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

Zhong Chen¹, Chunpeng Zhang¹, Dayong Wu¹, Hongyan Chen¹, Anna Rorick¹, Xiaoting Zhang² and Qianben Wang¹,*

¹Department of Molecular and Cellular Biochemistry and the Comprehensive Cancer Center, The Ohio State University College of Medicine, Columbus, OH, USA and ²Department of Cancer and Cell Biology, College of Medicine, University of Cincinnati, Cincinnati, OH, USA

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 checkpoint (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.
Results

Uptregulation 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 5α-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 BglII 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 (≥20) fragments with greater interactions (≥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, E2-2, E2-3, E2-4, E2-5 and E2-6 were significantly enhanced after androgen treatment in LNCaP cells compared with LNCaP and 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
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 E1/C0 + 60 kb regions and/or attenuated UBE2C mRNA stability) may be responsible for the...

**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) ± 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) ± 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.

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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-α-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...
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 phosphorylation. However, U0126 significantly decreased MED1 phosphorylation in AR-negative CRPC cells. Moreover, the phosphorylation efficiency of MED1 was found to be increased in LNCaP cells compared with AR-negative CRPC cells. Importantly, phospho-T antibodies strongly recognized MED1 immunoprecipitated by either MED1 antibodies or p-MED1 antibodies. This suggests that MED1 phosphorylation drives chromatin looping by enhancing protein recruitment and protein–protein interactions on chromatin.
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

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 μM 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) ± 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) ± 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) ± 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) ± 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).
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 was performed with p-MED1 antibodies. 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 epo-ptide-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 delete 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 (>1.5-fold) recruitment of ectopic phosphorylated MED1 to E1, E3-a, E3-b and the UBE2C promoter in PC-3/WT MED1 cells versus PC-3/DM 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, E3-b, E3-a and the UBE2C promoter regions was markedly higher (>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 (>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 (>1.5-fold) Pol II and TBP loading on the UBE2C promoter in PC-3/DM MED1 compared with PC-3/WT MED1 cells.
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 attenuated 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 (>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-
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 remodelers (e.g., BRG1) and HATs (e.g., CRP/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 remodelers 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 (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-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
To analyse cell-cycle profiles, FACS analysis was performed (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, UBEC2 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 a 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® Green PCR Master Mix (Applied Biosystems) on the StepOnePlus™ Real-Time PCR System (Applied Biosystems) following the manufacturer’s instructions. The primers were designed using the Primer Express™ 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 are listed in Supplementary Table I.

FACS analysis
To analyse cell-cycle profiles, 1 x 10⁶ 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.

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 reverse 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.

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

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Conflict of interest
The authors declare that they have no conflict of interest.

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