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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 & Experimental Therapeutics and Greenebaum Cancer Center, University of Maryland School of Medicine, Baltimore, MD 21201

[2]Department of Epidemiology & Preventive Medicine, University of Maryland School of Medicine, Baltimore, MD 21201

[3]Department of Pathology, New York University School of Medicine, New York, NY 10016

[4]Medical Biotechnology Center, University of Maryland Biotechnology Institute, Baltimore, MD 21201

[5]Laboratory of Proteomics and Analytical Technologies, SAIC-Frederick, Inc., National Cancer Institute at Frederick, Frederick, MD 21702

Summary

The androgen receptor (AR) plays a critical role in prostate cancer. We have identified an ubiquitin E3 ligase RNF6 as one of AR-associated proteins 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 human hormone-refractory 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 co-factor recruitment.

*Correspondence should be addressed to: Yun Qiu, Department of Pharmacology & Experimental Therapeutics, University of Maryland School of Medicine, 655 W Baltimore St, BRB Rm 4-002 Baltimore, MD 21201 Tel: 410-706-4535 Fax: 410-706-0032 yqiu@som.umaryland.edu.

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 patients. However, most patients inevitably develop deadly recurrent cancer which are resistant to current therapy. In this study, we showed 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 the ubiquitination machinery, such as RNF6, may potentially be effective in treatment of advanced prostate cancer.

Accession Number

The raw data of the microarray analysis were deposited in the GEO repository (submission # GSE14575).

INTRODUCTION

Androgen receptor (AR) is the key transcription factor mediating androgen-induced signaling, which is required for prostate cell survival and proliferation. It has been 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 nuclear receptor family for steroid hormones, 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 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 co-factor repertoire and posttranslational modifications of AR as well as local chromatin modifications of the target genes may play a role in 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 for a broad range of intracellular signaling, 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 ubiquitin molecule can form chains on substrates with various lengths and elongating at different lysine (K) residues within the ubiquitin molecule. All of 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 is responsible to mono-ubiquitinate 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 was studied previously (Poukka et al., 2000; Verma et al., 2004), but detailed mechanisms remain quite obscure. Recent evidence showed that the E3 ligase MDM2 can induce polyubiquitination of AR, which leads to AR degradation via 26S proteasome (Lin et al., 2002). This modification reduces the steady 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 turn-over as the ubiquitin system was reported to directly regulate the transcriptional activity of p53 independent of protein degradation (Le Cam et al., 2006). It has yet to be examined whether such mechanism may be applicable in regulation of AR activity.

In an effort to search 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). *RNF6* 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 which is responsible for its ubiquitin ligase activity. The murine homologue of human *RNF6* was demonstrated to activate *Inha* gene expression by participating in a protein complex binding to the promoter region (Lopez et al., 2002). Recent study demonstrated that a serine/threonine kinase LIM kinase 1 (LIMK1) is a substrate of RNF6 and the RNF6-induced LIMK1 polyubiquitination is mediated via K48 of ubiquitin and leads 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 AR-interacting proteins in hormone refractory prostate cancer CWR-R1 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 bind to AR but not to the GST control, RNF6 is one of the top-scored proteins identified by mass spectrometry (MS) analysis (Figure 1A). The interaction between AR and RNF6 was confirmed by reciprocal co-immunoprecipitation 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 two 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 as 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 the mutant lacking 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 MDM2C464A, suggesting that both RNF6 and MDM2 could induce AR ubiquitination in a RING-domain dependent manner. Consistent with previous studies, the steady-state level of AR in cells co-expressing wild-type MDM2 was dramatically reduced compared to that in cells expressing AR alone or co-expressing MDM2C464A 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 RNF6-induced AR ubiquitination appeared 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 only detected in the presence of the active RNF6 (Figure 2B). We then examined which lysine residue(s) in the ubiquitin molecule may be involved in assembly of the atypical polyubiquitin chains on AR induced by RNF6. AR and RNF6 were co-expressed with His-tagged ubiquitin, ubiquitin mutant without lysines (K0) or with a single lysine in COS-1 cells. It appears that when overexpressing His-Ub in cells, the transfected AR was heavily mono- or oligo-ubiquitinated 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 effects 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 assembly polyubiquitination chains at K6 and K27. This is supported by in vitro

ubiquitination assays shown in Figure 2D. Although all single-K Ub mutants were able to be conjugated in the mono- or di-Ub ARc, only Ub-K6 and Ub-K27 mutants could assembly 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 nonproteolytic effect on its target proteins (Ben-Saadon et al., 2006). This finding may help to explain our observation that RNF6-induced AR polyubiquitination did 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 R1881, suggesting that ubiquitination of AR is accompanied with its activation (Figure S3). Consistent with our earlier observation that RNF6 induces AR ubiquitination, knock-down 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 -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 the ARR2-Luc reporter as a readout. Figure 3B shows that 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 androgens or in the presence of low level of androgen (0.1 nM), AR activity was also significantly reduced in RNF6 knocked-down CWR-R1 cells ($p < 0.01$), suggesting that RNF6 may also be required for AR transcriptional activity under androgen-depleted conditions. This result is further supported by the observation that knocking-down endogenous RNF6 in CWR-R1 and C4-2B cells under the serum free conditions diminished the expression level of a well-established AR target gene product PSA, concurrent with the reduction of AR ubiquitination (Figure 3C). In addition, an increase of RNF6 protein level in the hormone-insensitive LNCaP derivative C4-2B compared to its parental line LNCaP was detected (Figure S4A). This is consistent with the report in a publicly available microarray database showing a 2-3 fold increase in RNF6 mRNA in C4-2 cells compared to LNCaP cells (Figure S4B). Overexpression of AR with RNF6 but not the RNF6ΔR, induced an approximately 2-3 fold higher activity of PSA promoter in COS-1 cells (Figure 3D). A similar enhancement on 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 the specific shRNA and microarray analysis was performed to determine altered gene profiling. As shown in Figure 3F (left panel), knock-down of RNF6 in LNCaP cells treated with 1nM DHT induced profound global changes on gene expression. The expression of some of genes was up-regulated while others down-regulated, suggesting that RNF6 may function as a general transcriptional regulator (either repressor or activator) in response to androgen, depending on the context of promoters. Many androgen-responsive genes are known to be regulated by AR (Figure 3F, right panel). Knock-down of RNF6 had dramatic effects on expression of a subset of these genes, such as *HIST1H4, TMEPAI, RLN1, KLK3, NKX3.1* and *BMF*, demonstrating that RNF6 modulates 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 such as *KLK2, PCANAP6, TMPRSS2* and *SORD*. Consistent with the microarray data, the ChIP assays shown in Figure 3G demonstrated that knock-down 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 effects 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 the reChIP assays using anti-RNF6 following the anti-AR antibody. As shown in Figure 3H, RNF6 appears to be detectable in the AR-containing protein complex assembled on the ARE sites in the regulatory regions of *PSA* (ARE III), *RLN1* and *BMF* but not present in the complex on the ARE sites of *PSA* (ARE I/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 MS/MS analysis revealed that two lysine residues, K845 and K847, were ubiquitinated in the samples derived from cells expressing AR and RNF6 (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 and ubiquitination of K845 may be a prerequisite for efficient ubiquitination of K847 and plays a more important role in regulation of AR activity. This result is supported by reporter assays demonstrating that the ARK845R mutant completely failed to be activated by RNF6 while the ARK847R 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 natural milieu, we replaced endogenous AR in LNCaP cells with the mutant AR lacking the ubiquitin acceptor site using the 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) was enhanced by overexpression of RNF6 under the serum free-condition while the ARcs-K845R mutant failed to do so. 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 knocked-down 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 the AR-replaced LNCaP cells demonstrated that K845R mutation abolished the capacity of AR binding to a subset of AR-responsive genes (Figure 4G), which is also consistent with the ChIP result 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 K845R mutation compromises the ligand binding activity since 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 co-factors, especially those

containing ubiquitin-interacting domains such as ARA54. ARA54 is a member of RING finger B-box protein family (Ito et al., 2001). It interacts with the C-terminal portion AR (a.a. 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 LBD. This possibility is supported by our observation that the association between AR and the endogenous ARA54 was completely abolished in CWR-R1 cells when either RNF6 was knocked down (Figure 5A) or 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 co-activator 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 at all 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.

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 naïve 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 those in benign (0.0278±0.0278) and hormone-naïve (0.2586±0.0502) samples ($p < 0.001$). Meanwhile, the frequency of detection of RNF6 positive nuclear staining was dramatically increased in hormone-resistant tissues (50%) compared to the benign (2.78%) and hormone-naïve (14.16%) samples. The changes of cytoplasmic staining of RNF6 followed a similar pattern as that of the nuclear staining. Figure 6B shows the 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 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 the specific shRNA in CWR-R1 and C4-2B. 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 indicated 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 was mediated through modification at K845 of AR, we substituted endogenous AR in CWR-R1 cells with either the 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. Such growth retardation was accompanied with 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 demonstrated 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 posttranslational modifications has been extensively studied. Polyubiquitination of AR induced by MDM2 has been shown to have profound impact on the stability and turnover of AR. In this report, we demonstrated that AR transcriptional activity and specificity is 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, the reason why RNF6-induced ubiquitination does not lead to AR degradation as MDM2 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 of the proteolysis-independent transactivation of AR by RNF6-induced polyubiquitination, implying that polyubiquitination as a general mechanism by which transcriptional factors are regulated. The biochemical mechanisms remain to be addressed as for how this mixed branching chain enhances AR transcriptional activity. 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 polyubiquitin chain as a recognition motif has also been reported for the K63-based polyubiquitin chain which is essential for the activation of NFk-B 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 that the ubiquitin acceptor site K845 appears to be not conserved among the steroid hormone nuclear receptor superfamily.

We 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 showing that expression of RNF6 is significantly elevated during prostate cancer progression in the publicly available Oncomine (Rhodes et al., 2007) database (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 an androgen-suppressed gene *BMF* is negatively regulated by RNF6. BMF is a BH3-domain-only protein and a proapoptotic member of BCL2 family proteins (Puthalakath et al., 2001). BMF 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 likely 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 carcinoma and cell lines (Cheung et al., 2005; Lo et al., 2002), it remains to be examined whether these mutations affect RNF6 activity. It is possible that RNF6 may have both oncogenic and tumor-suppressive effects depending on cellular context.

In addition, we showed that RNF6 can induce polyubiquitination at K845 of AR and the assembled polyubiquitin chains in AF2 domain of AR may serve as a platform for recruiting additional transcription coregulators that are required for stabilization of transcription complex and activation of a subset of AR regulated genes. One example of such coregulators is the RING-finger protein ARA54, a ligand-dependent AR coactivator. We showed that 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 miminal 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 on AR target genes. It is still unknown how ubiquitination determines the selectivity on ARE binding. Besides the specific coregulators binding to the polyubiquitin chains, other elements present in cellular microenvironment such as local chromatin context and availability/accessibility of certain cofators may likely be involved. ARA54 contains RING-like sequence domains which 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 and such ubiquitination cascade may amplify androgen-AR signaling and provide another layer of regulation on transcription magnitude and specificity. Based on the current study, our working model is that RNF6 induces an atypical polyubiquitination of AR and such modification may serve as a platform to recruit additional co-regulators containing the ubiquitin interacting domain(s) to facilitate transcription 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 is regulated. Such mechanism may also be applicable to regulation of other nuclear receptors. Given that RNF6 is overexpressed in hormone refractory prostate cancer and 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.

MATERIALS AND METHODS

DNA Plasmids 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). GST fusion protein of AR C-terminal domains (a.a. 622-919) was generously provided by Dr. Eric Xu. 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 given by Dr. Wei Gu. The shRNAs for AR and RNF6 were constructed as described previously (Guo et al., 2006). The target sequences of human RNF6 shRNAs are: shRNF6-1, 5’-GAAGCCAAACCTCAGTGAA-3’, and shRNF6-2, 5’-GAGGCCTATTATCAGTTTA-3’. The antibodies used in immunoblotting, immunoprecipitation and immunofluorescence include anti-AR (N-20), anti-actin (C-2), anti-

PSA (A67-B/E3), anti-ubiquitin (P4D1), anti-ARA54 (H-300), anti-GAPDH and anti-MDM2 (SMP14) (Santa Cruz), anti-FLAG antibody M2 (Sigma). The monoclonal antibody for RNF6 (anti-RNF6) was developed by immunizing the mice with a purified fusion protein containing N-terminal residues of RNF6 (amino acids 1-246), and hybridoma clones were isolated and maintained in RPMI 1640 medium containing 25 mM HEPES (Invitrogen). The conditional medium was used in the study.

Cell Culture and Transfection

All cell lines (293T, COS-1, human prostate cancer cell lines except for CWR-R1) used in this study were purchased from American Tissue Culture Collections (Rockville, MD). CWR-R1 was kindly provided by Drs. Christopher Gregory and Elizabeth Wilson (University of North Carolina at Chapel Hill) (Gregory et al., 2001). 293T and COS-1 cells were routinely maintained in Dulbeccos Modified Eagle Medium (DMEM), while prostate cancer cells were cultured in RPMI 1640. The mediums were supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin (P/S) unless other conditions were specified. Transfections were performed by using Fugene HD (Roche) or the calcium phosphate precipitation method (Biological Mimetics Inc.) according to the manufacturer's instruction.

In Vitro Ubiquitination Assay

In vitro ubiquitination assay was carried out as described previously with minor modifications (Fang et al., 2000; Guan et al., 2008). Briefly, 10ng of purified GST-ARc was mixed with 300ng of either ligase-active GST-RNF6 (a.a 246-685) or the ligase-inactive GST-RNF6 (a.a. 1-585) in a total volume of 30 μl containing 50 mM HEPES (pH7.9), 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 h 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 the lysine-null (K0) or a single-lysine-containing mutant (K6, K11, K27, K29, K33, K48, 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 with *lenti*-viral constructs as indicated in figure legends for 24 h. After serum starvation for 16 h, cells were pretreated as described in figure legends before being cross-linked with 1% formaldehyde and then sonication. The soluble chromatins were immunoprecipitated as de scribed previously (Louie et al., 2003; Shang et al., 2002). PSA Promoter ARE (ARE I/II), 5'-AGGGATCAGGGAGTCTCACA-3' and 5'-GCTAGCACTTGCTGTTCTGC-3'; PSA Enhancer ARE (ARE III), 5'-ACAGACCTACTCTGGAGGAAC-3' and 5'-AAGACAGCAACACCTTTTT-3'; TMPRSS2, 5'-TGGTCCTGGATGATAAAAAAAGTTT-3' and 5'-GACATACGCCCCACAACAGA-3' (Wang et al., 2007); RLN1, 5'-GAGAGTCCCAAAGGCTAGCAGAG-5' and 5'-GAACTTCCTCGGCTTCTGTTTGG-3'; BMF, 5'-GCTGGAGTGCACCACCTCAC-3' and 5'-CCAGAAGCAGCAACCACCTG-3'.

ReChIP analysis was performed as described previously (Mal and Harter, 2003). Briefly, ubiquitin antibody was added to chromatin extracts for overnight at 4°C followed by the addition of 60 μl salmon sperm/protein A agarose (Upstate Biotechnology) to recover immunocomplexes. Ubiquitinated protein complexes were eluted by 10 mM DTT at room temperature for 30 minutes, and the elution was then diluted ten times with reChIP buffer [0.5 mM DTT, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl and 20 mM Tris (pH 8.1)] and

subsequently re-immunoprecipitated by the addition of anti-FLAG or control IgG antibodies for 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). The primer sequences were: PSA, 5'-TCTGCGGCGGTGTTCTG-3' and 5'-GCCGACCCAGCAAGATCA-3' (Patel et al., 2004); TMPRSS2, 5'-GGACAGTGTGCACCTCAAAGAC-3' and 5'-TCCCACGAGGAAGGTCCC-3' (Wang et al., 2007); RLN1, 5'-CGGCCAAATGGAAGGAC-3' and 5'-GTGGCAAATTAGCAATGAATTCCA-3'; BMF, 5'-GAGGTACAGATTGCCCGAAA-3' and 5'-TTCAAAGCAAGGTTGTGCAG-3'; Actin, 5'-GCTATCCAGGCTGTGCTATC-3' and 5'-TGTCACGCACGATTTCC-3'. The relative abundance of each target transcript was quantified by using the comparative ΔΔCt 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 contain 287 cases including 18 hormone-refractory (HR) and 18 age and Gleason matched hormone-naive (HN) transurethral resection (TURP) specimens of prostate from patients with clinically advanced prostate cancer, and 36 cases of non-tumor-containing tissue from patients with benign prostatic hypertrophy. Additionally included are 215 radical prostatectomy cases representing additional hormone naïve prostate cancer cases. The determination of HN and HR was as follows: (1) patients who had earlier undergone surgical orchiectomy or medical hormone-suppressive therapy at least 6 months prior to the procedure were considered as HR; (2) 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 upon collection into the resource (under an IRB-approved protocol) had repeat pathology characterization of tissues and review of medical records. The 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 (Harvey et al., 1999). The immunoreactive score for each case was quantified by the average of four cores. The statistical analyses were carried out by using the 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 *lenti*-viruses encoding shRNAs specific for RNF6 or GFP control. After 48h, cells were maintained in fresh phenol-red-free RPMI 1640 medium with 5% charcoal-stripped FBS, and allowed the cells to grow for 7-10 days. The cells were then fixed with 1% formaldehyde, and stained with Coomassie Blues. The tumor growth in the SCID/nude mice were carried out as described previously (Craft et al., 1999). All procedures involving animals were approved by IACUC of University of Maryland. Tumor cells were allowed to grow in five to ten intact or castrated male mice for 4–10 weeks, to reach the sizes around 300 mm^3. The tumor volumes were calculated by the formula $0.5236 \times r_1^2 \times r_2$ ($r_1 < r_2$) (Long et al., 2000).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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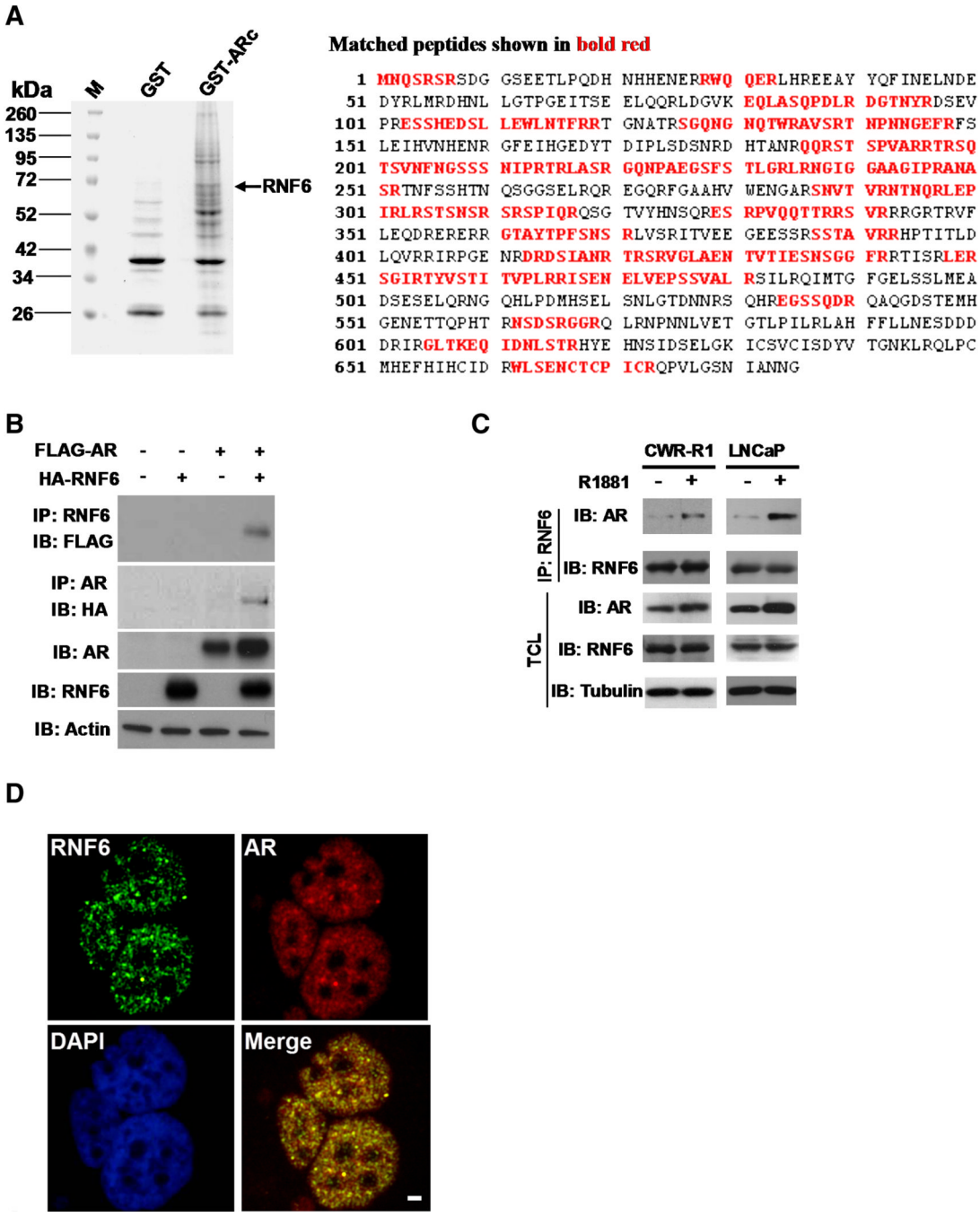

Figure 1. RNF6 interacts with AR in prostate cancer cells

(A) GST pull-down assays were performed using GST or GST-ARc as described in Methods. The bound proteins were eluted and resolved by SDS-PAGE, then visualized by Coomassie Blue staining. Discrete bands were excised and subjected to tryptic digestion and mass spectrometry (MALDI-TOF) analysis. M, marker; GST-ARc, GST-AR fusion protein containing the residues 622 to 919 of AR. Arrow indicates the band in which RNF6 was identified. Protein sequence of RNF6 is given, and the matched peptides are shown in bold red. (B) 293T cells were transfected with AR, RNF6 or both. At 48 h posttransfection, cells were lysed and immunoprecipitation was performed using anti-AR or anti-RNF6 antibody, followed by immunoblotting with indicated antibodies. (C) CWR-R1 and LNCaP cells were

subjected to immunoprecipitation using control IgG or anti-RNF6. Immunoblotting was followed using either precipitated samples or total cell lysates (TCL) with indicated antibodies. Arrows indicates the presence of IgG. (D) Immunofluorescence confocal microscopy was carried out by costaining CWR-R1 cells with anti-AR (red) and anti-RNF6 (green) antibodies. The nucleus was counterstained with DAPI. Bar, 1μm.

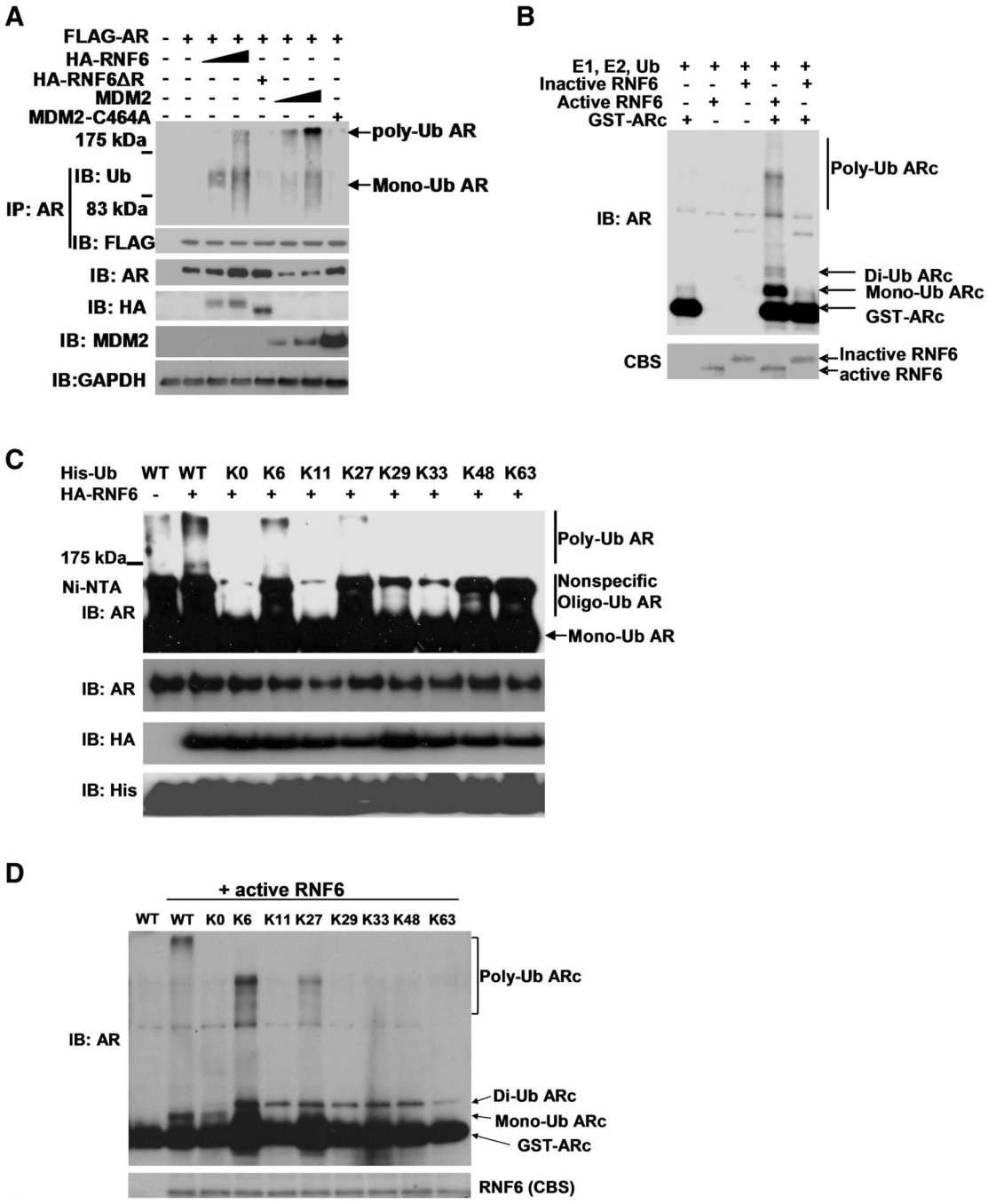

Figure 2. RNF6 induces atypical polyubiquitination of AR

(A) COS-1 cells were transfected with the plasmids as indicated. At 36 h, cells were lysed and subjected to immunoprecipitation using anti-AR under denaturing conditions, followed by immunoblotting with indicated antibodies. (B) The *in vitro* ubiquitination assays were carried out as described in Materials and Methods. 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 the His×6-tagged wild-type (WT) ubiquitin, lysine-null (K0) or a single-lysine-containing mutant as indicated, along with FLAG-AR and HA-RNF6. At 36 h posttransfection, cells were lysed, and lysates were incubated with Ni-NTA beads rotating at 4°C for overnight. Immunoblotting was followed

using indicated antibodies. (D) The *in vitro* ubiquitination assays was carried out as 2B, except that ubiquitin was replaced with the lysine-null (K0) or a single-lysine-containing mutant as indicated.

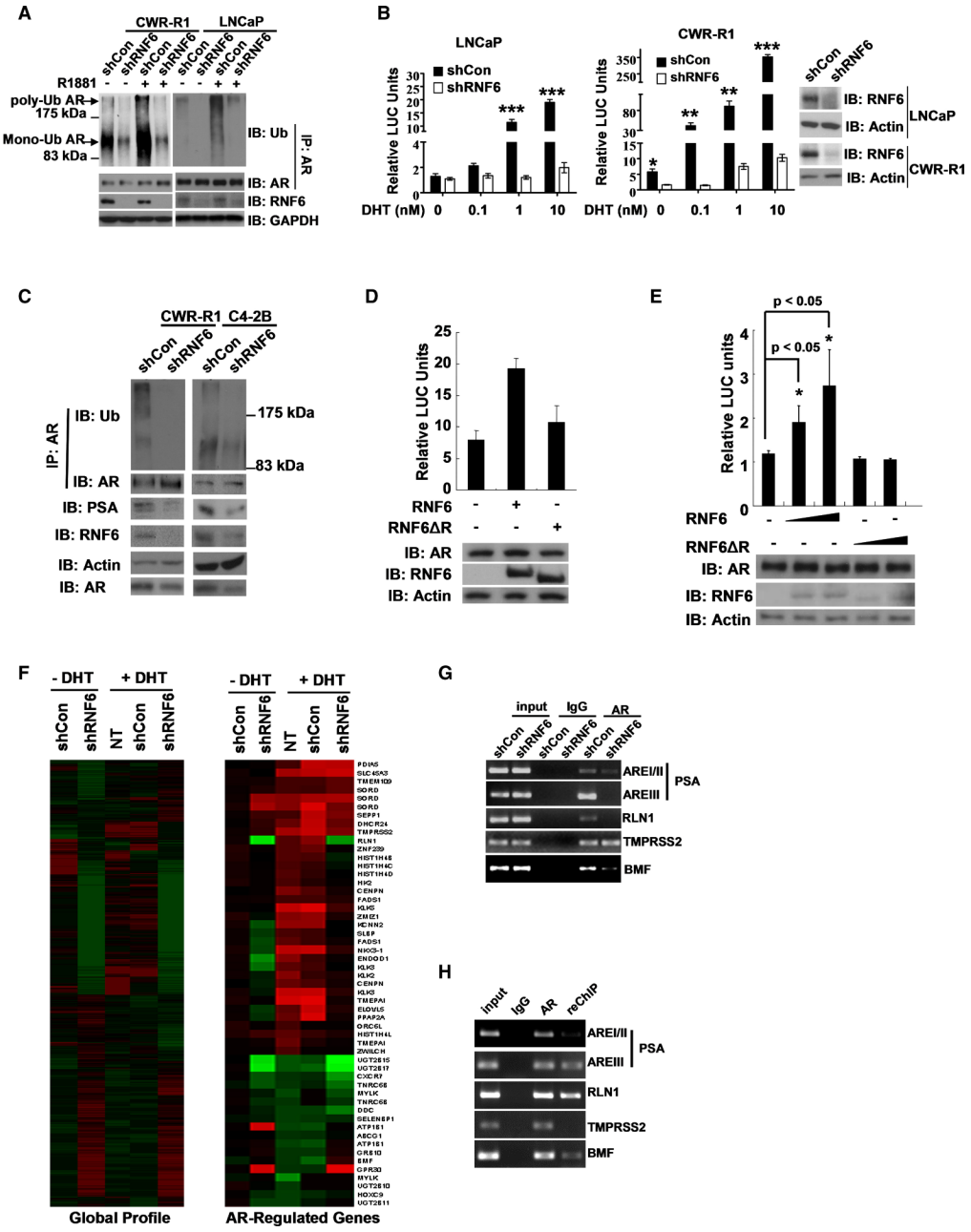

Figure 3. RNF6 modulates AR transcriptional activity and specificity

(A) CWR-R1 and LNCaP cells were infected with *lenti*-viruses encoding shRNAs for control vector (shCon) or RNF6 (shRNF6). After serum starvation, cells were pretreated with 10 nM R1881 for 16 h. Then cells were lysed and subjected to immunoprecipitation with anti-AR under denaturing conditions, followed by immunoblotting with indicated antibodies. (B) CWR-R1 and LNCaP cells were transfected with ARR2-LUC reporter and infected with *lenti*-viruses encoding shRNAs for control (shCon, solid bars) or RNF6 (shRNF6, open bars). After serum-starvation, cells were pretreated with indicated doses of DHT for 16 h before the luciferase activity was measured. The results were presented as the mean relative light units (RLU)±SD of the triplicate samples. * $p < 0.01$, ** $p < 0.005$, *** $p < 0.001$. Insert, expression of RNF6 in LNCaP and CWR-R1 cells, respectively. (C) CWR-R1 and C4-2B cells were infected with *lenti*-viruses encoding shRNAs for control vector (shCon) or RNF6 (shRNF6).

After serum-starvation for 16 h, cells were lysed and subjected to immunoprecipitation with anti-AR under denaturing conditions, followed by Western blots 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 h before the luciferase activity was measured. The results were presented as the mean relative light units (RLU)±SD of triplicate samples. * $p < 0.001$. (E) LNCaP cells were transfected with ARR2-LUC reporter, together with increasing doses of wild-type RNF6 (RNF6) or RING-deleted mutant (RNF6ΔR). Cells were serum-starved for 16 h before luciferase activity was measured. The results were presented as the mean relative light units (RLU)±SD of the triplicate samples. (F) LNCaP cells were infected with or without *lenti*-viruses encoding shRNAs for either control (shCon) or RNF6 (shRNF6). At 16 h post-infection, cells were incubated in phenol-red-free media with 5% charcoal-stripped FBS and pretreated with (+DHT) or without (−DHT) 1 nM DHT for 16 h. RNAs were then extracted and microarray assay was performed as described in Supplementary Materials and Methods. (G) LNCaP cells were infected with *lenti*-viruses encoding shRNAs for control vector (shCon) or RNF6 (shRNF6). After serum-starvation for 16 h, LNCaP cells were treated with 10 nM DHT for 1 h before ChIP assay was carried out to examine the binding of AR to the AREs located at the regulatory region of *PSA, RLN1, BMF* or *TMPRSS2.* PCR products amplified from the input, immunoprecipitation with control antibody (IgG), and with anti-AR (AR) were resolved on agarose gels. (H) LNCaP cells were serum-starved for 16 h before reChIP assay was performed using anti-AR followed by anti-RNF6. The recruitment of the protein complex to promoter (ARE I/II) or enhancer (ARE III) regions of *PSA*, *RLN1*, *BMF* or *TMPRSS2* gene was tested. PCR products from input, immunoprecipitation with control antibody (IgG), first round ChIP with anti-AR (AR), and with experimental antibodies (reChIP) was resolved on agarose gels.

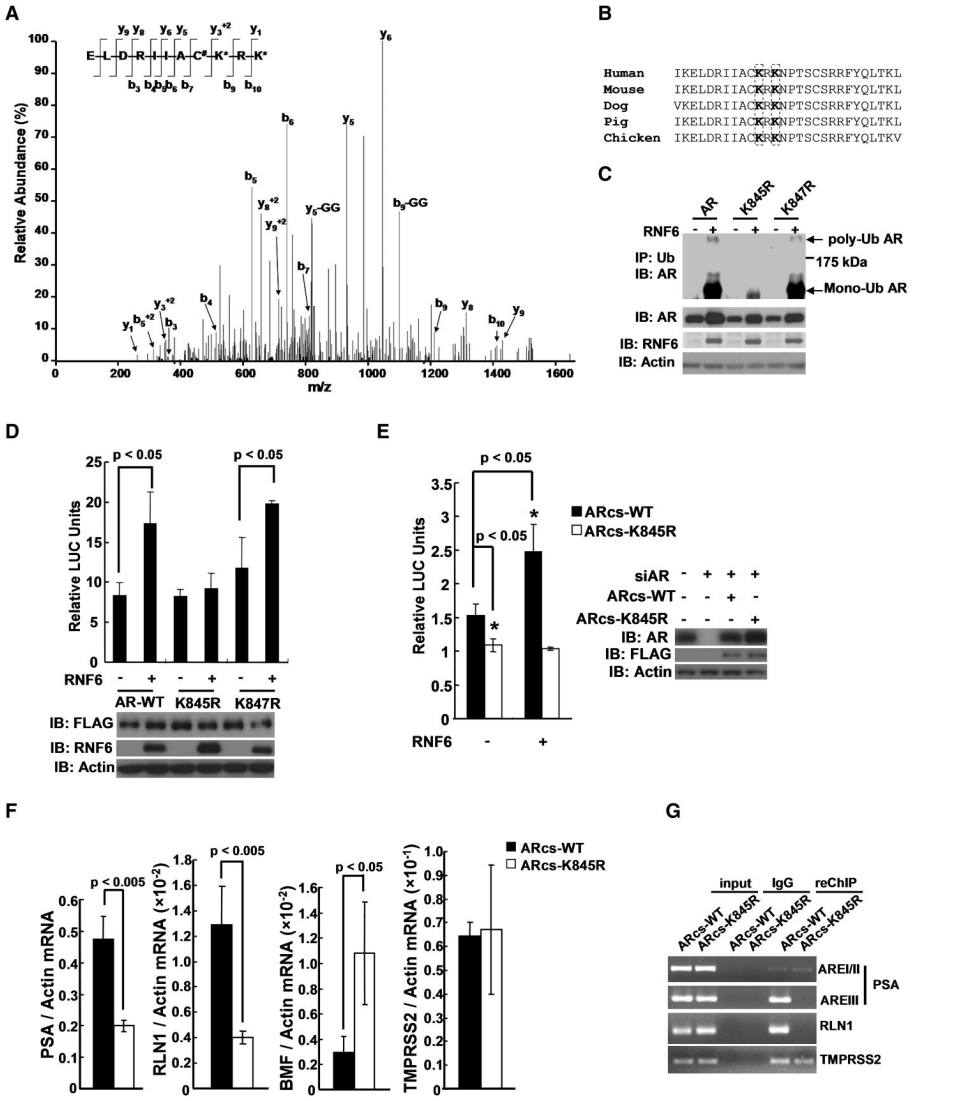

Figure 4. RNF6 induced polyubiquitination of AR at K845 and K847

(A) Tandem mass spectrometry (MS/MS) spectrum of AR peptide showing ubiquitination at K845 and K847. K* inducates the lysine residues that are ubiquitinated. (B) Alignment of AR protein sequences. K845 & K847 (boxed) are evolutionarily conserved through different species. (C) COS-1 cells were transfected with wild-type AR (AR) or AR mutants (AR-K845R, AR-K847R), together with RNF6. At 36 h posttransfection, cells were lysed and subjected to immunoprecipitation using anti-AR under denaturing conditions. Immunoblotting was followed using indicated antibodies.

(D) COS-1 cells were transfected with ARR2-LUC reporter and indicated plasmids. After serum-starvation for 16 h, luciferase activity was measured. The results were presented as the mean relative light units (RLU)±SD of the triplicate samples. Insert, expression of wild-type AR and AR mutants. (E) Following AR replacement, LNCaP cells were transfected with ARR2-LUC reporter, and serum-starved for 16 h before luciferase activity was measured. The results were presented as the mean relative light units (RLU)±SD of the triplicate samples. (F) Following AR replacement strategy, LNCaP cells were serum-starved and then treated with 1nM DHT for 16 h. Total RNA was extracted and subjected to quantitative real-time PCR. The results were presented as the mean values±SD of three independent experiments. (G) After AR

replacement, LNCaP cells were serum-starved for 16 h and then pretreated with 1nM DHT for 1 h before re-ChIP assay was performed using anti-Ub followed with anti-Flag. Binding of AR to promoter (ARE I/II) or enhancer (ARE III) regions of *PSA*, *RLN1* or *TMPRSS2* gene was tested. PCR products from input, immunoprecipitation with control antibody (IgG), and with experimental antibodies (reChIP) were resolved on agarose gels.

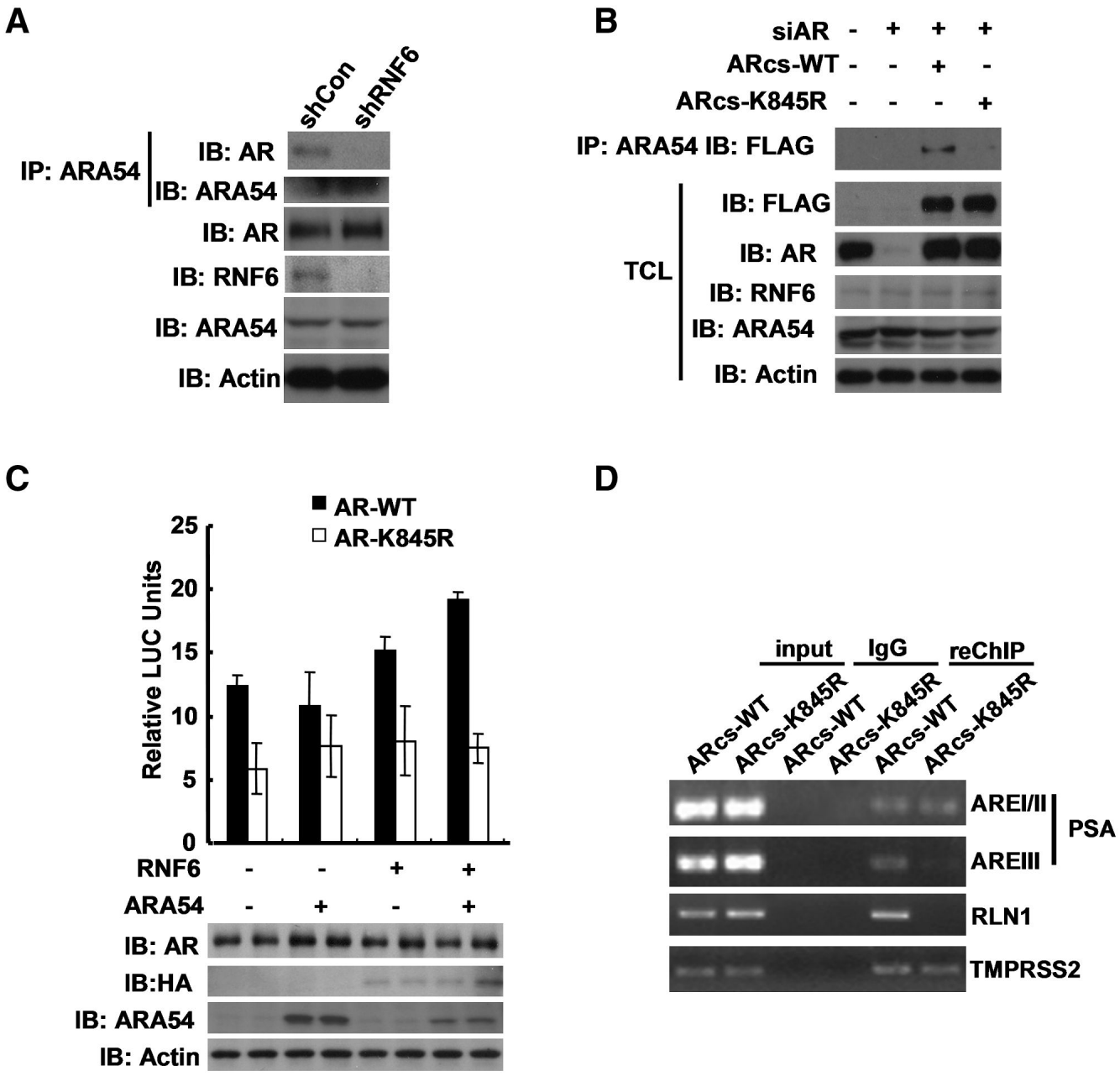

Figure 5. RNF6-induced polyubiquitination of AR facilitates its binding to coactivators on a subset of AREs

(A) CWR-R1 cells were infected with *lenti*-viruses encoding shRNAs specific for RNF6 (shRNF6) or the control vector (shCon). Immunoprecipitation was performed using anti-ARA54, followed by immunoblotting with indicated antibodies. (B) Following AR replacement experiment, immunoprecipitation was carried out in CWR-R1 cells using anti-ARA54 antibody, followed by immunoblotting with indicated antibodies. TCL, total cell lysates. (C) COS-1 cells were transfected with ARR2-Luc reporter as well as the indicated plasmids. Cells were serum-starved for 16 h before luciferase activity was measured. The results were presented as the mean relative light units (RLU)±SD of triplicate samples. (D) After AR replacement, LNCaP cells were serum-starved for 16 h before ChIP assay was performed using anti-ARA54. Binding of AR to the promoter (ARE I/II) or enhancer (ARE

III) regions of *PSA*, *RLN1* or *TMPRSS2* gene was tested. PCR products from input, immunoprecipitation with control antibody (IgG), and with experimental antibodies (AR) were resolved on agarose gels.

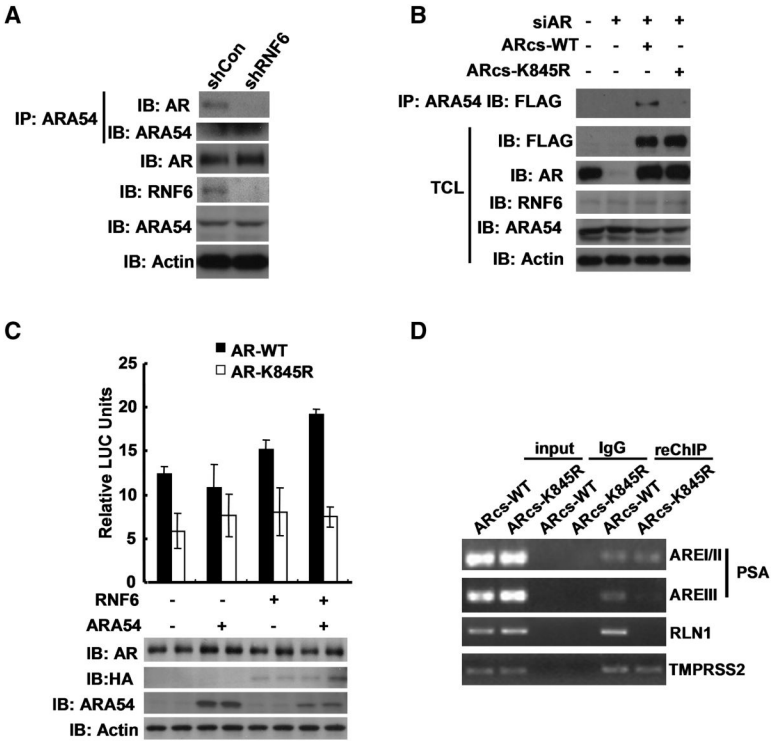

Figure 6. Expression of RNF6 is associated with progression of prostate cancer

(A) Statistical analysis of human prostate tissue arrays stained with anti-RNF6. Immunostaining scores (mean±SD) for RNF6 in benign epithelium, hormone-naïve (HN), and hormone-refractory (HR) prostate tissues were summarized. The first numbers in brackets represent the sample sizes; the second ones represent the percentage of positive staining cases (+%). The statistical differences were determined by Wilcoxon rank sum test. (B) Representative fields of human prostate tissue arrays. Immunohistochemical staining was performed using anti-RNF6 antibody.

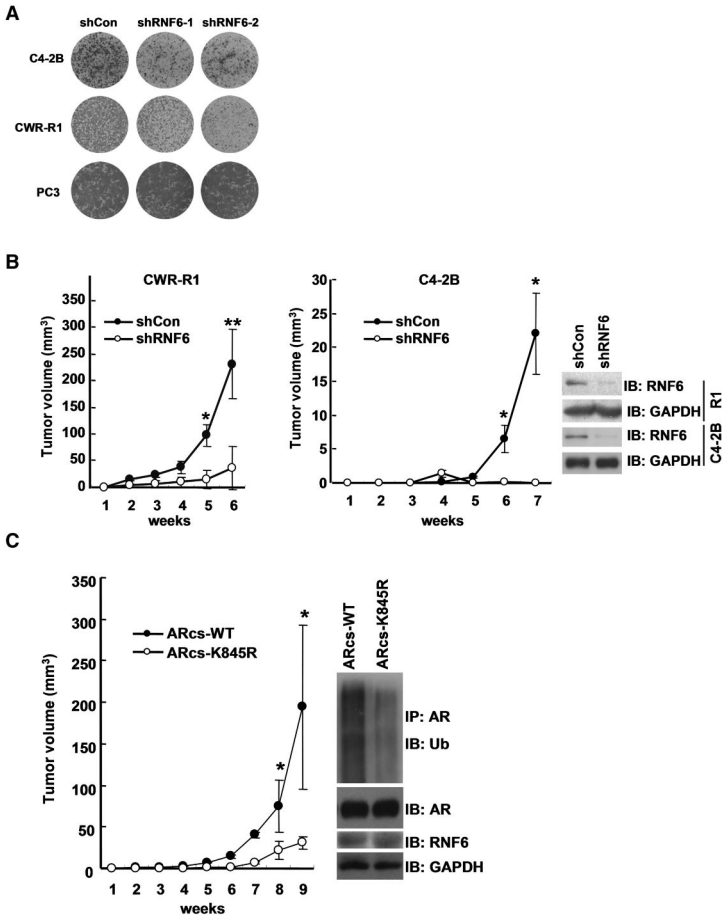

Figure 7. RNF6 is required for prostate cancer cell growth under androgen-depleted conditions

(A) Prostate cancer cells were infected with *lenti*-viruses encoding shRNAs specific for RNF6 (shRNF6) or the control vector (shCon). At 16 h post-infection, cells were incubated in fresh phenol-red-free media with 5% charcoal-stripped FBS, and maintained for 7-10 days. The cell colonies were visualized by Coomassie blue staining. (B) CWR-R1 (left panel) and C4-2B (right panel) were infected with *lenti*-viruses encoding shRNAs specific for RNF6 (shRNF6) or the control vector (shCon). At 48 h post-infection, cells were injected into left (shCon) and right (shRNF6) flanks of the castrated male nude mice. Growth of tumors was examined as described in Methods. The results represent the mean tumor sizes±SD (n= 5 mice/group). * $p < 0.05$, ** $p < 0.001$. Insert, expression of RNF6 in CWR-R1 and C4-2B cells, respectively. (C) After AR replacement, CWR-R1 cells expressing ARcs-WT (solid) or ARcs-K845R (open) were injected into castrated male nude mice. Tumor sizes were measured as described. The result represents the mean tumor volume±SD (n=5 mice/group). Insert, immunoprecipitation with anti-AR antibody using xenograft mouse tissues, followed by immunoblotting with antibodies as indicated. * $p < 0.05$.

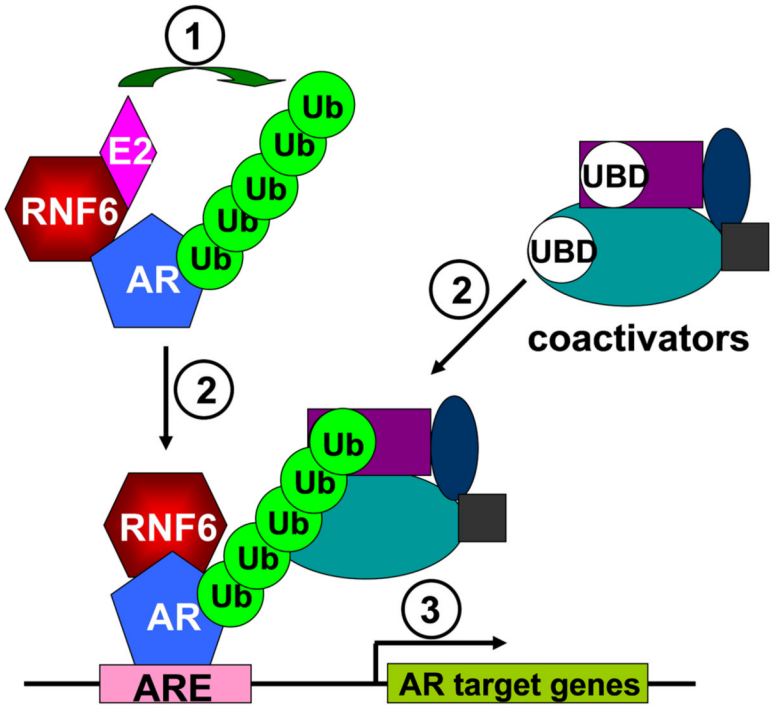

Figure 8. A postulated model of regulation of AR activity by RNF6

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 (UBD), and stabilizes the transcription complex at a subset of ARE sites in a polyubiquitin dependent manner; Step-3, Modulation of transcription of AR targeted gene by the polyubiquitination dependent complex.