The lysine demethylase, KDM4B, is a key molecule in androgen receptor signalling and turnover

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ABSTRACT

The androgen receptor (AR) is a key molecule involved in prostate cancer (PC) development and progression. Post-translational modification of the AR by co-regulator proteins can modulate its transcriptional activity. To identify which demethylases might be involved in AR regulation, an siRNA screen was performed to reveal that the demethylase, KDM4B, may be an important co-regulator protein. KDM4B enzymatic activity is required to enhance AR transcriptional activity; however, independently of this activity, KDM4B can enhance AR protein stability via inhibition of AR ubiquitination. Importantly, knockdown of KDM4B in multiple cell lines results in almost complete depletion of AR protein levels. For the first time, we have identified KDM4B to be an androgen-regulated demethylase enzyme, which can influence AR transcriptional activity not only via demethylation activity but also via modulation of ubiquitination. Together, these findings demonstrate the close functional relationship between AR and KDM4B, which work together to amplify the androgen response. Furthermore, KDM4B expression in clinical PC specimens positively correlates with increasing cancer grade ($P < 0.001$). Consequently, KDM4B is a viable therapeutic target in PC.

INTRODUCTION

Currently, the control of gene transcription by chromatin dynamics and interplay between histone modifications is the subject of detailed investigation. There is much debate relating to the role of histone modifications in the control of gene transcription, and it is becoming evident that in many cases, the role of a particular histone modification is dependent on the context in which it is found. For example, tri-methylation on histone H3 at lysine 9 (H3K9Me3) has long been considered a mark of inactive chromatin; however, this mark can also be found in the chromatin of active genes (1,2). It seems that rather than one mark being able to determine the functional output of an area of chromatin, a combination of marks are required, and cross-talk between them is important. In many cases, these marks can act as docking sites for other proteins to start the chain reaction to transcriptional activation or repression. This was demonstrated recently, where to achieve activation of hormone-dependent genes, the chromatin remodelling NURF complex is recruited and anchored to chromatin via an H3K4Me3 signal deposited by MLL2 and MLL3 and the displacement of the demethylase, KDM5B. The NURF complex then allows the recruitment of transcription factors to the promoter and displacement of the linker histone H1 followed by displacement of H2A/H2B and the generation of a more open chromatin structure (3).

Lysine methyltransferases (KMT) and demethylases (KDM) are proving to be important enzymes that can control the activity of non-histone proteins. For example, NF-κB has been found to be mono-methylated by the KMT, SETD7, to result in increased protein degradation (4). SETD7 can also methylate p53 to increase its transcriptional activity (5). It is becoming clear that methylation of non-histone proteins can also functionally interact with other post-translational modifications, such as acetylation, suggesting that a whole network of interactions are involved in the regulation of signalling pathways. It is, therefore, conceivable that both histone and protein modifications, particularly within transcription factors, can also interact to assist in transcriptional control.

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The androgen receptor (AR) signalling pathway is a key factor in the development and progression of prostate cancer (PC). This molecule is subject to many post-translational modifications, including acetylation (6), ubiquitination (7) and sumoylation (8), to regulate its transcriptional activity. Recently, we reported that the AR can be methylated at lysine 632 (9), which was followed by the identification of a second methylation site at lysine 630 (10). Therefore, we propose that methylation and demethylation may be important in the control of AR-regulated gene transcription, whether it be at the level of the histone, the level of the AR or interplay between the two. Some KDM enzymes have already been reported as co-activators of the AR, including KDM4D, KDM4A (11) and KDM1A (12).

KDM4B, first identified in 2004 (13), is a member of the Jmj family of KDMs. Recently, there has been a surge in KDM4B publications describing its role in transcription. For example, KDM4B can antagonize H3K9 tri-methylation at pericentric heterochromatin (14), influence H3K4/H3K9 methylation in hormonally responsive breast cancer (15) and function as an oestrogen receptor (ER) co-factor (16). In this study, we identified KDM4B as an AR co-activator. This is achieved by multiple mechanisms, including H3K9Me3 demethylation and H3K9Ac, as well as increasing AR transcriptional activity and stability. Furthermore, KDM4B is the first identified AR-regulated KDM, suggesting this enzyme has a major role in regulating AR activity and the cellular response to androgens. Collectively, our data suggest that KDM4B is a viable therapeutic target for the treatment of PC.

MATERIALS AND METHODS

Antibodies and constructs

AR (Santa Cruz Biotechnology C-19), KDM4B (Bethyl Laboratories A301-478A; Thermo Scientific PA5-25354; Santa Cruz H-200 sc-97192), tri-methyl histone H3 lysine 9 (H3K9Me3) (AbCam), acetylated histone H3 lysine 9 (H3K9Ac) (AbCam), H3 (AbCam), α-tubulin (Sigma), PSA (Santa Cruz C-19), Ub (Santa Cruz P4D1), MDM2 (Santa Cruz N-20), p(ARE)$_3$Luc, pCMV-β-gal (17), pCMV-HA-KDM4B, pCMV-HA-KDM4B$^{(H189G/E191Q)}$, pGL2-KDM4B$_{(-1022/+12)}$, pGL2-KDM4B$_{(-700/+12)}$, pGL2-KDM4B$_{(-518/+12)}$ and pGL2-KDM4B$_{(-400/+12)}$ were described previously (18). Flag-AR and pSG-AR were kind gifts from Ralf Janknecht (Mayo Clinic, USA) (11). pCDNA3 His Ubiquitin WT was a kind gift from David Lane (Singapore) (19).

Cell culture

CWR22rv1 (20) and LNCaP cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in RPMI 1640 media supplemented with 10% (v/v) fetal calf serum and 2 mM L-glutamine (Sigma) at 37°C in 5% CO_2 atmosphere. LNCaP-CdxR cells were generated and maintained as described previously (21,22); LNCaP-AI cells were generated in-house by serially maintaining LNCaP cells in steroid-depleted media (SDM) for >9 months. LNCaP-shKDM4B was generated as described in Supplementary Materials and Methods.

siRNA screen

In this siRNA screen, 78 methylase and demethylase enzymes were targeted alongside control siRNAs against Prostate Specific Antigen (PSA), AR and non-silencing controls. All siRNA sequences were designed by Sigma using the Rosetta algorithm. Every target had three independent sequences generated, which were pooled together for use in the screen. Individual siRNA sequences are available in Supplementary Information.

LNCaP cells (4000 cells/well) were reverse transfected with 25 nM siRNA, consisting of three independent sequences pooled together, in SDM using RNAiMax (Invitrogen) in 96-well plates. Dihydrotestosterone (DHT) (100 nM) was applied 24 h post-transfection and incubated for 72 h. PSA secretion and expression were then assayed as described later in the text. Further details can be found in Supplementary Materials and Methods.

PSA enzyme-linked immunosorbent assay

The concentration of PSA secreted into the media was assessed using a human Kallikrein 3/PSA ELISA (enzyme-linked immunosorbent assay) (DuoSet ELISA Development System, R&D Systems) according to the manufacturer's protocol. Standard curves were generated, and PSA concentrations were determined by linear regression of $\log_{(10)}$ normalized absorbance at 450 nm. This allowed R^2 values and the slope of the standard curve to be calculated, so that interexperimental variation could be taken into account. Screening with this experimental end point was performed in duplicate at least three times.

Quantitative polymerase chain reaction

RNA was isolated using TRIzol (Invitrogen) according to the manufacturer's protocol. RNA was quantified using Nanodrop spectrophotometer, and 200 ng of RNA was reverse transcribed using $1\times$ Moloney Murine Leukemia Virus (MMLV) buffer (Promega), 60 U MMLV reverse transcriptase, 0.5 μM oligo d(T)$_{18}$ (Bioline) and 0.4 mM dNTPs (Bioline) per reaction. Quantitative reverse transcriptase–polymerase chain reaction (QRT–PCR) was performed in triplicate for genes of interest using Platinum SYBR$^®$ green qPCR SuperMix-UDG (Invitrogen) in 384-well clear optical reaction plates using the ABI 7900HT real-time PCR system (Applied Biosystems) according to the manufacturer's protocol. Data were normalized against the house keeping gene *hypoxanthine phosphoribosyltransferase 1* (*hprt1*). Primers sequences are described in Supplementary Materials and Methods.

Chromatin immunoprecipitation assays

Chromatin immunoprecipitation (ChIP) assays were performed in LNCaP cells and LNCaP-shKDM4B cells as described previously (7). For assays in LNCaP-shKDM4B

cells, $1 \mu g \, ml^{-1}$ doxycycline was added when cells were placed into SDM for 72 h to ensure efficient KDM4B knockdown. For immunoprecipitation, $2 \mu g$ of the appropriate antibody was used, specifically, AR (Santa Cruz Biotechnology C-19), KDM4B (Bethyl Laboratories), tri-methyl histone H3 lysine 9 (H3K9Me3) (AbCam), acetylated histone H3 lysine 9 (H3K9Ac) (AbCam) and non-specific isotype control antibodies (Dako). Purified DNA fragments were then analysed by QRT-PCR. Primer sequences are detailed in Supplementary Information. Data represent mean fold change in percentage input from three independent experiments normalized to 0 h DHT stimulation.

Reporter assays

For all reporter assays, 8×10^3 Cos7 cells were routinely plated per well of a 24-well plate (Corning). After 24 h, cells were transfected using LT-1 reagent (Geneflow) according to the manufacturer's protocol. Assays used to determine the effect of KDM4B on AR activity incorporated 200 ng of p(ARE)$_3$-Luc (17) together with 0–75 ng of either pCMV-HA-KDM4B or pCMV-HA-KDM4B$^{(H189G/E191Q)}$. Assays used to determine AR regulation of KDM4B transcription incorporated 20 ng of pGL2-hKDM4B with 0–100 ng of Flag-AR (a gift from R. Janknecht) or with 50 ng pSGFlag-AR with increasing concentrations of DHT for 48 h. To determine the area of the promoter that is responsive to AR stimulation, deletion constructs of pGL2-hKDM4B were used of the indicated sizes with 0–100 ng of Flag-AR. Cells were harvested in all cases 48 h post-transfection, and luciferase and β-galactosidase assays were performed as previously described (23). Each experiment was performed in triplicate and repeated a minimum of three times. Mean fold change represents induction with DHT compared with SDM alone normalized to the activity of the pCMV-driven vector control. Data represent an average of three repeats ± standard error.

Immunoprecipitation

To determine whether AR can interact with KDM4B and whether the demethylase activity is required for the interaction, Cos7 cells grown in RPMI-1640 media containing 10% fetal calf serum were transfected with 1 μg of Flag-AR and 1 μg of pCMV-HA-KDM4B or 1 μg of pCMV-HA- KDM4B$^{(H189G/E191Q)}$ after 24 h using LT-1 reagent (Geneflow) according to the manufacturer's protocol. Cells were harvested after 48 h and subjected to immunoprecipitation using anti-AR antibody (Santa Cruz Biotechnology: C-19) followed by western blotting with KDM4B antibody (Bethyl Laboratories).

To determine whether AR can interact with endogenous KDM4B, LNCaP cells were collected and lysed, and AR was immunoprecipitated using anti-AR antibody (Santa Cruz Biotechnology: C-19) followed by western blotting with KDM4B antibody (Bethyl Laboratories). Immunoprecipitation with anti-HA antibody (Santa Cruz Biotechnology) was used as a control.

Denaturing immunopreciptitation using Ni-NTA agarose beads

Cos7 cells were seeded out onto 90-mm dishes and transfected with 1 μg of Flag-AR and 1 μg of pcDNA3 His Ubiquitin and increasing concentrations (0, 1, 100 and 500 ng) of either pCMV-HA-KDM4B or pCMV-HA-KDM4B$^{(H189G/E191Q)}$ as described earlier in the text. After 24 h, 20 μM MG132 (Enzo Life Sciences, UK) was applied for 16 h. Cells were washed twice with ice cold phosphate-buffered saline and collected. Input samples were taken as a 10% volume of cell suspension and resuspended in sodium dodecyl sulphate (SDS) loading buffer [0.125 M Tris, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 10% (v/v) β-mercaptoethanol and 0.01% (w/v) bromophenol blue]. Remaining cells were then lysed in 6 ml of lysis buffer (6 M guanidine–HCl, 0.1 M Na$_2$HPO$_4$/NaH$_2$PO$_4$, 0.01 M Tris–HCl, pH 8, 5 mM imidazole and 10 mM β-mercaptoethanol) on ice for 30 min. Ni-NTA agarose beads (Qiagen) were added, and samples were incubated at $4°C$ overnight with agitation. Agarose beads were washed for 5 min with agitation with each of the following buffers. Buffer 1 (6 M guanidinium–HCl, 0.1 M Na$_2$HPO$_4$/NaH$_2$PO$_4$, 0.01 M Tris–HCl, pH 8 and 10 mM β-mercaptoethanol), buffer 2 (8 M urea, 0.1 M Na$_2$HPO$_4$/NaH$_2$PO$_4$, 10 mM β-mercaptoethanol and 0.01 M Tris, pH 8), buffer 3 [8 M urea, 0.1 M Na$_2$HPO$_4$/NaH$_2$PO$_4$, 10 mM β-mercaptoethanol, 0.01 M Tris, pH 6.3 and 0.2% (v/v) Triton X-100] and buffer 4 [8 M urea, 0.1 M Na$_2$HPO$_4$/NaH$_2$PO$_4$, 10 mM β-mercaptoethanol, 0.01 M Tris, pH 6.3 and 0.1% (v/v) Triton X-100]. Samples were then eluted from the beads in 50 μl of elution buffer [200 mM imidizole, 0.1 M Na$_2$HPO$_4$/NaH$_2$PO$_4$, 0.15 M Tris–HCl, pH 6.7, 10 mM β-mercaptoethanol, 30% (v/v) glycerol and 5% (w/v) SDS] for 20 min with agitation at room temperature. Elutes were mixed 1:1 with SDS loading buffer and analysed by western blotting using AR antibody (BD).

Denaturing antibody-mediated immunoprecipitation

Cos7 cells were seeded, transfected and treated with MG132 as described earlier in the text. Cells were collected in IP lysis buffer containing 20% SDS and boiled for 10 min. Ten per cent inputs were then taken, and SDS sample buffer was added at a 1:1 ratio. The remaining cell lysate was then diluted in non-SDS containing lysis buffer to allow antibody use. Two micrograms of either anti-AR or anti-Ubiquitin antibody was then added and incubated at $4°C$ overnight with gentle rotation. Protein G Sepharose was added for 4 h. Bead–antibody complexes were then washed 4–6 times in 0.5 M KCl then once in 0.1 M KCl. Beads were re-suspended in SDS loading buffer and analysed by western blotting.

Proliferation assays

PC cell lines were seeded out in full media onto 96-well plates and reverse transfected with 25 nM siRNA using RNAiMax according to the manufacturer's protocol. Oligonucleotide concentrations were normalized using N/S siRNA. After three doubling times, proliferation

was assessed using WST-1 reagent (Roche) according to the manufacturer's protocol. Experiments were performed in repeats of six and performed three times. All data were normalized to N/S for each cell line.

Immunohistochemistry

IHC was performed on tissue microarray (TMA) containing 0.6-mm cores of benign prostatic hyperplasia (BPH) and PC tissue from each sample and control tissue, such as breast, kidney, adrenal, placenta, ovary, lung and liver. Slides were baked for 2 h at 60°C to remove moisture, de-paraffinized with xylene and hydrated with varying concentrations of ethanol. Endogenous peroxidase activity was quenched with 0.5% H_2O_2 in methanol. Epitope retrieval by pressure cooking in 0.01 M citrate buffer, pH 6.0, was performed before primary antibody application. Slides were incubated with goat serum for 30 min; then primary KDM4B antibody (Bethyl laboratories) was applied (1:250) in phosphate-buffered saline and incubated overnight at 4°C. Biotinylated goat anti-rabbit secondary antibody was used to detect the bound primary. ABC kit (Vector Laboratories) and 3,3-Diaminobenzidine (DAB) were used for detection of immuno-reactivity. The immuno-reactivity scoring was done by two experienced uro-pathologists. The signal intensity of the epithelial cancer cells was scored according to the intensity and was categorized into weak, moderate and strong cytoplasmic signal groups.

Statistical analyses

Mann–Whitney U-test and Kruskal–Wallis test were used to correlate expression of KDM4B with benign prostate and grades of PC. Patient survival analysis used Kaplan–Meier plots with log-rank statistical testing. Tests were undertaken using SPSS, version 11.0, computer software (SPSS Inc.). A P-value of <0.05 was taken to indicate statistical significance.

RESULTS

KDM4B regulates AR-mediated transcription

To identify which KDM enzymes are involved in AR signalling, an siRNA screen was performed in the PC cell line, LNCaP, using a pool of three independent siRNA sequences per enzyme. After knockdown for 48 h in SDM, DHT (100 nM) was applied to stimulate the AR signalling pathway. Media were collected 72 h later, and PSA ELISA was used as a high-throughput method to determine whether AR signalling was affected. KDM4B was identified as an AR co-regulator, as knockdown decreased PSA secretion by 38% (Figure 1A). QRT-PCR analysis confirmed a 33% reduction in *PSA* mRNA levels (Figure 1B), which was authenticated further using three independent siRNA sequences (Figure 1C) ($P < 0.005$) to knockdown KDM4B (Figure 1D). In comparison with the knockdown of AR, the effect of KDM4B depletion on PSA levels may be considered modest. However, there are many reasons why this may occur. For example, there may be other factors in the

pathway that can affect the expression of PSA independently of KDM4B, whereas AR is the key regulator; therefore, AR knockdown will have the greatest impact on PSA levels. Second, knockdown of KDM4B is not the same as knockout. Therefore, any residual KDM4B in the cells may contribute to PSA expression. However, we can rule out the effects of other KDM4 family members, as these remain unaltered in response to KDM4B knockdown (Supplementary Figure S1G).

To confirm that KDM4B is involved in androgen responsive gene expression as a whole and not just *PSA* expression, DHT stimulation time courses were performed post-KDM4B knockdown in a number of AR positive PC cell lines, including LNCaP (Figure 2), LNCaP-CdxR (22) and LNCaP-AI cells (Supplementary Figure S1). A panel of androgen-regulated genes were investigated by QRT-PCR including *PSA*, *Kallikrein-related peptidase 2* (*KLK2*), *transmembrane protease, serine 2* (*TMPRSS2*), *NKX3.1* and *N-myc downstream regulated 1* (*NDRG1*). As shown in Figure 2, knockdown of KDM4B inhibits androgen responsive gene expression of all the genes tested, robustly demonstrating a role for KDM4B in the AR signalling pathway.

KDM4B regulates AR-mediated transcription by modulating histone post-transcriptional modifications in response to androgens

KDM4B has been shown to demethylate histone H3 at lysine 9, albeit with a weaker activity than other KDM4 family members (14). It can remove tri-methyl marks to leave either a mono- or a di-methyl species. The response to this removal can include transcriptional activation, and indeed removal of methyl marks on lysine 9 has already been implicated in activation of androgen-regulated genes (24,25). To determine whether this could be the mechanism through which KDM4B functions here, a series of ChIP assays were performed to determine the status of particular histone marks in response to DHT, and whether KDM4B is found at the promoters of androgen responsive genes. In response to DHT stimulation, a 6.5-fold enrichment of AR was observed at the AREIII sequence within the *PSA* promoter (Figure 3A). KDM4B was found to be present and enriched by ~2.7-fold after 30 min of DHT stimulation. However, after 120 min, KDM4B was absent (Figure 3B), whereas AR continued to be enriched, suggesting KDM4B functions early in the transcriptional response to DHT. Alternatively, KDM4B may cycle on and off the chromatin similar to other transcription factors including the AR (26). Furthermore, in response to DHT, a significant reduction in the repressive H3K9Me3 (Figure 3C) was observed ($P < 0.005$) further supporting a role for KDM4B in histone modification at androgen responsive genes. As there have been many links made between methylation and acetylation of histones, the levels of H3K9Ac were also investigated. Increases in H3K9Ac are associated with transcriptional activation, and indeed increases were observed at the *PSA* promoter in response to DHT (Figure 3D). It seems that once the tri-methyl mark has been removed, the acetylation mark increases at ~120 min post-DHT stimulation.
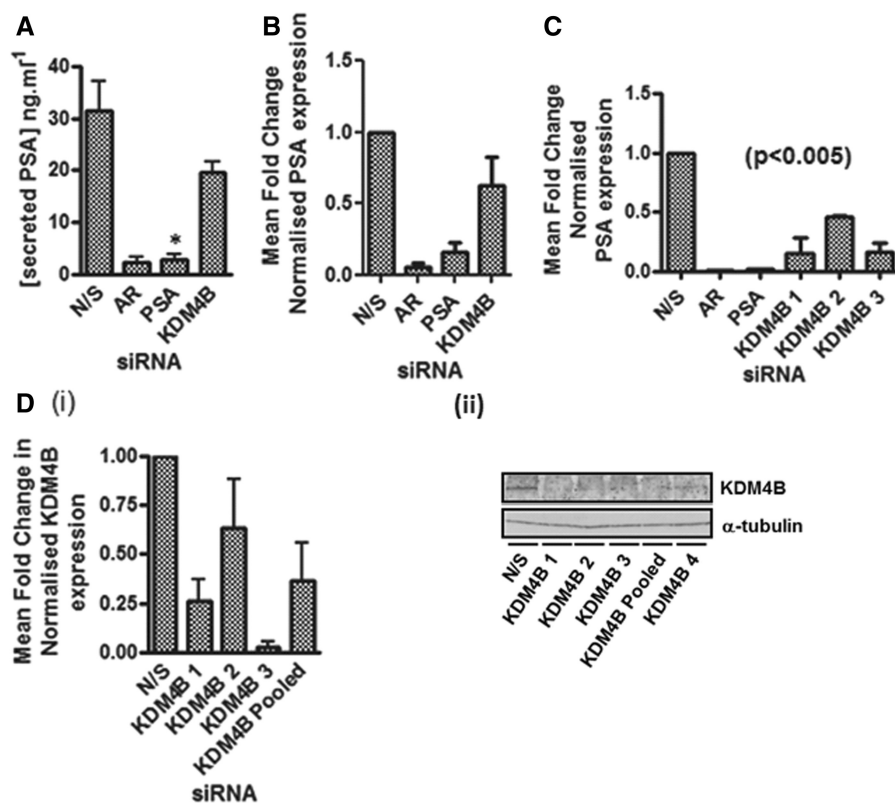
Figure 1. KDM4B is a putative AR activity regulator. (**A**) LNCaP cells grown in SDM were transiently transfected with a pool of three independent siRNA sequences targeting KDM4B in SDM for 24 h before DHT (100 nM) stimulation for a further 72 h. Non-silencing (N/S), AR and PSA siRNAs were also included as controls. PSA ELISA (R&D systems DuoSet) and (**B**) QRT-PCR was performed to detect *PSA* mRNA levels. (**C**) LNCaP cells grown in SDM were transiently transfected with individual siRNA oligos targeting KDM4B in SDM 24 h before DHT (100 nM) stimulation followed by RNA extraction and QRT-PCR for *PSA* mRNA expression (*$P < 0.05$). KDM4B knockdown was confirmed by (**Di**) QRT-PCR and (**Dii**) western blotting. All QRT-PCR data were normalized to *HPRT1*, and α-tubulin was used as a loading control on western blots. All experiments were performed twice or more times, and data represent the mean fold change ± SE.

This may suggest a requirement for interplay between demethylation and acetylation to achieve efficient androgen-stimulated gene expression.

Removal of H3K9Me3 in response to DHT may be a consequence of other enzymes functioning at the same time point, which has been demonstrated previously for KDM4C and KDM1A (25). To further demonstrate that KDM4B plays a role in events observed on the chromatin in response to DHT, a stably inducible shRNA LNCaP cell line was generated: LNCaP-shKDM4B. When doxycycline (1 μg ml^{-1}) is applied to this cell line, the shRNA against KDM4B is expressed, which results in KDM4B knockdown (Supplementary Figure S2A) and inhibition of *PSA* expression in response to DHT stimulation (Supplementary Figure S2B). ChIP assays were performed in LNCaP–shKDM4B cells stimulated with doxycycline on starvation in SDM for 72 h. DHT (100 nM) was applied to the cells for 0 and 120 min followed by formaldehyde fixation and chromatin collection. In the absence of doxycycline, a 2-fold enrichment of AR was found at the AREIII sequence within the *PSA* promoter (Figure 3E) and a 6-fold enrichment at *KLK2* and *TMPRSS2* promoters (Supplementary Figure S2C and D). However, on KDM4B knockdown (Figure 3F), this was reduced to basal levels supporting the theory that KDM4B may be required for AR to efficiently associate

with chromatin. It also seems that KDM4B functions in the removal of H3K9Me3 in response to androgens, as when KDM4B is knocked down, an increase in H3K9Me3 was observed (Figure 3G). In addition, when KDM4B was knocked down, H3K9Ac was considerably reduced (Figure 3H), indicating that the removal of H3K9Me3 and the appearance of H3K9Ac are linked. As the effect on H3K9Me3 was not seen at the global level (Figure 3I), this effect seems to be specific to this region of the chromatin.

KDM4B interacts with the AR and enhances its transcriptional activity

Although KDM4B may play a role in this signalling pathway at the level of the histone, it may also be able to coactivate the AR. Previously, other members of the KDM4 family have been shown to interact with, and coactivate the AR (25); however, this has not been shown for KDM4B to date. To address this, Cos7 cells were transiently transfected with wild-type AR (Flag-AR) and wild-type KDM4B, followed by AR immunoprecipitation. Western analysis demonstrated that KDM4B can interact with AR under these conditions (Figure 4A). Furthermore, an endogenous interaction between KDM4B and AR was confirmed in LNCaP
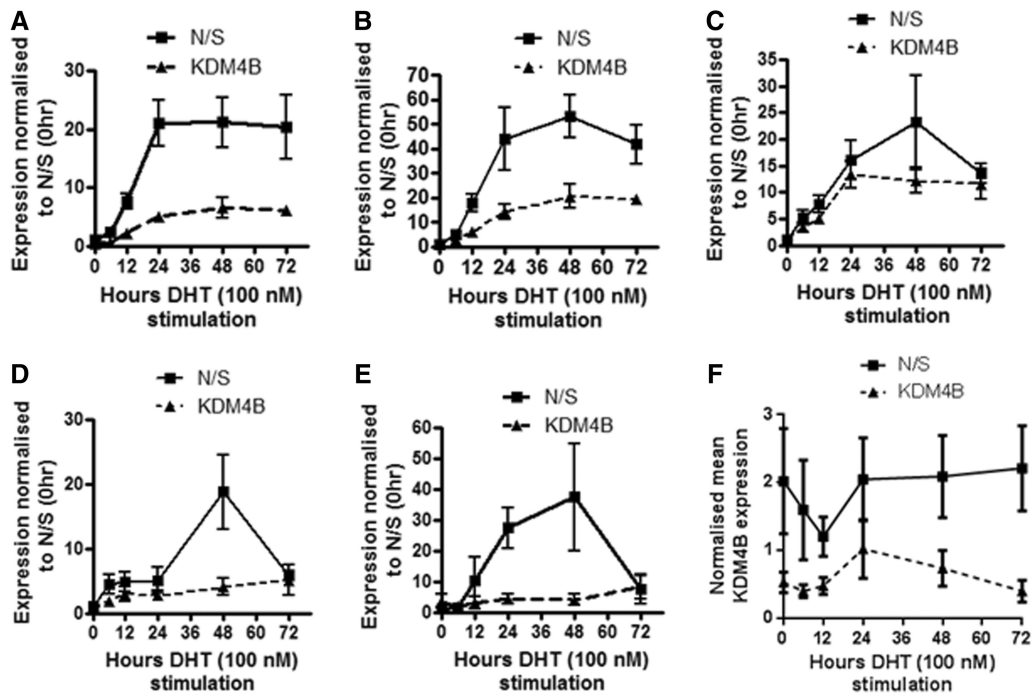
Figure 2. KDM4B is important for AR-mediated transcription. LNCaP cells with KDM4B knockdown were stimulated with DHT (100 nM) for 72 h. (**A**) *PSA*, (**B**) *KLK2* (**C**) *TMPRSS2*, (**D**) *NKX3.1*, (**E**) *NDRG1* and (**F**) *KDM4B* levels were assessed >72 h by QRT-PCR and normalized to *HPRT1*. A non-silencing (N/S) oligo was used as a control. Data are expressed as mean fold change compared with N/S without DHT stimulation ± SE.

cells (Figure 4B). To determine whether the catalytic activity is required for this interaction between AR and KDM4B, a KDM4B construct that has been mutated within the JmjC domain (H189G/E191Q), to render it catalytically inactive (18), was used. Western blotting revealed that the catalytic activity is not required for KDM4B to interact with the AR (Figure 4A).

To investigate whether KDM4B can increase the transcriptional activity of the AR, reporter assays were performed using p(ARE)$_3$-luc (17) in conjunction with AR and increasing amounts of KDM4B. When KDM4B was overexpressed, AR activation induced by DHT increased from 2.7- to 5.3-fold, suggesting that KDM4B can coactivate the AR (Figure 4C). To test whether the demethylase activity is required for this increased AR transactivation, the demethylase null form of the enzyme was used instead of the wild-type. Here, there was no change in the transactivation of AR, suggesting that the demethylase activity is important for the effects on AR activity (Figure 4C).

KDM4B can increase AR protein stability by inhibiting AR ubiquitination

On western blotting to demonstrate expression of Flag-AR with increasing levels of KDM4B in Cos7 cells, we observed that although eqimolar quantities of AR were transfected, AR levels increased (Figure 5A). Similarly, AR became stabilized by KDM4B[(H189G/E191Q)], but AR transactivation was absent (Figure 4C), suggesting that the KDM activity is required to maximally enhance AR activity. Similar results were obtained when the AR was expressed from a Simian vacuolating virus 40 (SV40)

promoter (Supplementary Figure S2E), ruling out increased levels being because of activation of the pCMV promoter. As overexpression of KDM4B seems to increase the levels of AR protein when AR is expressed from a pCMV (or SV40) promoter suggesting an effect on protein stability, this raised the question of whether KDM4B knockdown can result in a de-stabilized AR and, therefore, reduced AR levels. To investigate this further, transient knockdown of KDM4B was performed in LNCaP cells for 48 h, and the levels of AR protein were assessed by western blotting. Indeed, this revealed that knockdown of KDM4B results in reduced AR levels (Figure 5B). Moreover, this was also tested in cell lines that are androgen independent (LNCaP-AI) and resistant to anti-androgen therapy (LNCaP-cdxR) to reveal similar findings (Figure 5C).

AR turnover is controlled via the ubiquitin–proteasome pathway (27), and in histone biology, there is increasing evidence that methylation and ubiquitination are closely linked (28). Therefore, the level of AR ubiquitination (AR-Ub) was assessed in the presence and absence of KDM4B by denaturing immunoprecipitation in Cos7 cells transfected with AR- and His-tagged ubiquitin. On stimulation with DHT, AR-Ub was reduced (Figure 5D, lane 4); however, when either KDM4B or KDM4B[(H189G/E191Q)] was added, AR-Ub was lost (Figure 5D, lanes 5–8). This was further confirmed by performing an antibody-based denaturing IP protocol detailed in 'Material and Methods' section demonstrating AR-Ub in the presence of MG132, which is abolished when KDM4B is added (Figure 5E). The reciprocal IP using an anti-Ubiquitin antibody also supported these data
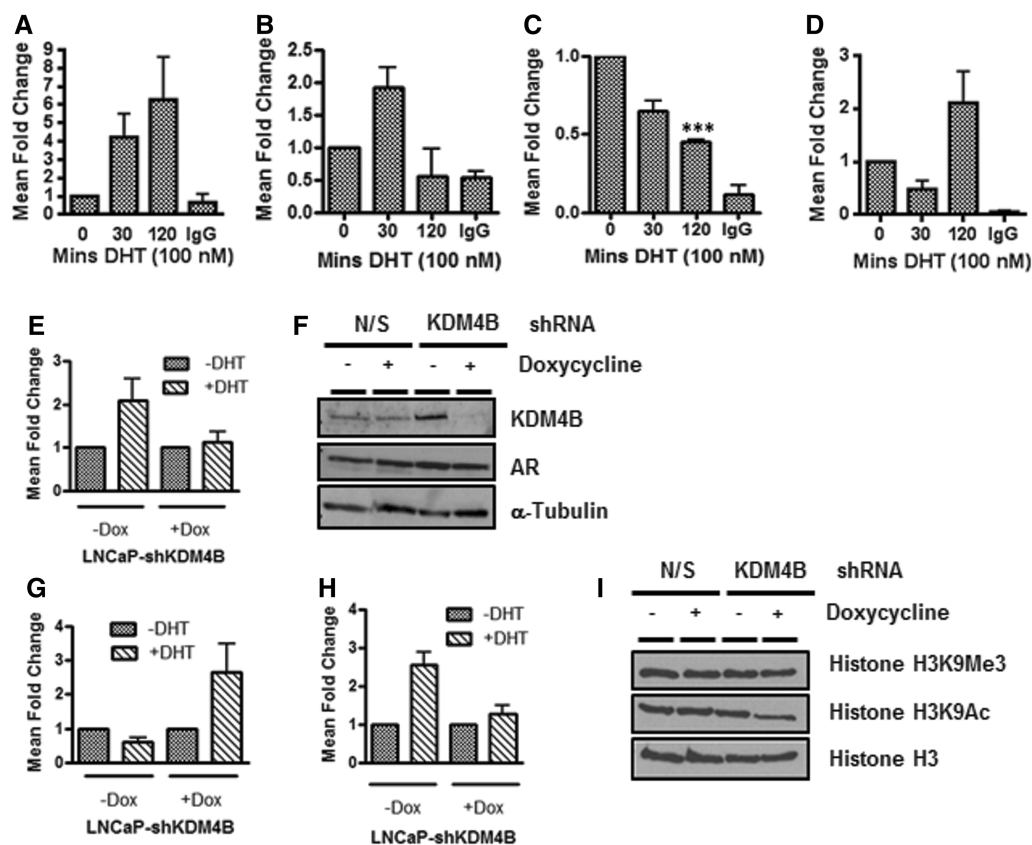
Figure 3. KDM4B is required for AR recruitment, H3K9Me3 removal and H3K9Ac addition at androgen-regulated genes. ChIP for (**A**) AR, (**B**) KDM4B, (**C**) H3K9Me3 and (**D**) H3K9Ac at the *PSA* promoter in LNCaP cells treated with DHT (100 nM) for 0, 30 and 120 min. Non-specific IgG was used as a negative control. (**E**) ChIP analysis for AR at the *PSA* promoter in LNCaP-shKDM4B cells treated with and without doxycycline for 72 h and 100 nM DHT for 0 and 120 min. Data are shown as mean fold change of IgG normalized data compared with time 0 from two or more independent experiments ± SE (***$P < 0.0005$). (**F**) KDM4B and AR levels were assessed by western blotting in both LNCaP-shKDM4B and the control cell line LNCaP-shN/S in the presence and absence of doxycycline. (**G**) ChIP analysis for H3K9Me3 and (**H**) H3K9Ac at the *PSA* promoter in LNCaP-shKDM4B cells treated with and without doxycycline for 72 h and 100 nM DHT for 0 and 120 min. (**I**) Global histone modifications. Histone H3 and α-tubulin were used as loading controls.

(Supplementary Figure S2F). This effect also seemed to be KDM4B dose dependent (Figure 5F), and interestingly, although KDM4B$^{(H189G/E191Q)}$ can also inhibit AR-Ub, it seems to be deficient in this process (Figure 5G), as both the mutant and wild-type forms are expressed at the same levels (Supplementary Figure S2G), with only higher KDM4B concentrations being able to influence AR-Ub. KDM4B was confirmed as ubiquitinated (Supplementary Figure S2H); however, overexpression of His-tagged Ubiquitin ensures that KDM4B should not be able to 'mop up' all the Ubiquitin within the cell. Therefore, we conclude that this decrease in AR-Ub is a genuine affect.

Because of the role of the E3 ubiquitin ligase, MDM2, in AR-Ub and that another KDM4 family member, KDM4C, has been described to enhance MDM2 levels (29), we questioned whether MDM2 levels were affected by KDM4B. However, increased AR stability seems to be via the prevention of AR-Ub, as we found no evidence of altered MDM2 levels with KDM4B knockdown (Figure 5H). We speculate that this is due to the structural interaction between KDM4B and AR masking ubiquitin acceptor sites. The novel role for KDM4B in ubiquitination inhibition and AR degradation warrants

further investigation, but it is encouraging to note that interplay between various modifications is just as important here as on histone proteins.

KDM4B is an androgen-regulated gene

As KDM4B and AR seem to be functionally linked, it would be logical for KDM4B levels to increase in response to DHT, allowing AR and KDM4B to cooperate and promote AR transcriptional events. To test this theory, *KDM4B* mRNA and protein levels were assessed in response to DHT in LNCaP cells revealing that, indeed *KDM4B* mRNA (Figure 6A) and protein (Figure 6B) are androgen regulated. This was further confirmed by gene reporter assays, where Cos7 cells were co-transfected with AR and pGL2-human KDM4B luciferase construct (18), containing the *KDM4B* promoter (−1022/+12) sequence upstream of a luciferase gene, where increasing doses of DHT (Figure 6C) and increasing AR levels (Figure 6D) significantly enhanced KDM4B reporter activity ($P < 0.05$). As this is the first example of a demethylase enzyme being androgen regulated, the KDM4B promoter was analysed for potential androgen response elements within its promoter sequence using Genomatix software
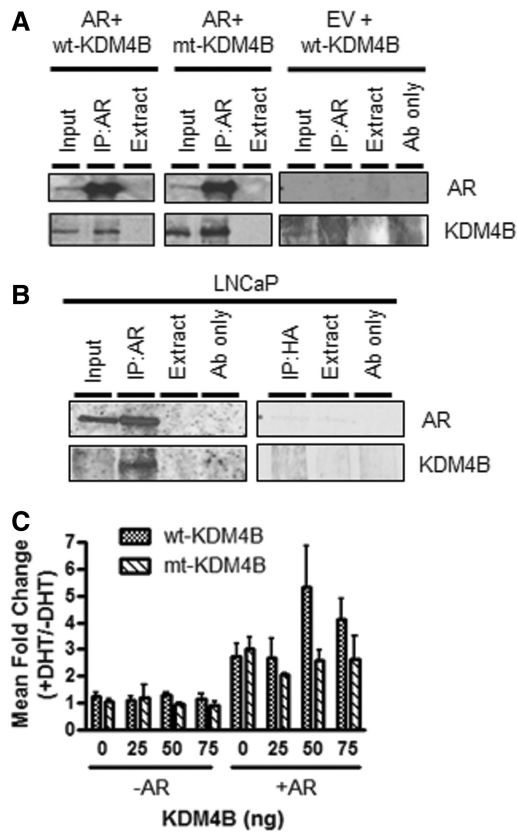
Figure 4. KDM4B interacts with AR and coactivates the AR via its demethylase activity. (**A**) AR was immunoprecipitated from Cos7 cells transfected with Flag-AR and pCMV-HA-KDM4B or pCMV-HA-KDM4B$^{(H189G/E191Q)}$ and analysed by western blotting with anti-AR and anti-KDM4B. (**B**) Endogenous AR was immunoprecipitated from LNCaP cells and analysed by western blotting with anti-AR and anti-KDM4B. Negative controls are included as extracts (cell lysates without antibody addition) and Ab only (an antibody only control in the absence of cell lysate), (**C**) Cos7 cells transfected with Flag-AR and increasing concentrations of either pCMV-HA-KDM4B (wt-KDM4B) or pCMV-HA-KDM4B$^{(H189G/E191Q)}$ (mt-KDM4B) together with ARE$_3$-luciferase and β-galactosidase reporter, were stimulated with DHT (100 nM) for 48 h followed by luciferase and β-galactosidase assays. All DNA concentrations were adjusted with empty vector plasmids. All experiments were performed twice or more times, and data are expressed as mean fold change ± SE.

(www.genomatix.de). This revealed three potential AR-binding sites −3000 bases from the transcription start site (TSS) (Supplementary Figure S3), one of which resides in the sequence incorporated within the luciferase construct. To identify whether AR can directly bind the DNA sequence incorporated within the luciferase construct, ChIP assays were performed in LNCaP cells in response to DHT over time, and primers were designed to span an area within the sequence. In response to androgen stimulation, the AR was significantly enriched by 6-fold at the *KDM4B* promoter ($P < 0.005$), confirming that *KDM4B* is indeed an AR-regulated gene (Figure 6E). To further isolate the region of androgen dependence, deletion constructs were used (18), which were all responsive to AR stimulation, suggesting that the site of androgen regulation lies within the first −400 base pairs upstream of the TSS (Figure 6F) contradictory to the

Genomatix predictions. However, after further inspection, a putative ARE was found within this region (Figure 6G). To test whether this site is functional, one of the half sites of the ARE was mutated within the luciferase construct that resulted in an ∼50% decrease in fold induction in response to DHT (Figure 6H), suggesting that the identified ARE is indeed functional (P-value = 0.006).

KDM4B is important for cell proliferation via its regulation of the AR

KDM4B regulates AR levels; therefore, KDM4B knock-down may result in a similar phenotype to AR knockdown. To test this hypothesis, a series of AR-positive cell lines were transfected with siRNA against N/S, AR, KDM4B or both KDM4B and AR together, and proliferation was assessed. As controls, two AR-negative cell lines, DU145 and PC3, were used. On AR or KDM4B knockdown, proliferation was reduced in all AR-positive cell lines but not in AR-negative cell lines (Figure 7A), suggesting that the effects on proliferation are mediated by AR function. Although AR knockdown has been reported to result in a 62% reduction in LNCaP proliferation (30), this was apparent after 11 days by colony forming as opposed to 3.5 days that was used here. However, LNCaP-shKDM4B cells demonstrated a reduced colony-forming ability (∼75%) on KDM4B knockdown (data not shown). When both AR and KDM4B were knocked down, proliferation was inhibited further, again a phenomenon not observed in AR-negative cell lines (Figure 7A). Interestingly, LNCaP-CdxR, a model of PC, which has relapsed treatment with Bicalutimide (Casodex^{TM}), showed ∼40% reduction in proliferation, demonstrating that decreased AR levels in response to KDM4B knockdown do result in a beneficial functional outcome.

KDM4B dysregulated expression can be associated with increasing grade of prostate cancer

As KDM4B seems to be a key molecule in the regulation of AR levels within PC cells, the expression of this demethylase was assessed in human PC clinical material (Figure 7B, Supplementary Figures S4–S6). In total, 78 BPH controls and 187 PC cases were included in this study, all of which have long-term follow-up clinical information available. Newly diagnosed, treatment naïve and paired relapse samples are included in this sample set. Increased immunoreactivity for KDM4B was seen in PC compared with BPH ($P < 0.001$) (Figure 7C), which correlates with the findings of Cloos *et al.* (31) where transcripts were found to be upregulated in PC compared with normal tissue. Moreover, a trend for increasing KDM4B cytoplasmic staining is associated with increasing Gleason grade ($P < 0.001$) (Figure 7D), suggesting a correlation with increasingly aggressive PC. On correlation of KDM4B staining intensity with clinical PC outcomes, time to hormone relapse (Figure 7E) and overall survival (Figure 7F), we observed divergent curves on Kaplan–Meier graphs; however, this pattern is not statistically significant. Although traditionally this KDM is described for its action against nuclear histone proteins, it is evident that in PC, there may be other cytoplasmic roles for this
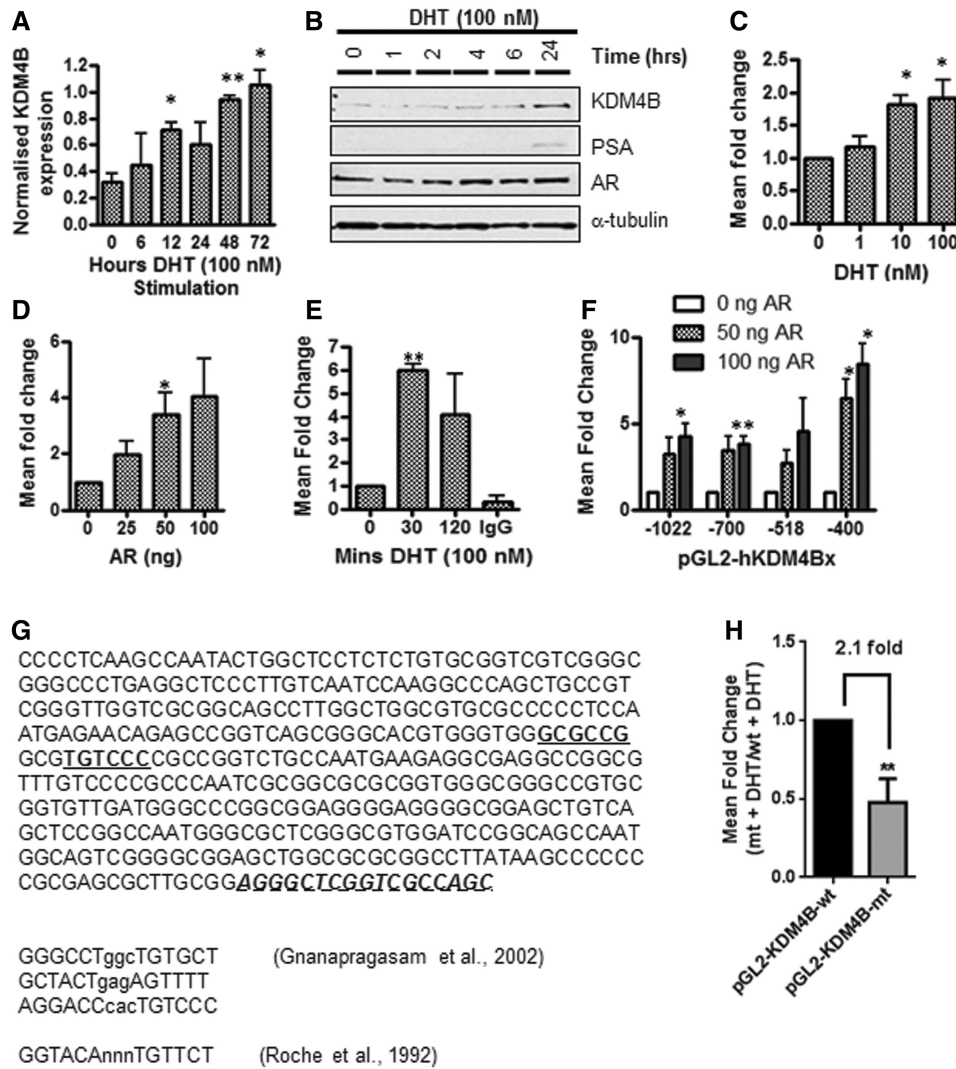
Figure 5. KDM4B inhibits AR ubiquitination. (**A**) Total cell lysate from Cos7 cells transfected with 1 μg of Flag-AR and increasing concentrations (0, 1, 100 and 500 ng) of either pCMV-HA-KDM4B or pCMV-HA- KDM4B$^{(H189G/E191Q)}$ were analysed by western blotting (WB) with anti-AR and anti-KDM4B. α-tubulin was used as a loading control. KDM4B was knocked down in (**B**) LNCaP, (**C**) LNCaP-AI and LNCaP-cdxR cells and analysed by WB using anti-AR, anti-KDM4B and anti-PSA. α-tubulin was used as a loading control. (**D**) Denaturing IP was performed on cell lysates from Cos7 cells transfected with Flag-AR, His-tagged ubiquitin (Ub), pCMV-HA-KDM4B or pCMV-HA-KDM4B$^{(H189G/E191Q)}$ then treated with DHT (100 nM) or vehicle control for 16 h in the presence of MG132 (20 μM). WB was carried out using anti-AR. (**E**) Ub was immunoprecipitated post-protein denaturation from Cos7 cells transfected with Flag-AR, His-tagged Ub and pCMV-HA-KDM4B and was treated with MG132 or vehicle control for 16 h using anti-Ub antibody and analysed by WB with anti-AR. Cos7 cells were transfected with Flag-AR, His-tagged Ub and increasing concentrations (0, 1, 100 and 500 ng) of pCMV-HA-KDM4B (**F**) or pCMV-HA-KDM4B$^{(H189G/E191Q)}$. (**G**) MG132 was applied for 16 h before denaturing IP and WB with anti-AR. (**H**) MDM2 levels in LNCaP cells with KDM4B knockdown. All DNA concentrations were adjusted with empty vector plasmids.

protein, as two other studies have also reported cytoplasmic localization (32,33). Indeed, strong cytoplasmic staining was associated, although not statistically significant, with decreased survival and time to relapse. This is an intriguing finding, as on cellular fractionation of LNCaP cells, KDM4B is found to be completely nuclear (data not shown). However, two independent antibodies have been tested and found to display the same staining patterns: a mixture of cytoplasmic and nuclear staining in prostate tissues (Supplementary Figure S5). The nuclear

staining that was observed (Figure 7, Supplementary Figures S4 and S6) showed no correlation in this data set, with Gleason grade.

DISCUSSION

The known regulatory mechanisms of the AR have substantially increased in complexity during the past decade. This has revealed the roles of many other proteins in the process, particularly those capable of post-translationally

Figure 6. KDM4B is an androgen responsive gene. KDM4B levels were assessed by (**A**) QRT-PCR and (**B**) western blotting in LNCaP cells stimulated with DHT (100 nM) >72 h. *HPRT1* and α-tubulin were used as respective loading controls. Cos7 cells were transfected with β-galactosidase (β-gal) and KDM4B-luciferase reporter (18) with either (**C**) AR with increasing DHT concentrations or (**D**) increasing AR concentrations for 48 h. Luciferase activity was normalized to β-gal controls. Data represent mean fold change of three independent experiments ± SE. (**E**) ChIP of AR at the *KDM4B* promoter in LNCaP cells treated with 100 nM DHT for 0, 30 and 120 min. (**F**) Cos7 cells were transiently transfected with KDM4B-luciferase deletion reporter constructs [pGL2-KDM4B$_{(-1022/+12)}$, pGL2-KDM4B$_{(-700/+12)}$, pGL2-KDM4B$_{(-518/+12)}$ and pGL2-KDM4B$_{(-400/+12)}$] (18) together with β-gal reporter and 0, 50 or 100 ng of AR for 24 h before DHT (100 nM) stimulation for 48 h. Luciferase activity was determined as described earlier in the text. (**G**) Putative androgen response element within the *KDM4B* promoter is underlined in bold. The TSS is denoted in italic. (**H**) Cos 7 cells were transfected with either pGL2-KDM4B$_{(-1022/+12)}$ or pGL2-KDM4Bmt[1] together with β-gal reporter and AR for 24 h before DHT (100 nM) stimulation for 48 h. Luciferase activity was determined as described earlier in the text. Data are expressed as mean fold change between wild-type and mutant reporter activity in response to DHT stimulation from four independent experiments (*$P < 0.05$; **$P < 0.005$).

modifying the receptor to impact on its activity. Furthermore, many of these additional factors have been shown to function as oncogenes in PC development and progression. In addition, as many castrate-resistant cancers still express the AR, targeting its regulatory proteins to indirectly manipulate the AR in disease is a viable therapeutic option. In this study, the KDM enzyme KDM4B was identified as a novel AR regulator. KDM4B can not only facilitate the demethylation of histone proteins at androgen-regulated chromatin, but it can also impact on other post-translational modifications of histone proteins to facilitate transcriptional activation. Furthermore, the

demethylase activity of this protein assists the transcriptional activity of the AR. Most interestingly, KDM4B can also regulate the levels of the AR protein independently of its demethylase activity. On knockdown of KDM4B in multiple cell lines, the levels of AR are significantly reduced, whereas overexpression of KDM4B can stabilize the AR protein. The mechanism through which this occurs seems to be via the prevention of AR-Ub. We speculate that this is due to the structural interaction between KDM4B and AR, masking the sites of ubiquitination or resulting in E3 ubiquitin ligases being unable to bind the AR. Consequently, knockdown of KDM4B results in a
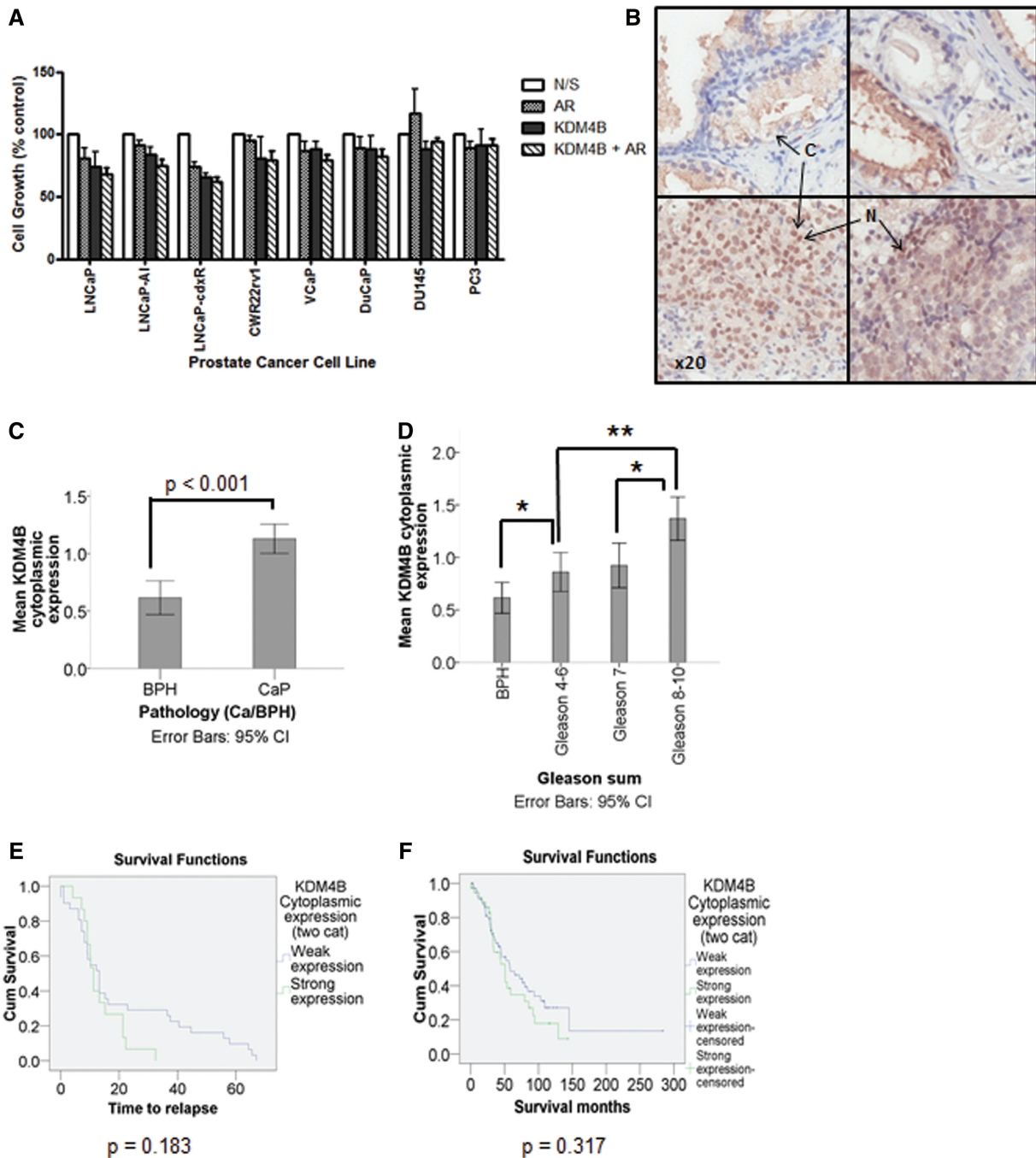
Figure 7. KDM4B associates with increased Gleason grade, decreased survival and reduced time to relapse. (**A**) Cell lines were reverse transfected with non-silencing (N/S), AR, KDM4B or KDM4B and AR together. After three doubling times, proliferation was determined by WST-1 assay (Roche). Data represent mean of three independent experiments with each cell line normalized to N/S controls ± SE. (**B**) Representative KDM4B staining of prostate tissue using anti-KDM4B antibody in immunohistochemistry. C denotes evidence of cytoplasmic staining and N denotes evidence of nuclear staining. (**C**) Analysis of TMA staining for KDM4B using a Mann–Whitney test shows increased KDM4B in CaP compared with BPH ($P < 0.001$), and (**D**) KDM4B increases with CaP grade (*$P < 0.05$; **$P < 0.005$). Kaplan–Meier analysis demonstrates statistically insignificant trend between KDM4B staining, (**E**) time to relapse and (**F**) survival.

reduction in proliferation similar to that of AR knockdown, whereas increased KDM4B expression was found in PC specimens that correlated with increasing grade of cancer. KDM4B itself was also found to be androgen regulated, suggesting the presence of an amplification strategy between the AR and KDM4B to achieve

a maximal response to androgen stimuli. This is similar to that proposed for ER signalling by Shi *et al.* (15), which was published during the preparation of this manuscript, although they do not mention the effects of KDM4B on ER ubiquitination. As KDM4B can increase the levels of AR and its demethylase activity is required to increase AR

activity, the development of a specific KDM4B inhibitor would be highly beneficial.

In response to androgen stimulation, KDM4B was found to be recruited to the promoters of androgen responsive genes, where it was able to demethylate H3K9Me3. We have also found that the activity of KDM4B is required for H3K9Ac to occur, which is not surprising because K9 methylation and acetylation are mutually exclusive (34). Cross-talk with other histone modifications is also likely to occur. For example, MLL2 has been described as a KDM4B interacting protein that can help to regulate the levels of H3K4 methylation at ER-regulated genes (15). Interestingly, MLL2 was also identified in our initial siRNA screen as a potential co-activator of the AR. MLL2 is enriched 3-fold at AR-regulated promoter regions in response to DHT stimulation and its knockdown results in the inhibition of AR-regulated gene expression, whereas the levels of AR themselves remain constant (data not shown). It would be interesting to determine whether KDM4B can also regulate the levels of H3K4Me3 and the activity of MLL2 in PC cell lines too, as H3K4Me3 marks have been described as binding surfaces for proteins containing PHD domains, specifically the ING family of acetyltransferases. As this study provides evidence for the interplay between methylation and acetylation, this leads us to speculate that MLL2 is required to produce H3K4Me3 for the appropriate acetyltransferase to bind and subsequently acetylate H3K9 after removal of H3K9Me3 to continue chromatin signalling towards a transcriptional response.

KDM4B was first suggested to be a p53 target gene via gene expression analysis in TP53-depleted cells treated with 5-fluorouracil (35). More recently, it has also been robustly described in two studies as an Hypoxia Inducible Factor 1-alpha (HIF-1 α) regulated gene (18,36), and most recently as an ER-regulated gene (33). To further add to the complexity of the regulation of this gene, evidence presented here demonstrates that KDM4B can also be regulated by AR. However, unlike the Oestrogen response Element (ERE) identified within intron 1 of KDM4B, a putative ARE resides within −400 bp of the TSS. After more detailed analysis of this sequence, a putative ARE was found −221 bp from the TSS. ChIP confirmed AR binding to this region of the chromatin, and a mutation in one of the half-site results in a reduction in response to AR by 50%, suggesting that this site is functional. Furthermore, other potential AR-binding sites were also identified upstream of the TSS, which have not been investigated, providing the potential for other sites to be functionally identified. ChIP-Seq studies have also identified KDM4B as a putative AR-regulated gene. Massie *et al.* (37) found KDM4B to be upregulated in response to androgens after 5 h, but found no change before this. On analysis of their data by Rivera-Gonzalez *et al.* (38), six androgen response elements were identified close to the KDM4B gene. Guseva *et al.* (39) also identified KDM4B as an AR-regulated gene by ChIP-Seq in LNCaP cells. Furthermore, Wyce *et al.* (40) identified an AR active region with a peak value of 2.612 in muscle cells stimulated with 30 nM DHT for 8 h. This was a similar peak height to TMPRSS2, which had one

active region with a peak value of 2.733. Yet, the majority of studies looking for AR-regulated genes did not identify KDM4B. This may be due to experimental variations. For example, Chen *et al.* (41) applied DHT for 2 h , whereas Urbanucci *et al.* (42) applied DHT for 2 and 24 h.

Given that the AR can exhibit greater stability when KDM4B is overexpressed, and AR can upregulate KDM4B; a positive feedback loop is evident between these two molecules to amplify the androgenic signal. This is similar to that proposed for ER signalling, which was published during the preparation of this manuscript (15). However, KDM4B is not only an androgen-regulated gene but, unlike the ER, it can also hinder AR ubiquitination and subsequent degradation to further amplify the androgenic signal. Indeed, it seems that KDM4B is required for the AR protein to function within the cell because of the extent of AR depletion when KDM4B is knocked down. This novel role for KDM4B involvement in the inhibition of ubiquitination and degradation of the AR warrants further study. Another KDM4 family member, KDM4C, has been described to enhance the levels of the E3 ubiquitin ligase, MDM2 (29), a phenomenon which does not hold true with KDM4B (Figure 5H). The mechanism by which the effect occurs requires further investigation, but it is encouraging to note that interplay between various modifications is just as important here as on histone proteins. Furthermore, it may be proposed that targeting the interaction between AR and KDM4B in a similar way to the interaction between MDM2 and p53 would be of benefit to PC patients. However, investigation into the viability of this strategy is required. Alternatively, the demethylase activity is required to enhance the transcriptional activity of the receptor rendering its enzymatic activity a drug target.

The links between KDM4B and hypoxic response are also important. It is well documented that androgen withdrawal in patients leads to hypoxia (43), and that hypoxia increases AR activity and androgen-regulated gene expression (44,45). Our studies suggest that the mediator of these effects could be KDM4B. KDM4B has been reported to be a transcriptional target of HIF (18); hence, if hypoxia results in increased expression of KDM4B that can stabilize the AR and increase its transcriptional activity and, therefore, enhance androgen-regulated gene expression, this would account for the findings in these studies. Furthermore, the finding that cytoplasmic levels of KDM4B are associated with increasing grade, decreased survival and reduced time to relapse in PC patients may be due to increased stability of the AR in this compartment. This could imply that the cells would be more responsive to lower levels of circulating androgens. This further supports the role of KDM4B as an oncogene in PC and provides a mechanism for KDM4B to be expressed under an androgen-ablated environment.

In summary, our data propose that to obtain efficient androgen-regulated gene transcription, there has to be coordination of events at the histone level, which must interplay with transcription factor post-translational modifications. First, the receptor is activated by its ligand and shuttled to the nucleus of the cell where,

together with its coactivator proteins, the local histone environment is altered by a flux of methylation, demethylation and acetylation events. KDM4B may be one of the initiator molecules in this pathway, as demethylation is certainly required to occur on lysine 9 of histone H3 for acetylation to occur. We have also identified methylases, not discussed here (manuscript in preparation), which may play a role in this chain of events. Because of the close relationship between AR and KDM4B identified here, we believe that KDM4B will prove to be a key therapeutic target in coming years not only for androgen-dependent PC but also for, more importantly, PCs that have relapsed conventional treatments.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figures 1–6 and Supplementary Materials and Methods.

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