDRG1 significantly decreased protein levels of the JAK family members, JAK2 and Tyk2, as well as STAT3 *versus* the relative VC cells under all incubation conditions (Fig. 7A). The activation of STAT3 at Ser727 (*i.e.*, p-STAT3^{Ser727}) is required for homo-dimerization and nuclear translocation, where it can promote AR protein stability and transactivation (84, 85). Overexpression of NDRG1 significantly decreased p-STAT3^{Ser727} levels *versus* the respective VC under all conditions (Fig. 7A). Incubation with EGF markedly and significantly increased p-STAT3^{Ser727} in VC cells, with the

ability of NDRG1 overexpression to inhibit p-STAT3^{Ser727} levels being most pronounced under this condition. This latter response in the presence of EGF led to a significant decrease in the p-STAT3/STAT3 ratio after NDRG1 overexpression relative to the VC (Fig. 7A).

These effects of NDRG1 overexpression on inhibiting JAK/STAT3 signaling were further examined using LNCaP CRISPR cells, where *NDRG1* silencing generally induced opposite effects as expected (Fig. 7B) to NDRG1 overexpression (Fig. 7A). In fact, *NDRG1* silencing resulted in significant upregulation of JAK2 expression *versus* the respective NC conditions in response to testosterone and EGF, while Tyk2 levels were upregulated by *NDRG1* silencing under all conditions (Fig. 7B). Unlike the effect of NDRG1 overexpression on decreasing STAT3 under all conditions (Fig. 7A), silencing *NDRG1* had no significant effect on total STAT3 levels (Fig. 7B). While NDRG1 overexpression decreased p-STAT3 levels under all conditions (Fig. 7A), silencing *NDRG1* increased p-STAT3^{Ser727} *versus* the NC upon incubation with testosterone or EGF (Fig. 7B). The ability of EGF to markedly induce p-STAT3^{Ser727} levels in VC cells was further enhanced upon *NDRG1* silencing, resulting in a significant increase of the ratio of p-STAT3 to total STAT3 *versus* the respective NC (Fig. 7B).

Generally similar results to those obtained after CRISPR silencing of *NDRG1* in androgen-dependent LNCaP cells (Fig. 7B) were also observed using *NDRG1* silencing in androgen-independent, 22Rv1 cells (Fig. S5A). In the latter cell type, there was little effect of *NDRG1* silencing on total STAT3 levels *versus* the Con-siRNA treatments, as found for LNCaP cells (Fig. 7B). EGF markedly and significantly increased p-STAT3 levels in the Con-siRNA-treated cells, with *NDRG1* silencing significantly enhancing p-STAT3^{Ser727} levels and the p-STAT3^{Ser727}/STAT3 ratio in response to EGF. Examining androgen-independent C4-2B cells (Fig. S5B), *NDRG1* silencing again did not affect total STAT3 levels when compared with the relative VC under each treatment condition. The levels of p-STAT3^{Ser727} in C4-2B cells were significantly increased after *NDRG1* silencing *versus* Con-siRNA treatment only under control conditions. As found for LNCaP and 22Rv1 cells (Figs. 7B and S5A), EGF markedly and significantly increased p-STAT3 levels in the Con-siRNA-treated C4-2B cells (Fig. S5B). However, under EGF treatment, *NDRG1* silencing had no significant effect on p-STAT levels or the p-STAT: STAT3 ratio (Fig. S5B).

Overall, the results in Figure 7, *A* and *B* and Fig. S5, *A* and *B* demonstrate that NDRG1 can effectively inhibit the JAK/STAT3 signal transduction pathway in PCa cells, which in turn could decrease AR activation.

NDRG1 inhibits testosterone and EGF-mediated NF-κB signaling in PCa cells

The transcription factor, nuclear factor-kappa B (NF-κB), is implicated in tumorigenesis, being an important downstream mediator of several cancer signaling pathways, including the PI3K/AKT and MAPK pathways (86). Interestingly, NF-κB has also been demonstrated to promote the activation of AR
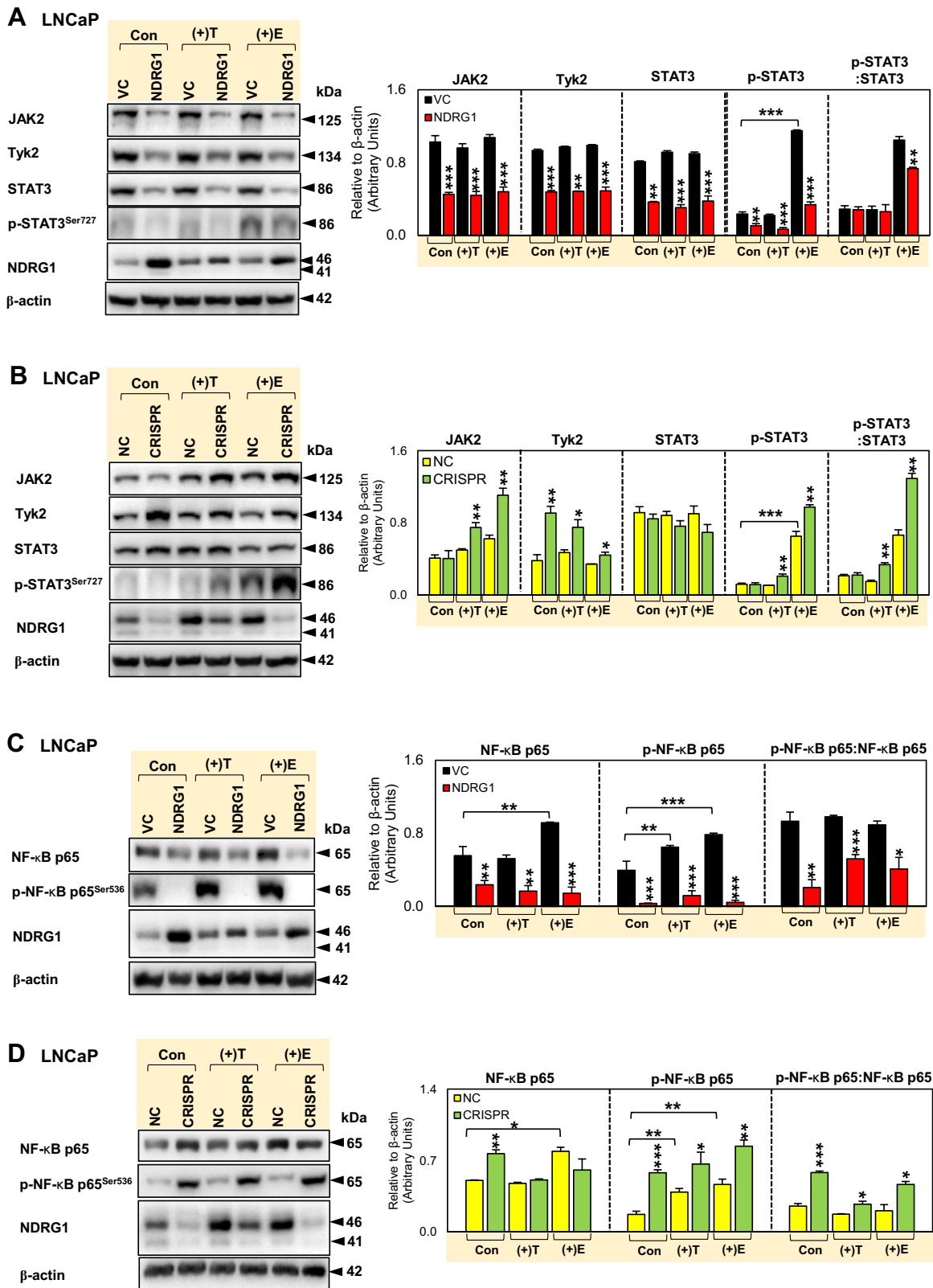
Figure 7. NDRG1 expression inhibits expression and activation of STAT3 and NF-κB p65 in LNCaP cells. LNCaP cells were transfected with either: (*A and C*) an NDRG1 expression vector (NDRG1) or the empty vector control (VC); or (*B and D*) CRISPR-Cas9 using three different guide RNAs targeting *NDRG1* (CRISPR) or the empty vector (NC). These cells were incubated with control medium (Con), or this medium containing testosterone (+T; 10 nM) for 24 h/37 °C; or EGF (+E; 100 μg/ml), which was added in the last 10 min of the 24 h/37 °C incubation. The following were then assessed using western blot, namely JAK2, Tyk2, STAT3, p-STAT3[Ser727], and NDRG1 (*A and B*); and NF-κB and p-NF-κB[Ser536] (*C and D*) *via* western blot. β-actin was used as the loading control. Densitometry results are mean ± S.E.M (n = 3). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ denote statistical significance compared with the relevant VC, or as otherwise indicated on the graphs.

signaling, leading to increased PSA levels in PCa (86). Hence, we next assessed the effects of NDRG1 overexpression on NF-κB p65 levels and its activation in LNCaP cells in response to testosterone or EGF.

As shown in Figure 7C, NDRG1 overexpression significantly downregulated the expression of total NF-κB p65 under all conditions relative to the respective VC cells. Interestingly, incubation with either testosterone or EGF significantly increased phosphorylation of NF-κB p65 at Serine 536 (*i.e.,* p-NF-κB p65^{Ser536}) in the VC cells relative to that observed with control medium. This latter phosphorylation of NF-κB p65 promotes PCa angiogenesis, invasiveness, and metastasis (87, 88). However, these effects were potently inhibited by NDRG1 overexpression, with a significant reduction in p-NF-κB p65^{Ser536} levels being observed under all conditions, resulting in a significant decrease in the p-NF-κB p65^{Ser536}: NF-κB ratio (Fig. 7C).

In contrast, when *NDRG1* was silenced by CRISPR in LNCaP cells (Fig. 7D), p-NF-κB p65^{Ser536} was significantly upregulated under all conditions. Silencing *NDRG1* also significantly increased the ratio of p-NF-κB p65^{Ser536} to total NF-κB p65, demonstrating increased NF-κB activation (Fig. 7D). A similar effect was also demonstrated in androgen-independent 22Rv1 (Fig. S6A) and C4-2B cells (Fig. S6B), where silencing *NDRG1* significantly increased total and phosphorylated NF-κB p65 levels under all incubation conditions. However, a significant increase in the ratio of p-NF-κB p65^{Ser536} to total NF-κB p65 was only observed after *NDRG1* silencing in 22Rv1 cells after incubation with testosterone or EGF.

Collectively, these results in Figure 7, Figs. S5 and S6 indicate the significant role of NDRG1 in regulating the expression and activation of STAT3 and NF-κB p65 signaling pathways in androgen-dependent and -independent PCa cells, both of which play important roles in promoting CRPC (86, 89, 90).

NDRG1 decreased IL-6 production by LNCaP cells

Many of the pathways examined above, including JAK/STAT and PI3K/AKT signaling, are activated by the cytokine, IL-6 (83). In fact, IL-6 was reported to increase AR activation in the absence of androgens *via* JAK/STAT, MAPK, and AKT signaling, thus contributing to androgen resistance in PCa (89–92). Further, IL-6 is a direct transcriptional target of NF-κB (93), which we demonstrate is potently inhibited by NDRG1 (Fig. 7, *C* and *D*). Hence, we next examined whether NDRG1 expression affected IL-6 production by LNCaP cells using an ELISA assay (Fig. 8A).

Under control conditions, overexpression of NDRG1 significantly decreased IL-6 production by LNCaP cells (Fig. 8A). While testosterone significantly induced IL-6 production by LNCaP VC cells relative to control medium, this effect was significantly reduced by NDRG1 overexpression (Fig. 8A). This finding suggests that NDRG1 may also inhibit AR transcriptional activation in PCa *via* inhibition of IL-6 production.

NDRG1 decreases PCa cellular proliferation and migration

As cellular proliferation and migration are important events in the progression of PCa and the development of CRPC, we next investigated how NDRG1 affects these properties in LNCaP cells in response to testosterone or EGF (Fig. 8B). To assess the effect of NDRG1 overexpression on proliferation, we examined Ki-67 levels, a well-established marker of cellular proliferation (94), in both VC and NDRG1 overexpressing cells using confocal immunofluorescence microscopy (Fig. 8B) These studies were performed upon treatment of LNCaP cells with control medium or this medium containing either testosterone or EGF (Fig. 8B). Under all conditions, including in the presence of testosterone or EGF, NDRG1 overexpression significantly decreased Ki-67 levels in LNCaP cells, demonstrating its potent antiproliferative activity (Fig. 8B).

Further studies also examined the migratory ability of LNCaP VC and NDRG1 cells in the presence or absence of testosterone using a transwell migration assay (Fig. 8C). While testosterone significantly increased migration of VC cells, this effect was significantly inhibited by NDRG1 overexpression after 24 h/37 °C (Fig. 8C). In fact, NDRG1 overexpression significantly suppressed the migration of these cells in the presence or absence of testosterone. In summary, these results in Figure 8 demonstrated that NDRG1 overexpression decreases IL-6 production, as well as proliferation and migration of PCa cells.

NDRG1 is negatively correlated with PSA levels in PCa patient specimens

The results above indicate the potent antioncogenic effects of NDRG1 in androgen-dependent and -independent PCa cells. Previous studies demonstrated that NDRG1 expression was negatively correlated with Gleason grade, metastasis, and overall survival (19, 22). However, p-NDRG1 levels in PCa specimens have not been previously examined, and how this and total NDRG1 expression correlate with PSA levels remains unknown. Examination of NDRG1 phosphorylation could be critical, as it is involved in inhibiting downstream oncogenic signaling (36), and its levels are markedly increased by agents that inhibit tumor growth and metastasis (37, 38). Thus, for the first time, we assessed both total NDRG1 and its phosphorylation at Ser330 (*i.e.,* p-NDRG1^{Ser330}) using patient specimens (*i.e.,* primary PCa tumors) obtained from predominantly prostate cancer stage T3, *i.e.,* the cancer has grown outside the prostate and may have spread to the seminal vesicles (95).

Two different PCa patient groups were examined, namely those that did not experience relapse and metastasis after prostatectomy (n = 18) and those who relapsed with metastatic disease (n = 13) 5 to 10 years following prostatectomy. Specifically, the patient cohort that did not develop any metastases following prostatectomy will henceforth be termed the "Remission" cohort, while those that relapsed with metastases within 5 to 10 years will be designated the "Relapsed" group. Importantly, our analysis was conducted using prostatectomy tissue from primary tumors obtained from treatment naïve
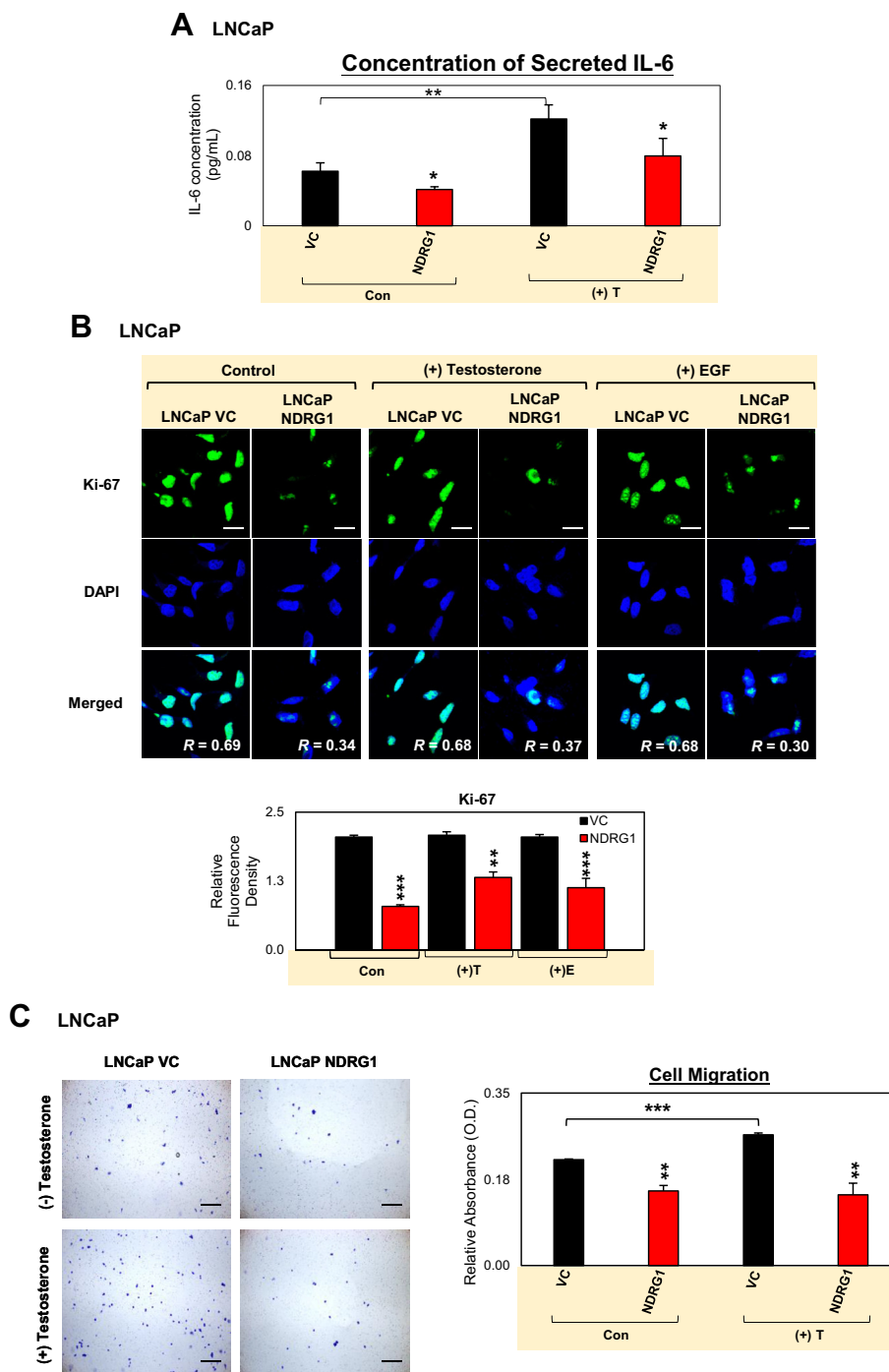
Figure 8. NDRG1 expression decreases IL-6 production, the proliferation-related marker, Ki-67, and testosterone-mediated migration of LNCaP cells. *A*, an IL-6 ELISA assay was used to determine the effect of NDRG1 overexpression on IL-6 secretion. In these studies, LNCaP cells transfected with the vector control (VC) or an NDRG1 expression vector (NDRG1) were incubated with either control medium (Control) for 24 h/37 °C; or this medium containing testosterone (+T; 10 nM) for 24 h/37 °C. The overlying medium was then decanted, centrifuged to remove cell debris, and used for the estimation of IL-6. *B*, LNCaP cells overexpressing NDRG1 (NDRG1) or their relevant vector control (VC) cells were incubated with either control medium (Control) for 24 h/37 °C; or this medium containing testosterone (+T; 10 nM) for 24 h/37 °C; or EGF (+E; 100 μg/ml), which was added in the last 10 min of the 24 h/37 °C incubation. The cells were then assessed for Ki-67 expression *via* confocal immunofluorescence microscopy. All images were taken using a 63× objective at the same exposure time (scale bar = 20 μm). *C*, LNCaP cells overexpressing NDRG1 or their relevant VC cells were incubated with control media or this media containing testosterone (10 nM) for 24 h/37 °C in transwell inserts, and the migrated cells were stained and visualized using crystal violet. Scale Bar = 20 μm. Results are mean ± S.E.M (n = 3). **$p < 0.01$ and ***$p < 0.001$ denote statistical significance compared with the relevant VC, or as otherwise indicated on the graphs.

patients, enabling examination of whether tumor baseline NDRG1 or p-NDRG1 levels could be used to predict whether patients will relapse with metastasis after prostatectomy.

To assess NDRG1 levels in patient samples, we analyzed total NDRG1 protein in the Remission and Relapsed cohorts using IHC staining (Fig. 9A). The staining of these sections was
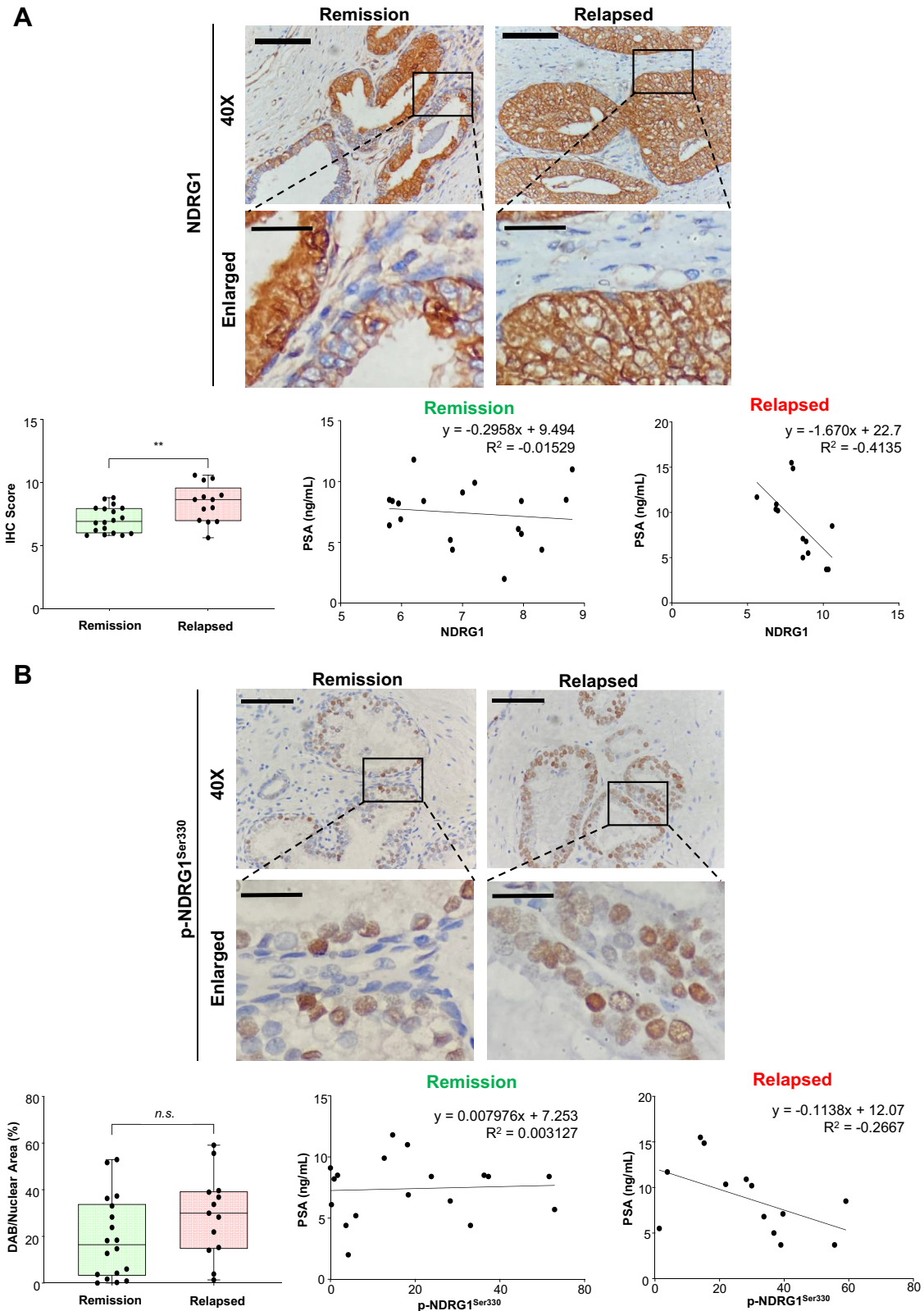
Figure 9. Total NDRG1 levels are negatively correlated with serum PSA levels in PCa patients that relapsed with metastasis following prostatectomy. PCa biopsies from primary tumors were examined from patients that either had no relapse (the "Remission" cohort), or those that went on to develop metastasis (the "Relapsed" group) 5 to 10 years following prostatectomy. These biopsies were assessed for: (*A*) NDRG1 and (*B*) p-NDRG1^{Ser330} levels using immunohistochemistry (IHC; scale bar = 20 μm (40×); scale bar = 5 μm (enlarged)). Scoring of NDRG1 and quantitation of p-NDRG1^{Ser330} were performed as described in the Experimental procedures. Results are mean ± S.E.M (n = 18 for the Remission cohort and n = 13 for the Relapsed group). The Mann–Whitney test was used to determine the significance of the: (*A*) IHC score and (*B*) DAB/nuclear area (%) between the two groups. Pearson's correlation coefficient was used to determine the correlation between NDRG1 or p-NDRG1^{Ser330} and serum PSA levels. Results are also presented in Tables 1 and 2.

then scored according to standard procedures (96, 97). In the samples from PCa patients in Remission, 15 (83%) had moderate and three (17%) had strong staining of NDRG1 (n = 18; Table 1). Examining the Relapsed cohort, six patients (46%) had moderate NDRG1 staining and seven (54%) had strong NDRG1 staining (n = 13; Table 1).

Total NDRG1 protein was most prominently detected in the cytoplasm and membrane of PCa cells (see enlarged images; Fig. 9A), with an average IHC score of 7.06 ± 0.25 in the Remission group (Table 1). However, relative to this latter cohort, NDRG1 was found to be significantly increased in the Relapsed patients with metastasis, with an average IHC score of 8.35 ± 0.42 (Fig. 9A and Table 1). Considering these results, while NDRG1 expression has been previously correlated to suppression of metastasis in PCa (19, 22), a direct comparison to the current investigation was not possible since different parameters were examined. These earlier reports (19, 22) demonstrated that NDRG1 expression was inversely correlated to Gleason grading or overall survival and that metastases demonstrated lower NDRG1 levels *versus* localized tumors. Furthermore, patient treatment status was not reported in these latter two publications, confounding interpretation relative to the current studies where patients were treatment naïve.

The preoperative blood PSA concentrations in patients were then correlated to NDRG1 levels. In the PCa patient cohort in Remission, PSA levels (7.41 ± 0.59 ng/ml; Table 1) did not correlate with NDRG1 (R^2 = −0.01529; p = 0.6250; Fig. 9A). However, examining the Relapsed cohort, these patients had higher average PSA (8.76 ± 1.08 ng/ml; Table 1) and a significant negative correlation (R^2 = −0.4135; p = 0.0178) was observed between NDRG1 expression and PSA levels (Fig. 9A and Table 1). These data suggest lower NDRG1 levels were correlated with increased serum PSA in PCa patients that relapsed with metastasis. This result is in good agreement with the current studies in cell culture that indicates NDRG1 expression suppresses PSA levels (Fig. 1A).

In contrast to total NDRG1 (Fig. 9A), p-NDRG1 staining was almost exclusively in the nuclei in PCa tissues from both patient groups (see enlarged images in Fig. 9B). Hence, the intensity of p-NDRG1 staining was quantified as the average percentage of DAB-positive to the total nuclear area, as previously performed (97). However, there was no significant difference in p-NDRG1 levels between the Remission and Relapsed PCa patient groups. Examining the preoperative blood PSA concentrations in the patient cohort in Remission demonstrated little correlation (R^2 = 0.0031) between p-NDRG1 and PSA, while in the Relapsed group, a weak negative correlation was observed (R^2 = −0.2667; Fig. 9B). However, this latter correlation did not reach statistical significance (p = 0.0708; Table 2). Overall, these results in Figure 9 indicate that the negative correlation between NDRG1 and PSA (Fig. 9A) may help predict patients likely to relapse and suffer metastasis after prostatectomy.

Discussion

For the first time, the current study examined the effect of the metastasis suppressor, NDRG1, and its ability to modulate AR expression and signaling. Phosphorylation of AR is required for mediating its activity and downstream signaling in PCa (98), especially phosphorylation at p-AR^{Ser213} and p-AR^{Ser81}, which are associated with the progression and development of advanced PCa (98). Previous studies examining patient specimens demonstrated that p-AR^{Ser213} was significantly increased as PCa progressed from hormone-sensitive to castration-resistant, and this was associated with poor overall survival (99, 100). Herein, we demonstrate for the first time that NDRG1 overexpression markedly decreased the activation of AR (*i.e.*, p-AR^{Ser213} and p-AR^{Ser81}). This response was accompanied by significantly reduced PSA expression in androgen-dependent and -independent PCa cells, suggesting the importance of NDRG1 in inhibiting AR transcriptional activity *via* androgen-dependent and -independent signaling pathways.

The current investigation focused on the dissection of mechanism at the functional protein level, which is of direct translational relevance to prostate cancer patients where PSA and NDRG1 protein levels were examined in tumors and serum (see Fig. 9). In fact, we comprehensively validate the inhibitory effect of NDRG1 on AR transcription, nuclear translocation, protein expression, and phosphorylation using four key indicators, including that NDRG1 expression: (1) significantly decreased ARE-luciferase activation (Fig. 1C); (2) potently reduced testosterone-mediated nuclear translocation of p-AR Ser^{81} (Fig. 2A); (3) decreased AR phosphorylation at two key sites that regulate AR transcriptional activity (*i.e.*, Ser^{81} and Ser^{213} (98); Fig. 1A); and (4) markedly decreased the key AR transcriptional target, PSA (Fig. 1A). Hence, we have convincingly demonstrated that NDRG1 expression negatively regulates AR-mediated activity.

Comparing the endogenous expression of NDRG1 between the three different cell types used (androgen-dependent LNCaP cells and androgen-independent 22Rv1 and C4-2B cells), all had similar endogenous NDRG1 levels and also expressed the AR. Notably, earlier studies have shown that the

Table 1

Tumor NDRG1 IHC scores and blood PSA levels in PCa patients that were successfully treated without further disease (Remission) following prostatectomy (n = 18) *versus* those that relapsed with metastases (Relapsed; n = 13)

Patient sample group	NDRG1 IHC score				Average IHC score	IHC significance compared with Remission	PSA levels	Significance (*p*-value) of NDRG1 and PSA correlation
	Absent	Mild	Moderate	Strong				
Remission	0	0	15	3	7.06 ± 0.25	—	7.41 ± 0.59	0.6250
Relapsed	0	0	6	7	8.35 ± 0.42	p < 0.0084	8.76 ± 1.08	0.0178[a]

Values are mean ± S.E.M.
[a] Significance (p < 0.01).

Table 2
Tumor p-NDRG1 (DAB/nuclear area %) and blood PSA levels in PCa patients that were successfully treated following prostatectomy (Remission; n = 18) *versus* those that relapsed with metastases (Relapsed; n = 13)

Patient sample group	p-NDRG1^{Ser330}		Average DAB staining (%)	IHC significance compared with Remission	PSA levels	Significance (*p*-value) of NDRG1 and PSA correlation
	Negative	Positive				
Remission	6	12	19.11 ± 4.11	—	7.41 ± 0.59	0.8256
Relapsed	2	11	29.11 ± 4.92	Not significant ($p > 0.05$)	8.76 ± 1.08	0.0708

Values are mean ± S.E.M.

AR directly regulates *NDRG1* transcription by binding to the ARE in the *NDRG1* promoter (101, 102), with this interaction being mediated by other cofactors, such as c-JUN or MLL5, to promote either positive or negative regulation of NDRG1 (103, 104). However, our current findings establish for the first time that NDRG1 interacts with and influences the activation and accumulation of the AR. This is the first evidence of a potential feedback loop between these two proteins, which is likely to have important implications for PCa progression and response to current therapies.

A potential mechanism identified in this study by which NDRG1 overexpression decreased AR activation involved c-Jun. c-Jun is a member of the activator protein-1 (AP-1) transcription complex and is a cofactor of AR (69). The relationship between c-Jun and AR remains somewhat controversial and is context-dependent (65–68). Recent work has elucidated that there are two distinct mechanisms by which c-Jun can influence AR activity. The first is the association of c-Jun to AR (coactivation), which can promote AR transcriptional activity, leading to increased PSA levels and PCa cell proliferation (66, 67). Importantly, phosphorylation of c-Jun as Ser63 was suggested to stabilize the c-Jun-AR interaction (68). The second mechanism by which c-Jun influences AR activity is not mediated by a direct interaction with AR, rather the downstream activity of c-Jun itself (transactivation), which can potently reduce AR transcriptional activity and cell proliferation (65, 66). It is likely that the balance between these two distinct mechanisms of c-Jun activity ultimately determines whether c-Jun inhibits or promotes AR activation and downstream signaling in PCa. We demonstrate herein that NDRG1 may influence this critical balance and promote the AR-inhibitory function of c-Jun. This is evidenced by the ability of NDRG1 to potently upregulate total c-Jun levels, while at the same time inhibiting c-Jun binding to the AR in the presence of testosterone. The reduced phosphorylation of c-Jun at Ser63, which stabilizes the c-Jun-AR interaction (68), likely contributes to the reduced c-Jun-AR interaction upon NDRG1 overexpression.

Phosphorylation of c-Jun is increased in a number of human cancers, especially PCa, where it is associated with invasive properties and shorter cancer patient survival (68, 69, 105, 106). Hence, the ability of NDRG1 overexpression to decrease EGF-mediated activation of AR and its transcriptional activity could be mediated *via* inhibition of p-c-Jun^{Ser63} (Fig. 10*A*).

Another mechanism by which NDRG1 inhibits AR activation is *via* its effects on HSP90. HSP90 directly binds AR, sequestering it in an inactive state in the cytoplasm (70), which is also important for AR functional maturation and stability (107). The current study has identified that NDRG1 expression promotes the direct interaction between HSP90 and AR, while at the same time reducing AR activation and nuclear translocation (Fig. 10*A*). Further, NDRG1 was demonstrated to associate with AR and HSP90, suggesting that it may stabilize the AR-HSP90 complex and increase the threshold for AR activation by testosterone and other ligands. The increased HSP90 levels in response to NDRG1 overexpression also suggest that NDRG1 associates with HSP90 to promote its accumulation.

No change in HSP90 expression was consistently observed upon *NDRG1* silencing, while its levels were potently increased upon NDRG1 overexpression. Similar effects were demonstrated with other NDRG1-regulated proteins in this investigation (*i.e.*, STAT3, c-Jun) and in other studies where NDRG1 overexpression and silencing did not result in opposite responses (29). This effect has been reported to occur when a protein is part of a complex that might control the expression or stability of other proteins (108). Our previous studies demonstrated that NDRG1 binds to other proteins and plays an important role in protein complexes that control proteasomal degradation and endosomal/lysosomal trafficking (40). Hence, it is conceivable that NDRG1 overexpression may enhance its association with HSP90, which could increase its stability, leading to HSP90 accumulation. In contrast, lower endogenous NDRG1 levels may not bind HSP90, so further silencing of *NDRG1* will have no effect compared with baseline.

To further delineate the mechanism by which NDRG1 inhibits AR activation, we examined which region of the NDRG1 protein was necessary for this activity. Using four deletion constructs of NDRG1, we identified that the CAP region in the α/β hydrolase fold of NDRG1 (amino acids 169–235) (38) was the most important for comprehensively inhibiting AR activation. Deletion of the CAP region prevented the ability of NDRG1 to decrease p-AR^{Ser213}, p-AR^{Ser81}, and PSA levels. Notably, deletion of the CAP region also prevented the ability of NDRG1 to promote AR-HSP90 association, highlighting the importance of this particular site in directly mediating AR expression and activation. Importantly, the crystal structure of NDRG1 was very recently published (109), revealing that the CAP domain is formed by three helices and covers the top of the α/β hydrolase fold. While this crystal structure lacked electron density for helices α-6 and α-8, which form part of the CAP domain, this region was suggested to have some flexibility, which is often associated with protein–protein interactions or ligand binding (109). Thus, we can hypothesize that the CAP region of NDRG1 may directly associate with the AR-HSP90 complex to regulate its function and prevent AR activation.
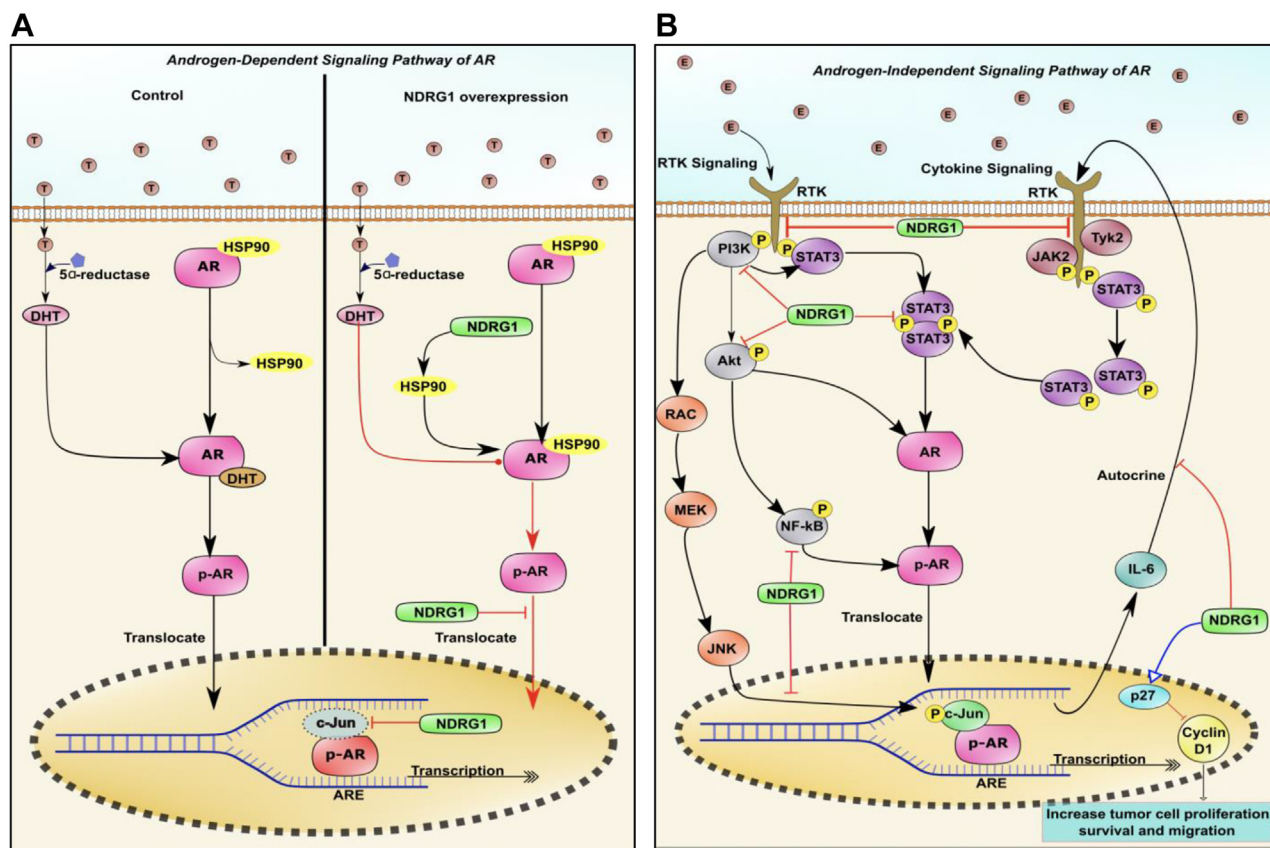
Figure 10. NDRG1 expression inhibits both androgen-dependent and -independent signaling pathways of the AR in prostate cancer cells. *A*, examining the androgen-dependent signaling pathway, testosterone binds to the AR, which leads to a conformational change and dissociation from HSP90, AR phosphorylation, and then nuclear translocation. Once in the nucleus, AR binds to androgen response elements (AREs) in the promoters of target genes and enhances PCa progression. AR transcriptional activity is enhanced by association with c-Jun. NDRG1 overexpression inhibits androgen-dependent AR activation *via* two major mechanisms, including: (i) stabilization of the AR-HSP90 complex in the cytoplasm, which reduces its activation in response to testosterone and attenuates AR nuclear translocation; and (ii) inhibiting the interaction between AR and its transcriptional co-activator, c-Jun. *B*, examining the androgen-independent signaling pathway of the AR, phosphorylation of receptor tyrosine kinases (RTKs) activates the PI3K/AKT, JAK/STAT3, and NF-κB downstream pathways. The autocrine feedback mechanism of IL-6 will further activate the JAK/STAT3 signaling pathway. This effect leads to the activating phosphorylation of AR (*i.e.*, p-AR^{Ser213} and p-AR^{Ser81}) and its subsequent nuclear translocation in an androgen-independent manner. Once in the nucleus, AR promotes transcription of oncogenic genes (*i.e.*, cyclin D1). NDRG1 inhibits EGF-mediated activation of PI3K/AKT, JAK/STAT3, and NF-κB signaling pathways, as well as their ability to activate the AR. NDRG1 expression also inhibits the autocrine feedback mechanism of IL-6, while markedly upregulating the tumor suppressor, p27, and inhibiting prooncogenic cyclin D1 expression. Finally, NDRG1 inhibits EGF-mediated phosphorylation of c-Jun (which is mediated by downstream PI3K/JNK signaling; (135)) to prevent AR transcriptional activity.

As AR can be activated *via* both androgen-dependent and -independent mechanisms, it was of significance that NDRG1 expression could attenuate testosterone and EGF-mediated AR activation. Herein, we identified that NDRG1 inhibited both testosterone and EGF-mediated activation of PI3K/AKT and NF-κB signaling in multiple PCa cell types that were both androgen-dependent and -independent (Fig. 10*B*). Further, NDRG1 also inhibited EGF-mediated activation of EGFR, HER2, HER3, and STAT3 signaling, which all promote downstream AR activation (85, 110, 111). This effect was accompanied by decreased cyclin D1 levels and upregulation of the tumor suppressor, p27, both of which are downstream of AKT (112, 113). These observations indicate that NDRG1 inhibits this oncogenic signaling hub in the presence of both testosterone and EGF. The ability of NDRG1 overexpression to inhibit these multiple oncogenic signaling pathways in response to EGF is likely to be mediated by the decreased EGFR activation (Fig. 5*A*). This hypothesis is suggested as EGFR is a master regulator of many downstream signaling

pathways including PI3K/AKT, NF-κB, STAT3, *etc.* (114). However, HSP90, which can also directly bind and regulate a diverse array of proteins (115), is potently upregulated by NDRG1 in the presence of testosterone (Fig. 3*A*) and may be involved in mediating the effects of NDRG1 on other proteins under this condition.

A subsequent decrease in PCa cell proliferation and migration was also observed in response to NDRG1 overexpression. This finding supports earlier studies demonstrating the potent ability of NDRG1 to inhibit invasion and metastatic progression of PCa *in vitro* and *in vivo* (19, 23, 27, 29, 33, 103, 104). As EGF-mediated activation of the abovementioned pathways plays a central role in androgen-independent AR activation (116–118), this suggests that NDRG1 may suppress the development of androgen resistance in PCa.

The IL-6 ligand can be produced by PCa cells in an autocrine manner (83) and can activate STAT3, MAPK, and AKT signaling, enabling enhanced AR transcriptional activity (92, 119–122). Herein, we demonstrated that NDRG1 expression

attenuated IL-6 production by PCa cells and inhibited the activation of STAT3 and AKT in PCa cells. Hence, this could be another potential mechanism by which NDRG1 attenuates AR activation. NDRG1 expression also attenuated NF-κB signaling, which can directly promote IL-6 transcription in PCa (93). Further, the IL-6/STAT3/NF-κB cascade has been reported to be responsible for PCa resistance to the anti-androgen, Enzalutamide (123–125). Hence, targeting NDRG1 with novel clinically trialed agents designed in our laboratory to upregulate its expression (126, 127) could offer an innovative approach to overcoming resistance to antiandrogens, such as Enzalutamide (49).

Considering its potent inhibitory effects on AR activity and potential to overcome androgen resistance, we further examined NDRG1 expression and phosphorylation in treatment naïve PCa patient specimens (primary tumors) that either had no relapse or relapsed with metastatic disease 5 to 10 years following prostatectomy. While earlier studies have demonstrated that NDRG1 expression is negatively correlated with Gleason grade and metastasis in PCa (19, 128–130), these earlier studies did not examine p-NDRG1 nor its correlation with PSA levels. Importantly, we observed a negative correlation between NDRG1 and PSA levels in patients that relapsed, with this observation being in good agreement with the findings from our cellular studies. However, no correlation between NDRG1 or p-NDRG1 and PSA levels was observed in patients that did not relapse. These findings indicate that the NDRG1/PSA signature may be useful in predicting the likelihood of PCa metastatic relapse after prostatectomy. As such, further investigations examining NDRG1 and PSA levels and how they relate to metastatic progression and treatment response are now warranted.

The antioncogenic activity of NDRG1 is not only confined to PCa cells expressing the AR, as shown in the current investigation. In fact, we have previously demonstrated its marked antioncogenic effects in AR-negative PCa cell-types, such as PC3 and DU145 cells, including its ability to inhibit the epithelial mesenchymal transition (28, 30, 33–35). This underscores the multieffector nature of NDRG1 expression on oncogenic signaling that is important to consider in terms of the broad antitumor activity of NDRG1-inducing chemotherapeutics (for reviews see (38, 58)).

Taken together, the findings from the current study reveal that NDRG1 downregulates both androgen-dependent and -independent signaling pathways of AR activation in PCa, the latter of which is responsible for deadly castration-resistant PCa that remains a critical problem. These studies indicate that NDRG1 could be a candidate as a cancer biomarker and a key molecular target in androgen signaling to develop innovative NDRG1 targeting therapies.

Experimental procedures

Cell culture

Prostate cancer cell lines (LNCaP, C4-2B, and 22Rv1) were obtained from the American Type Culture Collection and were maintained in Roswell Park Memorial Institute 1640 media (RPMI1640; Invitrogen). All media were supplemented with 10% FCS (Sigma-Aldrich), 100 μg/ml penicillin/streptomycin/glutamine (Invitrogen), 0.1 mM nonessential amino acids (Invitrogen), and 1 mM sodium pyruvate (Invitrogen).

Cell transfection and treatment: NDRG1 overexpression and CRISPR-Cas9

For stable transfection of NDRG1, LNCaP cells were transfected with either wild-type NDRG1 or the empty vector control (VC) using a 72 h/37 °C incubation with Lipofect-amine 3000 (L3000008; Sigma-Aldrich). This procedure was followed by treatment with G418 (ALX-380-013-G005) at a final concentration of 400 μg/ml for maintaining vector expression.

For stable silencing of *NDRG1*, LNCaP cells were transfected with either CRISPR-Cas9 using three different guide RNAs targeting *NDRG1* (CRISPR) or nontargeting control guide RNA (NC; Sigma-Aldrich) for 24 h/37 °C. Successfully transfected cells were sorted using flow cytometry, according to the standard manufacturer's protocol. Testosterone was purchased from Sigma-Aldrich (T1500-5G) and used at a final concentration of 10 nM (131). The EGF (Cell Signaling Technology) was incubated with cells at a final concentration of 100 μg/ml for 10 min/37 °C.

The 22Rv1 and C4-2B cell types were transfected with either siRNA against *NDRG1* (50 nM; Cat. #4392422; Life Technologies) or negative control siRNA (Cat. # AM4635; Life Technologies;) implementing the manufacturer's protocol using a 72 h/37 °C incubation, followed by incubation with testosterone (10 nM) or EGF (100 μg/ml) for another 24 h/37 °C. Transient transfection was performed using a 72 h/37 °C incubation of LNCaP or LNCaP CRISPR cells with truncated NDRG1 constructs (ΔC, Δ3xR, ΔCAP, ΔHTH) or WT-NDRG1 and analyzed using western blotting. The NDRG1 constructs were a kind gift from Dr Y. Sadovsky (University of Pittsburg, PA; (72)).

Protein extraction: Whole cell protein and fractionation

Whole cell protein lysates were extracted, as described previously (33). Briefly, cells were washed once with ice-cold PBS. Then, an appropriate volume of lysis buffer at 4 °C was added to the monolayer, and the cells scraped from the culture dish using a policeman. The cell suspension was then sonicated and centrifuged at 13,200g for 40 min/4 °C. The supernatant containing total cell protein was collected and protein concentration measured using the BCA protein assay (Cat. #: 23225; Thermo Fisher) and analyzed *via* western blotting. Nuclear and cytoplasmic extraction was performed, following the manufacturer's protocol (Cat. #: 78835; Thermo Fisher).

Western blot

Western blotting was performed *via* established methods (132). Primary antibodies (diluted in 1:1000–1:2000) and secondary antibodies (diluted in 1:10,000) used are summarized in Table S1. Proteins were visualized using a ChemiDoc Gel

Imaging System from Bio-Rad and quantified using ImageLab (Bio-Rad).

Coimmunoprecipitation

Co-IP was performed using Dynabeads Protein G (Cat. #: 10003D; Invitrogen) following the standard manufacturer's protocol. Briefly, cells were washed with ice-cold PBS once and lysed using the immunoprecipitation lysis buffer (Pierce) containing protease inhibitors (Roche Diagnostics). Protein (300–400 μg) was incubated with either monoclonal AR antibody or NDRG1 antibody (1:50) overnight at 4 °C with gentle rotation. This mixture was then added to 40 μl of Dynabeads Protein G and incubated for 4 h/4 °C with gentle rotation. The beads were then washed three times with ice-cold lysis buffer. The beads were then mixed with 20 μl of loading dye, heated for 5 min/95 °C, and placed on a magnet to separate beads from supernatant. Then equal amounts of supernatant were loaded and separated using a 10% SDS-PAGE gel. For input samples, 30 to 40 μg of the original protein lysate was also loaded for each sample as a comparison. Expression of HSP90, AR, and NDRG1 was detected by western blotting.

Luciferase assay

LNCaP VC and LNCaP NDRG1 cells were transfected with an AR reporter construct (Cignal Androgen Receptor Reporter Kit; Cat. #: 336841; Qiagen) for 72 h/37 °C, followed by treatment with either testosterone (24 h/37 °C) or EGF (10 min/37 °C) and a dual-Luciferase assay performed following the manufacturer's protocol (Cat. #: E1910; Promega). A CLARIOstar Plus monochromator microplate reader (BMG Labtech) was used to read Firefly (550 nm) and Renilla (488 nm) luminescence, and the results were normalized by Renilla luminescence. All assays were repeated in triplicate and independently performed three times.

IL-6 ELISA

Culture media from LNCaP cells (VC, NDRG1) treated with or without testosterone were collected and centrifuged at 300g for 5 min/20 °C to remove cell debris, and an IL-6 ELISA assay (Cat. #: 46027, Abcam) was used following the manufacturer's protocol.

Cell migration assay

Cell migration assays were performed by using Corning Transwell cell culture inserts following the manufacturers' protocol (Cat. #: CLS3464; Corning). Briefly, a 6.5 mm transwell with 8.0 μm pore polycarbonate membrane insert was placed in a 24-well culture plate, and LNCaP cells (VC and NDRG1 overexpression clones) in serum-free media were pipetted carefully onto the insert. The wells were filled with complete media (10% FBS as a chemo-attractant) and incubated for 24 h/37 °C. The inserts were then washed with PBS and fixed with 10% paraformaldehyde for 10 min/20 °C before staining with crystal violet (Sigma-Aldrich) for another 10 min/20 °C. These inserts were then imaged using an Olympus BX51 bright-field microscope (Olympus). The stained cells were then incubated with 10% acetic acid for 10 min/20 °C with orbital shaking, and the absorbance measured at 590 nm using a microplate reader to quantify the number of migrated cells.

Confocal immunofluorescence

Confocal immunofluorescence microscopy was performed using a standard protocol (33), and the expression of Ki-67 assessed using the primary and secondary antibodies listed in Table S1. Nuclei were stained with DAPI (Cat. #: P36962: Invitrogen). The slides were then photographed using a Zeiss LSM510 Meta fluorescence microscope (Carl Zeiss AG) and images analyzed using ImageJ software (NIH).

Immunohistochemistry

Immunohistochemistry was performed on 31 treatment naïve, PCa patient primary tumor samples from 18 patients who had no relapse 5 to 10 years after prostatectomy and 13 patients that experienced metastatic relapse within 5 to 10 years following prostatectomy. The average age of men in each group was 61. All tissue samples were acquired from the Garvan Institute of Medical Research and then stained as previously described (41). The levels of NDRG1 and p-NDRG1^{Ser330} were assessed using primary antibodies listed in Table S1. The antibodies were diluted according to the manufacturer's protocol by using a diluent from Dako (Cat. #: S0809). The tumor sections were visualized using EnVision+System (Cat. #: K4003) and DAB (Cat. #: K3408) from Dako. Images were taken using an Olympus BX51 microscope with camera system (Olympus).

Evaluation of immunohistochemical staining

NDRG1 staining was scored based on standard procedures, as previously implemented (96, 97). Two independent researchers scored each patient sample in a blinded manner, with average IHC scores being presented. The scoring system was as follows: ≤10% = 1; 11 to 25% = 1; 25 to 50% = 2; 50 to 75% = 3; and ≥75% = 3. The scoring for intensity was recorded as: absent = 0; weak staining = 1; moderate staining = 2; and strong staining = 3. The individual scores were then multiplied to obtain a final IHC score, as previously reported (96, 97). The overall IHC scores were then grouped based on: 0 to 1 = absence; 2 to 3 = mild; 4 to 8 = moderate; and 9 to 12 = strongly positive, as described previously (133).

As p-NDRG1^{Ser330} localization was almost exclusively nuclear, we quantified the intensity using ImmunoRatio plug-in in ImageJ (NIH), as previously described (134). The background for each sample was corrected with a blank field image, and these data were presented as an average value of the DAB/nuclear area (%) from five random images/sample.

Densitometry and statistical analysis

Densitometry was performed using Image Lab software (Bio-Rad) and normalized using the relative β-actin as

the loading control for all western blots or normalized using the corresponding protein for all Co-IP assays. All results are presented as typical of three independent experiments (unless specified) and presented at mean ± standard error of the mean (S.E.M) or as otherwise specified. Data were compared using the Student's *t* test, one-way ANOVA test, Mann–Whitney test, and Pearson's correlation coefficient (for IHC slides). Results were considered statistically significant when $p < 0.05$.

Data availability

This study includes no data deposited in external repositories.

Abbreviations—The abbreviations used are: ADT, androgen deprivation therapy; AKT, protein kinase B; AR, androgen receptor; ARE, androgen response element; CAP, cysteine-rich secretory protein/antigen 5/pathogenesis related-1; Co-IP, coimmunoprecipitation; CRISPR, clustered regularly interspaced short palindromic repeats; CRPC, castration-resistant prostate cancer; DAB, 3,3′-diaminobezidine; DAPI, 4,6-diamidino-2-phenylindole; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ELISA, enzyme-linked immunosorbent assay; EMT, epithelial-to-mesenchymal transition; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HDAC, histone deacetylase; HSP90, heat shock protein 90; IHC, immunohistochemistry; IL-6, interleukin-6; JAK, Janus kinase; MAPK, mitogen-activated protein kinase; NDRG1, N-myc downstream regulated gene 1; NF-κB, nuclear factor kappa light chain enhancer of activated B cells; PBS, phosphate-buffered saline; PCa, prostate cancer; PI3K, phosphoinositide 3-kinase; PSA, prostate-specific antigen; S.E.M, standard error of the mean; STAT3, signal transducer and activator of transcription 3; T, testosterone; Tyk2, tyrosine kinase 2.

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