

# Phospho-MED1-enhanced UBE2C locus looping drives castration-resistant prostate cancer growth

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The *UBE2C* oncogene is overexpressed in many types of solid tumours including the lethal castration-resistant prostate cancer (CRPC). The underlying mechanisms causing *UBE2C* gene overexpression in CRPC are not fully understood. Here, we show that CRPC-specific enhancers drive *UBE2C* overexpression in both AR-negative and -positive CRPC cells. We further show that co-activator MED1 recruitment to the *UBE2C* enhancers is required for long-range *UBE2C* enhancer/promoter interactions. Importantly, we find that the molecular mechanism underlying MED1-mediated chromatin looping involves PI3K/AKT phosphorylated MED1-mediated recruitment of FoxA1, RNA polymerase II and TATA binding protein and their subsequent interactions at the *UBE2C* locus. MED1 phosphorylation leads to *UBE2C* locus looping, *UBE2C* gene expression and cell growth. Our results not only define a causal role of a post-translational modification (phosphorylation) of a co-activator (MED1) in forming or sustaining an active chromatin structure, but also suggest that development of specific therapies for CRPC should take account of targeting phosphorylated MED1.

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## Introduction

The *UBE2C* protein is an anaphase-promoting complex/cyclosome (APC/C)-specific E2 ubiquitin-conjugating enzyme (Ye and Rape, 2009) that has a critical role in APC/C-dependent M-phase cell-cycle progression by inactivating the M-phase check point (Reddy *et al.*, 2007) or increasing the pool of active APC/C (van Ree *et al.*, 2010). *UBE2C* mRNA and protein are overexpressed in various types of solid tumours

including breast cancer, colon cancer, gastro-esophageal cancer, lung cancer, ovary cancer, thyroid cancer and late-stage prostate cancer (Wagner *et al.*, 2004; Lin *et al.*, 2006; Fujita *et al.*, 2009; Wang *et al.*, 2009). Functional studies have found that overexpression of *UBE2C* accelerates cancer cell proliferation in cancer model systems (Lin *et al.*, 2006; Fujita *et al.*, 2009; Wang *et al.*, 2009) and leads to the development of a broad spectrum of tumours in transgenic mice (van Ree *et al.*, 2010), suggesting that *UBE2C* is a prominent oncogene in solid tumours. However, the underlying mechanisms causing *UBE2C* gene overexpression are not fully understood.

Cell-specific enhancers have a critical role in driving cell-specific gene expression (Crawford *et al.*, 2006; Pennacchio *et al.*, 2007; Heintzman *et al.*, 2009; Bulger and Groudine, 2011). Thus, cancer cell-specific *UBE2C* enhancers may trigger the *UBE2C* overexpression in solid tumour cells. In prostate cancer, *UBE2C* is highly overexpressed in fatal castration-resistant prostate cancer (CRPC) compared with earlier stage androgen-dependent prostate cancer (ADPC) (Varambally *et al.*, 2005; Wang *et al.*, 2009). As a heterogeneous disease, CRPC exists in two forms: androgen receptor (AR)-positive CRPC and AR-negative CRPC (Shah *et al.*, 2004; Li *et al.*, 2008). Our recent studies comparing genome-wide AR binding sites in AR-positive CRPC cells and ADPC cells identified two CRPC-specific AR-bound enhancers located –32.8 and +41.6 kb away from the transcription start site (TSS) of the *UBE2C* gene. AR, an enhancer-bound transcription factor (Bolton *et al.*, 2007; Wang *et al.*, 2007) that has a critical role in prostate cancer growth (Heinlein and Chang, 2004), functions through these two CRPC cell-specific enhancers, leading to increased expression of *UBE2C* in AR-positive CRPC (Wang *et al.*, 2009). However, *UBE2C* enhancers in AR-negative CRPC have not been characterized. Furthermore, the molecular mechanisms underlying *UBE2C* enhancer/promoter interactions in AR-negative and -positive CRPC have not been fully elucidated.

By using a *UBE2C* locus-centric chromosome conformation capture (3C) approach, we identified three distal regions whose interaction with the *UBE2C* promoter is greater in AR-negative CRPC compared with ADPC cells. We further demonstrate enhancer activities of these distal regions in AR-negative CRPC, but not in ADPC cells. Importantly, we determined that a selective post-translational modification of co-activator Mediator 1 (MED1), PI3K/AKT-induced MED1 T1032 phosphorylation in AR-negative CRPC cells, enhanced *in vivo* long-range interactions between the three *UBE2C* enhancers and the *UBE2C* promoter, resulting in *UBE2C* overexpression and AR-negative CRPC cell growth. Finally, we established that phosphorylated MED1-enhanced *UBE2C* locus looping also drives AR-positive CRPC cell growth. These results, in addition to elucidating the transcriptional regulatory mechanisms of *UBE2C* in AR-negative CRPC cells, identify a novel and general role for phosphorylated MED1 in establishing and/or maintaining *UBE2C* locus looping in both AR-negative and -positive CRPC cells.

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## Results

### **Upregulation of UBE2C expression is necessary for AR-negative CRPC cell growth**

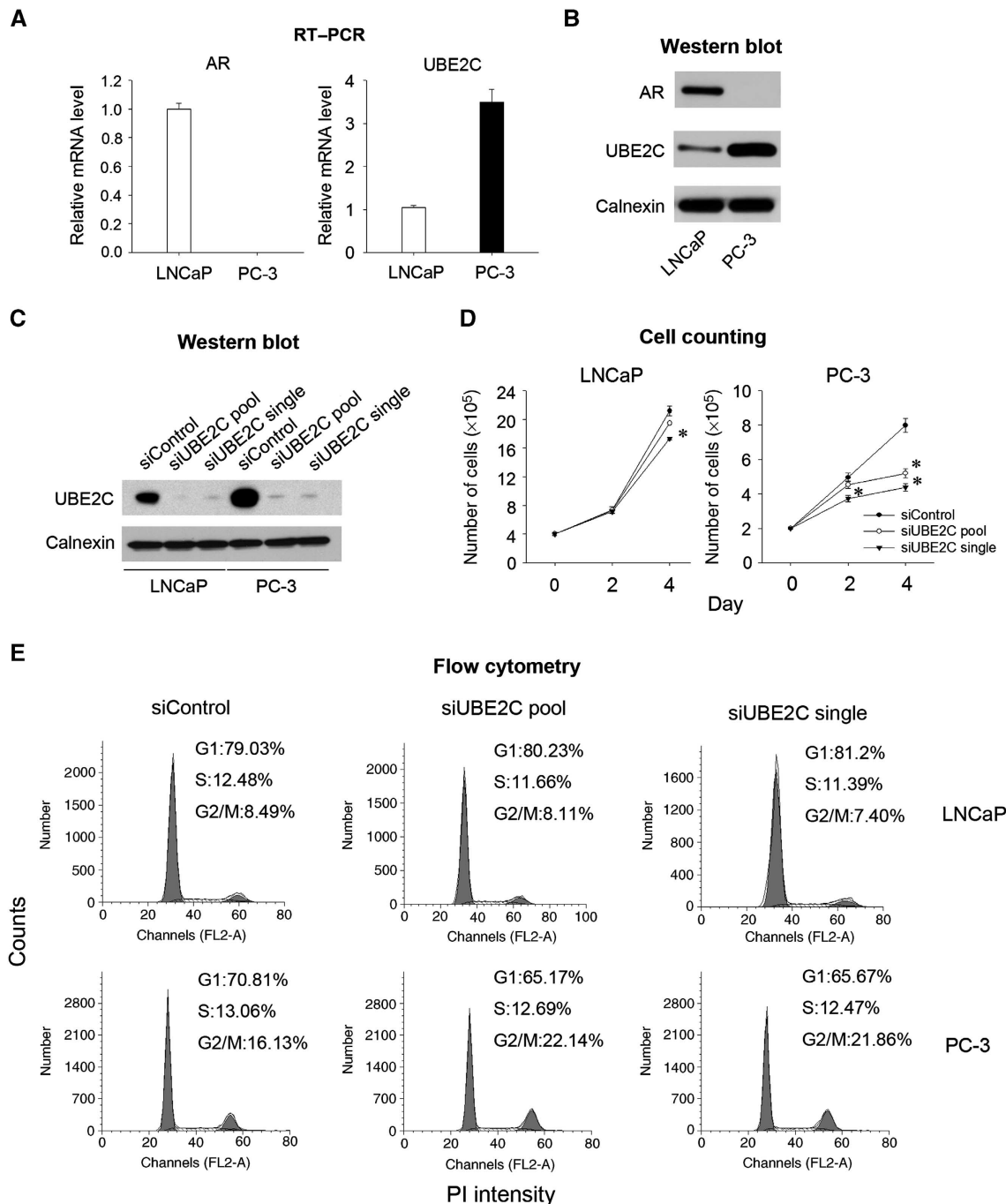
We first compared mRNA expression of UBE2C in the AR-positive ADPC cell line LNCaP with the AR-negative CRPC cell line PC-3 by quantitative RT-PCR. LNCaP is a lymph node-derived ADPC cell line that expresses a cellular differentiation marker prostate-specific antigen (PSA), whereas the CRPC cell line PC-3 is derived from a prostate cancer lumbar vertebral metastasis and does not express AR and PSA (Sobel and Sadar, 2005). LNCaP and PC-3 cells were treated with the physiological androgen 5 $\alpha$ -dihydrotestosterone (DHT) for 4 h. UBE2C mRNA level was significantly greater in PC-3 cells versus LNCaP cells (Figure 1A) and not affected by DHT treatment (Supplementary Figure S1A). As positive controls, DHT treatment significantly increased mRNA expression levels of two well-characterized AR target genes *PSA* and *TMPRSS2* in LNCaP cells (Wang *et al*, 2005, 2007; Supplementary Figure S1B). To rule out the possibility increased UBE2C expression in PC-3 cells was the result of increased RNA stability, LNCaP and PC-3 cells were treated with the transcription inhibitor actinomycin D, and quantitative RT-PCR analysis was performed. UBE2C mRNA stability between LNCaP and PC-3 cells was not different (Supplementary Figure S1C). We next examined AR and UBE2C protein expression levels. Western blot analysis showed that UBE2C protein level was significantly higher in AR-negative PC-3 cells versus AR-positive LNCaP cells (Figure 1B; Supplementary Figure S1D). To investigate the functional role of UBE2C overexpression in PC-3 cells, we tested the effects of UBE2C silencing on cell proliferation and cell-cycle progression. Silencing of UBE2C decreased proliferation of PC-3 compared with LNCaP cells (Figure 1C and D). Consistent with the role of UBE2C in driving cell-cycle G2/M progression (Reddy *et al*, 2007; van Ree *et al*, 2010), fluorescence-activated cell sorting (FACS) analysis revealed that the decreased PC-3 proliferation after UBE2C silencing was due to a G2/M-phase blockage (Figure 1E; Supplementary Figure S1E). Collectively, these data suggest that overexpression of UBE2C has an essential role in AR-negative CRPC cell growth.

### **Identification and characterization of UBE2C enhancers in AR-negative CRPC cells**

The higher expression of UBE2C in AR-negative PC-3 compared with LNCaP motivated us to further investigate the mechanism underlying UBE2C gene regulation in AR-negative PC-3 cells. Given that cell-specific enhancers drive cell-specific gene expression (Crawford *et al*, 2006; Pennacchio *et al*, 2007; Heintzman *et al*, 2009; Bulger and Groudine, 2011), we hypothesized that PC-3-specific UBE2C enhancers direct the upregulation of the UBE2C gene in PC-3 cells. The findings that distal enhancers communicate with target gene promoters through chromatin looping (Wang *et al*, 2005, 2007, 2009; Dekker, 2008) suggested that distal regions forming loops with specific gene promoters may potentially function as gene-specific enhancers. As 3C technology allows for the detection of physical interactions between such distal/proximal regions (Dekker *et al*, 2002), we performed quantitative 3C assays (Hagege *et al*, 2007) for the UBE2C locus in LNCaP and PC-3 cells, in order to identify potential

PC-3-specific UBE2C enhancers. LNCaP and PC-3 cells were treated with vehicle or DHT for 4 h. The formaldehyde crosslinked chromatin was digested with *Bgl*III and 3C was performed at the UBE2C locus extending ~120 kb (–60 to +60 kb of TSS). Analysis of the 3C results identified seven crosslinking frequencies-high ( $\geq 20$ ) fragments with greater interactions ( $\geq 1.5$ -fold) between these fragments and the UBE2C promoter in PC-3 than in LNCaP: the –36 kb (1.66-fold), –20 kb (1.94-fold), –17 kb (1.52-fold), –14 kb (1.57-fold), +2 (1.75-fold), +19 kb (1.52-fold) and +25 kb (1.53-fold) fragments (Figure 2A; Supplementary Figure S2A). Although no effect of DHT on UBE2C locus chromatin looping was observed, interactions between four distal AR binding regions (Wang *et al*, 2007; Yu *et al*, 2010) were significantly enhanced after androgen treatment in the *TMPRSS2* locus and the *TMPRSS2* promoter (Supplementary Figure S2B). No difference in crosslinking frequencies between the –7 and +46 kb fragments and the UBE2C promoter was observed between the two cell lines. Interestingly, our previous studies found that an AR-bound UBE2C enhancer (termed Enhancer-1) was located within the –20 kb fragment in an AR-positive CRPC cell line LNCaP-abl (Wang *et al*, 2009). Thus, Enhancer-1 might be a shared UBE2C enhancer between LNCaP-abl cells and PC-3 cells, though different Enhancer-1 binding proteins may exist in these two cell lines.

We next selected the fragment with the highest fold change (–20 kb fragment, hereafter called E1), and the closest upstream and downstream fragments (–14 kb (hereafter called E2) and +2 kb fragments (hereafter called E3)) for further analysis. We addressed whether the Enhancer-1 within the E1 fragment (fragment size 1.2 kb), the E2 fragment (fragment size 2.3 kb) and the E3 fragment (fragment size 1.8 kb) contained specific enhancer elements in PC-3 cells. Using genomic DNA from LNCaP and PC-3 cells as PCR templates, we systematically subcloned three ~400 bp regions in Enhancer-1 (hereafter called E1-1, E1-2 and E1-3), six ~400 bp regions in the –14 kb fragment (hereafter called E2-1, E2-2, E2-3, E2-4, E2-5 and E2-6) and five ~400 bp regions in the +2 kb fragment (hereafter called E3-1, E3-2, E3-3, E3-4 and E3-5) in front of a minimal E4 TATA promoter driving the expression of luciferase. These constructs generated using LNCaP and PC-3 genomic DNA were transfected into LNCaP cells and PC-3 cells, respectively. The luciferase was measured after 24 h treatment with vehicle or DHT. As shown in Supplementary Figure S2C, E1-3, E2-1, E2-4, E3-1, E3-2 and E3-5 displayed significant enhancer activity ( $> 2.5$ -fold) in PC-3 cells in the presence or absence of DHT. In contrast, no enhancer activity of these regions was observed in LNCaP cells. As a positive control for DHT-stimulated transcription, treatment of LNCaP cells with DHT increased transcriptional activation of the PSA enhancer reporter (Wang *et al*, 2005; Supplementary Figure S2D). To address whether sequence differences between LNCaP-generated and PC-3-generated constructs may account for their differential enhancer activity in LNCaP and PC-3 cells, sequencing analysis of these constructs was performed. Sequences of the LNCaP-generated and PC-3-generated E1-3, E2-1, E3-1, E3-2 and E3-5 were not different, but a C to A point mutation in the E2-4 sequence was seen in the PC-3 cells (Supplementary Table I). However, the LNCaP-generated E2-4 construct demonstrated similar enhancer activity to that



**Figure 1** Increased UBE2C expression is required for PC-3 cell growth. (A) The UBE2C mRNA level is higher in PC-3 cells than in LNCaP cells. Total RNA was isolated and amplified with primers recognizing AR mRNA and UBE2C mRNA (mean ( $n = 3$ )  $\pm$  s.d.). (B) The UBE2C protein expression level is higher in PC-3 cells than in LNCaP cells. Western blots analyses were performed using the indicated antibodies. (C) Suppression of UBE2C protein levels by RNAi. LNCaP and PC-3 cells were transfected with a siUBE2C ON-TARGET pool including four siRNAs and a single siUBE2C not included in the siUBE2C ON-TARGET pool, and western blots were performed using an anti-UBE2C antibody. (D) UBE2C silencing significantly decreases PC-3 growth. The cell numbers were determined by a direct viable cell count assay (mean ( $n = 2$ )  $\pm$  s.d.) (two-sided *t*-test,  $*P < 0.05$ ). (E) UBE2C silencing arrests cell cycle in G2/M-phase. Ninety-six hours after UBE2C siRNAs transfection, LNCaP and PC-3 cells were analysed by FACS. A representative result of three independent experiments was shown.

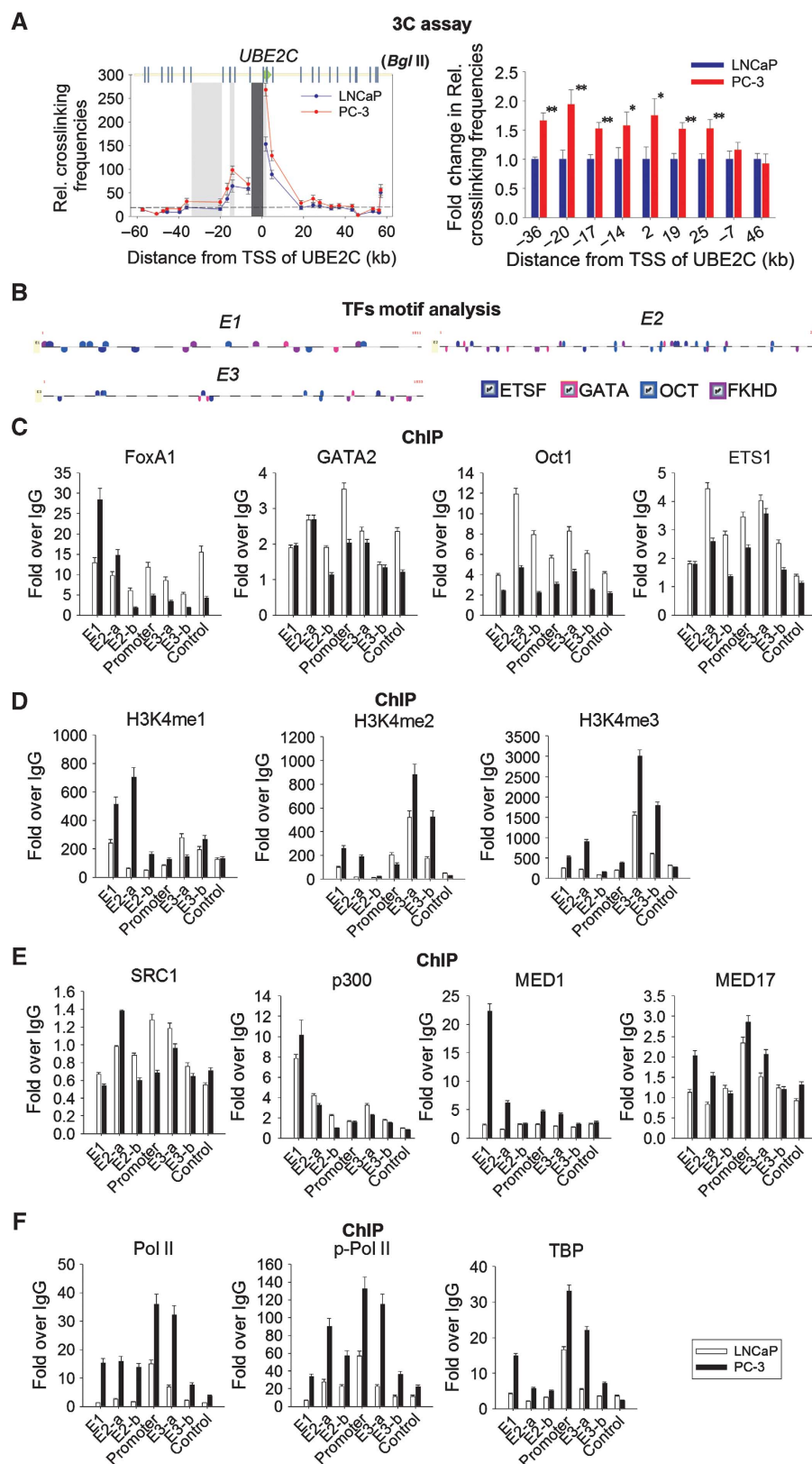
of PC-3-generated E2-4 construct in PC-3 cells (data not shown), suggesting that sequence variation in this construct does not contribute to enhancer activity variation. Collectively, these results demonstrate that E1, E2 and E3 contain PC-3-specific functional enhancers.

We further investigated whether E1, E2 and E3 could regulate UBE2C expression in another AR-negative CRPC cell line, DU-145, derived from a different tissue (brain)

(Sobel and Sadar, 2005). Although enhancer activity ( $> 1.5$ -fold) of E1-1 and E3-1 was significant in reporter gene assays conducted in DU-145 cells (Supplementary Figure S2E), no difference in UBE2C locus looping was observed between LNCaP and DU-145 cells (Supplementary Figure S2F), suggesting that other mechanisms (e.g., enhancers beyond the  $-60$  to  $+60$  kb regions and/or attenuated UBE2C mRNA stability) may be responsible for the

increased UBE2C mRNA and protein expression (Supplementary Figures S2G and H) in DU-145 cells. These findings, however, are consistent with recent genome-wide studies demonstrating that a large proportion of enhancers are cell specific (Heintzman *et al*, 2009; Bulger and Groudine, 2011).

The observation that almost identical sequences in E1, E2 and E3 display greater interactions with the UBE2C promoter on chromatin and drive higher expression of reporter genes in PC-3 cells compared with LNCaP cells indicated that PC-3-specific transcription complexes bind and function through these enhancers. We first investigated whether specific



transcription factors are recruited to E1, E2 and E3 regions. Because motif analysis of E1, E2 and E3 sequences identified a large number of motifs, we focused on specific transcription factors that recognize these motifs and are expressed in prostate cancer cells or prostate tissues: FoxA1, GATA2, Oct1 and ETS1 (BioGPS) (Wu *et al*, 2009; Figure 2B). The expression levels of these transcription factors in PC-3 cells were similar to or slightly lower than those in LNCaP cells (Supplementary Figure S3A). To examine whether these transcription factors were differentially recruited to E1, E2 and E3 on chromatin, chromatin immunoprecipitation (ChIP) assays were performed using specific antibodies against FoxA1, GATA2, Oct1 and ETS1 in LNCaP and PC-3 cells treated with or without DHT. The immunoprecipitated DNA was analysed by quantitative PCR using primers spanning E1, E2 and E3. As the average sizes of sonicated fragments in ChIP assays were around 1–1.5 kb and the sizes of E2 and E3 were larger than 1.5 kb, two primer sets were designed to cover E2 and E3 regions (termed E2-a, E2-b, E3-a and E3-b). A LNCaP-abl-specific +41.6 kb AR-bound enhancer (termed Enhancer-2) (Wang *et al*, 2009) served as a negative control. Recruitment of GATA2, Oct1 and ETS1 to E1, E2, E3, the UBE2C promoter, and the control region was similar or even lower in PC-3 cells compared with LNCaP cells (Figure 2C; Supplementary Figure S3B). In contrast, increased FoxA1 binding (>1.5-fold) at E1 and E2-a regions but not at the control region was seen in PC-3 cells compared with LNCaP cells (Figure 2C; Supplementary Figure S3B). Consistent with our recent findings that higher levels of enhancer histone methylation marks H3K4 mono- and di-methylation (H3K4me1 and H3K4me2) (Heintzman *et al*, 2007, 2009) led to increased FoxA1 binding in breast cancer cells and AR-positive CRPC cells (Lupien *et al*, 2008; Wang *et al*, 2009), we found that higher FoxA1 recruitment to E1 and E2-a regions was correlated with higher levels (>1.5-fold) of H3K4me1 and H3K4me2 on E1 and E2-a regions in PC-3 cells than in LNCaP cells treated with or without DHT (Figure 2D; Supplementary Figure S3C). Although we did not identify specific transcription factors that show stronger binding to E2-b, E3-a and E3-b regions in PC-3 cells than in LNCaP cells, we found that higher levels (>1.5-fold) of H3K4me1 and/or H3K4me2 on these regions but not the control region in PC-3 cells compared with LNCaP cells (Figure 2D; Supplementary Figure S3C). Interestingly, we also found that H3K4 tri-methylation (H3K4me3) level was significantly higher (>2-fold) on E3-a and E3-b regions in PC-3 cells than in LNCaP cells (Figure 2D; Supplementary Figure S3C), consistent with previous reports showing that H3K4me3 was also detected at some enhancers (Barski *et al*, 2007; Wang *et al*, 2008).

We next examined whether transcription co-activators are differentially recruited to E1, E2 and E3 regions. Transcription co-activators are generally classified into two families: (a) chromatin remodelling or modifying enzymes (e.g., histone acetyltransferases (HATs) SRC1 and p300) and (b) the Mediator complex that functions to bridge transcription factors with preinitiation complex (PIC), and regulates the assembly and function of PIC (Malik and Roeder, 2005; Taatjes, 2010). We first examined SRC1, p300 and the Mediator complex subunits MED1 and MED17 protein levels using western blot analysis. Levels of SRC1, p300 and MED17 protein were similar between LNCaP and PC-3 cells, whereas MED1 protein level was significantly higher in PC-3 cells versus LNCaP cells (Supplementary Figure S3A). To examine the recruitment of SRC1, p300, MED1 and MED17 to E1, E2 and E3 regions in LNCaP and PC-3 cells, ChIP assays were performed, both in the presence and absence of hormone. While the levels of SRC1 and p300 on E1, E2, E3, the UBE2C promoter and the control region were similar or even lower in PC-3 compared with LNCaP, significantly higher levels of MED1 and/or MED17 (>1.5-fold) was observed on E1, E2-a, E3-a and the promoter region but not on the control region in the absence and/or presence of DHT (Figure 2E; Supplementary Figure S3D). Consistent with the notion that Mediator facilitates the recruitment of RNA polymerase II (Pol II) and TATA binding protein (TBP) to target gene promoter regions (Malik and Roeder, 2005; Taatjes, 2010), we found higher levels of Pol II (>2-fold), phosphorylated Pol II (>2-fold) and TBP (>1.5-fold) occupancy at the UBE2C promoter in PC-3 compared with LNCaP in the presence or absence of androgen (Figure 2F; Supplementary Figure S3E). In agreement with previous findings showing Pol II, phosphorylated Pol II and TBP are also present at active enhancers (Louie *et al*, 2003; Wang *et al*, 2005; Kim *et al*, 2010), the recruitment of Pol II, phosphorylated Pol II and TBP to E1, E2 and E3 regions was also higher (>1.5-fold) in PC-3 cells (Figure 2F; Supplementary Figure S3E). Collectively, these data show greater recruitment of FoxA1 and Mediator to AR-negative CRPC cell-specific UBE2C enhancers in PC-3 versus LNCaP, which was also correlated with increased interactions between the UBE2C enhancers and the UBE2C promoter, and enhanced PIC assembly in PC-3 cells.

#### **FoxA1 and MED1 mediated long-range chromatin interactions between the UBE2C enhancers and the UBE2C promoter lead to UBE2C gene expression in AR-negative CRPC cells**

To investigate whether increased expression and/or binding of FoxA1 and the Mediator at the PC-3-specific UBE2C enhancers have a causal role in long-range interactions

**Figure 2** Identification and characterization of UBE2C enhancers in PC-3 cells. (A) Greater interactions between seven distal regions and the UBE2C promoter in PC-3 cells than in LNCaP cells. Left panel: 3C assays were performed in LNCaP and PC-3 cells in the absence of DHT. The black shading shows the position of the fixed fragment (the UBE2C promoter). The grey shading indicates three fragments that were selected for further functional analysis. Right panel: The results of 3C assays were presented as fold changes in relative crosslinking frequencies (two-sided *t*-test, \**P*<0.05, \*\**P*<0.01). The –7 and +46 kb regions were used as controls. (B) Schematic representation of ETSF, GATA, OCT and FKHD motifs within the E1, E2 and E3 regions. (C) Increased FoxA1 binding at the UBE2C enhancers in PC-3 cells than in LNCaP cells. ChIP assays were performed using antibodies against FoxA1, GATA2, Oct1 and ETS1 in LNCaP and PC-3 cells in the absence DHT (mean (*n*=3) ± s.d.). (D) Higher levels of H3K4 methylation on the UBE2C enhancers in PC-3 cells compared with LNCaP cells. ChIP assays were performed as above using antibodies against H3K4me1, H3K4me2 and H3K4 me3 (mean (*n*=3) ± s.d.). (E) Increased recruitment of MED1 and/or MED17 to the UBE2C enhancers and promoter. ChIP assays were conducted as above using SRC1, p300, MED1 and MED17 antibodies. (F) Higher levels of Pol II and TBP occupancy on the UBE2C promoter and enhancers in PC-3 cells than in LNCaP cells. ChIP assays were performed as above using antibodies against Pol II, phosphorylated Pol II and TBP (mean (*n*=3) ± s.d.).

between the UBE2C enhancers and the UBE2C promoter, we examined the effect of silencing of FoxA1 and MED1 on loop formation. PC-3 cells were transfected with siFoxA1, siMED1 or siControl. Seventy-two hours after siRNA transfection, 3C assays were performed at the UBE2C locus to measure crosslinking frequencies between PC-3-specific UBE2C enhancers and the UBE2C promoter. As shown in Figure 3A, FoxA1 silencing caused a modest decrease of crosslinking frequencies between E1, E2, E3 and the UBE2C promoter. However, MED1 silencing significantly decreased the interactions between E1, E2, E3 and the UBE2C promoter. Western blot analysis showed that siFoxA1 and siMED1 correspondingly decreased protein expression levels (Figure 3B), indicating that the greater silencing effect of MED1 than FoxA1 on

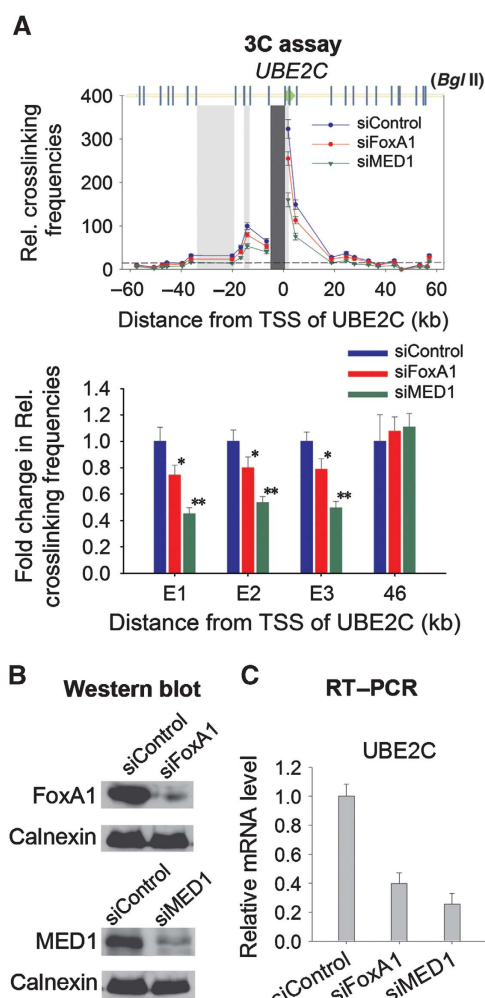
looping formation was not due to differences in siRNA efficiency. Consistent with the notion that formation of chromatin loops is a prerequisite for transcription (Vakoc *et al*, 2005; Cai *et al*, 2006), silencing of FoxA1 or MED1 (but more notably MED1) significantly decreased UBE2C mRNA expression (Figure 3C). These findings indicated that MED1 was a crucial mediator of chromatin looping and gene expression at the UBE2C locus in AR-negative CRPC cells.

### Enhanced expression and recruitment of PI3K/AKT phosphorylated MED1 strongly correlates with increased protein–protein interactions at the UBE2C locus in AR-negative CRPC cells

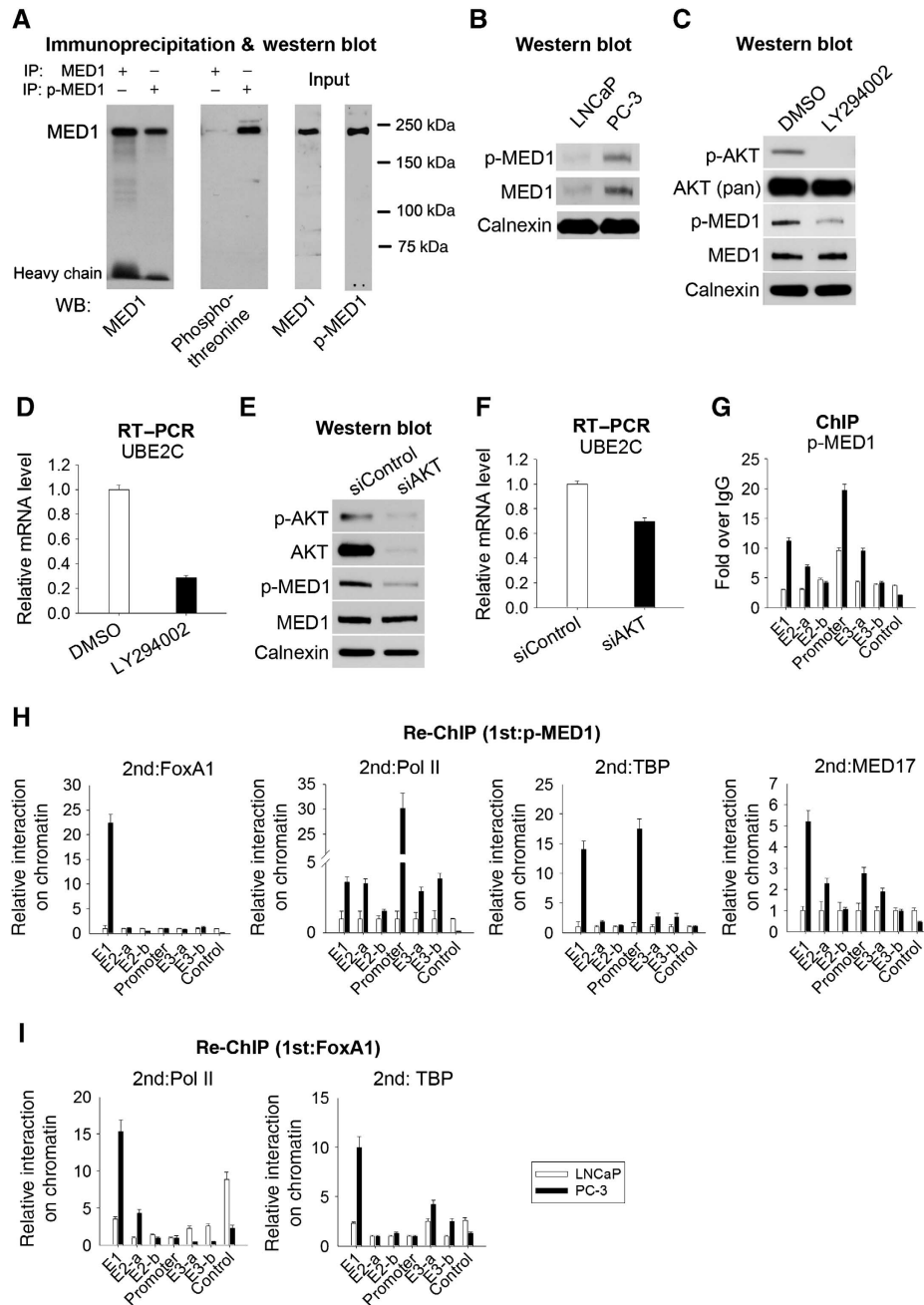
We next investigated the underlying mechanisms for MED1-mediated looping in the UBE2C locus in AR-negative CRPC cells. As previous studies strongly suggested that protein–protein interactions between enhancer-bound proteins and promoter-bound proteins mediate chromatin looping (Vakoc *et al*, 2005; Miele and Dekker, 2008; Nolis *et al*, 2009), and in view of the fact that Mediator facilitates the recruitment of RNA Pol II and TBP to target gene promoter regions (Malik and Roeder, 2005; Taatjes, 2010), we reasoned that MED1 drives the formation of chromatin looping by enhancing protein recruitment and protein–protein interactions at the UBE2C locus. Consistent with the idea that protein phosphorylation significantly affects protein–protein interaction (Sims and Reinberg, 2008), MED1 phosphorylation on threonine (T) 1032 and 1457 was recently shown to promote its association with Pol II and other subunits within the Mediator complex in solution (Pandey *et al*, 2005; Belakavadi *et al*, 2008). We thus further hypothesized that MED1 phosphorylation drives chromatin looping by enhancing protein recruitment and protein–protein interactions on chromatin.

To investigate the role of MED1 phosphorylation in UBE2C locus looping in AR-negative CRPC cells, we generated a phosphorylated T1032-specific MED1 (p-MED1) antibody. To confirm that the p-MED1 antibody is phosphorylation specific, MED1 was immunoprecipitated from PC-3 cells using p-MED1 antibodies or a commercial MED1 antibody. The immunoprotein complexes were then probed by western blot using MED1 or phospho-T antibodies. As shown in Figure 4A, MED1 antibodies recognized MED1 immunoprecipitated by either MED1 antibodies or p-MED1 antibodies. Importantly, phospho-T antibodies strongly recognized MED1 immunoprecipitated by p-MED1 antibodies, but only weakly detected MED1 immunoprecipitated by commercial MED1 antibodies, demonstrating the specificity of the p-MED1 antibody. Subsequent western blot analysis using the p-MED1 antibody showed greater expression of phosphorylated MED1 in PC-3 versus LNCaP (Figure 4B), which was mostly due to the increased total MED1 expression in PC-3 (Figure 4B; Supplementary Figure S4A).

As previous studies have shown that MED1 is phosphorylated at T1032 and T1457 by extracellular signal-regulated kinase (ERK) of the mitogen-activated protein kinase (MAPK) family in HeLa cells (Pandey *et al*, 2005; Belakavadi *et al*, 2008), we next investigated which kinases, in PC-3 cells, are responsible for phosphorylating MED1. We first examined whether inhibition of MAPK pathway influences MED1 phosphorylation. Treatment of PC-3 cells with U0126, an inhibitor for MAPK kinase 1/2 (MEK1/2), had no effect on MED1



**Figure 3** Silencing of FoxA1 and MED1 impairs long-range interactions at the UBE2C locus and decreases UBE2C gene expression in PC-3 cells. (A) Upper panel: knocking down of FoxA1 and MED1 decreases crosslinking frequencies between the UBE2C enhancers and the UBE2C promoter. 3C assays were performed in siControl, siFoxA1 or siMED1 transfected PC-3 cells. Lower panel: the 3C results were presented as fold changes in relative crosslinking frequencies (two-sided *t*-test, \**P* < 0.05, \*\**P* < 0.01). (B) Suppression of FoxA1 and MED1 levels by siRNAs. PC-3 cells were transiently transfected with siControl, siFoxA1 or siMED1 and protein levels were determined by western blot analysis. (C) Silencing of FoxA1 and MED1 decreases UBE2C gene expression in PC-3 cells. PC-3 cells were transfected with siRNA targeting FoxA1 and MED1. Seventy-two hours after siRNA transfection, real-time RT-PCR was performed (mean (*n* = 3) ± s.d.).



**Figure 4** Enhanced interactions between PI3K/AKT-induced T1032 phosphorylated MED1 and UBE2C enhancer/promoter-bound proteins on chromatin in PC-3 cells. (A) Characterization of a phosphorylated T1032-specific MED1 (p-MED1) antibody. Whole cell lysates from PC-3 cells were immunoprecipitated with a commercial MED1 antibody or p-MED1. Western blot analyses were then performed using indicated antibodies. (B) Comparisons of phosphorylated MED1 and MED1 protein levels in LNCaP and PC-3 cells. Western blot analyses were performed with the p-MED1 antibody. The same membrane was then reprobed with the MED1 antibody. (C) Effects of LY294002 on MED1 phosphorylation at T1032. PC-3 cells were treated with 50  $\mu$ M LY294002 or vehicle for 24 h, and western blot analyses were performed using antibodies indicated. (D) Effects of LY294002 on UBE2C mRNA expression. Total RNA was isolated from LY294002 or vehicle-treated PC-3 cells, and amplified with UBE2C primers (mean ( $n = 3$ )  $\pm$  s.d.). (E) Silencing of AKT decreases MED1 phosphorylation at T1032 in PC-3 cells. Seventy-two hours after siRNA transfection, western blot analyses were performed using antibodies indicated. (F) AKT silencing decreases UBE2C mRNA expression. Real-time RT-PCR was performed 72 h after siRNA transfection (mean ( $n = 3$ )  $\pm$  s.d.). (G) Higher recruitment of phosphorylated MED1 to the UBE2C enhancers and promoter in PC-3 cells than in LNCaP cells. ChIP assays were conducted with the p-MED1 antibody (mean ( $n = 3$ )  $\pm$  s.d.). (H) Increased protein–protein interactions between phosphorylated MED1 with UBE2C enhancer and promoter-bound proteins in PC-3 cells compared with LNCaP cells. ChIPs were first performed with the p-MED1 antibody. The immunoprecipitated complexes were eluted and the second ChIPs were performed with antibodies against FoxA1, Pol II, TBP and MED17 (mean ( $n = 3$ )  $\pm$  s.d.). (I) Increased protein–protein interactions between FoxA1 and Pol II/TBP. Re-ChIP assays were performed using antibodies against FoxA1 (for first ChIP) and Pol II/TBP (for second ChIP).

T1032 phosphorylation (Supplementary Figure S4B). However, because phosphorylation sites for both MAPK (P-X-S/T-P, where X represents any amino acid) (Gonzalez *et al*,

1991; Pearson *et al*, 2001) and AKT (R-X-X-S/T, where X represents any amino acid) (Basu *et al*, 2003; Fang *et al*, 2007) are contained with the T1032 region of MED1, it was of

interest to examine the effect of phosphatidylinositol 3-kinase (PI3K)/AKT pathway inhibition on MED1 phosphorylation. Interestingly, exposure of PC-3 to the PI3K inhibitor LY294002 decreased AKT phosphorylation at Serine (S) 473 and MED1 phosphorylation at T1032 (Figure 4C), leading to a reduced UBE2C mRNA expression (Figure 4D). The decreased T1032 phosphorylated MED1 protein expression (Figure 4E) and UBE2C mRNA expression (Figure 4F) in siAKT transfected versus siControl transfected PC-3 cells further supported that PI3K/AKT phosphorylated MED1 at T1032 in PC-3 cells.

We next examined the functional significance of PI3K/AKT-induced MED1 T1032 phosphorylation in PC-3. ChIP analysis revealed the increased (>1.5-fold) recruitment of T1032 phosphorylated MED1 to E1, E2-a, E3-a and the UBE2C promoter region in PC-3 versus LNCaP (Figure 4G; Supplementary Figure S4C and D). We then used serial ChIP experiments (re-ChIP) to further investigate whether greater phosphorylated MED1 recruitment to the UBE2C enhancers and promoters correlated with increased protein-protein interaction on the same DNA fragments at the UBE2C locus in PC-3. First-round ChIP was with p-MED1 antibodies, followed by second-round ChIP using antibodies against enhancer-bound FoxA1, promoter-bound Pol II and TBP, and another Mediator subunit MED17. Interactions between phosphorylated MED1 and FoxA1, Pol II, TBP and MED17, on UBE2C enhancers and/or UBE2C promoters were much stronger (>2-fold) in PC-3 cells than in LNCaP cells (Figure 4H). To investigate whether such increased interactions between phosphorylated MED1 and enhancer/promoter-bound proteins lead to enhanced interactions between enhancer-bound proteins and promoter-bound proteins, FoxA1 ChIP followed by re-ChIP with Pol II and TBP was performed. As anticipated, the FoxA1–Pol II and FoxA1–TBP interactions were significantly increased (>1.5-fold) in PC-3 cells compared with LNCaP cells in the UBE2C locus (Figure 4I; Supplementary Figure S4E), further supporting the hypothesis that phosphorylated MED1 mediates UBE2C locus looping through enhancing its interactions with the enhancer-bound transcription factor FoxA1, and promoter-bound Pol II and TBP.

#### **Phosphorylation of MED1 in AR-negative CRPC cells has a causal role in UBE2C locus looping, UBE2C gene expression and cell growth**

To further substantiate the critical role of phosphorylation of MED1 for UBE2C locus looping and UBE2C gene expression, retrovirus-mediated gene transfer was used to establish stable

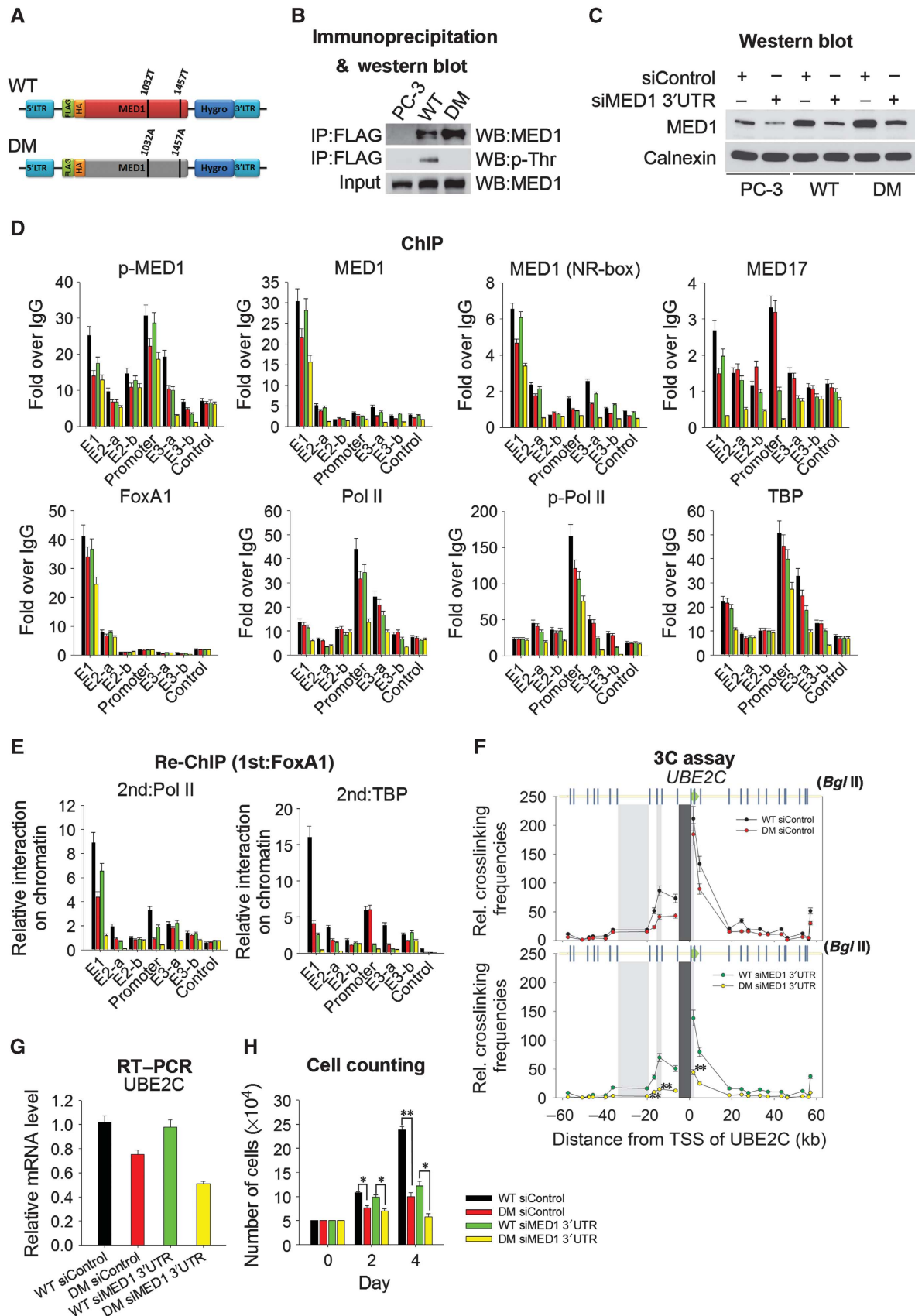
PC-3 cell lines expressing a FLAG/HA epitope-tagged wild-type (WT) MED1 (termed PC-3/WT MED1) and a FLAG/HA epitope-tagged double phosphomutant (T1032A/T1457A) MED1 (termed PC-3/DM MED1) (Figure 5A; Supplementary Figure S5). We performed a FLAG immunoprecipitation/western blot analysis to examine the protein expression levels of epitope-tagged MED1 in PC-3/WT MED1 and PC-3/DM MED1. Consistent with previous reports showing that co-activator (e.g., MED1 and PGC-1) phosphomutants with alanine substitutions have increased protein expression levels compared with WT co-activators due to altered interactions with protein turnover factors (Puigserver *et al*, 2001; Pandey *et al*, 2005), we found that the epitope-tagged MED1 expression level was higher in PC-3/DM MED1 cells than in PC-3/WT MED1 cells (Figure 5B). Importantly, phospho-T antibodies only recognized WT MED1 but not DM MED1 immunoprecipitated by FLAG antibodies (Figure 5B).

To examine the effect of MED1 phosphorylation on protein binding at the UBE2C locus, a siRNA targeting MED1 3' untranslated region (UTR) was used to deplete endogenous MED1 (Figure 5C), as ectopic epitope-tagged WT MED1 and DM MED1 constructs lack 3'UTR (Figure 5A). ChIP assays were then performed to examine transcription complex binding at the UBE2C enhancers and promoter between PC-3/WT MED1 and PC-3/DM MED1 cell lines. Using this knocking down approach, we observed significantly lower ( $\geq 1.5$ -fold) recruitment of ectopic phosphorylated MED1 to E1, E3-a, E3-b and the UBE2C promoter in PC-3/DM MED1 cells versus PC-3/WT MED1 cells (Figure 5D). While the protein expression level of epitope-tagged MED1 was lower in PC-3/WT MED1 cells than in PC-3/DM MED1 cells, recruitment of epitope-tagged WT MED1 and/or MED17 to E1, E2-a, E2-b, E3-a, E3-b and the UBE2C promoter regions was markedly higher ( $\geq 1.5$ -fold) in PC-3/WT MED1 cells than in PC-3/DM MED1 cells (Figure 5D), demonstrating that Mediator complex binding to chromatin was enhanced by phosphorylation of MED1. Interestingly, FoxA1 binding at the E1 region was significantly decreased ( $\geq 1.5$ -fold) in PC-3/DM MED1 cells compared with PC-3/WT MED1 cells (Figure 5D), indicating that phosphorylated MED1 may assist FoxA1 binding. Consistent with the essential role of the Mediator complex in facilitating Pol II and TBP to target gene promoters (Malik and Roeder, 2005; Taatjes, 2010), the attenuated recruitment of MED1, phosphorylated MED1 and MED17 to the UBE2C enhancers and/or promoter led to significantly decreased ( $\geq 1.5$ -fold) Pol II and TBP loading on the UBE2C promoter in PC-3/DM MED1 compared with PC-3/WT MED1 cells

**Figure 5** Phosphorylation of MED1 in PC-3 cells is required for UBE2C locus looping, UBE2C gene expression and cell growth. (A) Schematic diagram of the retroviral vectors expressing FLAG/HA-tagged wild-type (WT) MED1 and T1032A/T1457A double-mutated (DM) MED1. (B) Immunoprecipitation of epitope-tagged WT MED1 and DM MED1 from PC-3/WT MED1 and PC-3/DM MED1 cells. Whole cell lysates were immunoprecipitated with an anti-FLAG antibody. Western blot analyses were then performed using an anti-MED1 antibody and an anti-phospho-threonine antibody. (C) Suppression of MED1 expression by RNAi. Parental PC-3, PC-3/WT MED1 and PC-3/DM MED1 cells were transfected with siMED1 targeting MED1 3'UTR, and western blot was performed with an anti-MED1 antibody. (D) Decreased recruitment of proteins to the UBE2C locus in PC-3/DM MED1 cells compared with PC-3/WT MED1 cells. PC-3/DM MED1 cells and PC-3/WT MED1 cells were transfected with siControl and siMED1 3'UTR. ChIP assays were performed using antibodies indicated (mean ( $n=3$ )  $\pm$  s.d.). (E) Decreased interactions between FoxA1 and Pol II/TBP at the UBE2C locus in PC-3/DM MED1 cells compared with PC-3/WT MED1 cells. PC-3/DM MED1 cells and PC-3/WT MED1 cells were transfected with siControl and siMED1 3'UTR. Re-ChIP assays were conducted with antibodies against FoxA1 (for first ChIP) and Pol II/TBP (for re-ChIP) (mean ( $n=3$ )  $\pm$  s.d.). (F) Disrupted UBE2C locus looping in PC-3/DM MED1 cells compared with PC-3/WT MED1 cells (two-sided *t*-test,  $^{**}P<0.01$ ). 3C assays were performed using siControl and siMED1 3'UTR transfected PC-3/DM MED1 cells and PC-3/WT MED1 cells. (G) UBE2C mRNA expression is lower in PC-3/DM MED1 cells than in PC-3/WT MED1 cells transfected with siMED1 3'UTR (mean ( $n=3$ )  $\pm$  s.d.). (H) PC-3/DM MED1 cells transfected with siMED1 3'UTR grow slower than PC-3/WT MED1 cells transfected with siMED1 3'UTR. The cell proliferation was measured using a direct viable cell count assay (mean ( $n=2$ )  $\pm$  s.d.) (two-sided *t*-test,  $^{*}P<0.05$ ,  $^{**}P<0.01$ ).

(Figure 5D). The effect of MED1 phosphorylation on protein-protein interaction at the UBE2C locus was further examined using re-ChIP assays (FoxA1 antibody for the first ChIP and

Pol II and TBP antibodies for second ChIP). Those experiments revealed that interactions between FoxA1 and Pol II/TBP were significant attenuated (>2-fold) on the UBE2C



enhancers and the UBE2C promoter (Figure 5E). Collectively, these findings suggested that FoxA1, Pol II and TBP recruitment to the UBE2C locus and their interactions on chromatin were enhanced by MED1 phosphorylation.

We next tested whether the decreased protein recruitment and protein–protein interactions at the UBE2C locus attenuate chromatin looping and *UBE2C* gene expression. PC-3/DM MED1 cells and PC-3/WT MED1 cells were transfected with siMED1 3'UTR to silence endogenously expressed MED1; cultured for 72 h; and 3C and RT-PCR assays were performed. As shown in Figure 5F, the crosslinking frequencies between all three UBE2C enhancers and the UBE2C promoter were significantly lower in PC-3/DM MED1 cells versus PC-3/WT MED1 cells, suggesting that MED1 phosphorylation was necessary for UBE2C locus looping. As expected, UBE2C mRNA level was significantly lower in PC-3/DM MED1 cells compared with PC-3/WT MED1 cells, paralleling the decreased interactions between the UBE2C enhancers and the UBE2C promoter (Figure 5G).

Having established a significant role for phosphorylation of MED1 in UBE2C locus looping and *UBE2C* gene expression (Figure 5F and G) and that the *UBE2C* gene was necessary for PC-3 cell proliferation (Figure 1D), the functional importance of MED1 phosphorylation was examined by comparing the growth of siMED1 3'UTR transfected PC-3/WT MED1 cells and PC-3/DM MED1 cells. Results of cell proliferation assays clearly demonstrated that PC-3/DM MED1 cells grow much slower than PC-3/WT MED1 cells (Figure 5H), strongly supporting a critical role for MED1 phosphorylation in AR-negative CRPC cell proliferation.

### Phosphorylated MED1 in AR-positive CRPC cells also drives UBE2C locus looping, UBE2C gene expression and cell growth

Our previous findings that MED1 binding to the two AR enhancers (Enhancer-1 and Enhancer-2) at the UBE2C locus is increased in an AR-positive CRPC cell model LNCaP-abl compared with LNCaP cells (Wang *et al*, 2009) prompted us to investigate whether MED1 and phosphorylated MED1 in LNCaP-abl cells also drive UBE2C locus looping, *UBE2C* gene expression and cell growth. MED1 protein level was higher in LNCaP-abl versus LNCaP cells (Figure 6A), and ChIP assays showed higher ( $\geq 1.5$ -fold) occupancy of MED1 to E1 (Enhancer-1), E2-b, E3-b, Enhancer-2, and the UBE2C promoter regions in LNCaP-abl versus LNCaP cells (Figure 6B), which was correlated with increased interactions between the UBE2C enhancers (E1, E2, E3 and Enhancer-2) and the UBE2C promoter (Figure 6C; Supplementary Figure S6A). Similar to our findings in PC-3 cells, we found that silencing of MED1 significantly decreased the interactions between the

UBE2C enhancers and promoter in LNCaP-abl cells (Figure 6D; Supplementary Figure S6B), strongly indicating that MED1 is also crucial for UBE2C locus looping in LNCaP-abl cells. Given that protein expression and binding of PI3K/AKT phosphorylated MED1 to UBE2C locus were higher in LNCaP-abl compared with LNCaP (Figure 6A and E; Supplementary Figure S6C), we next examined the functional role of phosphorylated MED1 in LNCaP-abl cells. Importantly, siMED1 3'UTR transfection followed by 3C assays revealed UBE2C locus looping was significantly decreased in LNCaP-abl/DM cells compared with LNCaP-abl/WT cells (Figure 6F; Supplementary Figure S6D), leading to decreased UBE2C mRNA expression (Figure 6G) and cell growth (Figure 6H) of LNCaP-abl/DM cells compared with LNCaP-abl/WT cells. Taken together, these findings demonstrate the general importance of MED1 and phosphorylated MED1 in UBE2C locus looping, *UBE2C* gene expression and CRPC growth.

While MED1 and phosphorylated MED1 are essential for UBE2C locus looping and *UBE2C* gene expression of both AR-negative and -positive CRPC cells (Figures 3, 5 and 6), overexpressed MED1 and phosphorylated MED1 in LNCaP cells were not sufficient to enhance UBE2C expression (Supplementary Figure S7A–C). Furthermore, silencing of endogenous MED1 had no effect on *UBE2C* gene expression in LNCaP cells (Supplementary Figure S7D), suggesting that MED1 is a crucial determinant for UBE2C expression in CRPC cells but not in ADPC cells. It is possible that during prostate cancer progression from ADPC to CRPC, altered expression of additional transcription factors and/or coregulators is required for MED1-mediated UBE2C expression in CRPC.

## Discussion

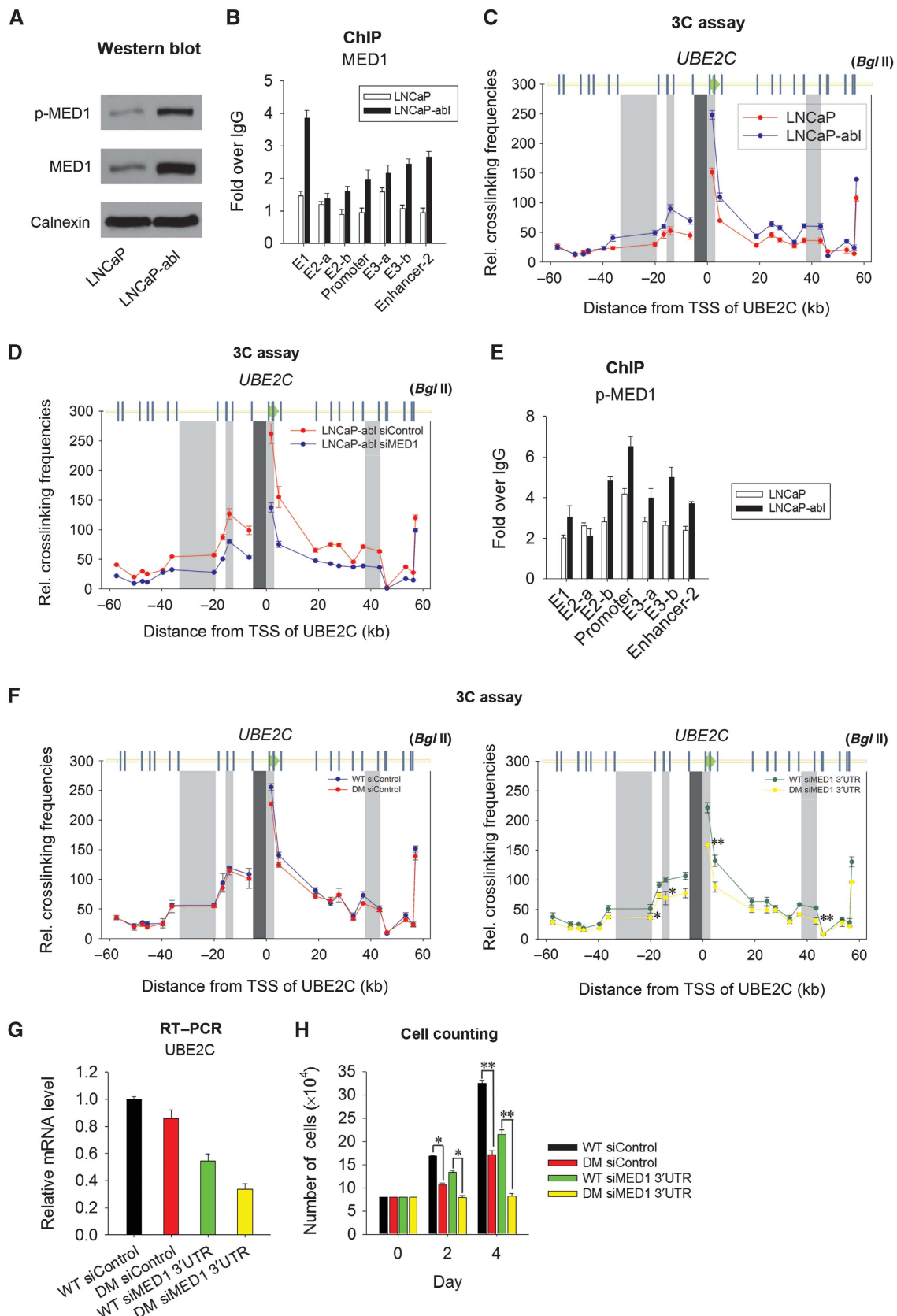
### Identification and characterization of CRPC cell-specific UBE2C enhancers

The identification of cell-specific enhancers has been the focus of several recently developed computational and experimental approaches, including sequence evolution conservation analysis (Pennacchio *et al*, 2006), and mapping of DNase hypersensitive sites (Crawford *et al*, 2006), histone marks such as H3K4me1 (Heintzman *et al*, 2007, 2009), or transcription co-activators such as p300 and MED1 (Heintzman *et al*, 2007, 2009; Visel *et al*, 2009) throughout the genome. However, assigning the distal enhancers identified using these approaches to their target genes remains a considerable experimental challenge. In this study, by using a UBE2C locus-centric 3C approach, we identified three distal regions (E1, E2 and E3) with greater interactions with the UBE2C promoter in both AR-negative and -positive CRPC cells (PC-3 and LNCaP-abl), compared with LNCaP (a well-

**Figure 6** Phosphorylated MED1 in LNCaP-abl cells enhances UBE2C locus looping, UBE2C mRNA expression and cell growth. (A) Comparison of MED1 and phosphorylated MED1 expression levels in LNCaP and LNCaP-abl cells. Western blot analyses were performed using the antibodies indicated. (B) Increased MED1 binding at the UBE2C enhancers and UBE2C promoter in LNCaP-abl compared with LNCaP cells. ChIP assays were performed using an anti-MED1 antibody (mean ( $n = 3$ )  $\pm$  s.d.). (C) Increased interactions between the UBE2C enhancers (E1, E2, E3 and Enhancer-2) and the UBE2C promoter in LNCaP-abl compared with LNCaP cells. 3C assays were performed in LNCaP-abl and LNCaP cells in the absence of DHT. (D) Silencing of MED1 decreases crosslinking frequencies between the UBE2C enhancers and the UBE2C promoter in LNCaP-abl cells. 3C assays were performed in siControl or siMED1 transfected LNCaP-abl cells. (E) Increased recruitment of phosphorylated MED1 to the UBE2C regulatory regions in LNCaP-abl compared with LNCaP cells. ChIP assays were performed with the p-MED1 antibody. (F) Disrupted UBE2C locus looping in LNCaP-abl/DM MED1 cells compared with LNCaP/WT MED1 cells (two-sided *t*-test  $*P < 0.05$ ,  $**P < 0.01$ ). 3C assays were performed following siControl or siMED1 3'UTR transfection. (G) Decreased UBE2C mRNA expression in LNCaP-abl/DM MED1 cells transfected with siMED1 3'UTR compared with LNCaP-abl/WT MED1 cells transfected with siMED1 3'UTR (mean ( $n = 3$ )  $\pm$  s.d.). (H) Decreased cell growth of LNCaP-abl/DM MED1 cells transfected with siMED1 3'UTR compared with LNCaP-abl/WT MED1 cells transfected with siMED1 3'UTR. (mean ( $n = 2$ )  $\pm$  s.d.) (two-sided *t*-test,  $*P < 0.05$ ,  $**P < 0.01$ ).

known model for ADPC) (Figures 2A and 6C). Greater levels of co-activator MED1, phosphorylated MED1 and Pol II binding, and active histone H3K4 methylation marks at

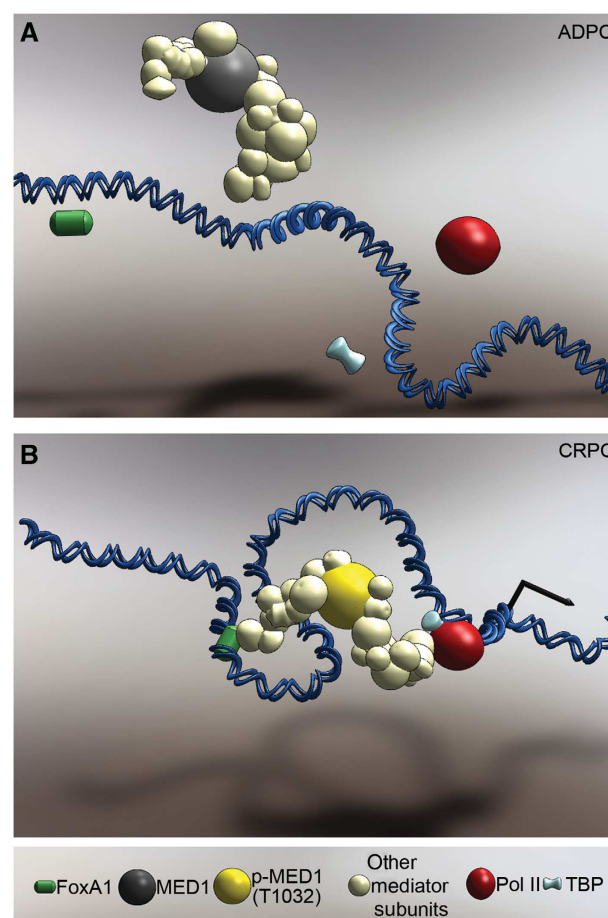
these distal regions indicate that these regions have important transcriptional regulatory roles in CRPC but not in ADPC cells (Figures 2 and 6; Wang *et al*, 2009).



### Phosphorylated MED1 enhances UBE2C locus looping in CRPC cells

Having identified CRPC-specific UBE2C enhancers that interact with the UBE2C promoter through chromatin looping, it was of interest to determine the transcription factors and co-activators that mediate loop formation. Based on the results using an integrative approach of ChIP assays combined with siRNA-3C assays, we assert that higher occupancy of MED1 binding at the UBE2C enhancers and promoter is required for the UBE2C locus looping in PC-3 and LNCaP-abl cells (Figures 2, 3 and 6), consistent with previous reports demonstrating that transcription factors (e.g., GATA-1 and oestrogen receptor) (Vakoc *et al*, 2005; Fullwood *et al*, 2009) and co-activators (e.g., Med12, p300/CBP and BRG1) (Park *et al*, 2005; Wang *et al*, 2005; Hu *et al*, 2008; Kim *et al*, 2009; Kagey *et al*, 2010) have important roles in looping establishment and/or maintenance. While previous studies, including our own, imply that MED1 is important for chromatin looping in other loci (Park *et al*, 2005; Wang *et al*, 2005; Degenhardt *et al*, 2009), the current study is the first to demonstrate that MED1 has a causal role in looping formation in a locus (Figures 3A and 6D).

Chromatin remodellers (e.g., BRG1) and HATs (e.g., CBP/p300) are thought to control looping via modifying chromatin structures (Kim *et al*, 2009). However, as Mediator lacks intrinsic chromatin modifying activities (Malik and Roeder, 2005; Taatjes, 2010), how this type of co-activator mediates looping is currently unknown. Studies reporting that Mediator recruitment to chromatin occurs after recruitment of chromatin remodellers and HATs suggest that the function of Mediator is subsequent to other modifiers (Sharma and Fondell, 2002; Metivier *et al*, 2003). However, these findings do not rule out the possibility that Mediator may function as a 'chromatin architectural' factor. Because protein phosphorylation has profound effect on protein-protein interaction (Sims and Reinberg, 2008) and MED1 is highly phosphorylated at T1032 by PI3K/AKT in PC-3 cells (Figure 4), we hypothesize that the Mediator complex may establish and/or sustain the looping through phosphorylated MED1-mediated protein recruitment and interactions between enhancer- and promoter-bound proteins. In support of this hypothesis, we first show that the higher expression and binding of phosphorylated MED1 is strongly correlated with its stronger interactions with FoxA1, Pol II, TBP and MED17, the stronger interactions between FoxA1 and Pol II and TBP, and enhanced interactions between the UBE2C enhancers and promoter in PC-3 cells versus LNCaP cells (Figure 4; Supplementary Figure S4). Importantly, we further demonstrate that the ability of a MED1 phosphomutant to facilitate FoxA1, Pol II and TBP recruitment to the UBE2C locus and their interactions on chromatin is compromised compared with a WT MED1 in PC-3 cells (Figure 5). The similar role of PI3K/AKT phosphorylated MED1 in enhancing UBE2C enhancer/promoter interactions in LNCaP-abl cells (Figure 6; Supplementary Figure S6) suggest that phosphorylated MED1 is of general importance for enhancing UBE2C locus looping in CRPC cells. Collectively, these data are the first to establish MED1 phosphorylation as a mechanism underlying the ability of the Mediator complex to form or sustain an active chromatin structure at the UBE2C locus in CRPC cells, but not in ADPC cells (Figure 7). Our future studies will address whether MED1 phosphorylation mediates looping in a locus-



**Figure 7** Models of phosphorylated MED1-mediated chromatin looping. (A) In ADPC cells, unphosphorylated MED1 does not mediate recruitment of FoxA1, Pol II and TBP and their subsequent interactions on chromatin. (B) In CRPC cells, phosphorylated MED1 at T1032 facilitates FoxA1, Pol II and TBP recruitment and mediates their interactions on chromatin leading to chromatin looping.

specific manner or results in a global change of higher-order structures. Given that MED1 is a 'master gene' (O'Malley, 2006), interacting with multiple transcription factors (e.g., AR and BRCA-1) (Wang *et al*, 2002; Wada *et al*, 2004) and other proteins (e.g., cohesin) (Kagey *et al*, 2010) that contribute to looping formation involved in diverse signalling pathways, it seems reasonable to speculate that phosphorylated MED1-mediated looping is not limited in the UBE2C locus.

### Targeting UBE2C and PI3K/AKT/phosphorylated MED1 pathway in CRPC therapy

As CRPC continues to express a functional AR, clinical studies targeting the receptor are ongoing (Knudsen and Penning, 2010). However, as AR expression in CRPC is highly heterogeneous, both within and between patients (Shah *et al*, 2004; Li *et al*, 2008), identifying growth-related genes functional in both AR-negative and -positive CRPC and studying their regulatory mechanisms has strong translational implications for the disease, particularly for developing new therapeutics. We report that UBE2C and phosphorylated MED1 are essential for both AR-negative and -positive CRPC cell growth (Figures 1, 5 and 6; Wang *et al*, 2009), identifying potential new therapeutic targets for CRPC. As PI3K/AKT pathway is constitutively active in a significant

portion of CRPC patients due to loss of phosphatase and tensin homologue (PTEN) activity (Li *et al*, 2005; Majumder and Sellers, 2005), identification of potent inhibitors for PI3K/AKT will be critical for inhibiting MED1 phosphorylation in both AR-negative and -positive CRPC. Finally, combining agents that target AR, UBE2C and phosphorylated MED1 pathway may be an effective strategy in future clinical trials for heterogeneous CRPC, a fatal disease.

## Materials and methods

### Cell culture

The AR-positive ADPC cell line (LNCaP) and AR-negative CRPC cell lines (PC-3 and DU-145) were obtained from the American Type Culture Collection. The AR-positive CRPC cell line LNCaP-abl was kindly provided by Zoran Culig (Innsbruck Medical University, Austria; Culig *et al*, 1999).

### Real-time RT-PCR

Real-time RT-PCR was performed as before (Wang *et al*, 2009). Briefly, total RNA was isolated using an RNeasy kit (Qiagen, Valencia, CA). cDNA was reverse transcribed from total RNA (2 µg) using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Real-time polymerase chain reaction was performed using Power SYBR<sup>®</sup> Green PCR Master Mix (Applied Biosystems) on the StepOnePlus<sup>™</sup> Real-Time PCR System (Applied Biosystems) following the manufacturer's instructions. The primers were designed using the Primer Express<sup>®</sup> Software v3.0 (Applied Biosystems). Primer sequences are listed in Supplementary Table I.

### Cell proliferation assay

Cell proliferation was measured by a direct viable cell count assay.

### RNA interference

siRNA duplexes were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Cells were analysed 48–72 h after transfection. The siRNA sequences were listed in Supplementary Table I.

### FACS analysis

To analyse cell-cycle profiles,  $1 \times 10^6$  cells were harvested, washed and fixed in ice-cold 70% ethanol overnight. Subsequently, fixed cells were stained with a solution containing 50 µg/ml propidium iodide, 100 µg/ml RNase and 0.1% Nonidet P-40 for 30 min at 37°C. The DNA content and percentage of cells in different phases of the cell cycle were determined using a FACS Calibur cell flow cytometer (Becton Dickinson Biosciences, San Diego, CA) and quantified using ModFit software (Verity Software House, Inc., Topsham, ME).

### Quantitative 3C

3C-qPCR assays were performed as described with minor modifications (Wang *et al*, 2009). Details are available in the Supplementary data.

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### ChIP and serial ChIP (re-ChIP)

ChIP and re-ChIP assays were performed as previously described (Wang *et al*, 2005, 2007). For ChIP assay, chromatin was crosslinked for 10 min at room temperature with 1% formaldehyde. After sonication, chromatin was immunoprecipitated with specific antibodies at 4°C overnight. The reversed ChIP DNA was purified and then analysed by real-time PCR. For re-ChIP assays, the first immunoprecipitated complexes were washed, eluted with 10 mM dithiothreitol at 37°C for 30 min and diluted 50 times with ChIP dilution buffer. The second immunoprecipitations were then performed. Each ChIP or re-ChIP assay was repeated at least three times with independent samples. Antibodies used are described in Supplementary data.

### Western blots and immunoprecipitation

Western blots and immunoprecipitation were performed as previously described (Wang *et al*, 2002). Antibodies used are described in Supplementary data.

### Plasmids construction and retrovirus-mediated gene transfer

The pWZL-hygro-Flag-HA TRAP220 wild-type (WT) vector was purchased from Addgene (Cambridge, MA) (Ge *et al*, 2008). To generate pWZL-hygro-Flag-HA TRAP220 double-mutant (DM) vector (T residue 1032 to A and T residue 1457 to A), site-directed mutagenesis was performed using the Quick Change (Stratagene) according to the manufacturer's instructions. The mutagenic oligonucleotides were listed in Supplementary Table I. PC-3 and LNCaP-abl cells were infected with retroviral particles containing constructs encoding a WT MED1 or a T1032A/T1457A double-mutated MED1, selected with hygromycin, and pooled drug-resistant clones were used for the experiments.

### Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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**Author contributions:** This study was designed by ZC and QW. The experiments were performed by ZC, CZ, DW, HC, AR and QW. The data analyses were performed by ZC, XZ and QW. ZC and QW wrote the manuscript.

## Conflict of interest

The authors declare that they have no conflict of interest.

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