Reconstruction of nuclear receptor network reveals that NR2E3 is a novel upstream regulator of ESR1 in breast cancer

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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 21 July 2011

Thank you for the submission of your manuscript to EMBO Molecular Medicine. We have now heard back from the two referees whom we asked to evaluate your manuscript. You will find their comments below. While the referees find the study of potential interest, some important concerns are raised and need to be convincingly addressed in a major revision.

As you will see below, the raised issues regard the experimental aspect of your work. In particular referee #1 suggests some additional but necessary experiments to strengthen the overall message of the study. We also would appreciate, as suggested by the referees, some re-writing to re-focus the attention of the manuscript to the biological and medical aspects of the data.

Should you fully address these criticisms within the time constraints outlined below, we would be happy to consider a revised version of your manuscript. Please note that it is EMBO Molecular Medicine policy to allow only a single round of revision and that, as acceptance or rejection of the manuscript will depend on another round of review, your responses should be as complete as possible.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions, except under exceptional circumstances in which a short extension is obtained from the editor.

I look forward to seeing a revised form of your manuscript as soon as possible.
Yours sincerely,

Editor
EMBO Molecular Medicine

REFEREE REPORTS:

Referee #1:

The current study by Park et al. demonstrates a novel role for the orphan nuclear receptor NR2E3 in the regulation of ESR1 expression and a potential role for NR2E3 as a predictive marker for recurrence free survival of patients with ER-positive breast cancer. The authors identify a correlation between NR2E3 and ESR1 expression profiles and their patterns of target gene expression using a systems-level analysis of publicly available gene expression datasets from NCI-60 cell lines. This correlation was additionally identified from two distinct cohorts of breast cancer patients (NKI and UNC). The authors further investigate this potential link between ESR1 and NR2E3 with functional studies. NR2E3 silencing by shRNA results in a reduction in ESR1 expression at both the mRNA and protein levels, demonstrating a role for NR2E3 in the regulation of ESR1 expression. The authors further show that NR2E3 silencing results in decreased expression of target genes known to be positively regulated by the estrogen receptor. The reciprocal knockdown of ESR1 by siRNA had no effect on NR2E3 mRNA levels, suggesting that ESR1 does not utilize NR2E3 expression to feedback-regulate its own expression. Chromatin immunoprecipitation experiments of NR2E3 clearly show the association of NR2E3 with the ESR1 promoter, further supporting a role for NR2E3 in the transcriptional regulation of the estrogen receptor. The NR2E3 coregulator and E3 SUMO ligase, PIAS3, was identified within the same region of the ESR1 promoter as NR2E3 by chromatin immunoprecipitation. Furthermore, siRNA knockdown of PIAS3 abolishes binding of NR2E3 in the ESR1 promoter, suggesting a possible role for PIAS3 in NR2E3 regulation of ESR1 expression.

Utilizing published data from the NKI and UNC breast cancer cohorts, the authors demonstrate a correlation between high NR2E3 expression and recurrence free survival (RFS). Importantly, NR2E3 expression levels consistently predict the likelihood for RFS in ER-positive patients (IJB cohort), ER-positive patients after tamoxifen treatment (subset of IJB cohort), and ER-positive patients that have lymph node negative breast cancer (subset of IJB cohort) - groups for which ESR1 expression level has no predictive value.

This study provides insight into the poorly understood mechanism for the transcriptional regulation of the estrogen receptor. NR2E3 has been identified as a novel regulator of estrogen receptor expression at the transcriptional level. Furthermore, this study shows that NR2E3 expression levels may have predictive value for RFS within groups of breast cancer patients for which there is currently no known predictive marker.

Major Comments:

1. The authors show by siRNA knockdown of ESR1 that NR2E3 protein levels remain unchanged, however they did not look at the effect of ESR1 knockdown on PIAS3 protein expression. If PIAS3 is critical for NR2E3 regulation of the estrogen receptor, then ESR1 could also feedback regulate through PIAS3. It would also be helpful to show protein levels of a known downstream target of ESR1 as a control.

2. The knockdown of the SRCs is a nice control showing that not all coregulators involved in ESR1 target gene expression also participate in transcriptional regulation of the estrogen receptor. While the levels of SRC-1 and SRC-2 knockdown look good, the ~25% knockdown shown for SRC-3 is not impressive (Figure 5A). To conclude anything about SRC-3, the authors must show that SRC-3 protein levels are significantly down under these conditions or to improve the knockdown at the mRNA level. This is especially important considering the fact that there is a significant effect of SRC-3 knockdown on PIAS3 expression, which is not known to be regulated by SRC-3.

3. It is interesting that the PIAS3 E3 SUMO ligase may play a role in transcriptional regulation of
ESR1 expression. While it is not clear from the methods and referenced paper (Iwasaki et al., 2007), it appears that only one siRNA was utilized for PIAS3 knockdown and at a final concentration of 1 M. Because off-target effects increase with the concentration of siRNA, if the 1 M siRNA concentration was utilized, then the knockdown should be repeated using a lower siRNA concentration (preferably less than 100 nM). Furthermore, the knockdown effects should be confirmed independently with a second siRNA.

4. The NR2E3/PIAS3 co-immunoprecipitation shows a weak interaction between the two proteins. The evidence for this interaction would be strengthened if the co-immunoprecipitation experiment was accompanied by a repeat using purified, recombinant NR2E3 and PIAS3.

5. Considering the results, it would be important to know if the E3 SUMO ligase activity of PIAS3 is involved in the transcriptional regulation of the estrogen receptor. The knockdown of the E2 SUMO ligase Ubc9 in the system utilized in Figure 4E could provide insight into this question. If Ubc9 knockdown abolishes the effect of PIAS3 overexpression on luciferase activity, this would suggest that PIAS3 SUMOylation activity is important for transcriptional regulation of ESR1.

6. The authors show a correlation between NR2E3 and recurrence free survival in patients with ER positive breast cancer. Since the authors suggest an important role for PIAS3 in the coregulation of ESR1 transcriptional activity through NR2E3, it would be interesting to see if there is also a correlation between PIAS3 expression levels and ER-positive breast cancer. The authors could investigate such a correlation from the IJB cohort.

Minor Comments:

1. In certain portions of the manuscript the sentence structure is cumbersome, making the manuscript difficult to follow. The results section in particular has portions (including the description of Figure 4 in the text) that should be re-written for clarity.

2. There are minor errors throughout the manuscript that should be corrected. There should be a space between a word and parentheses. There is a period after al in et al. There are many cases of the improper use or absence of articles (a, an, the). Improper usage of singular/plural nouns should be corrected. In the methods section, the UNC cohort data are from the public (with an "l") Merck website. "In trans" should be italicized throughout the manuscript and figure legends.

3. The descriptions of the methods used in this study need to be stated more explicitly. The siRNAs that were utilized as well as what concentration of siRNA was used for knockdown experiments should be clearly stated in the manuscript or be easy to understand from a referenced protocol. Antibodies used for PIAS3 should be specified for ChIP and co-immunoprecipitation experiments. It is unclear whether cells were in phenol red free medium as well as charcoal stripped serum for stimulation with E2. The authors include a description of microarray in the materials and methods, however it is unclear from the text when the authors carried out a microarray themselves. It is presented in the manuscript that all gene expression data were obtained from publicly available datasets. Please clarify this whole area of the manuscript.

4. The introduction focuses on the value of genome-wide analysis and utilizing publicly available gene expression datasets to make new connections between cellular factors. While these points are relevant to the current study, it would also be helpful for more time to be spent introducing ESR1 and NR2E3 which are the focus of the manuscript. Minimally, NR2E3 should be introduced as an orphan nuclear receptor and not simply identified as a novel regulator of ESR1 expression. Furthermore, PIAS3 should be introduced in the introduction since the authors propose a critical role for PIAS3 in NR2E3 regulation of ESR1 expression.

5. In the last paragraph of the introduction the authors state that there is a novel interaction between NR2E3 and ESR1, which suggests a physical interaction. Since this is not the demonstrated relationship between NR2E3 and ESR1, it would be helpful for this statement to re-stated so that it more accurately describes the findings of the study.

6. Defining the terms "in trans significance" and "in trans correlation" would contribute to the clarity of the manuscript.
7. The lack of effect of NR2E3 silencing on FOXA1 expression in MCF7 cells (Figure 3B) should be discussed. Since NR2E3 silencing decreases ESR1 expression at both mRNA and protein levels, it seems that all downstream targets of the estrogen receptor would be affected. Consistent with this, FOXA1 expression is reduced due to NR2E3 silencing in T47D cells (Figure 3D).

8. ChIP data show that both NR2E3 and PIAS3 are bound within the same region of the ESR1 promoter, which supports the authors hypothesis that both proteins are involved in ESR1 transcriptional regulation. However, ChIP only indicates that a protein is present within a certain region on a large piece (usually ~500-800 bp) of chromatin, therefore it cannot provide evidence for the direct interaction of two proteins on DNA. While NR2E3 and PIAS3 may interact in solution, there is no direct evidence in this manuscript for an interaction between these proteins on DNA. Either show it, or change the statement.

9. Figure 1: the y-axis label (ITS) is an acronym that is not defined anywhere in the manuscript. While it probably stands for "in trans significance," this should be clarified.

10. Figure 4A:
1. Need to specify both 5' and 3' boundaries of the promoter region used in the luciferase construct. A map of these different ESR1 promoter regions might be helpful.

2. A description of the experiment in Figure 4A is necessary to understand the results. While the experiment looks as though it involves the overexpression of NR2E3, this should be explained in the results section and figure legend.

3. The authors should indicate significant changes in luciferase activity between the different promoter constructs. By eye it is unclear whether there are significant differences between the different constructs. This information is important to evaluate possible differences in NR2E3 binding between the different ESR1 promoter constructs.

11. Figure 8: The differences between the different log rank tests shown in C should be described. While they are all significant, it is not clear what the different tests mean.

Referee #2 (Comments on Novelty/Model System):

The authors have used bioinformatic analyses to show that the expression of the nuclear receptor NR2E3, previously largely associated with a function in photoreceptor cells, is associated with estrogen receptor (ESR1) expression in breast cancer cells. Usual techniques are used to demonstrate that NR2E3 regulates ESR1 expression in breast cancer cells and further bioinformatic as well as microarray and RPPA analyses are used to provide in vivo confirmation. These analyses are of high quality. Data for RPPA analysis using breast cancer tissue are provided, but no statement is made regarding ethics approval, which I assume exists as this patient cohort has been previously used. Clarification may be required.

Referee #2 (Other Remarks):

The estrogen receptor (ESR1) is expressed in the majority of breast cancers. It functions as a transcription factor, acting by regulating the expression of genes that promote breast cancer growth. As such ESR1 is a key therapeutic target in breast cancer, hormonal therapies working by inhibiting its function. Understanding the mechanisms of ESR1 expression, as well as the genes it regulates is key to understanding its role in breast cancer. ESR1 is a member of the nuclear receptor (NR) superfamily of transcription factors that play important roles in development and human disease including cancer. The authors have used bioinformatics approaches to analyse publicly available gene expression datasets to show that another member of the NR superfamily, NR2E3 (aka photoreceptor cell-specific NR (PNR)) is associated with gene expression profiles highly similar to those of ESR1 in a panel of cancer cell lines (NCI60 panel) and in breast cancer gene expression profiles. The subsequent work is a straightforward, but well-controlled demonstration that NR2E3 can regulate ESR1 expression in breast cancer cell lines and highlights the involvement of the PIAS3 transcriptional coregulator in the regulation of ESR1 gene expression by NR2E3. This is
followed up by standard analysis of public datasets to show that for some of the breast cancer microarray expression datasets high level NR2E3 expression is associated with better relapse-free survival, as also observed for ESR1, a finding that the authors confirm at the protein level in a further patient cohort. The study also reports on gene expression microarray analysis for MCF-7 breast cancer cells following shRNA for NR2E3 and described NR2E3 gene signatures whose expression profiles are predictive for patient relapse.

This is a well-written paper, describing interesting and novel findings. The work is well controlled and although the in vivo confirmation is largely based on public microarray datasets, some confirmation has been provided by RPPA analysis of a distinct tumor set. There are just a few specific points, detailed below:

1. Abstract: Whilst ESR1 is expressed in the majority of breast tumors and inhibition of its action is the basis for adjuvant hormonal therapies, I am not aware that ESR1 is an "oncogene" according to the definition of the term. This should be removed.
2. Introduction: The authors refer to FoxA1 as a "downstream partner" to ESR1 in breast cancer cells. As a factor that regulates ESR1 binding to gene promoters, is this really the correct description for the action of FoxA1 in breast cancer?
3. P11: As a regulator of ESR1 expression, the association of NR2E3 with longer RFS is intriguing in datasets in which ESR1 expression is not associated with RFS. This may indicate a role for NR2E3 which is independent of its role in regulating ESR1, as is proposed by the authors elsewhere. Does it therefore have a role in ESR1 negative breast cancer? Is this evident from analysis of gene expression profile datasets? For the genes identified in the microarray analysis for NR2E3 shRNA, are these genes predictive of RFS or OS in ESR1 negative breast cancer or only in ESR1 positive breast cancer?
4. Figure 7: Were ESR1 and NR2E3 proteins independently associated with RFS and OS in the MDACC RPPA analysis?
5. Figure 8: It would be helpful if compound covariate predictor (CCP), INN, 3NN, etc are more clearly defined. Genes associated with these groups should be described in the supplementary information.

1st Revision - Authors' Response
13 September 2011

Referee #1:

We are very pleased with the positive comments of this reviewer. All of the suggestions are addressed and incorporated in the revised manuscript.

Major Comments:

1. The authors show by siRNA knockdown of ESR1 that NR2E3 protein levels remain unchanged, however they did not look at the effect of ESR1 knockdown on PIAS3 protein expression. If PIAS3 is critical for NR2E3 regulation of the estrogen receptor, then ESR1 could also feedback regulate through PIAS3. It would also be helpful to show protein levels of a known downstream target of ESR1 as a control.

Response: As reviewer suggested, we knocked down ESR1 in MCF-7 cells and measured PIAS3 protein level. As shown in Figure 5 E, PIAS3 protein level was not changed; suggesting that ESR1 does not regulate PIAS3 via feedback loop while Cyclin D1 expression was decreased as a positive control.

2. The knockdown of the SRCs is a nice control showing that not all coregulators involved in ESR1 target gene expression also participate in transcriptional regulation of the estrogen receptor. While the levels of SRC-1 and SRC-2 knockdown look good, the ~25% knockdown shown for SRC-3 is not impressive (Figure 5A). To conclude anything about SRC-3, the authors must show that SRC-3
protein levels are significantly down under these conditions or to improve the knockdown at the mRNA level. This is especially important considering the fact that there is a significant effect of SRC-3 knockdown on PIAS3 expression, which is not known to be regulated by SRC-3.

Response: We agree with the reviewer. Using SMART Pool siRNA from Dharmacon, we knocked down NCOA (SRC) 1, 2, and 3. As shown in Figure 5A and B, expression of NCOA1, 2, and 3 was significantly reduced by indicated siRNAs. While ESR1 expression was not changed by siNCOA, ESR1 downstream gene expression level was changed. In addition, PIAS3 mRNA expression level was slightly changed by siNCOA2 and 3.

3. It is interesting that the PIAS3 E3 SUMO ligase may play a role in transcriptional regulation of ESR1 expression. While it is not clear from the methods and referenced paper (Iwasaki et al., 2007), it appears that only one siRNA was utilized for PIAS3 knockdown and at a final concentration of 1 µM. Because off-target effects increase with the concentration of siRNA, if the 1 µM siRNA concentration was utilized, then the knockdown should be repeated using a lower siRNA concentration (preferably less than 100 nM). Furthermore, the knockdown effects should be confirmed independently with a second siRNA.

Response: When we performed the siRNA experiments, we used 20nM concentration of siRNA. As the reviewer suggested, we used another siRNA targeting PIAS3 from Dharmacon SMART Pool. As shown in Supplementary Fig. S4 when we silenced siPIAS by SMART Pool, ESR1 and ESR1 downstream genes expression level was decreased. These results are similar with the result of Figure 5C.

4. The NR2E3/PIAS3 co-immunoprecipitation shows a weak interaction between the two proteins. The evidence for this interaction would be strengthened if the co-immunoprecipitation experiment were accompanied by a repeat using purified, recombinant NR2E3 and PIAS3.

Response: As suggested, we carried out additional interaction experiments with purified recombinant proteins. We purified GST-NR2E3 and His-Tag PIAS3 proteins from E. coli and tested direct interaction with NR2E3 and PIAS3 with recombinant proteins. As shown in Figure 5H, NR2E3 directly interacts with PIAS3 in vitro.

5. Considering the results, it would be important to know if the E3 SUMO ligase activity of PIAS3 is involved in the transcriptional regulation of the estrogen receptor. The knockdown of the E2 SUMO ligase Ubc9 in the system utilized in Figure 4E could provide insight into this question. If Ubc9 knockdown abolishes the effect of PIAS3 overexpression on luciferase activity, this would suggest that PIAS3 SUMOylation activity is important for transcriptional regulation of ESR1.

Response: As suggested, we carried out new experiments with silencing expression of UBC9. When the expression of UBC9 was silenced by specific siRNA, overall transcription activity was not changed as shown in a new reporter assay (Figure 5F). Thus, we concluded that E3 ligase activity of PIAS3 is not necessary for regulation of ESR1 expression.

6. The authors show a correlation between NR2E3 and recurrence free survival in patients with ER positive breast cancer. Since the authors suggest an important role for PIAS3 in the coregulation of ESR1 transcriptional activity through NR2E3, it would be interesting to see if there is also a correlation between PIAS3 expression levels and ER positive breast cancer. The authors could investigate such a correlation from the IJB cohort.

Response: As the reviewer suggested, we investigated whether there is a correlation between NR2E3 and PIAS in ESR1-positive breast cancer cohort (IJB cohort). As shown in Supplementary Fig. S7, NR2E3 is significantly correlated with PIAS in ER-positive breast cancer. (r=0.206, p=2.4x10^-5) and PIAS3 also correlated with ESR1 (r=0.255, p=1.5x10^-7).
Minor Comments:
1. In certain portions of the manuscript the sentence structure is cumbersome, making the manuscript difficult to follow. The results section in particular has portions (including the description of Figure 4 in the text) that should be re-written for clarity.

Response: We re-wrote and corrected the manuscript to improve clarity.

2. There are minor errors throughout the manuscript that should be corrected. There should be a space between a word and parentheses. There is a period after al in et al. There are many cases of the improper use or absence of articles (a, an, the). Improper usage of singular/plural nouns should be corrected. In the methods section, the UNC cohort data are from the public (with an "l") Merck website. "In trans" should be italicized throughout the manuscript and figure legends.

Response: These minor errors have now been corrected.

3. The descriptions of the methods used in this study need to be stated more explicitly. The siRNAs that were utilized as well as what concentration of siRNA was used for knockdown experiments should be clearly stated in the manuscript or be easy to understand from a referenced protocol. Antibodies used for PIAS3 should be specified for ChIP and co-immunoprecipitation experiments. It is unclear whether cells were in phenol red free medium as well as charcoal stripped serum for stimulation with E2. The authors include a description of microarray in the materials and methods, however it is unclear from the text when the authors carried out a microarray themselves. It is presented in the manuscript that all gene expression data were obtained from publicly available datasets. Please clarify this whole area of the manuscript.

Response: We agree with the referee and the methods have been rewritten and clarified.

4. The introduction focuses on the value of genome-wide analysis and utilizing publicly available gene expression datasets to make new connections between cellular factors. While these points are relevant to the current study, it would also be helpful for more time to be spent introducing ESR1 and NR2E3 which are the focus of the manuscript. Minimally, NR2E3 should be introduced as an orphan nuclear receptor and not simply identified as a novel regulator of ESR1 expression. Furthermore, PIAS3 should be introduced in the introduction since the authors propose a critical role for PIAS3 in NR2E3 regulation of ESR1 expression.

Response: In our design of investigation, NR2E3 and PIAS3 was not intended gene of interest from the beginning of study. We discovered the importance of these genes during the course of informatical approaches and validation experiments. We did not include a brief introduction of these genes in the introduction section because of the logical flow of our study. If we emphasize importance of NR2E3 and PIAS3 in introduction, it may give readers a wrong or confused impression. However, we absolutely agree with the reviewer that we needed a brief introduction of NR2E3 and PIAS3. Thus, we put it in the results section.

5. In the last paragraph of the introduction the authors state that there is a novel interaction between NR2E3 and ESR1, which suggests a physical interaction. Since this is not the demonstrated relationship between NR2E3 and ESR1, it would be helpful for this statement to be re-stated so that it more accurately describes the findings of the study.

Response: We agree with the reviewer. Since NR2E3 physically does not interact with ESR1, interaction is not the proper expression. We re-state, “We uncovered an unexpected relationship between NR2E3 and ESR1”.
6. Defining the terms "in trans significance" and "in trans correlation" would contribute to the clarity of the manuscript.

Response: We changed "in trans correlation" to “Correlated Genes”.

7. The lack of effect of NR2E3 silencing on FOXA1 expression in MCF7 cells (Figure 3B) should be discussed. Since NR2E3 silencing decreases ESR1 expression at both mRNA and protein levels, it seems that all downstream targets of the estrogen receptor would be affected. Consistent with this, FOXA1 expression is reduced due to NR2E3 silencing in T47D cells (Figure 3D).

Response: As the reviewer pointed out, we described it in the result section.

8. ChIP data show that both NR2E3 and PIAS3 are bound within the same region of the ESR1 promoter, which supports the authors’ hypothesis that both proteins are involved in ESR1 transcriptional regulation. However, ChIP only indicates that a protein is present within a certain region on a large piece (usually ~500-800 bp) of chromatin, therefore it cannot provide evidence for the direct interaction of two proteins on DNA. While NR2E3 and PIAS3 may interact in solution, there is no direct evidence in this manuscript for an interaction between these proteins on DNA. Either show it, or change the statement.

Response: We agree with the reviewer. We changed the sentence to “PIAS3 is directly or indirectly recruited to NR2E3 on the ESR1 promoter region”.

9. Figure 1: the y-axis label (ITS) is an acronym that is not defined anywhere in the manuscript. While it probably stands for "in trans significance," this should be clarified.

Response: We changed the ITS expression to “Correlated Genes”.

10. Figure 4A:
1. Need to specify both 5' and 3' boundaries of the promoter region used in the luciferase construct. A map of these different ESR1 promoter regions might be helpful.

Response: Following the reviewer’s comment, we added a map of our deletion construct (Figure 4A).

2. A description of the experiment in Figure 4A is necessary to understand the results. While the experiment looks as though it involves the overexpression of NR2E3, this should be explained in the results section and figure legend.

Response: Figure 4A is moved to Figure 4B and 4B is moved to 4C. We re-wrote the results and figure legend section in detail.

3. The authors should indicate significant changes in luciferase activity between the different promoter constructs. By eye it is unclear whether there are significant differences between the different constructs. This information is important to evaluate possible differences in NR2E3 binding between the different ESR1 promoter constructs.

Response: We used three different types of deletion construct reporters (ESR1 Promoter; -2769 ~ +212, -735 ~ +212, -245 ~ +212) as shown in Figure 4A. Figure 4B and C shows that luciferase
activity still remain although we deleted up to -245 bp on the ESR1 promoter-suggesting that NR2E3 binding site for ESR1 is located within -245 bp. Thus, we designed primers within these regions (-245 ~ +212; Figure 4D) and performed the ChIP assay. As shown in Figure 4E, NR2E3 bound ESR1 promoter region from -245 to +212.

11. Figure 8: The differences between the different log rank tests shown in C should be described. While they are all significant, it is not clear what the different tests mean.

Response: To predict patients with ESR1-independent NR2E3 gene expression signature, we used a prediction model. We used six different classifiers as shown in Figure 8C. Depending on the classifier, the algorithm is different. The reason we used different classifiers is to strengthen the prediction power. Although we used different types of classifier, the results were all significant-suggesting that ESR1-independent NR2E3 gene expression signature possesses a strong prediction power. In the Supplementary Method, we described in what individual classifiers are different.

Referee #2 (Comments on Novelty/Model System):

We are grateful to this reviewer for the positive comments and for making constructive suggestions for improving the quality our work and thus strengthening the manuscript considerably.

1. Abstract: Whilst ESR1 is expressed in the majority of breast tumors and inhibition of its action is the basis for adjuvant hormonal therapies, I am not aware that ESR1 is an "oncogene" according to the definition of the term. This should be removed.

Response: We agree with the reviewer’s comment. As pointed out, we removed the “oncogene” expression and re-wrote, “ESR1 is one of the most important transcription factor and therapeutic targets in breast cancer”.

2. Introduction: The authors refer to FoxA1 as a "downstream partner" to ESR1 in breast cancer cells. As a factor that regulates ESR1 binding to gene promoters, is this really the correct description for the action of FoxA1 in breast cancer?

Response: We agree with the reviewer’s comment. ESR1 bound on FOXA1 promoter region and regulates FOXA1 downstream genes such as CCND1 and TFF1 (Laganière J et al., Proc Natl Acad Sci U S A. 2005 Aug 16;102 (33):11651-6). FOXA1 is a downstream binding partner of ESR1 rather than downstream gene. Thus, we re-write “FOXA1 as a new estrogen receptor (ESR1) partner bound to ESR1 promoter region in breast cancer cells”.

3. P11: As a regulator of ESR1 expression, the association of NR2E3 with longer RFS is intriguing in datasets in which ESR1 expression is not associated with RFS. This may indicate a role for NR2E3 which is independent of its role in regulating ESR1, as is proposed by the authors elsewhere. Does it therefore have a role in ESR1 negative breast cancer? Is this evident from analysis of gene expression profile datasets? For the genes identified in the microarray analysis for NR2E3 shRNA, are these genes predictive of RFS or OS in ESR1 negative breast cancer or only in ESR1 positive breast cancer?

Response: As the reviewer suggested, we tested whether NR2E3 might be a predictive marker in ER-negative cancers using NKI and UNC cohort. As shown in Supplementary Fig. S11, NR2E3 was not associated with RFS in ER negative BC. Our Microarray data showed that 1847 genes are NR2E3-specific but ESR1-independent (Figure 8A) and pathway analysis revealed that the 1847 genes are involved in pivotal biological pathways. To test the significance of the 1847 gene signature, we applied the same prediction models to patients with ER-positive or ER-negative breast
cancer independently. Supplementary Fig. S12 and S13 show that those genes have predictive power in ER-positive BC but not in ER-negative BC.

4. Figure 7: Were ESR1 and NR2E3 proteins independently associated with RFS and OS in the MDACC RPPA analysis?

Response: We did a survival analysis in MDACC cohort. Supplementary Fig. S6 shows that ESR1 or NR2E3 independently associated with RFS and OS.

5. Figure 8: It would be helpful if compound covariate predictor (CCP); INN, 3NN, etc are more clearly defined. Genes associated with these groups should be described in the supplementary information.

Response: We described the classifiers in Supplementary Method 1 and added the gene lists used in Figure 8C as Supplementary Table 1.

2nd Editorial Decision 17 October 2011

Thank you for the submission of your revised manuscript to EMBO Molecular Medicine. We have now received the enclosed reports from the referees that were asked to re-assess it. As you will see the reviewers are supportive and I am pleased to inform you that we will be able to accept your manuscript pending the following final editorial amendments.

- Please double check The Paper Explained attached here. For clarity, we took the liberty to slightly modify it. Please feel free to correct it too. Be reminded that it should be written in a way accessible to specialists and non-specialists in the field.
- Please correct typos in the main text of the manuscript, specifically add spaces between the last word of a sentence and the "(" of the corresponding citation.
- Concerning Figures; Figures 3 and 4 have odd black and grey bars above barplots- Please modify. It is our standard procedure to request the original files from which the figures were obtained to clarify potential conflict, please provide us with the original uncropped western blots corresponding to Figure 5G when you return a revised version of your manuscript.
- In the materials and methods section: Please provide an ethical statement regarding the patients samples used.
- Supplementary material should be assembled in a single pdf file in its final format - except the table that should be provided as a separate dataset in an excel format. Supplementary material will not be further edited should your paper be accepted.

I look forward to reading a new revised version of your manuscript as soon as possible.

Yours sincerely,

Editor
EMBO Molecular Medicine
REFEREE REPORTS:

Referee #1:

This manuscript identifies NR2E3 as a novel regulator of ESR1 gene expression and demonstrates a requirement for PIAS3 in this function of NR2E3. The authors further show a correlation between NR2E3 expression and recurrence free survival of breast cancer patients. The authors have adequately addressed the major comments on the manuscript. Briefly, the authors demonstrated that ESR1 does not regulate PIAS3 expression in a feedback loop, controls for siRNA knockdown were included and a detailed description of how knockdown was carried out is now described in the methods, the interaction between NR2E3 and PIAS3 has been confirmed using purified proteins, and the authors provided initial evidence that sumoylation of NR2E3 by PIAS3 is not important for NR2E3 regulation of ESR1 gene expression. Furthermore, the clarity of the text is now significantly improved. While there are still quite a few typos in the text, the manuscript demonstrates a convincing and novel role for NR2E3 in the regulation of ESR1 expression and that NR2E3 could be an important predictive marker for recurrence free survival in patients with breast cancer.

Referee #2:

The authors have addressed the issues I raised, to my satisfaction.

2nd Revision - Authors' Response 17 October 2011

Enclosed please find our revised manuscript entitled “Reconstruction of nuclear receptor network reveals that NR2E3 is a novel upstream regulator of ESR1 in breast cancer”. We sincerely appreciate your time and effort for efficient handing of this manuscript. We revised the manuscript according to suggestions.

Please double check The Paper Explained attached here. For clarity, we took the liberty to slightly modify it. Please feel free to correct it too. Be reminded that it should be written in a way accessible to specialists and non-specialists in the field.

Response: We agree with all of modification in “Paper Explained”.

- Please correct typos in the main text of the manuscript. specifically add spaces between the last word of a sentence and the "(" of the corresponding citation.

Response: We checked the manuscript intensively.

- Concerning Figures; Figures 3 and 4 have odd black and grey bars above barplots. Please modify. It is our standard procedure to request the original files from which the figures were obtained to clarify potential conflict, please provide us with the original uncropped western blots corresponding to Figure 5G when you return a revised version of your manuscript.

Response: We removed the odd black and grey bars above bar-plots in Figure 3 and 4. As the editor requested, we added the original western image file regarding Figure 5G.

- In the materials and methods section: Please provide an ethical statement regarding the patients samples used.

Response: We added a description regarding human samples in Methods section. We wrote that “Human breast tumors were obtained from Tumor Banks following pathologist review under the auspices of Institutional Review Board-approved protocols at the M. D. Anderson Cancer Center”.
- Supplementary material should be assembled in a single pdf file in its final format except the table that should be provided as a separate dataset in an excel format. Supplementary material will not be further edited should your paper be accepted.

Response: We made the supplementary file as one single pdf file and Supplementary Table 1 was separated as one independent pdf file.