Cancer Cell
Article

Regulation of Androgen Receptor Transcriptional Activity and Specificity by RNF6-Induced Ubiquitination

Kexin Xu,[1] Hermela Shimelis,[1] Douglas E. Linn,[1] Richeng Jiang,[1] Xi Yang,[1] Feng Sun,[1] Zhiyong Guo,[1] Hege Chen,[1] Wei Li,[1] Hegang Chen,[2] Xiangtian Kong,[3] Jonathan Melamed,[3] Shengyun Fang,[4] Zhen Xiao,[5] Timothy D. Veenstra,[5] and Yun Qiu[1,*]
[1]Department of Pharmacology and Experimental Therapeutics and Greenebaum Cancer Center
[2]Department of Epidemiology and Preventive Medicine
University of Maryland School of Medicine, Baltimore, MD 21201, USA
[3]Department of Pathology, New York University School of Medicine, New York, NY 10016, USA
[4]Medical Biotechnology Center, University of Maryland Biotechnology Institute, Baltimore, MD 21201, USA
[5]Laboratory of Proteomics and Analytical Technologies, SAIC-Frederick, Inc., National Cancer Institute at Frederick, Frederick, MD 21702, USA
*Correspondence: yqiu@som.umaryland.edu
DOI 10.1016/j.ccr.2009.02.021

SUMMARY

The androgen receptor (AR) plays a critical role in prostate cancer. We have identified a ubiquitin E3 ligase, RNF6, as an AR-associated protein in a proteomic screen. RNF6 induces AR ubiquitination and promotes AR transcriptional activity. Specific knockdown of *RNF6* or mutation of RNF6-induced ubiquitination acceptor sites on AR selectively alters expression of a subset of AR target genes and diminishes recruitment of AR and its coactivators to androgen-responsive elements present in the regulatory region of these genes. Furthermore, RNF6 is overexpressed in hormone-refractory human prostate cancer tissues and required for prostate cancer cell growth under androgen-depleted conditions. Our data suggest that RNF6-induced ubiquitination may regulate AR transcriptional activity and specificity through modulating cofactor recruitment.

INTRODUCTION

The androgen receptor (AR) is the key transcription factor mediating androgen-induced signaling, which is required for prostate cell survival and proliferation. It is well accepted that AR plays an important role in development of prostate cancer as well as progression to androgen-independent disease (Chen et al., 2004; Zegarra-Moro et al., 2002). Like other members of the steroid hormone nuclear receptor superfamily, AR is translocated into the nucleus upon ligand stimulation and binds to specific DNA sequences known as androgen-responsive elements (AREs), where it recruits the basic transcription machinery as well as the cofactors to modulate the transcription of androgen-responsive genes (Agoulnik and Weigel, 2006). Several recent studies have suggested that AR may preferentially recognize different AREs in different cell contexts and in response to different extracellular stimuli (Bolton et al., 2007; Guo et al., 2006). The different cofactor repertoire and posttranslational modifications of AR as well as local chromatin modifications of the target genes may play a role in the regulation of AR transcriptional activity and specificity. As a key regulator of prostate homeostasis, AR activity is precisely modulated at multiple levels, including posttranslational modifications such as phosphorylation, acetylation, sumoylation, and ubiquitination (Faus and Haendler, 2006; Gregory et al., 2004; Guo et al., 2006; Lin et al., 2002; Mahajan et al., 2007; Ueda et al., 2002).

Ubiquitination is one of the most abundant protein modifications in eukaryotic cells, and it has emerged as a vital mediator

SIGNIFICANCE

The androgen receptor (AR) is the key transcription factor mediating androgen signaling in prostate cells. Androgen ablation therapy is the most common treatment for advanced prostate cancer. However, most patients inevitably develop deadly recurrent cancers, which are resistant to current therapy. In this study, we show that RNF6 is overexpressed in hormone-resistant prostate cancer and induces ubiquitination of AR to promote its transcriptional activity toward a subset of AR target genes under androgen-depleted conditions. Our work suggests that ubiquitination of AR, and possibly other transcription factors, may function as the scaffold for cofactor recruitment to modulate transcriptional activity and specificity. Targeting components of the ubiquitination machinery, such as RNF6, may potentially be effective in treatment of advanced prostate cancer.

for a broad range of intracellular signaling activities, such as proteasomal degradation, endocytosis, subcellular localization, and kinase activation (Haglund and Dikic, 2005). Its involvement in versatile cellular functions possibly comes from the fact that the ubiquitin (Ub) molecule can form chains on substrates with various lengths and can elongate at different lysine (K) residues within the ubiquitin molecule. All seven conserved lysine residues within ubiquitin can mediate polyubiquitin chain formation (Kim et al., 2007), and different types of branching chains have distinct biological effects (Chastagner et al., 2006; Hofmann and Pickart, 1999; Morris and Solomon, 2004). For example, K48-branched chains are well accepted as the signals for proteasome degradation, while K6-mediated polyubiquitin chains can protect the substrates from proteolysis (Shang et al., 2005). K63 linkages, however, mainly have nonproteolytic functions such as DNA repair (Spence et al., 1995), kinase activation (Deng et al., 2000), and endocytosis (Bonifacino and Weissman, 1998). The attachment of multiple ubiquitin moieties is mediated via a three-step mechanism involving the sequential actions of E1, E2, and E3 enzymes (Hershko and Ciechanover, 1998; Pickart and Eddins, 2004). The ubiquitin chain topology is believed to be tightly controlled, and at least two different classes of E2 are required to attach a polyubiquitin chain of a particular topology to a substrate. The first E2 monoubiquitinates the acceptor lysine residue of the substrate; the second E2 elongates a polyubiquitin chain using a defined lysine residue of ubiquitin in concert with a specific E3 ligase (Kim et al., 2007; Rodrigo-Brenni and Morgan, 2007; Windheim et al., 2008). Regulation of AR nuclear trafficking and activity by the ubiquitin system has been studied previously (Poukka et al., 2000; Verma et al., 2004), but detailed mechanisms remain obscure. Recent evidence has shown that the E3 ligase MDM2 can induce polyubiquitination of AR, which leads to AR degradation via the 26S proteasome (Lin et al., 2002). This modification reduces the steady-state level of AR and attenuates AR transcriptional activity as well as androgen/AR-mediated cell growth. However, modulation of androgen/AR signaling by ubiquitination may not be limited to regulation of protein turnover, as the ubiquitin system has been reported to directly regulate the transcriptional activity of p53 independently of protein degradation (Le Cam et al., 2006). It has yet to be determined whether such a mechanism may be applicable in regulation of AR activity.

In a search for AR-associated proteins in prostate cancer cells, we identified a RING domain-containing E3 ligase, RNF6. *RNF6* was originally cloned in a genetic study of chromosomal rearrangements in myeloproliferative disorders, mapping to chromosome 13q12.12 (Macdonald et al., 1999). It encodes a 685 amino acid protein containing a coiled-coil domain at the N terminus and a RING-H_2 finger at the C terminus that is responsible for its ubiquitin ligase activity. The murine homolog of human *RNF6* has been demonstrated to activate *Inha* gene expression by participating in a protein complex binding to the promoter region (Lopez et al., 2002). A recent study demonstrated that the serine/threonine kinase LIM kinase 1 (LIMK1) is a substrate of RNF6 and that RNF6-induced LIMK1 polyubiquitination is mediated via K48 of ubiquitin, leading to proteasomal degradation of the kinase (Tursun et al., 2005). In the current study, we intended to examine the mechanisms by which RNF6 may regulate AR transcriptional activity and its potential role in prostate cancer progression.

RESULTS

RNF6 Is Associated with AR in Prostate Cancer Cells

To search for AR-interacting proteins in hormone-refractory CWR-R1 prostate cancer cells, we performed in vitro GST pull-down assays using the GST fusion protein containing the AR C-terminal region (aa 622–919) (GST-ARc). Among a number of proteins that specifically bound to AR but not to the GST control, RNF6 was one of the top scored proteins identified by mass spectrometry (MS) analysis (Figure 1A). The interaction between AR and RNF6 was confirmed by reciprocal coimmunoprecipitation experiments in 293T cells overexpressing both AR and RNF6 (Figure 1B). In addition, the association of endogenous RNF6 with AR was enhanced by androgen treatment in the prostate cancer cell lines CWR-R1 and LNCaP (Figure 1C). Immunofluorescence staining revealed that endogenous AR and RNF6 were colocalized in punctate structures spreading throughout in the nucleus (Figure 1D). It is well known that active transcription foci are distributed in the nucleus in a punctate pattern; therefore, RNF6 and AR may form a complex to regulate transcription in prostate cancer cells.

RNF6 Induces Ubiquitination of AR

To test whether AR is a substrate of RNF6, AR and HA-tagged wild-type RNF6 or a mutant lacking the RING domain (HA-RNF6ΔR) were coexpressed in COS-1 cells. Another known AR E3 ligase, MDM2, was included as a positive control. As shown in Figure 2A, accumulation of ubiquitinated AR was detected in cells expressing either RNF6 or MDM2, but not in cells expressing inactive mutant HA-RNF6ΔR or MDM2-C464A, suggesting that both RNF6 and MDM2 can induce AR ubiquitination in a RING domain-dependent manner. Consistent with previous studies, the steady-state level of AR in cells coexpressing wild-type MDM2 was dramatically reduced compared to that in cells expressing AR alone or that in cells coexpressing MDM2-C464A mutant as a result of polyubiquitination-associated proteasomal degradation. However, AR protein level was not decreased in cells overexpressing RNF6, suggesting that these two ubiquitin ligases have distinct effects on AR stability and that RNF6-induced AR ubiquitination appears not to be involved in AR degradation. The in vitro ubiquitination assays suggested that AR might be a direct substrate of RNF6, as the higher-molecular-weight polyubiquitinated AR was detected only in the presence of the active RNF6 (Figure 2B). We then examined which lysine residue (or residues) in the ubiquitin molecule might be involved in assembly of the atypical polyubiquitin chains on AR induced by RNF6. AR and RNF6 were coexpressed with His-tagged ubiquitin, a ubiquitin mutant without lysines (K0), or ubiquitin mutants with a single lysine in COS-1 cells. It appears that when overexpressing His-Ub in cells, the transfected AR is heavily mono- or oligoubiquitinated even in the absence of exogenous RNF6, possibly due to the activity of some endogenous E2 or E3 ligases. Addition of RNF6 seemed to have little effect on mono-Ub or oligo-Ub AR but did significantly enhance polyubiquitination of AR in the presence of wild-type Ub, Ub-K6, and possibly Ub-K27, suggesting that RNF6 may preferentially assemble polyubiquitination chains at K6 and K27. This was supported by the in vitro ubiquitination assays shown in Figure 2D. Although all single-K Ub mutants were able to be conjugated in

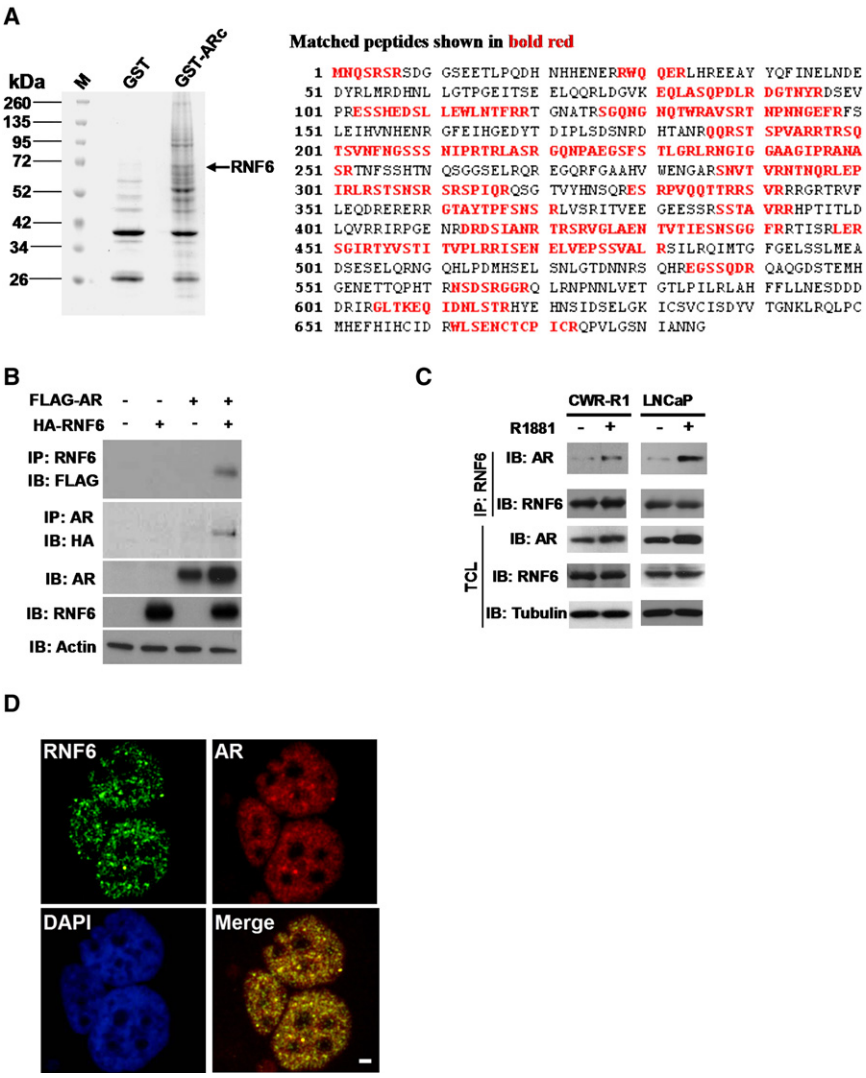

Figure 1. RNF6 Interacts with the Androgen Receptor in Prostate Cancer Cells

(A) Left: GST pull-down assays were performed using GST or GST-ARc as described in Experimental Procedures. Bound proteins were eluted and resolved by SDS-PAGE and then visualized by Coomassie blue staining. Discrete bands were excised and subjected to tryptic digestion and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) analysis. M, marker; GST-ARc, GST-AR fusion protein containing residues 622–919 of the androgen receptor (AR). Arrow indicates the band in which RNF6 was identified. Right: protein sequence of RNF6, with matched peptides shown in bold red.

(B) 293T cells were transfected with AR, RNF6, or both. At 48 hr posttransfection, cells were lysed and immunoprecipitation was performed using anti-AR or anti-RNF6 antibody, followed by immunoblotting with the indicated antibodies.

(C) CWR-R1 and LNCaP cells were subjected to immunoprecipitation using control IgG or anti-RNF6. Immunoblotting was performed using either precipitated samples or total cell lysates (TCL) with the indicated antibodies.

(D) Confocal immunofluorescence microscopy was carried out by costaining CWR-R1 cells with anti-AR (red) and anti-RNF6 (green) antibodies. Nuclei were counterstained with DAPI. Scale bar = 1 μm.

the mono- or di-Ub ARc, only the Ub-K6 and Ub-K27 mutants could assemble detectable poly-Ub chains on ARc under our experimental conditions. These data suggest that only K6 and/or K27 are able to assemble polyubiquitin chains on AR protein in the presence of RNF6. The atypical polyubiquitin chain has recently been reported to have a nonproteolytic effect on its target proteins (Ben-Saadon et al., 2006). This finding may help to explain our observation that RNF6-induced AR polyubiquitination does not lead to its destabilization.

RNF6 Modulates AR Transcriptional Activity and Specificity

It is well known that androgens induce transactivation of AR in prostate cancer cells. Interestingly, a dramatic increase in ubiquitination of AR was also observed in CWR-R1 and LNCaP cells treated with the synthetic androgen R1881, suggesting that ubiquitination of AR is accompanied by its activation (see Figure S3 available online). Consistent with our earlier observation that RNF6 induces AR ubiquitination, knockdown of endogenous *RNF6* in these cells significantly diminished both basal and androgen-induced ubiquitination of AR (Figure 3A), implying that RNF6 may modulate AR transcriptional activity via ubiquitination under both androgen-responsive and androgen-depleted conditions. To test whether RNF6 is involved in AR activation, we examined the effects of *RNF6* knockdown on AR transcriptional activity in LNCaP and CWR-R1 cells using ARR2-LUC reporter as a readout. As shown in Figure 3B, dihydrotestosterone (DHT)-induced AR activation was dramatically inhibited by *RNF6* shRNA in these cells, suggesting that RNF6 is required for transactivation of AR in response to androgen. It should be noted that in the absence of androgen or in the presence of a low level of androgen (0.1 nM), AR activity was also significantly reduced in *RNF6* knockdown CWR-R1 cells ($p < 0.01$), suggesting that RNF6 may also be required for AR transcriptional activity under androgen-depleted conditions. This result was further supported by the observation that knocking down endogenous *RNF6* in CWR-R1 and C4-2B cells under serum-free conditions diminished the expression level of a well-established AR target gene product, prostate-specific antigen (PSA), concurrent with the reduction of AR ubiquitination (Figure 3C). In addition, an increase in RNF6 protein level was detected in the hormone-insensitive LNCaP derivative C4-2B compared to its parental line LNCaP (Figure S4A). This is consistent with a report in a publicly available microarray database showing a 2- to 3-fold increase in *RNF6* mRNA in C4-2 cells compared to LNCaP cells (Figure S4B). Overexpression of AR with RNF6, but not with RNF6ΔR, induced approximately 2- to 3-fold higher activity of

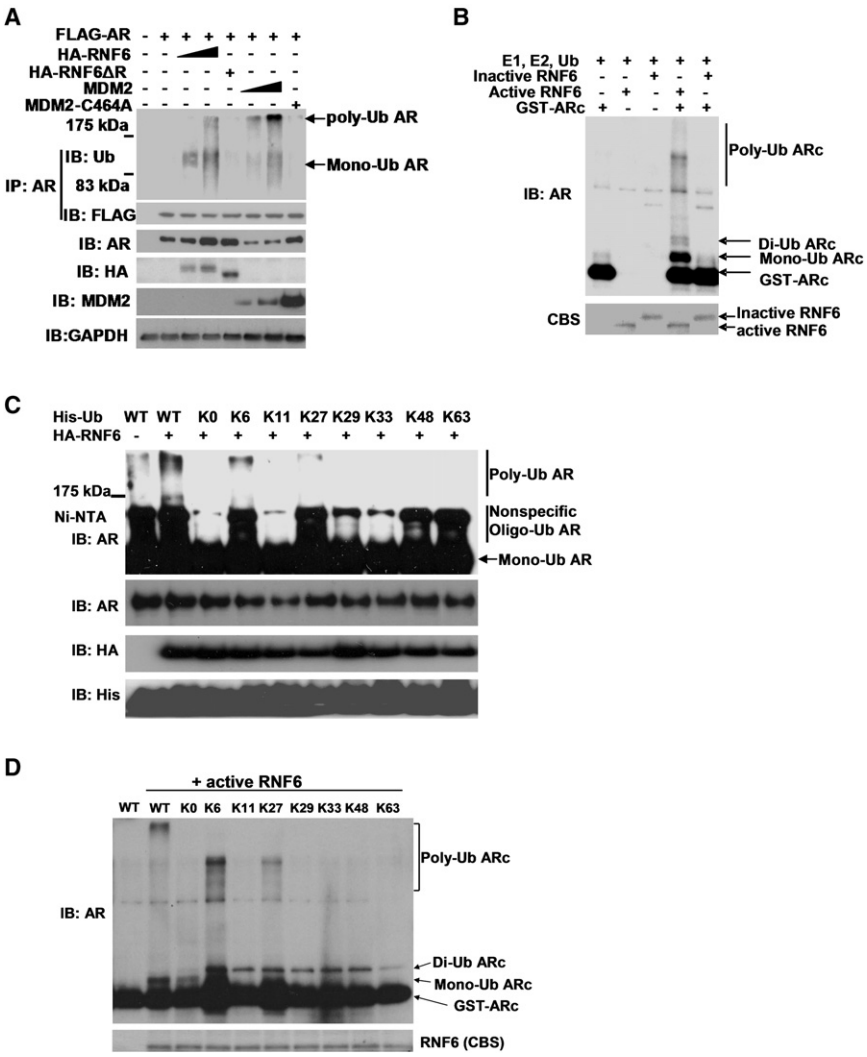

Figure 2. RNF6 Induces Atypical Polyubiquitination of AR

(A) COS-1 cells were transfected with plasmids as indicated. At 36 hr, cells were lysed and subjected to immunoprecipitation using anti-AR under denaturing conditions, followed by immunoblotting with the indicated antibodies.

(B) In vitro ubiquitination assays were carried out as described in Experimental Procedures. Top: immunoblot of anti-AR to detect AR and AR conjugates. Bottom: Coomassie blue staining (CBS) of the gel to monitor the amount of E3 ligases present in the reactions.

(C) COS-1 cells were transfected with His_6-tagged wild-type (WT) ubiquitin, a lysine null mutant (K0), or a single-lysine-containing mutant as indicated, along with FLAG-AR and HA-RNF6. At 36 hr post-transfection, cells were lysed, and lysates were incubated with Ni-NTA beads rotating at 4°C for overnight. Immunoblotting was performed using the indicated antibodies.

(D) In vitro ubiquitination assays were carried out as in (B), except that ubiquitin was replaced with the lysine null mutant (K0) or a single-lysine-containing mutant as indicated.

the *PSA* promoter in COS-1 cells (Figure 3D). A similar enhancement of endogenous AR transcriptional activity was also observed in LNCaP cells (Figure 3E). Taken together, these results demonstrate that RNF6 modulates AR transcriptional activity under hormone-deprived conditions.

To examine the global effect of RNF6 on gene transcription in prostate cancer cells, *RNF6* expression was knocked down in LNCaP cells by specific shRNA, and microarray analysis was performed to determine altered gene profiling. As shown at left in Figure 3F, knockdown of *RNF6* in LNCaP cells induced profound global changes in gene expression in response to androgen. Expression of some of genes was upregulated while expression of others was downregulated, suggesting that RNF6 may function as a general transcriptional regulator (either repressor or activator) depending on promoter context. Many androgen-responsive genes are known to be regulated by AR (Figure 3F, right). Knockdown of *RNF6* had dramatic effects on expression of a subset of these genes, including *PPAP2A*, *TMEPAI*, *RLN1*, *KLK3*, *NKX3.1*, and *BMF*, suggesting that RNF6 may modulate AR transcriptional activity on these target genes. Meanwhile, inhibition of RNF6 appeared to have little or no effect on another subset of AR target genes including *PDIA5*, *SLC45A3*, *TMPRSS2*, and *SORD*. A similar change induced by shRNF6 was observed in cells without DHT treatment, suggesting that RNF6 may also modulate the basal activity of AR under androgen-depleted conditions. To test whether the altered gene expression was mediated via AR, we examined the effects of *RNF6* knockdown on AR recruitment to the regulatory region of these genes. Consistent with the microarray data, the chromatin immunoprecipitation (ChIP) assays shown in Figure 3G demonstrated that knockdown of *RNF6* in LNCaP abolished AR recruitment to a subset of AREs (including those located in the enhancer region of *PSA* [AREIII] and the promoter region of *RLN1* and *BMF*) but had little effect on the other subset of AREs such as those in the promoter region of *PSA* (AREI/II) and *TMPRSS2*. Thus, RNF6 may modulate the specificity of AR-mediated transcription, and overexpression of RNF6 in prostate cancer cells may preferentially modulate a subset of AR target genes, such as *RLN1* (relaxin 1), to promote progression to an androgen-independent state. In addition, we performed reChIP assays using anti-RNF6 following the anti-AR antibody. As shown in Figure 3H, RNF6 appeared to be detectable in the AR-containing protein complex assembled on the ARE sites in the regulatory regions of *PSA* (AREIII), *RLN1*, and *BMF* but not present in the complex on the ARE sites of *PSA* (AREI/II) and *TMPRSS2*.

To further investigate how RNF6-induced ubiquitination modulates AR activity, we set out to identify the ubiquitin acceptor sites in AR. Our tandem MS analysis revealed that two lysine residues, K845 and K847, were ubiquitinated in samples derived from cells expressing AR and RNF6

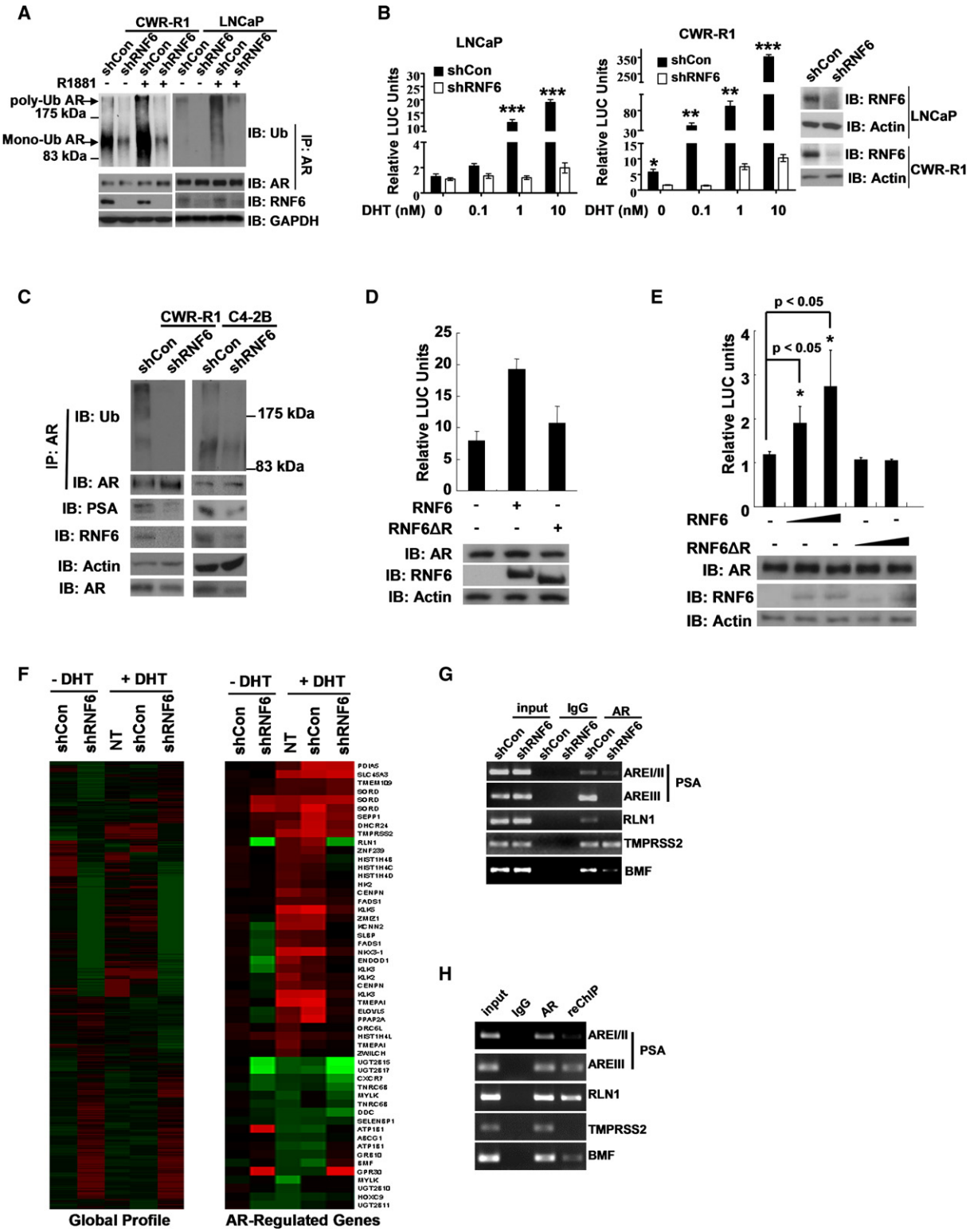

Figure 3. RNF6 Modulates AR Transcriptional Activity and Specificity

(A) CWR-R1 and LNCaP cells were infected with lentiviruses encoding shRNAs for control vector (shCon) or *RNF6* (shRNF6). After serum starvation, cells were pretreated with 10 nM R1881 for 16 hr. Cells were then lysed and subjected to immunoprecipitation with anti-AR under denaturing conditions, followed by immunoblotting with the indicated antibodies.

(B) CWR-R1 and LNCaP cells were transfected with ARR2-LUC reporter and infected with lentiviruses encoding shRNAs for control (shCon, black bars) or *RNF6* (shRNF6, white bars). After serum starvation, cells were pretreated with the indicated doses of dihydrotestosterone (DHT) for 16 hr before luciferase activity was

(Figure 4A). Alignments of AR sequences from various species showed that K845 and K847 were highly conserved (Figure 4B), implying the functional importance of these two residues. Substitution of K845 with an arginine residue (K845R mutation) completely abolished AR polyubiquitination even in the presence of RNF6, while K847R mutation only partially reduced AR polyubiquitination, possibly due to the loss of the K847 acceptor site (Figure 4C), suggesting that both lysines are ubiquitinated but that ubiquitination of K845 may be a prerequisite for efficient ubiquitination of K847 and may play a more important role in regulation of AR activity. This result was supported by reporter assays demonstrating that the AR-K845R mutant completely failed to be activated by RNF6 while the AR-K847R mutant functioned similarly to the wild-type AR (Figure 4D). Thus, ubiquitination of K845 is essential for AR transcriptional activity promoted by RNF6.

To study the physiological role of the ubiquitinated AR under conditions similar to the natural milieu, we replaced endogenous AR in LNCaP cells with the mutant AR lacking the ubiquitin acceptor site using a strategy described previously (Guo et al., 2006). As shown in Figure 4E, the transcriptional activity of the replaced codon-switched wild-type AR (ARcs-WT), but not the ARcs-K845R mutant, was enhanced by overexpression of RNF6 under the serum-free condition. Real-time PCR analysis was also carried out to compare the effects of ARcs-K845R with ARcs-WT on several AR target genes. Consistent with the microarray data obtained in the *RNF6* knockdown cells, the integrity of K845 appeared to be required for expression of both *PSA* and *RLN1* genes, but not for *TMPRSS2* (Figure 4F). Furthermore, the reChIP assay using anti-ubiquitin in AR-replaced LNCaP cells demonstrated that the K845R mutation abolished the capacity of AR binding to a subset of AR-responsive genes (Figure 4G), which was also consistent with the ChIP results obtained in *RNF6* knockdown cells. Taken together, these data suggest that RNF6-mediated polyubiquitination at AR-K845 is required for AR recruitment to a subset of AREs and thus modulates transcription of the cognate target genes.

Ubiquitination of AR Is Required for Recruitment of AR and Its Cofactor to a Subset of AREs

As the ubiquitination acceptor site of AR is located within the ligand-binding domain (also known as the AF2 domain), mutation of K845 may affect its ligand binding and/or transactivation activity associated with the AF2 domain. However, it is unlikely that the K845R mutation compromises the ligand binding activity because the ARK845R mutant still responded to DHT treatment and translocated into the nucleus upon ligand stimulation as the wild-type AR did (Figure S5). Because RNF6-induced ubiquitination of AR modulates androgen-responsive genes by facilitating its binding to a subset of ARE sites, we hypothesized that this posttranslational modification may be necessary for AR binding to its coactivators that interact specifically with AR at its C-terminal region through the polyubiquitin chains. We then tested whether ubiquitination of AR plays a role in the recruitment of cofactors, especially those containing ubiquitin-interacting domains such as ARA54. ARA54 is a member of the RING finger B box protein family (Ito et al., 2001) that interacts with the C-terminal portion of AR (aa 652–919) and enhances androgen-dependent transactivation of AR (Kang et al., 1999). The hydrophobic segments flanking the ring between ring fingers (RBR) region of ARA54 cooperate with the conserved domain for ubiquitin binding (Eisenhaber et al., 2007). It is possible that ARA54 may interact with AR through the polyubiquitin chain at the C-terminal ligand-binding domain. This possibility is supported by our observation that the association between AR and endogenous ARA54 was completely abolished in CWR-R1 cells either when *RNF6* was knocked down (Figure 5A) or when endogenous AR was replaced with the ARcs-K845R mutant (Figure 5B). Since ARA54 is reported to promote transactivation of AR in an androgen-dependent manner, we examined whether overexpression of RNF6 is sufficient to enhance ARA54 coactivator activity for AR in the absence of ligand. Figure 5C shows that, consistent with previous reports, ARA54 alone did not promote AR activity in the absence of hormone, while a synergistic increase of AR activity was detected in cells expressing both ARA54 and RNF6 together compared to either one alone. This increase appeared to depend on the integrity of the ubiquitin acceptor site K845, as the AR-K845R mutant was not affected under these conditions. Furthermore, the ChIP assay again demonstrated that the recruitment of ARA54 to a subset of AREs was dependent on ubiquitination of K845 (Figure 5D). These data strongly suggest that RNF6-induced ubiquitination of AR K845 functions as the scaffold to recruit its coactivators such as ARA54.

measured. Results are presented as mean relative LUC units ± SD of triplicate samples. *$p < 0.01$, **$p < 0.005$, ***$p < 0.001$. Right: expression of RNF6 in LNCaP and CWR-R1 cells.

(C) CWR-R1 and C4-2B cells were infected with lentiviruses encoding shRNAs for control vector (shCon) or *RNF6* (shRNF6). After serum starvation for 16 hr, cells were lysed and subjected to immunoprecipitation with anti-AR under denaturing conditions, followed by western blotting with the indicated antibodies.

(D) COS-1 cells were transfected with PSA-LUC reporter together with AR, wild-type RNF6 (RNF6), or RING-deletion mutant (RNF6ΔR). Cells were maintained in serum-free media for 16 hr before luciferase activity was measured. Results are presented as mean relative LUC units ± SD of triplicate samples.

(E) LNCaP cells were transfected with ARR2-LUC reporter, together with increasing doses of wild-type RNF6 (RNF6) or RING-deletion mutant (RNF6ΔR). Cells were serum-starved for 16 hr before luciferase activity was measured. Results are presented as mean relative LUC units ± SD of triplicate samples. *$p < 0.001$.

(F) LNCaP cells were left untreated (NT) or infected with lentiviruses encoding shRNAs for either control (shCon) or *RNF6* (shRNF6). At 16 hr postinfection, cells were incubated in phenol red-free medium with 5% charcoal-stripped FBS and pretreated with (+DHT) or without (−DHT) 1 nM DHT for 16 hr. RNAs were then extracted and microarray assay was performed as described in Supplemental Experimental Procedures.

(G) LNCaP cells were infected with lentiviruses encoding shRNAs for control vector (shCon) or *RNF6* (shRNF6). After serum starvation for 16 hr, LNCaP cells were treated with 10 nM DHT for 1 hr before ChIP assay was carried out to examine the binding of AR to the androgen-responsive elements (AREs) located in the regulatory region of *PSA*, *RLN1*, *TMPRSS2*, and *BMF*. PCR products amplified from the input and immunoprecipitation with control antibody (IgG) and anti-AR (AR) were resolved on agarose gels.

(H) LNCaP cells were serum-starved for 16 hr before reChIP assay was performed using anti-AR followed by anti-RNF6. The recruitment of the protein complex to the promoter (AREI/II) or enhancer (AREIII) regions of *PSA*, *RLN1*, *BMF* or *TMPRSS2* was tested. PCR products from the input, immunoprecipitation with control antibody (IgG), first-round ChIP with anti-AR (AR), and immunoprecipitation with experimental antibodies (reChIP) were resolved on agarose gels.

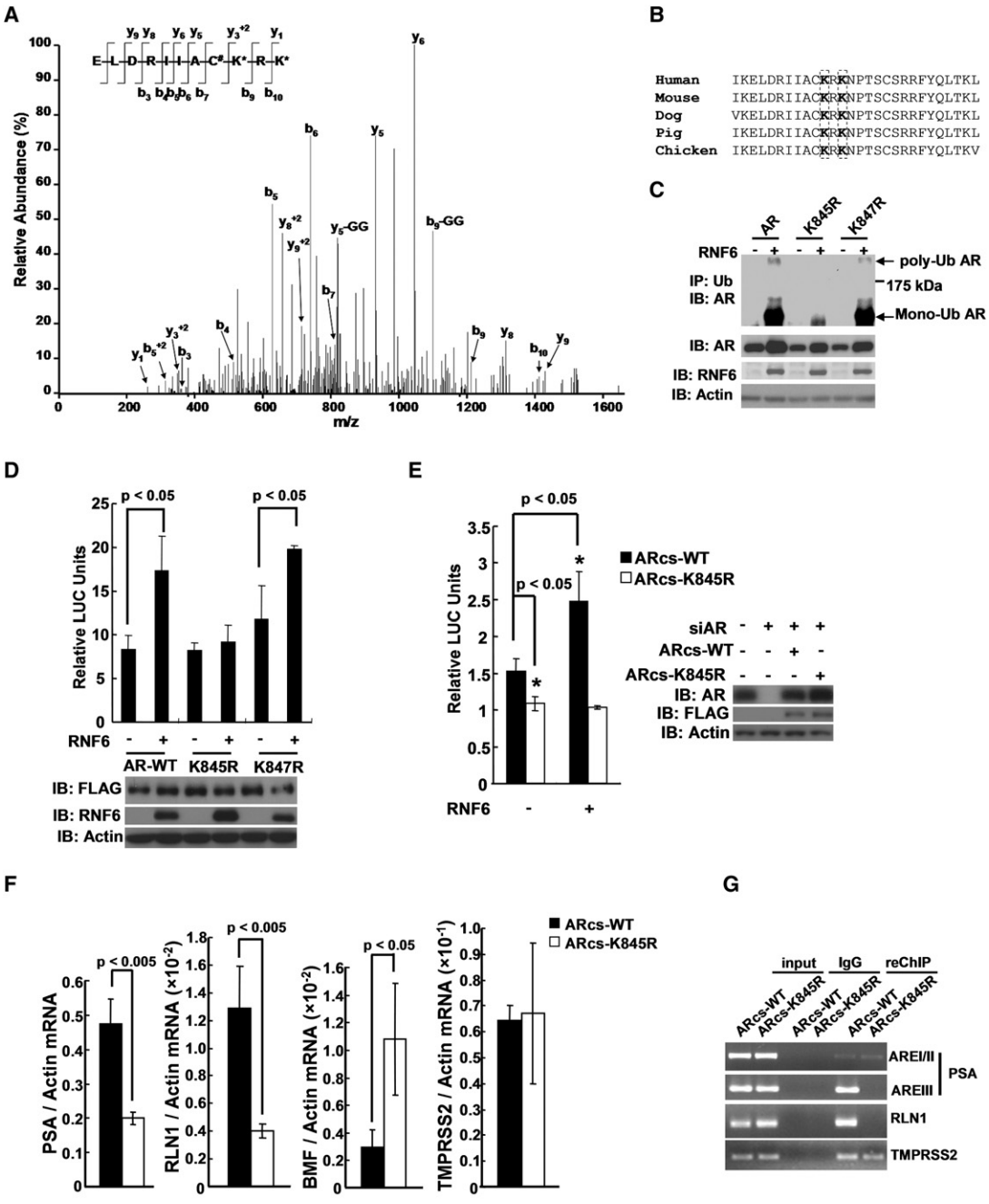

Figure 4. RNF6-Induced Polyubiquitination of AR at K845 and K847

(A) Tandem mass spectrometry (MS/MS) spectrum of AR peptide showing ubiquitination at K845 and K847. K* indicates lysine residues that are ubiquitinated.

(B) Alignment of AR protein sequences. K845 and K847 (boxed) are evolutionarily conserved across different species.

(C) COS-1 cells were transfected with wild-type AR (AR) or AR mutants (K845R and K847R), together with RNF6. At 36 hr posttransfection, cells were lysed and subjected to immunoprecipitation using anti-AR under denaturing conditions. Immunoblotting was performed using the indicated antibodies.

(D) Top: COS-1 cells were transfected with ARR2-LUC reporter and the indicated plasmids. After serum starvation for 16 hr, luciferase activity was measured. Results are presented as mean relative LUC units ± SD of triplicate samples. Bottom: expression of wild-type AR and AR mutants.

(E) Left: following AR replacement, LNCaP cells were transfected with ARR2-LUC reporter and serum-starved for 16 hr before luciferase activity was measured. Results are presented as mean relative LUC units ± SD of triplicate samples. Right: AR or AR mutant expression in these cells was monitored by western blotting.

(F) Following AR replacement, LNCaP cells were serum-starved and then treated with 1 nM DHT for 16 hr. Total RNA was extracted and subjected to quantitative real-time RT-PCR. Results are presented as mean values ± SD of three independent experiments.

(G) Following AR replacement, LNCaP cells were serum-starved for 16 hr and then pretreated with 1 nM DHT for 1 hr before reChIP assay was performed using anti-Ub followed with anti-FLAG. Binding of AR to the promoter (AREI/II) or enhancer (AREIII) regions of *PSA*, *RLN1*, and *TMPRSS2* was tested. PCR products from input and immunoprecipitation with control antibody (IgG) and experimental antibodies (reChIP) were resolved on agarose gels.

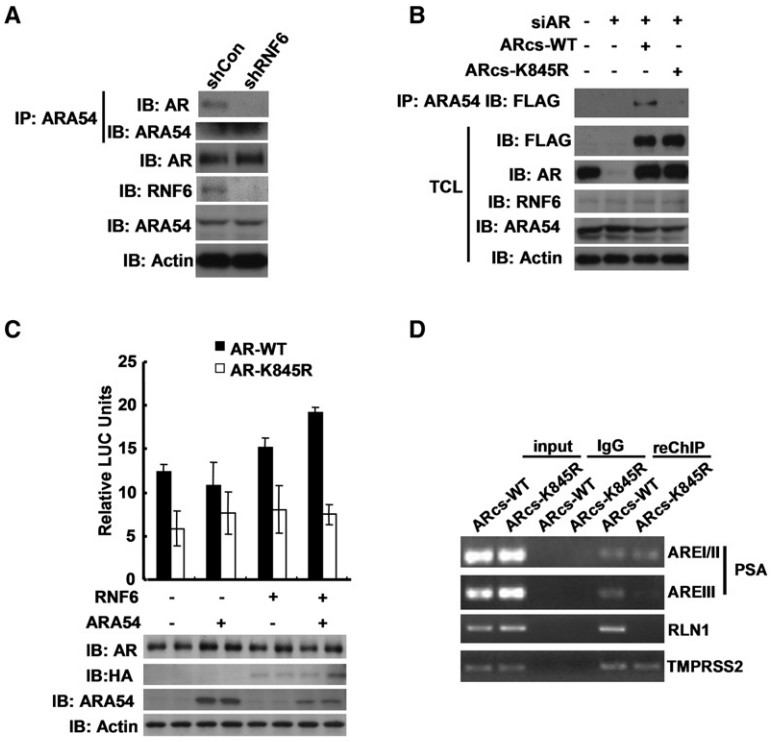

Figure 5. RNF6-Induced Polyubiquitination of AR Facilitates Its Binding to Coactivators on a Subset of AREs

(A) CWR-R1 cells were infected with lentiviruses encoding shRNAs specific for control vector (shCon) or *RNF6* (shRNF6). Immunoprecipitation was performed using anti-ARA54 followed by immunoblotting with the indicated antibodies.

(B) Following AR replacement, immunoprecipitation was carried out in CWR-R1 cells using anti-ARA54 antibody followed by immunoblotting with the indicated antibodies. TCL, total cell lysates.

(C) COS-1 cells were transfected with ARR2-LUC reporter and the indicated plasmids, and cells were serum-starved for 16 hr before luciferase activity was measured. Results are presented as mean relative LUC units ± SD of triplicate samples.

(D) Following AR replacement, LNCaP cells were serum-starved for 16 hr before ChIP assay was performed using anti-FLAG followed by anti-ARA54. Binding of AR and ARA54 to the promoter (AREI/II) or enhancer (AREIII) regions of *PSA*, *RLN1*, or *TMPRSS2* was tested. PCR products from input and immunoprecipitation with control antibody (IgG) and experimental antibodies (reChIP) were resolved on agarose gels.

RNF6 Is Upregulated during Prostate Cancer Progression and Required for Tumor Growth

To obtain more insights into the role of RNF6 in prostate carcinoma development, we performed immunohistochemistry (IHC) analysis on human prostate tissue arrays containing 36 benign, 233 hormone-naive, and 18 hormone-refractory samples. RNF6 was detected in both nucleus and cytoplasm of luminal epithelial cells. As summarized in Figure 6A, the mean score for nuclear staining of RNF6 in hormone-refractory samples (2.1944 ± 0.5884) was significantly higher than that in benign (0.0278 ± 0.0278) and hormone-naive (0.2586 ± 0.0502) samples ($p < 0.001$). Meanwhile, the frequency of detection of RNF6-positive nuclear staining increased dramatically in hormone-resistant tissues (50%) compared to the benign (2.78%) and hormone-naive (14.16%) samples. The changes in cytoplasmic staining of RNF6 followed a pattern similar to that of the nuclear staining. Figure 6B shows representative fields of the human prostate tissue arrays. Taken together, these data suggest that RNF6 is significantly upregulated in prostate cancer, especially in hormone-resistant

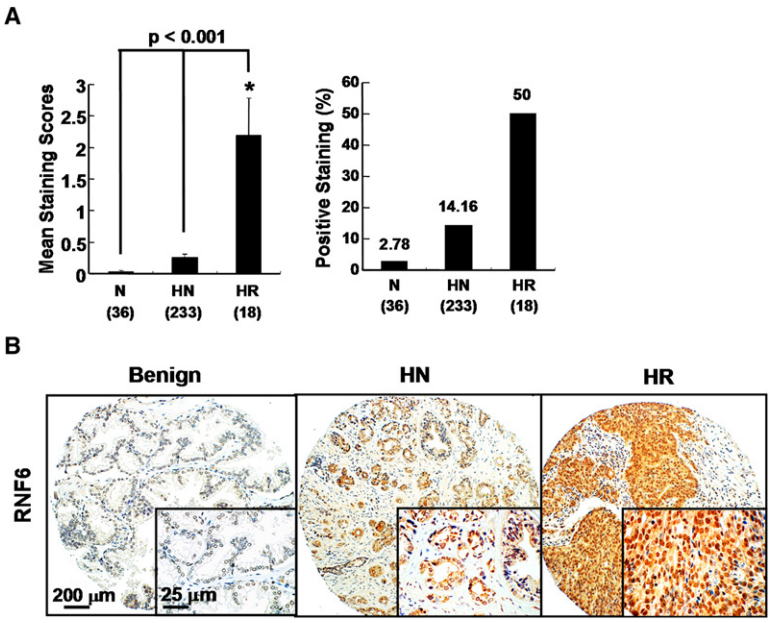

Figure 6. Expression of RNF6 Is Associated with Progression of Prostate Cancer

(A) Statistical analysis of human prostate tissue arrays stained with anti-RNF6. Immunostaining scores (mean ± SD) for RNF6 in benign epithelium (N), hormone-naive (HN), and hormone-refractory (HR) prostate tissues are summarized. Numbers in parentheses represent sample sizes; numbers above the bars in the right panel represent the percentage of positive-staining cases (+%). Statistical differences were determined by Wilcoxon's rank-sum test.

(B) Representative fields of human prostate tissue arrays. Immunohistochemical staining was performed using anti-RNF6 antibody.

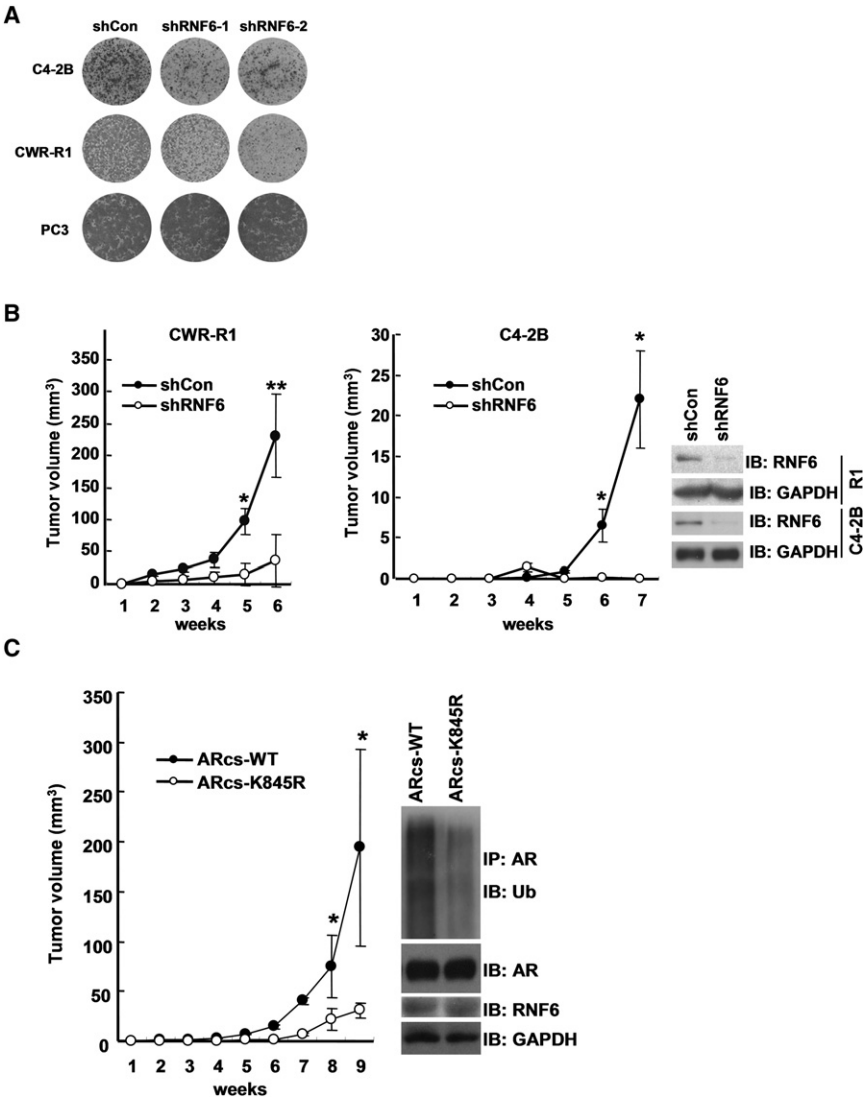

Figure 7. RNF6 Is Required for Prostate Cancer Cell Growth under Androgen-Depleted Conditions

(A) Prostate cancer cells were infected with lentiviruses encoding shRNAs specific for the control vector (shCon) or *RNF6* (shRNF6-1 and -2). At 16 hr postinfection, cells were incubated in fresh phenol red-free medium with 5% charcoal-stripped FBS and maintained for 7–10 days. Cell colonies were visualized by Coomassie blue staining.

(B) CWR-R1 (left graph) and C4-2B (right graph) were infected with lentiviruses encoding shRNAs specific for the control vector (shCon) or *RNF6* (shRNF6). At 48 hr postinfection, cells were injected into left (shCon) and right (shRNF6) flanks of castrated male nude mice. Growth of tumors was examined as described in Experimental Procedures. Results represent mean tumor sizes ± SD ($n = 5$ mice per group). $^*p < 0.05$, $^{**}p < 0.001$. Right: expression of RNF6 in CWR-R1 and C4-2B cells.

(C) Left: following AR replacement, CWR-R1 cells expressing ARcs-WT (●) or ARcs-K845R (○) were injected into castrated male nude mice. Tumor volume was measured as described in Experimental Procedures. Results represent mean tumor volume ± SD ($n = 5$ mice per group). $^*p < 0.05$. Right: immunoprecipitation with anti-AR antibody using xenograft mouse tissues, followed by immunoblotting with antibodies as indicated.

prostate cancer. The elevated expression of RNF6 displays a close correlation with prostate cancer progression.

To test whether the overexpressed RNF6 in hormone-refractory prostate cancer cells is required for tumor cell growth under androgen-depleted conditions, *RNF6* expression was knocked down by specific shRNA in C4-2B and CWR-R1 cells. Figure 7A shows that growth of these cells in androgen-depleted media was significantly attenuated. Such growth inhibition was also observed in xenograft models in castrated immunodeficient mice (Figure 7B). These data indicate that RNF6 is required for prostate tumor cell growth in both cell culture and xenograft models under androgen-depleted conditions.

To further demonstrate that the effect of RNF6 on tumor growth is mediated through modification of K845 of AR, we substituted endogenous AR in CWR-R1 cells with either ARcs-WT or ARcs-K845R mutant, and tumor growth was examined in castrated male nude mice. As shown in Figure 7C, growth of CWR-R1 cells expressing ARcs-K845R was dramatically compromised compared to that of cells expressing ARcs-WT. This growth retardation was accompanied by a reduction in ubiquitination of the ARcs-K845R mutant in tumors, while the levels of AR protein appeared to be similar. Taken together, our data demonstrate that RNF6-induced ubiquitination of AR at K845 is important for prostate cancer cell growth under androgen-depleted conditions.

DISCUSSION

Modulation of AR activity by various post-translational modifications has been extensively studied. Polyubiquitination of AR induced by MDM2 has been shown to have a profound impact on the stability and turnover of AR. In this report, we have demonstrated that AR transcriptional activity and specificity are regulated by an atypical polyubiquitination induced by RNF6. The RNF6-induced AR polyubiquitination appears to be different from the previously reported MDM2-mediated ubiquitin modification. One possible explanation for this noncanonical function of ubiquitination is the different topology of polyubiquitin chains induced by RNF6. RNF6 appears to preferentially promote the assembly of K6- and K27-mediated polyubiquitination of AR, possibly through partnering with an E2 enzyme different from that for the LIMK1 substrate. The K6/27-mediated ubiquitin chains have recently been shown to play a nonproteolytic function (Ben-Saadon et al., 2006), and the K6-mediated ubiquitin chain is known to prevent modified substrate from degradation (Shang et al., 2005). This observation may explain at least in part why RNF6-induced ubiquitination does not lead to AR degradation as MDM2-induced ubiquitination does. Although RNF6 is able to confer K48-mediated polyubiquitination of LIMK1 and induces

its degradation in neuronal cells, we did not detect an appreciable level of K48-mediated polyubiquitination of AR and associated degradation. It is possible that RNF6 may partner with a different E2 for distinct substrates in various cell contexts. The role of ubiquitination in transcription regulation has been demonstrated previously, such as in the cases of p53 (Le Cam et al., 2006) and Met4 (Kaiser et al., 2000). In both cases, ubiquitination of these transcription factors does not lead to protein degradation but rather modulates their binding capacity with chromatin or cofactors. Here, we have added another example, the proteolysis-independent transactivation of AR by RNF6-induced polyubiquitination, implying that polyubiquitination is a general mechanism by which transcriptional factors are regulated. The biochemical mechanisms by which this mixed branching chain enhances AR transcriptional activity remain to be addressed. Nevertheless, it would be reasonable to postulate that the chain may serve as a docking site for recruitment of important transcriptional activators such as ARA54. This function of the polyubiquitin chain as a recognition motif has also been reported for the K63-based polyubiquitin chain, which is essential for the activation of NF-κB pathway instead of protein degradation (Ea et al., 2006). On the other hand, the effect of RNF6 on AR transcriptional activity appears to be quite specific, as RNF6 does not promote the activity of the closely related nuclear receptor glucocorticoid receptor (GR) (Figure S6), possibly due to the fact that the ubiquitin acceptor site K845 appears not to be conserved among the steroid hormone nuclear receptor superfamily.

We have also demonstrated that RNF6 is upregulated in hormone-refractory prostate cancer and may play an important role in promoting prostate cancer growth under androgen-depleted conditions. Our findings are supported by at least three independent microarray studies in the publicly available Oncomine database (Rhodes et al., 2007) showing that expression of RNF6 is significantly elevated during prostate cancer progression (Figure S7). These microarray analyses suggest that RNF6 may function as a potential oncogene in promoting prostate cancer progression, especially to the androgen-independent state. RNF6 may exert its biological activity at least in part by preferentially regulating a subset of AR target genes that are known to be involved in progression to the hormone-refractory state. Deregulation of some of the genes regulated by RNF6 (e.g., *RLN1*) has been shown to play a causal role in development of hormone-refractory prostate cancer (Feng et al., 2007; Liu et al., 2008; Wang et al., 2007). This observation is supported by our microarray analysis and validation experiments including real-time PCR and ChIP assays. In addition, our microarray analysis also uncovered that the androgen-suppressed gene *BMF* is negatively regulated by RNF6. BMF is a BH3 domain-only protein and a proapoptotic member of the BCL2 family of proteins (Puthalakath et al., 2001) and is a key mediator of anoikis and luminal cell death during mouse mammary acinar morphogenesis (Schmelzle et al., 2007). Suppression of *BMF* expression by RNF6 in prostate cancer cells may prevent apoptosis induced by androgen ablation and promote hormone-independent growth. Although somatic mutations of *RNF6* have been detected in some primary esophageal squamous cell carcinomas and cell lines (Lo et al., 2002), it remains to be determined whether these mutations affect RNF6 activity. It is possible that RNF6 may have both oncogenic and tumor-suppressive effects, depending on the cellular context.

In addition, we have shown that RNF6 can induce polyubiquitination at K845 of AR and that the assembled polyubiquitin chains in the AF2 domain of AR may serve as a platform for recruiting additional transcription coregulators that are required for stabilization of the transcription complex and activation of a subset of AR-regulated genes. One such coregulator is the RING-finger protein ARA54, a ligand-dependent AR coactivator. We showed that an RNF6-mediated polyubiquitin chain is required for recruiting ARA54 to a subset of androgen-responsive genes under androgen-depleted conditions. Mutation of AR at K845 completely abolished binding of ARA54 to a subset of AREs including those located in the regulatory region of *RLN1* and the distal ARE of *PSA* but had very minimal effects on the AREs located in the regulatory region of *TMPRSS2* and the proximal AREs of *PSA*. These results suggest that RNF6-induced polyubiquitination of AR may play a role in determining promoter specificity of AR target genes. It is still unknown how ubiquitination determines the selectivity of ARE binding. Besides the specific coregulators binding to the polyubiquitin chains, other aspects of the cellular microenvironment such as local chromatin context and availability/accessibility of certain cofactors are likely to be involved. ARA54 contains RING-like sequence domains that lead to its autoubiquitination (Ito et al., 2001), but its cellular substrates have yet to be identified. Here, we demonstrated that ARA54 can promote the activity of AR together with RNF6 under serum-free conditions, suggesting a synergistic cooperation between these two cofactors. Such a ubiquitination cascade may amplify androgen/AR signaling and provide another layer of regulation of transcription magnitude and specificity. Based on the current study, our working model is that RNF6 induces an atypical polyubiquitination of AR, and this modification may serve as a platform to recruit additional coregulators containing the ubiquitin-interacting domain (or domains) to facilitate transcriptional regulation of AR target genes (Figure 8). Our work has suggested that ubiquitination is one of the mechanisms by which AR transcription activity and specificity are regulated. Such a mechanism may also be applicable to regulation of other nuclear receptors. Given that RNF6 is overexpressed in hormone-refractory prostate cancer and that its activity is required for tumor cell growth under androgen-depleted conditions, it may potentially be a new drug target for treatment of advanced prostate cancer.

EXPERIMENTAL PROCEDURES

DNA Plasmid Constructs and Antibodies

Two human EST clones of *RNF6* (GI 12 769 059 and 22 815 435) were obtained from American Type Culture Collection (ATCC). The full-length human *RNF6* cDNA was amplified by PCR using the EST clones as the template and then subcloned into pcDNA3-based vector. Mammalian expression constructs of FLAG-tagged AR and its mutants were cloned as described previously (Kurita et al., 2001). The GST fusion protein of AR C-terminal domains (aa 622–919) was generously provided by E. Xu (Van Andel Research Institute). RNF6 and AR mutants were generated by a PCR-based method using a QuikChange kit (Stratagene) and confirmed by DNA sequencing. Mammalian expression constructs of His_6-ubiquitin molecules (both the wild-type and lysine-free mutant) were generously gifts of W. Gu (Columbia University). The shRNAs for *AR* and *RNF6* were constructed as described previously (Guo et al.,

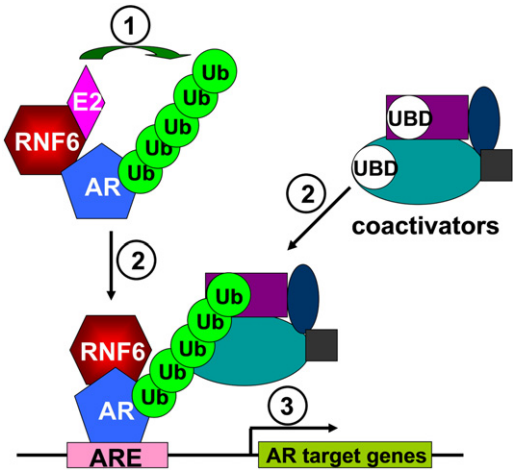

Figure 8. A Postulated Model of Regulation of AR Activity by RNF6
Our working model is that RNF6-induced ubiquitination of AR may serve as a scaffold for cofactor recruitment. Step 1: RNF6 binds to AR and induces polyubiquitination of the AF2 domain of AR in a RING domain-dependent manner. Step 2: the RNF6-induced polyubiquitin chain facilitates the recruitment of AR coactivators containing ubiquitin-binding domains (UBDs) and stabilizes the transcription complex at a subset of ARE sites in a polyubiquitin-dependent manner. Step 3: the polyubiquitination-dependent complex modulates transcription of AR-targeted genes. The E2 or E2s involved in this process have yet to be identified.

2006). The target sequences of the human *RNF6* shRNAs are 5′-GAAGCC AAACCTCAGTGAA-3′ (shRNF6-1) and 5′-GAGGCCTATTATCAGTTTA-3′ (shRNF6-2). Antibodies used for immunoblotting, immunoprecipitation, and immunofluorescence included anti-AR (N-20), anti-actin (C-2), anti-PSA (A67-B/E3), anti-ubiquitin (P4D1), anti-ARA54 (H-300), anti-GAPDH, anti-MDM2 (SMP14) (Santa Cruz), and anti-FLAG M2 (Sigma). The monoclonal antibody for RNF6 (anti-RNF6) was developed by immunizing mice with a purified fusion protein containing N-terminal residues of RNF6 (aa 1–246), and hybridoma clones were isolated and maintained in RPMI 1640 medium containing 25 mM HEPES (Invitrogen). The conditioned medium was used in the study.

Cell Culture and Transfection

293T, COS-1, and all human prostate cancer cell lines except for CWR-R1 used in this study were purchased from ATCC. CWR-R1 was kindly provided by C. Gregory and E. Wilson (University of North Carolina at Chapel Hill) (Gregory et al., 2001). 293T and COS-1 cells were routinely maintained in DMEM, while prostate cancer cells were cultured in RPMI 1640. Media were supplemented with 10% FBS and 1% penicillin/streptomycin unless otherwise specified. Transfections were performed using FuGENE HD (Roche) or the calcium phosphate precipitation method (Biological Mimetics, Inc.) according to the manufacturer's instructions.

In Vitro Ubiquitination Assay

In vitro ubiquitination assay was carried out as described previously (Fang et al., 2000; Guan et al., 2008), with minor modifications. Briefly, 10 ng of purified GST-ARc was mixed with 300 ng of either ligase-active GST-RNF6 (aa 246–685) or ligase-inactive GST-RNF6 (aa 1–585) in a total volume of 30 μl containing 50 mM HEPES (pH 7.9), 4 mM ATP, 5 mM $MgCl_2$, 15 μM $ZnCl_2$, 150 μM ubiquitin (Boston Biochem, cat. #U-100H), 30 nM rabbit E1 (Boston Biochem, cat. #E302), and 200 nM UbcH5a (Boston Biochem, cat. #E2-616). The reaction was incubated at 37°C for 4 hr, and reactions were terminated by addition of SDS sample buffer. Samples were resolved by SDS-PAGE and immunoblotted with anti-AR antibody (C-19). In some experiments, ubiquitin was replaced with a lysine null mutant (K0) or a single-lysine-containing mutant (K6, K11, K27, K29, K33, K48, and K63) (Boston Biochem) as indicated in the figures. In some cases, the E2 UbcH5a was replaced with UbcH2, UbcH10, or Use1 (Boston Biochem) as indicated in the figures.

Chromatin Immunoprecipitation

LNCaP cells were cultured in complete RPMI 1640 medium and infected for 24 hr with lentiviral constructs as indicated in figure legends. After serum starvation for 16 hr, cells were pretreated as described in the figure legends before being crosslinked with 1% formaldehyde and then sonicated. Soluble chromatin was immunoprecipitated as described previously (Louie et al., 2003; Shang et al., 2002). Primers used for ChIP were *PSA* promoter ARE (AREI/II), 5′-AGGGATCAGGGAGTCTCACA-3′ and 5′-GCTAGCACTTGCTGTT CTGC-3′; *PSA* enhancer ARE (AREIII), 5′-ACAGACCTACTCTGGAGGAAC-3′ and 5′-AAGACAGCAACACCTTTTT-3′; *TMPRSS2*, 5′-TGGTCCTGGATGATA AAAAAAGTTT-3′ and 5′-GACATACGCCCCACAACAGA-3′ (Wang et al., 2007); *RLN1*, 5′-GAGAGTCCCAAAGGCTAGCAGAG-5′ and 5′-GAACTTC CTCGGCTTCTGTTTGG-3′; *BMF*, 5′-GCTGGAGTGCACCACCTCAC-3′ and 5′-CCAGAAGCAGCAACCACCTG-3′.

ReChIP analysis was performed as described previously (Mal and Harter, 2003). Briefly, first-round antibody was added to chromatin extracts and incubated overnight at 4°C followed by addition of 60 μl salmon sperm/protein A agarose (Upstate Biotechnology) to recover immunocomplexes. The bound protein complexes were eluted by 10 mM dithiothreitol (DTT) at room temperature for 30 min, and the elution was then diluted ten times with reChIP buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris [pH 8.1]) and subsequently reimmunoprecipitated by addition of the second-round or control IgG antibodies overnight at 4°C. Recovery and preparation of DNA was performed and followed by PCR using the oligonucleotides described above.

Quantitative Real-Time RT-PCR

Quantitative real-time RT-PCR was performed as described previously (Zhang et al., 2005). Primer sequences used were *PSA*, 5′-TCTGCGGCG GTGTTCTG-3′ and 5′-GCCGACCCAGCAAGATCA-3′ (Patel et al., 2004); *TMPRSS2*, 5′-GGACAGTGTGCACCTCAAAGAC-3′ and 5′-TCCCACGAGGA AGGTCCC-3′ (Wang et al., 2007); *RLN1*, 5′-CGGCCAAATGGAAGGAC-3′ and 5′-GTGGCAAATTAGCAATGAATTCCA-3′; *BMF*, 5′-GAGGTACAGATTGCCC GAAA-3′ and 5′-TTCAAAGCAAGGTTGTGCAG-3′; and *Actin*, 5′-GCTATCCAG GCTGTGCTATC-3′ and 5′-TGTCACGCACGATTTCC-3′. The relative abundance of each target transcript was quantified using the comparative ΔΔCt method, with *Actin* as an internal control.

Immunohistochemical Analysis

Several intermediate-density prostate tissue arrays were prepared by the NYU Cooperative Prostate Cancer Tissue Resource. These arrays contained 287 cases, including 18 hormone-refractory (HR) and 18 age- and Gleason-matched hormone-naive (HN) transurethral resection (TURP) prostate specimens from patients with clinically advanced prostate cancer, 36 cases of non-tumor-containing tissue from patients with benign prostatic hypertrophy, and 215 radical prostatectomy cases representing additional HN prostate cancer cases. The determination of HR versus HN was as follows: patients who had previously undergone surgical orchiectomy or medical hormone-suppressive therapy at least 6 months prior to the procedure were considered as HR, and patients who did not receive hormonal therapy prior to the TURP or radical prostatectomy were considered as HN. Tissue specimens were from the archival paraffin block inventory of the NYU Cooperative Prostate Cancer Tissue Resource. All cases were collected into the resource under an Institutional Review Board-approved protocol and underwent repeated pathological characterization of tissues and review of medical records. A Vectastain Elite ABC Kit (Vector Laboratories) was used for immunohistochemical staining according to the protocol recommended by the manufacturer. Immunostaining was evaluated manually and graded using a two-score system based on intensity score (IS) and proportion score (PS) as described previously (Harvey et al., 1999). The immunoreactive score for each case was quantified by the average of four cores. Statistical analyses were carried out using SAS version 9.1 statistics software.

In Vitro Cell Growth Assay and In Vivo Tumor Growth in Xenograft Models

Prostate cancer cells were cultured in complete RPMI 1640 and infected with lentiviruses encoding shRNAs specific for *RNF6* or *GFP* control. After 48 hr, cells were maintained in fresh phenol red-free RPMI 1640 medium with 5% charcoal-stripped FBS and allowed to grow for 7–10 days. The cells were then fixed with 1% formaldehyde and stained with Coomassie blues. Tumor growth in SCID/nude mice was monitored as described previously (Craft et al., 1999). All procedures involving animals were approved by the Institutional Animal Care and Use Committee of the University of Maryland. Tumor cells were allowed to grow in 5–10 intact or castrated male mice for 4–10 weeks until the tumors reached a volume of ~300 mm^3. Tumor volume was calculated by the formula $0.5236 \times r_1^2 \times r_2$ (where $r_1 < r_2$) (Long et al., 2000).

ACCESSION NUMBERS

The raw data from the microarray analysis have been deposited in the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) with the accession number GSE14575.

SUPPLEMENTAL DATA

The Supplemental Data include Supplemental Experimental Procedures and nine figures and can be found with this article online at http://www.cancercell.org/supplemental/S1535-6108(09)00073-7.

ACKNOWLEDGMENTS

We would like to thank B. Guan and C. Bieberich for their assistance with ubiquitination assays. This work was supported in part by NIH grant CA106504 and US Department of Defense (DOD) grants W81XWH-06-1-0199 and W81XWH-08-1-0126 to Y.Q., NIH grant NCI UO1-CA86772 to J.M., NIH contract NO1-CO-12400 to T.D.V., DOD Predoctoral Fellowship W81XWH-06-1-0005 to K.X., and DOD Predoctoral Fellowship W81XWH-08-1-0068 to D.E.L.

Received: July 22, 2008
Revised: December 8, 2008
Accepted: February 23, 2009
Published: April 6, 2009

REFERENCES

Agoulnik, I.U., and Weigel, N.L. (2006). Androgen receptor action in hormone-dependent and recurrent prostate cancer. J. Cell. Biochem. *99*, 362–372.

Ben-Saadon, R., Zaaroor, D., Ziv, T., and Ciechanover, A. (2006). The polycomb protein Ring1B generates self atypical mixed ubiquitin chains required for its in vitro histone H2A ligase activity. Mol. Cell *24*, 701–711.

Bolton, E.C., So, A.Y., Chaivorapol, C., Haqq, C.M., Li, H., and Yamamoto, K.R. (2007). Cell- and gene-specific regulation of primary target genes by the androgen receptor. Genes Dev. *21*, 2005–2017.

Bonifacino, J.S., and Weissman, A.M. (1998). Ubiquitin and the control of protein fate in the secretory and endocytic pathways. Annu. Rev. Cell Dev. Biol. *14*, 19–57.

Chastagner, P., Israel, A., and Brou, C. (2006). Itch/AIP4 mediates Deltex degradation through the formation of K29-linked polyubiquitin chains. EMBO Rep. *7*, 1147–1153.

Chen, C.D., Welsbie, D.S., Tran, C., Baek, S.H., Chen, R., Vessella, R., Rosenfeld, M.G., and Sawyers, C.L. (2004). Molecular determinants of resistance to antiandrogen therapy. Nat. Med. *10*, 33–39.

Craft, N., Shostak, Y., Carey, M., and Sawyers, C.L. (1999). A mechanism for hormone-independent prostate cancer through modulation of androgen receptor signaling by the HER-2/neu tyrosine kinase. Nat. Med. *5*, 280–285.

Deng, L., Wang, C., Spencer, E., Yang, L., Braun, A., You, J., Slaughter, C., Pickart, C., and Chen, Z.J. (2000). Activation of the IkappaB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain. Cell *103*, 351–361.

Ea, C.-K., Deng, L., Xia, Z.-P., Pineda, G., and Chen, Z.J. (2006). Activation of IKK by TNFalpha requires site-specific ubiquitination of RIP1 and polyubiquitin binding by NEMO. Mol. Cell *22*, 245–257.

Eisenhaber, B., Chumak, N., Eisenhaber, F., and Hauser, M.T. (2007). The ring between ring fingers (RBR) protein family. Genome Biol. *8*, 209.

Fang, S., Jensen, J.P., Ludwig, R.L., Vousden, K.H., and Weissman, A.M. (2000). Mdm2 is a RING finger-dependent ubiquitin protein ligase for itself and p53. J. Biol. Chem. *275*, 8945–8951.

Faus, H., and Haendler, B. (2006). Post-translational modifications of steroid receptors. Biomed. Pharmacother. *60*, 520–528.

Feng, S., Agoulnik, I.U., Bogatcheva, N.V., Kamat, A.A., Kwabi-Addo, B., Li, R., Ayala, G., Ittmann, M.M., and Agoulnik, A.I. (2007). Relaxin promotes prostate cancer progression. Clin. Cancer Res. *13*, 1695–1702.

Gregory, C.W., Johnson, R.T., Jr., Mohler, J.L., French, F.S., and Wilson, E.M. (2001). Androgen receptor stabilization in recurrent prostate cancer is associated with hypersensitivity to low androgen. Cancer Res. *61*, 2892–2898.

Gregory, C.W., Fei, X., Ponguta, L.A., He, B., Bill, H.M., French, F.S., and Wilson, E.M. (2004). Epidermal growth factor increases coactivation of the androgen receptor in recurrent prostate cancer. J. Biol. Chem. *279*, 7119–7130.

Guan, B., Pungaliya, P., Li, X., Uquillas, C., Mutton, L.N., Rubin, E.H., and Bieberich, C.J. (2008). Ubiquitination by TOPORS regulates the prostate tumor suppressor NKX3.1. J. Biol. Chem. *283*, 4834–4840.

Guo, Z., Dai, B., Jiang, T., Xu, K., Xie, Y., Kim, O., Nesheiwat, I., Kong, X., Melamed, J., Handratta, V.D., et al. (2006). Regulation of androgen receptor activity by tyrosine phosphorylation. Cancer Cell *10*, 309–319.

Haglund, K., and Dikic, I. (2005). Ubiquitylation and cell signaling. EMBO J. *24*, 3353–3359.

Harvey, J.M., Clark, G.M., Osborne, C.K., and Allred, D.C. (1999). Estrogen receptor status by immunohistochemistry is superior to the ligand-binding assay for predicting response to adjuvant endocrine therapy in breast cancer. J. Clin. Oncol. *17*, 1474–1481.

Hershko, A., and Ciechanover, A. (1998). The ubiquitin system. Annu. Rev. Biochem. *67*, 425–479.

Hofmann, R.M., and Pickart, C.M. (1999). Noncanonical MMS2-encoded ubiquitin-conjugating enzyme functions in assembly of novel polyubiquitin chains for DNA repair. Cell *96*, 645–653.

Ito, K., Adachi, S., Iwakami, R., Yasuda, H., Muto, Y., Seki, N., and Okano, Y. (2001). N-terminally extended human ubiquitin-conjugating enzymes (E2s) mediate the ubiquitination of RING-finger proteins, ARA54 and RNF8. Eur. J. Biochem. *268*, 2725–2732.

Kaiser, P., Flick, K., Wittenberg, C., and Reed, S.I. (2000). Regulation of transcription by ubiquitination without proteolysis: Cdc34/SCF(Met30)-mediated inactivation of the transcription factor Met4. Cell *102*, 303–314.

Kang, H.Y., Yeh, S., Fujimoto, N., and Chang, C. (1999). Cloning and characterization of human prostate coactivator ARA54, a novel protein that associates with the androgen receptor. J. Biol. Chem. *274*, 8570–8576.

Kim, H.T., Kim, K.P., Lledias, F., Kisselev, A.F., Scaglione, K.M., Skowyra, D., Gygi, S.P., and Goldberg, A.L. (2007). Certain pairs of ubiquitin-conjugating enzymes (E2s) and ubiquitin-protein ligases (E3s) synthesize nondegradable forked ubiquitin chains containing all possible isopeptide linkages. J. Biol. Chem. *282*, 17375–17386.

Kurita, T., Wang, Y.Z., Donjacour, A.A., Zhao, C., Lydon, J.P., O'Malley, B.W., Isaacs, J.T., Dahiya, R., and Cunha, G.R. (2001). Paracrine regulation of apoptosis by steroid hormones in the male and female reproductive system. Cell Death Differ. *8*, 192–200.

Le Cam, L., Linares, L.K., Paul, C., Julien, E., Lacroix, M., Hatchi, E., Triboulet, R., Bossis, G., Shmueli, A., Rodriguez, M.S., et al. (2006). E4F1 is an atypical ubiquitin ligase that modulates p53 effector functions independently of degradation. Cell *127*, 775–788.

Lin, H.K., Wang, L., Hu, Y.C., Altuwaijri, S., and Chang, C. (2002). Phosphorylation-dependent ubiquitylation and degradation of androgen receptor by Akt require Mdm2 E3 ligase. EMBO J. *21*, 4037–4048.

Liu, S., Vinall, R.L., Tepper, C., Shi, X.B., Xue, L.R., Ma, A.H., Wang, L.Y., Fitzgerald, L.D., Wu, Z., Gandour-Edwards, R., et al. (2008). Inappropriate activation of androgen receptor by relaxin via beta-catenin pathway. Oncogene *27*, 499–505.

Lo, H.S., Hu, N., Gere, S., Lu, N., Su, H., Goldstein, A.M., Taylor, P.R., and Lee, M.P. (2002). Identification of somatic mutations of the RNF6 gene in human esophageal squamous cell carcinoma. Cancer Res. *62*, 4191–4193.

Long, B.J., Grigoryev, D.N., Nnane, I.P., Liu, Y., Ling, Y.Z., and Brodie, A.M. (2000). Antiandrogenic effects of novel androgen synthesis inhibitors on hormone-dependent prostate cancer. Cancer Res. *60*, 6630–6640.

Lopez, P., Vidal, F., Martin, L., Lopez-Fernandez, L.A., Rual, J.F., Rosen, B.S., Cuzin, F., and Rassoulzadegan, M. (2002). Gene control in germinal differentiation: RNF6, a transcription regulatory protein in the mouse sertoli cell. Mol. Cell. Biol. *22*, 3488–3496.

Louie, M.C., Yang, H.Q., Ma, A.H., Xu, W., Zou, J.X., Kung, H.J., and Chen, H.W. (2003). Androgen-induced recruitment of RNA polymerase II to a nuclear receptor-p160 coactivator complex. Proc. Natl. Acad. Sci. USA *100*, 2226–2230.

Macdonald, D.H., Lahiri, D., Sampath, A., Chase, A., Sohal, J., and Cross, N.C. (1999). Cloning and characterization of RNF6, a novel RING finger gene mapping to 13q12. Genomics *58*, 94–97.

Mahajan, N.P., Liu, Y., Majumder, S., Warren, M.R., Parker, C.E., Mohler, J.L., Earp, H.S., and Whang, Y.E. (2007). Activated Cdc42-associated kinase Ack1 promotes prostate cancer progression via androgen receptor tyrosine phosphorylation. Proc. Natl. Acad. Sci. USA *104*, 8438–8443.

Mal, A., and Harter, M.L. (2003). MyoD is functionally linked to the silencing of a muscle-specific regulatory gene prior to skeletal myogenesis. Proc. Natl. Acad. Sci. USA *100*, 1735–1739.

Morris, J.R., and Solomon, E. (2004). BRCA1: BARD1 induces the formation of conjugated ubiquitin structures, dependent on K6 of ubiquitin, in cells during DNA replication and repair. Hum. Mol. Genet. *13*, 807–817.

Patel, K., Whelan, P.J., Prescott, S., Brownhill, S.C., Johnston, C.F., Selby, P.J., and Burchill, S.A. (2004). The use of real-time reverse transcription-PCR for prostate-specific antigen mRNA to discriminate between blood samples from healthy volunteers and from patients with metastatic prostate cancer. Clin. Cancer Res. *10*, 7511–7519.

Pickart, C.M., and Eddins, M.J. (2004). Ubiquitin: structures, functions, mechanisms. Biochim. Biophys. Acta *1695*, 55–72.

Poukka, H., Karvonen, U., Yoshikawa, N., Tanaka, H., Palvimo, J.J., and Janne, O.A. (2000). The RING finger protein SNURF modulates nuclear trafficking of the androgen receptor. J. Cell Sci. *113*, 2991–3001.

Puthalakath, H., Villunger, A., O'Reilly, L.A., Beaumont, J.G., Coultas, L., Cheney, R.E., Huang, D.C.S., and Strasser, A. (2001). Bmf: a proapoptotic BH3-only protein regulated by interaction with the myosin V actin motor complex, activated by anoikis. Science *293*, 1829–1832.

Rhodes, D.R., Kalyana-Sundaram, S., Mahavisno, V., Varambally, R., Yu, J., Briggs, B.B., Barrette, T.R., Anstet, M.J., Kincead-Beal, C., Kulkarni, P., et al. (2007). Oncomine 3.0: genes, pathways, and networks in a collection of 18,000 cancer gene expression profiles. Neoplasia *9*, 166–180.

Rodrigo-Brenni, M.C., and Morgan, D.O. (2007). Sequential E2s drive polyubiquitin chain assembly on APC targets. Cell *130*, 127–139.

Schmelzle, T., Mailleux, A.A., Overholtzer, M., Carroll, J.S., Solimini, N.L., Lightcap, E.S., Veiby, O.P., and Brugge, J.S. (2007). Functional role and oncogene-regulated expression of the BH3-only factor Bmf in mammary epithelial anoikis and morphogenesis. Proc. Natl. Acad. Sci. USA *104*, 3787–3792.

Shang, F., Deng, G., Liu, Q., Guo, W., Haas, A.L., Crosas, B., Finley, D., and Taylor, A. (2005). Lys6-modified ubiquitin inhibits ubiquitin-dependent protein degradation. J. Biol. Chem. *280*, 20365–20374.

Shang, Y., Myers, M., and Brown, M. (2002). Formation of the androgen receptor transcription complex. Mol. Cell *9*, 601–610.

Spence, J., Sadis, S., Haas, A.L., and Finley, D. (1995). A ubiquitin mutant with specific defects in DNA repair and multiubiquitination. Mol. Cell. Biol. *15*, 1265–1273.

Tursun, B., Schluter, A., Peters, M.A., Viehweger, B., Ostendorff, H.P., Soosairajah, J., Drung, A., Bossenz, M., Johnsen, S.A., Schweizer, M., et al. (2005). The ubiquitin ligase Rnf6 regulates local LIM kinase 1 levels in axonal growth cones. Genes Dev. *19*, 2307–2319.

Ueda, T., Mawji, N.R., Bruchovsky, N., and Sadar, M.D. (2002). Ligand-independent activation of the androgen receptor by interleukin-6 and the role of steroid receptor coactivator-1 in prostate cancer cells. J. Biol. Chem. *277*, 38087–38094.

Verma, S., Ismail, A., Gao, X., Fu, G., Li, X., O'Malley, B.W., and Nawaz, Z. (2004). The ubiquitin-conjugating enzyme UBCH7 acts as a coactivator for steroid hormone receptors. Mol. Cell. Biol. *24*, 8716–8726.

Wang, Q., Li, W., Liu, X.S., Carroll, J.S., Janne, O.A., Keeton, E.K., Chinnaiyan, A.M., Pienta, K.J., and Brown, M. (2007). A hierarchical network of transcription factors governs androgen receptor-dependent prostate cancer growth. Mol. Cell *27*, 380–392.

Windheim, M., Peggie, M., and Cohen, P. (2008). Two different classes of E2 ubiquitin-conjugating enzymes are required for the mono-ubiquitination of proteins and elongation by polyubiquitin chains with a specific topology. Biochem. J. *409*, 723–729.

Zegarra-Moro, O.L., Schmidt, L.J., Huang, H., and Tindall, D.J. (2002). Disruption of androgen receptor function inhibits proliferation of androgen-refractory prostate cancer cells. Cancer Res. *62*, 1008–1013.

Zhang, Y., Wang, X.W., Jelovac, D., Nakanishi, T., Yu, M.H., Akinmade, D., Goloubeva, O., Ross, D.D., Brodie, A., and Hamburger, A.W. (2005). The ErbB3-binding protein Ebp1 suppresses androgen receptor-mediated gene transcription and tumorigenesis of prostate cancer cells. Proc. Natl. Acad. Sci. USA *102*, 9890–9895.