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AP-2 regulates estrogen receptor-mediated long-range chromatin interaction and gene transcription

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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 16 November 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has been now been evaluated by three referees and I enclose their reports below, as you will see they currently find the study describing a role for AP-2gamma in regulating ERalpha binding, long range genomic interactions and transcription to be very interesting.

The referees are in good agreement with some additional experimental analysis that is required to support the mans conclusions of the study, these include expression profiling to determine the role of AP-2 in regulating the global expression of ERalpha target genes, and also Co-IP, ChIP and ChIP-reChIP experiments to demonstrate the colocalisation of AP2alpha, FoxA1 and ERalpha. These main concerns raised here are central to the conclusions proposed in the study. Furthermore, referee #2 feels that the study would be significantly strengthened by addition of AP-2alpha ChIA-PET data, which they feel should be relatively straightforward to acquire, and in addition it would also address some of the above concerns. It is clear that the additional ChIA-PET analysis would significantly strengthen the study, but I am not clear how easy it will be to perform this analysis and this is something we can further discuss. Given the interest in the study should you be able to address these issues, we would be wiling to consider a revised manuscript.

I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. When you submit a revised version to the EMBO Journal, please make sure you upload a letter of response to the referees' comments.

Please note that when preparing your letter of response to the referees' comments that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

Tan et al._29253
'AP-2γ is required for estrogen receptor-mediated interactome organization and gene transcription'

In the present manuscript, the authors identified and functionally characterized AP-2γ, a transcription factor involved in breast cancer oncogenesis, as a novel collaborative factor of ERα. They show that ERα binding sites (ERBS) identified from their ChIA-PET experiments (previous study, Fullwood et al., 2009) are enriched for AP-2 motifs and that AP-2γ binds to these sites in a ligand independent manner and is required for transcription of estrogen target genes such as GREB1 and RET. AP-2γ appears to enhance the recruitment of ERα at ERBS and to facilitate the subsequent formation of long-range chromatin interactions by working together with FoxA1. Finally, they show on a global scale that most ERBS associated with long-range chromatin interactions are co-localized with AP-2γ and FoxA1 and that binding of AP-2γ and FoxA1 at these ERBS are dependent on each other.
The authors combine molecular, genomics and computational approaches. They use data from a previous study that identified ERα binding sites (ERBS) and long-range interactions using ChIA-PET; (Fullwood et al., 2009) and they produce new data for the AP-2γ and FoxA1global profiles using ChIP-seq. Their experiments are well performed and with proper controls.

Major points:

1. Most of the AP-2γ ChIP-qPCR experiments show very low recoveries (less than 0.2%).

2. All data for the role of AP-2γ on the transcription of estrogen target genes are from two targets (GREB1 and RET; Figure 1 and data not shown). A global analysis of estrogen target genes in the presence or absence of AP-2γ is required in order to claim that 'AP-2γ is essential for efficient transcription of estrogen-regulated genes'.

3. The authors should use sequential ChIP-qPCR (ChIP-re-CHIP-qPCR) to prove that AP-2γ and FoxA1 co-localize with ERBS.

Specific comments:

1. Figure 1B: The legend of Y axis should be more informative (like in 1D and 1F).

2. Figure 2D: the authors use the RET-associated ERBS, subcloned into luciferase reporter constructs, in transient transfection assays to prove that AP-2γ works together with ERα to induce maximal transcriptional activation. These luciferase expression data are consistent with the ERα binding but not the AP-2γ binding on the ERBS. This assay may be indicative for transcriptional mechanisms however, does not always reflect real mechanisms for endogenous proteins/genes.

3. Fig. 4B: the venn diagram show that a significant fraction (23%) of AP-2γ binding sites are

gained upon E2 stimulation. However, the authors do not comment on these sites. The authors should modify their statement that 'AP-2γ binds to ERBS in a ligand independent manner' and 'AP-2γ binding to chromatin in the breast cancer genome is independent of estrogen signaling'.

4. Figure 5: The authors compare the ERα, AP-2γ and FoxA1 global profiles obtained with different approaches (ChIA-PET for ERα and ChIP-seq for AP-2γ and FoxA1) and this may affect their conclusions. In addition, it is not clear what sites they compare in 5A, the ones identified in the presence of EtOH or E2?

5. Figure 7C: it should be explained in the figure legend that A and F indicate AP-2γ and FoxA1, respectively

6. In the description of the results, authors should refer to the precise parts of supplementary figures. For example: 'Supplementary Fig. 1a' instead of 'Supplementary Fig. 1' (page 6, line 7).

7. Supplementary Fig. 2: the screenshot part should be 'A' and not 'a' (to be consistent with the rest)

8. Supplementary Table S1: the authors should explain what they mean with '** after resampling' for the mapping tags of FoxA1/EtOH

9. The discussion starts with a rather long re-cap of the findings. This should be condensed.

Referee #2 (Remarks to the Author):

Tan et al. show that ER binding sites (ERSBs) are enriched for AP-2 motifs. They show for one locus (RET) that its expression is critically dependent on the presence of ERa and AP-2. Furthermore they show a change in the 3D conformation of the locus upon estradiol stimulation, a change that is not found in ERa and AP-2gamma knock-down cell lines. By doing ChIP-Seq for AP-2gamma and FoxA1 they show that these factors overlap with ERa binding sites. Importantly the sites where all three factors come together (ERa, FoxA1 and AP-2g) are sites that show 3D chromatin interactions.

This is an interesting paper, but there are a number of points that need to be addressed.

The group recently published a novel strategy, called ChIA-PET, for the simultaneous genome-wide assessment of protein binding sites and chromatin loops (Fullwood et al., Nature 2009). It was advertised as being superior to existing strategies like ChIPseq and genome-wide 3C methods, as a single assay simultaneously uncovers a protein's binding profile and the chromatin loops formed. After reading the title that claims insight into the 'interactome' I was expecting more genome-wide conformation analyses. I was therefore surprised to learn that the investigators used ChIPseq and returned to 3C technology to study loops a single gene locus. Given the general claims they want to make here, and their previous claim that ChIA-PET is ChIPseq-plus, why did the authors not perform ChIA-PET? AP-2gamma seems to be the ultimate relevant protein for ChIA-PET, as, unlike ERa, it appears chromatin bound prior to E2 stimulation, therefore uniquely allowing the assessment of de novo formed chromatin loops upon E2 stimulation. If their data are correct, the prediction would be that after stimulation AP-2gamma ChIA-PET would give similar results as ER ChIA-PET, with de novo loops formed exclusively at those AP-2gamma sites that also attract ERa.

It should not be difficult for the authors to perform ChIA-PET on AP2-gamma before and after E2 stimulation (they may even have done this already?). The data need to be shown to justify the general claims on the genome-wide role of AP2-gamma in chromatin loop formation. Moreover, a demonstration that ChIA-PET studies on AP-2gamma support and extend the previous ER ChIA-PET findings (Fullwood et al., 2009) will be important for the many groups that are now setting up this technique in their own laboratories.

Other concerns:

1. Without any knowledge of the background distribution the knowledge that 39% percent of ERBSs contain an AP-2 motif is meaningless. The authors should perform a statistical analysis by selecting

similar sized sites with equal CG content from the genome and determine the AP-2 motif occurrence.

2. Figure 2. The authors state that "AP-2gamma was enriched at all six binding sites", pertaining to ERBSs. Without a proper control locus where AP-2gamma does not bind this cannot be assessed truly.

3. In figure 3 the authors present highly reproducible 3C results. However, if I compare 3D with 3E there seems to be a strong difference in interactions between siCtrl + EtOH between the experiments. How do the authors explain this difference given that their error bars represent data from independent experiments? All identical 3C experiments need to be combined to have consistent values and error bars in the different figures.

4. The authors show overlap between the ChIP-Seq profiles (Fig. 4B, 4E, 5A). The authors should perform a thresholding analysis to determine to what extent their results are influenced by the chosen thresholds.

5. This is particularly relevant since the authors talk about high confidence ERBS, which apparently are a subset of the reported 14,468 ERBS identified with ChIA-PET. The authors should explain in the result section or introduction how these sites were selected.

6. Authors, please explain in the main text why only 16.79% of the ERBS high confidence binding sites contain the ERE motif (Suppl. Fig. 1A)? Please provide the same table for all 14,468 binding sites and discuss this percentage.

7. It would be interesting to know if AP-2gamma and FoxA1 directly interact. The authors should do a co-IP of these proteins to assess this.

8. A screenshot of the ChIP-Seq/ChIA-PET data should be added to the main figures.

9. At the first mention of E2 they should mention this is a estradiol analog.

10. "Since the above results show the binding profile of AP-2gamma...is similar to that of FoxA1" This sentence is very unclear. I take it the authors mean that AP-2gamma is also a pioneer factor. If this is the case the authors should make this explicit, preferably not at the end of the paragraph.

11. The authors should provide more technical details. For example, for each relevant figure panel it should be clear how long the E2 treatment was.

12. Other necessary controls seem missing. For example, the cyclohexamide experiment (Fig. 1F) is meaningless without a control showing that translation was blocked. I also failed to find a description of the CHX experiment; details should be provided.

Referee #3 (Remarks to the Author):

Tan et al. present an interesting study in which they identify AP-2 gamma as a novel pioneering factor for ER alpha using bioinformatics analysis of ERalpha ChIA-PET data. They show that it is essential for ER alpha and FOXA1 binding, E2-dependent long-range chromatin interactions, and regulation of RET and GREB1 gene expression upon E2 treatment. Using ChIP-PET approach, they show that AP-2gamma sites overlap with FOXA1 and these sites associate with long-range chromatin interactions.
In terms of bioinformatics, it is a very strong study. Also the experiments appear to be well done. However, to make this a more complete study with more depth and completeness, the authors should (1) show more genes that are affected by this factor- a microarray study where they knock-down AP-2 gamma (and FOXA1 in parallel) in MCF-7 cells and compare E2-mediated gene transcription relative to control cells would be ideal- and (2) they should do some additional interaction studies (CoIP, ChIP-reChIP) for AP-2gamma and ERalpha (and FOXA1).

1st Revision - authors' response 14 February 2011

We thank all the reviewers for their comments and suggestions. In this revised manuscript, we have included experiments as requested by the reviewers and provide additional data and explanation that specifically address their major concerns.

Referee #1 (Remarks to the Author):

Major points:

1. Most of the AP-2γ; ChIP-qPCR experiments show very low recoveries (less than 0.2%).

We agree with the reviewer that most of the AP-2γ ChIP-qPCR results show low recoveries. We think this is most likely due to the antibody recognizing AP-2γ relatively weakly, however, we believe the ChIP results show the binding of AP-2γ at most ERBS are significantly and reproducibly enriched compared to control regions (we have now included a non-specific genomic region as a control for our all ChIP assays as requested by Reviewer #2). From our AP-2γ ChIP-seq data, we have validated other AP-2γ binding sites that are stronger (more than 0.3% input). Furthermore, when we performed motif occurrence in this data set we found that more than 80% of the AP2GBS contain AP-2 motifs. These observations in combination with our other bioinformatic data in the manuscript suggest that although the antibody is relatively weak it is specific for AP-2γ.

2. All data for the role of AP-2γ; on the transcription of estrogen target genes are from two targets (GREB1 and RET; Figure 1 and data not shown). A global analysis of estrogen target genes in the presence or absence of AP-2γ; is required in order to claim that ' AP-2γ; is essential for efficient transcription of estrogen-regulated genes'.

We agree with the reviewer and have now included a genome-wide profile of MCF-7 cells transfected with and without AP-2γ siRNA, in the presence and absence of E2 (Fig. 1D). From our microarray analysis, we have identified 676 E2-responsive genes with a cut-off of 1.5 fold change when comparing siCtrl ETOH vs. siCtrl E2. We observed that almost equal proportion of E2-regulated genes are activated or repressed. Out of 349 E2-upregulated genes, approximately 60% are dependent on AP-2γ for their activation. On the other hand, out of 327 E2-downregulated genes, about 30% were dependent on AP-2γ for their repression. Hence, our data suggest that AP-2γ plays a major role in regulating estrogen-dependent genes by functioning as a transcriptional activator and repressor.

3. The authors should use sequential ChIP-qPCR (ChIP-re-CHIP-qPCR) to prove that AP-2γ; and FoxA1 co-localize with ERBS.

We agree with the reviewer and we have now included ChIP-re-ChIP-qPCR experiments. Our results (Supplementary Fig. 10) indicate that FoxA1 and AP-2γ are indeed co-localized together with ERα on chromatin after E2 induction.

Specific comments:

1. Figure 1B: The legend of Y axis should be more informative (like in 1D and 1F).

We have changed the y-axis of Figure 1B. The y-axis now describes the fold change of E2 versus the ETOH condition for both RET 9 and RET 51 gene expression at different time points.

2. Figure 2D: the authors use the RET-associated ERBS, subcloned into luciferase reporter constructs, in transient transfection assays to prove that AP-2γ works together with ERα to induce maximal transcriptional activation. These luciferase expression data are consistent with the ERα binding but not the AP-2γ binding on the ERBS. This assay may be indicative for transcriptional mechanisms however, does not always reflect real mechanisms for endogenous proteins/genes.

This is a valid point. We agree with the reviewer on the limitations of this assay and have now included a statement in the main text of the manuscript to address this point.

3. Fig. 4B: the venn diagram show that a significant fraction (23%) of AP-2γ; binding sites are gained upon E2 stimulation. However, the authors do not comment on these sites. The authors should modify their statement that ' AP-2γ binds to ERBS in a ligand independent manner' and ' AP-2γ binding to chromatin in the breast cancer genome is independent of estrogen signaling'.

We agree with the reviewer and have now included comments on these E2 specific sites when we described the AP-2γ ChIP-seq data in the main text of the manuscript. We have also modified the statements, ' AP-2γ binds to ERBS in a ligand independent manner'to'AP-2γ binds to ERBS associated with RET in a ligand independent manner', and ' AP-2γ binding to chromatin in the breast cancer genome is independent of estrogen signaling' to ' Majority of AP-2γ binding to chromatin in the breast cancer genome is independent of estrogen signaling'.

4. Figure 5: The authors compare the ERα, AP-2γ and FoxA1 global profiles obtained with different approaches (ChIA-PET for ERα and ChIP-seq for AP-2γ and FoxA1) and this may affect their conclusions. In addition, it is not clear what sites they compare in 5A, the ones identified in the presence of EtOH or E2?

This is a valid point. Recently, we have published a ChIP-seq of ERα in MCF-7 cells under the same E2 condition as the ERα ChIA-PET (Joseph et al, 2010). We found that over 60% (8,862 out of 14,468) of the ERBS from the ERα ChIA-PET can be found in the ERα ChIP-seq data (15,306 binding site). When we overlapped the ERα ChIP-seq peaks with AP-2γ and FoxA1 ChIP-seq peaks, we found the three factors were co-localized at 4,406 genomic sites (compared with 5,166 ERBS from the ERα ChIA-PET). More importantly, 87 % (3,844 out of 4,406) of the triple overlapped sites from the ERα ChIP-seq were also found in the triple overlap from ERα ChIA-PET, suggesting that even though the ERBS obtained from the two different techniques differ, their overlap with the AP-2γ and FoxA1 were very similar and therefore does not alter our general conclusion that ERα, FoxA1, and AP-2γ share many common binding regions across the genome. Binding sites from E2 conditions were used for the triple comparison and this has been clarified in the Figures and Figure legends.

5. Figure 7C: it should be explained in the figure legend that A and F indicate AP-2γ; and FoxA1, respectively.

We have changed the legend as requested.

6. In the description of the results, authors should refer to the precise parts of supplementary figures. For example: 'Supplementary Fig. 1a' instead of 'Supplementary Fig. 1' (page 6, line 7).

We have made the changes to the rest of the text as requested.

7. Supplementary Fig. 2: the screenshot part should be 'A' and not 'a' (to be consistent with the rest)

We have changed the labeling. Please note, Supplementary Fig. 2 is now Supplementary Fig. 3.

*8. Supplementary Table S1: the authors should explain what they mean with '** after resampling' for the mapping tags of FoxA1/EtOH*

We have provided the description for resampling of the CHM046 library in the footnote of Supplementary Table S1.

9. The discussion starts with a rather long re-cap of the findings. This should be condensed.

We have summarized our findings in a significantly shorter paragraph (see first paragraph of discussion).

Referee #2 (Remarks to the Author):

Major point:

The reviewer suggests that we perform AP-2γ ChIA-PETs before and after E2 stimulation.

We agree with the reviewer that AP-2γ is a good candidate for ChIA-PET analysis and the results obtained from this analysis would greatly improve the manuscript and extend our previous ERα ChIA-PET findings. We have attempted to generate ChIA-PETs for AP-2γ, but due to technical difficulties we have been unable to obtain these results. This is because the success of the ChIA-PET experiment largely depends on the antibody used and we think in contrast to the ERα antibody used in our ERα ChIA-PET, the antibody that we are currently using for AP-2γ is not strong and specific enough for ChIA-PET analysis. As noted by reviewer #1, the ChIP recovery for AP-2γ is on the low side. Compared to the ERα antibody (using % input at the same binding site as a rough gauge of strength), we think the AP-2γ antibody is up to 15-20x weaker in recovering protein bound DNA. We are continually exploring other ideas to improve this including the addition of protein-protein crosslinkers and also generating new AP-2γ antibodies.

Specific comments:

1. Without any knowledge of the background distribution the knowledge that 39% percent of ERBSs contain an AP-2 motif is meaningless. The authors should perform a statistical analysis by selecting similar sized sites with equal CG content from the genome and determine the AP-2 motif occurrence.

This is a valid point. We agree with the reviewer and we have now included this analysis with detailed description in Supplementary Fig. 2. Our results showed an expected AP-2 motif occurrence of 25% in the genome, which is 15% lesser than the observed AP-2 motif occurrence (40%) in ChIA-PET ERBS. The probability that the expected motif occurrence will be the same or more than the observed occurrence is very low (*p*-value of 1E-1039), implying that the higher occurrence of AP-2 motif in ChIA-PET ERBS is significant.

2. Figure 2. The authors state that "AP-2gamma was enriched at all six binding sites", pertaining to ERBSs. Without a proper control locus where AP-2gamma does not bind this cannot be assessed truly.

We agree and we have now included a control locus in Figure 2 and in other ChIP-qPCR experiments.

3. In figure 3 the authors present highly reproducible 3C results. However, if I compare 3D with 3E there seems to be a strong difference in interactions between siCtrl + EtOH between the experiments. How do the authors explain this difference given that their error bars represent data from independent experiments? All identical 3C experiments need to be combined to have consistent values and error bars in the different figures.

We would like to note that the strong difference observed for the siCtrls between Figure 3D and 3E is because the knockdown-3C experiments for ERα and AP-2γ were performed independently and at different times. Specifically, we performed all the ERα knockdown experiments first and then subsequently the AP2-γ knockdown experiments. Because of this, we represented the experiments independently as depicted in Figure 3D and 3E. We apologize for the confusion caused.

4. The authors show overlap between the ChIP-Seq profiles (Fig. 4B, 4E, 5A). The authors should perform a thresholding analysis to determine to what extent their results are influenced by the chosen thresholds.

This is a good point. The overlaps shown in Figures 4B, 4E, and 5A were generated using AP-2γ and FoxA1 peaks called using a very stringent FDR cut-off of 0.005. We have now performed similar analyses using AP-2γ and FoxA1 peaks called using more stringent and less stringent FDR cut-offs ranging from 0.002 to 0.05. Overall, our results show that the extent of overlap between the three factors does not change significantly regardless which FDR was used for peak calling (Supplementary Figs. 7, 8 and 9).

5. This is particularly relevant since the authors talk about high confidence ERBS, which apparently are a subset of the reported 14,468 ERBS identified with ChIA-PET. The authors should explain in the result section or introduction how these sites were selected.

We have now included in the introduction to say that high confidence ERBS from the ERα ChIA-PET are binding sites that contain at least 50 or more self-ligation PET counts per cluster.

6. Authors, please explain in the main text why only 16.79% of the ERBS high confidence binding sites contain the ERE motif (Suppl. Fig. 1A)? Please provide the same table for all 14,468 binding sites and discuss this percentage.

The 'score' in the table does not reflect the actual motif occurrence in the ChIA-PET ERBS, but is actually an attempt to achieve a rough estimate of the number of positive predictions using motif scanning. Specifically, the entire genome was first scanned with the PWM of motifs from Transfac and the locations of top-scoring 300,000 motif hits or predictions were recorded (such that the expected occurrence of hits is 1 in 10,000 bp). Next, we intersected the 2,513 ERBS (high confidence ChIA-PET ERBS) or 14, 468 ERBS (all ChIA-PET ERBS) with the motif hits to obtain the histogram of the hits occurrence around the ERBS within -/+ 5 kb window. In order to ensure the validity of motif enrichment, we attempt to account for the false positive motifs by subtracting the number of motif hits around the ERBS (approximately -/+250 bp) by the expected false positive motifs estimated in the flanking background. This in turn gives us the number of true predictions and we represented it as a 'score' as shown in Supplementary Fig. 1A. Under the assumption that each binding site is associated with at most one true prediction, the 'score' may be interpreted as the number of binding sites containing one "true prediction". We have included the details of this analysis in the Materials and Methods section to describe the meaning of 'score'. We apologize to the reviewer for any confusion caused. We have also included analysis for both high confidence and all ERα binding sites from the ERα ChIA-PET. Our results show that AP-2 motifs are highly enriched in both datasets.

7. It would be interesting to know if AP-2gamma and FoxA1 directly interact. The authors should do a co-IP of these proteins to assess this.

We agree with the reviewer. We have attempted the co-IP experiment to check if AP-2γ interact with FoxA1. Unfortunately, we could not detect physical interaction between these two proteins, which may suggests that the interaction may be very weak in solution, most of the proteins are bound to the chromatin fraction, or the antibodies we used for the pull-downs are not good enough for this assay. However, we have performed sequential ChIP/ChIP-reChIP assay (Supplementary Fig. 10) to show the two factors are bound onto the same stretch of chromatin at the same time. Taken together, our results suggest that AP-2γ and FoxA1 may influence each other by being in close proximity on chromatin rather than in direct physical contact in solution.

8. A screenshot of the ChIP-Seq/ChIA-PET data should be added to the main figures.

We have now included the screenshot of ChIA-PET and ChIP-seq of AP-2γ and FoxA1 at the RET gene locus as Fig. 5A.

9. At the first mention of E2 they should mention this is an estradiol analog.

We have now mentioned that E2 is 17β-estradiol in the results section.

10. "Since the above results show the binding profile of AP-2gamma...is similar to that of FoxA1" This sentence is very unclear. I take it the authors mean that AP-2gamma is also a pioneer factor. If this is the case the authors should make this explicit, preferably not at the end of the paragraph.

That is a valid point. We agree with the reviewer and have now removed the sentence and replaced it with, 'Our data therefore suggest that AP-2γ is a pioneering factor of ERα with similar characteristics as FoxA1'.

11. The authors should provide more technical details. For example, for each relevant figure panel it should be clear how long the E2 treatment was.

We have now included additional information in the figure legends.

12. Other necessary controls seem missing. For example, the cycloheximide experiment (Fig. 1F) is meaningless without a control showing that translation was blocked. I also failed to find a description of the CHX experiment; details should be provided.

We agree with the reviewer and we have now included secondary estrogen target genes such as E2F1 and CDC6, which have been reported previously to be affected by cycloheximide treatment in MCF-7 cells (Bourdeau et al, 2008) as positive control genes for the cycloheximide treatment. Details of the CHX experiment are provided in the figure legend. Please note, Fig. 1F is now Supplementary Fig. 4C.

Referee #3 (Remarks to the Author):

Tan et al. present an interesting study in which they identify AP-2 gamma as a novel pioneering factor for ER alpha using bioinformatics analysis of ERalpha ChIA-PET data. They show that it is essential for ER alpha and FOXA1 binding, E2-dependent long-range chromatin interactions, and regulation of RET and GREB1 gene expression upon E2 treatment. Using ChIP-PET approach, they show that AP-2gamma sites overlap with FOXA1 and these sites associate with long-range chromatin interactions.

In terms of bioinformatics, it is a very strong study. Also the experiments appear to be well done. However, to make this a more complete study with more depth and completeness, the authors should (1) show more genes that are affected by this factor- a microarray study where they knock-down AP-2 gamma (and FOXA1 in parallel) in MCF-7 cells and compare E2-mediated gene transcription relative to control cells would be ideal- and (2) they should do some additional interaction studies (CoIP, ChIP-reChIP) for AP-2gamma and ERalpha (and FOXA1).

This is a good suggestion from the reviewer. We have performed genome-wide profiling of MCF-7 transfected with negative control and AP-2γ siRNA, in presence and absence of E2 (See Fig. 1D and our explanation above). We could not perform parallel FoxA1 knockdown experiments because in our hands FoxA1 appears to affect ERα expression and thus any effects we see from the microarray could be an indirect effect due to changes in ERα levels and not FoxA1. We have also performed the sequential ChIP/ChIP-reChIP in three combinations: 1st ChIP ERα, 2nd ChIP AP-2γ; 1st ChIP FoxA1, 2nd ChIP AP-2γ and 1st ChIP FoxA1, 2nd ChIP ERα (Supplementary Fig. 10). Overall, our results suggest that ERα, FoxA1, and AP-2γ exist as a protein complex on chromatin after E2 stimulation.

2nd Editorial Decision 07 March 2011

Your manuscript has been reviewed once more by two of the original referees, who find that you have satisfactorily addressed their initial concerns. We would be happy to publish your manuscript in The EMBO Journal once the remaining issues are addressed.

When you send us your revision, please include a cover letter with an itemised list of all changes made, or your rebuttal, in response to comments from review.
When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website:
http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to reading the revised manuscript.

Yours sincerely,

Editor

The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

Tan et al._29253_revised
'AP-2γ is required for estrogen receptor-mediated interactome organization and gene transcription'

In the revised manuscript, the authors have responded to all our major points and specific comments by adding new experiments, providing explanations and modifying the text/figures/figure legends accordingly.

Specific comments:

- Fig. 1D (new figure): in response to our major point #2, the authors have included a genome-wide profile of MCF-7 cells transfected with and without AP-2γ siRNA and describe the effect of AP-2γ siRNA on the E2-activated genes (page 8). The authors should also present/discuss in the Results the effect of AP-2γ siRNA on the E2-repressed genes (as they do in the 'point-by-point response' to reviewers).

- The author's response to our specific comment #5 (overlap of ChIA-PET for ERα and ChIP-seq for AP-2γ and FoxA1 as compared to overlap of ChIP-seq for all three factors) should be included in the Results (as a short statement in the description of Fig. 5B, page 12).

- Page 13, 3rd paragraph, line 2: the authors should use the term 'sequential ChIP-qPCR' or 'ChIP-re-ChIP-qPCR' instead of 'sequential ChIP/ChIP re-ChIP'.

- Page 14, first paragraph, lines 4-5: '..AP-2γ and FoxA1 are both recruited to chromatin in a ligand-independent manner..'. This is true for the majority of AP-2γ and FoxA1, however, E2-dependent binding has been also observed.

- Page 14, middle of second paragraph: it is not clear why the authors refer to Supplementary fig. 6.

The revised version of the manuscript is now suitable for EMBO Journal.

Referee #2 (Remarks to the Author):

The authors correctly addressed all the issues I raised, with the unfortunate exception of the suggested ChIA-PET experiment.

They state in their rebuttal that the antibody, which works in ChIPseq, is not good enough for ChIA-PET, which may be fine. However, since they are the inventors of ChIA-PET, highly advertise this technique also in this manuscript, and work in this manuscript on a factor, AP-2gamma, that is ideally suited for CHIA-PET (since loop formation apparently does not depend on the binding of this factor per se), they should explain in the results section why ChIA-PET could not be performed. It will be relevant for the community to understand that an antibody suitable for ChIPseq may not be good enough for ChIA-PET.

Related to this: without ChIA-PET data I find the title too strong. The term 'interactome' really suggests that loops have been studied throughout the genome, which is not the case. Only a selected number of loci have been studied by 3C experiments.

Suggested title:
A role for AP-2gamma in estrogen receptor-mediated gene transcription and chromatin loop formation

Referee #1 (Remarks to the Author):

Specific comments:

1. Fig. 1D (new figure): in response to our major point #2, the authors have included a genome-wide profile of MCF-7 cells transfected with and without AP-2γ siRNA and describe the effect of AP-2γ siRNA on the E2-activated genes (page 8). The authors should also present/discuss in the results the effect of AP-2γ siRNA on the E2-repressed genes (as they do in the 'point-by-point response' to reviewers).

This is a valid point. We have now included a statement in the Results section discussing the effect of AP-2γ depletion on E2-repressed genes.

2. The author's response to our specific comment #5 (overlap of ChIA-PET for ERα and ChIP-seq for AP-2γ and FoxA1 as compared to overlap of ChIP-seq for all three factors) should be included in the Results (as a short statement in the description of Fig. 5B, page 12).

We have now included the data in Supplementary Fig. 10 and added a description of the analysis in the Results section.

3. Page 13, 3rd paragraph, line 2: the authors should use the term 'sequential ChIP-qPCR' or 'ChIP-re-ChIP-qPCR' instead of 'sequential ChIP/ChIP re-ChIP'.

We have changed the term 'sequential ChIP/ChIP re-ChIP' to 'sequential ChIP-qPCR' as requested.

4. Page 14, first paragraph, lines 4-5: '.. AP-2γ and FoxA1 are both recruited to chromatin in a ligand-independent manner..'. This is true for the majority of AP-2γ and FoxA1, however, E2-dependent binding has been also observed.

We agree with the reviewer. We have now modified the statement to, 'AP-2γ and FoxA1 recruitment to chromatin are mostly independent of ligand stimulation.'

5. Page 14, middle of second paragraph: it is not clear why the authors refer to Supplementary fig. 6.

We apologize for the confusion. We referred to Supplementary Fig. 6 to indicate to the readers that FoxA1 protein expression remains unaffected by AP-2γ depletion. Therefore, it is not the change in protein expression that has caused the decrease in FoxA1 recruitment after AP-2γ knockdown. We have modified the sentence to indicate this.

Referee #2 (Remarks to the Author):

1. The reviewer suggests that we should explain in the results section why AP-2γ ChIA-PET could not be performed.

We agree, however, we believe this would be more suited in the Discussion section of the manuscript. In the Discussion section, we have now included an explanation on the technical issues of generating AP-2γ ChIA-PET.

2. Without the ChIA-PET data, the reviewer suggests our current title is too strong and should be changed.

We agree. We have now changed the title of our manuscript to 'AP-2γ regulates estrogen receptor-mediated long-range chromatin interaction and gene transcription.'

3rd Editorial Decision 15 April 2011

I have looked through your revised manuscript and find that you have satisfactorily addressed all the remaining minor issues. I am happy to accept the study for publication in THe EMBO Journal. You will receive the official acceptance letter in the next day or so.

Yours sincerely,

Editor
The EMBO Journal

