

Regulation of androgen receptor and histone deacetylase 1 by Mdm2-mediated ubiquitylation

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Received November 12, 2004; Revised and Accepted December 1, 2004

ABSTRACT

The androgen receptor (AR) is a member of the nuclear hormone receptor family of transcription factors and plays a critical role in regulating the expression of genes involved in androgen-dependent and -independent tumour formation. Regulation of the AR is achieved by alternate binding of either histone acetyltransferase (HAT)-containing co-activator proteins, or histone deacetylase 1 (HDAC1). Factors that control AR stability may also constitute an important regulatory mechanism, a notion that has been confirmed with the finding that the AR is a direct target for Mdm2-mediated ubiquitylation and proteolysis. Using chromatin immunoprecipitation (ChIP) and re-ChIP analyses, we show that Mdm2 associates with AR and HDAC1 at the active androgen-responsive PSA promoter in LNCaP prostate cancer cells. Furthermore, we demonstrate that Mdm2-mediated modification of AR and HDAC1 catalyses protein destabilization and attenuates AR activity, suggesting that ubiquitylation of the AR and HDAC1 may constitute an additional mechanism for regulating AR function. We also show that HDAC1 and Mdm2 function co-operatively to reduce AR-mediated transcription that is attenuated by the HAT activity of the AR co-activator Tip60, suggesting interplay between acetylation status and receptor ubiquitylation in AR regulation. In all, our data indicates a novel role for Mdm2 in regulating components of the AR transcriptosome.

INTRODUCTION

The androgen receptor (AR) is a member of the nuclear hormone receptor (NR) superfamily of transcription factors,

and in response to androgenic signals derived from the testes, plays a pivotal role in regulating genes involved in growth, development and transformation of the prostate (1,2). Similar to other NR family members, the AR contains several independently functioning domains which facilitate the mechanics of receptor activity, including nuclear localization, DNA- and ligand-binding and transcriptional activation (3,4). Although predominantly cytoplasmic in the absence of its cognate ligand androgen, the AR rapidly translocates to the nucleus upon hormone association and subsequently binds and activates numerous androgen-responsive genes (5,6).

AR-mediated transcription requires the concerted function of two inherent transcriptional activation domains, namely the N-terminal activation function 1 (AF-1) and the C-terminal activation function 2 (AF-2) (3). Although not clearly defined, several lines of evidence suggest that the N- and C-termini of the AR interact in a ligand-dependent manner to provide a suitable platform for co-accessory protein interactions that are requisites for AR regulation (7,8). To date, numerous co-accessory proteins have been characterized to either enhance (co-activate) or reduce (co-repress) AR-mediated transcription [for review, see (9–11)]. Intriguingly, most co-activators and co-repressors share the capacity to influence transcriptional potential of the receptor by regulating the acetylation status of androgen responsive genes and/or the AR itself, via their respective histone acetyltransferase (HAT) or histone deacetylase (HDAC) activities. Indeed, we and others have demonstrated that the co-activators Tip60 (12), p300 and PCAF (13) enhance the inherent transcriptional activity of the AR by direct receptor acetylation and up-regulate transcriptional rate by histone acetylation of AR target genes. Conversely, AR activity has been shown to be down-regulated by histone deacetylase 1 (HDAC1) in a deacetylase-dependent manner (12,14), suggesting that reversal of HAT activity is important for abrogating AR function.

Ubiquitin-dependent proteolysis represents an important mechanism for controlling protein turnover and is pertinent to the regulation of numerous transcription factors (15,16). Protein ubiquitylation is catalysed by a trimeric enzymatic

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reaction containing an E1 ubiquitin-activating enzyme, which acts to prime the 76 amino acid ubiquitin peptide for attachment; an E2 conjugating enzyme which aids in ubiquitin conjugation to lysine residues within the target; and an E3 ubiquitin ligase protein which is important for establishing reaction specificity (17). In most cases, catalysis terminates after numerous cycles resulting in the formation of long polyubiquitin chains upon the target protein that are duly recognized by the 26S proteasome as a signal for destruction.

The recent evidence that several NRs are targets for direct ubiquitylation and subsequent destruction provides an additional mechanism for receptor regulation. For example, both the oestrogen receptor (ER) (18–20) and glucocorticoid receptor (21,22) are rapidly ubiquitylated and destroyed after binding to their cognate hormone, suggestive of a highly acute transcriptional regulatory process. Furthermore, additional data regarding the ER have indicated that while the proteasomal machinery is required for ER destruction, it also plays a vital role in ER-mediated transcription (20), indicating that there is an intimate relationship between the activation and destruction of the ER that are both regulated in part by components of the proteolytic machinery.

In keeping with other NR family members, the AR has recently been shown to be a target for ubiquitylation and destruction by the 26S proteasome (23), revealing another level of AR down-regulation in addition to that imposed by HDAC1. Furthermore, the demonstration that inhibition of proteasomal activity by the small molecule inhibitor MG132 abolishes productive rounds of AR-mediated transcription upon the androgen-responsive PSA promoter (24) suggests that AR turnover is intrinsically linked to transcriptional activity. This notion was further supported with the finding that the S1 subunit of the 19S proteasome sub-complex is recruited to the active PSA promoter with association kinetics parallel to the AR (24).

Of the many candidate E3 ligases for AR ubiquitylation, the *mdm2* proto-oncogene Mdm2 E3 ligase has recently been shown to catalyse AR ubiquitylation and proteolysis *in vivo* (25). Although this work confirmed a role for Mdm2 as an E3 ligase for the AR, the mechanics of androgen-dependent Mdm2 function remain largely ill-defined. Given that Mdm2 has recently been shown to associate directly with the active oestrogen-responsive pS2 promoter in complex with the ER (20), we sought to investigate a potential involvement of Mdm2 in AR-mediated transcription at the PSA promoter. Using a combination of chromatin immunoprecipitation (ChIP) and re-ChIP analyses, we show that Mdm2 associates directly with the active PSA promoter and is a component of a promoter-bound multimeric complex containing AR and HDAC1. By western analysis and immunoprecipitation experiments, we demonstrate that both AR and HDAC1 are ubiquitylated in response to androgen and that HDAC1 is a direct target for modification by Mdm2. Furthermore, we provide evidence that the deacetylase activity of HDAC1 is required for optimal Mdm2-mediated AR ubiquitylation and destruction that is attenuated by the acetyltransferase activity of Tip60, suggesting competition between ubiquitylation and acetylation status is a mechanism of receptor regulation, as seen for p53 (26). In all, our data provide a novel insight into the function of Mdm2 in the acute regulation of AR-mediated gene expression that may provide new targets for therapeutic intervention.

MATERIALS AND METHODS

Plasmids

The following plasmids have been described previously: pPSALuc, pCMV- β -gal, pcDNA3-AR, pCMV-Tip60, pCMV-Tip60_{Q377E/G380E} (27), pBJ5-FLAG-HDAC1 and pBJ5-FLAG-HDAC1_{H141A} (gifts from Stuart Schreiber, Harvard Medical School) (28,29), pxj41-Mdm2 and the ubiquitylase-dead mutant pxj41-Mdm2_{C462A} (gifts from Bohdan Wasylyk, Institute of Genetics and Molecular and Cellular Biology, Illkirch Cedex, France) (30,31), pcDNA3-His-Ubiquitin (gift from David Lane, University of Dundee, UK) and pCMV-c-abl (gift from Ygal Haupt, The Hebrew University Hadassah Medical School, Jerusalem) (32).

Cell culture and DNA transfection

Cell culture and DNA transfection were performed as described previously (12). COS-7 cells were maintained in RPMI 1640 media containing 10% fetal calf serum (FCS) (Gibco BRL), 1% penicillin and 1% streptomycin. For reporter assays, 1×10^4 COS-7 cells were routinely plated per well in 48 microtitre plates (Corning). After 24 h, the cells were transfected using Superfect (Qiagen) according to the manufacturer's recommendations. After 2 h, the cells were washed and incubated in RPMI 1640 containing 10% FCS which had been stripped of steroids by treatment with dextran-coated charcoal prior to experimentation with 10 nM R1881 (synthetic androgen analogue). After 48 h, the cells were harvested and assayed for luciferase activity according to the manufacturer's guidelines (Promega). Luciferase activity was corrected for the corresponding β -galactosidase activity to give relative activity, as described previously (27). For immunoprecipitation of ectopically expressed proteins, 5×10^5 COS-7 cells were plated per 90 mm dish (Corning), and transfected as above with 5 μ g of each expression vector.

Unless indicated, co-transfection experiments for reporter assays using COS-7 cells incorporated 50 ng of each expression vector and 200 ng of each reporter construct. Fold increases were determined for 50 ng expression vector by comparing activity with empty pCMV-driven vector. Each experiment was performed in triplicate and repeated a minimum of three times. Transfection of 90 mm dishes incorporated 10 μ g of each expression vector for COS-7 cells. An aliquot of 10 nM R1881, 1 μ M of the proteasomal inhibitor MG132, 1 μ M of the deacetylase inhibitor trichostatin A (TSA) and 1 μ M of the translation inhibitor cycloheximide (CHX) were routinely used for all experiments.

Western blotting and antibodies

Western analysis was performed as described previously (12). Monoclonal antibodies for AR (BD Pharmingen), HDAC1 (Upstate Biotechnology), Mdm2 and ubiquitin (Santa Cruz Biotechnology) were used according to the manufacturer's recommendations.

Immunoprecipitation

AR immunoprecipitation from LNCaP cells was performed as described previously (12) using a polyclonal anti-AR antibody (Santa Cruz), while HDAC1 was immunoprecipitated from the same cell lines using a monoclonal HDAC1 antibody (Upstate

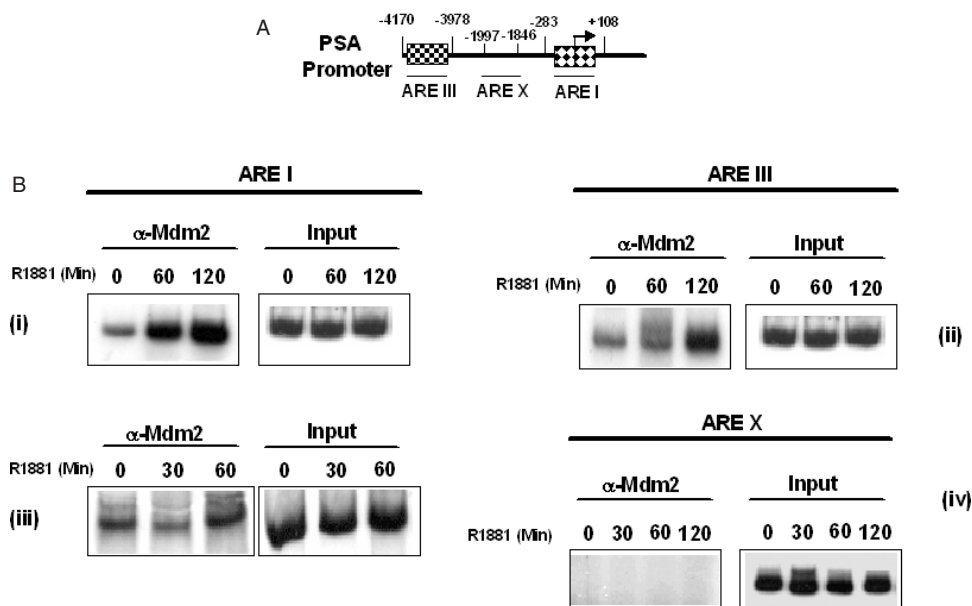


Figure 1. Mdm2 association with the PSA promoter is up-regulated in an androgen-dependent manner. ChIP assays were performed in LNCaP cells to investigate Mdm2 association with the PSA promoter in the presence and absence of androgen (R1881). (A) Schematic representation of PSA promoter and ChIP PCR: length of PCR product is shown by black bars and numbers. (B) Soluble chromatin extracts from LNCaP cells treated with or without 10 nM androgen for 0–120 min were immunoprecipitated with antibodies against Mdm2 followed by semi-quantitative PCR incorporating primers specific for ARE I (i and iii), ARE III (ii) or AREX (iv). Input samples containing crude chromatin extracts prior to immunoprecipitation were also analysed.

Biotechnology). Immunoprecipitates were boiled in SDS sample buffer (10% β -mercaptoethanol, 125 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol and 0.005% bromophenol blue) for 10 min and subject to western analysis (see above). To assess AR ubiquitylation by Mdm2, plasmids for AR, Mdm2 or the ubiquitylase-dead Mdm2_{C462A} mutant together with pcDNA3-His-ubiquitin were transiently transfected into COS-7 cells and after 48 h, lysed in His-lysis solution (6 M guanidinium-Cl, 0.1 M, Na₂HPO₄/NaH₂PO₄, 0.01 M Tris-HCl, pH 8, 5 mM imidazole and 10 mM β -mercaptoethanol). To immunoprecipitate ubiquitylated proteins, 50 μ l equilibrated nickel-agarose (Ni-NTA Qiagen) was added to each sample and incubated at 4°C for 8 h, followed by stepwise washes of the pelleted agarose in wash solutions 1 (8 M urea, 0.1 M Na₂HPO₄/NaH₂PO₄, 0.01 M Tris-HCl, pH 8, 10 mM β -mercaptoethanol) and 2 (8 M urea, 0.1 M Na₂HPO₄/NaH₂PO₄, 0.01 M Tris-HCl, pH 6.3, 10 mM β -Mercaptoethanol and 0.1% triton). Proteins were eluted from the agarose using 70 ml elution solution (200 mM imidazole, 0.15 M Tris-HCl, pH 6.7, 30% glycerol, 0.72 M β -mercaptoethanol and 5% SDS), followed by boiling in SDS sample buffer as above. Immunoprecipitates were subject to western analysis using an anti-AR antibody.

ChIP assays

ChIP assays were performed as described previously (12). For immunoprecipitation, 2 μ g of polyclonal AR antibody and 2 μ g monoclonal HDAC1, Mdm2 and ubiquitin antibodies were used as indicated. Re-ChIP analysis was performed as described previously (20). Briefly, AR, Mdm2 and ubiquitin antibodies were added to chromatin extracts for 5 h followed by the addition of 60 μ l salmon sperm/protein A agarose (Upstate Biotechnology) to recover immunocomplexes.

AR-, Mdm2- and ubiquitin-containing complexes were eluted by 1 h incubation in re-ChIP 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 2 μ g antibodies for AR, Mdm2, HDAC1 or anti-VP16 for control, to an equal volume of eluted material. Recovery and preparation of DNA was performed as described previously (12). Semi-quantitative PCR was performed with 10 μ l of DNA, BioTaq DNA polymerase and [α -³²P]dATP, using the following primers: ARE IF: TCT GCC TTT GTC CCC TAG AT and ARE IR: AAC CTT CAT TCC CCA GGA CT to amplify 235 bp of the proximal PSA promoter, encompassing the ARE I (see Figure 1A); ARE IIIF: CCT CCC AGG TTC AAG TGA TT and ARE IIIR: GCC TGT AAT CCC AGC ACT TT to amplify the distal ARE III; ARE XF: CTG TGC TTG GAG TTT ACC TGA and ARE XR: GCA GAG GTT GCA GTG AGC C to amplify a non-ARE containing portion of the PSA promoter. PCR products were resolved, dried and then exposed to X-ray film for 2–12 h. ChIP data are representative of triplicate experiments performed using similar passage number LNCaP cells.

RESULTS

Mdm2 associates with the AR-responsive PSA promoter in an androgen-dependent manner

The demonstration that abrogation of proteasomal activity by the small molecule inhibitor MG132 increases AR stability provided evidence that AR proteolysis constitutes an additional mechanism for AR regulation (23). This notion was confirmed with the finding that the AR is a direct target for Mdm2-mediated ubiquitylation and proteasomal destruction (33). In light of the fact that Mdm2 has been identified

to associate with the ER at the active ER-responsive pS2 promoter (20), suggestive of a role for the E3 ligase in ER function, we figured that Mdm2 may also have the potential to associate with the AR at active androgen-responsive genes as a mechanism of transcriptional regulation. Using ChIP assays in AR-expressing LNCaP prostate cancer cells, incorporating an Mdm2 antibody, we examined the association of the E3 ligase with the AR-responsive PSA promoter in the presence and absence of the synthetic androgen, R1881. Using primers specific to both the proximal ARE I and distal ARE III (see Figure 1A), we sought to examine potential differences in Mdm2 association at these AR-binding sites. As shown in Figure 1B, in the absence of androgens, a small amount of Mdm2 was detected at both ARE I and ARE III, but not at a region lacking a functional androgen response element (ARE X), indicating that Mdm2 may associate with the proximal and distal AREs of the inactive promoter. However, the fact that both AR and HDAC1 associate with the promoter in the absence of androgen (Figure 2A) suggests that it is a common phenomenon and may be explained by the promiscuity of the endogenous AR_{T877A} mutant, expressed in LNCaP cells, for binding trace levels of androgens and other steroids within our experimental system (34,35). A 60 min exposure

of cells to androgen caused a robust increase in Mdm2 association at ARE I that was increased further after 120 min hormone treatment [Figure 1B (i), compare lanes 2 and 3 with 1]. A similar pattern of Mdm2 recruitment was observed at the AREIII site [Figure 1B (ii)], but not at AREX [Figure 1B (iv)], suggesting that Mdm2 association is conserved at both the proximal and enhancer regions of the PSA promoter and is dependent upon AR-binding sites.

Mdm2 associates with AR and HDAC1 at the PSA promoter

Our previous findings that both AR and HDAC1 are recruited to ARE I of the PSA promoter after 20 min androgen treatment was suggestive of an acute mechanism for AR regulation (12). To assess a role for Mdm2 in AR regulation at the promoter, we first extended our ChIP analysis to examine AR and HDAC1 association over a period of 120 min. As shown in Figure 2A, both AR and HDAC1 recruitment to ARE I increased after 30 min hormone treatment as expected, decreased after 60 min and subsequently increased after 120 min stimulation, suggesting in a manner similar to that observed for the ER and components of the ER transcriptosome (20), active AR and HDAC1

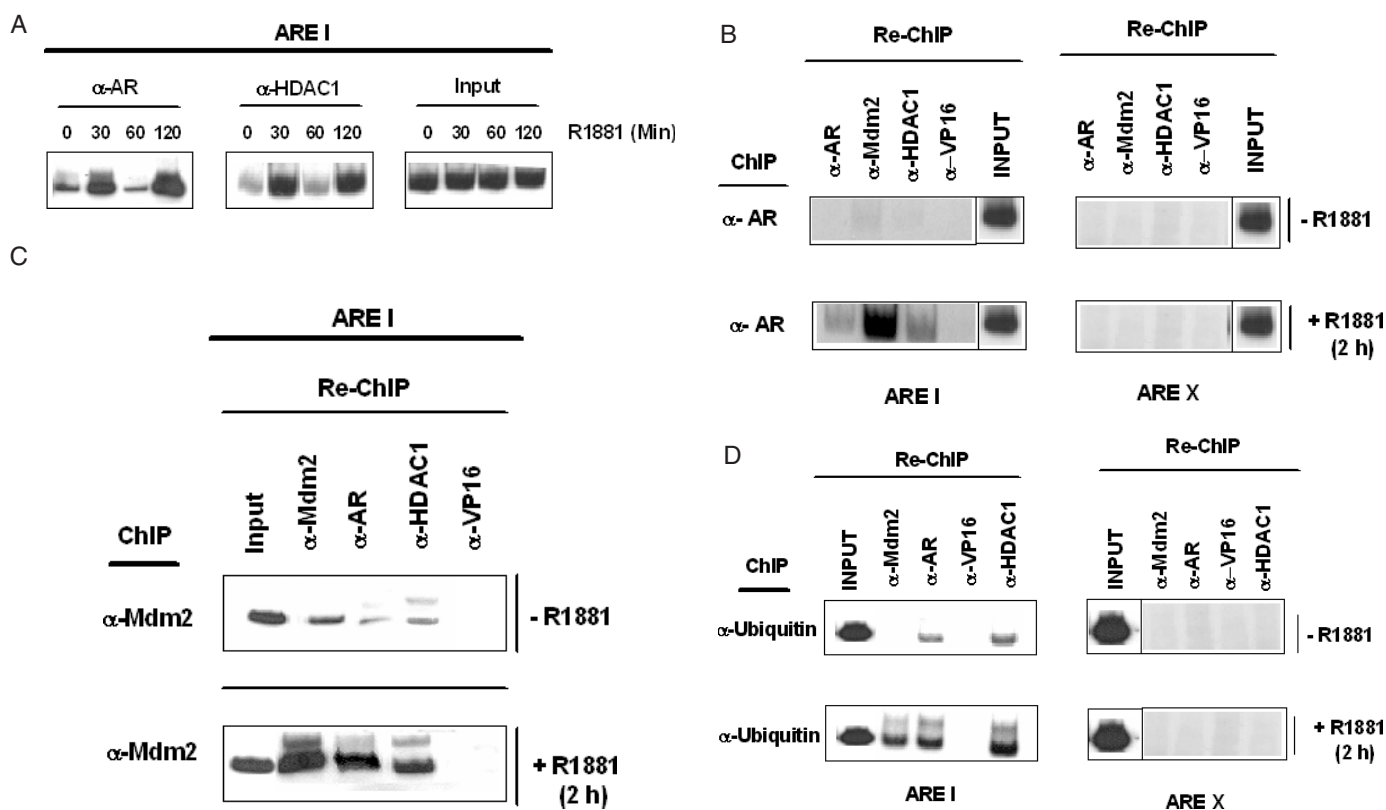


Figure 2. Mdm2 co-associates with AR and HDAC1 at the PSA promoter. ChIP assays were performed in LNCaP cells, using primers specific to ARE I, to assess HDAC1 and AR association with the PSA promoter. (A) Soluble chromatin extracts from LNCaP cells treated with or without 10 nM androgen for 0–120 min were immunoprecipitated with antibodies against AR or HDAC1 followed by semi-quantitative PCR. (B and C) To investigate potential interaction between AR, Mdm2 and HDAC1 at the PSA promoter, re-ChIP analysis was performed using LNCaP cells treated with or without androgen for 120 min. Chromatin extracts immunoprecipitated with anti-AR (B) or -Mdm2 (C) antibodies were re-immunoprecipitated with antibodies to AR, Mdm2, HDAC1 or VP16 for control followed by semi-quantitative PCR incorporating primers specific for ARE I and ARE X. (D) To examine changes to the ubiquitylation status of the AR transcriptosome upon the PSA promoter, ChIP analysis was performed using an anti-ubiquitin antibody followed by re-ChIP analysis using antibodies for AR, Mdm2, HDAC1 and VP16.

display dynamic association-dissociation kinetics upon the PSA promoter. In contrast, Mdm2 recruitment to ARE I is minimal after 30 min androgen stimulation [Figure 1B (iii)], but increases after 60 min and further by 120 min hormone addition. In all, while AR and HDAC1 display altered association kinetics to that of Mdm2 from 0 to 60 min androgen treatment, data from the 120 min time-point provide evidence that AR, Mdm2 and HDAC1 may co-associate upon the proximal PSA promoter. Given the recent evidence regarding the cyclical nature of Mdm2 upon the ER-responsive pS2 reporter (20), it is likely that our broad time-frames for the analysis are unable to resolve the true nature of Mdm2 kinetics, and by incorporating additional time-points, we speculate that AR, Mdm2 and HDAC1 co-association is a more common event.

To test whether AR, Mdm2 and HDAC1 are associated in the same complex on the ARE I of the PSA promoter, we performed ChIP analysis in LNCaP cells with an antibody against AR and then re-chromatin immunoprecipitated (re-ChIP) the crosslinked AR-containing complexes with antibodies specific to AR, Mdm2 and HDAC1 as well as an anti-VP16 antibody for control. In the absence of androgen, antibodies to AR and HDAC1 failed to generate PCR products for the ARE I region of the PSA promoter, while the Mdm2 antibody produced a weak signal that may suggest the presence of a sub-population of Mdm2 that is not directly associated with the AR, but does interact with ARE I of the PSA promoter. After 120 min androgen stimulation, the amount of AR, Mdm2 and HDAC1 immunoprecipitated from the initial AR-containing complex increased at ARE I (Figure 2B, +R1881), but not at ARE X, indicating that AR, Mdm2 and HDAC1 co-associate specifically at AR-responsive regions of the PSA promoter in response to androgen stimulation. The fact that the control anti-VP16 antibody failed to generate PCR products both in the presence and absence of ligand provides evidence that our experimental system is specific in identifying components of the AR complex at the PSA promoter and indicates no cross-reactivity between antibodies used in the primary ChIP with those used to re-ChIP the immunocomplex.

To confirm the notion that AR, Mdm2 and HDAC1 co-associate at the active PSA promoter, we performed the reciprocal experiment using an anti-Mdm2 antibody for ChIP, and antibodies for AR, Mdm2, HDAC1 and VP16 in re-ChIP, followed by PCR using primers for ARE I. As shown in Figure 2C, in the absence of ligand, we observed a small amount of Mdm2, AR and HDAC1 at ARE I, which was increased robustly upon androgen treatment, confirming that AR, Mdm2 and HDAC1 co-associate upon the PSA promoter in response to AR activation.

Given that Mdm2 is an E3 ubiquitin ligase, and that AR (23,33), HDAC1 (36,37) and Mdm2 (38) are each targets for ubiquitylation, we next sought to establish whether these components of the AR transcriptosome are ubiquitylated at the PSA promoter. The recent demonstration that components of the ER signalling cascade are subject to ubiquitylation at the oestrogen-responsive pS2 promoter indicated that ubiquitylase activity occurs at an early stage in the transcriptional process and is likely mediated by promoter-bound E3 ligases, such as Mdm2 (20). Using an anti-ubiquitin antibody in ChIP analysis, and then antibodies against Mdm2, AR and HDAC1 in re-ChIP analysis, we examined the association of Mdm2, AR and HDAC1 with the ubiquitylated protein fraction at ARE I

and ARE X of the PSA promoter in the absence and presence of androgens. In the absence of hormone, AR and HDAC1, but not Mdm2 or the negative control VP16, were identified in the ubiquitylated fraction (Figure 2D, -R1881), suggesting that the small amount of AR and HDAC1 identified at the inactive ARE I (Figure 2A) is either directly ubiquitylated or is associated with a modified chromatin complex. After 120 min hormone treatment, AR, Mdm2 and HDAC1 association with the ubiquitylated fraction increased strongly (Figure 2D, +R1881), indicating that the extent of ubiquitylation at ARE I is responsive to the ligand-bound status of the AR, and may suggest that Mdm2, AR and HDAC1 are all targets for ubiquitylation at this early stage of the transcriptional process. Furthermore, the failure of the ARE X primers to generate a PCR signal in both the presence and absence of androgens indicates that changes to the ubiquitylation status at the PSA promoter is specific to sites of AR association.

AR is ubiquitylated in response to androgen treatment

The correlation between increased association of AR with the ubiquitylated protein fraction and androgen treatment led us to investigate changes in the ubiquitylation status of the receptor upon hormone stimulation. Considering that AR (and HDAC1) were potentially modified after 2 h AR activation in re-ChIP analysis, we monitored changes to AR mobility by western analysis, incorporating an anti-AR antibody, following 0–5 h androgen exposure. Furthermore, to enhance visualization of potentially ubiquitylated receptor proteins, LNCaP cells were treated with 1 μ M MG132 prior to ligand treatment. After 1 h androgen exposure, we observed the appearance of two higher molecular weight AR species, \sim 110 and 135 kDa in size, indicative of mono- and tri-ubiquitylated receptor proteins (Figure 3A, compare lanes 2–5 with lane 1). The appearance and increase in intensity of an additional slower-migrating receptor form of \sim 250 kDa between 3 and 5 h post-hormone addition (Figure 3A, see lanes 4 and 5) was suggestive of a polyubiquitylated AR protein whose presence, like both the 120 and 135 kDa species, was androgen dependent. To confirm androgen-dependent AR ubiquitylation, we immunoprecipitated AR from 5 h androgen-treated LNCaP cells and examined AR ubiquitylation using an anti-ubiquitin antibody. As shown in Figure 3B, AR ubiquitylation is markedly enhanced after hormone treatment compared with AR immunoprecipitated from cells not stimulated with androgen, indicating that AR activity correlates with ubiquitylation status, and may initiate at the promoter level.

Given that polyubiquitination, in general, constitutes a destructive signal recognized by the proteasome, it was pertinent to examine changes to AR protein levels during hormone stimulation. Extending the time-course in Figure 3A to 8 h androgen exposure, we show that receptor levels decrease modestly between 2 and 4 h, but fail to decrease further after 8 h hormone addition (Figure 3C, left panel). The fact that the AR gene is a direct target for AR-mediated transcription (39) may suggest that the active receptor maintains its own protein levels by up-regulating the AR production in response to increased proteolysis. To test this hypothesis, we analysed changes to AR protein levels as before, but from cells pre-treated with 1 μ M of the translation inhibitor CHX. We figured that analysis of

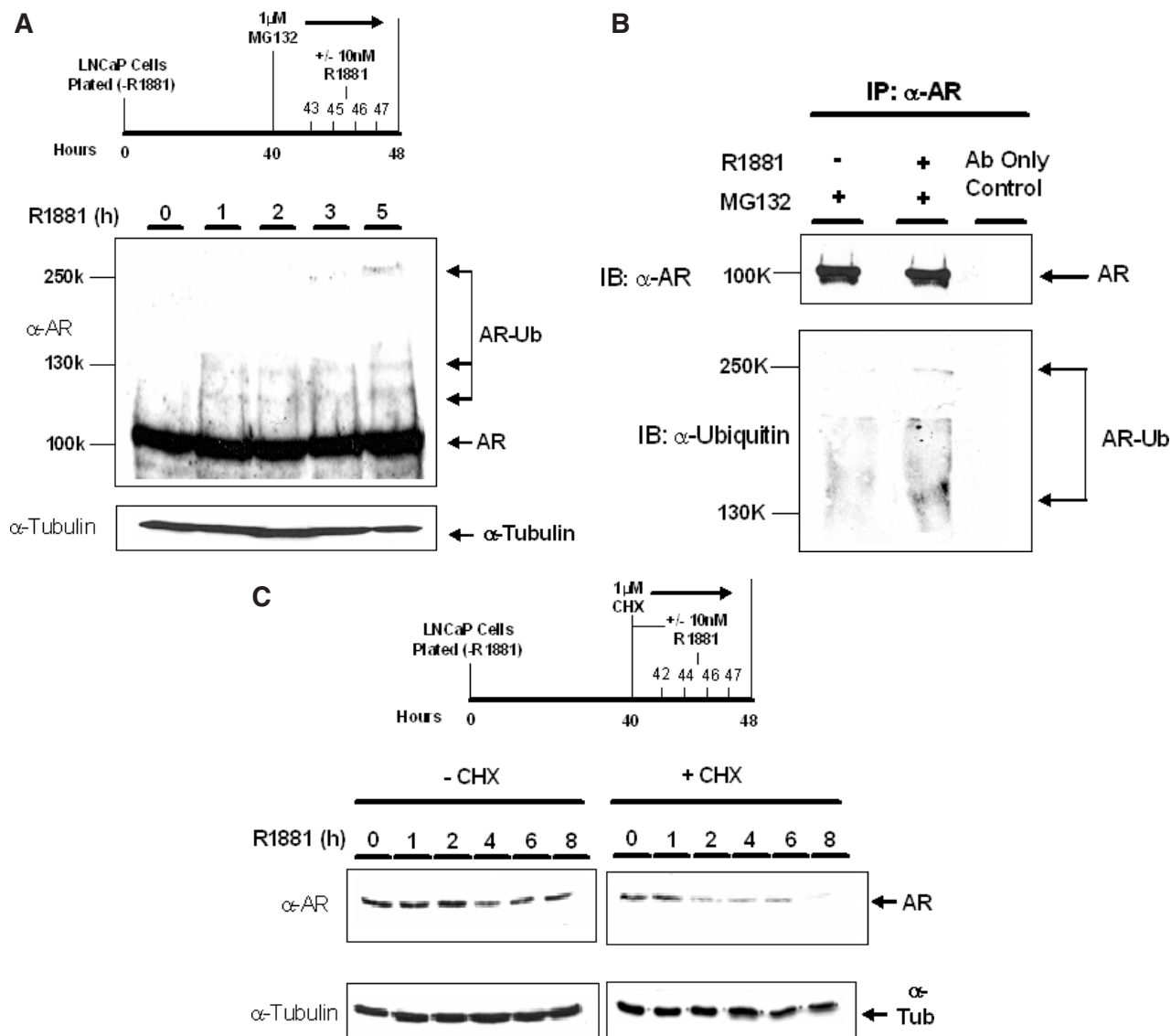


Figure 3. AR is a target for androgen-dependent ubiquitylation. (A) To investigate whether androgen treatment stimulates AR modification, LNCaP cells were treated with 1 μ M MG132 for 8 h and 10 nM R1881 for 0–5 h, and higher molecular weight AR species were identified by western analysis using an anti-AR antibody. (B) Following 0 or 5 h R1881 and 8 h MG132 treatment, LNCaP cells were subject to immunoprecipitation using an anti-AR antibody and ubiquitylated species were detected by western analysis using an anti-ubiquitin antibody. (C) To assess whether hormone-dependent AR ubiquitylation confers AR destabilization, we examined AR protein levels in LNCaP cells treated with androgen for 0–8 h by western analysis as above. AR synthesis was attenuated with 1 μ M CHX treatment for 8 h to assess turnover of a single receptor population.

a single receptor population may provide a more decisive model for testing the effects of androgen-dependent ubiquitylation. As shown in Figure 3C (right panel), AR protein levels decreased rapidly after 2 h androgen treatment leading to a near complete loss of the receptor 8 h post-hormone addition, indicating androgen-stimulated receptor ubiquitylation confers a signal for protein destabilization that is seen to be amplified when examining a single AR population in the absence of *de novo* protein synthesis.

Mdm2-mediated AR ubiquitylation and destruction is blocked by c-abl

We next tested the ability for Mdm2 to directly ubiquitylate the AR by immunoprecipitation in an effort to confirm

previous reports (33). COS-7 cells transiently transfected with AR, His-tagged ubiquitin and either Mdm2, a ubiquitylase-inactive Mdm2 mutant, Mdm2_{C462A}, or an empty vector for control, in the presence of androgen and 1 μ M MG132, were subject to immunoprecipitation using nickel-agarose (to bind his-ubiquitylated proteins) followed by western analysis incorporating an AR antibody. As shown in Figure 4A, ectopic expression of Mdm2, but not the ubiquitylase-dead Mdm2_{C462A} mutant, resulted in increased levels of higher molecular weight AR species that was not due to unequal loading of the samples (Figure 4A, see input). In the absence of MG132, AR protein levels were reduced in the presence of ubiquitylase activity (Figure 4B), confirming the previous finding that Mdm2-mediated AR ubiquitylation is necessary for receptor destruction (33).

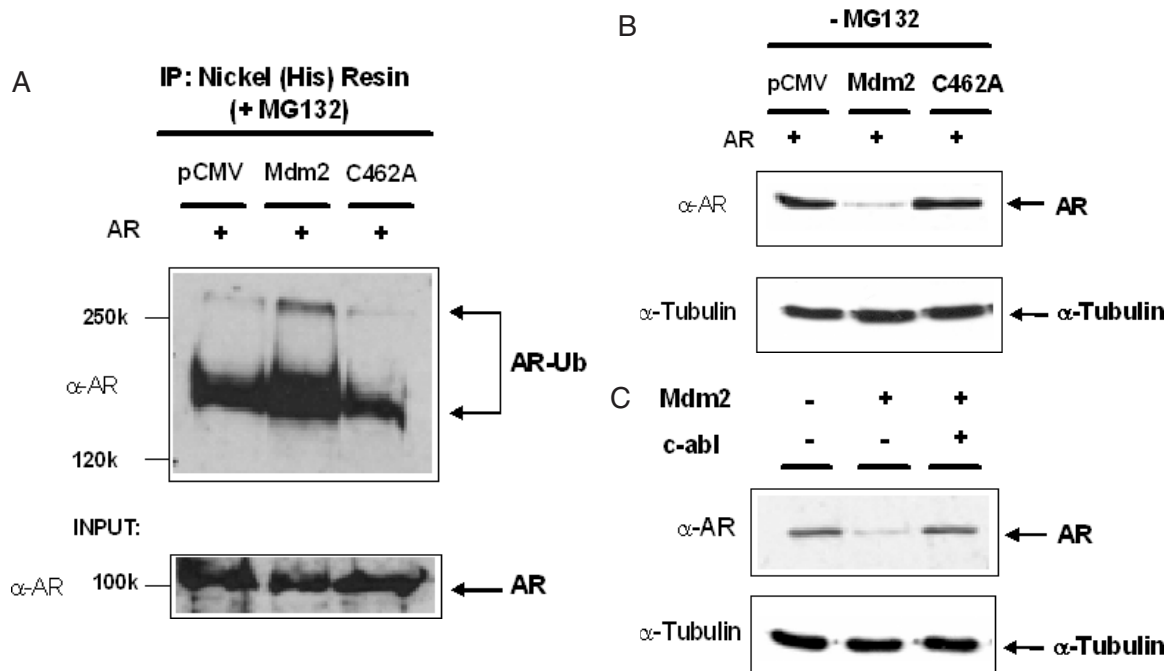


Figure 4. AR is targeted for Mdm2-mediated ubiquitylation and destruction. (A) To confirm Mdm2 catalyses AR ubiquitylation, COS-7 cells transfected with AR, His-tagged ubiquitin, and either wild-type Mdm2 or an Mdm2 ubiquitylase-dead mutant Mdm2_{C462A} was treated with 1 μ M MG132 for 8 h prior to immunoprecipitation with nickel-agarose (His-ubiquitin-bound proteins) followed by immunoblotting with an anti-AR antibody. (B) To assess a role for the enzymatic activity of Mdm2 in AR destabilization, COS-7 cells were transfected as above and AR protein levels analysed by western analysis. (C) To examine potential cross-talk between p53 and AR pathways, the effect of c-abl kinase on Mdm2-mediated AR destruction was examined. COS-7 cells were transiently transfected with AR and combinations of Mdm2 and c-abl followed by western analysis using an anti-AR antibody.

The ubiquitylase activity of Mdm2 is regulated, in part, by the c-abl kinase. Direct c-abl-mediated Mdm2 phosphorylation reduces Mdm2 ubiquitylase activity and has been demonstrated to stabilize p53 protein levels in response to numerous cellular insults, including ionizing irradiation (32,40,41). To investigate a role for c-abl in regulating Mdm2-mediated AR destruction, COS-7 cells transiently transfected with combinations of expression vectors for AR, Mdm2 and c-abl were analysed by western analysis incorporating an anti-AR antibody. As expected, in the presence of Mdm2, AR protein levels are greatly reduced compared with cells transfected with AR alone (Figure 4C, compare lanes 1 and 2). Interestingly, in the presence of c-abl kinase activity, AR protein levels are increased to near normal levels (compare lanes 1 and 3) indicating, in a manner similar to that observed for p53, attenuation of Mdm2 activity by c-abl enhances receptor stability, suggesting potential cross-talk between regulation of p53 and AR.

HDAC1 is a target for Mdm2-mediated ubiquitylation and destruction

The recent demonstration that HDAC1 is a target for direct ubiquitylation provided evidence of an additional regulatory mechanism for HDAC1 activity (36). Our re-ChIP analysis provided evidence that either direct HDAC1 ubiquitylation or association with a ubiquitylated complex at the PSA promoter is enhanced in the presence of hormone (Figure 2D). To assess the potential of direct HDAC1 ubiquitylation in response to androgen treatment, LNCaP cells treated with or without

10 nM androgen were subject to immunoprecipitation using an anti-HDAC1 antibody and then analysed by western blotting incorporating antibodies against HDAC1 (Figure 5A, top panel) and ubiquitin (Figure 5A, lower panel). To increase the potential for HDAC1 ubiquitylation, we extended androgen treatment from 2 h, as used in the re-ChIP analysis, to 4 h and also pre-treated cells with 1 μ M MG132 to prevent potential HDAC1 proteolysis. As shown in Figure 5A, no ubiquitylated HDAC1 species were observed in the absence of androgen. However, after 4 h hormone treatment, we identified three higher molecular weight HDAC1 species of ~120, 130 and 160 kDa, indicative of hyperubiquitylated HDAC1 proteins. In contrast, unmodified HDAC1 protein levels were comparable in the presence and absence of androgen, indicating that the differences observed using the anti-ubiquitin antibody were not due to unequal protein loading.

In keeping with our AR experiments, we next investigated whether increased ubiquitylation could confer HDAC1 destabilization. As shown in Figure 5B, 4 h androgen treatment of LNCaP cells, grown in the absence of MG132, caused a marked reduction in HDAC1 protein levels (compare lanes 1 and 2), which was attenuated in the presence of 1 μ M MG132 (Figure 5B, compare lanes 1 and 3), implicating a role for the proteasome in ubiquitylated HDAC1 turnover.

The correlation between androgen-facilitated HDAC1 and Mdm2 co-association at the PSA promoter (Figure 2B and C) with elevated HDAC1 ubiquitylation (Figures 2D and 5A) suggests that HDAC1 may be modified by the ubiquitylase activity of Mdm2. To test this, the ubiquitylation status of ectopically expressed HDAC1 was analysed in the presence

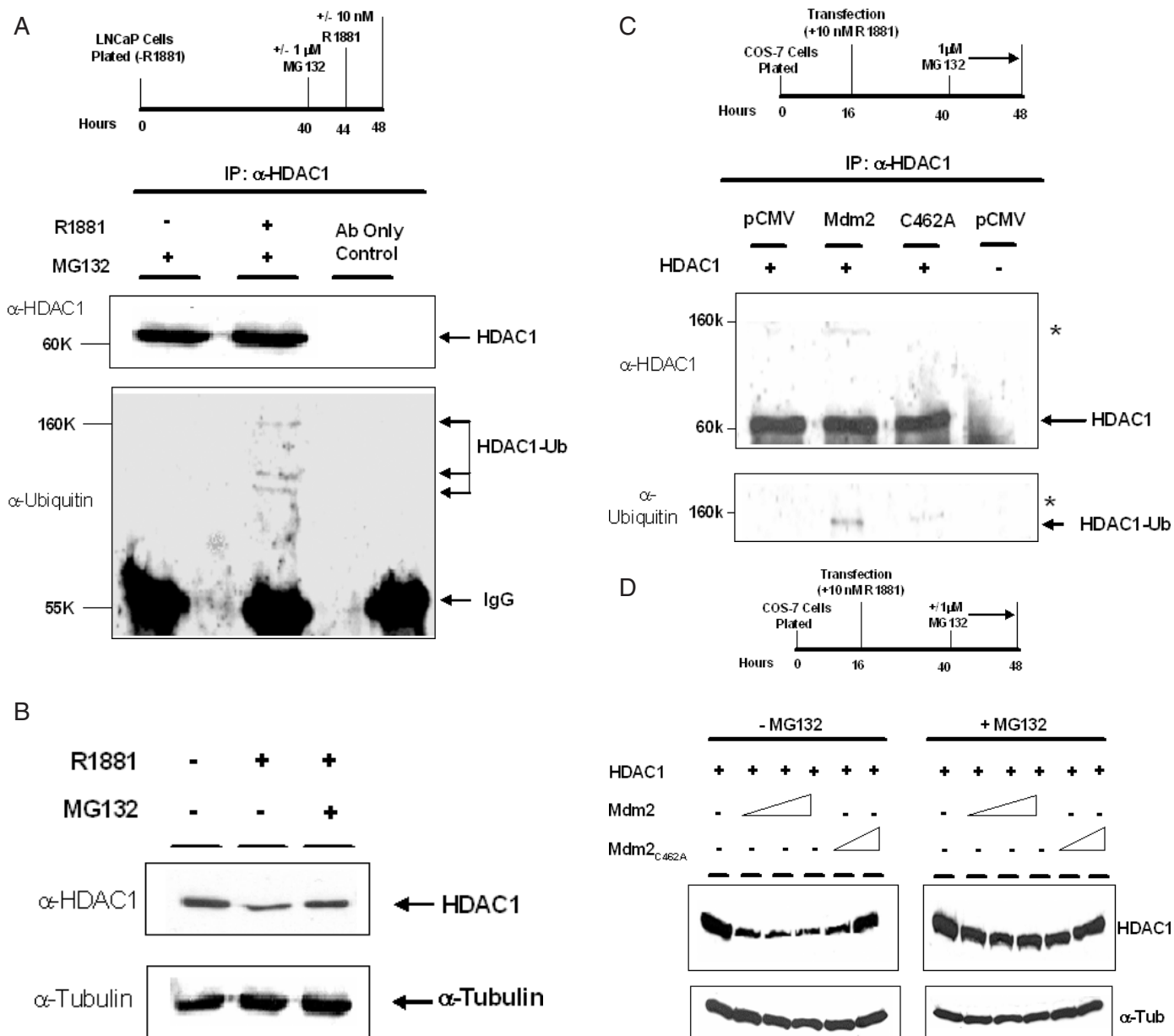


Figure 5. HDAC1 is a target for androgen-dependent ubiquitylation and destruction by Mdm2. Given that HDAC1 associates with Mdm2 and is associated with a ubiquitylated complex at the PSA promoter, we investigated changes to the ubiquitylation status and stability of HDAC1 after hormone treatment and in the presence of Mdm2. (A) HDAC1 was immunoprecipitated from LNCaP cells treated with or without 10 nM androgen for 4 h and 1 μ M MG132 for 8 h, followed by western analysis using an anti-ubiquitin antibody. (B) LNCaP cells were treated with 10 nM androgen, as before, with or without 1 μ M MG132 and HDAC1 protein analysed by western analysis. (C) To assess a role for Mdm2 in HDAC1 ubiquitylation, COS-7 cells were transiently transfected with HDAC1 together with either Mdm2, Mdm2_{C462A} or empty vector, in the presence of 10 nM MG132 and treated with 1 μ M MG132 for 8 h, and subject to immunoprecipitation using an anti-HDAC1 antibody followed by western analysis incorporating an antibody against ubiquitin. (D) COS-7 cells were transiently transfected with HDAC1, increasing amounts of Mdm2 or Mdm2_{C462A} in the presence of 10 nM R1881 and treated with or without 1 mM MG132 for 8 h prior to immunoblot analysis using an anti-HDAC1 antibody.

of transiently transfected wild-type Mdm2 or Mdm2_{C462A} in COS-7 cells. As before, the cells were treated with 1 μ M MG132 prior to immunoprecipitation with an HDAC1 antibody to maximize the detection of ubiquitylated proteins. As shown in Figure 5C (upper panel), we observed an additional HDAC1-specific band at ~160 kDa in the presence of Mdm2, but not by the inactive Mdm2_{C462A} mutant (compare lanes 2 and 3 with 1), suggesting that Mdm2 has the potential to ubiquitylate HDAC1. This was confirmed using an

anti-ubiquitin antibody to probe the HDAC1 immunocomplex; a band corresponding to 160 kDa was observed in the presence of the active E3 ligase (Figure 5C, lower panel, lane 2). This data indicate that the overexpression of Mdm2 *in vivo* enhances HDAC1 ubiquitylation, thus extending the substrate specificity of Mdm2 to modify HDAC1. Furthermore, it is the first identification to date of a known E3 ligase for HDAC1.

To assess the functional significance of Mdm2-mediated HDAC1 ubiquitylation, we examined changes to the stability

of HDAC1 in the presence and absence of Mdm2 ubiquitylase activity (Figure 5D). Increasing amounts of Mdm2 (50–200 ng) correlated with decreased HDAC1 stability (see upper left panel, compare lanes 2–4 with lane 1) that was reduced in the presence of MG132 (see upper right panel, compare lanes 2–4 with lane 1), indicating that Mdm2-mediated HDAC1 destruction is via the proteasome. To provide evidence that the observed effect was due to the ubiquitylase activity of Mdm2, we replaced wild-type Mdm2 with 50 and 200 ng of Mdm2_{C462A} and monitored HDAC1 protein levels. Unexpectedly, in the presence of 50 ng Mdm2_{C462A}, HDAC1 was reduced to levels equivalent to that observed upon expression of 50 ng wild-type Mdm2, suggesting that Mdm2 may lower HDAC1 protein levels in an ubiquitylase-independent manner (Figure 5D, upper left panel, compare lanes 1 and 5). However, recent evidence has suggested that at low levels, ubiquitylase-inactive Mdm2 mutants may retain the ability to dimerize with their endogenous wild-type counterparts (42) and direct target protein modification and destruction, while in greater amounts, we speculate that the mutants act in a dominant negative fashion and thus perturb Mdm2 activity. We confirmed this notion with the finding that 200 ng Mdm2_{C462A} resulted in stabilization of HDAC1 protein levels (upper left panel, compare lanes 1 and 6), and thus indicated that Mdm2 reduces HDAC1 protein levels in an ubiquitylase-dependent manner.

Mdm2 and HDAC1 work co-operatively to down-regulate AR protein levels

The recent demonstration that HDAC1 activity is required for Mdm2-mediated ubiquitylation and destruction of transcriptionally active p53 indicated that deacetylase and ubiquitylase activities synergize to provide an efficient down-regulatory mechanism for p53 function (26). The fact that lysine residues of the conserved KSKK motif of p53 are targeted for both acetylation and ubiquitylation (43) suggests that HDAC1 facilitates p53 ubiquitylation by directly deacetylating active p53 and exposing the lysine-rich sequence to Mdm2-mediated modification. Although the residues for AR ubiquitylation are presently unknown, the similarity between the KSKK motif of p53 and the KLKK motif within the hinge domain of the AR, which is also a target for acetylation (12,13), suggests that the lysine-rich sequence of the AR is modified by ubiquitylation. Therefore, in keeping with mechanisms of p53 repression, we hypothesized that deacetylase activity may be important for ubiquitylation and destruction of the activated receptor.

To assess a role for HDAC activity in hormone-dependent receptor ubiquitylation, AR protein was immunoprecipitated from LNCaP cells grown in androgen-containing media in the presence and absence of 100 nM of the HDAC inhibitor TSA (as indicated in Figure 6A) and subjected to western analysis incorporating an anti-ubiquitin antibody. According to our hypothesis, HDAC inhibition would reduce AR modification and so to facilitate identification of flux to receptor ubiquitylation status, the cells were grown in the presence and absence of 1 μ M MG132. As shown in Figure 6A, 12 h MG132 treatment greatly enhanced endogenous AR ubiquitylation over background levels, with the appearance of a smear between 130 and 250 kDa, indicative of hyperubiquitylated AR species (Figure 6A, compare lanes 1 and 2). A combination of

MG132 and TSA dramatically reduced AR ubiquitylation to near basal levels (Figure 6A, compare lanes 1 and 3), indicating that deacetylase activity is important for AR ubiquitylation *in vivo*.

We next sought to examine the potential co-operativity between HDAC1 and Mdm2 in regulating AR stability. COS-7 cells ectopically expressing AR and combinations of Mdm2, HDAC1 and a deacetylase-inactive HDAC1 mutant, HDAC1_{H141A}, were subjected to western analysis, incorporating an anti-AR antibody, to examine changes to receptor degradation. As expected, AR turnover was increased robustly in the presence of Mdm2 (Figure 6B, compare lanes 1 and 2), which was further enhanced upon co-expression of Mdm2 and HDAC1 (Figure 6B, compare lanes 2 and 3), suggesting that HDAC1 and Mdm2 function in synergy to enhance receptor turnover. In contrast, the failure of the HDAC1_{H141A} mutant to stimulate Mdm2-mediated receptor destruction (Figure 6B, compare lanes 3 and 4) suggests that the deacetylase activity of HDAC1 is required to facilitate the function of Mdm2.

To assess the existence of a trimeric complex between AR, Mdm2 and HDAC1, LNCaP cells grown in the presence and absence of hormone were subject to immunoprecipitation using both AR and HDAC1 antibodies followed by western analysis using antibodies for AR, HDAC1 and Mdm2. As shown in Figure 6C, both HDAC1 and Mdm2 were co-immunoprecipitated with the AR that was enhanced in a ligand-dependent manner, while both Mdm2 and AR were shown to interact with HDAC1, indicating that AR, Mdm2 and HDAC1 are likely components of a hormone-dependent complex.

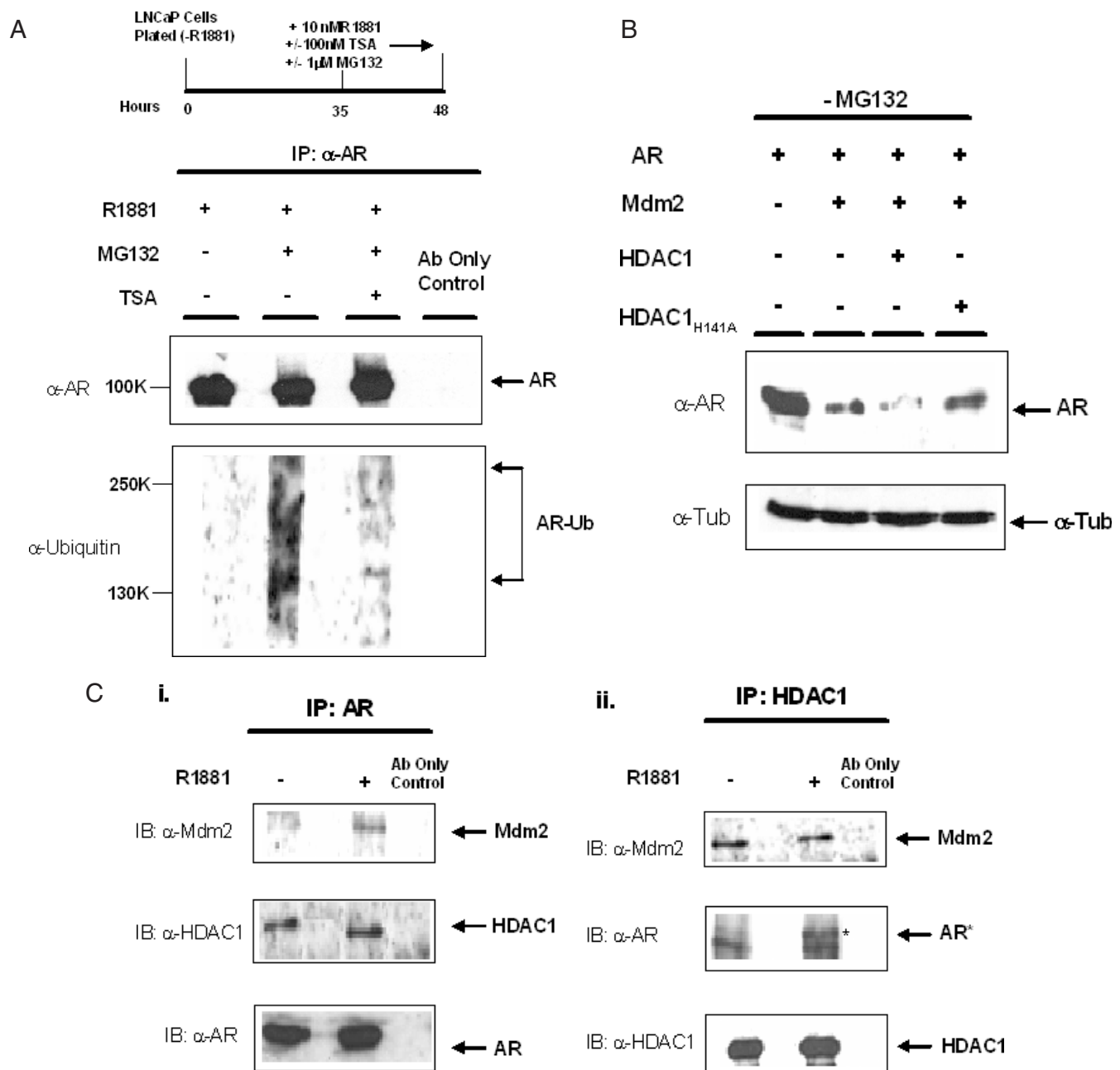
We next analysed the effect of a combination of deacetylase and ubiquitylase activities on AR-mediated gene expression using the androgen-responsive PSA luciferase reporter as described previously (12). As shown in Figure 6D (i), the 3-fold induction of AR activity in response to androgen was reduced by ~50% in the presence of Mdm2 and 60% by HDAC1, but was not effected by the overexpression of HDAC1_{H141A}, as reported previously (12,33). The co-expression of Mdm2 with HDAC1 reduced AR-mediated transcription to basal levels, while HDAC1_{H141A} attenuated the repressive effect of Mdm2, indicating that not only do deacetylase and ubiquitylase activities function co-operatively, the failure for Mdm2 to function in the presence of the dominant negative HDAC1_{H141A} mutant suggests that HDAC1 activity is necessary to facilitate Mdm2-mediated AR ubiquitylation.

To examine potential interplay between ubiquitylation and acetylation in regulating AR activity, the above experiment was repeated with the inclusion of wild-type Tip60 and a HAT-inactive Tip60_{Q377E/G380E} mutant. We figured that if deacetylation was required for Mdm2-mediated AR inactivation, overexpression of Tip60, an established co-activator and factor acetyltransferase protein for AR, would cause hyperacetylation of the receptor to counteract the effect of Mdm2 upon AR function. In keeping with this notion, overexpression of wild-type, but not HAT-defective Tip60 attenuated Mdm2-mediated AR inactivation [Figure 6D (ii)], providing evidence that the acetylase activity of Tip60 competes with HDAC1/Mdm2 in AR regulation and further credits the hypothesis that acetylation and ubiquitylation share overlapping sites within the receptor.

DISCUSSION

The AR, similar to many NRs, is a target for several post-translational modifications that govern numerous facets of receptor function. Of these, two have recently emerged as major mechanisms for regulating AR-mediated transcription and stability. Acetylation and the interplay between HAT and HDAC proteins constitutes an important transcriptional switch that controls the transcriptional potential of the AR (12,13,44), while ubiquitylation has been demonstrated to signal receptor destruction providing an absolute mechanism of AR inactivation (33). Intriguingly, factors that catalyse acetylation, deacetylation and ubiquitylation have been shown to co-exist in macromolecular complexes upon active NR-responsive

genes, indicating that changes to acetylation and ubiquitylation status is likely to enable acute control of transcriptional activity and protein stability at the promoter (20). The data presented here demonstrate that the E3 ligase Mdm2 associates with the active PSA promoter and is in complex with both the AR and HDAC1 suggesting that, in a manner similar to other NR family members, factors involved in regulating acetylation and ubiquitylation are present upon active androgen-responsive genes. Although the time-frames for ChIP analysis were less detailed in comparison with reports regarding ER transcriptosome recruitment to the active pS2 gene (20), our data unequivocally show that Mdm2 recruitment to the endogenous PSA promoter increases robustly over a period of 2 h and Mdm2 co-associates with AR and HDAC1 at the 2 h



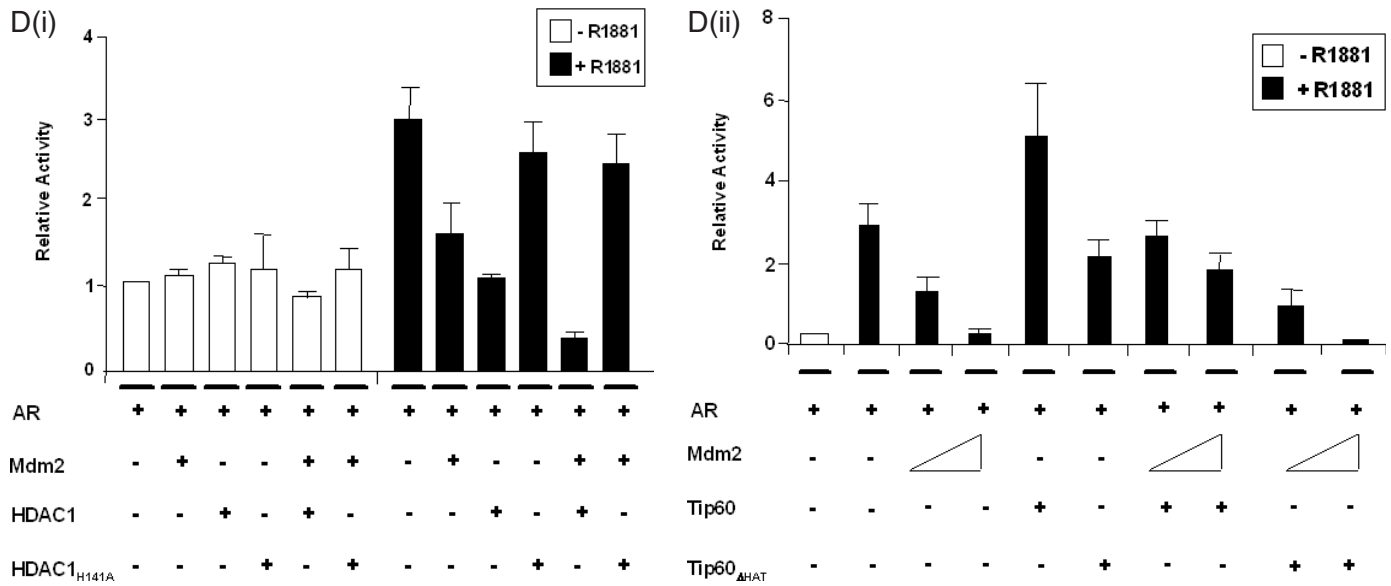


Figure 6. HDAC1 and Mdm2 co-operate to down-regulate AR activity and stability. (A) To assess whether HDAC activity is important for AR ubiquitylation, LNCaP cells treated with 10 nM androgen, and combinations of 1 μ M TSA and 1 μ M MG132 were subject to immunoprecipitation using an anti-AR antibody followed by western analysis incorporating an anti-ubiquitin antibody. (B) To gain an insight into Mdm2 and HDAC1 co-operativity, COS-7 cells were transiently transfected with AR together with Mdm2 and either HDAC1 or a deacetylase-inactive HDAC1_{H141A} mutant prior to western analysis using an anti-AR antibody. (C) The formation of a trimeric complex between AR, Mdm2 and HDAC1 was examined by immunoprecipitation of AR or HDAC1 from LNCaP cells, grown in the presence and absence of 10 nM R1881 for 8 h, followed by immunoblotting using AR, Mdm2 and HDAC1 antibodies. (D) (i) To assess the impact of Mdm2 and HDAC1 co-operation upon AR activity, COS-7 cells were transfected as before and including the androgen-responsive PSA-luciferase reporter, and β -galactosidase reporter for control. Results shown are the average of three independent experiments performed in quadruplicate \pm SD. (ii) To examine potential interplay between acetylation and ubiquitylation in AR regulation, the above experiment was repeated, but with the inclusion of wild-type or a HAT-inactive Tip60 mutant

time-point. However, given the recent evidence indicating rapid association/dissociation kinetics of Mdm2 and other ER-binding factors to ER-responsive promoters (20), it will be pertinent to extend our ChIP analysis by including additional time-frames, to identify potential overlap in recruitment between Mdm2, AR, HDAC1 and other AR transcriptosome components. Although Mdm2 associated with both the proximal ARE I and distal ARE III of the PSA promoter (Figure 1B), suggesting an equal affinity for the two distinct response elements, examining other AR-responsive genes, such as p21 and phosphatidic acid phosphatase (PAP), could confirm Mdm2 as a bona fide component of the AR transcriptional complex.

The emergence of re-ChIP analysis as a tool for identifying both transcription factor associations and post-translational modifications at promoter elements has increased our understanding of the divergent processes involved during initiation and elongation of transcription. Indeed, we successfully used re-ChIP analysis to confirm our ChIP data that Mdm2, AR and HDAC1 are closely associated at the PSA promoter (Figure 2B and C). Furthermore, incorporating an anti-ubiquitin antibody into our analysis, we provided evidence that the AR, HDAC1 and Mdm2 were potentially hyperubiquitylated in response to androgen stimulation and suggesting that the ubiquitylase activity of Mdm2 may be active during the transcriptional process (Figure 2D).

While polyubiquitination of proteins is widely acknowledged as a signal for destruction by the proteasome (45), monoubiquitination, although presently ill-defined, has been shown to influence numerous cellular events, including endocytosis and meiosis (46,47). To extrapolate our re-ChIP data

and to assess the propensity for mono- or polyubiquitylation of the AR and HDAC1, we analysed androgen-dependent changes to the ubiquitylation status of both AR and HDAC1 in LNCaP cells by western analysis and immunoprecipitation. The rapid appearance of two slower-migrating immunoreactive AR species (110 and 130 kDa) after 1 h androgen treatment indicated that the AR is potentially mono- and tri-ubiquitylated upon activation (Figure 3A). Whether this mono- and tri-ubiquitylation of the AR is a genuine modification or is a result of inefficient polyubiquitination will need to be addressed. Recently, Burgdorf and co-workers (48) have identified tumour susceptibility gene 101 (TSG 101) as an AR-interacting partner that enhances the stability of a mono-ubiquitylated form of the receptor in MCF-7 breast cancer cells. It is thought that upon androgen stimulation, receptor ubiquitylation is limited to a single site by the binding of TSG101 that prevents polyubiquitylation and destruction of the AR. It is interesting to speculate that in LNCaP cells, a similar scenario exists in which hormone-dependent AR ubiquitylation is limited to mono- or tri-modification by the binding of TSG101 or a related factor. This hypothesis is further strengthened with the demonstration that AR protein levels remain stable between 1 and 2 h androgen treatment (Figure 3C). Moreover, it was suggested that additional signals during transcription may signal the release of TSG101 and allow hyperubiquitylation and destruction of the AR (48). In keeping with this notion, the formation of numerous higher molecular weight AR species after 3 h hormone treatment, ranging from 150 to 250 kDa in size, was indicative of polyubiquitination and resulted in a correlative destabilization of the receptor protein (Figure 3). We suggest that the concerted

increase in Mdm2 association at the PSA gene between 0 and 2 h androgen treatment may negate potential anti-ubiquitylase activities present at the active promoter and subsequently polyubiquitylate the AR rendering it a target for destruction.

In a manner similar to AR, HDAC1 protein levels were decreased robustly between 3 and 4 h hormone stimulation as a result of hyperubiquitylation of the protein (Figure 5). In addition to androgens, glucocorticoids have recently been shown to increase HDAC1 turnover, albeit after 24 h stimulation (49), indicating that destruction of HDAC1 may be a common event in transcriptional regulation of NRs. The demonstration that HDAC1 is a target for androgen-dependent Mdm2-mediated ubiquitylation and proteolysis provides the first demonstration that HDAC1 is directly ubiquitylated by a known E3 ligase (Figure 5C and D). Together with our re-ChIP analysis, our data indicate both HDAC1 and AR are targets for ubiquitylation by Mdm2, potentially at the promoter level, that is likely to confer protein destabilization and provide an additional mechanism for AR and HDAC1 regulation.

At present, the target sites for Mdm2-mediated AR and HDAC1 ubiquitylation are unknown. For HDAC1, a C-terminal lysine-rich sequence corresponding to residues 432–482 downstream of a PEST sequence has been shown to be hyperubiquitylated *in vitro* (36). For the AR, evidence suggests that the KLKK motif, and possibly other flanking lysine residues, is directly modified by Mdm2. First, the KSKK of p53 and KLKK motif of AR are both targets for p300- and PCAF-mediated acetylation (13,50,51), suggesting these sequences are conducive to identical catalytic events. Second, the demonstration that deacetylase activity is required to facilitate Mdm2-mediated AR ubiquitylation, as previously reported for p53 (26), implies acetylation and ubiquitylation share overlapping lysine sites, with the KLKK motif a likely candidate for both.

The finding that the deacetylase activity of HDAC1 is required to enhance AR ubiquitylation and turnover by Mdm2 provided a novel insight into a co-operative role of HDAC1 and Mdm2 in AR regulation (Figure 6). Although the exact mechanism of interplay between the two factors is ill-defined, evidence from our ChIP analysis may indicate a sequential mode of AR inhibition at the PSA promoter. HDAC1 recruitment to the active promoter occurs after 20 min androgen exposure, suggesting that catalytic activity may be employed at the very early stages of transcription to negate AR activity by directly deacetylating the receptor and concurrently exposing the ubiquitin target sites. The AR is then primed for ubiquitylation by a rapidly associating Mdm2 population at the PSA promoter between 1 and 2 h androgen treatment that is likely to target both AR and HDAC1 for ubiquitylation and destruction. Thus, it may be pertinent to suggest that the tandem ubiquitylation and destruction of AR and HDAC1 between 2 and 4 h androgen treatment, as demonstrated in Figures 3 and 5, respectively, may act to clear the promoter allowing for new rounds of transcription to be initiated. However, the predominance of HAT proteins at active promoter elements complicates this simplified model. We have previously shown Tip60 co-associates with HDAC1 upon the active PSA promoter directly acetylates the AR and attenuates HDAC1-mediated AR deacetylation (12). We have also demonstrated that the HAT activity of Tip60 can counteract the repressive effect of

Mdm2 [Figure 6E (ii)], indicating that interplay between HATs and HDACs at the PSA and other AR-responsive genes may render the AR either active or a target for destruction. An additional consideration is the fact that Mdm2 and other E3 ligase enzymes have broad substrate specificities. Of interest is the recent finding that Mdm2 catalyses ubiquitylation and destruction of Tip60 (52) and PCAF (53), both co-activators for the AR. Therefore, it is likely that at active genes, the recruitment of Mdm2 results in a rapid, but discriminate modification of numerous members of the AR transcriposome, including both positive and negative regulatory factors, which likely pre-disposes the AR for ubiquitylation and proteolysis.

Finally, monoubiquitylation of histone H2B has been associated with transcriptionally active foci in several eukaryotic organisms (54). During transcriptional activation of the *Gall* gene in *Saccharomyces cerevisiae*, histone H2B succumbs to Rad6/Bre-1-mediated ubiquitylation that induces histone H3 methylation, indicating that ubiquitylase activity at the promoter facilitates transcription by altering the histone code (55,56). Although the role of histone H2B ubiquitylation during transcriptional activation in mammals is not clearly understood, the limited evidence implicates roles in both transcriptional activation and repression (54). The association of Mdm2 at the active PSA promoter may be deemed as a predominantly negative event for AR-mediated transcription. The concurrent increase in ubiquitylation at the gene, as shown by re-ChIP analysis, may indicate that in addition to members of the AR transcriptional complex, histone proteins may constitute a bona fide Mdm2 target that facilitates attenuation of receptor function. At present, we are establishing a re-ChIP procedure to examine changes to histone ubiquitylation at the active PSA promoter.

In all, our data have provided a novel insight into the role of Mdm2 in regulating AR-mediated transcription and the interplay between HDAC and HAT proteins in the mechanics of Mdm2 function. Further investigation may focus upon the relative levels of these proteins in different stages of prostate cancer to identify whether flux to ubiquitylase, HAT and HDAC activities exerts an influence upon cellular transformation.

ACKNOWLEDGEMENTS

We are very grateful for the various plasmids received from Stuart Schreiber, Ygal Haupt, David Lane and Bohdan Wasylyk. This work was supported by the Association for International Cancer Research and the NCRI (DoH, CRUK and MRC; grant no. G0100100/64424). Funding to pay the Open Access publication charges for this article was provided by the Association for International Cancer Research (AICR).

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