THE EMBO JOURNAL

Manuscript EMBO-2014-90306

Agonist and Antagonist Switch DNA Motifs Recognized by Human Androgen Receptor in Prostate Cancer

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Review timeline:

Submission date:	13 October 2014
Editorial Decision:	07 November 2014
Revision received:	20 November 2014
Accepted:	26 November 2014

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.)

Editor: Thomas Schwarz-Romond

1st Editorial Decision 07 November 2014

Thank you for submitting your study on agonist versus antagonist switching of AR-binding in prostate cancer for publication in The EMBO Journal.

The enclosed comments from two scientists reveal definitive interest in the reported results. Both referees also offer a few minor suggestions as to increase general accessibility as well as improving immediate value of the very complex dataset. Ref #1 asks to present a full, statistically relevant inventory of the AR-occupancy pattern. Subsequently, s/he requests to focus and present data for at least one (if not a few) novel target with experimentally validated biological effects (for instance Enzalutamide-responses?).

I would also appreciate if you were to position yourself to the two minor issues raised by the second referee before submitting your finally amended dataset for ultimate presentation in The EMBO Journal, please!
(I am certain that experiments addressing most of these remarks are readily available in the lab as to enable relatively timely pursuit).

Please do not hesitate to get in touch in case I can be of any assistance/to discuss feasibility, amount and timeline of such final adjustments.

REFEREE REPORTS:

Referee #1:

The authors use the ChIP-exo method to map AR binding sites in cell-lines treated with an agonist and two antagonists. The ChIP-exo method has been previously reported to provide greater spatial resolution in the mapping of binding sites and thus enhance the ability to detect enriched motifs directly linked to the recruitment or activity of the ChIPped factor. The principal novel finding in the paper is that antagonist-treated cells have a distinct AR binding profile compared to agonist-treated cells. The AR binding profile observed upon enzalutamide treatment is entirely novel and the authors associate these sites with genes predictive of clinical outcome. They also perform ChIP-exo on tissue samples and find that AR-occupied regions overlap with cell-line data where the cells have been treated with enzalutamide. The authors propose that targeting genes that are prognostic and dependent on these sites will enhance treatment response. The paper does therefore have novelty and a potentially translational impact. However I have some major concerns which should be addressed:-

1. Clarity

In reviewing the manuscripts a number of key questions arose. What are the motifs that co-enrich with distinct AR sites in the treatment conditions used? The authors focus on Forkhead motifs and emphasises FOXA1 and they also mention CTCF motifs. Surprisingly I can find no inventory of co-enriched motifs in the paper. Does this mean that none are present or relevant? Using a higher resolution method I would perhaps expect to find fewer but the AR is pleiotropic in its TF binding partners and the authors should be more comprehensive and categoric on this point. I expect a full inventory and full statistical analysis.

It is supposed by the field that the AR will bind to distinct sites in tissue versus cell-lines owing to heterogeneity of sample type and a multitude of other factors. The authors have a great opportunity to directly compare AR site patterns between cell-lines and tissues (NATs and tumours) in the various treatment conditions used. The authors use AR binding locations rather ARBEs for their analyses. What is the resolution of an AR binding location and how is it defined? This is of course essential information prior to comparing AR recruitment patterns or performing motif enrichment. It is also essential for those seeking to replicate the findings in subsequent studies. Having clarified these points I would like to see straightforward Venn diagrams depicting numerical overlaps in AR binding locations or ARBEs (whichever they feel is more useful) for various conditions and cell/tissue types.

The authors comment that in tissue due to heterogeneity there is a lower ChIP-exo signal/noise ratio. They therefore used a strategy to initially identify AR-enriched locations followed by identification of ARBEs in these locations. What is that strategy? Does this mean that peaks and sites aren't otherwise clear or detectable?

The authors comment that agonist-bound ARBEs resided less frequently in promoter regions in tumour enhanced versus common AR binding locations. They then set a window of 50kb to TSS to assign gene annotation. Does this exclude tumour-specific ARBEs in enhancers? 50kb is fairly conservative if the implication is that enhancers are important.

The authors need to show that using AR site information in some way enhances gene discovery with associated prognostication versus transcriptomic data alone.

2. Additional experiments

Can targeting genes identified in this study using siRNA or drugs if available significantly enhance/inhibit the cellular response to Enzalutamide?

In short the authors have undertaken a significant body of work. Revisions are desirable to make the study more accessible and the findings more translatable. The authors should critically re-read the

manuscript and ask for each key finding how the research community can use that result to design better and more insightful experiments. In some cases the data complexity may limit this but there should be at least one compelling example that has been taken to a stage where it has been experimentally tested and will therefore be explored further by researchers in the field.

Referee #2:

This revised study utilized Chip-Exo to assess agonist (DHT) vs. antagonist (Bicalutamide or Enzalutamide) bound AR occupancy at sites of action in a hormone-therapy sensitive cell line (LNCaP), with validation studies performed using published datasets from clinical specimens. From the exhaustive Chip-Exo, ChIP-Seq, DNAse-Seq, and RNA-Seq data, it is concluded that antagonist-bound AR recognizes distinct motifs and resultant altered transcriptional outcomes. The studies are generally well performed, novel, and will be of significant impact for the nuclear receptor and prostate cancer fields. Only minor concerns remain, which should be addressed:

1. Enzalutamide has been proposed to function by preventing AR translocation to the nucleus. In this study, a different mechanism is proposed. The impact of enzalutamide on nuclear enrichment should be addressed.

2. From the description in the text it sounds like gene expression profile comparisons in Fig 6 were performed using a previously reported dataset. The present study would be strengthened by inclusion of gene expression analyses performed in parallel it the ChIP-Exo.

1st Revision - authors' response 20 November 2014

Reviewer 1:

Summary points
"*The principal novel finding in the paper is that antagonist-treated cells have a distinct AR binding profile compared to agonist-treated cells. The AR binding profile observed upon enzalutamide treatment is entirely novel and the authors associate these sites with genes predictive of clinical outcome. They also perform ChIP-exo on tissue samples and find that AR-occupied regions overlap with cell-line data where the cells have been treated with enzalutamide. The authors propose that targeting genes that are prognostic and dependent on these sites will enhance treatment response. The paper does therefore have novelty and a potentially translational impact.*"

Response: We thank the reviewer for appreciating the novelty and significance of our study.

1. "*What are the motifs that co-enrich with distinct AR sites in the treatment conditions used? The authors focus on Forkhead motifs and emphasises FOXA1 and they also mention CTCF motifs. Surprisingly I can find no inventory of co-enriched motifs in the paper. Does this mean that none are present or relevant? Using a higher resolution method I would perhaps expect to find fewer but the AR is pleiotropic in its TF binding partners and the authors should be more comprehensive and categoric on this point. I expect a full inventory and full statistical analysis.*"

Response: In addition to Forkhead motifs with strong border signals, many other TF motifs with less clear or weak border signals were co-enriched with ARBE in agonist- and antagonist-responsive regions. We have provided a full inventory of ARBE co-enriched motifs with statistical analysis in Table E2.

2. "*It is supposed by the field that the AR will bind to distinct sites in tissue versus cell-lines owing to heterogeneity of sample type and a multitude of other factors. The authors have a great opportunity to directly compare AR site patterns between cell-lines and tissues (NATs and tumours) in the various treatment conditions used. The authors use AR binding locations rather ARBEs for*

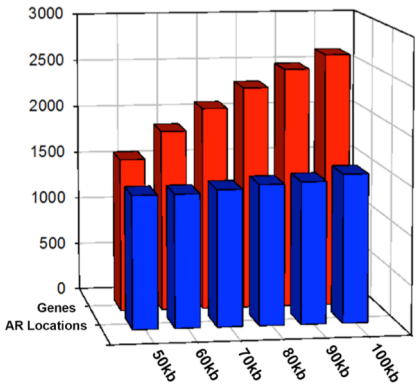
their analyses. What is the resolution of an AR binding location and how is it defined? This is of course essential information prior to comparing AR recruitment patterns or performing motif enrichment. It is also essential for those seeking to replicate the findings in subsequent studies. Having clarified these points I would like to see straightforward Venn diagrams depicting numerical overlaps in AR binding locations or ARBEs (whichever they feel is more useful) for various conditions and cell/tissue types."

Response: We appreciate the opportunity to clarify this. The resolution of an AR binding peak/location in our data is 288±155 bp. All peaks/locations were called with BELT using Read 2 from our paired-end sequencing analysis, which was described in "Materials and Methods" on page 24. The Venn diagram depicting numerical overlaps in AR binding locations for various conditions and cells/tissues was included in Fig E6F (see page 17).

3. "*The authors comment that in tissue due to heterogeneity there is a lower ChIP-exo signal/noise ratio. They therefore used a strategy to initially identify AR-enriched locations followed by identification of ARBEs in these locations. What is that strategy? Does this mean that peaks and sites aren't otherwise clear or detectable?*"

Response: From paired-end sequencing reads, our strategy is to identify AR enriched locations in tissues using the BELT program with Read 2 followed by detecting border signals within the enriched locations using the MALD model with Read 1. This strategy is detailed in the Materials and Methods section, and we have provided a graphical depiction of paired-end read generation in Fig E1. The AR enriched peaks in tissues are very clear, but ChIP-exo border signals in tissues are less clear than in LNCaP cells.

4. "*The authors comment that agonist-bound ARBEs resided less frequently in promoter regions in tumour enhanced versus common AR binding locations. They then set a window of 50kb to TSS to assign gene annotation. Does this exclude tumour-specific ARBEs in enhancers? 50kb is fairly conservative if the implication is that enhancers are important.*"



Response: To ease the concern of this reviewer that 50 kb to a TSS is a conservative distance, we have performed an analysis to examine the effect of distance on AR binding location near associated genes. As shown in the inserted Figure, while increasing the distance from 50 kb to 100 kb significantly increased the numbers of AR-associated genes, the number of AR binding locations utilized for annotation only increased very slightly (from 82% to 89% of all AR binding locations). These results suggest that our analysis has already included the vast majority of tumour-specific ARBEs in enhancers, thus 50 kb is an appropriate distance for binding/gene association analysis.

5. "*The authors need to show that using AR site information in some way enhances gene discovery with associated prognostication versus transcriptomic data alone.*"

Response: Previous work has shown the ability of the estrogen receptor cistrome to define genes predictive of clinical outcomes of breast cancer patients (Ross-Innes et al. *Nature*, 481: 389-393, 2012), suggesting advances in our understanding of prostate cancer prognosis can be made by using primary events (AR binding) to find important secondary outcomes (gene expression) rather than using transcriptomic data alone. Indeed, we have found that only 60.7% of genes within the most significantly enriched gene set associated with AR binging locations, "prostatic neoplasms" (117 genes; Fig 7C) were regulated by DHT in LNCaP cells (Fig. 7D). The strong predictive power of the

117-gene signature highlights the importance of primary AR binding events in survival prediction for prostate cancer patients.

6. "*Can targeting genes identified in this study using siRNA or drugs if available significantly enhance/inhibit the cellular response to Enzalutamide?" "…there should be at least one compelling example that has been taken to a stage where it has been experimentally tested and will therefore be explored further by researchers in the field.*"

Response: We have performed experiments to investigate whether silencing of enzalutamide-liganded AR target genes, *CPEB4*, *RANBP9* or *PKIB* affects the growth of enzalutamide-treated LNCaP cells. Our results showed that silencing of these three genes markedly enhanced the cell growth inhibitory effects of enzalutamide. These new data were included in Fig 6I and E5L.

Reviewer 2

Summary points
"*From the exhaustive Chip-Exo, ChIP-Seq, DNAse-Seq, and RNA-Seq data, it is concluded that antagonist-bound AR recognizes distinct motifs and resultant altered transcriptional outcomes. The studies are generally well performed, novel, and will be of significant impact for the nuclear receptor and prostate cancer fields.*"

Response: We thank this reviewer for appreciating the novelty and impact of our study.

1. "*Enzalutamide has been proposed to function by preventing AR translocation to the nucleus. In this study, a different mechanism is proposed. The impact of enzalutamide on nuclear enrichment should be addressed.*"

Response: We would like to point out that a very recent study has already demonstrated that enzalutamide treatment is able to induce nuclear translocation of AR in LNCaP cells (Myung et al. *J Clin Invest*, 123: 2948-2960, 2013). Indeed, that enzalutamide-liganded AR translocates to the nucleus allows AR to recognize an enzalutamide-liganded ARBE. We have discussed this issue in our revised manuscript (see page 20).

2. "*From the description in the text it sounds like gene expression profile comparisons in Fig 6 were performed using a previously reported dataset. The present study would be strengthened by inclusion of gene expression analyses performed in parallel it the ChIP-Exo.*"

Response: In this study we have re-analysed a very recent gene expression dataset (Guerrero et al. *Prostate*, 73: 1291-1305, 2013) from LNCaP cells treated with enzalutamide (1 μM or 10 μM) for 16 h. This is the same enzalutamide dose used in our AR ChIP-exo study and the longer treatment time compared to ChIP assays allows for analysis of mRNA expression subsequent to AR binding in LNCaP cells (Wang et al. *Mol Cell*, 19: 631-642, 2005; Tran et al. *Science*, 324: 787-790, 2009), making this gene expression dataset very appropriate for integrative analysis with our AR ChIP-exo dataset. Importantly, we have also experimentally validated 3 enzalutamide-liganded AR target genes, *CPEB4*, *RANBP9* and *PKIB*, by parallel ChIP and RT-PCR analysis (Figure 6F-H and Figure E5I-K).

