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

Zhong Chen, Chunpeng Zhang, Dayong Wu, Hongyan Chen, Anna Rorick, Xiaoting Zhang, and Qianben Wang

Corresponding author: Qianben Wang, The Ohio State University

Review timeline:

<table>
<thead>
<tr>
<th>Event</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Submission date</td>
<td>14 October 2010</td>
</tr>
<tr>
<td>Editorial Decision</td>
<td>05 November 2010</td>
</tr>
<tr>
<td>Revision received</td>
<td>29 January 2011</td>
</tr>
<tr>
<td>Editorial Decision</td>
<td>21 February 2011</td>
</tr>
<tr>
<td>Rebuttal</td>
<td>23 February 2011</td>
</tr>
<tr>
<td>Additional Editorial</td>
<td>24 February 2011</td>
</tr>
<tr>
<td>Author Correspondence</td>
<td>25 February 2011</td>
</tr>
<tr>
<td>Revision received</td>
<td>18 April 2011</td>
</tr>
<tr>
<td>Accepted</td>
<td>18 April 2011</td>
</tr>
</tbody>
</table>

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 05 November 2010

Thank you very much for submitting your research manuscript for consideration to The EMBO Journal editorial office.

As you will see from the comments enclosed below, the referees appreciate the potential functional contribution of phospho-MED towards chromatin looping and thus UBE2C regulation in the absence of AR. However, their detailed comments also reveal the very preliminary state of the study when it comes to upstream regulatory mechanisms convincing support for the proposed proliferation model and importantly physiological significance in the context of other hormone-independent cell lines. Thus, all three are currently not convinced that the study corroborates the proposed model experimentally to justify publication in a rather more general journal such as The EMBO Journal. Despite the current obvious limitations that are explicitly spelled out in the major points from the referees (so no need for me to repeat them here in full), we decided to offer you the chance to follow their detailed suggestions and amend the paper in a way that should convince the referees from the significance of your findings. I do have to urge you to take their comments serious and invest the necessary time and experimental efforts to avoid later disappointments. Given the strong demands I would also understand if you would decide to submit the study in its current form elsewhere.

In case you decide to pursue revisions for the EMBO Journal, I have to remind you that it is EMBO_J policy to allow a single round of major revisions but we are able to grant additional time for necessary experimentation upon authors request. Finally, please keep in mind that the final
decision on acceptance or rejection entirely depends on the content and strength of the final version of your manuscript that will be assessed by some of the original referees!

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1:

This manuscript entitled "Phospho-MED1-induced chromatin looping drives androgen receptor negative prostate cancer growth" attempts to establish how a key oncogene, UBE2C, is regulated in absence of androgen receptor. The authors report that Med1 phosphorylation is required for enhancer looping and gene expression and subsequent activation of UBE2C.

The concept of mediator influencing chromatin architecture and gene expression is not particularly novel, but the mechanisms regulating that function remained to be explored. Furthermore, the discovery of new avenues for treatment of AR-independent cancers is highly clinically relevant. The authors provide some valuable insight into this area, but need to improve on several major aspects of their manuscript in order for this reviewer to recommend publication.

In particular, this paper would be greatly improved if the authors could provide insight into the signaling pathway(s) that is regulating Med1 phosphorylation and show that this pathway(s) is physiologically relevant. For instance does inhibition or activation of the MAPK kinase pathway influence the phosphorylation status of Med1 and UBE2C expression levels? It would also be interesting to know if the same enhancers are active in other hormone-independent cancer cell lines.

Major points:

1) A key element of the proposed manuscript is establishing the dependency on UBE2C for PC-3 proliferation. However, in the current form, it is far from convincing. The WST-1 assay is not a "cell number" assay, but a metabolism-based assay. The block in G2/M is represented by a 2-3% increase, which is very minimal compared to the results presented for the WST-1 assay. Another interpretation of the data would be that UBE2C KD decreases metabolism, but does not affect proliferation. To start with, the authors should use a direct viable cell count assay and display the full cell cycle profiles. Then, they should try to support their conclusions with statistical analysis of the observed differences. Finally, they need to comment on how a 2-3% delay in G2/M explains their phenotype.

2) The 3C data in Fig 2A, Fig 4A and 6F are not convincing. There are no error bars for the 3C graph. Was this experiment only done once? Was the real-time PCR at least done in triplicate? If the real-time PCR was done in triplicate there should at least be error bars for that. This reviewer understands that 3C experiments are very difficult to do and hard to interpret, but it's not clear why the 3 putative enhancers regions were selected when there are additional fragments (besides -20 kb, -14 kb and +2 kb) with a greater interaction frequency in PC-3 cells relative to LNCaP cells (Fig 2A).

When the authors state in the text that the crosslinking frequencies for in the PC-3/DM Med1 cells were significantly lower relative to the PC-3/WT Med1 cells (Fig 6F). Is this actually significant? It is hard to tell without a calculated P-value or at least error bars on the graph.

3) What is the control in Fig 2B? Does it include a positive enhancer region? This information should be in the figure legend. It seems strange that the cloned enhancer fragments are having a negative effect relative to the control in LNCaP cells.
4) The validation of the phospho-Med1 antibody needs to be more rigorous.

It doesn't make any sense to this reviewer why the total Med1 and the phospho-specific antibody give such different results in ChIP. For instance, why does the Med1 Ab not detect any signal at the promoter (Fig 6D) while a lot of enrichment is detected for the phospho-Med1 Ab? Furthermore, all regions tested following the phospho-Med1 ChIP gave at least 20-fold enrichment including the control region. This reviewer is left wondering if this antibody is really specific. If possible, a competition with different phospho-peptide in ChIP conditions would increase confidence in the antibody specificity. The authors should add a full WB of the input in addition to the IP in Fig 5 to show that the antibody recognizes only 1 band.

Figure 5B and the text accompany it is misleading. "Western blots analysis using the phospho-MED1 antibody showed that the expression of phosphorylated MED1 was significantly higher in PC-3 cells than in LNCaP cells (Figure 5B)." According to Figure 3A, Med1 is expressed at much higher levels in PC-3 cells, so it is not surprising that there is more phospho-Med1 in PC-3 cells. However the text and figure imply that the ratio of phosphorylated to unphosphorylated Med1 is greater in PC-3 cells, which most likely is not the case. The text should be changed to reflect this. However, if the ratio actually is greater this would be usual information to know since it would indicate that the signaling pathway resulting in phospho-Med1 is more active in PC-3 cells. The authors should probe the same lysates in Figure 5B for total Med1 to determine this.

5) The authors overstate this conclusion: "...demonstrating that phosphorylation of MED1 was required for the Mediator complex to bind to chromatin." First the Med1 double phosphorylation mutant still binds DNA (Fig 6D). It is just reduced relative to wildtype Med1. Second, based on the data of one additional mediator subunit (Med17) the authors cannot conclude that the binding of the whole mediator complex (approximately 30 subunits) requires phosphorylation of Med1. The text needs to be revised.

6) Throughout the text and figures, the authors use the androgen-dependent cancer cell line LNCaP. However, it doesn't seem to respond to DHT treatment in any assay. For example, why does the DHT treatment not increase UBE2C expression for the LNCaP cell line in Fig 1A? Shouldn't there be some DHT-induced looping events specific to the LNCaP cells in Fig 2A since these cells are AR-positive? Is there any reason to present the DHT treatment data at all if it doesn't affect the cells or the message of the paper. One option would be to omit this data or move it to supplemental since it would simplify the figures substantially.

Minor points:

1) Page 11: "Seventy-two h" should be Seventy-two hr

2) Page 16: Decreased should be changed to increased in the following statement in the text: "Interestingly, FoxA1 binding at the E1 region was significantly decreased (~1.5-fold) in PC-3/WT MED1 cells as compared to PC-3/DM MED1 cells (Fig 6D), suggesting that phosphorylated MED1 may assist FoxA1 binding."

3) Page 21: "fetal" should be "fatal"

4) The author's should more explicitly state that the results of their serial ChIP experiments (Fig 5D) indicate that these factors are interacting on the same DNA fragments in the same cell.

5) The authors state in the discussion that the presence of Pol2 at the E1, E2 and E3 enhancers likely indicates the presence of eRNAs that facilitate mRNA synthesis. I realize this is a possibility even though very little experimental evidence in this paper (i.e. detection of eRNAs) is provided. A more realistic interpretation is that the presence of Pol2 at the enhancers is resulting from the enhancer region looping to the promoter and being crosslinked within close proximity to promoter bound Pol2.

6) The authors state: "Significant suppression of UBE2C expression level, determined by Western blot analysis, was achieved after transfection of two independent short interfering RNA (siRNA) targeting UBE2C (siUBE2C) (Fig 1C)." However based on the labeling in Figure 1C this might not
be the case. I am assuming that siUBe2C Pool is a mix of siRNAs. Is siUBE2C single included in the siUBe2C Pool? I am assuming that it is not but if it is then this experiment does not demonstrate reproducibility with two different siRNAs. The labeling or figure legend should be clearer. Also, the text and figure legend is a little misleading by stating that two different siRNAs were used independently of each other, which is not the case if one of the transient transfections was done with a pool of siRNAs. The text should reflect this difference.

7) For Fig 4C what are the expression levels relative to? Maybe non-transfected cells? For instance the siControl transfection causes a 2.5-3 fold increase in UBE2C expression relative to what? Shouldn't the data be presented relative to the siControl transfection?

8) Fig. 3A could be moved to supplemental and replaced by a schematic representation of the 3 enhancers and the different DNA-binding motif.

Referee #2:

The authors identify regulatory regions and associated factors in the ubiquitin ligase UBE2C gene that is overexpressed in certain tumours. Regulatory elements were identified using the 3C technology and characterized using ChIP and gene knockdown experiments. The authors note that MED1 is overexpressed and phosphorylated in a cell line that actively expresses UBE2C. A combination of 3C, ChIP and siRNA data led the authors to suggest that MED1 phosphorylation is directly involved in looping.

This is an extensive study of regulatory elements and associated mechanisms of an interesting gene. Conceptually, the investigation aims at unravelling pathways that lead to UBE2C activation in the absence of AR function. The role of the identified elements is convincingly shown. While there is substantial information about the composition and interaction of the networks bound to these elements in one specific cell line, overall this analysis remains in many respects preliminary. The experimental design, general relevance of model systems and the main interpretation are subject to questions.

Major criticism:

1) The introduction should be written more concise. The relevant question is not worked out clearly. The authors should in the intro (as well as in the result section) focus more on facts instead of discussing hypotheses. The language in some parts is unfortunate: i.e. page 4, 3rd para "three distal regions that have increased proximity...". It seems the abbreviations CRPC and ADPC are used for defined cell lines and for disease phenotypes, which is confusing. Their relevance as a model for prostate cancer (which form, which stadium) and their specific biological role (differentiation status etc) should be better explained.

2) The link to AR function and the previous work is difficult to follow. It is unclear as to why the authors compare AR-negative CRPC with AR-positive ADPCs. In neither of these cells UBE2C reacts on the activation of AR. In fact, one is wondering why is UBE2C expression not affected by DHT in the AR-positive ADPC "LNCaP" (Fig. 1A). Why is hormone included throughout the paper although it shows no effect in any experiment? If the authors wish to clarify an AR-independent pathway the paper should be rewritten and the selection of experiments should be revisited.

3) Also unclear, why is cellular proliferation not affected by UBE2C siRNA treatment in LNCaP (Fig. 1D).

4) Contrary to the statement of the authors the luciferase data show that E1 is active in both cell lines, whereas solely E2 and E3 are exclusively active in PC-3 (Fig. 2B)? Further contradictory to this functional analysis the 3C data in the first place uncover a related (although activated) architecture in PC-3 versus LNCaP cells. That should be explained! Furthermore, the suitability of +46 kb as a control - with very little crosslinking at it - remains unclear.
5) Altogether the reader is wondering whether the authors eventually uncover the truly critical factors and mechanisms at their model gene. For example, the ChIP analysis in Fig. 3 leaves substantial doubt. The focus on regulatory factors that are equally present in both cell lines is surprising. Not unexpectedly, there are little differences in gene occupancy. The choice of potential downstream effectors seem similarly arbitrary. MED1 seems to play a role and perhaps also a phosphorylated form of it. However, the role of other cofactors and the impact of the Mediator complex has not been looked at. Interactions of Mediator and RNAPII are well established. It is not unexpected that GTFs are increased on active genes. Altogether, it seems difficult to draw any novel general conclusion from the ChIP and siRNA data.

6) Most notably, to conclude on looping mechanism based upon reduction of 3C crosslinking upon silencing of MED1 is preliminary. The data in this study merely suggest that Mediator could play (a possibly entirely indirect) role in establishing structures that facilitate such interactions. However, Mediator could equally well just recruit RNAPII (or another factor), which then mediates interactions between elements. For the same reason further conclusions on a specific role of a phosphorylated form of MED1 in looping remain preliminary. Reduction of 3C crosslinking in the presence of mt versus wt MED1 merely indicates a role in formation of an active complex, which in turn may lead to less frequent interactions and obviously to reduced binding of general factors (Fig. 6F).

7) Although there is a wealth of data in Fig. 5 and 6, also their novelty is limited. Moreover, specific aspects remain obscure. For example, why is the difference in occupancy between wt and mt (MED1) proteins bigger in the presence of endogenous protein than in its absence (siRNA control in the p-MED1 panel on E1). The levels of overexpression of retroviral proteins relative to endogenous is not shown? Fig. 6C lacks this important (wt PC-3) control. In summary, it is preliminary to conclude that phosphorylation is required for binding of Mediator complex to chromatin. Beyond it, many specific issues arise: Why is MED1 exclusively at E1 whereas p-MED1 and MED17 are present on all elements? Why are p-MED1 levels 20-fold over IgG level at the control element? How can the authors generate data without an error bar on MED17 at levels that are below (roughly 30% of) the IgG control? One severe limitation is that absolute levels are never shown.

8) There is little mechanistic insight coming from the re-ChIP analyses. This method is notoriously difficult: how did the authors normalize their results?

Minor criticism:
1) Fig. 1B: Calnexin concentrations should be identical in the two cell lines or another control should be used.
2) The font size should be kept constant within one figure if possible.
3) The authors speak of a G2/M blockage and an essential role of the enzyme and show somewhat reduced cell numbers and an increase of 2% in G2/M? How accurate are these small changes (describe method).
4) The luciferase data in Fig. 2B seem to suggest that at least E1 also works in LNCaP.
5) The term MED1 phosphorylation codes for looping, even if it was true, is completely inappropriate. An interaction (which is actually not shown) is not equivalent to a code.

Referee #3:

This manuscript describes the regulation of UBE2C by the Mediator complex in AR negative prostate cancer cells. The authors take a detailed approach to identify the regulatory elements around the UBE2C gene region, which they show possesses enhancer activity and can be occupied by various regulatory factors, including the mediator protein MED1. They also perform detailed CCC assays to confirm the specific enhancers that interact with the UBE2C promoter region. They perform detailed siRNA and rescue experiments to identify the various parameters (enhancers, regulatory factors etc) involved in transcriptional regulation of UBE2C. Importantly they raise a phospo-specific MED1 antibody and confirm that phosphorylated MED1 is the critical regulator of protein binding and transcription of UBE2C.

Overall this is a very nice study. The authors have taken a comprehensive approach to definitively
prove that MED1 is a critical regulator of UBE2C. The experiments are well controlled for and the conclusions are generally valid. I do however have a few issues that need to be addressed before this can be published. Although there is a wealth of data and the authors should be commended on producing a solid body of work, my concerns revolve around issues that are central to the overall biological and clinical significance.

- The WST1 assay is an indirect measure of cell growth and can be influenced by a number of factors that don't actually impact proliferation. The change in G2/M fraction in Figure 1E is so small (less than 3% total change), I would consider this background and not significant. What does %S phase look like in PC3 cells Vs LNCaP cells and when UBE2C is silenced? Alternatively, if the authors perform cell counting at different time points, is there a difference between the cell lines and between siControl and siUBE2C transfected cells? I believe that the same argument is true for the siRNA and overexpression experiments with MED1 (Figure 6H). If modulation of MED1 and UBE2C does not have an appreciable influence on cell growth (which I am currently not convinced about), this would suggest that their detailed experiments are interesting, but not relevant to prostate cancer cell growth.

- I am a little concerned about the CCC experiments. It is well established that CCC interactions within a 20kb window are difficult to interpret due to false positives. The authors are finding marginal differences in their relative cross-linking frequencies, which are not overly convincing. The most convincing experiment would be to engineer out the E1 region from a BAC and determine if this impacts expression of UBE2C. The use of a control region 46kb away from the TSS is not a good control. At a minimum, the authors should use a control within the 20kb region that is more prone to false positive interactions.

- Out of curiosity, is the ChIP and Re-ChIP data from Figure 5 independent of ligand treatment?

- Does MED1 and UBE2C expression correlate in AR negative prostate cancer samples, even using a publicly available database, such as Oncomine? Or would this only occur between phopho-MED1 and UBE2C levels?

- If the authors are correct and MED1 is a key regulator of UBE2C expression and this subsequently alters cell growth, does MED1 expression in LNCaP cells (hormone deprived) result in increased UBE2C expression and increased cell growth?

1st Revision - Authors' Response 29 January 2011

Response to reviewers’ comments

Reviewer 1

Summary points:

1. “In particular, this paper would be greatly improved if the authors could provide insight into the signaling pathway(s) that is regulating Med1 phosphorylation and show that this pathway(s) is physiologically relevant. For instance does inhibition or activation of the MAPK kinase pathway influence the phosphorylation status of Med1 and UBE2C expression levels?”

Response: We thank the reviewer for the insight by suggesting this critical experiment. We investigated which kinases are capable of phosphorylating MED1 in AR-negative CRPC cell line PC-3. As previous studies have shown that MED1 is phosphorylated on T1032 and T1457 (Pandey et al. Mol Cell Biol, 25:10695-10710, 2008; Belakavadi et al. Mol Cell Biol, 28:3932-3942, 2008) by ERK of the MAPK family in HeLa cells, we treated PC-3 cells with U0126 (a highly specific MEK1/2 inhibitor), and examined the effect of MAPK kinase pathway inhibition on MED1 phosphorylation. Western blot analysis using a p-MED1 antibody specifically recognizing T1032 showed that MEK inhibition had no effect on MED1 phosphorylation at T1032 (see new Supplementary Figure 4B). We would like to point out that p-MED1 antibody specificity has been validated using p-MED1 immunoprecipitation/p-Threonine western blot analysis [new Fig.5A], the new p-MED1 western blot analysis [new Fig.5A], and the new peptide competition assay in ChIP conditions [new Supplementary Figure 4C]). In addition, treatment of PC-3 cells with Bisindolylmaleimide 1 (PKC inhibitor), Tyrphostin AG 1478 (EGFR inhibitor) and SB203580 (p38 inhibitor) had no effect on MED1 phosphorylation at T1032 (data not shown)
Because T1032 region of MED1 contains phosphorylation sites for both MAPK (P-X-S/T-P, where X represents any amino acid) (Gonzalez, et al. J Biol Chem, 266: 22159-22163, 1991; Pearson et al. Endocr Rev, 22:153-183, 2001) and AKT (R-X-S/T, where X represents any amino acid) (Basu, et al. Mol Cell, 11:11-23, 2003; Fang et al. J Biol Chem, 15:11221-11229, 2007), we examined the effect of PI3K/AKT pathway inhibition on MED1 phosphorylation. Exposure of PC-3 cells to the PI3K inhibitor LY294002 significantly decreased AKT phosphorylation at S473 and MED1 phosphorylation at T1032, leading to a striking attenuation of UBE2C mRNA expression. The decreased T1032 phosphorylated MED1 protein and UBE2C mRNA expression in siAKT transfected versus siControl transfected PC-3 cells further supported that PI3K/AKT phosphorylated MED1 at T1032 in PC-3 cells. These new data are included in new Figs. 5C to 5F. We have also discussed the significance of PI3K/AKT-induced MED1 phosphorylation in prostate cancer in “Discussion” part (see pages 23).

2. “It would also be interesting to know if the same enhancers are active in other hormone-independent cancer cell lines.”

Response: We have now investigated whether E1, E2 and E3 regulate UBE2C expression in another AR-negative CRPC cell line DU-145 derived from different tissue

(DU-145 is derived from brain, whereas PC-3 is derived from lumbar vertebra [Sobel and Sadar, J Urol, 173:342-359, 2005]). Although in reporter gene assays in DU-145 cells E1-1 and E3-1 demonstrated significant enhancer activities (>1.5-fold) (new Supplementary Figure 2E), no difference in UBE2C locus looping was observed in LNCaP versus DU-145 cells (new Supplementary Figure 2F), suggesting that the increased UBE2C mRNA and protein expression (new Supplementary Figures 2G and 2H) in DU-145 cells might result from other mechanisms (e.g. enhancers beyond the -60 kb ~ +60 kb of TSS and/or attenuated UBE2C mRNA stability). Based on these new data, we suggest that E1, E2 and E3 are tissue-specific AR-negative CRPC UBE2C enhancers. Additionally, our previous studies have demonstrated that E1 is functional in an AR-positive CRPC cell line LNCaP-abl (Wang et al. Cell, 138: 245-256, 2009).

Major points:

1. A key element of the proposed manuscript is establishing the dependency on UBE2C for PC-3 proliferation. However, in the current form, it is far from convincing. The WST-1 assay is not a "cell number" assay, but a metabolism-based assay. The block in G2/M is represented by a 2-3% increase, which is very minimal compare to the results presented for the WST-1 assay. Another interpretation of the data would be that UBE2C KD decreases metabolism, but does not affect proliferation. To start with, the authors should use a direct viable cell count assay and display the full cell cycle profiles. Then, they should try to support their conclusions with statistical analysis of the observed differences. Finally, they need to comment on how a 2-3% delay in G2/M explains their phenotype.

Response: We thank all three reviewers for suggesting these critical experiments. We have now examined the effect of UBE2C silencing on PC-3 and LNCaP proliferation by using a direct viable cell count assay. PC-3 and LNCaP cells were transfected with siControl ON-TARGET pool, siUBE2C ON-TARGET pool or siUBE2C single (The siUBE2C ON-TARGET pool is a mix of 4 siRNAs [purchased from Dharmacon], and siUBE2C single is a siRNA not included in the siUBE2C ON-TARGET pool [Reddy, et al. Nature, 446:921-925, 2007]). Silencing of UBE2C decreased (p<0.05) PC-3 cell proliferation, whereas silencing of UBE2C did not significantly affect LNCaP cell proliferation (see new Fig.1D). The slightly decreased cell proliferation observed in siUBE2C single transfected LNCaP cells may be caused by an off-target effect of this single unmodified siRNA. We believe that the most relevant comparison is siUBE2C ON-TARGET pool versus siControl ON-TARGET pool. These "ON-TARGET " siRNAs are chemically modified to reduce off target effect (Jackson et al. RNA, 12:1197-1205, 2006). In addition, ON-TARGET siRNAs also reduce miRNA-like off-target effects mediated by seed region matched to the 3′-UTR of other non-target genes (Birmingham et al. Nat Methods, 3: 199-204, 2006).
We next re-performed cell-cycle analysis. Our new data showed that silencing of UBE2C in PC-3 cells resulted in an increase of ~ 6% in G2/M phase (see revised Fig.1E lower panel), which is similar to published data showing that UBE2C silencing led to an increase of 6~7% in G2/M phase in LoVo colorectal cancer cells and DLD1 colon cancer cells (Chen et al. Clin Exp Pharmacol Physiol, 37:525-529, 2010; Fujita et al. BMC Cancer, 9:87, 2009). By contrast, silencing of UBE2C did not significantly change cell distribution over cell-cycle phases in LNCaP cells (see revised Fig.1E upper panel), suggesting that UBE2C in LNCaP cells might be redundant with another E2 enzyme.

To further substantiate that UBE2C is critical for G2/M phase cell-cycle progression in PC-3 cells, we synchronized PC-3 cells to G2/M phase by using a thymidine-nocodazole block (Fang et al. Mol Cell, 2:163-171, 1998) and released for 1.5 hours. UBE2C silencing led to an increase in the G2/M phase and a decrease in the G1 phase after releasing from G2/M synchronization, suggesting that UBE2C silencing delayed G2/M phase to G1 phase transition. These new data are included in new Supplementary Figure 1E.

2. “The 3C data in Fig 2A, Fig 4A and 6F are not convincing. There are no error bars for the 3C graph. Was this experiment only done once? Was the real-time PCR at least done in triplicate? If the real-time PCR was done in triplicate there should at least be error bars for that.”

Response: Our 3C experiments were performed in triplicate. We have now added error bars to all 3C data (Fig. 2A, Supplementary Figures 2A, 2B and 2F, Fig. 4A and Fig. 6F).

This reviewer understands that 3C experiments are very difficult to do and hard to interpret, but it's not clear why the 3 putative enhancer regions were selected when there are additional fragments (besides -20 kb, -14 kb and +2 kb) with a greater interaction frequency in PC-3 cells relative to LNCaP cells (Fig 2A).”

Response: We have revised Fig. 2 and the text (see pages 6-7) to better interpret 3C data. Our analysis of the 3C results identified seven cross linking frequency-high (≥ 20) fragments with greater interactions (≥1.5-fold, P<0.05 or P<0.01) between these fragments and the UBE2C promoter in PC-3 cells than in LNCaP cells: 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 (Fig. 2A and Supplementary Figure 2A). We next selected the fragment with the highest fold change (-20 kb fragment), and the closest upstream and downstream fragments (-14 kb fragment and +2 kb fragment) for further analysis.

“When the authors state in the text that the cross linking frequencies for in the PC-3/DM Med1 cells were significantly lower relative to the PC-3/WT Med1 cells (Fig 6F). Is this actually significant? It is hard to tell without a calculated P-value or at least error bars on the graph.”

Response: The looping difference between WT and DM is significant (P<0.01). We have added error bars to Fig. 6F and P-value to Fig. 6 legend.

3. “What is the control in Fig 2B? Does it include a positive enhancer region? This information should be in the figure legend.”

Response: We have now included the PSA enhancer as a positive control (Wang, et al. Mol Cell, 19:631-642, 2005). We showed that the transcriptional activity of the PSA enhancer reporter gene was significantly increased in the presence of DHT. These new data are included in new Supplementary Figure 2D.
It seems strange that the cloned enhancer fragments are having a negative effect relative to the control in LNCaP cells.

Response: We think that some transcription repressors might bind to these elements in LNCaP cells but not in PC-3 cells. Alternatively, specific factors in LNCaP cells but not in PC-3 cells may block the TATA binding sites (We used a minimal E4 TATA promoter driving the expression of luciferase).

4. The validation of the phospho-Med1 antibody needs to be more rigorous.

It doesn't make any sense to this reviewer why the total Med1 and the phospho-specific antibody give such different results in ChIP. For instance, why does the Med1 Ab not detect any signal at the promoter (Fig 6D) while a lot of enrichment is detected for the phospho-Med1 Ab? Furthermore, all regions tested following the phospho-Med1 ChIP gave at least 20-fold enrichment including the control region. This reviewer is left wondering if this antibody is really specific. If possible, a competition with different phospho-peptide in ChIP conditions would increase confidence in the antibody specificity. The authors should add a full WB of the input in addition to the IP in Fig 5 to show that the antibody recognizes only 1 band.

Response: As suggested by the reviewer, we have now performed a peptide competition assay in ChIP conditions using 1032T phospho-peptide and 1032T non-phospho-peptide (cold peptide). The cross linked chromatin from PC-3 cells was sonicated and diluted, and cold peptide or 1032T phospho-peptide of 10-fold, 100-fold or 1000-fold molar ratio antigen-to-antibody were added to ChIP diluted buffer before immunoprecipitation with the p-MED1 antibody. Significantly, adding 1032T phospho-peptide but not cold peptide completely abolished p-MED1 recruitment to the UBE2C enhancers and promoter. In addition, we have performed a p-MED1 western blot analysis. Both p-MED1 antibody and the commercial total MED1 antibody recognize only one major band. These new data further demonstrate the specificity of the p-MED1 antibody (see new Supplementary Figure 4C and revised Fig. 5A).

Although the commercial total MED1 antibody is specific, we doubt that the low sensitivity of this antibody led to the significant difference between the p-MED1 antibody and the commercial total MED1 antibody in ChIP assays (Fig.6D). We have tested another total MED1 antibody (anti-MED1 NR box) with high sensitivity and specificity (Zhang, et al. Mol Cell, 19:89-100, 2005) in ChIP assays. ChIP analysis with the anti-MED1 (NR box) antibody showed that MED1 was not only recruited to E1 region, but also to E2-a, E3-a and the promoter regions (see revised Fig.6D).

With regard to the +46 kb control region used in ChIP assay (Fig. 6D), while the cross linking frequency between this control region and the UBE2C promoter is very low (Figs. 2A, 4A and 6F), we could not rule out the possibility that this region contains some transcriptional activity (e.g. some level of coactivator binding) required for activation of other genes. We also apologize for an error in ChIP normalization. The control region in p-MED1 ChIP in Fig. 6D now gave less than 10-fold enrichment (see revised Fig. 6D).

Figure 5B and the text accompany it is misleading. "Western blots analysis using the phospho-MED1 antibody showed that the expression of phosphorylated MED1 was significantly higher in PC-3 cells than in LNCaP cells (Figure 5B)." According to Figure 3A, Med1 is expressed at much higher levels in PC-3 cells, so it is not surprising that there is more phospho-Med1 in PC-3 cells. However the text and figure imply that the ratio of phosphorylated to unphosphorylated Med1 is greater in PC-3 cells, which most likely is not the case. The text should be changed to reflect this. However, if the ratio actually is greater this would be usual information to know since it would
indicate that the signaling pathway resulting in phospho-Med1 is more active in PC-3 cells. The authors should probe the same lysates in Figure 5B for total Med1 to determine this.

Response: We have re-performed western blots to compare p-MED1 expression between LNCaP and PC-3 cells by using the p-MED1 antibody. The same membrane was then reprobed with a total MED1 antibody. The band intensities in LNCaP and PC-3 cells were quantified using Image J (V 1.43, NIH). We found that the relative ratio of p-MED1/total MED1 was only slightly higher in PC-3 than in LNCaP cells (1.3-fold), although p-MED1 expression was significantly higher in PC-3 compared to LNCaP cells (8.4-fold). We have revised the text to “Subsequent Western blot analysis using the p-MED1 antibody showed greater expression of phosphorylated MED1 in PC-3 versus LNCaP (Figure 5B), which was due mostly to the increased total MED1 expression in PC-3 (Figure 5B and Supplementary Figure 4A)” (see page 14).

5. “The authors overstate this conclusion: "...demonstrating that phosphorylation of MED1 was required for the Mediator complex to bind to chromatin." First the Med1 double phosphorylation mutant still binds DNA (Fig 6D). It is just reduced relative to wildtype Med1. Second, based on the data of one additional mediator subunit (Med17) the authors cannot conclude that the binding of the whole mediator complex (approximately 30 subunits) requires phosphorylation of Med1. The text needs to be revised.”

Response: We agree with the reviewer and have revised the text to “…demonstrating that Mediator complex binding to chromatin was enhanced by phosphorylation of MED1” (see pages 17-18).

6. “Throughout the text and figures, the authors use the androgen-dependent cancer cell line LNCaP. However, it doesn't seem to respond to DHT treatment in any assay. For example, why does the DHT treatment not increase UBE2C expression for the LNCaP cell line in Fig 1A?

Response: Our published data (Wang, et al. Cell, 138: 245-256, 2009) and the data in this manuscript consistently showed that UBE2C is not an androgen-regulated gene in LNCaP. With regard to response of LNCaP to DHT treatment, we have now performed reporter gene assays using the PSA enhancer control (Wang, et al. Mol Cell, 19:631-642, 2005) in the presence and absence of DHT. We found that DHT treatment significantly increased transcriptional activation of the PSA enhancer reporter gene in LNCaP cells (new Supplementary Figure 2D). As positive controls to Fig. 1A, we showed that DHT treatment significantly increased PSA and TMPRSS2 mRNA expression in LNCaP cells (new Supplementary Figure 1B).

Shouldn't there be some DHT-induced looping events specific to the LNCaP cells in Fig 2A since these cells are AR-positive?

Response: We have now performed 3C assays to examine the effect of DHT on chromatin looping in the TMPRSS2 locus in LNCaP cells. Interestingly, we found that DHT treatment significantly increased the interactions between four distal AR binding sites (Wang, et al. Mol Cell, 27:380-392, 2007; Yu, et al. Cancer Cell, 17:443-454, 2010) and the TMPRSS2 promoter. These new data are included in new Supplementary Figure 2B.

Is there any reason to present the DHT treatment data at all if it doesn’t affect the cells or the message of the paper. One option would be to omit this data or move it to supplemental since it would simplify the figures substantially.”

Response: As suggested by this reviewer, we have moved all DHT-treated data to Supplementary figures.
Minor points:

1. “Page 11: "Seventy-two h" should be Seventy-two hr”

Response: The style for EMBO J uses “h” rather than “hr”.

2. “Page 16: Decreased should be changed to increased in the following statement in the text: "Interestingly, FoxA1 binding at the E1 region was significantly decreased (>~1.5-fold) in PC-3/WT MED1 cells as compared to PC-3/DM MED1 cells (Fig 6D), suggesting that phosphorylated MED1 may assist FoxA1 binding.”

Response: We have revised this.

3. “Page 21: "fetal" should be "fatal"”

Response: We have corrected this.

4. “The author’s should more explicitly state that the results of their serial ChIP experiments (Fig 5D) indicate that these factors are interacting on the same DNA fragments in the same cell.”

Response: We have revised the text to more explicitly explain serial ChIP assays (see pages 15-16).

5. “The authors state in the discussion that the presence of Pol2 at the E1, E2 and E3 enhancers likely indicates the presence of eRNAs that facilitate mRNA synthesis. I realize this is a possibility even though very little experimental evidence in this paper (i.e. detection of eRNAs) is provided. A more realistic interpretation is that the presence of Pol2 at the enhancers is resulting from the enhancer region looping to the promoter and being cross linked within close proximity to promoter bound Pol2.”

Response: We agree with the reviewer and have removed the discussion on eRNA. The text was now revised to “…PC-3 cells showed higher levels of coactivator MED1, phosphorylated MED1, and Pol II binding, and active histone H3K4 methylation marks at E1, E2 and E3 regions, indicative of enhanced transcriptional activation in PC-3 cells versus LNCaP cells (Figures 3 and 5)” (see page 20).

6. “The authors state: "Significant suppression of UBE2C expression level, determined by Western blot analysis, was achieved after transfection of two independent short interfering RNA (siRNA) targeting UBE2C (siUBE2C) (Fig 1C).” However based on the labeling in Figure 1C this might not be the case. I am assuming that siUBe2C Pool is a mix of siRNAs. Is siUBE2C single included in the siUBE2C Pool? I am assuming that it is not but if it is then this experiment does not demonstrate reproducibility with two different siRNAs. The labeling or figure legend should be clearer. Also, the text and figure legend is a little misleading by stating that two different siRNAs were used independently of each other, which is not the case if one of the transient transfections was done with a pool of siRNAs. The text should reflect this difference.”

Response: The siUBE2C ON-TARGET pool is a mix of 4 siRNAs (purchased from Dharmacon), and siUBE2C single is a siRNA not included in the siUBE2C ON-TARGET pool (Reddy, et al.
The sequencés of siUBE2C ON TARGET pool and siUBE2C single are listed in Supplementary Table 1. We have revised the figure legend (see page 28) to clarify this.

7. “For Fig 4C what are the expression levels relative to? Maybe non-transfected cells? For instance the siControl transfection causes a 2.5-3 fold increase in UBE2C expression relative to what? Shouldn’t the data be presented relative to the siControl transfection?”

Response: We apologize if the original Figure 4C was misleading. Actually, the UBE2C mRNA level in siControl transfected PC-3 cells is 3-fold higher than that in siControl transfected LNCaP cells (data not shown). Since we only presented the UBE2C mRNA data in PC-3 here, we have now normalized the UBE2C mRNA level in siFoxA1 and siMED1 transfected cells to siControl transfected cells (see revised Fig. 4C).

8. “Fig. 3A could be moved to supplemental and replaced by a schematic representation of the 3 enhancers and the different DNA-binding motif.”

Response: As this reviewer suggested, we have replaced Fig. 3A (now Supplementary Figure 3A) with a schematic representation of the three enhancers with ETSF, GATA, OCT and FKHD motifs (see new Fig. 3A).

Reviewer 2

Major point:

1. “The introduction should be written more concise. The relevant question is not worked out clearly. The authors should in the intro (as well as in the result section) focus more on facts instead of discussing hypotheses. The language in some parts is unfortunate: i.e. page 4, 3rd para "three distal regions that have increased proximity..". It seems the abbreviations CRPC and ADPC are used for defined cell lines and for disease phenotypes, which is confusing. Their relevance as a model for prostate cancer (which form, which stadium) and their specific biological role (differentiation status etc) should be better explained.”

Response: We agree with the reviewer. 1) The introduction has been revised to make it more concise and more focused on facts (see pages 3-4). Part of the “introduction” has been removed to the “discussion” (see page 20). 2) “three distal regions that have increased proximity...” has been revised to “...three distal regions that have greater interactions with...” 3) We have now used “CRPC cells” or “PC-3” and “ADPC cells” or “LNCaP” for cell lines, and “CRPC” or “ADPC” for disease phenotypes in the text. 4) We have now explained more on the relevance of the cell models to prostate cancer (see pages 5 and 9).

2. “The link to AR function and the previous work is difficult to follow. It is unclear as to why the authors compare AR-negative CRPC with AR-positive ADPCs. In neither of these cells UBE2C reacts on the activation of AR. In fact, one is wondering why is UBE2C expression not affected by DHT in the AR-positive ADPC "LNCaP" (Fig. 1A).”

Response: Our previous studies have found that UBE2C is a basal AR-regulated but not liganded-AR regulated gene in AR-positive CRPC cells (abl). In LNCaP cells, UBE2C is neither an androgen-regulated gene nor a basal AR-regulated gene (Wang et al. Cell, 138:245-256, 2009).
Since AR expression in CRPC is very heterogeneous, in this paper we asked how UBE2C is up-regulated in AR-negative CRPC by comparing AR-negative CRPC with ADPC (AR is expressed in almost all ADPC).

Why is hormone included throughout the paper although it shows no effect in any experiment? If the authors wish to clarify an AR-independent pathway the paper should be rewritten and the selection of experiments should be revisited.”

Response: As pointed by this reviewer and Reviewer 1, we have now moved 3C, RT-PCR, Western blots, reporter gene assays, and ChIP data in the presence of hormone to Supplementary figures.

3. “Also unclear, why is cellular proliferation not affected by UBE2C siRNA treatment in LNCaP (Fig. 1D)”.

Response: As this reviewer and Reviewers 1 and 3 suggested, we have now examined the effect of UBE2C silencing on PC-3 and LNCaP proliferation by using a direct viable cell count assay. PC-3 and LNCaP cells were transfected with siControl ON-TARGET pool, siUBE2C ON-TARGET pool or siUBE2C single (The siUBE2C ON-TARGET pool is a mix of 4 siRNAs [purchased from Dharmacon], and siUBE2C single is a siRNA not included in the siUBE2C ON-TARGET pool [Reddy, et al. Nature, 446:921-925, 2007]). Silencing of UBE2C decreased (p<0.05) PC-3 cell proliferation, whereas silencing of UBE2C did not significantly affect LNCaP cell proliferation (see new Fig.1D). The slightly decreased cell proliferation observed in siUBE2C single transfected LNCaP cells may be caused by an off-target effect of this single unmodified siRNA. We believe that the most relevant comparison is siUBE2C ON-TARGET pool versus siControl ON-TARGET pool. These "ON-TARGET " siRNAs are chemically modified to reduce off target effect (Jackson et al. RNA, 12:1197-1205, 2006). In addition, ON-TARGET siRNAs also reduce miRNA-like off-target effects mediated by seed region matched to the 3'-UTR of other non-target genes (Birmingham et al. Nat Methods, 3: 199-204, 2006).

We next re-performed cell-cycle analysis. Our new data showed that silencing of UBE2C in PC-3 cells resulted in an increase of ~ 6% in G2/M phase (see revised Fig.1E lower panel), which is similar to published data showing that UBE2C silencing led to an increase of 6~7% in G2/M phase in LoVo colorectal cancer cells and DLD1 colon cancer cells (Chen et al. Clin Exp Pharmacol Physiol, 37:525-529, 2010; Fujita et al. BMC Cancer, 9:87, 2009). By contrast, silencing of UBE2C did not significantly change cell distribution over cell-cycle phases in LNCaP cells (see revised Fig.1E upper panel), suggesting that UBE2C in LNCaP cells might be redundant with another E2 enzyme.

4. “Contrary to the statement of the authors the luciferase data show that E1 is active in both cell lines, whereas solely E2 and E3 are exclusively active in PC-3 (Fig. 2B)? Further contradictory to this functional analysis the 3C data in the first place uncover a related (although activated) architecture in PC-3 versus LNCaP cells. That should be explained!

Response: We are afraid that this is an oversight by this reviewer. Our reporter gene assay data in Fig. 2B showed that all three enhancers (E1, E2 and E3) are not functional compared with the control empty vector, which is consistent with the low interaction frequencies between these fragments with the UBE2C promoter (Fig. 2A).
Furthermore, the suitability of +46 kb as a control - with very little cross linking at it - remains unclear.”

Response: Since our previous studies found that an AR-bound enhancer is located with the +46 kb region in AR-positive CRPC cells (abl) but not in ADPC cells (LNCaP) (Wang et al., Cell, 138:245-256, 2009), we used this region as a control for AR-negative CRPC cells (PC-3). However, as this reviewer and Reviewer 3 suggested, we have added the -7 kb region with relative high cross linking frequency (>20) as another control region within the -20 kb region. No significant difference in cross linking frequencies between this fragment and the UBE2C promoter was observed between LNCaP and PC-3 (see revised Fig. 2).

5-6 “Altogether the reader is wondering whether the authors eventually uncover the truly critical factors and mechanisms at their model gene. For example, the ChIP analysis in Fig. 3 leaves substantial doubt. The focus on regulatory factors that are equally present in both cell lines is surprising. Not unexpectedly, there are little differences in gene occupancy.

Response: We agree with this reviewer that equal protein expression of GATA2, Oct1 and ETS1 between PC-3 and LNCaP are associated with the similar or even lower binding of these factors in PC-3 cells versus LNCaP cells (Supplementary Figure 3A and Fig. 3B). However, we do found that FoxA1 binding at E1 and E2-a is higher in PC-3 cells than in LNCaP cells, though the protein expression between these two cell lines is similar (Fig. 3B and Supplementary Figure 3B). Consistent with our recent findings that higher levels of enhancer histone methylation marks H3K4me1 and H3K4me2 lead to increased FoxA1 binding in breast cancer cells and AR-positive CRPC cells (Lupien et al. Cell, 132:958-970, 2008; Wang et al. Cell, 138:245-256, 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 (Fig.3C and Supplementary Figure 3C).

The choice of potential downstream effectors seem similarly arbitrary. MED1 seems to play a role and perhaps also a phosphorylated form of it. However, the role of other cofactors and the impact of the Mediator complex has not been looked at. Interactions of Mediator and RNAPII are well established. It is not unexpected that GTFs are increased on active genes. Altogether, it seems difficult to draw any novel general conclusion from the ChIP and siRNA data.”

“Most notably, to conclude on looping mechanism based upon reduction of 3C cross linking upon silencing of MED1 is preliminary. The data in this study merely suggest that Mediator could play (a possibly entirely indirect) role in establishing structures that facilitate such interactions. However, Mediator could equally well just recruit RNAPII (or another factor), which then mediates interactions between elements. For the same reason further conclusions on a specific role of a phosphorylated form of MED1 in looping remain preliminary. Reduction of 3C cross linking in the presence of mt versus wt MED1 merely indicates a role in formation of an active complex, which in turn may lead to less frequent interactions and obviously to reduced binding of general factors (Fig. 6F).”

Response: We apologize if we did not explain clearly the rationale for selecting MED1 for further mechanistic studies. The ChIP data in Fig. 3 [Please note we have add the new MED17 ChIP and western blot data [see revised Fig. 3D and Supplementary Figure 3A] following this reviewer’s suggestion to examine the role of the Mediator complex] found that the protein expression and binding of MED1 but not other cofactors was significantly increased in PC-3 versus LNCaP, which prompted us to further investigate the role of MED1 in looping formation/maintenance (Figs. 4 to 6). Although the concept that transcription cofactors play important roles in looping is not novel and the interaction of Mediator and Pol II is well studied, the potential role of MED1 as a chromatin architectural factor is novel (Taatjes. Trends Biochem Sci, 35:315-322, 2010; Malik and Roeder. Nat Rev Genet, 11:761-772, 2010). While this manuscript in preparation, we noted a recent publication...
demonstrating that Mediator/cohesin complex plays an essential role in looping formation/stabilization in murine embryonic stem cells (Kagey et al. Nature, 467:430-435, 2010), whereas our study first demonstrated a critical role of MED1 phosphorylation in chromatin looping in human cancer cells. Nonetheless, we fully agree with this reviewer that the role of MED1 and phosphorylated MED1 in mediating looping maybe indirect. The interactions of phosphorylated MED1 with other proteins (e.g. FoxA1, Pol II, TBP and cohesin) may facilitate the formation/maintenance of chromatin looping.

7. Although there is a wealth of data in Fig. 5 and 6, also their novelty is limited. Moreover, specific aspects remain obscure. For example, why is the difference in occupancy between wt and mt (MED1) proteins bigger in the presence of endogenous protein than in its absence (siRNA control in the p-MED1 panel on E1).

Response: One explanation for this observation (Fig. 6D) is that binding ratios of endogenous MED1 to transfected WT/DM MED1 vary among different genomic regions. Specifically, the binding ratio of endogenous p-MED1 to WT MED1 may be higher than that of endogenous p-MED1 to DM MED1 at E1 region.

The levels of over expression of retroviral proteins relative to endogenous is not shown? Fig. 6C lacks this important (wt PC-3) control.

Response: We agree with this reviewer and have added this important control (see revised Fig. 6C).

In summary, it is preliminary to conclude that phosphorylation is required for binding of Mediator complex to chromatin.

Response: We agree with this reviewer and Reviewer 1 and have revised the text to “…demonstrating that Mediator complex binding to chromatin was enhanced by phosphorylation of MED1” (see pages 17-18).

Beyond it, many specific issues arise: Why is MED1 exclusively at E1 whereas p-MED1 and MED17 are present on all elements?

Response: We doubt that the low sensitivity of the commercial total MED1 antibody led to the significant difference between the p-MED1 antibody and the commercial total MED1 antibody in ChIP assays (Fig.6D). We have then tested another total MED1 antibody (anti-MED1 NR box) with high sensitivity and specificity (Zhang, et al. Mol Cell, 19:89-100, 2005) in ChIP assays. ChIP analysis with this anti-MED1 (NR box) antibody showed that MED1 was not only recruited to E1 region, but also to E2-a, E3-a and the promoter regions (see revised Fig. 6D).

Why are p-MED1 levels 20-fold over IgG level at the control element?

Response: The +46 kb control region used in ChIP assay (Fig. 6D) is the same one used in 3C assays (Figs. 2A, 4A and 6F). While the cross linking frequency between this control region and the UBE2C promoter is very low, we could not rule out the possibility that this region contains some transcriptional activity (e.g. some level of coactivator binding) required for activation of other genes. We also apologize for an error in ChIP normalization. The control region in p-MED1 ChIP in Fig. 6D now gave less than 10-fold enrichment (see revised Fig. 6D).
How can the authors generate data without an error bar on MED17 at levels that are below (roughly 30%) of the IgG control? One severe limitation is that absolute levels are never shown.

Response: All of our ChIP data have error bars, but the standard deviations for MED17 ChIP on E1, E2-b and the promoter regions are too small to be seen. With regard to presentation of ChIP data, both absolute (% of Input) and relative (Fold over IgG) methods have been used to in published studies. In this paper we decided to use the relative method, as we think that this could minimize the bias resulted from immunoprecipitation difference between two different cell lines.

8. “There is little mechanistic insight coming from the re-ChIP analyses. This method is notoriously difficult: how did the authors normalize their results?”

Response: The re-ChIP data tell us whether the enhancer-bound proteins interact with the promoter-bound proteins on the same DNA fragments at the UBE2C locus in the same cell. We agree with the reviewer that it is complicated to analyze re-ChIP data. In our current study, the relative interaction on chromatin (relative enrichment) was calculated as follows: Fold enrichment = 2^(Ct input – Ct ChIP). Ten percent of the first-eluted sample was used as input for re-ChIP. The values of PC-3 were then normalized to those of LNCaP.

Minor points:

1. “Fig. 1B: Calnexin concentrations should be identical in the two cell lines or another control should be used.”

Response: We agree with this reviewer and have re-performed Western blot analysis of AR, UBE2C and calnexin (see revised Fig. 1B and Supplementary Figure 1D).

2. “The font size should be kept constant within one figure if possible.”

Response: We agree with this reviewer and have now kept the font size constant within one figure.

3. “The authors speak of a G2/M blockage and an essential role of the enzyme and show somewhat reduced cell numbers and an increase of 2% in G2/M? How accurate are these small changes (describe method).”

Response: We have now re-performed cell-cycle analysis. Our new data show that silencing of UBE2C in PC-3 cells resulted in an increase of ~ 6% in G2/M phase (see revised Fig. 1E), which is similar to published data showing that UBE2C silencing led to an increase of 6~7% in G2/M phase in LoVo colorectal cancer cells and DLD1 colon cancer cells (Chen et al. Clin Exp Pharmacol Physiol, 37:525-529, 2010; Fujita et al. BMC Cancer, 9:87, 2009).

To further substantiate that UBE2C is critical for G2/M phase cell-cycle progression in PC-3 cells, we synchronized PC-3 cells to G2/M phase by using a thymidine-nocodazole block (Fang et al. Mol Cell, 2:163-171, 1998) and released for 1.5 hours. UBE2C silencing led to an increase (~ 6%) in the G2/M phase after releasing from G2/M synchronization, suggesting that UBE2C silencing delayed G2/M phase to G1 phase transition. These new data are included in new Supplementary Figure 1E.

4. “The luciferase data in Fig. 2B seem to suggest that at least E1 also works in LNCaP.”
Response: We are afraid that this is an oversight by this reviewer. Our reporter gene assay data in Fig. 2B showed that E1 is not functional compared with the control empty vector.

5. “The term MED1 phosphorylation codes for looping, even it was true, is completely inappropriate. An interaction (which is actually not shown) is not equivalent to a code.”

Response: We agree with this reviewer. We have changed “... codes for looping” to “enhances” or “drives” looping (see pages 4, 14 and 21).

Reviewer 3

1. “The WST1 assay is an indirect measure of cell growth and can be influenced by a number of factors that don’t actually impact proliferation. The change in G2/M fraction in Figure 1E is so small (less than 3% total change), I would consider this background and not significant. What does %S phase look like in PC3 cells vs LNCaP cells and when UBE2C is silenced? Alternatively, if the authors perform cell counting at different time points, is there a difference between the cell lines and between siControl and siUBE2C transfected cells? I believe that the same argument is true for the siRNA and over expression experiments with MED1 (Figure 6H). If modulation of MED1 and UBE2C does not have an appreciable influence on cell growth (which I am currently not convinced about), this would suggest that their detailed experiments are interesting, but not relevant to prostate cancer cell growth.”

Response: As this reviewer and Reviewers 1 and 2 suggested, we have now examined the effect of UBE2C silencing on PC-3 and LNCaP proliferation by using a direct viable cell count assay. PC-3 and LNCaP cells were transfected with siControl ON-TARGET pool, siUBE2C ON-TARGET pool or siUBE2C single (The siUBE2C ON-TARGET pool is a mix of 4 siRNAs [purchased from Dharmacon], and siUBE2C single is a siRNA not included in the siUBE2C ON-TARGET pool [Reddy, et al. Nature, 446:921-925, 2007]). Silencing of UBE2C decreased (p<0.05) PC-3 cell proliferation, whereas silencing of UBE2C did not significantly affect LNCaP cell proliferation (see new Fig.1D). The slightly decreased cell proliferation observed in siUBE2C single transfected LNCaP cells may be caused by an off-target effect of this single unmodified siRNA. We believe that the most relevant comparison is siUBE2C ON-TARGET pool versus siControl ON-TARGET pool. These "ON-TARGET " siRNAs are chemically modified to reduce off target effect (Jackson et al. RNA, 12:1197-1205, 2006). In addition, ON-TARGET siRNAs also reduce miRNA-like off-target effects mediated by seed region matched to the 3'-UTR of other non-target genes (Birmingham et al. Nat Methods, 3: 199-204, 2006).

We next re-performed cell-cycle analysis. Our new data showed that silencing of UBE2C in PC-3 cells resulted in an increase of ~ 6% in G2/M phase (see revised Fig.1E lower panel), which is similar to published data showing that UBE2C silencing led to an increase of 6~7% in G2/M phase in LoVo colorectal cancer cells and DLD1 colon cancer cells (Chen et al. Clin Exp Pharmacol Physiol, 37:525-529, 2010; Fujita et al. BMC Cancer, 9:87, 2009). By contrast, silencing of UBE2C did not significantly change cell distribution over cell-cycle phases in LNCaP cells (see revised Fig.1E upper panel), suggesting that UBE2C in LNCaP cells might be redundant with another E2 enzyme.

To further substantiate that UBE2C is critical for G2/M phase cell-cycle progression in PC-3 cells, we synchronized PC-3 cells to G2/M phase by using a thymidine-nocodazole block (Fang et al. Mol Cell, 2:163-171, 1998) and released for 1.5 hours. UBE2C silencing led to an increase in the G2/M phase and a decrease in the G1 phase after releasing from G2/M synchronization, suggesting that UBE2C silencing delayed G2/M phase to G1 phase transition. These new data are included in new Supplementary Figure 1E.
In addition, we have re-performed cell proliferation experiment in Fig. 6H using a direct viable cell count assay. This demonstrated that PC-3/DM MED1 cells grow much slower than PC-3/WT MED1 cells (see revised Fig. 6H), strongly supporting a critical role for MED1 phosphorylation in AR-negative CRPC cell proliferation.

2. “I am a little concerned about the CCC experiments. It is well established that CCC interactions within a 20kb window are difficult to interpret due to false positives. The authors are finding marginal differences in their relative cross-linking frequencies, which are not overly convincing. The most convincing experiment would be to engineer out the E1 region from a BAC and determine if this impacts expression of UBE2C. The use of a control region 46kb away from the TSS is not a good control. At a minimum, the authors should use a control within the 20kb region that is more prone to false positive interactions.”

Response: As this reviewer suggested, we have added the -7 kb region with relatively high cross linking frequencies (>20) as a control region within the -20 kb region (see revised Fig. 2). No significant difference in cross linking frequencies between this fragment and the UBE2C promoter was observed between LNCaP and PC-3. Since our previous studies found that an AR-bound UBE2C enhancer is located with the +46 kb region in AR-positive CRPC cells (abl) but not in ADPC cells (LNCaP) (Wang et al, Cell, 138:245-256, 2009), we used this region as another control for AR-negative CRPC cells (PC-3).

3. “Out of curiosity, is the ChIP and Re-ChIP data from Figure 5 independent of ligand treatment?”

Response: We have now performed ChIP assays (p-MED1) and re-ChIP assays (FoxA1 antibody for the first ChIP and PolII/TBP antibodies for the second ChIP) in the presence of androgen. As expected, the results in the presence of androgen were similar to those in the absence of androgen. These new data are included in new Supplementary Figures 4D and 4E.

4. “Does MED1 and UBE2C expression correlate in AR negative prostate cancer samples, even using a publicly available database, such as Oncomine? Or would this only occur between phospho-MED1 and UBE2C levels?”

Response: We agree with this reviewer that it will be very interesting to correlate MED1 and UBE2C expression to clinicopathological parameters using Oncomine. However, we could not find AR-negative prostate cancer clinical datasets in Oncomine. In fact, frequent AR+ and AR- tumor populations are often found within the same CRPC patient (Shah et al. Cancer Res, 64: 9209-9216, 2004).

5. “If the authors are correct and MED1 is a key regulator of UBE2C expression and this subsequently alters cell growth, does MED1 expression in LNCaP cells (hormone deprived) result in increased UBE2C expression and increased cell growth?”

Response: We have generated LNCaP cell lines stably expressing a MED1 or a GFP. We found that over expression of MED1 in LNCaP cells was not able to increase UBE2C expression and accelerate cell proliferation in the absence of hormone. These results suggest that while MED1 and phosphorylated MED1 are necessary for UBE2C expression and cell growth of PC-3 cells, they are not sufficient alone to enhance UBE2C expression and androgen-independent growth of LNCaP cells. These new data are included in new Supplementary Figures 5B-5D.
Thank you very much for submitting a revised version of your research paper (EMBOJ-2010-76254). I did receive comments from two of the original referees that both appreciate modifications and changes compared to the original submission.

As you will learn from the remarks, ref#3 is still not convinced from the significance of the major conclusion despite overall support for publication. However, ref#2 is very explicit NOT recommending publication in a broad and more general journal as general significance and overall conclusiveness still remain rather uncertain. Together with the issue that the characterized enhancer element turns out to be of tissue specific rather than general importance, I am afraid I do have no other choice to finally reject the paper. Please understand that with strong criticisms remaining after two rounds of peer-review and consistent with our strict guidelines that allow a single round of revisions only (as outlined in my initial decision), I am sorry to have to return the paper to you at this point with the message that we are unable to offer further proceedings.

I still hope that the revisions demanded for our journal might enable rapid publication of the work in a more appropriate, specialized title. I also hope that you still consider our journal for publication of your future studies.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #2 :

I have looked at the revised manuscript. There was a substantial degree of criticism from all three referees. The authors have resolved some of the questions in their revision. However, for many individual aspects there remain open questions (ie for the discrepancies in mediator complex subunits versus MED1, antibodies, 3C technology, ChIP technology). Most importantly, the main message, the proposed role of phosphorylation of MED1 in looping (title) has not been convincingly demonstrated.

In summary, I do not recommend publication.

Referee #3 :

The authors have addressed all of my concerns and have included the required controls. The new data on changes in growth following silencing of overexpression of MED1 (or mutant MED1) is convincing. However, the fact that modulation of MED1 does not influence UBE2C expression would suggest that it is an important component of a potentially massive protein complex, but not a crucial determinant. In my opinion this detracts from the overall conclusions. The wording of the text should be modified to better reflect this. That said, this manuscript does provide a number of novel findings that would be of potential interest to a general readership. As such, I am willing to support publication.

Rebuttal

Thank you for considering our manuscript entitled, “Phospho-MED1-induced chromatin looping drives androgen receptor negative prostate cancer growth”. After extensively revising the manuscript according to the specific suggestions and addressing all previous concerns, I was
surprised by the decision rendered (reject). Nonetheless, I respectfully request the opportunity to resubmit the manuscript, based on the following reasons:

1. Reviewer 3 fully supports publishing the study in the EMBO Journal. Furthermore, the reviewer comments that the manuscript provides novel findings of interest to a general readership and he/she supports publication. In response to the only comment of Reviewer 3, and based on our new data that MED1 modulation influences UBE2C locus looping in both AR-positive and AR-negative CRPC cells and MED1 is a crucial component of the complex (see attached Response letter), we propose a new title: “Phospho-MED1-enhanced UBE2C locus looping drives castration-resistant prostate cancer growth”

2. Reviewer 2 comments, including the “open questions”, were in fact addressed in the revised manuscript but may not have been entirely clear to the reviewer (see attached Response letter). These include:
   a. A crucial role of MED phosphorylation in looping has been demonstrated.
   b. There are no discrepancies in mediator complex subunits vs. MED1.
   c. There are no issues with antibodies, 3C technology, ChIP technology.

3. We provide convincing evidence that characterized enhancer elements are of general importance (see attached Response letter).

4. All previous concerns raised by Reviewer 1 were addressed experimentally, including comments on upstream regulatory mechanisms and physiological significance in other hormone-independent prostate cancer cell lines. These were specifically pointed out in your initial decision letter. In addition, our new 3C data highlight the general importance of the UBE2C enhancers in CRPC cells (see Figure 1 in attached Response letter). I assume that the revised paper was assessed by Reviewer 1 and found to be acceptable for publication.

We believe that by re-clarifying these points, which are fully addressed in attached Response letter, the manuscript is now suitable for publication in your esteemed journal.

Thank you in advance for your consideration, I look forward to a positive reply. Please let me know if I can provide any further information.

Attached Point-by-Point-Response

Appeal

Editor’s point:

“Together with the issue that the characterized enhancer element turns out to be of tissue specific rather than general importance”

Response: Our findings that UBE2C enhancers are functional in PC-3 cells, which are derived from a prostate cancer lumbar vertebral metastasis, but not in DU-145 cells (derived from a prostate cancer brain metastasis) are consistent with the recent genomewide studies demonstrating that enhancers but not promoters are mostly cell-specific (Heintzman et al. Nature, 459:108-112, 2009; Heintzman, et al. Nature Genet, 39:311-318, 2007). Nonetheless, our new data (see Appeal Figure 1) demonstrate that the UBE2C enhancers are functional in at least a subset of CRPC cells, highlighting the general importance of these enhancers.
While the revised manuscript was under review, we continued to make significant progress supporting this important study. Our new 3C data shows a significantly greater interaction between E1, E2 and E3 identified from PC-3 cells and the UBE2C promoter in an androgen receptor (AR) positive CRPC cell line LNCaP-abl (derived from lymph node) than in LNCaP cells (Appeal Figure 1). The previously identified +46 kb Enhancer 2 in LNCaP-abl cells (Wang et al. Cell, 138:245-256, 2009) served as a positive control. The clinical properties of a significant proportion of CRPC patients are observed in LNCaP-abl cells. For example, recent studies reported that AR upregulates cell cycle genes in LNCaP-abl cells, mimicking the pattern of upregulated genes observed in human CRPC vs. ADPC (Wang et al. Cell, 138:245-256, 2009; Sharma et al. J Clin Invest, 120: 4478-4492, 2010; Varambally et al. Cancer Cell, 8:393-406, 2005; Stanbrough et al. Cancer Res, 66:2815-2825, 2006).

Importantly, silencing of MED1 significantly decreased the interactions between E1, E2 and E3 and the UBE2C promoter in LNCaP-abl cells (Appeal Figure 1B), strongly indicating that MED1 is also crucial for UBE2C looping in LNCaP-abl cells. In addition, Western blot analysis showed greater protein expression of phospho-MED1 and total MED1 in LNCaP-abl cells compared to LNCaP cells (Appeal Figure 1C).

Proposed change in manuscript title: Given that MED1 is also important for UBE2C locus looping (Appeal Figure 1B) and UBE2C gene expression (Wang et al. Cell, 138:245-256, 2009) in the AR-positive CRPC cell line LNCaP-abl, and in view of the general importance of E1, E2 and E3 in at least a subset of AR-positive and AR-negative CRPC cells (Appeal Figure 1A and manuscript Figure 2), we would like to change the title of the manuscript to, “Phospho-MED1-enhanced UBE2C locus looping drives castration-resistant prostate cancer growth”, which we believe better describes the general importance and impact of our study.

Reviewer 3:

“However, the fact that modulation of MED1 does not influence UBE2C expression would suggest that it is an important component of a potentially massive protein complex, but not a crucial determinant. In my opinion this detracts from the overall conclusions.

The wording of the text should be modified to better reflect this.”

Response: While reviewer 3 is correct that overexpression of MED1 in ADPC cell line LNCaP does not enhance UBE2C mRNA expression, we clearly demonstrate that silencing of MED1 significantly decreases UBE2C locus looping and UBE2C gene expression in both AR-negative (PC-3) and AR-positive (LNCaP-abl) CRPC cells (Appeal Figure 1B, manuscript Figure 4, and Wang et al. Cell, 138:245-256, 2009). This observation supports the hypothesis that MED1 is critical for UBE2C expression in CRPC but not in ADPC cells. Our new data showing that silencing
of MED1 (and FoxA1) had no effect on endogenous UBE2C expression in LNCaP cells further supports our hypothesis (Appeal Figure 2). It is possible that the altered signaling pathways (e.g. PI3K/AKT) during prostate cancer progression from ADPC to CRPC significantly affect the role of MED1 in mediating UBE2C expression, a point that could be more fully addressed in the “Discussion” section. Thus, we now have convincing additional experimental data and evidence in the published literature to support the conclusion that MED1 is a crucial determinant for UBE2C expression in CRPC cells and that the UBE2C enhancers are of general importance for CRPC cells.

Reviewer 2

“Most importantly, the main message, the proposed role of phosphorylation of MEDI in looping (title) has not been convincingly demonstrated.”

Response: The question of whether Mediator complex mediates chromatin looping is a topical theme highlighted by recent publications in high impact journals (Park et al. Mol Cell, 19:643-653, 2005; Kagey et al. Nature, 467:430-435, 2010; Malik and Roeder. Nat Rev Genet, 11:761-762, 2010). Although it has been demonstrated that Mediator mediates looping of several loci, the molecular mechanisms underlying Mediator-mediated looping are unknown. We propose that phosphorylation of MED1 is a mechanism for Mediator-mediated looping. We believe that our study convincingly demonstrates that phosphorylation of MED1 enhances UBE2C locus looping. Previous studies have shown that comparing wild-type and phosphorylation-defective mutants in functional assays is a powerful way to reveal the role of phosphorylation in coactivator activity (Wu et al. Mol Cell, 15:937-949, 2004; Li et al. Mol Cell, 31:835-849, 2008). Importantly, our 3C assay revealed significantly (P<0.01) decreased UBE2C looping in PC-3/DM (phospho-doublemutants) cells compared to PC-3/WT (wild-type) cells (see manuscript Figure 6).

In order to further demonstrate the general importance of phospho-MED1 in UBE2C looping, we plan to examine the effect of MED1 phosphorylation on UBE2C looping in LNCaP- abl cells. We should be able to complete this experiments within 4–5 weeks.

However, for many individual aspects there remain open questions (ie for the discrepancies in mediator complex subunits versus MEDI, antibodies, 3C technology, ChIP technology).

Response: While we appreciate the point of view of the reviewer, we respectfully disagree that there are “open questions” or “discrepancies” in any of these experimental aspects of our study, for the following reasons.

1) Discrepancies in Mediator complex subunits vs. MED1: I assume that this reviewer questioned the different binding patterns of MED1 and MED17 on UBE2C locus (Figures 3D and 6D).
However, these results are consistent with the recent findings that MED1 but not MED17 only exists in a subpopulation of Mediator complex (Malik and Roeder, Nat Rev Genet, 11:761-772, 2010).

2) Antibodies: All commercial antibodies have been used in other published studies. With regard to our generated phosphorylated MED1 antibody, the specificity has been rigorously validated using p-MED1 immunoprecipitation/p-Threonine western blot analysis (manuscript Figure 5A), the new p-MED1 western blot analysis (manuscript Figure 5A), and the new peptide competition assay in ChIP conditions (manuscript Supplementary Figure 4C).

3) 3C technology and ChIP technology: We have significant experience on these two technologies, as evidenced by our recent publications (Wang et al. Cell, 138:245-256, 2009; Wang et al. Mol Cell, 27:380-392, 2007; and Wang et al. Mol Cell, 19: 631-642, 2005). The 3C and ChIP experiments were carefully performed with all necessary controls.

---

Additional Editorial Correspondence 24 February 2011

Thank you very much for your recent correspondence that outlines further experimentation in favour of a more general role of the characterized enhancer (so far only applicable in PC3 cells). As expansion to other (hormone-independent) cancer cell lines was already requested during the first round of reviews, this was indeed the major reason for my recent rejection. I can see that the new data provided in your rebuttal address this point to a certain extend, and might also generalize your findings. I would suggest to include these data on the role of MED1 phosphorylation in UBE2C looping in LNCaP-abl cells. Only with such a complete and obviously experimentally convincing dataset would we be able to re-assess a revised version for suitability here. This might however involve once more one of the original referees. I like to add that in contrast to your assumption ref#1 was unavailable to judge your initial revisions.

Based on these conditions and framing your conclusions in a way that are fully supported by the presented data, we would indeed be willing to reconsider your...

Yours sincerely,

Editor
The EMBO Journal

Additional Author Correspondence 25 February 2011

Thanks a lot for your positive response on our rebuttal. We will investigate the role of phosphorylated MED1 in UBE2C looping in LNCaP-abl cells. We should be able to complete this experiment in about 4-5 weeks.

Could you provide us a link so that we could submit a revised manuscript?

---

2nd Revision - Authors' Response 18 April 2011

Editor’s point:
“...the characterized enhancer element turns out to be of tissue specific rather than general importance...”
Response: Our findings that UBE2C enhancers are functional in an AR-negative CRPC cell model PC-3, but not in another AR-negative cell model DU-145 are consistent with the recent genomewide studies demonstrating that a large proportion of enhancers (but not promoters) are cell-specific (Bulger and Groudine. Cell, 144:327-339, 2011; Heintzman et al. Nature, 459:108-112, 2009). However, to fully address the point raised, we examined the AR-positive CRPC cell model LNCaP-abl, which closely models clinical CRPC (Wang et al. Cell, 138:245-256, 2009; Sharma et al. J Clin Invest, 120: 4478-4492, 2010; Varambally et al. Cancer Cell, 8:393-406, 2005). We demonstrated that the UBE2C enhancers are also functional in LNCaP-abl and that MED1/phosphorylated MED1 mediates UBE2C enhancer/promoter interactions in this cell line. The data are described in detail below. These new findings highlight the general importance of UBE2C enhancers, and the general role of MED1 and phosphorylated MED1 in mediating UBE2C locus looping, UBE2C gene expression and CRPC growth.

Our previous findings that MED1 binding to the two AR enhancers (Enhancer-1 and Enhancer-2) in the UBE2C locus is increased in an AR-positive CRPC cell model LNCaP-abl compared to LNCaP cells (Wang et al. Cell, 138:245-256, 2009) provided the rationale for us to investigate whether MED1 and phosphorylated MED1 in LNCaP-abl cells can drive UBE2C locus looping, UBE2C gene expression and cell growth. Western blot analysis revealed that MED1 protein level was higher in LNCaP-abl versus LNCaP cells (new Figure 6A). Results of 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 (new Figure 6B), which was correlated with increased interactions between the UBE2C enhancers (E1, E2, E3 and Enhancer-2) and the UBE2C promoter (new Figure 6C and new Supplementary Figure 6A). Similar to our findings in PC-3 cells, we found that silencing of MED1 significantly decreased the interactions between the UBE2C enhancers and the UBE2C promoter in LNCaP-abl cells (new Figure 6D and new Supplementary Figure 6B), 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 to LNCaP (new Figures 6A and 6E and new Supplementary Figure 6C), we next examined the functional role of phosphorylated MED1 in LNCaP-abl cells. Importantly, siMED1 3'UTR transfection followed by 3C assays revealed significantly (P<0.05 or P<0.01) decreased UBE2C locus looping in LNCaP-abl/DM cells compared to LNCaP-abl/WT cells (new Figure 6F), which led to decreased UBE2C mRNA expression (new Figure 6G) and cell growth (new Figure 6H) of LNCaP-abl/DM cells compared to LNCaP-abl/WT cells. Taken together, these new findings demonstrate the general importance of MED1 and phosphorylated MED1 in UBE2C locus looping, UBE2C gene expression and CRPC growth.

Based on our new findings in LNCaP-abl cells, we have now changed the title of the manuscript to, “Phospho-MEDI1-enhanced UBE2C locus looping drives castration-resistant prostate cancer growth”, which we believe better describes the general importance and impact of our study.

Reviewer 3:

“Howver, the fact that modulation of MED1 does not influence UBE2C expression would suggest that it is an important component of a potentially massive protein complex, but not a crucial determinant. In my opinion this detracts from the overall conclusions. The wording of the text should be modified to better reflect this.”

Response: To address this important point, we have generated new data which clearly demonstrates that MED1 silencing significantly decreased UBE2C locus looping and UBE2C gene expression in both AR-negative (PC-3) and AR-positive (LNCaP-abl) CRPC cells (Figure 3, new Figure 6D) in addition to our previous report (Wang et al. Cell, 138:245-256, 2009). Furthermore, silencing of endogenous MED1 had no effect on UBE2C gene expression in LNCaP cells (new Supplementary...
These findings suggest that MED1 is a crucial determinant for UBE2C expression in CRPC 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, a possibility that is now clarified in the revised text (see page 21).

Reviewer 2

“Most importantly, the main message, the proposed role of phosphorylation of MED1 in looping (title) has not been convincingly demonstrated.”

Response: The important issue of whether Mediator complex mediates chromatin looping has been highlighted in several recent publications (Park et al. Mol Cell, 19:643-653, 2005; Kagey et al. Nature, 467:430-435, 2010; Malik and Roeder. Nat Rev Genet, 11:761-762, 2010). Although Mediator mediates looping of several loci, the molecular mechanisms remain unknown. We propose that phosphorylation of MED1 is a mechanism for Mediator-mediated looping and believe that our study convincingly demonstrates that phosphorylation of MED1 enhances UBE2C locus looping. Previous studies have shown that comparing wild-type and phosphorylation-defective mutants in functional assays is a powerful approach for demonstrating the role of phosphorylation in coactivator activity (Wu et al. Mol Cell, 15:937-949, 2004; Li et al. Mol Cell, 31:835-849, 2008). Importantly, our 3C assay revealed that UBE2C looping was significantly (P<0.05 or P<0.01) decreased in PC-3/DM (phospho-double-mutants) cells compared to PC-3/WT (wild-type) cells (Figure 5F), and in LNCaP-abl/DM cells compared with LNCaP-abl/WT cells (new Figure 6F). To our knowledge, our study is the first to address the molecular mechanism of Mediator-mediated looping.

“However, for many individual aspects there remain open questions (ie for the discrepancies in mediator complex subunits versus MED1, antibodies, 3C technology, ChIP technology).”

Response: For all experimental aspects of our study, we have made every effort to address these important concerns. With regard to the specific points:

1) Discrepancies in Mediator complex subunits vs. MED1: If we assume that the Reviewer is referring to the different binding patterns of MED1 and MED17 on UBE2C locus (Figures 2E and 5D), we would like to suggest that our results are completely consistent with the recent findings that MED1 but not MED17 only exists in a subpopulation of Mediator complex (Malik and Roeder, Nat Rev Genet, 11:761-772, 2010).

2) Antibodies: We have used commercially available antibodies that have been used in other published studies and suggest that these are “proven” antibodies. Furthermore, we are confident regarding the specificity of our phosphorylated MED1 antibody, which has been rigorously validated using p-MED1 immunoprecipitation/p-Threonine western blot analysis (Figure 4A), p-MED1 western blot analysis (Figure 4A), and the peptide competition assay in ChIP conditions (Supplementary Figure 4C).

3) 3C technology and ChIP technology: While these are complex and technically difficult approaches, we have significant experience with both technologies as evidenced by our recent publications in highly stringent journals (Wang et al. Cell, 138:245-256, 2009; Wang et al. Mol Cell, 27:380-392, 2007; and Wang et al. Mol Cell, 19: 631-642, 2005). Appropriate controls were included in all 3C and ChIP experiments.