Deubiquitinating enzyme Usp12 is a novel co-activator of the Androgen Receptor*

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*Running title: Usp12 is a novel positive regulator of the AR

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Keywords: Androgen Receptor, Usp12, prostate cancer, deubiquitination, Uaf-1, WDR20

•**Background:** Androgen Receptor (AR) is the principle therapeutic target in prostate cancer.

•**Result:** We have established that Usp12 deubiquitinates and stabilises the AR resulting in increased transcriptional and pro-proliferative activity.

•**Conclusion:** We have identified Usp12 to be a novel positive regulator of AR.

•**Significance:** Usp12 presents a therapeutic target upstream of AR that could enable bypassing the limitations of therapeutics aimed specifically at AR.

SUMMARY

The androgen receptor (AR), a member of the nuclear receptor family, is a transcription factor involved in prostate cell growth, homeostasis and transformation. AR is a key protein in growth and development of both normal and malignant prostate making it a common therapeutic target in prostate cancer (PCa). AR is regulated by an interplay of multiple post-translational modifications including ubiquitination. We and others have shown that the AR is ubiquitinated by a number of E3 ubiquitin ligases, including MDM2, CHIP and NEDD4 which can result in its proteosomal degradation or enhanced transcriptional activity. As ubiquitination of AR causes a change in AR activity or stability and impacts both survival and growth of PCa cells, deubiquitination of these sites has an equally important role. Hence deubiquitinating enzymes (DUBs) could offer novel therapeutic targets. We performed an siRNA screen to identify DUBs that regulate AR, in that screen ubiquitin specific protease 12 (Usp12) was identified as a novel positive regulator of AR. Usp12 is a poorly characterised protein with few known functions and requires the interaction with two cofactors, Uaf-1 and WDR20, for its enzymatic activity. In this report we demonstrate that Usp12, in complex with Uaf-1 and WDR20, deubiquitinates the AR to enhance receptor stability and transcriptional activity. Our data shows that Usp12 acts in a pro-proliferative manner by stabilising AR and enhancing its cellular function.

The androgen receptor (AR) belongs to the nuclear hormone receptor superfamily, and plays a key role in the transcriptional regulation of numerous genes important in the development of both normal and malignant prostate [1]. Ligand binding of the AR in the cytoplasm results in dimerisation, translocation to the nucleus and transcription of androgen responsive genes. Deregulation of AR signalling leads to the development of

prostate cancer (PCa) and as such the receptor represents the most common therapeutic target in PCa [1]. The current clinical strategies in PCa centre around anti-hormone therapies and aim to pharmacologically decrease serum androgen levels. This is currently achieved through the use of luteinising hormone-releasing hormone (LHRH) agonists such as goserelin or anti-androgens which can be either steroidal (cyproterone acetate) or non-steroidal (flutamide) and function by competitively inhibiting the AR [2]. However, despite initial success most of anti-androgen therapies will invariably fail resulting in castrate-resistant prostate cancer (CRPCa). Importantly, the AR signalling cascade remains functional in CRPCa. Treatment failure can be caused by changes in AR signalling including alterations in AR cofactor levels and activity as well as emergence of AR mutations that permit receptor activity in the presence of non-androgenic steroids and anti-androgens [2]. In CRPCa, AR still remains a crucial target, with increased AR levels and gene amplification observed in 30% of patients. Increased levels of the downstream gene Prostate Specific Antigen (PSA) in the serum are also associated with PCa recurrence [3]. Previous research demonstrated that even at CRPCa stage, depletion of AR by siRNA treatment decreased tumor growth. Similarly, AR overexpression in PCa xenograft in castrate animals still enhanced cancer growth [4].

AR function and activity is known to be regulated by a number of post-translational modifications. In response to androgens, AR is phosphorylated by kinases including AKT [5] and TFIIH [6] resulting in changes to transcriptional activity and cellular localisation. This can be reversed by phosphatases such as protein phosphatases 1 and 2A [7]. Acetylation by p300 [8] and Tip60 [9] enhances transcriptional activity which can be reversed by the histone deactylases HDAC1 [10] and SIRT1 [11]. AR is also subjected to SUMOylation by PIAS1 and PIASxα, a process which is believed to decrease its transcriptional activity [12]. We and others have shown that the AR is also ubiquitinated by a number of E3 ubiquitin ligases, including MDM2 [13, 14], CHIP [15, 16] and NEDD4 [17, 18] which results in proteosomal degradation. AR ubiquitination can also lead

to the increase in transcriptional activity; RNF6 has been reported to ubiquitinate AR at K845 and K847 promoting its activity by allowing ARA54 co-activator recruitment [19]. Further details of AR post-translational modifications have been recently reviewed [20].

Ubiquitination of AR causes a change in AR activity and stability and impacts both survival and growth of PCa cells. As a result deubiquitination of those sites has an equally important role.

Currently, very little is known about the enzymes that deubiquitinate AR. It has been reported that Usp26 can directly bind and deubiquitinate AR, acting as a co-regulator of AR by reversing AR activation and degradation by MDM2 ubiquitination depending on cellular context [21]. Additionally, Usp10 has been reported to bind AR resulting in increased transcriptional activity [22].

In this study we focused on Usp12, a deubiquitinating enzyme identified in an siRNA screen as a positive regulator of AR. Usp12 has three reported targets; histones H2B, H2A [23] and non-activated Notch [24]. Usp12 is highly homologous to Usp46 and Usp1 and its activity, similarly to that of Usp46, is enhanced by binding to its cofactors Uaf-1 and WDR20, with Uaf-1 required for enzymatic activity of Usp12 [25, 26, 27]. It has been reported that Uaf-1 binds and stabilises Usp12 and this complex is bound by WDR20. This interaction is both stoichiometric and evolutionarily conserved and has been shown to play the same role in *S. pombe* [28]. Usp12 and Uaf-1 containing complex was shown to deubiquitinate all types of ubiquitin chains apart from linear chains [27]. Our search of Oncomine profiles revealed that Usp12 is differentially expressed in bladder, brain, CNS, cervical, kidney, lymphoma and ovarian cancer samples compared to healthy controls.

We now show that Usp12, in complex with Uaf-1 and WDR20, interacts with and deubiquitinates the AR resulting in increased protein stability and transcriptional activity. Moreover, we report that Usp12 depletion reduces PCa cell proliferation and up-regulates cell apoptosis suggesting it is an additional

regulator of the AR that may represent a novel target for therapy.

EXPERIMENTAL PROCEDURES

Antibodies and plasmids - Antibodies used were anti-Flag (Sigma), anti-Usp12 (Dundee Cell Products), anti-AR (Santa Cruz, N20 clone), anti-HA (Santa Cruz, Y11 clone), anti-α tubulin (Sigma) and anti-ubiquitin (Santa Cruz). Plasmids used were pPSA-Luc, pARE3-Luc, pCMV-β-gal, pFlag-His-AR [9], pFlag-Usp12 wild type and C48A mutant generated by *in vitro* mutagenesis (Quickchange, Stratagene), pHA-Ubiquitin and pHA-Flag-WDR20 and pFlag-Uaf-1 [25]; [26] which were kind gifts from Professor Alan D'Andrea (Dana-Farber Cancer Institute, Boston).

Cell culture, transfections and reporter assays - LNCaP, HEK293T and COS-7 cells were obtained from American Type Culture Collection (Manassas, USA). VCaP cells were kindly donated by Professor Guido Jenster (Erasmus Medical Centre, Rotterdam). Cells were cultured in RPMI 1640 media with 2 mM L-glutamine (Invitrogen) supplemented with 10% (v/v) foetal calf serum (FCS) at 37°C in 5% CO_2. LNCaP-AI variant cell line was derived in-house by culturing LNCaP cells in steroid-depleted media (DCC) to allow for the development of androgen independence [30]. LNCaP-7B7 cells stably overexpressing pPSA-Luc vector were kindly donated by Professor Jan Trapman (Erasmus Medical Centre, Rotterdam) and cultured with the addition of 25 μg/ml zeocin. Transfections were performed using TransIT-LT1 reagent (MirusBiol) following the manufacturer's instructions.

For luciferase assays, cells were transfected with 50 ng pARE3-luc, 50 ng pCMV-β-gal and 10 ng of pFlag-His-AR, pFlag-Usp12 and pFlag-Uaf-1 as required. All reactions were balanced with pCMV empty vector. Cells were cultured under steroid depleted conditions for 48h followed by supplementation with dihydrotestosterone (DHT), at a range of concentration of 5 and 10 nM with comparable results obtained for both concentrations, for an additional 24h. Cells were lysed and incubated in 1x Promega luciferase assay reagents according to the manufacturer's instruction and luciferase counts per second were established and normalised to β-galactosidase activity. Results were normalised to AR expression alone in steroid depleted conditions.

siRNA gene silencing and gene expression analysis - The generation and DUB siRNA screening methodology for AR regulators screen using an ELISA against PSA protein as a readout of AR activity in LNCaP cells has been described previously [29]. Usp12 targeting siRNA sequences were (A) GAAACUCUGUGCAGUGAAU[dTdT] (B) CAGAUCUCUUCCAUAGCAU[dTdT] and (C) CAUCAGAUAUCUCAAAGAA[dTdT], WDR20 was silenced with siRNAs (A) CGAGAAAGAUCACAAGCGA[dTdT] and (B) GUUUGACCCUUAUACCACU[dTdT] and Uaf-1 with (A) CAAAUUGGUUCUCAGUAGA[dTdT] and (B) CAUUGACUGCCUCAAAUAA[dTdT]. Initial DUB screen used a pool of siRNAs against Usp12, further experiments were performed using siRNA (B). Uaf-1 A achieved 61.5% knockdown with Uaf-1 B 61% similarly, WDR20 A achieved 67.4% and WDR20 B 57.4% in qPCR validation (data not shown). As a result siRNAs (A) were selected for silencing of both Uaf-1 and WDR20.

LNCaP cells were reverse transfected with siRNA using RNAiMax (Invitrogen) according to manufacturer's instructions and incubated in culture media for 96h prior to cell lysis and analysis by Western blotting as described previously [31] or qPCR. For qPCR RNA was extracted using the EZ RNA isolation kit (Biological Industries) and cDNA synthesis and data analysis was performed as described previously [32]. Proliferation was measured by cell counting 96h post gene silencing. To measure colony forming ability cells were reverse transfected with siRNA for 72h, followed by re-seeding at varying cell densities and incubated for 14 days to allow colony formation and stained with crystal violet. Colonies were counted and the surviving fraction calculated [31].

Flow cytometry - Cell cycle profiles were generated by propidium iodide (PI) staining, cells were permeabilised with 1% Triton X-100 and incubated with 1 μg/ml RNaseA and PI followed by analysis on BD FACScan [33]. Levels of apoptosis were analysed after 96h of

gene silencing by Annexin V assay (BD) according to manufacturer's instruction and analysed on BD FACScan. Cells were stained for both annexin V and PI positivity and during analysis divided into quarters representing normal cells, necrotic cells and apoptotic cells.

Immunoprecipitations (IP) - Cells were seeded at 5×10^5 cells per 90 mm dish, transfected 24h later with 1µg of each plasmid as indicated, incubated for 48h and lysed directly into lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.2 mM Na_3VO_4, 1% NP-40, 1 mM PMSF, 1 mM DTT and 1x protease inhibitors (Roche)). Lysates were incubated with 1 µg of antibodies as indicated for 16h at 4°, antibodies were pulled down using Protein G Sepharose beads. For denaturing IPs, cells were subject to 20 µM of MG132 proteosomal inhibitor treatment for the final 16h followed by collection into lysis buffer with an addition of 2% SDS and denatured at 100°C for 10 minutes [29]. Following denaturation samples were diluted 10x in lysis buffer without SDS and processed as in native IP. Immunoprecipitants were analysed using Western blotting.

Chromatin immunoprecipitations (ChIP) were performed as described previously [10]. LNCaP cells were transfected in steroid-depleted media for 72h followed by DHT treatment for 120 minutes. Data is presented as % Input using the following formula: % Input = 100 x 2^(CT Adjusted Input sample - CT immunoprecipitated sample), CT refers to cycle threshold.

Immunohistochemistry (IHC) - Tissue microarray (TMA) containing 0.6-mm cores of benign prostatic hyperplasia (BPH) (n=7), PCa (n=7) and control tissues including breast, kidney, placenta, ovary and liver was used. Antigens were retrieved by pressure cooking the TMA in 0.01M Citrate buffer pH 6.0 followed by staining the tissues with rabbit polyclonal anti-Usp12 antibody (Dundee Cell Products) [29].

RESULTS

Identification of Usp12 as a positive regulator of AR - To identify DUBs that regulate AR we conducted an siRNA screen in LNCaP cells using an ELISA detecting the levels of Prostate Specific Antigen (PSA), which is an AR target gene product and as such its levels will be affected by any change in AR activity, as a surrogate readout of AR activity. All samples were normalised against the scrambled control (SCR) and readouts of +/- two fold PSA protein level compared to SCR were taken for further validation. To confirm that target DUBs regulate AR transcriptional activity and not only PSA secretion, further validation included the assessment of PSA mRNA levels upon DUB depletion eliminating DUBs that solely affect PSA rather than AR activity. Of the seventy-eight screened DUBs, four were identified as potentially involved in androgen-dependant regulation of AR. Silencing of one of the targets, Usp12, resulted in 73% reduction in PSA protein levels (fig 1A, highlighted panel). This result was also confirmed by Western blotting (fig 1B). Usp12 siRNA was shown to significantly decrease *Usp12* transcript levels in three PCa cell lines namely, LNCaP, LNCaP-AI and VCaP (fig 1C). Additionally depletion of Usp12 resulted in significant decrease of *PSA* transcript in LNCaP, LNCaP-AI and VCaP cells confirming that Usp12 affects AR transcriptional activity and as a result PSA gene expression rather than protein secretion (fig 1C). To summarise, Usp12 has been identified as a positive regulator of AR in multiple PCa cell lines and it was selected for further validation and analysis.

Usp12 stabilises AR at the protein level - Ubiquitination of AR causes a change in AR activity and stability and impacts both survival and growth of PCa cells, as a result deubiquitination of those sites has an equally important role. We hypothesised that Usp12 affects AR activity by deubiquitinating it and as a result stabilising AR protein. To determine the effects of Usp12 on AR activity and protein stability we analysed the levels of endogenous AR in PCa cells depleted of Usp12. Silencing of Usp12 resulted in a decrease of AR protein compared to SCR in three PCa cell lines (fig 2A-2C). Stabilisation of AR by Usp12 relies on enzymatic activity of this DUB as mutant $Usp12_{C48A}$ was not able to stabilise AR protein levels to the same extent as the wild type (fig 2D). Additionally as little as 1:10 of Usp12 (50ng) to AR (500ng) was sufficient to cause this effect (fig 2D). Similarly when HEK293T cells were

transfected with AR and co-transfected with Usp12, Uaf-1 and WDR20 the levels of AR increased compared to AR alone (fig 2E). Even the increase in AR protein levels observed in cells transfected with Uaf-1 and WDR20 can be attributed to the stabilisation of endogenous Usp12 caused by the overexpression of its cofactors as illustrated by Usp12 protein levels (figure 2E). To assess if reduced receptor levels were a consequence of compromised *AR* gene expression we analysed *AR* transcript level in LNCaP cells depleted of Usp12. Knockdown of Usp12 or either one of its interacting partners had no statistically significant effect on *AR* transcript levels suggesting that Usp12 may function to enhance the stability of the AR in PCa cells (data not shown). Additionally, we investigated the effects of Usp12 depletion on AR half-life in PCa cells. In cells with depleted Usp12 the pool of AR was smaller than in controls even before the cycloheximide treatment and there was a trend of decreased half-life of AR (fig 2F). In conclusion we have shown that Usp12, with its interacting partners Uaf-1 and WDR20, increases AR protein levels rather than regulating its gene expression.

AR stabilisation by Usp12 results in increased AR activity - To assess the effects of increased AR protein stability HEK293T cells were transfected with an androgen-responsive luciferase reporter containing three adjacent Androgen Responsive Elements (AREs) upstream from the *luciferase* gene. Additionally, combinations of mammalian expression vectors including dual Flag-AR, Flag-Usp12 and Flag-Uaf-1 were co-transfected and receptor activity was assessed. Cells transfected with Usp12 showed a trend of increased AR transcriptional activity which was not statistically significant (fig 3A) however, when Usp12 and Uaf-1 were co-transfected, AR transcriptional activity was significantly increased to levels comparable to that caused by the known AR co-regulator p300 (fig 3A). This result is in agreement with previous reports that Usp12 requires the presence of Uaf-1 for its enzymatic activity [27]. To confirm a transcriptional co-regulatory role of Usp12 in the AR signalling cascade, we assessed AR activity upon depletion of Usp12 in the LNCaP variant cell line, LNCaP-7B7, which have a stably

integrated *luciferase* gene under the control of the *PSA* promoter. Usp12 silencing caused a significant decrease in AR transcriptional activity both in steroid depleted conditions and after DHT stimulation (fig 3B). Additional confirmation of Usp12 effects on AR transcriptional activity came from qPCR analysis, where we observed that Usp12 silencing caused a significant decrease of transcript levels of AR regulated genes *PSA* and *TMPRSS2* (fig 3C and 1C) in multiple PCa cell lines. Further analysis of Usp12 silencing in LNCaP cells cultured in steroid depleted conditions followed by DHT stimulation for 0-48h showed a significant decrease in transcript levels of all five androgen regulated genes that were tested (fig 3D). Our results confirm that the stabilisation of AR caused by Usp12 results in enhanced transcriptional activity of AR and increased transcript levels of AR regulated genes. To determine the underlying cause of the decreased transcriptional activity of AR we performed ChIP analysis of AR recruitment to AREIII of *PSA* enhancer after DHT stimulation. AR recruitment was shown to be significantly decreased after Usp12 silencing in comparison to SCR treated control further confirming the role of Usp12 in the regulation of AR activity (fig 3E).

Stabilisation of AR by Usp12 affects survival and proliferation of prostate cells - We have shown that Usp12 enhances AR activity by up-regulating receptor stability. We next assessed the role of Usp12 in regulating the survival and proliferation of PCa cell lines. Proliferation of three PCa cell lines was quantified by cell counting 96h post Usp12 depletion. It was observed that Usp12 silencing caused significant decrease in cellular proliferation in all three cell lines (fig 4A). To examine the impact of Usp12 knockdown on LNCaP cell survival, we measured apoptosis by Annexin V staining by flow cytometry. Silencing of the Usp12 complex and Usp12 alone resulted in a significant increase of apoptotic cells that was comparable to the value obtained for cells with depleted AR (fig 4B).

Cell cycle analysis by propidium iodine staining revealed that depletion of the Usp12 complex results in a significant increase of G1 arrested PCa cells (fig 4C). Additionally, silencing of the Usp12 complex significantly

increased the number of cells in subG1 phase confirming that it causes an increase in cellular apoptosis (fig 4D).

One of the crucial properties of cancer cells is their ability to form colonies which allows for cancer survival. We evaluated the ability of LNCaP cells to form colonies after depletion of Usp12. Cells with silenced Usp12 had a significantly decreased ability to form colonies in comparison to SCR treated control (fig 4E). In conclusion, we have shown that AR stabilisation by Usp12 has a significant effect on proliferation and survival of PCa cells. Usp12 is pro-proliferative and cells deficient in it have decreased ability to proliferate, increased apoptosis, G1 arrest and are deficient in their ability to form colonies. This data suggests that Usp12 has a key role in PCa disease progression.

Usp12 interacts with AR and stabilises it via deubiquitination - We have shown that Usp12 stabilises AR at the protein level and as a result increases transcriptional activity acting in a pro-survival manner in prostate cells. To determine if Usp12 interacts with AR we immunoprecipitated AR and its interacting partners from the whole cell lysate. These immunoprecipitants were immunoblotted for the presence of Usp12. Both wild type Usp12 and its Cys48 catalytically deficient mutant Usp12$_{C48A}$ were shown to directly interact with AR (fig 5A). Additionally, we confirmed the interaction between the endogenous AR and Usp12 proteins in LNCaP PCa cells (fig 5B). To determine if this interaction affects the AR ubiquitination status we transfected COS-7 cells with ubiquitin, AR and wild type and the deubiquitinase dead C48A mutant Usp12. Cells were treated with proteosomal inhibitors and lysed under denaturing conditions which ensures that only covalent bonds will remain unaffected and as a result only direct ubiquitination of the AR rather than its interacting partners can be visualised. Lysates were subsequently immunoprecipitated using an anti-ubiquitin antibody which allowed separation of all directly ubiquitinated proteins and the levels of ubiquitinated AR visualised by immunoblotting the precipitant for AR protein. Wild type but not Cys48 deficient mutant of Usp12 caused deubiquitination of AR (fig 5C).

To assess potential co-localisation of the AR and Usp12 both proteins were ectopically expressed in COS-7 cells. Immunofluorescence and confocal microscopy were employed to visualise their cellular localisation. It was observed that Usp12 and AR co-localise in the cytoplasm in steroid depleted conditions (fig 5D). We have shown that enhanced levels of Usp12 result in increased AR protein stability and transcriptional activity. To confirm if this increase in Usp12 can be observed *in vivo* we analysed Usp12 positivity in benign and PCa patient samples. Usp12 protein levels were increased in PCa samples compared to benign controls (fig 5E). To summarise, AR and Usp12 interact and enzymatically functional Cys48 of Usp12 is not required for this interaction. Usp12 deubiquitinates AR through this interaction and for this intact Cys48 of Usp12 is required, as a result AR is stabilised at the protein level and its transcriptional activity is enhanced resulting in the activation of pro-survival pathways in prostate cells.

DISCUSSION

It is widely accepted that the post-translational regulation of AR protein stability is a crucial mechanism that regulates both normal and malignant prostate cells. Even in CRPCa AR still plays a major role and remains the main focus of therapeutic strategies, highlighting its importance as a drug target. Therapies aimed at AR invariably fail as a result of AR becoming promiscuous through mutations and acquiring the ability to become activated by a variety of steroid based ligands as well as antiandrogens [2]. As a result more focus is needed on upstream co-regulators of AR as they could be potential drug targets.

Our study identified Usp12 as a novel positive regulator of AR through an siRNA screen of DUBs. Usp12 was initially identified as a histone H2B and H2A deubiquitinase [25, 23], recently it was also reported to be a negative regulator of Notch signalling by impairing Notch trafficking and decreasing its cell surface levels [24]. Still little is known about the role of Usp12 in humans but the role of the Usp12 homologue in yeast has been researched in more detail with the enzyme shown to be involved in actin dynamics, cell polarity and endocytosis [28]. In *S. pombe,*

silencing of the Usp12 homologue in combination with Myosin-1 or Wiskott-Aldrich Syndrome protein (WAS) was reported to be lethal. Our data also points to the importance of Usp12 in cell survival as Usp12 depletion significantly increased the percentage of apoptotic cells and caused G1 arrest in PCa cells suggesting an evolutionarily conserved role of Usp12 in cell survival.

Usp12 DUB activity was previously shown to be very low in the absence of its binding partners, WD40 proteins Uaf-1 and WDR20 [27]. Our reporter gene assays confirm this role of Uaf-1, we have shown that without Uaf-1, Usp12 was not able to increase AR transcriptional activity while Usp12 knockdown alone was sufficient to decrease it. This confirms the role Uaf-1 plays in regulating the activity of Usp12. In *S. pombe,* binding partners were shown to be necessary not only for DUB activity but also to affect cellular localisation of Usp12. Uaf-1 deletion traps Usp12 in the nucleus and analogously WDR20 deletion traps Usp12 in the cytoplasm [28]. It remains to be established if their role in Usp12 localisation is conserved in human.

DUBs have been previously shown to affect AR stability. Usp10 is reported to bind to AR and positively regulate its transcriptional activity [22]. Recently Usp10 effects on AR were proposed to be indirect via Usp10 mediated deubiquitination of H2A.Z leading to transcriptional activation of AR [35]. This hypothesis requires further investigation in the context of Usp12 as it was also shown to deubiquitinate histones H2A and H2B [23]. It

is possible that Usp12 effect on AR could be a combination of both direct deubiquitination and stabilisation of the receptor and indirect regulation through deubiquitination of histones which allows transcriptional activation. Similarly, Usp26 is reported to assemble with AR and additional cofactors in subnuclear foci resulting in AR deubiquitination [21]. We have also observed that Usp12 and AR colocalise and interact resulting in deubiquitination of AR. Co-localisation of AR and Usp12 occurs in the cytoplasm which can be explained by the large portion of the proteasome being present in the cytoplasm, it is also possible that for their co-localisation in the nucleus additional Usp12 co-factors would need to be co-transfected to facilitate its shuttling similarly to what was observed in *S. pombe*. This causes stabilisation of AR at a protein level. It has been previously published that silencing of AR results in G1 arrest and increased apoptosis, interestingly we have observed that Usp12 silencing has the same effects on PCa cells. These cellular effects might be mediated by a combination of both AR-mediated and AR-unrelated effects. AR stabilisation by Usp12 enhances transcriptional activity of AR and as a result increases cellular proliferation. We report that Usp12 acts in a pro-proliferative manner in PCa cells, this suggests that Usp12 may play a crucial role in prostate cancer development, progression and metastasis. It remains to be established if mutations in Usp12 that are associated with PCa occurrence and progression. Detailed impact of Usp12 in PCa and its potential as a therapeutic target need further assessment in the   future.

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FOOTNOTES

FIGURE LEGENDS

FIGURE 1 Usp12 is a co-regulator of AR as shown by an siRNA screen

(A) Results of the siRNA screen on the effects of DUBs knock down on AR activity presented as a readout of PSA protein levels measured by an ELISA in LNCaP cells. (B) LNCaP cells were treated with siRNA as indicated for 48h prior to DHT stimulation for 0-72 hours as indicated followed by lysis and immunoblotting. (C) LNCaP, LNCaP-AI and VCaP cells were treated with siRNA as indicated for 96h. Total RNA was extracted and quantitative RT-PCR performed to analyse the transcript levels of Usp12 and PSA normalised to *HPRT1*. Data are presented as mean +/- SEM of three independent experiments normalised to scrambled (SCR), statistical significance was analysed using t-test.

FIGURE 2 Usp12 stabilises AR at the protein level

(A-C) LNCaP, LNCaP-AI and VCaP cells respectively, were treated with siRNA as indicated. LNCaP and VCaP cells were cultured in full media and LNCaP-AI in DCC. At 96h cells were lysed followed by immunoblotting. (D) COS-7 cells were transfected with 500 ng of p-Flag-His-AR and increasing amounts (10-500 ng) of Wt and C48A mutant Usp12 for 48h. Reactions were balanced with empty pCMV vector. (E) HEK293T cells were transfected with pFlag-His-AR, pFlag-Usp12, pFlag-Uaf-1 and pHA-Flag-WDR20 plasmids as indicated, at 48h cells were lysed followed by Western blotting. (F) LNCaP cells were treated with siRNA as indicated for 96 hours followed by treatment with 1μM of cycloheximide for 0-4 hours.

FIGURE 3 Stabilisation of AR by Usp12 results in increased transcriptional activity of AR

(A) HEK293T cells were transfected with pARE3-luc, pCMV-β-gal, pFlag-AR pFlag-Usp12, and pFlag-Uaf-1 as indicated and cultured for 48h in steroid depleted conditions followed by addition of DHT for 24h. Results are represented as a luciferase count per second normalised to β-galactosidase activity. Data are a mean +/- SEM of three independent experiments normalised to AR alone,

statistical significance was analysed with t-test. (B) LNCaP-7B7 cells were incubated in steroid-depleted media followed by gene silencing after 24h. At 48h DHT was applied and luciferase quantity determined 48h later. Data was normalised to total protein content determined using a BCA assay. Data is displayed as the fold change in normalised luciferase activity compared to non-transfected cells without DHT treatment. Error bars represent SEM of three independent experiments. (C) LNCaP, LNCaP-AI and VCaP cells were treated with siRNA as indicated for 96h, total RNA was extracted and quantitative RT-PCR performed to analyse the transcript levels normalised to *HPRT1*. Data are a mean +/- SEM of three independent experiments normalised to SCR, statistical significance was analysed with t-test. (D) LNCaP cells were treated with siRNA for 48h followed by stimulation with DHT for 0-48h. Total RNA was extracted and quantitative RT-PCR performed to analyse the transcript levels normalised to *HPRT1*. Data are a mean +/- SEM of three independent experiments normalised to SCR, statistical significance was analysed using t-test. (E) LNCaP cells were treated with siRNA for 72h in steroid-depleted media. DHT was applied for 2h before cells were harvested and ChIP for AR recruitment to AREIII of *PSA* enhancer or control IgG performed. Data is represented as percentage recruitment relative to input and is the mean of three independent experiments +/- SEM.

FIGURE 4 Stabilisation of AR by Usp12 affects prostate cancer cell survival and proliferation

(A) LNCaP, LNCaP-AI and VCaP cells were treated with siRNA as indicated for 96h, following silencing cellular proliferation was assessed by cell counting and normalised to SCR. Data are a mean +/- SEM of three independent experiments, statistical analysis was undertaken with t-test; ***p<0.001, *p<0.05. (B) LNCaP cells were treated with siRNA as indicated for 96h, following silencing levels of apoptotic cells were determined by flow cytometry detection of Annexin V positive cells and results were normalised to SCR. Data are a mean +/- SEM of four independent experiments, statistical analysis was undertaken with ANOVA test. (C) LNCaP cells were treated with siRNA as indicated for 96h, following silencing cell cycle status was determined by flow cytometry analysis of PI staining. Ratio of cells in G1 vs S phase was determined and results were normalised to SCR. Data are a mean +/- SEM of three independent experiments, statistical analysis was undertaken with ANOVA test. (D) LNCaP cells were treated with siRNA as indicated for 96h, following silencing the number of cells in subG1 phase was determined by flow cytometry analysis of PI staining. Results were normalised to SCR. Data are a mean +/- SEM of three independent experiments, statistical analysis was undertaken with ANOVA test. (E) LNCaP cells were treated with siRNA as indicated for 72h, following silencing cells were seeded at equal numbers with varied densities in 6-well plates and incubated for 14 days. At day 14 cells were fixed and stained with crystal violet, number of colonies in each sample was determined and normalised to SCR. Data are a mean +/- SEM of three independent experiments, statistical analysis was undertaken with t-test. Images show growth 14 days after seeding 4,000 cells per 2mm well.

FIGURE 5 Usp12 interacts with AR resulting in AR deubiquitination and AR stabilisation

(A) HEK293T cells were transfected with plasmids as indicated, wild type (Usp12) and Cys48 deficient mutant (Usp12M) of Usp12 were used. 48h post transfection cells were harvested and lysates were immunoprecipitated (IP) with 1μg of AR antibody followed by immunoblotting. (B) LNCaP cells were harvested and lysates immunoprecipitated for AR and with a non-specific IgG. (C) COS-7 cells were transfected with plasmids as indicated, wild type (Usp12) and Cys48 deficient mutant (Usp12M) of Usp12 were used. 72h post transfection cells were treated with MG132 and harvested 16h later. Lysates were denatured and subsequently immunoprecipitated (IP) with 1μg of Ubiquitin antibody followed by immunoblotting. (D) COS-7 cells were grown in steroid depleted media and transfected with equal amounts of Usp12-Flag and AR plasmids, 48h post transfection cells were fixed and immunofluorescence followed by confocal microscopy were used to visualise both proteins cellular localisation, DAPI was used as a nuclear stain. Scale bar = 20μm. (E) Usp12 immunohistochemistry of PCa and benign patient samples. Images are representatives of n=7 for each group. Bar = 50μm.
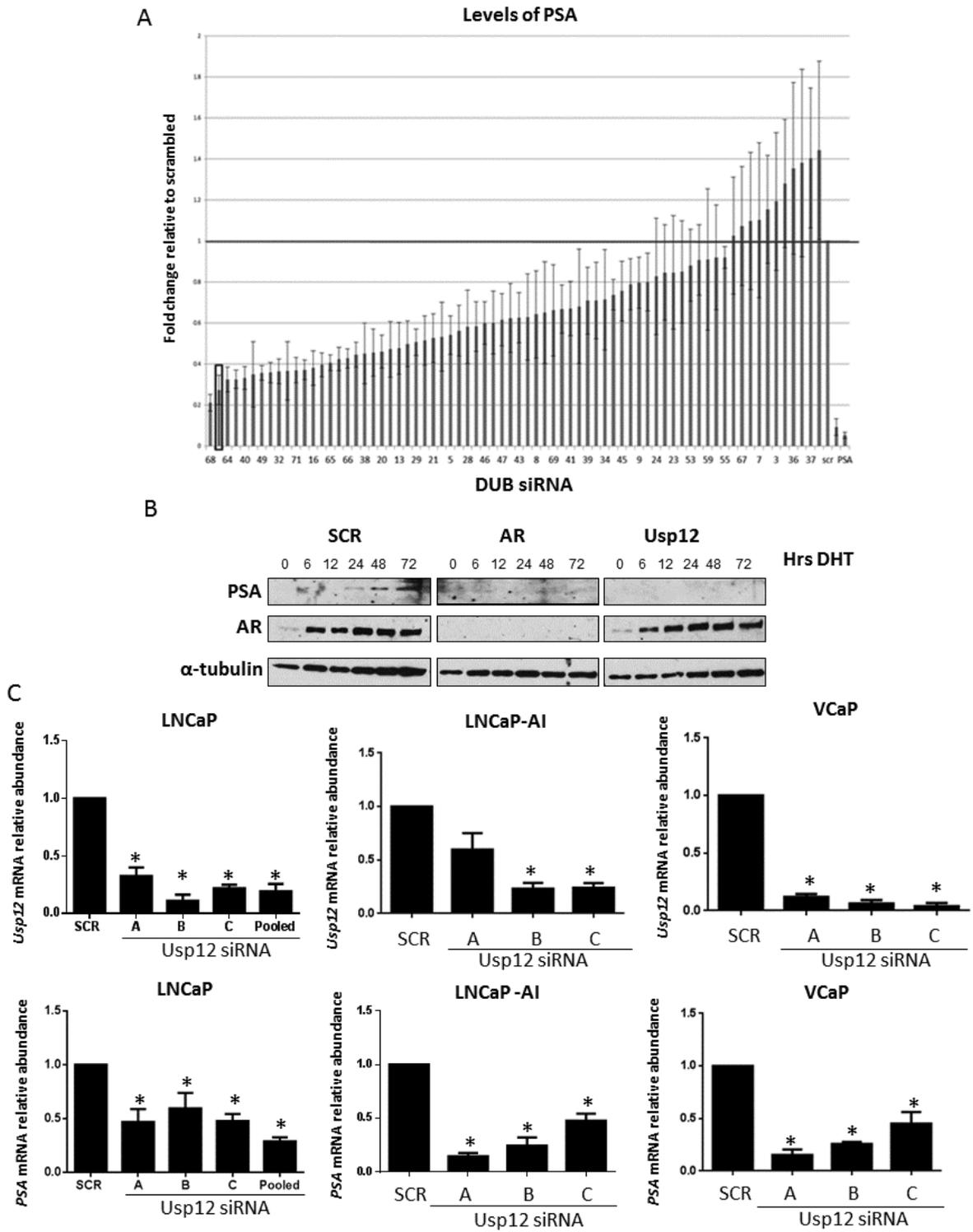
Figure 1

Figure 2

A

LNCaP

Usp12

	SCR	A	B	C
Usp12				
AR				
α-tubulin				

B

LNCaP-AI

Usp12

	SCR	A	B	C
Usp12				
AR				
α-tubulin				

C

VCAP

Usp12

	SCR	A	B	C
Usp12				
AR				
α-tubulin				

D

Usp12$_{C48A}$ Usp12

AR

Usp12

α-tubulin

E

AR	+	+	+	+	+
Usp12	-	+	+	-	+
Uaf-1	-	+	-	+	+
WDR20	-	-	+	+	+

AR

Usp12

α-tubulin
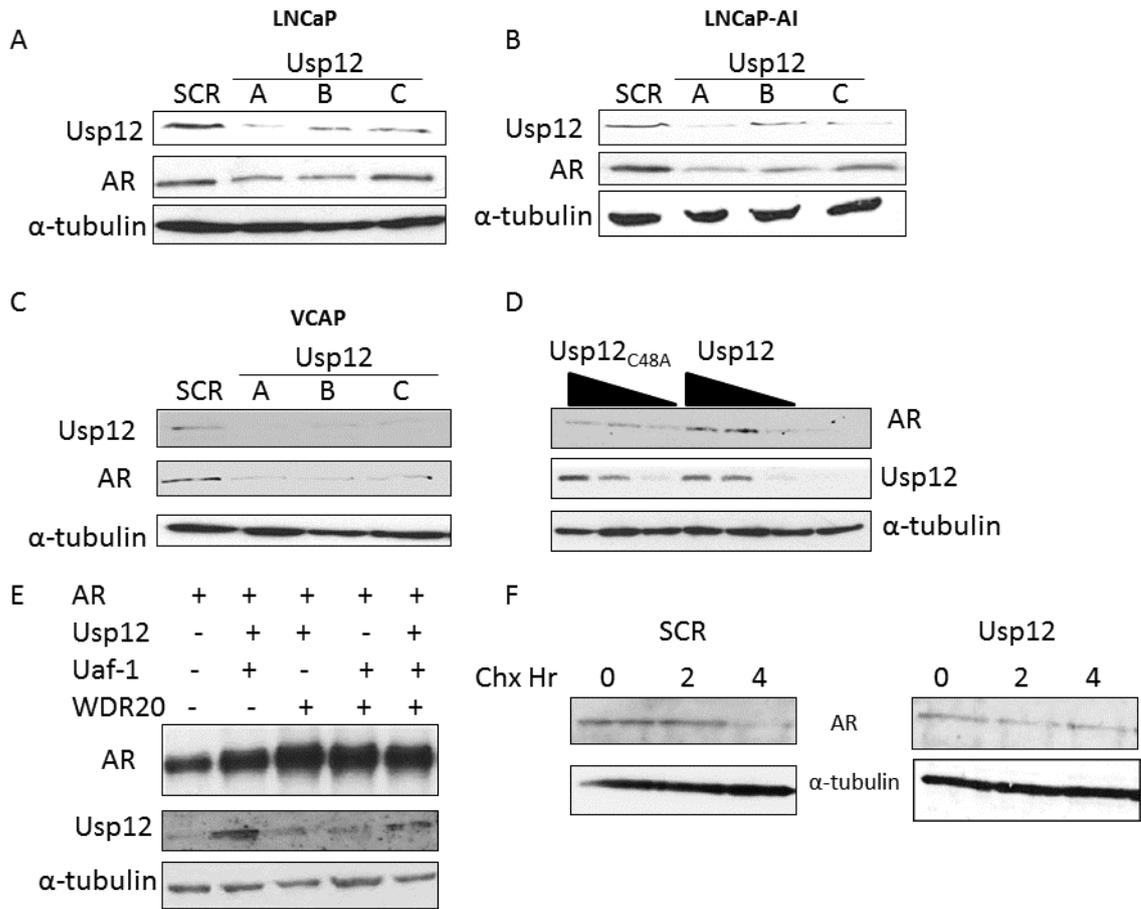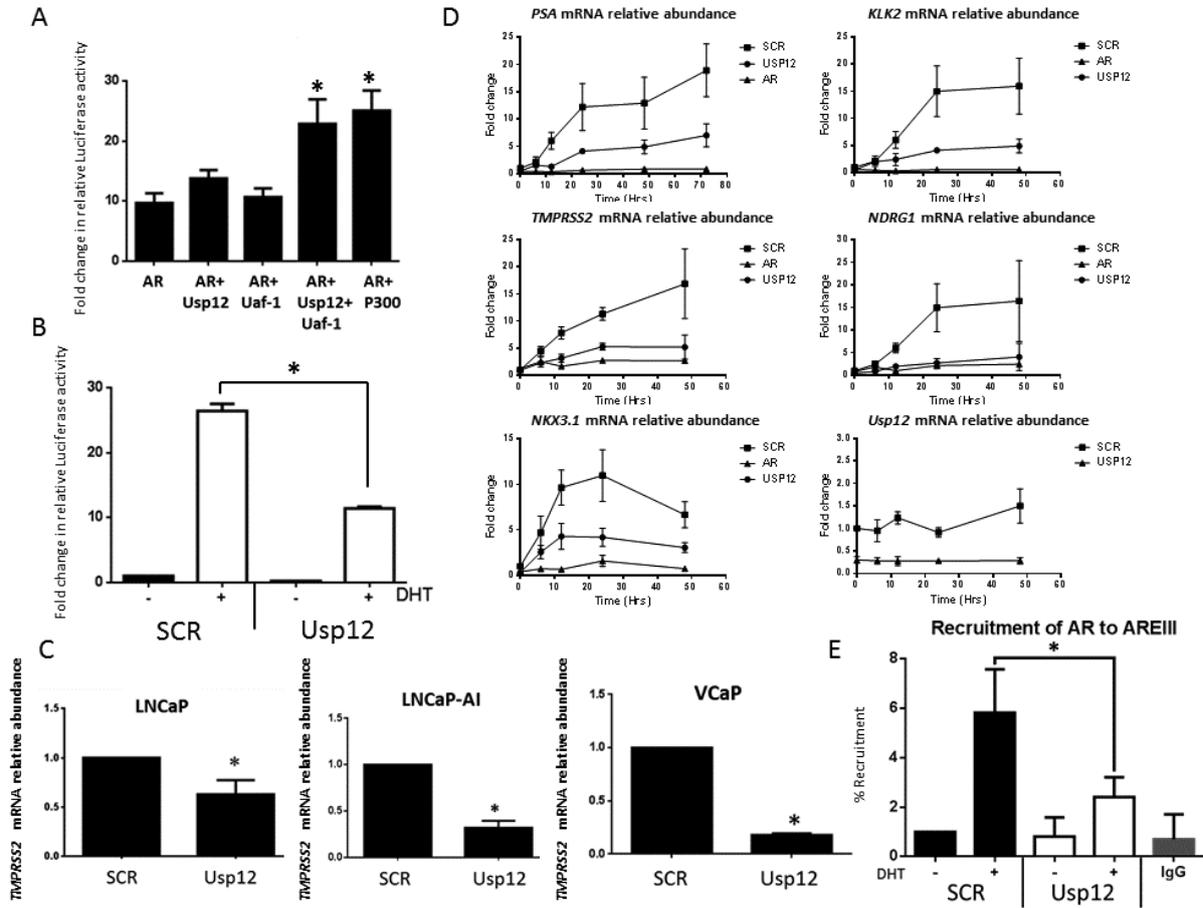
F

SCR

Chx Hr	0	2	4

AR

α-tubulin

Usp12

	0	2	4

Figure 3

Figure 4

Figure 5