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Dual Role of FoxA1 in Androgen Receptor Binding to Chromatin, Androgen Signaling and Prostate Cancer

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(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 19 May 2011

Thank you very much for submitting your research paper that reports genome-wide, dynamic changes of AR-chromatin binding depending FoxA1.

As you will see from the enclosed reports, all three scientists appreciate your results. At the same time, refs #1and #3 recommend further experimentation to increase the significance of the study. Particularly, the presented time-points/DHT-dosages are issues that need to be addressed. Further, corroborating the AR-target gene categorization in prostate cancer models (ref#3 point 1) as well as thoroughly connecting the IHC-results to the proposed regulatory networks by confirming at least some to the targets in the examined tissue (ref#3 point 4, respective parts of refs#1 point 5) would strengthen the clinical relevance and thus be essential. Overall, we would be delighted to receive an adequately revised version of your study for final scientific assessment.

Please be reminded that it is EMBO Journal policy to allow a single round of revisions only and that the ultimate decision on acceptance depends on the content and strength of the revised manuscript.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

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Referee #1:

Sahu et al., address the interplay between FoxA1 and chromatin binding by the androgen receptor and the glucocorticoid receptor in the LNCaP-F15 cell-line using ChIP-seq. They conclude that binding is highly dynamic and that, in contrast to the estrogen receptor, knocking down FoxA1 creates news binding events for both the AR and GR. This is novel and unreported in the interest. It is an observation that merits publication. The crux of my comments therefore relates to the data analysis and the mechanistic context for this change. I should stress at this point that because this paper is heavily dependent on ChIP-seq and expression array datasets I have been significantly hampered in my evaluation by the fact that none of the datasets have been deposited in a secure but accessible way. To quote 'the raw ChIP-seq and microarray data have been submitted to the Gene Expression Omnibus (GEO) repository under the accession number xxxyy'. It is possible to deposit this data so that it remains private and accessible only to the referees during the review process. I would also recommend deposition of both raw and processed (.bed and/or .wig files) for the ChIP-seq datasets.

1. The authors comment extensively on changes in binding site number and distribution with and without FoxA1 siRNA upon ligand treatment. Binding site variations due to the timepoints chosen for ligand treatment are to be expected, as are variations due to peak calling algorithms selected. I would strongly suggest some changes related to these general comments:

A. A careful justification of the treatment timepoints chosen (eg. 2 hours with DHT).
B. A meta-analysis of AR binding sites, comparing your datasets with other published datasets. For example what proportional of the additional AR binding sites observed upon FoxA1 knockdown are to be found in other published datasets where longer ligand treatment times have been used?
C. The use of at least two peak-calling algorithms and working with only those sites that called by both at a suitably stringent threshold/FDR (eg. 2%).
D. The authors should comment on the number of replicates that have been sequenced and the number of alignable/non-duplicate reads obtained per condition.
E. PCR validation of a selection of called binding sites not based on linkage to genes but based on peak height/intensity (eg. Ten sites from each of three peak height/intensity thresholds) to generate a limited experimental assessment of FDR.

2. Many of the same points then apply to the expression array datasets too. Here the authors have elected to use a 24-hour ligand treatment timepoint. Following on from my previous remarks:

A. The authors should carefully justify their reasons for using this rather than a more detailed time course.
B. The authors should comment on the significant difference between the number of binding sites (1000s) and the number of altered transcripts (100s). Early in the paper they map binding sites to the nearest RefSeq genes and use a 100kb window. Are there other windows that better reflect the numerical differences between site number and differential transcript expression? Why use the 100kb window?
C. Many expression datasets exists for hormone-treated cell-lines. If a meta-analysis is undertaken are there timepoints or subgroups of genes that associate more significantly with the new sites generated by FoxA1 knockdown or other conditions used in this study?
D. Are there particular pathways/biological processes that associate with AR binding sites in the FoxA1 knockdown condition that are not significant when FoxA1 is present?

3. The authors correctly imply that a mechanism to explain the generation of new sites upon FoxA1 would significantly enhance the impact of the work. Based on their motif analysis it would be interesting to know whether a subset of these sites also emerge if CTCF or a Groucho complex protein such as TLE1 is knocked down. Motif analysis speaks somewhat for CTCF. I agree that this is risky but constitutes hypothesis-testing and would enhance the impact of the paper and subsequent follow-up work by other groups if done robustly.
4. The authors propose that AR binding is dependent on the FoxA1 expression level. This is testable. A rescue experiment in which siRNA-resistant FoxA1 is introduced under an androgen-inducible promoter (eg. Probasin) would allow ChIP PCR or even ChIP-seq to be undertaken at a number of timepoints with increasing FoxA1 expression without resorting to a sledgehammer such as a CMV promoter. Not essential for this paper but this or something similar should be considered and of

course it would be great if the authors felt able to attempt this also to control for off-target effects from the siRNA.
5. The immunohistochemical data in the paper currently looks like something of an outlier. The cohort is impressive in both patient number and the duration of the clinical follow-up data. However, this component of the paper lacks a clear bridge back to the gene regulation and binding site data generated in the cell-line. The clearest of bridges would be tissue ChIP-seq on a limited number of samples. This is possible for the AR using fresh-frozen material (Yu et al., Cancer Cell 2010) and for FFPE samples for histone marks (Fanelli et al., PNAS 2010) but may pose too great a technical challenge if the authors wish to look at TFs in FFPE samples. As an alternative please test for IHC correlates abstracted from your gene network analysis. Candidates could include CDK6, FOXO1 and FOXO3. Without some additional linkage the IHC data can be placed in supplementary information.

The manuscript presents a novel new observation on the interplay between nuclear hormone receptors and FoxA1. To be accepted it needs a more careful justification of the conditions used and some additional experiments. Extrapolating into clinical material/a clinical context is always fraught with risks and this element of the work should at this stage either be given less emphasis or augmented with additional work.

Referee #2:

This manuscript addresses the genomic properties of AR in prostate cancer cells. The authors maps AR binding in prostate cancer cells and finds the presence of the Forkhead motif, which provided the impetus to map FoxA1 binding. This resulted in a global definition of AR and FoxA1 overlap, which is extended to assess the pioneer role of FoxA1 for AR binding. Silencing of FoxA1 resulted in a loss of AR binding and gene expression, but remarkably a simultaneous gain of AR binding and gene expression. As such, FoxA1 both pioneers AR binding, but also blocks potential AR binding sites. The loss of FoxA1 alters H3K4me2 at some sites, (a finding that is distinct from previous findings) and results in increased histone marks at some of the gained sites. Interestingly the absence of FoxA1 also alters GR binding. These findings are followed up in clinical samples by staining for FoxA1 in prostate cancers and correlating with outcome and AR status.

Overall this is an outstanding manuscript. The datasets are rock solid and impressive. The combination of siFoxA1 with AR binding, GR binding and both histone and DHS is excellent. I thoroughly enjoyed reading this manuscript and it will have a profound impact on the scientific community. Not only do I recommend publishing this manuscript as it is (the first time I have recommended this in a long time), but I would also recommend fast tracking this manuscript due to its importance in a competitive and rapidly evolving field.

Referee #3:

FoxA1 has been identified as a pioneer factor for nuclear receptors including the androgen receptor (AR), and is posited to play a role in prostate cancer. The present study challenges this premise on a genome wide scale through ChIP-seq and bioinformatic analyses. FoxA1 depletion resulted in a reprogramming of AR binding in response to androgen, allowing subclassification into sites that are FoxA1 dependent, FoxA1 independent, and those that are unmasked upon FoxA1 depletion. Parallel analyses of H3K4-me2 revealed that FoxA1 guides AR to this mark, and interestingly, depletion of FoxA1 can also lead to new sites of methylation. DNase hypersensitivity assays revealed that most AR/FoxA1 binding sites occur in sites of open chromatin, and hypersensitivity was also observed in the "new" sites observed after FoxA1 depletion. To begin to address specificity, the effects of FoxA1 depletion of GR was assessed, wherein it was shown that redistribution of this receptor also occurs after FoxA1 knockdown. Finally, relevance was considered, in that FoxA1 levels were shown to be enriched in prostate cancer specimens and associated with poor outcome.

Comments: This is an elegant study that illuminates new functions for FoxA1 in prostate cancer. The genome-wide assessment has been thoughtfully planned and the stated conclusions are largely

supported by the data shown. However, there are concerns that the depth of investigation limits the impact of the findings. If the following concerns can be addressed, the impact of the observations would be significantly bolstered:

1. Specificity is an issue. The study is largely limited to analyses of a single cell line, thus reducing confidence in the general applicability of the findings. The classification of AR target genes into the 3 major categories should be validated in prostate cancer model systems not derived from LNCaP (eg LAPC4 or VCAP cells) using ChIP-qPCR. In addition, ChIP-qPCR analyses to validate the findings in LNCaP (currently shown in Supp Fig S4) should be moved to the main figures of the paper, as these studies provide some of the only validation of the ChIP-Seq data.

2. ChIP-qPCR should be used to validate the proposed alterations in AR and FoxA1 binding for the 3 categories of AR binding sites shown exemplified by the genes examined in S4. These studies should also be performed using lower doses of DHT ---the doses of DHT used to perform the ChIP-Seq studies are extraordinarily high (well beyond the range of physiological relevance), and are within the dose that is strongly anti-proliferative in LNCaP cells.

3. The changes in gene expression appear to have been assessed after 24 hrs of exposure to DHT, yet the ChIP-Seq studies were performed with considerably shorter timepoints. The possibility that the classification into 3 categories (FoxA1-independent, FoxA1-pioneered, and new genes in FoxA1-depleted) is an artifact of having examined a single timepoint for binding and gene expression should be ruled out through kinetic analyses of binding and mRNA expression.

4. The IHC from human cancer tissue is of interest and adds strength to the concept that FoxA1 is of relevance for human disease. However, these exciting observations are not well connected with the rest of the study. Associations with the products of at least a subset of identified FoxA1 dependent AR target genes should be examined in these tissues.

5. The study clearly identifies a subset of AR targets that are FoxA1 dependent, and establishes FoxA1 elevation as occurring with frequency in human disease. It should be determined whether mimicking the tumor-associated events (ie upregulation of FoxA1) in LNCaP and/or other hormone therapy sensitive lines results in altered expression of representative genes within the 3 categories.

1st Revision - Authors' Response 14 July 2011

Our point-to-point responses to the issues raised by the reviewers are as follows.

Referee #1:

We thank the reviewer for his/her comments and suggestions.

To quote 'the raw ChIP-seq and microarray data have been submitted to the Gene Expression Omnibus (GEO) repository under the accession number xxxyy'. It is possible to deposit this data so that it remains private and accessible only to the referees during the review process. I would also recommend deposition of both raw and processed (.bed and/or .wig files) for the ChIP-seq datasets.

Our response: ChIP-seq and gene expression microarray data have been deposited in the Gene Expression Omnibus database with accession number GSE30624.

1. The authors comment extensively on changes in binding site number and distribution with and without FoxA1 siRNA upon ligand treatment. Binding site variations due to the timepoints chosen for ligand treatment are to be expected, as are variations due to peak calling algorithms selected. I would strongly suggest some changes related to these general comments:

A. A careful justification of the treatment timepoints chosen (eg. 2 hours with DHT).

Our response: The two-hour time point for ChIP assays was selected on the basis of our previous work (*e.g.,* Kang et al, Mol Endocrinol 18: 2633–2648, 2004; Thompson et al, EMBO J 25: 2757–

2767, 2006) and that of others (*e.g.,* Wang et al, Mol Cell 19: 631–642, 2005). These results indicate that AR loading onto chromatin peaks in cultured cells by two h of hormone exposure, and it stays after that relatively stable at least for the ensuing 12 h. This is now mentioned in the revised text (p. 5).

B. A meta-analysis of AR binding sites, comparing your datasets with other published datasets. For example what proportional of the additional AR binding sites observed upon FoxA1 knockdown are to be found in other published datasets where longer ligand treatment times have been used?

Our response: We have performed these analyses on new ARBs in siFoxA1 cells, and the results show that a very limited number of these sites (<10%) are found in published data sets. This is now mentioned on p. 6 (last line onwards) of the revised manuscript. We feel that a more thorough meta-analysis of ARBs in parental cells is not germane in the present work that addressed the role of FoxA1 in the AR pathway. We will compare our ARB data sets to those published by other groups in another manuscript cited in the text (Sahu *et al*, manuscript in preparation).

C. The use of at least two peak-calling algorithms and working with only those sites that called by both at a suitably stringent threshold/FDR (eg. 2%).

Our response: We have analyzed our data by another peak calling algorithm (SPP peak caller; http://compbio.med.harvard.edu/Supplements/ChIP-seq), and the results are very similar to those we have seen by using the MACS algorithm for all three categories of ARBs and GRBs. Since the MACS algorithm has been used in a number of recent genome-wide analyses and is one of the best-performing algorithms for ChIP-seq peak detection (Wilbanks EG, Facciotti MT, Plos one: e11471, 2010), we do not see a justifiable reason for changing our current peak identification. Moreover, when *de novo* motif search was conducted on the AR- or GR-binding sites identified by the SPP algorithm, exactly the same motifs for different classes of binding sites were identified as those shown in the original version in Figs. 3 and 6.

D. The authors should comment on the number of replicates that have been sequenced and the number of alignable/non-duplicate reads obtained per condition.

Our response: This information is now mentioned in the supplementary results section (Supplementary Table S13).

E. PCR validation of a selection of called binding sites not based on linkage to genes but based on peak height/intensity (eg. Ten sites from each of three peak height/intensity thresholds) to generate a limited experimental assessment of FDR.

Our response: We show now ChIP-qPCR validation of selected ARBs from the three different categories of binding sites (8 sites in each category) in Supplementary Fig. S5. Validation results are mentioned on p. 7 of the revised text.

2. Many of the same points then apply to the expression array datasets too. Here the authors have elected to use a 24-hour ligand treatment timepoint. Following on from my previous remarks:

A. The authors should carefully justify their reasons for using this rather than a more detailed time course.

Our response: We have performed time-course and DHT dose-response experiments. Collectively, the results show that maximal responses, in terms of androgen-regulated mRNA accumulation, take place at 24 h of hormone exposure and at 100 nM DHT. This is now mentioned in the revised text (p. 8). These results are shown as Figs. S7A–S7C.

B. The authors should comment on the significant difference between the number of binding sites (1000s) and the number of altered transcripts (100s). Early in the paper they map binding sites to the nearest RefSeq genes and use a 100kb window. Are there other windows that better reflect the numerical differences between site number and differential transcript expression? Why use the 100kb window?

Our response: Previous genome-wide studies have used a window of 200 kb to map the binding site

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of a transcription factor to the nearest gene (Carroll *et al*, 2005; Wang *et al*, 2007; Carroll *et al*, 2006, Wang *et al*, 2009). We employed 200-kb and 50-kb windows in our preliminary analysis, but chose to use the 100-kb window, as it reflected the best correlation between the number of binding sites and the number of transcripts. While we agree that this is a somewhat arbitrary number, we would like to point out that there is no useful convention in the field at present.

C. Many expression datasets exists for hormone-treated cell-lines. If a meta-analysis is undertaken are there timepoints or subgroups of genes that associate more significantly with the new sites generated by FoxA1 knockdown or other conditions used in this study?

Our response: As mentioned above, only a very limited number of ARBs in siFoxA1 cells are present in any of the publicly available data resources.

D. Are there particular pathways/biological processes that associate with AR binding sites in the FoxA1 knockdown condition that are not significant when FoxA1 is present?

Our response: We have included information on pathways/biological processes associated with the three classes on androgen-responsive genes regulated by FoxA1 depletion, *i.e.,* those shared by parental and siFoxA1 cells (=FoxA1-independent), those unique to parental cells (=FoxA1-pioneered), and those unique to siFoxA1 cells. These results are shown in Supplementary Table S6 and mentioned on p. 10 of the revised manuscript.

3. The authors correctly imply that a mechanism to explain the generation of new sites upon FoxA1 would significantly enhance the impact of the work. Based on their motif analysis it would be interesting to know whether a subset of these sites also emerge if CTCF or a Groucho complex protein such as TLE1 is knocked down. Motif analysis speaks somewhat for CTCF. I agree that this is risky but constitutes hypothesis-testing and would enhance the impact of the paper and subsequent follow-up work by other groups if done robustly.

Our response: We agree with the reviewer that these experiments would be of interest. However, we feel that they would be beyond the scope of the present work.

4. The authors propose that AR binding is dependent on the FoxA1 expression level. This is testable. A rescue experiment in which siRNA-resistant FoxA1 is introduced under an androgen-inducible promoter (eg. Probasin) would allow ChIP PCR or even ChIP-seq to be undertaken at a number of timepoints with increasing FoxA1 expression without resorting to a sledgehammer such as a CMV promoter. Not essential for this paper but this or something similar should be considered and of course it would be great if the authors felt able to attempt this also to control for off-target effects from the siRNA.

Our response: Once again, we agree with the reviewer that these studies would be of interest. Despite this, we feel that they would be beyond the scope of the present work.

5. The immunohistochemical data in the paper currently looks like something of an outlier. The cohort is impressive in both patient number and the duration of the clinical follow-up data. However, this component of the paper lacks a clear bridge back to the gene regulation and binding site data generated in the cell-line. The clearest of bridges would be tissue ChIP-seq on a limited number of samples. This is possible for the AR using fresh-frozen material (Yu et al., Cancer Cell 2010) and for FFPE samples for histone marks (Fanelli et al., PNAS 2010) but may pose too great a technical challenge if the authors wish to look at TFs in FFPE samples. As an alternative please test for IHC correlates abstracted from your gene network analysis. Candidates could include CDK6, FOXO1 and FOXO3. Without some additional linkage the IHC data can be placed in supplementary information.

Our response: The reviewer points out important future experiments. We have not tried to use ChIP-seq for tissue specimens, since all our primary prostate cancer specimens are archival FFPE samples on tissue microarrays. However, we have now looked at FOXO1 and FOXO3 staining using selected TMA slides containing 10 low-moderate FoxA1/high AR spots and compared their staining intensities to 30 randomly selected high FoxA1/high AR spots on the same slides. The results are shown in Supplementary Table 13 and indicate that the low-moderate FoxA1 spots have higher FOXO1 and FOXO3 staining intensity that the high FoxA1 spots.

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The manuscript presents a novel new observation on the interplay between nuclear hormone receptors and FoxA1. To be accepted it needs a more careful justification of the conditions used and some additional experiments. Extrapolating into clinical material/a clinical context is always fraught with risks and this element of the work should at this stage either be given less emphasis or augmented with additional work.

Referee #2:

Overall this is an outstanding manuscript. The datasets are rock solid and impressive. The combination of siFoxA1 with AR binding, GR binding and both histone and DHS is excellent. I thoroughly enjoyed reading this manuscript and it will have a profound impact on the scientific community. Not only do I recommend publishing this manuscript as it is (the first time I have recommended this in a long time), but I would also recommend fast tracking this manuscript due to its importance in a competitive and rapidly evolving field.

Our response: We are very pleased indeed by these very positive and supportive comments of reviewer 2.

Referee #3:

We thank the reviewer for his/her constructive comments and support.

Comments: This is an elegant study that illuminates new functions for FoxA1 in prostate cancer. The genome-wide assessment has been thoughtfully planned and the stated conclusions are largely supported by the data shown. However, there are concerns that the depth of investigation limits the impact of the findings. If the following concerns can be addressed, the impact of the observations would be significantly bolstered:

1. Specificity is an issue. The study is largely limited to analyses of a single cell line, thus reducing confidence in the general applicability of the findings. The classification of AR target genes into the 3 major categories should be validated in prostate cancer model systems not derived from LNCaP (eg LAPC4 or VCAP cells) using ChIP-qPCR. In addition, ChIP-qPCR analyses to validate the findings in LNCaP (currently shown in Supp Fig S4) should be moved to the main figures of the paper, as these studies provide some of the only validation of the ChIP-Seq data.

Our response: We have included ChIP-seq results on VCaP cells (supplementary results, Fig. S6; revised text p. 7). These data show the same phenomenon as with LNCaP-1F5 cells, in that FoxA1 depletion generates three classes of ARBs; those that are independent of FoxA1, those that are pioneered by FoxA1, and those that appear upon FoxA1 depletion. As was the case with LNCaP-1F5 cells, the number of new ARBs in VCaP cells emerging upon FoxA1 depletion is very high.

The original supplementary results in Fig. S4 (now Fig. S8) depicted qPCR validation of mRNA expression levels, not ChIP-qPCR validation of AR-binding sites. We have performed ChIP-qPCR validation of the ChIP-seq data for a total of 24 sites of the three ARB classes in LNCaP-1F5 cells (FoxA-independent, pioneered by FoxA1, and new in siFoxA1 cells; 8 sites for each class). These data are shown in supplementary results section (Fig. S5). Owing to the large number of figures already in the main text, we prefer to present these latter validation results in the supplementary information section.

2. ChIP-qPCR should be used to validate the proposed alterations in AR and FoxA1 binding for the 3 categories of AR binding sites shown exemplified by the genes examined in S4. These studies should also be performed using lower doses of DHT ---the doses of DHT used to perform the ChIP-Seq studies are extraordinarily high (well beyond the range of physiological relevance), and are within the dose that is strongly anti-proliferative in LNCaP cells.

Our response: For validation of AR-binding sites, see our response to point 1. We have performed DHT dose-response experiments using ChIP-qPCR on *PSA* and *TMPRSS2* enhancers in parental and FoxA1-depleted cells (Fig. S1). The data show that half-maximal effect on AR loading occurs at

DHT concentration between 1–3 nmol/l that is commensurate with the K_d of the interaction between AR and DHT. With regard to FoxA1-binding sites, we believe that our ChIP-seq data in siFoxA1 cells provides compelling genome-wide validation for their authenticity.

These dose-response results are now mentioned on p. 5 of the revised text. With regard to the DHT concentration used in gene expression profiling experiments, the dose-response results (Figs. S7A–S7C, p. 8 in the revised text) show that steroid concentrations lower than 100 nM DHT elicit effects very similar to those of 100 nM DHT. Even though there are some differences in the androgen sensitivity and temporal expression patterns, the data show nevertheless that maximal responses were achieved at 24 h and with 100 nM DHT. Extensive time-course analyses by qPCR on 379 androgen-regulated genes in a related cell line (LNCaP cells) by Ngan et al. (Oncogene 28: 2051–2063, 2009) showed that in almost every instance, the summit (up-regulation) or nadir (down-regulation) of the response takes place at 24 h of androgen exposure.

3. The changes in gene expression appear to have been assessed after 24 hrs of exposure to DHT, yet the ChIP-Seq studies were performed with considerably shorter timepoints. The possibility that the classification into 3 categories (FoxA1-independent, FoxA1-pioneered, and new genes in FoxA1-depleted) is an artifact of having examined a single timepoint for binding and gene expression should be ruled out through kinetic analyses of binding and mRNA expression.

Our response: See our responses to points 1 and 2. In view of the time-course and dose-response experiments, we do not believe that there is a reason to assume that our original results represent an artifact due to the time points used in these experiments.

4. The IHC from human cancer tissue is of interest and adds strength to the concept that FoxA1 is of relevance for human disease. However, these exciting observations are not well connected with the rest of the study. Associations with the products of at least a subset of identified FoxA1 dependent AR target genes should be examined in these tissues.

Our response: We have examined FOXO1 and FOXO3 staining using selected TMA slides containing 10 low-moderate FoxA1/high AR spots and compared their staining intensities to 30 high FoxA1/high AR spots on the same slides. The results are shown in Supplementary Table 13 and indicate that low-moderate FoxA1 spots have higher FOXO1 and FOXO3 staining intensity that the high FoxA1 spots. We feel that this information now connects the results in FoxA1-depleted cells to the IHC data, in that FOXO1 and FOXO3 mRNA accumulation was up-regulated upon FoxA1 depletion.

5. The study clearly identifies a subset of AR targets that are FoxA1 dependent, and establishes FoxA1 elevation as occurring with frequency in human disease. It should be determined whether mimicking the tumor-associated events (ie upregulation of FoxA1) in LNCaP and/or other hormone therapy sensitive lines results in altered expression of representative genes within the 3 categories.

Our response: We agree with the reviewer that it would be of interest to examine dose-response effects of FoxA1 in a cell-based system. However, just mere overexpression of FoxA1 in LNCaP or other prostate cancer-derived cells lines is not, in our mind, a preferred way to examine this issue, as these cells already express FoxA1 to relatively high levels. It is our aim to address this issue in our upcoming studies by first creating cell lines in which FoxA1 is depleted in a stable and/or inducible fashion. These cells would subsequently provide a suitable background to examine reprogramming of the AR pathway in a FoxA1 concentration dependent fashion. Despite their potential interest, we feel nevertheless that these experiments would be beyond the scope of our present work.

And finally, we would like to thank the editor and the reviewers for their thoughtful and supportive comments and believe that by addressing the issues raised during the review, we have considerably improved and strengthened this manuscript.

2nd Editorial Decision 29 July 2011

I received final comments on your study from one of the original referees. As you will see, a couple of further amendments would be needed to enable final acceptance of the study. I would thus be grateful if you pursue the indicated experiments and provide us with a final version of your study as soon as you can.

Yours sincerely,

Editor
The EMBO Journal

P.S. I do like to add that the re-review took a bit longer as we preparing a small feature highlighting your study as well as related papers recently published in EMBO Journal and other titles on this topic.

I still urge you carefully attend to the issues raised by the referee and remain with best regards!

REFEREE REPORT

Referee #3:

This paper highlights an important new role for FOXA1 in prostate cancer development and progression, shifting the conventional view of how pioneer factors contribute to transcriptional programs regulated by nuclear receptors. Extensive new data have been included, and a large number of concerns have been addressed. Although there is substantial enthusiasm for the study, there are a few remaining, significant concerns that should be addressed more carefully:

1) Internal inconsistencies:
Inclusion of the dose response curves and time course studies significantly improved the study. While it is true (as stated in the text) that for most genes, a maximal response is seen at 24 hours with 100nM DHT, several inconsistencies are seen at lower doses and shorter time points which have dramatic implications for interpretations. For example, PSA and NFKB1A levels (classified as a FOXA1-independent genes in figure S7A) only appear to be FOXA1 "independent" at a concentration of 100nM. At all other doses (including 0nM) knock-down of FOXA1 appears to induce these transcripts (as compared to in control). This is supported by the ChIP data presented in figure S1 which demonstrates that AR occupancy at the PSA enhancer increases not only with the concentration of DHT, but with loss of FOXA1 as well. Thus the term "FOXA1-independent" should be expanded upon and clarified in the text, to make such distinctions clear.

2) Specificity:
Validation of novel AR binding sites induced upon FOXA1 knockdown in the VCaP cell line provides much needed depth and impact for the study. Determining which AR binding sites are shared between LNCaP and VCaP cell lines upon FOXA1 knockdown could provide extremely useful information in identifying FOXA1 programs that are conserved across all prostate cell lines, and are important for disease specific AR regulatory pathways. This validation should be expanded beyond bioinformatics, and should include ChiP validation of class specific targets (much like the compelling data in figure S5). Western blots of FOXA1 knockdown in the VCaP model should be provided to ensure a similar degree of FOXA1 knockdown between cell lines.

Minor Concerns:
1. The LNCaP-1F5 cell line should be described in more detail in the introduction, and the consequence of rat GR over-expression on AR directed programs discussed.

We thank the Reviewer 3 for his/her support and constructive comments.

This paper highlights an important new role for FOXA1 in prostate cancer development and progression, shifting the conventional view of how pioneer factors contribute to transcriptional programs regulated by nuclear receptors. Extensive new data have been included, and a large number of concerns have been addressed. Although there is substantial enthusiasm for the study, there are a few remaining, significant concerns that should be addressed more carefully.

Our response: We appreciate these comments.

1) Internal inconsistencies:
Inclusion of the dose response curves and time course studies significantly improved the study. While it is true (as stated in the text) that for most genes, a maximal response is seen at 24 hours with 100nM DHT, several inconsistencies are seen at lower doses and shorter time points which have dramatic implications for interpretations. For example, PSA and NFKB1A levels (classified as a FOXA1-independent genes in figure S7A) only appear to be FOXA1 "independent" at a concentration of 100nM. At all other doses (including 0nM) knock-down of FOXA1 appears to induce these transcripts (as compared to in control). This is supported by the ChIP data presented in figure S1 which demonstrates that AR occupancy at the PSA enhancer increases not only with the concentration of DHT, but with loss of FOXA1 as well. Thus the term "FOXA1-independent" should be expanded upon and clarified in the text, to make such distinctions clear.

Our response: This is an important issue. We have now mentioned the fact that some FoxA1-independent AR-binding sites (or androgen-regulated genes) are more androgen-sensitive in siFoxA1 cells and become totally FoxA1-independent only at high androgen concentrations (p. 8, lines 11–15). In addition, we bring up the androgen sensitivity issue of some FOXA1-independent sites again in the Discussion section on p. 18, lines 6–8 and p. 19, last line onwards to p. 20, lines 1–4.

2) Specificity:
Validation of novel AR binding sites induced upon FOXA1 knockdown in the VCaP cell line provides much needed depth and impact for the study. Determining which AR binding sites are shared between LNCaP and VCaP cell lines upon FOXA1 knockdown could provide extremely useful information in identifying FOXA1 programs that are conserved across all prostate cell lines, and are important for disease specific AR regulatory pathways. This validation should be expanded beyond bioinformatics, and should include ChiP validation of class specific targets (much like the compelling data in figure S5). Western blots of FOXA1 knockdown in the VCaP model should be provided to ensure a similar degree of FOXA1 knockdown between cell lines.

Our response: We have expanded the text pertaining to results on VCaP cells (p. 7, lines 6–15) and also included two new figures: Fig. 2 shows now data on FoxA1 depletion in VCaP cells, a Venn diagram for ARBs in parental and FoxA1-depleted VCaP cells and results from directed ChIP validation of nine new ARBs in siFoxA1 VCaP cells. These same sites are also among the new ARBs in siFoxA1 LNCaP-1F5 cells.

Supplementary Fig. S6 shows validation of ARBs in VCaP cells that are either pioneered by FoxA1 or independent of FoxA1. We also performed pathway and gene ontology analyses on FoxA1-independent ARBs and new ARBs in siFoxA1 cells that are shared by LNCaP-1F5 and VCaP cells. The data are shown in Supplementary Tables S7 and S8, and the results mentioned in the text (p. 10, lines 19–22)

Minor Concerns:
1. The LNCaP-1F5 cell line should be described in more detail in the introduction, and the consequence of rat GR over-expression on AR directed programs discussed.

Our response: We have described the LNCaP-1F5 cells line in more detail in the Introduction section and included information about its growth-response and gene expression profiles after androgen treatment – in comparison to the parental LNCaP cell line – on p. 22, lines 17–20, in the

Materials and Methods section.

We would like to thank the editor and the reviewer for their thoughtful and supportive comments and believe that by addressing the issues raised during the review, we have improved and strengthened this manuscript.

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