Cellular reprogramming by the conjoint action of ERα, FOXA1, and GATA3 to a ligand-inducible growth state

Say Li Kong, Guoliang Li, Siang Lin Loh, Wing-Kin Sung, Edison Liu

Corresponding author: Edison Liu, Genome Institute of Singapore

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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 March 2011

Thank you again for submitting your work to Molecular Systems Biology. First of all, I have to apologize for the delay in getting back to you. We have now heard back from two out of the three referees whom we asked to evaluate your manuscript. Since the last reviewer failed so far to return a report, I prefer to make a decision now rather than delaying further the process.

As you will see from the reports below, the referees find the topic of your study of potential interest. However, they raise several concerns on your work, which should be convincingly addressed in a revision of the present work. The major concerns raised by the present reviewers refer to the need of some additional analyses and data to support the concept of ER- FOXA1 and GATA3-mediated reprogramming. The recommendations provided by the reviewers are very clear in this regard.

On a more editorial note, we would kindly ask you to prepare supplementary information according to our instructions. Essentially, we strongly recommend that Supplementary Information including text, figures and small tables (less than 50 rows) is combined into a single PDF starting with a Table Of Content listing the items included in the file. Each legend should be written clearly beneath its figure. *Large tables (more than 50 rows) should be submitted as MS Excel spreadsheet documents (.xls) or tab-delimited text (.txt).*

*** PLEASE NOTE *** As part of the EMBO Publications transparent editorial process initiative (see our Editorial at http://www.nature.com/msb/journal/v6/n1/full/msb201072.html), Molecular Systems Biology will publish online a Review Process File to accompany accepted manuscripts. When preparing your letter of response, please be aware that in the event of acceptance, your cover letter/point-by-point document will be included as part of this File, which will be available to the scientific community. More information about this initiative is available in our Instructions to Authors. If you have any questions about this initiative, please contact the editorial office

msb@embo.org.

If you feel you can satisfactorily deal with these points and those listed by the referees, you may wish to submit a revised version of your manuscript. Please attach a covering letter giving details of the way in which you have handled each of the points raised by the referees. A revised manuscript might be once again subject to review and you probably understand that we can give you no guarantee at this stage that the eventual outcome will be favourable.

I apologize again for the lengthy process and look forward to receiving your revised work.

Best wishes,

Editor
Molecular Systems Biology

REFEREE REPORTS

Reviewer #1 (Remarks to the Author):

In this report, Kong et al investigated the role of FOXA1 and GATA-3 in reprogramming breast cancer cells to respond to estrogen. Extensive series of experiments have been performed and data presented for the most part support their conclusions. Although a recent report showed reprogramming of normal breast epithelial cells to E2 stimulated growth upon transfection of ER and BMI-1 (Breast Cancer Research 2007, 9:R38), this is the first report demonstrating the role of FOXA1 and GATA-3 in this reprogramming process and is highly relevant in breast cancer as ER, FOXA1 and GATA-3 are coexpressed in a subset of estrogen responsive breast cancer. However, there are few issues authors need to address to enhance the impact of the manuscript.

1) Authors demonstrate genes with ERalpha, FOXA1 and GATA3 binding sites are most responsive to E2-both repression and induction. Instead of listing all genes induced or repressed by E2 in the supplementary file, it will be beneficial to other researchers to provide the list of E2 regulated genes in relation to ER, FOXA1, and GATA-3 binding sites. Further subclassification of these genes, which showed E2-dependent in increase in Pol II binding, would be useful.

2) ER, FOXA1, and GATA-3 are co-expressed in Luminal type A breast cancer and this co-expression is associated with better prognosis. It will be clinically relevant to show the enriched expression of E2-inducible genes with ERalpha, FOXA1 and GATA3 binding sites in luminal breast cancers compared to other subtypes using publicly available gene expression databases.

3) Hua et al (Cell 137:1259) have previously shown enrichment of FOXA1 and GATA-3 binding sites with ER binding sites, although using an artificial system. It is important that authors discuss their results in the context of the above report.

4) Authors have observed enrichment of BACH binding sites in ER ChIP regions. This was also reported previously (MCB 28:7487-7503). May be appropriate to reference that publication.

5) Hurtado et al., (Nature Genetics 43:27-33) reported the need for FOXA1 for ER binding and suggested that chromatin opening by FOXA1 is essential for ER binding. Most of the results, although not statistically analyzed (see below) presented in this study show ER playing a role in FOXA1 and GATA-3 recruitment. These discrepant results need to be discussed.

6) Decline in FOXA1 binding in vehicle treated cells (Figure 3C) is surprising. Is this result reproducible or is it one time experiment? It does not appear that results of Figure 3B and C have been statistically analyzed although ligand-dependent increase in binding is described in the text. The statement in most figure legends "Means of two independent experiments with consistent reproducible results are compared and standard errors are shown" is confusing. Does this mean that results of only experiments where data show the same trend were handpicked for presentation or the

experiments were only twice?

7) Figure 1 is poorly described in the text. Figure 1C in page 6 should be Figure 1C-E, whereas Figure 1D should be Figure 1F.

8) It is critical to show ER, FOXA1 and GATA-3 expression in transfected MDA-MB-231 cells and demonstrate whether these cells have acquired luminal cell phenotype by expressing epithelial markers such as E-cadherin and lowering mesenchymal markers. This is necessary to substantiate the claim of reprogramming of cells by these three factors.

Reviewer #3 (Remarks to the Author):

In this manuscript, the authors describe a study of genome-wide binding sites for three key transcriptional regulators, ERalpha, FOXA1, and GATA3, of estrogen signaling and breast cancer biology and provide evidence for their importance in establishing estrogen-dependent proliferation of previously independent breast cancer cells. They present detailed and well executed analyses of the binding sites and potential mechanistic insights. This study contributes to the accumulating body of data which will ultimately provide a systems perspective of the gene regulatory networks in hormonal carcinogenesis and should be of interest to basic and clinical researchers involved in studies of transcriptional regulatory mechanisms and their roles in cancer cell proliferation. There are a few issues the authors should address, however, before this manuscript is suitable for publication:

1. The data sets generated for this study will be valuable resources for investigators in related fields of research. The authors should consider depositing the microarray and binding site data, with corresponding technical and annotation information, with a public database such as the Gene Expression Omnibus (GEO) at the NCBI.

2. A key finding of this study is the effect of co-expressing all three transcription factors on the establishment of estrogen-dependent growth in previously ER-negative cell lines, but some control experiments are missing and additional data are needed to support their conclusion. Specifically, the authors should provide western analysis results showing the expression levels of the three factors prior to and following transfections with the expression constructs to confirm that the corresponding changes in cellular behavior are due to the expression and activity of these factors. Additionally, the authors drew their conclusion on the effects on cell growth and proliferation on a tetrazolium salt-based assay which measures the metabolic activity of the cells and may or may not reflect changes in cell number. Their conclusion would be further strengthened if they include results from experiments which monitor changes in cell number as well, especially for the triple-transfection experiments which show estrogen-dependent changes.

3. The authors describe a "striking" correlation between the estrogen-responsive gene expression profiles of triple-transfected ER-negative cells and those observed for the ER-positive MCF-7 cells, but only provide dot-plots and R-values to support their assertion. It would be more informative, if the authors can show the expression levels and identities of the recapitulated responsive genes and comment on whether they include previously known estrogen-regulated genes and their putative or demonstrated roles in mediating the estrogen-dependent pro-proliferative effects of ERalpha, FOXA1, and GATA3.

1st Revision - authors' response 17 June 2011

We would like to thank the reviewers for their thoughtful comments, which have helped us further improve our manuscript. Following their and your suggestions, the analysis and validation were significantly extended and various parts of the manuscript were rewritten. We have also re-organized our supplemental information according to your recommendation. We hope that the revised paper is now acceptable for publication in Molecular Systems Biology.
The specific comments of the reviewers are addressed as follows.

Reviewer #1 (Remarks to the Author):

In this report, Kong et al investigated the role of FOXA1 and GATA-3 in reprogramming breast cancer cells to respond to estrogen. Extensive series of experiments have been performed and data presented for the most part support their conclusions. Although a recent report showed reprogramming of normal breast epithelial cells to E2 stimulated growth upon transfection of ER and BMI-1 (Breast Cancer Research 2007, 9:R38), this is the first report demonstrating the role of FOXA1 and GATA-3 in this reprogramming process and is highly relevant in breast cancer as ER, FOXA1 and GATA-3 are coexpressed in a subset of estrogen responsive breast cancer. However, there are few issues authors need to address to enhance the impact of the manuscript.

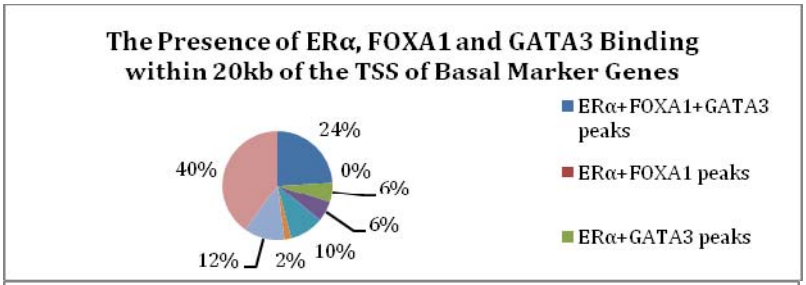
1) *Authors demonstrate genes with ERalpha, FOXA1 and GATA3 binding sites are most responsive to E2-both repression and induction. Instead of listing all genes induced or repressed by E2 in the supplementary file, it will be beneficial to other researchers to provide the list of E2 regulated genes in relation to ER, FOXA1, and GATA-3 binding sites. Further subclassification of these genes, which showed E2-dependent in increase in Pol II binding, would be useful.*

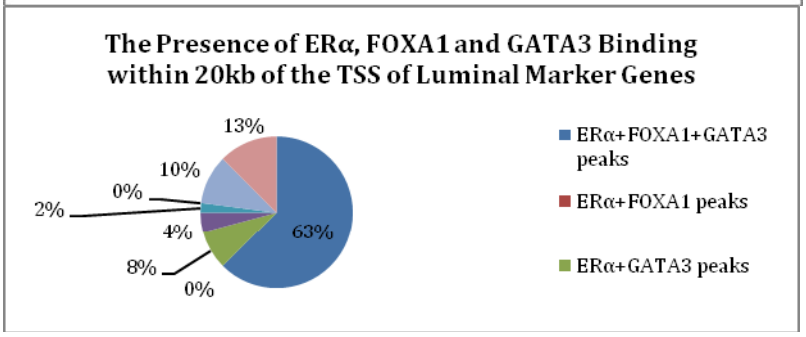
Response: We have included the list of E2-regulated genes supplemented with ER , FOXA1, GATA3 as well as RNA Pol II binding sites information in Supplemental Table VIII and IX.

2) *ER, FOXA1, and GATA-3 are co-expressed in Luminal type A breast cancer and this co-expression is associated with better prognosis. It will be clinically relevant to show the enriched expression of E2-inducible genes with ERalpha, FOXA1 and GATA3 binding sites in luminal breast cancers compared to other subtypes using publicly available gene expression databases.*

Response: We assessed the presence of ER , FOXA1 and GATA3 bindings within 20kb of the TSS for the luminal or basal marker genes defined by Kao et al (Kao et al, 2009)We observed that 63% of the luminal genes are associated with conjoint binding of the three transcription factors ER +FOXA1+ GATA3 within 20 kb of the TSS, and only 13% of these luminal genes showing no proximity binding of any of these 3 TFs. On the other hand, 24% of the basal genes are associated with proximate conjoint ER +FOXA1+ GATA3 binding with 40% of these genes are not associated with any ER , FOXA1 or GATA3 binding (see Supplemental Figure S15, Table XIII). This suggests that ER +FOXA1+GATA3 binding exerts greater impact on regulating the transcription of luminal marker genes as compared to the basal marker genes.

Supplemental Figure S15

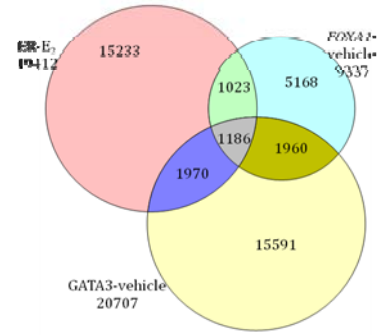




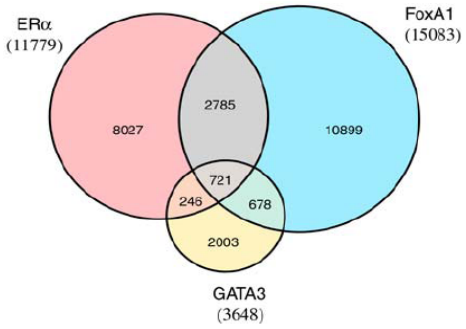
3*) Hua et al (Cell 137:1259) have previously shown enrichment of FOXA1 and GATA-3 binding sites with ER binding sites, although using an artificial system. It is important that authors discuss their results in the context of the above report.*

Response: The FOXA1 and GATA3 binding sites reported by Hua et al. (Hua et al, 2009) were not carried out under the estrogenic condition. In order to compare our finding with theirs, we have overlapped our binding sites from ER -E2, FOXA1-vehicle and GATA3-vehicle (see the below figures). We observed that 6% of our ER sites co-localized with FOXA1 and GATA3 binding sites ñ this is comparable to their finding. The work by Hua et al. has described the genomics impacts of RARs, FOXA1 and GATA3 bindings under the retinoid acid signaling and ER binding under the estrogen signaling. We posit that FOXA1 and GATA3 could have a broader "universal" co-regulator function for nuclear hormone binding (see page 19, first paragraph).

Co-localization of our ER-E2, FOXA1-vehicle and GATA3-vehicle binding sites



Co-localization of ER-E2, FOXA1 and GATA3 binding sites by Hua et al



4) Authors have observed enrichment of BACH binding sites in ER ChIP regions. This was also reported previously (MCB 28:7487-7503). May be appropriate to reference that publication.

Response: We thank you for drawing our attention to this paper. We have further elaborated on the enrichment of BACH and AP1 motifs in ER binding sites and cited the work by Bhat-Nakshatri et al.(Bhat-Nakshatri et al, 2008) in our motif analysis (page 6, paragraph 2).

5) Hurtado et al., (Nature Genetics 43:27-33) reported the need for FOXA1 for ER binding and suggested that chromatin opening by FOXA1 is essential for ER binding. Most of the results, although not statistically analyzed (see below) presented in this study show ER playing a role in FOXA1 and GATA-3 recruitment. These discrepant results need to be discussed.

Response: Our finding is not contradictory but in fact supports the notion by Hurtado et al. (Hurtado A. et al, 2011) that FOXA1 is required for a subset of ER binding (refer to Figure 3B, as

shown below) and we have cited Hurtado finding in our new manuscript (page 7, paragraph 2). Besides the recruitment of ER pioneered by FOXA1 binding, we examined the progressive recruitment of ER and FOXA1 from another angle ñ whether ER can recruit FOXA1 binding. This is not studied in (Hurtado et al, 2010). Our results indeed showed that ER also functions as a pioneering factor for a subset of FOXA1 binding as well (refer to Figure 3C, as shown below). This is defined as those sites occupied by ER only before ligand that then recruits FOXA1 with E2 exposure.

Though the number of sites where ER is the pioneering factor is less than those where FOXA1 is the pioneering factor, the numbers represent a significant proportion. This issue is discussed in both the results and the discussion sections of the manuscript. In summary, our results suggest that ER is as likely to be a pioneering factor to recruit FOXA1 as the converse.

Figure 3B

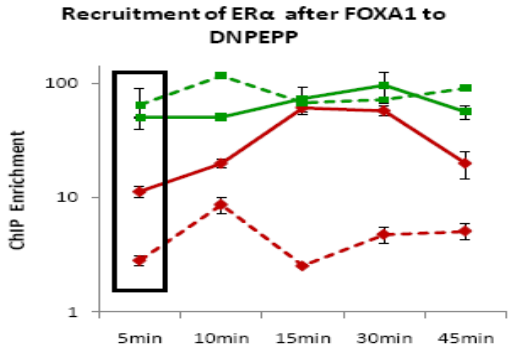
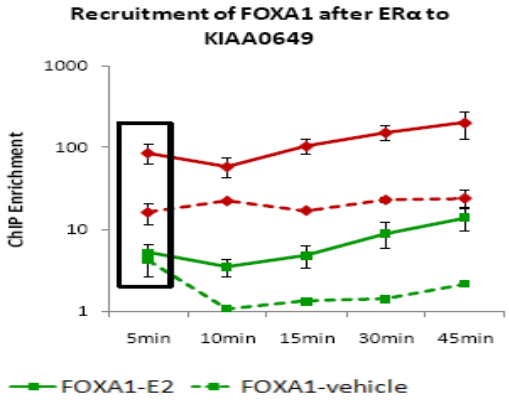


Figure 3C

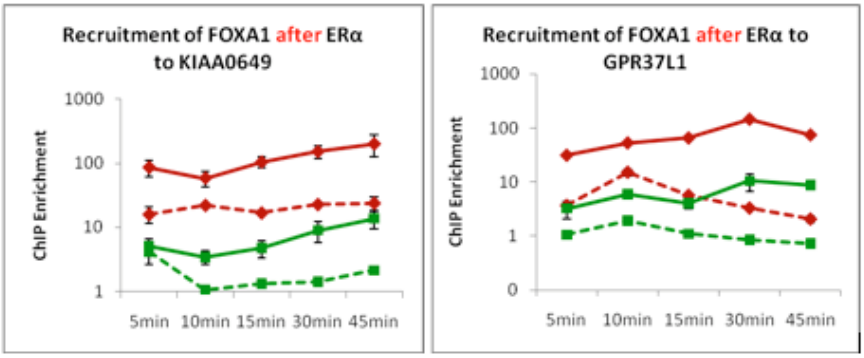


6) Decline in FOXA1 binding in vehicle treated cells (Figure 3C) is surprising. Is this result reproducible or is it one time experiment? It does not appear that results of Figure 3B and C have been statistically analyzed although ligand-dependent increase in binding is described in the text. The statement in most figure legends "Means of two independent experiments with consistent reproducible results are compared and standard errors are shown" is confusing. Does this mean that results of only experiments where data show the same trend were handpicked for presentation or the experiments were only twice?

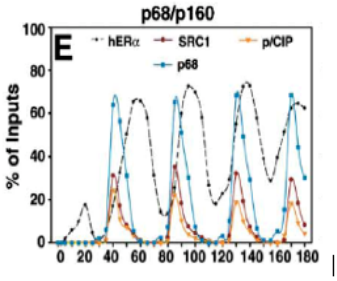
Response: In order to further assess the binding intensity of FOXA1 in vehicle-treated condition, we have tested another site nearest to GPR37L1 gene and observed marginal increased FOXA1 binding at early time point followed by reduced binding at the later time point. This suggests that the FOXA1 binding intensity fluctuates upon different sites and time points albeit in a minor manner. More importantly, however, is that the results from these two target sites revealed increased FOXA1 binding after estrogen stimulation that further supports our claim of recruitment. We also posit that the transient decline in FOXA1 binding could be partly due to the cyclic effect by -amanitin treatment that induces fluctuations in FOXA1 recruitment after synchronization. The cyclical recruitment for other transcription factors have also been evidenced in the work by Me¥ tivier R et al., Cell 2003, Vol. 115, 751ñ763 (an example is shown, see below). The difference in amplitude between our results and those of Metivier is because our protocol and design was not optimized for detection of cyclic binding but would indeed sense some of the changes.

The results shown are from the mean of two independent biological replicates carried out in two separate experiments with consistent reproducible results.

Figure 3C Additional tested site nearest to GPR37L1.



This figure displayed the dynamics recruitment of cofactors to pS2 promoter in a cyclic manner (Cell 2003, 115:751-763).



7) Figure 1 is poorly described in the text. Figure 1C in page 6 should be Figure 1C-E, whereas Figure 1D should be Figure 1F.

Response: We apologize for this error, the description for Figure 1 has been corrected (see page 6).

8) It is critical to show ER, FOXA1 and GATA-3 expression in transfected MDA-MB-231 cells and demonstrate whether these cells have acquired luminal cell phenotype by expressing epithelial markers such as E-cadherin and lowering mesenchymal markers. This is necessary to substantiate the claim of reprogramming of cells by these three factors.

Response: We have provided the western results showing the expression of ER , FOXA1 and GATA3 in MDA-MB-231 and BT-549 cells prior and after transfection (see page 12 in this main text, Supplemental Figure S6). We have analyzed the expression of luminal and basal marker genes in MDA-MB-231 transfected cells and observed that there is on-average up-regulation of luminal marker genes (see Supplemental Figure S14A) and concommitant down-regulation of basal marker genes (see Supplemental Figure S14B) after transfection of ER +FOXA1+GATA3-in MDA-MB-231 cells (originally basal phenotype) as compared to the ER -only and vector-control MDA-MB-231 cells. When we further assessed the differential expression of luminal and basal marker genes between ER +FOXA1+GATA3-expressing and vector control MDA-MB-231 cells, we observed that there is an average induction of luminal genes and average suppression of basal genes (Supplemental Figure S14C). This suggests that the enhanceosome component is competent to transcriptionally reprogramme the basal cells to luminal cells albeit to a modest degree.

Figure S14A (Luminal genes)

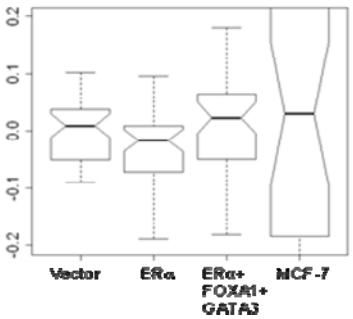


Figure S14B (Basal genes)

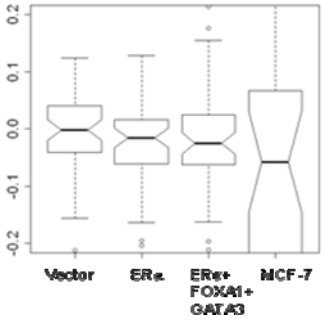
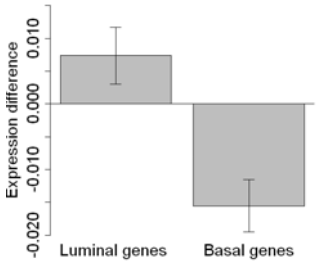


Figure S14C (average difference in gene expression between ER+FOXA1+GATA3 and vector-MDA-MB-231 cells)



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1. The data sets generated for this study will be valuable resources for investigators in related fields of research. The authors should consider depositing the microarray and binding site data, with corresponding technical and annotation information, with a public database such as the Gene Expression Omnibus (GEO) at the NCBI.

Response: We have deposited our microarray, binding sites data in the GEO database (GSE23701, GSE23893, GSE26831 and GSE29073 and GSE xxx) and will be released to public access when the manuscript is accepted for publication.

2. A key finding of this study is the effect of co-expressing all three transcription factors on the establishment of estrogen-dependent growth in previously ER-negative cell lines, but some control experiments are missing and additional data are needed to support their conclusion. Specifically, the authors should provide western analysis results showing the expression levels of the three factors prior to and following transfections with the expression constructs to confirm that the corresponding changes in cellular behavior are due to the expression and activity of these factors. Additionally, the authors drew their conclusion on the effects on cell growth and proliferation on a

tetrazolium salt-based assay which measures the metabolic activity of the cells and may or may not reflect changes in cell number. Their conclusion would be further strengthened if they include results from experiments which monitor changes in cell number as well, especially for the triple-transfection experiments which show estrogen-dependent changes.

Response: We have provided the western results showing the expression of ER , FOXA1 and GATA3 in MDA-MB-231 and BT-549 cells prior and after transfection (see page 12, Supplemental Figure S6A, S6B). We have also performed additional cell growth assay by monitoring the cell number counts in response to estrogen stimulation assayed by the Hoechst nuclei stain assessed by the high content screening platform on Cellomics ArrayScan VTi. The cell growth measured by WST-1 and cell number count by Hoechst stain is comparable. This has further strengthened our claim that the enhanceosome component by ER , FOXA1 and GATA3 is essential to reinstate the induced growth upon estrogen treatment in the ER +FOXA1+GATA3-expressing MDA-MB-231 and BT-549 cells (see page 13, Supplemental Figure S7-11).

Figure S6A

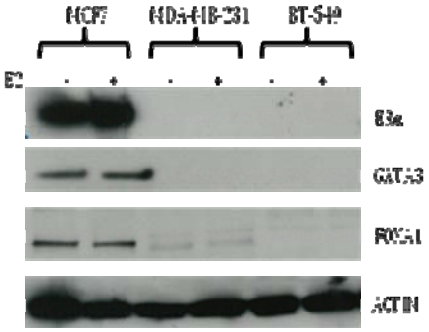


Figure S6B

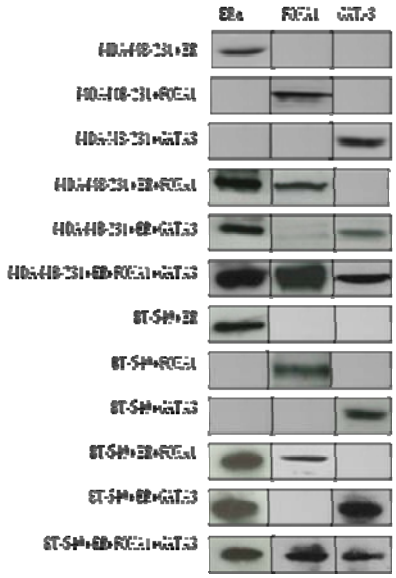


Figure S11A

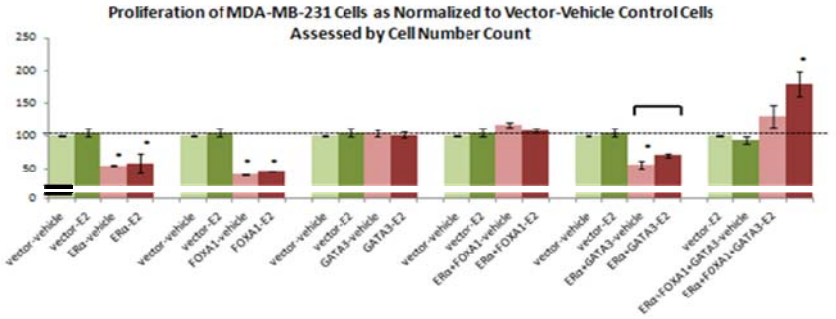
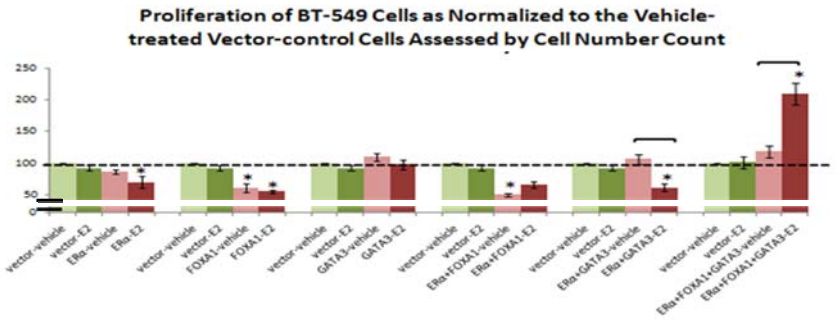


Figure S11B



Note: * means p-value ≤ 0.05 compared to vector-vehicle computed from the Student's t Test
— shows significant E2-dependent induced or repressed growth with p-value ≤ 0.05

3. The authors describe a "striking" correlation between the estrogen-responsive gene expression profiles of triple-transfected ER-negative cells and those observed for the ER-positive MCF-7 cells, but only provide dot-plots and R-values to support their assertion. It would be more informative, if the authors can show the expression levels and identities of the recapitulated responsive genes and comment on whether they include previously known estrogen-regulated genes and their putative or demonstrated roles in mediating the estrogen-dependent pro-proliferative effects of ERalpha, FOXA1, and GATA3.

Response: We have provided the list of estrogen-responsive genes and their expression levels in the transfected MDA-MB-231 cells in Supplemental Table X. We then assessed whether the estrogen-responsive genes found in the transfected MDA-MB-231 cells play a role in the cell proliferation using the Ingenuity Pathway Analysis. The result revealed that there is significant association of these genes with the cell cycle, cellular proliferation and DNA replication functionalities (p-value = 7.27E-12 - 1.28E-04, see Supplemental Figure S13A). Moreover, our expression data demonstrated that there is up-regulation of proliferative cell cycle genes in the ER +FOXA1+GATA3-expressing MDA-MB-231 cells as compared to the ER -only cells in responsed to estrogen stimulation (see Supplemental Figure S13B). By taking all the differentially expressed genes in MDA-MB-231 sublines, we demonstrated that the expression profile of E2 induced ER +FOXA1+GATA3 expressing MDA-MB-231 cells display a good positive correlation (R=0.42) with the E2 induced expression profile of MCF-7. By contrast, we observed a negative correlation between the expression profiles of MDA-MB-231 transfected with ER only (R= -0.21, p-value 2.08E-11) (Figure 6C-D, see Supplemental Table X for detailed subanalyses). In addition, when only cell cycle, DNA replication, and proliferation genes were examined, again, there was positive correlation between MDA-MB-231 transfected with ER +FOXA1+GATA3 and MCF-7 but no correlation between ER -only MDA-MB-231 cells and MCF-7 (Supplemental Figure S12). This has further strengthen our claim that the presence of ER , FOXA1 and GATA3 has partially reprogrammed the ER -negative MDA-MB-231 cells to transcriptionally resemble the ER -positive MCF-7 cells by recapitulating the estrogen responsive cassette and manifesting the proliferative phenotype. We also found that specific genes previously known to be E2-regulated in the ER

responsive cell lines (Frasor J. et al, 2003; Fullwood et al, 2009) such as CCND1, STC2, ADCY9 and BTG1 were also regulated in the same direction by ligand in the triple factor transfected MDA-MB-231 cells (Supplemental Table XI).

Figure S13B

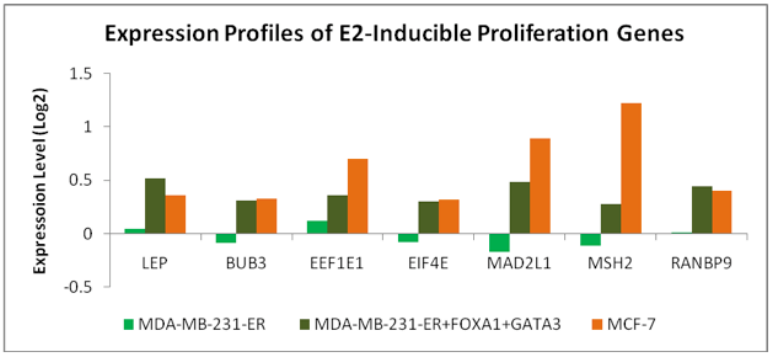
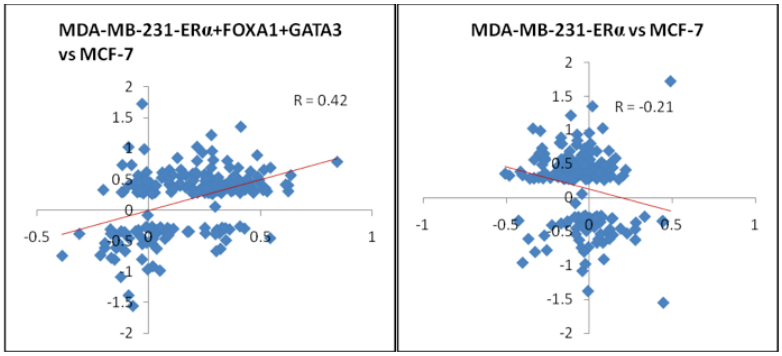
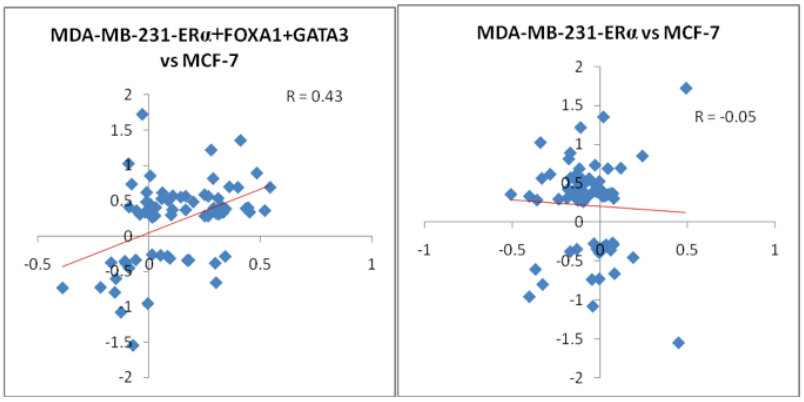


Figure 6C-D



Supplemental Figure S12B-C



References:
Bhat-Nakshatri P, Wang G, Appaiah H, Luktuke N, Carroll JS, Geistlinger TR, Brown M, Badve S, Liu Y, Nakshatri H (2008) AKT alters genome-wide estrogen receptor alpha binding and impacts estrogen signaling in breast cancer. Mol Cell Biol 28: 7487-7503.

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Thank you again for sending us your revised manuscript. As you will see, the reviewers are not satisfied with the modifications made and full supportive. I am pleased to inform you that your paper has been accepted for publication.

NOTE The reviewers make a few minor suggestions for small amendments. We would be grateful if you could send us the word file of the main text with the appropriate edits.

Thank you very much for submitting your work to Molecular Systems Biology.

Sincerely,

Editor
Molecular Systems Biology

Reviewer #1 (Remarks to the Author):

This revised manuscript has addressed many of the concerns. Clarifications, as noted below, are required.
1) Explanation provided for Figure 3B for DNPEPP gene is still not clear. Authors state "we observed enrichment of FOXA1 occupancy as early as 5 minutes upon E2 stimulation, followed by progressively increasing ER occupancy at later times points". However, this is not apparent in the figure. Similar levels of FOXA1 are seen in untreated and E2 treated cells after 5 minutes treatment. Data for FOXA1 recruitment after ER occupancy is very clear.
2) Words "interaction clusters" repeated at the end of page 10 and beginning of page 11.
3) Page 4, lane 8 studied instead of studies.
4) Page 20, lane 5, authors state that attenuation of FOXA1 reduces the levels of ERalpha. This is already reported previously. May be worth referring this article (Bernardo et al., Development 137:2045-2054)

Reviewer #3 (Remarks to the Author):

The authors adequately addressed the issues raised by the previous reviews with additional experimental results and revisions. The manuscript is now ready for acceptance for publication.

Minor note: The GEO accession number for the gene expression data described in the paper should be included in the final version of the manuscript.

