

## The Deubiquitinating Enzyme USP26 Is a Regulator of Androgen Receptor Signaling

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### Abstract

The androgen receptor (AR) is a member of the nuclear receptor superfamily and is essential for male sexual development and maturation, as well as prostate cancer development. Regulation of AR signaling activity depends on several posttranslational modifications, one of these being ubiquitination. We screened a short hairpin library targeting members of the deubiquitination enzyme family and identified the X-linked deubiquitination enzyme USP26 as a novel regulator of AR signaling. USP26 is a nuclear protein that binds to AR via three important nuclear receptor interaction motifs, and modulates AR ubiquitination, consequently influencing AR activity and stability. Our data suggest that USP26 assembles with AR and other cofactors in subnuclear foci, and serves to counteract hormone-induced AR ubiquitination, thereby contributing to the regulation of AR transcriptional activity. *Mol Cancer Res*; 8(6); 844–54. ©2010 AACR.

### Introduction

The androgen receptor (AR) is a member of the steroid receptor subfamily of nuclear receptors (NR). These are characterized by their homodimerization and nuclear translocation upon ligand binding, followed by binding to hormone response elements in the DNA. Subsequently, multicomponent transcriptional complexes, which consist of general and NR-specific transcriptional repressors and activators, as well as chromatin remodelers, are assembled in a highly organized manner. The final consequence is a fine-tuned transcriptional regulation of NR target genes (1–3).

The AR signaling pathway is essential for male sexual differentiation and maturation, maintenance of spermatogenesis, prostate development, and normal prostate function. Deregulation of this signaling pathway is highly associated with prostate cancer development and progression. This type of cancer is characterized by the transition from a hormone-dependent state, which is treatable, to a hormone-refractory state, at which point, the disease is incurable (4, 5).

Two main routes have been suggested by which the hormone-refractory state may arise. First, AR itself might develop mutations that provide it with increased affinity for its testosterone ligands, broader substrate specificity, altered cofactor interactions, or increased stability and expression.

Second, the general AR signaling axis might be tilted by changes in cofactor levels or activities. Any of these events are proposed to lead to AR-independent signaling and aberrant prostate cell growth (6, 7).

Posttranslational modifications such as phosphorylation, acetylation, methylation, and ubiquitination influence signaling at all levels of the AR pathway. Although K48-linked ubiquitination of proteins leads to proteasomal degradation, other types of ubiquitination have also proven to regulate important cellular events such as DNA damage sensing and repair, receptor internalization, vesicular transport, and signal transduction (8–11). Monoubiquitination, multiple monoubiquitination, and polyubiquitination modifications function as molecular switches that are specifically regulated by ubiquitin ligases and deubiquitinating enzymes (DUB), analogous to the regulation of protein phosphorylation mediated by kinases and phosphatases (12). Several hundred ubiquitin ligases have been identified, and these largely determine substrate specificity in the three-step ubiquitin conjugation process (13, 14). At present, the DUB family consists of approximately 100 members, and although the function of the majority of these is at present unknown, it is believed that DUBs are quite substrate-specific and therefore may be important regulators within distinct pathways (15, 16).

A number of ubiquitin ligases that influence AR signaling have been identified. In the cytoplasm, C-terminal Hsp-interacting protein (CHIP) forms a complex with Hsp70/Hsp90 and AR, and influences the stability of cytoplasmic AR (17, 18). Likewise, several ubiquitin ligases interact physically with nuclear AR. One of these, TRIM68 is specifically upregulated in prostate cancer, and its ubiquitination activity influences AR signaling (19). Other examples include PIRH2 and E6-AP, both of which bind to AR, while ubiquitinating HDAC1 and the steroid receptor coactivator Src-3, respectively

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doi: 10.1158/1541-7786.MCR-09-0424

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(20, 21). Interestingly, upon phosphorylation by GSK3, Src-3 is also subject to SCF<sup>Fbw7 $\alpha$</sup> -mediated multiple monoubiquitination followed by polyubiquitination that targets Src-3 for proteasomal degradation. This sequence of events serves to specifically regulate the transcriptional activity of Src-3 in estrogen receptor and AR signaling (22).

The activity of the AR itself seems to be regulated in a similar way. AR is ubiquitinated by Mdm2 subsequent to Akt phosphorylation (23), and more recently, chromatin immunoprecipitation assays have shown that Mdm2 is bound to androgen-responsive elements (ARE) in a complex with, among others, AR and HDAC1. Mdm2 mediates the ubiquitination of both HDAC1 and AR, the latter of which is first subjected to multiple monoubiquitination within hours of hormone stimulation, triggering transcriptional activation, and after a further delay, polyubiquitination and proteasomal degradation (24). In addition, it has been proposed that TSG101 may stabilize monoubiquitinated AR by binding to ubiquitin moieties and preventing polyubiquitination, thus further fine-tuning pathway regulation (25).

Recently, an additional layer of regulation has been added with the discovery of RNF6, a ubiquitin ligase that promotes the conjugation of K6/K27 mixed ubiquitin chains on AR. These do not target the protein for degradation but might serve as scaffolds recruiting transcriptional cofactors that regulate a subset of AR target genes specifically involved in progression to the hormone-refractory state (26).

The obvious importance of ubiquitination, and the number of ubiquitin ligases identified within AR signaling, prompted us to ask the question of whether DUB enzymes exist, which contribute to AR pathway regulation. Two previous reports have suggested the involvement of the DUBs USP10 and USP2a in AR signaling and prostate cancer development, respectively (27, 28). We used a plasmid-based short hairpin library, constructed in our lab, targeting a subset of all human DUBs to perform a loss-of-function screen in AR signaling (29–31). We present evidence that the X-linked DUB USP26 is a regulator of AR signaling, binding to AR in the nucleus after hormone stimulation, and reversing ubiquitination of AR, and thus regulating target gene activation.

## Materials and Methods

### Materials, antibodies, and plasmids

The generation of the plasmid-based DUB knockdown library has been described previously (30, 31). The target sequences of the three active USP26 hairpins are (A) GATATCCTGGCTCCACACA, (C) TCCCATCGTTTGC-TGATGA, and (E) CCTCCTAAGACCTACAAAA. These were cloned into pSUPER as described in ref. (32). Mouse p19<sup>ARF</sup>, with the target sequence GTTCGTGCG-ATCCCGGAGA, was used as a negative control as this sequence does not target any human open reading frame.

The Luciferase plasmid pGL3-ARE-E<sub>1</sub>B-Luc and pSV-hAR were gifts from Henk van der Poel (Division of Surgical Oncology, Netherlands Cancer Institute, Amsterdam, the Netherlands). CMV-Renilla was purchased from Promega. pVlag-AR and pEGFP-AR were generated by PCR amplification of pSV-AR. The pEGFP-AR<sup>F582,583A</sup> mutant was constructed by PCR with mutagenesis primers. pEGFP-USP26 and pVlag-USP26 were cloned by PCR amplification of IMAGE clone 7216887. Mutagenesis PCR primers were used to construct the C/S mutant, the panel of NR box mutants, and the patient mutants. GFP-USP1 WT and C/S control plasmids are described in ref. (30).

Antibodies used were anti-GFP (FL) and (B-2), anti-cyclin D1 (M-20) from Santa Cruz Biotechnology, anti-flag (M2) from Sigma, and anti-HA (12CA5) from a hybridoma culture supernatant grown in our lab. Normal mouse IgG from Santa Cruz Biotechnology was used as a nonimmune control in immunoprecipitation experiments.

### Cell cultures, transient transfections, and reporter assays

HepG2 and HEK293 cells were cultured in DMEM, supplemented with 10% FCS. LnCap cells were cultured in RPMI, supplemented with 10% FCS. All cell lines were acquired from the American Type Culture Collection. Transfections of HepG2 and HEK293 were done with the calcium phosphate method. For luciferase reporter assays, 0.6  $\mu$ g of pGL3-ARE-E<sub>1</sub>B-Luc, 10 ng of CMV-Renilla in HepG2s, or 1 ng of SV40-renilla in HEK293s, 0.5  $\mu$ g of pSV-hAR, and 2.25  $\mu$ g of pSUPER DUB plasmid were used per six-wells. Forty-eight hours after transfection, cells were stimulated with 10 nmol/L of dihydrotestosterone and luciferase activity was measured 72 hours after transfection.

### Small inhibiting RNA transfection and quantitative reverse transcription-PCR

LnCap cells were transfected with control and USP26 small inhibiting RNAs (siRNA; Dharmacon) according to the protocols of the manufacturer (siControl: siGENOME Non-Targeting siRNA Pool no. 1 D-001206-13-05, siUSP26 1, CCACAAAGCUGGAGGAAAA; siUSP26 2, GAAGAUACCUCACUUUGUC; siUSP26 3, GCAC-AAGACUCCGUUGGA; siUSP26 4, CCACACA-UUGGAUCAGAU). Total RNA was isolated using TRIzol (Invitrogen). cDNA was generated using Superscript II (Invitrogen) and random primers (Invitrogen). cDNA was diluted and quantitative reverse transcription (qRT) reactions were done using various TaqMan probes (USP26, hs01058061\_s1; TMPRSS2, hs01122334\_m1; Applied Biosystems) or specific oligo DNA primers (PSA, ATATCGTAGAGCGGGTGTGG/CCAGTGGGTCCTCACAGC; NKX3.1, CTCGATCACCTGAGTGTGGG/ACTTGGGGTCTTATCTGTTGGA) and FAST SYBR green (Invitrogen). All qRT reactions were run in parallel with glyceraldehyde-3-phosphate dehydrogenase probes to control for input of cDNA. Due to

the low expression of USP26, verification of knockdown was repeated in cells expressing exogenous USP26. The results shown are representative of three independent experiments.

### Immunoblotting and immunoprecipitation

Western blots were done using whole cell extracts, separated on 8% to 10% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes (Millipore). Western blots were probed with the indicated antibodies. For coimmunoprecipitation experiments, HEK293 cells were transfected by calcium phosphate precipitation with the indicated plasmids. Seventy-two hours after transfection, cells were lysed in ELB buffer (31), supplemented with “complete” protease inhibitors (Roche), and proteins were immunoprecipitated with 2  $\mu$ g of the indicated antibody conjugated to protein G sepharose beads. To detect Flag-AR ubiquitination, immunoprecipitations were done in the more denaturing radioimmunoprecipitation assay buffer, with vortexing during cellular lysis (31), after overnight incubation with 10  $\mu$ g/mL of proteasome inhibitor Z-leu-leu-leu-al (Cbz-LLL; Sigma), and 2 hours of treatment with 10 nmol/L of dihydrotestosterone.

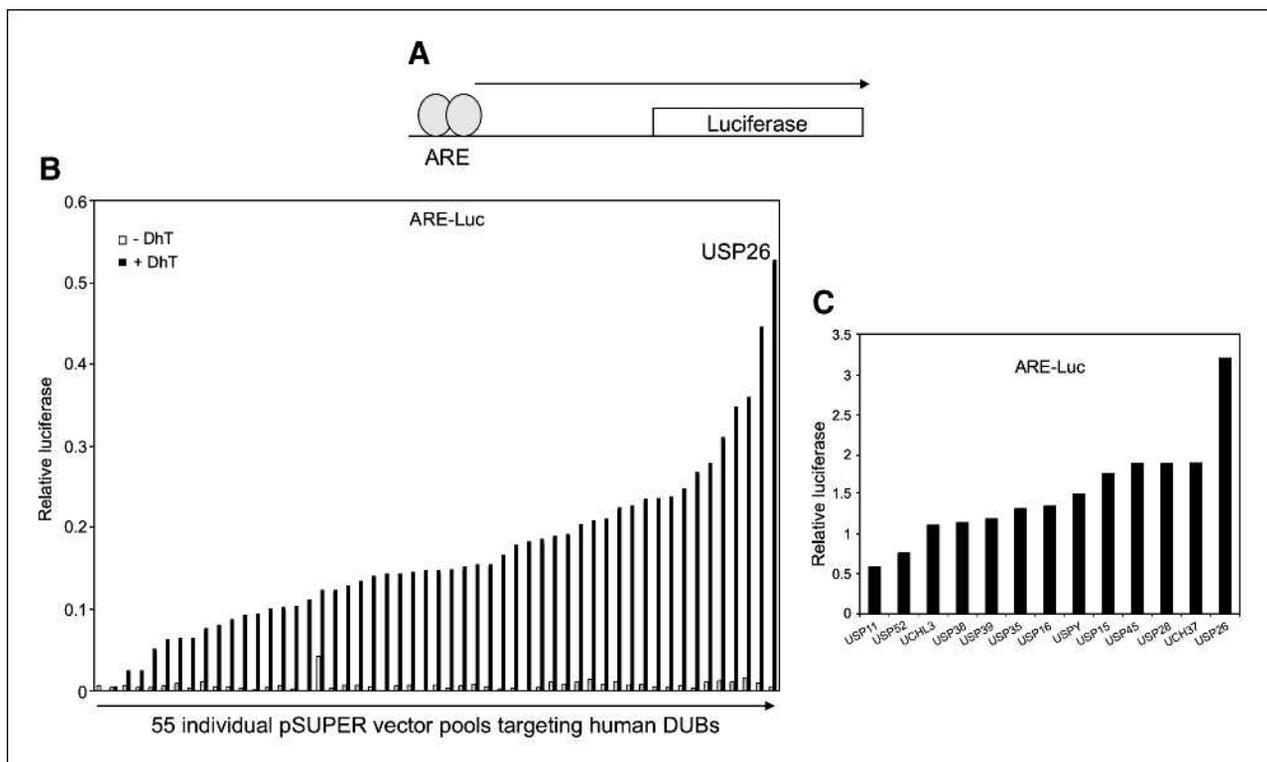
### Immunofluorescence microscopy

Immunofluorescence microscopy was carried out essentially as in ref. (33). Twenty-four hours after transfection, cells were plated on eight-well chamber slides (Nutacon), and after 48 hours, they were subjected to overnight treatment with 10 nmol/L of dihydrotestosterone, after which they were fixed with 4% formaldehyde in PBS for 15 minutes, permeabilized with 0.2% Triton X (Sigma), blocked and incubated with anti-flag rabbit (M2) antibody from Sigma followed by detection with Texas red-conjugated anti-rabbit secondary antibody (Vector Laboratories).

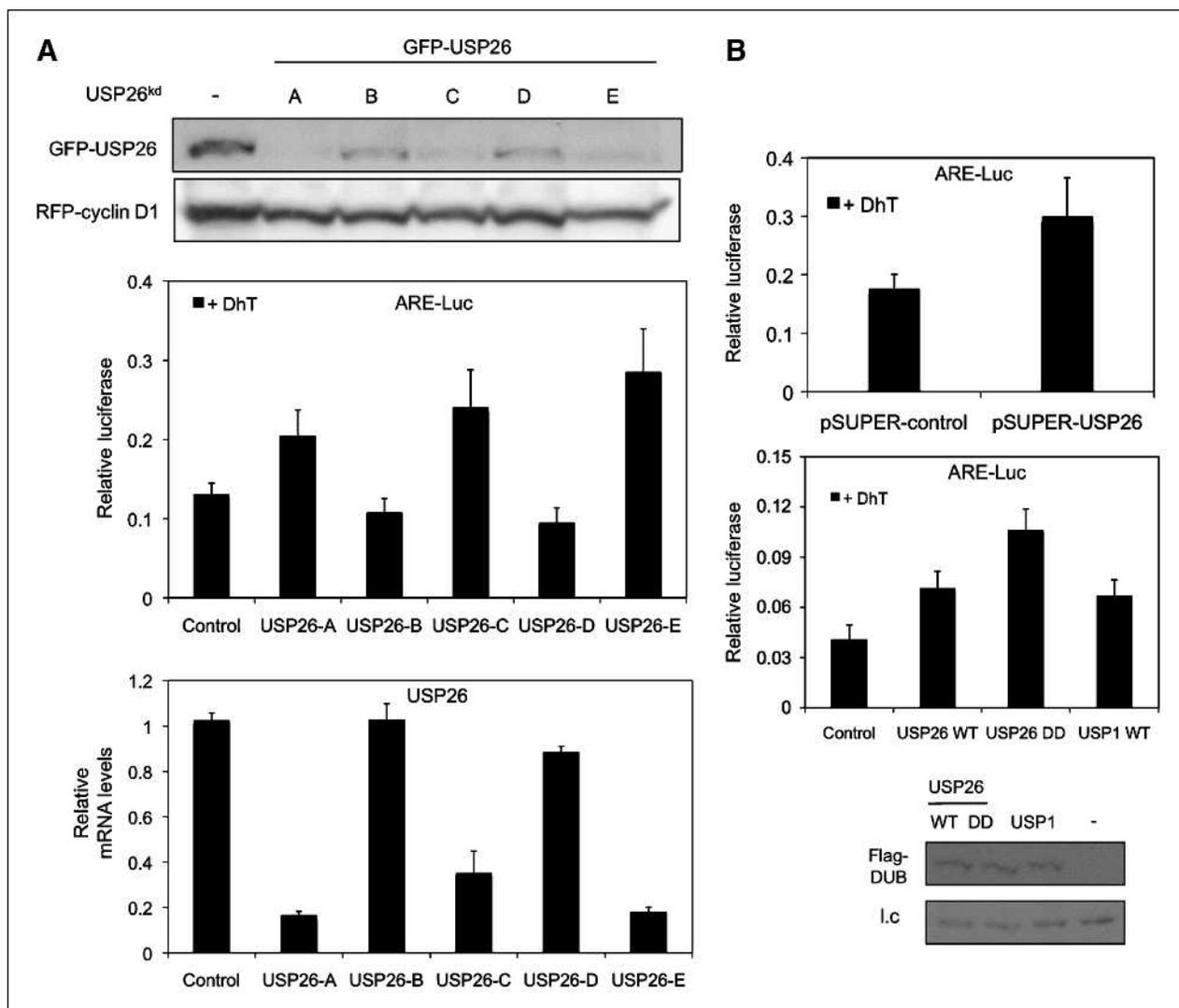
## Results

### Identification of USP26 as a regulator of AR signaling

To identify DUBs involved in AR signaling, we used an androgen-responsive firefly luciferase reporter (Fig. 1A). This vector was transiently transfected with a Renilla control vector, a cDNA expressing the human AR, and the 55 individual pools of an RNA interference library targeting members of the DUB family, which has previously been made in our lab (30, 31). We used the hepatocellular carcinoma cell line HepG2, which is androgen-responsive upon overexpression of AR. Forty-eight hours after transfection, cells



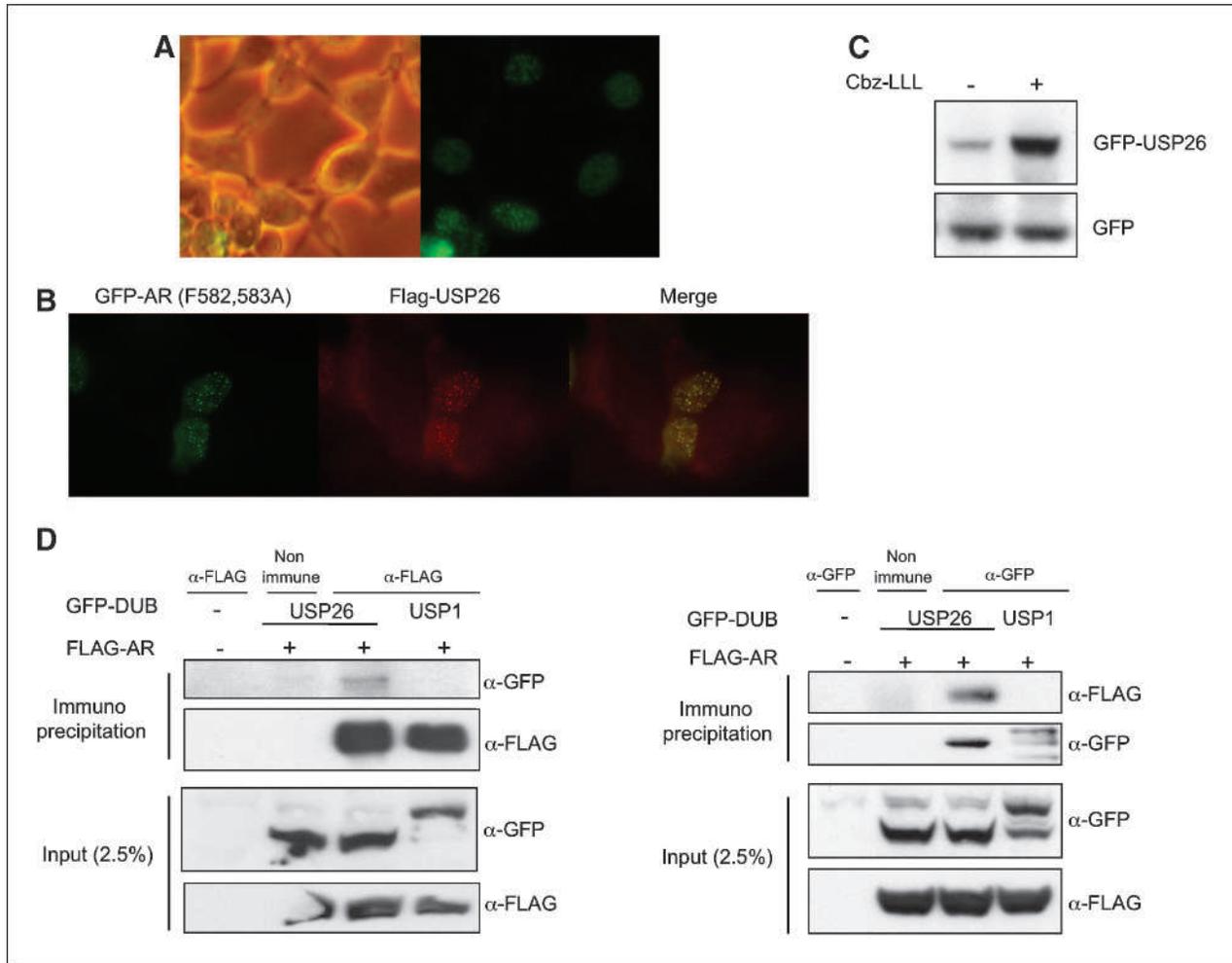
**FIGURE 1.** A DUB knockdown screen identifies USP26 as a regulator of AR signaling. A, schematic representation of the AR-responsive reporter gene construct used in the genetic screen. B, HepG2 cells transfected with an ARE-Luc reporter, CMV-Renilla, pSV-hAR, and individual pools of the pSUPER-DUB library. Cells were stimulated with dihydrotestosterone (DhT) and luciferase activity was measured 72 h after transfection for all individual pools of the library (white and black columns). C, rescreening of initial outliers establishes that knockdown of the X-linked DUB USP26 results in hyperactivation of the ARE-Luc reporter.



**FIGURE 2.** A, assessment of five individual hairpins targeting USP26. Top, HEK293 cells cotransfected with GFP-USP26, pSUPER-USP26 hairpin vectors, and RFP-cyclin D1 as a transfection control, followed by Western blots against GFP and cyclin D1 48 h posttransfection. Middle, luciferase assays in HepG2 cells assessing functionality of individual USP26 hairpin vectors cotransfected with the ARE-Luc reporter, CMV-Renilla, and pSV-hAR. Bottom, HEK293 cells transfected with pSUPER-USP26 hairpin vectors. cDNA was synthesized from TRIzol-extracted RNA, and USP26 expression was measured by qRT. The pSUPER-USP26 vectors A, C, and E inhibit the expression of GFP-USP26 protein and USP26 mRNA, and superactivate the ARE-Luc reporter. B, ARE-Luc assays in HepG2 cells. Bottom, ARE-Luc reporter, CMV-Renilla, and pSV-hAR were cotransfected with empty vector, flag-tagged USP26 wild-type (WT), USP26 DUB-dead (DD), or USP1 WT. Inset, similar expression of all three DUBs. In the absence of dihydrotestosterone, luciferase values were below the detection levels and were therefore omitted from the figure. Overexpression of the DUB-dead mutant hyperactivates the ARE-Luc reporter to a similar extent as USP26 knockdown (top).

were stimulated with dihydrotestosterone for 24 hours, whereupon relative luciferase activity was measured (Fig. 1B). We discarded a number of pools that conferred general toxicity, resulting in extremely low renilla luciferase values, and focused on pools that hyperactivate the ARE reporter. USP10, which has previously been reported as a positive regulator of AR signaling, was not an outlier in our screen (27). Possibly due to the lack of expression in HepG2 cells, or nonfunctionality of the short hairpin RNA pool targeting USP10. After performing a secondary screen retesting the top outliers, we found one pool that reproducibly superac-

tivates the ARE reporter (Fig. 1C). This pool targets USP26, an X-linked DUB. Interestingly, mutations in the *USP26* gene have been linked to impaired spermatogenesis and testicular development (34, 35). We identified several hairpins capable of inhibiting the expression of a GFP-fusion of USP26, and depleting USP26 mRNA, and found that these hairpins also affect AR signaling (Fig. 2A). To assess whether the phenotype observed by USP26 inhibition was due to DUB activity, we generated a catalytically inactive USP26 mutant in which the active site cysteine has been substituted with a serine residue, USP26<sup>C/S</sup> (36). Overexpression of this



**FIGURE 3.** USP26 colocalizes with, and binds to, AR in the nucleus. A, HEK293 cells were transfected with GFP-USP26. Twenty-four hours later, phase contrast and fluorescence (GFP) photos were taken. B, HEK293 cells were cotransfected with GFP-AR<sup>F582,583A</sup> and Flag-USP26, stimulated with dihydrotestosterone, and subjected to immunofluorescence and confocal microscopy. Both proteins colocalize to subnuclear foci. C, HEK293 cells were transfected with GFP-USP26 and GFP as a transfection control. Twenty-four hours later, cells were treated with Cbz-LLL. Whole cell lysates were immunoblotted with GFP antibody. D, HEK293 cells were transfected as indicated and 48 h later were treated with Cbz-LLL and dihydrotestosterone overnight. Cells were lysed in ELB. Left, immunoprecipitation of flag AR with flag antibody and visualization of coimmunoprecipitated fusion DUBs with GFP antibody. Right, immunoprecipitation with GFP antibody and visualization of coimmunoprecipitated AR with flag antibody. Flag-AR interacts specifically with GFP-USP26.

DUB-dead (DD) mutant also activated the ARE-Luc reporter compared with controls, as well as with wild-type (WT) USP26, but the phenotype was somewhat less pronounced compared with USP26 knockdown (Fig. 2B). This may be due to the fact that the USP26DD mutant is dominant negative over the wild-type protein, which remains present in this experiment. We conclude that the regulation of AR signaling by USP26 depends on its DUB activity.

#### USP26 is a nuclear protein that colocalizes with AR upon hormone stimulation

USP26 contains a putative nuclear localization signal. Indeed, when we transfected GFP-USP26 into HEK293 cells, we observed nuclear localization with an accumula-

tion in subnuclear foci, independently of hormone stimulation (Fig. 3A). Such subcellular localization is similar to that reported for a specific AR mutant that confers androgen insensitivity syndrome in patients. In an article published by Black et al., AR<sup>F582,583A</sup> was shown to arrest within subnuclear foci upon hormone stimulation and also recruit the transcriptional coregulators GRIP-1 and CBP that, in the presence of wild-type AR, are distributed throughout the nucleus (37). We speculated that these transcriptional cofactor-containing foci might be identical to the observed USP26-containing foci. To address this, we cotransfected HEK293 cells with GFP-AR<sup>F582,583A</sup> and Flag-USP26, followed by overnight stimulation with dihydrotestosterone. Immunofluorescence by confocal microscopy was used to detect Flag-USP26 with a Texas

red-conjugated secondary antibody, whereas GFP-AR<sup>F582,583A</sup> was visualized directly (Fig. 3B). As expected, both AR<sup>F582,583A</sup> and USP26 localize to the nuclear foci; furthermore, the merged image of the two proteins shows that they also colocalize.

#### USP26 physically interacts with AR and influences AR ubiquitination and transcriptional activation

After determining that USP26 and AR colocalize, the next question we wanted to answer was whether the two proteins also bind to each other. We transfected HEK293 cells with Flag-tagged AR and GFP-tagged USP26, as well as a control nuclear DUB, GFP-USP1. We note that full-length GFP-USP26 expressed from sequence-verified plasmids runs at a somewhat lower molecular weight than expected, possibly due to its specific charge or posttranslational processing. We then did immunoprecipitation experiments. As is the case for other DUBs (30), USP26 seems to be subject to rapid proteasomal degradation, as its abundance is increased by treatment with proteasome inhibitor (Fig. 3C). We therefore did immunoprecipitations in the presence of proteasome inhibitors. Furthermore, cells were stimulated with dihydrotestosterone overnight to induce AR nuclear translocation and colocalization with USP26. We first used a flag antibody to immunoprecipitate Flag-AR, we could coimmunoprecipitate GFP-USP26 but not the control DUB, GFP-USP1 (Fig. 3D, left). Subsequently, we did the reverse experiment. Using a GFP antibody, we pulled down GFP-USP26 and GFP-USP1, and in this case, Flag-AR was bound to GFP-USP26 but not USP1 (Fig. 3D, right). These results indicate that there is a specific physical interaction between AR and USP26 upon hormone stimulation.

Having established that AR and USP26 interact within transcription factor-containing foci, we wanted to investigate whether USP26 might influence AR signaling, possibly counteracting the Mdm2-mediated ubiquitination of AR, which takes place after short-term hormone stimulation (24). HEK293 cells were transfected with Flag-tagged AR, HA-ubiquitin, and GFP-USP26 WT or GFP-USP26 DD. Cells were treated overnight with proteasome inhibitor to visualize ubiquitination ladders, and either left unstimulated or stimulated for 2 hours with dihydrotestosterone prior to harvest. To avoid possible ubiquitination contamination by coimmunoprecipitated proteins, cells were lysed in denaturing radioimmunoprecipitation assay buffer. We then did Flag immunoprecipitations and immunoblots for HA-ubiquitin. We found that there was a significant increase in AR ubiquitination after 2 hours of hormone stimulation, and that this ubiquitination was counteracted by the overexpression of USP26 WT (Fig. 4A). To ascertain AR ubiquitination levels in the absence of ectopically expressed USP26, and whether deubiquitination of AR was specific for USP26, we did an additional experiment in which USP26 WT and USP26 DD were compared with our control DUB USP1, both WT and DD mutants. Here, we find that only USP26

WT overexpression reverses AR ubiquitination, implying substrate specificity (Fig. 4B).

Upon establishing that USP26 specifically binds to, and deubiquitinates AR, we wanted to investigate the effect on transcriptional activity in HEK293 cells. We did reporter experiments under conditions that were identical to the original screen. Surprisingly, we found that overexpression of WT USP26 superactivates the ARE-Luc reporter, which contradicts our observations in HepG2 cells (Fig. 4C).

To further probe these different responses, we used the hormone-responsive prostate cancer cell line LnCap. Cells were transfected with a pool of four synthetic siRNAs targeting USP26, and by qRT-PCR, we monitored the effect of USP26 inhibition on the activation of three different AR target genes following hormone stimulation. Although the effect of USP26 inhibition in the absence of hormone stimulation is subject to some experimental variation (Fig. 5A versus B), we found that USP26 inhibition consistently represses hormone-induced expression of all three measured target genes by approximately 2-fold (Fig. 5A). To further verify this finding, the four siRNAs targeting USP26 were tested individually. All four significantly inhibited USP26 mRNA and were able to repress the AR target gene PSA almost 2-fold upon hormone stimulation (Fig. 5B). In conclusion, these results suggest that the regulatory function of USP26 is cell line- and context-dependent.

#### Four common USP26 mutations found in patients with fertility defects do not affect AR signaling

As mentioned previously, several reports have suggested a correlation between mutations in USP26 and a variety of male infertility syndromes (34, 35). To investigate whether patient mutations might actually influence AR signaling, we introduced four of the most common mutations (494T-C, 1037T-A, 1090C-T, and 1423C-T) into USP26 cDNA. Neither AR binding, ubiquitination, or transcriptional activation were affected significantly by any of the mutants in our experiments in HEK293 cells (data not shown). It is possible that these mutations give highly subtle phenotypes, or that they need to be combined with additional less common mutations to have an effect. However, we chose not to investigate this further. In an alternative strategy to assess whether the reported USP26 patient mutations might be involved in prostate cancer development, we sequenced the *USP26* gene in a panel of eight prostate cancer cell lines. We did not find the patient mutations in any of these, nor did we find any other mutations within the *USP26* gene, suggesting that any involvement of USP26 in prostate cancer development was more likely to be caused by altered expression levels.

#### USP26 contains several NR interaction motifs that influence AR binding, ubiquitination, and transcriptional activation

Upon studying the amino acid sequence of USP26, we found that this protein contains several NR boxes, which are short motifs found in many coregulators that

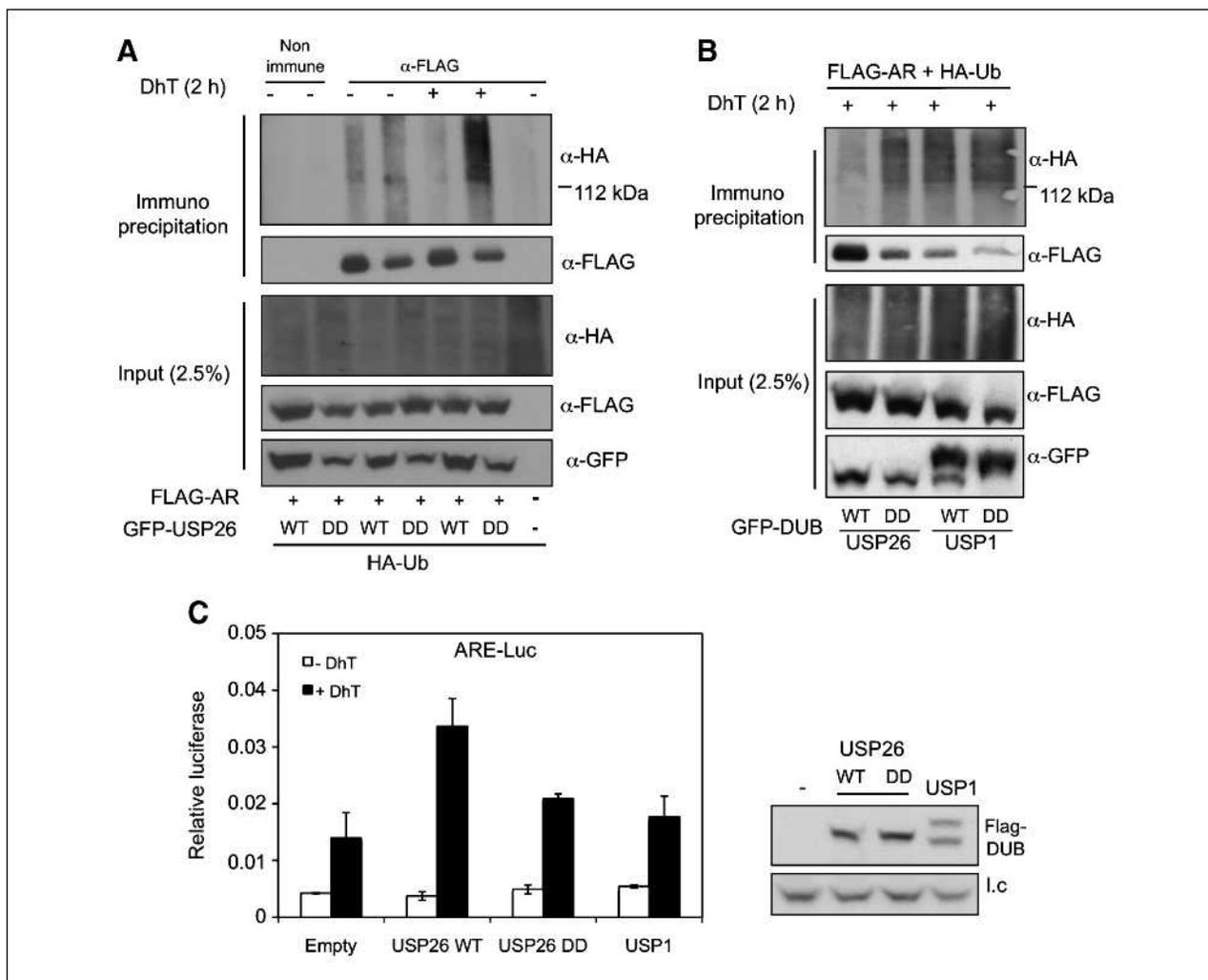
specifically mediate interactions with NRs (Fig. 6A). Three of these are classic LXXLL motifs (reviewed in ref. 38), whereas the other two are FXXLF and FXXFF motifs. AR has been shown to display increased affinity for FXXLF and FXXFF over LXXLL motifs (39, 40).

We mutated each of the five NR interaction motifs to establish whether any of these are essential for AR-USP26 interactions, AR ubiquitination, and AR transcriptional activity. Point mutations were introduced to change conserved leucine and phenylalanine residues into valine residues. We then did Flag-AR coimmunoprecipitation experiments in HEK293 cells. We found that three NR box mutants (L2, L3, and F2) displayed severely diminished binding to AR (Fig. 6B). Furthermore, these

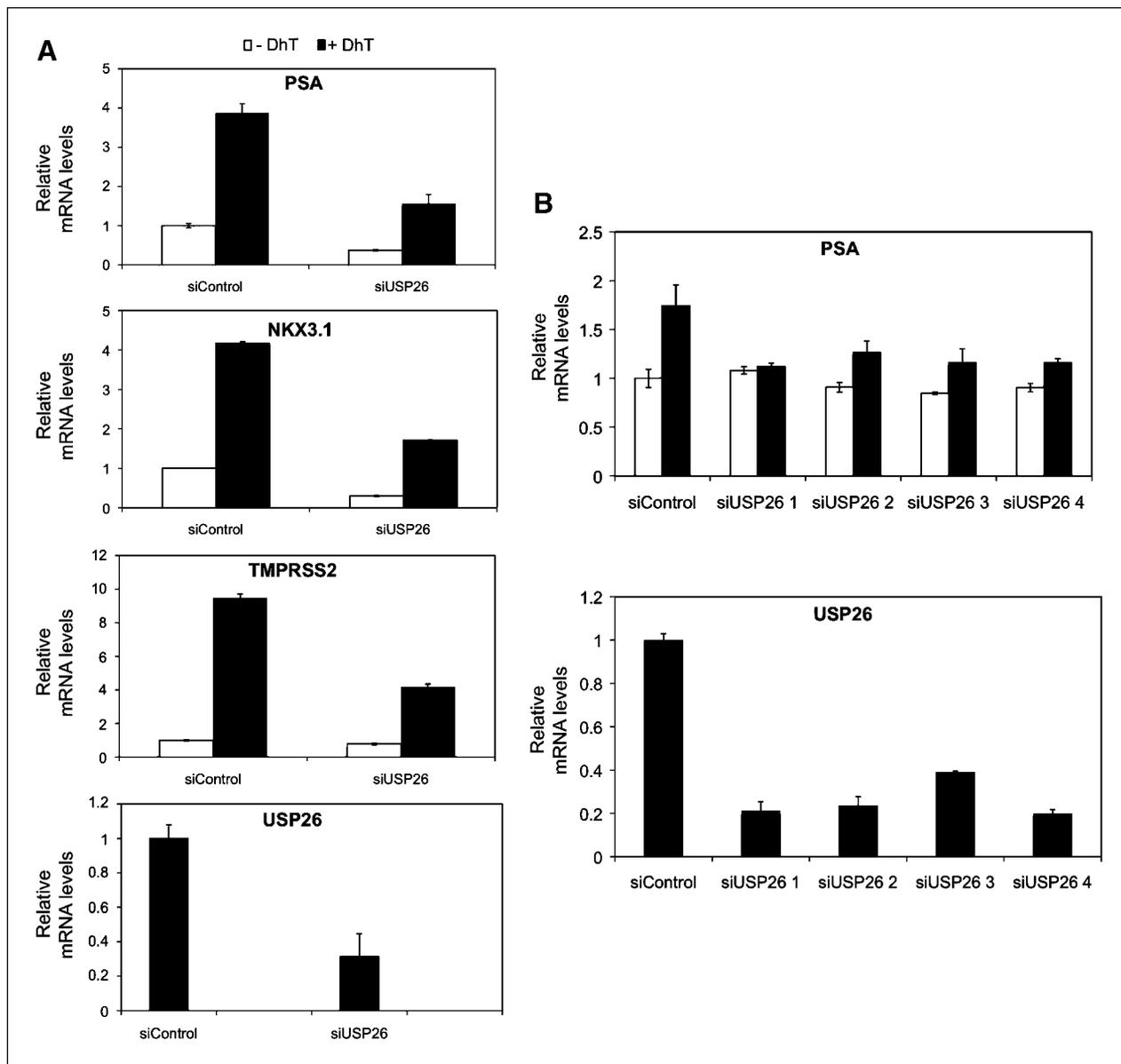
three mutants, like the catalytically inactive USP26<sup>C/S</sup> mutant, were unable to deubiquitinate Flag-AR in dihydrotestosterone-treated cells (Fig. 6C). Finally, we found that the inability of the mutants to deubiquitinate AR after short-term hormone stimulation correlated perfectly with the inability to superactivate the ARE-Luc reporter, also in HEK293 cells (Fig. 6D).

## Discussion

In this report, we used an RNA interference library targeting the family of DUB enzymes to search for novel regulators of AR signaling. We identified an X-linked DUB, USP26, which colocalizes with, and binds to AR



**FIGURE 4.** USP26 promotes deubiquitination of AR and stimulates AR signaling in HEK293 cells. **A**, HEK293 cells were transfected as indicated and 48 h later were treated with Cbz-LLL overnight, and either given dihydrotestosterone (DhT) for 2 h or no hormone. Cells were lysed in radioimmunoprecipitation assay buffer and immunoprecipitations were done with Flag antibody. AR ubiquitination was assessed with HA antibody. **B**, similar to **A**, but all cells were treated with dihydrotestosterone and a comparison was made between USP26 and USP1. AR ubiquitination is stimulated upon hormone stimulation, and counteracted specifically by overexpression of wild-type GFP-USP26. **C**, ARE-Luc assays in HEK293 cells cotransfected with ARE-Luc reporter, CMV-Renilla, and pSV-hAR and either empty vector, flag-USP26 WT or DD, or USP1 WT. Luciferase activity was measured 72 h posttransfection following overnight dihydrotestosterone stimulation. Inset, similar expression of all three DUBs.



**FIGURE 5.** Inhibition of USP26 lowers AR signaling in the LnCap cell line. A, LnCap prostate cancer cells were transfected with a control synthetic siRNA, or a pool of four synthetic siRNAs targeting USP26, and 48 h after transfection, cells were stimulated with dihydrotestosterone overnight or left untreated. cDNA was synthesized from TRIzol-extracted RNA, and expression of three AR target genes was measured by qRT. B, similar to A, but the four USP26 siRNAs were tested individually for their ability to repress the AR target gene PSA. Inhibition of USP26 represses AR target gene expression.

via three important NR interaction motifs. USP26 modulates AR ubiquitination levels, and as a consequence, affects AR signaling.

Within recent years, the importance of ubiquitination in transcriptional regulation has become evident. A number of transcription factors such as Myc, Src-3, estrogen receptor, and AR have been subjected to biphasic ubiquitination, in which (multiple) monoubiquitination initially stimulates transcriptional activation, and is followed by polyubiquitination leading to proteasomal degradation, and transcriptional switch-off (22, 41, 42). In the case of

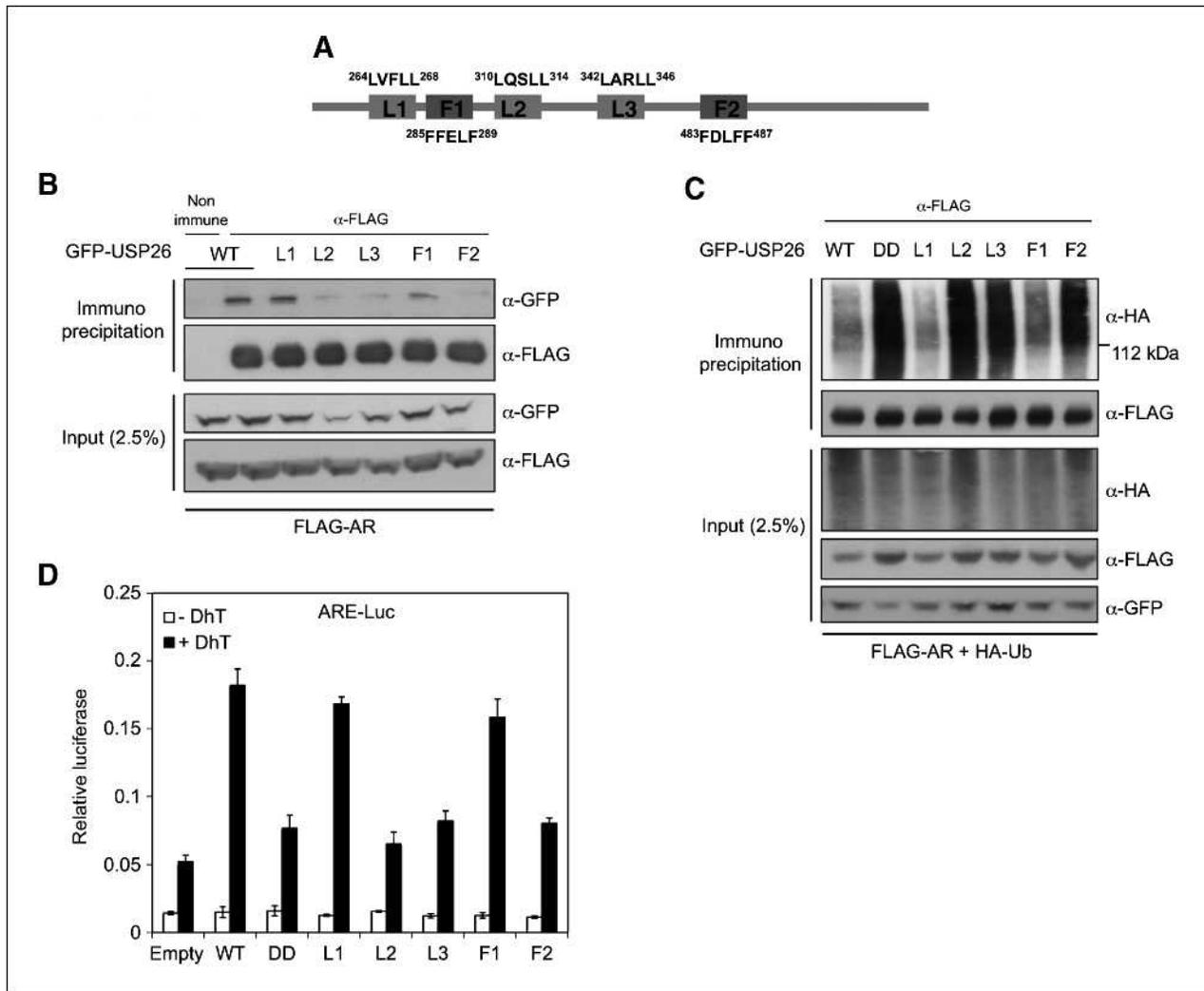
AR, such biphasic ubiquitination is mediated by Mdm2, which is recruited to AREs upon hormone stimulation (23, 24). TSG101 has been proposed to prevent the switch from AR monoubiquitination to polyubiquitination, and thus prolong transcriptional activity (25). The work presented here suggests that USP26 is a novel regulator of hormone-induced AR ubiquitination.

Activation of AR signaling is initiated by hormone binding to the AR ligand-binding domain. A subsequent structural rearrangement of the ligand-binding domain has been described that opens a binding pocket to which many

NR-specific coregulators, e.g., p160 family members, bind (43). The majority of interactions are dependent on so-called NR interaction motifs in the coregulators. We identified five such NR interaction motifs in USP26, three of which are essential for AR binding, ubiquitination, and signaling activation.

The androgen insensitivity mutant AR<sup>F582, 583A</sup> has been used to show the existence of subnuclear foci, which are normally transient in the presence of wild-type AR. These subnuclear foci are proposed to function as assembly platforms for transcriptional cofactors such as GRIP-1 and

CBP prior to chromatin binding (37). We found that USP26 colocalizes with AR<sup>F582, 583A</sup> to such subnuclear foci upon hormone stimulation. Based on our observations, we propose a model in which USP26 is an AR coregulator that is brought to sites of transcriptional initiation as part of AR-containing multicomponent protein complexes. At such sites, Mdm2 mediated the ubiquitination of AR functions as a transcriptional on-switch, and subsequent off-switch as a result of proteasomal degradation. USP26 is able to reverse both outcomes of ubiquitination, preventing activation or degradation depending on context as discussed



**FIGURE 6.** USP26 has three NR interaction motifs essential for AR binding, deubiquitination, and signaling. A, overview of the five USP26 NR interaction motifs. F1 and F2, phenylalanine-containing NR interaction motifs that are preferentially bound by AR. B, five GFP-USP26 NR interaction motif mutants were constructed and transfected as indicated in HEK293 cells. Following overnight treatment with Cbz-LLL and dihydrotestosterone, cells were lysed in ELB 72 h after transfection. Immunoprecipitations were done with Flag antibody and coimmunoprecipitated GFP-USP26 constructs were visualized with GFP antibody. C, HEK293 cells were transfected as indicated, following overnight Cbz-LLL treatment, and a 2-h stimulation with dihydrotestosterone, cells were lysed in radioimmunoprecipitation assay buffer 72 h after transfection. Flag immunoprecipitations were done and the influence of the various GFP-USP26 constructs on AR ubiquitination was visualized with HA antibody. D, ARE-Luc assays in HEK293 cells cotransfected with empty vector Flag-USP26 wild-type (WT), DUB-dead (DD), and five Flag-USP26 NR interaction motif mutants. Luciferase activity was measured 72 h posttransfection, following overnight dihydrotestosterone stimulation. The USP26 L2, L3, and F2 NR interaction motifs are essential for AR binding and deubiquitination, as well as AR signaling activity.

below, in this way, contributing to further fine-tuning of AR signaling activity.

We observed that USP26 inhibition stimulates AR signaling in HepG2 cells, whereas signaling is repressed in HEK293s and the hormone-responsive prostate cancer cell line LnCap. Discrepancies of this kind in AR cofactor function in HepG2s versus other cell lines have been observed previously, however, no functional explanations have been proposed (44). In the case of USP26, it is likely that deubiquitination activity targets monoubiquitinated AR in HepG2s, thus preventing transcriptional activation, whereas polyubiquitinated AR is targeted in LnCaps and HEK293s. Indeed, we see a strong inhibition of AR polyubiquitination upon overexpression of wild-type USP26 in the latter cell line, the consequence of which is augmented AR signaling (Fig. 4). One might speculate that the differences in timing of USP26 deubiquitination activity could be caused by variations in the composition and/or dynamics of assembly of multicomponent AR transcriptional complexes in the examined cell lines, as reviewed by Aoyagi and Archer (45).

The hunt for novel X-linked genes contributing to male infertility has been relatively fruitless thus far (46). *USP26* is one candidate gene whose possible involvement in spermatogenesis and testicular development has been discussed in several articles (34, 35, 47–51). Because AR signaling is essential for these processes, we investigated whether four described USP26 mutations from patients with fertility defects might affect signaling in any way. We did not measure any significant differences in any of our assays, which

supports claims that changes in the *USP26* gene are not restricted to infertile men and therefore cannot be causally involved (34, 35, 46–51). Interestingly, one of the mutants, 1037T-A, introduces a leucine to histidine amino acid change in the L3 NR interaction motif, which is highly important for AR binding, ubiquitination, and signaling activity.

It remains intriguing though that this gene has a clear role in the regulation of AR activity. When we sequenced the USP26 gene from a number of prostate cancer cell lines, we did not discover one single mutation. We therefore propose that any involvement of USP26 in prostate cancer development is more likely to be due to alterations in expression levels, which seems to be the case for the majority of NR coregulators (reviewed in ref. 52). However, this remains to be studied further in the future.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

### Grant Support

The Netherlands Genomics Initiative/Netherlands Organization for Scientific Research, the Dutch Cancer Society, and the EU Network of Excellence "RUBICON."

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Received 09/18/2009; revised 04/02/2010; accepted 04/22/2010; published OnlineFirst 05/25/2010.

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