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Genome-wide analysis reveals a role for BRCA1 and PALB2 in transcriptional co-activation

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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.)

Editor:

1st Editorial Decision 13 June 2013

Thank you for submitting your research manuscript entitled "Genome-wide analysis reveals a role for BRCA1 and PALB2 in NF-kB and retinoic acid signaling" (EMBOJ-2013-85567) to our editorial office. It has now been seen by three referees and their comments are provided below. I apologize for the delay in reaching a decision, which was caused by one delayed report.

All referees are supportive of your work, noting that the dataset is interesting, novel and well executed. Nevertheless, they do raise several issues, which will require substantial revision as detailed below. Given these evaluations, I would like to give you the opportunity to revise your manuscript, with the understanding that the referees concerns must be addressed by additional experimentation where necessary, and that acceptance of the manuscript is likely to entail validation with a subset of the referees.

Specific issues
Ref #1
Pt.1/2 should be addressed experimentally. However, should the re-ChIP prove technically challenging, we are open to discuss this experiment further.

Pt.3/5 are related and address the specificity of the effects you observe - an issue that is also stressed by referee #3. It will require inclusion of data that you might already have available.

Pt.4 raises an interesting question that would allow you to strengthen the physiological significance of your observations. Therefore, we strongly encourage addressing this issue.

Ref #2

Pt.1 If you do have data on hand, please include it, but it is not essential.

Pt.2 In our view, the establishment of the precise mechanism of BRCA1 and PALB2 to their target loci is beyond the scope of this study. Mapping of the functional domains - albeit interesting - is a further reaching point that is not crucial for publication.

Pt.3 suggests addition of evidence that BRCA1 and PALB2 can be recruited by RAR - we encourage this development.

Pt.4 This control needs to be added.

Ref #3
Pt.1 raises the important issue of antibody specificity that needs to be addressed.

Pt.2 relates back to concerns regarding the specificity of the BRCA1/PALB2 transcriptional effects. This important issue is raised by all three referees and should be further investigated.

Pt.3 should be addressed.

I would like to add that it is our policy to allow only a single major round of revision and that it is therefore important to address all criticism at this stage. Please do not hesitate to contact me should any particular point require further clarification.

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, please visit our website:
http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

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

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REFEREE REPORTS:

Referee #1:

This manuscript describes the genomic occupancy, using ChIP-Seq, of the BRCA1 and PALB2, proteins usually associated with DNA repair. Recruitment of these proteins to DNA is associated with the elongating form of RNA polymerase and binding of both is seen throughout the coding region of their target genes (albeit with BRCA1 binding peaking at the transcription start site (TSS)). The authors find a strong association of their binding with genes regulated by the NF-kB transcription factor and retinoic acid. Both BRCA1 and PALB2 are shown, by shRNA depletion, to be functionally required for a subset of NF-kB target genes. Moreover, the p65 NF-kB subunit is found to be required for recruitment of BRCA1, PALB2 and Pol II at NF-kB target genes.

As the authors note, transcriptional effects of BRCA1 have been previously reported. Moreover, BRCA1 has also been identified as a regulator of NF-kB transcriptional activity. Nonetheless, this is an interesting and significant study as it reports for the first time the genome wide association of NF-kB with BRCA1 (as well as PALB2), thereby underlining the potential significance of this regulatory mechanism. However, in its current form, there are some aspects of this report that require strengthening.

Specific comments

An important conclusion of this report is the link between BRCA1/PALB2 and the elongating form of RNA Polymerase. As the authors note, they were unable to see an interaction between p65 and BRCA1/PALB2 by co-IP (although this has been reported by others). This raises the possibility that the recruitment of BRCA1/PALB2 by p65 is indirect and merely a consequence of its ability to recruit to PolII to target gene promoters. Might similar data be seen with other proteins that associate with elongating Pol II. Therefore:

(1) Re-ChIP analysis should be performed to see if p65 and BRCA1/PALB2 co-occupy their target gene promoters (at the TSS).

(2) BRCA1/PALB2 depletion does not affect p65 binding - but does it affect p65 dependent Pol II recruitment to the TSS of those genes dependent on this pathway for their TNF induced expression? Or is initial recruitment of Pol II by p65 unaffected, with elongation being where they exert their regulatory effects?

(3) BRCA1/PALB2 depletion affects a subset of TNF inducible NF-kB target genes. It was unclear to me if they were still recruited to the genes that were not affected (implying functional redundancy at those genes) or if recruitment is selective. If the latter, it may imply recruitment dependent upon a specific post-translational modification of p65 (such as S536 or S468), which have been shown to have gene specific effects.

(4) BRCA1 and PALB2 are encoded by genes whose mutation is associated with breast and ovarian cancer. An important implication of this data, therefore, is that NF-kB function will be impaired in breast and ovarian cancer cells with BRCA1/PALB2 mutations. Is NF-kB dependent gene expression affected in cell lines containing such mutations?

(5) Inclusion of some more negative controls would be useful. For example, Fig. 4D shows effects of p65 depletion on BRCA1/PALB2 recruitment at specific target genes. Some genes where p65 did not affect BRCA1/PALB2 recruitment would demonstrate the specificity of these effects (e.g. RA regulated genes).

Other point

(6) The authors should cite the recent paper by Harte et al (Oncogene 2013) that also links NF-kB and BRCA1 function.

Referee #2:

In the present communication Gardini and colleagues performed genome-wide analysis of chromatin occupancy of two breast and ovarian cancer susceptibility proteins BRCA1 and PALB2 and their roles in gene expression responsiveness in breast epithelial cells. They found that BRCA1 and PALB2 co-reside in a substantial number of genes and the occupancy associates with high transcriptional activity at those loci. The authors further showed that BRCA1 and PALB2 are important for transcriptional response to activation of NF B by the pro-inflammatory cytokine TNF and to retinoic acid (RA). The findings are novel and provide certain sights into the role of BRCA1 and PALB2 as guardian of the genome.

Major concerns:
1. Although the overall pattern of Pol II occupancy on chromatin seems similar to those of BRCA1 and PALB2, it is much closer to BRCA1 than PALB2 (Fig. 2A). Thus, in Fig. 2C the authors should also compare the occupancy pattern of P-Ser2 with that of BRCA1, findings from which may provide certain sights into the observation that PALB2 plays a role to a smaller extent than BRCA1 in response to stimuli such as TNF (Fig. 5D).
2. The authors demonstrated that depletion of p65/RelA abolished the binding of BRCA1, PALB2 and Pol II at NF B response promoters examined. In contrast, they found that depletion of BRCA1 or PALB2 did not affect the occupancy of p65. They concluded that p65 mediates recruitment of BRCA1 and PALB2. These are very interesting findings. However, a number of outstanding issues

remain. First, how does p65 recruit BRCA1 and PALB2 into their target loci and what are the underlying mechanisms? Second, what's the role for BRCA1 in p65 mediated gene activation in response to TNF stimulation? At least the authors should provide information regarding which functional domain(s) of BRCA1 or PALB2 are required for p65-mediated transactivation.
3. Does BRCA1 or PALB2 utilize similar mechanisms to mediate gene activation in response to retinoic acid as to TNF ? If so, can BRCA1 or PALB2 be recruited by retinoic acid receptor into target loci and how?
4. In Fig. 6E, the authors showed that depletion of BRCA1 or PALB2 diminished RA-induced inhibition of cell growth. However, it is unclear if this is a RA specific effect or simply caused by the general effect of BRCA1 or PALB2 depletion on cell cycle progression and cell proliferation.

Minor concerns:
1. IgG ChIP should be included in Figs. 4D and 5C.
2. The authors stated that depletion of BRCA1 or PALB2 resulted in inhibition of transcription of 101 and 92 class I genes, respectively, including 56 co-regulated genes. In this case, the numbers in the Venn diagram in Fig. 3C should be corrected.

Referee #3:

This m/s reports a genome-wide ChIP-seq analysis of BRCA1 and PALB2 in the human breast epithelial cell line MCF10A. The authors report association of BRCA1 and PALB2 with actively transcribed genes, with accumulation of the ChIP signal within gene bodies and/or at the transcribed gene's transcription start site (TSS). The distribution appears to match that of RNA Pol II, including the actively transcribing form of the RNA Pol complex. Indeed, high occupancy by BRCA1 and PALB2 (373 genes) correlates with high levels of gene expression and knock-down of BRCA1 or of PALB2 reduces levels of transcription of these genes. The BRCA1 and PALB2 signal at actively transcribed genes is suppressed by flavopiridol, an inhibitor of cdks and of the transcription elongation factor P-TEFb. BRCA1 ChIP signal was also found to be associated with a fraction of tRNA genes and some other PolIII genes. Using a prediction algorithm that assumes specific regulation of gene expression by BRCA1 and PALB2, the authors focus on NFkappaB signaling. The statistics underlying the use of this algorithm are not clear. They report that siRNA-mediated depletion of p65/RelA reduces the BRCA1, PALB2 and RNA PolII ChIP signal at p65 target genes, although the specificity of this effect is not clear (no negative controls are shown). Conversely, treatment of cells with TNFalpha promotes recruitment of BRCA1 and PALB2 to target genes. The authors also study the response to retinoic acid (RA), for reasons that are not clear, and report that RA-induced activation of target promoters increases the ChIP signal of BRCA1 and PALB2 at these loci.

A role for BRCA1 in general transcription has been suggested by previous work (cited in the m/s). However, the mapping of BRCA1 and PALB2 to gene bodies has not been previously reported and is of general interest. Certain major issues need to be clarified:

1. Antibody specificity. The anti-BRCA1 and anti-PALB2 Abs used may cross-react with other cellular proteins. The data reported using other anti-BRCA1 Abs (Suppl Fig 2) is not adequate validation. Full ChIP-seq localization of BRCA1 should be repeated with at least one different BRCA1 antibody and with a one different PALB2 Ab. An alternative is to use epitope-tagged BRCA1 and PALB2 proteins, and to demonstrate that the localization by ChIP-seq (not just by a small number of selected ChIP experiemnts, as in Suppl Fig 2) is similar to that identified for the current set of ChIP-seq experiments.

2. Biological specificity. The findings suggest that BRCA1 and PALB2 co-localize with elongating RNA PolII as well as at the TSS of PolII and PolIII -regulated genes. The manipulation of TNFalpha, NFkappaB and RA responses suggest that BRCA1 and PALB2 support gene activation in response to these signals, but the experiments presented do not speak to the specificity of this effect. In particular, the RA signaling experiments seem to have a weak rationale. In the absence of convincing negative controls, one could conclude that BRCA1 and PALB2 support transcriptional activation in a non-specific fashion-this is indeed suggested by the close co-localization with PolII by ChIP-seq and by pre-existing publications on BRCA1. The concept that BRCA1 and PALB2 support transcriptional activation of a specific subset of genes would be strengthened if the authors

could report detailed negative control examples of signal-induced transcription activation responses that are not dependent upon BRCA1 or PALB2.

3. Interaction with the DNA damage response. One interpretation of the flavopiridol experiments is that the drug induces a DNA damage response that recruits BRCA1 and PALB2 away from their basal locations at highly expressed genes. It would be desirable to study the effect of other transcription inhibitors (e.g., DRB). It would also be valuable to determine whether the localization of BRCA1/PALB2 ChIP-seq signal is perturbed by acute extrinsic DNA damage. Repeat of the ChIP-seq experiments following exposure of cells to ionizing radiation would be one way to elicit such a response.

1st Revision - authors' response 11 December 2013

Response to Reviewer 1:

We thank the reviewer for finding our work interesting and significant.

An important conclusion of this report is the link between BRCA1/PALB2 and the elongating form of RNA Polymerase. As the authors note, they were unable to see an interaction between p65 and BRCA1/PALB2 by co-IP (although this has been reported by others). This raises the possibility that the recruitment of BRCA1/PALB2 by p65 is indirect and merely a consequence of its ability to recruit to PolII to target gene promoters. Might similar data be seen with other proteins that associate with elongating Pol II. Therefore:

(1) Re-ChIP analysis should be performed to see if p65 and BRCA1/PALB2 co-occupy their target gene promoters (at the TSS).

We have tried to address this point by performing Re-ChIP experiments using p65, PALB2 and BRCA1 antibodies, either in MCF7 or MCF10A cells. Despite our effort, this has proved technically difficult and the results were not reliable due to large variability in the qChIP deltaCt values.

(2) BRCA1/PALB2 depletion does not affect p65 binding - but does it affect p65 dependent Pol II recruitment to the TSS of those genes dependent on this pathway for their TNF induced expression? Or is initial recruitment of Pol II by p65 unaffected, with elongation being where they exert their regulatory effects?

We have performed the analysis on RNAPII recruitment using antibodies against total and phosphorylated RNAPII in MCF7 cells. New Figure 7B presents the results obtained with the group of genes where p65 occupancy was previously examined (Figure. 6C). As shown in Figure. 6D, BRCA1 depletion impairs TNFalpha activation of IL8, CCL20, CXCL1 and CXCL3. PALB2 depletion has a strong effect on IL8, but does not impact activation of the other genes. ChIP analysis revealed that RNAPII recruitment is severely impaired when BRCA1 or PALB2 depletion resulted in decreased activation of TNFalpha responsive genes, similar to that seen following p65 depletion. Therefore, while we find a functional association between BRCA genes and elongating form of RNAPII, the diminished responsiveness of NF-kB genes following depletion of BRCA1 or PALB2 stem from the lack of RNAPII recruitment reflecting a defect at initiation. This is now discussed in the revised manuscript.

(3) BRCA1/PALB2 depletion affects a subset of TNF inducible NF-kB target genes. It was unclear to me if they were still recruited to the genes that were not affected (implying functional redundancy at those genes) or if recruitment is selective. If the latter, it may imply recruitment dependent upon a specific post-translational modification of p65 (such as S536 or S468), which have been shown to have gene specific effects.

We assessed BRCA1 and PALB2 recruitment at three genes (IRF1, BCL3 and MAP3K8) that are responsive to TNFalpha in our screening (Table S4) and are dependent on p65 but not BRCA1 and PALB2 for their activation. In all 3 cases, BRCA1 and PALB2 are still recruited to the TSS in a stimulus-dependent manner, mirroring the recruitment of p65 (Supplementary Figure. 5B). We agree with the reviewer that this might be reflective of functional redundancy at those genes. This is now discussed in the revised version of the manuscript.

(4) BRCA1 and PALB2 are encoded by genes whose mutation is associated with breast and ovarian cancer. An important implication of this data, therefore, is that NF-kB function will be impaired in breast and ovarian cancer cells with BRCA1/PALB2 mutations. Is NF-kB dependent gene expression affected in cell lines containing such mutations?

We compared the TNF-alpha responsiveness of HCC1937 cell lines (it synthesizes a truncated BRCA1 protein that is the product of a disease-producing mutant allele (5382insC) that were reconstituted by wild type BRCA1 or vector alone (Scully e t al., 1999). Importantly, we observed an increased TNF-alpha responsiveness of HCC1937 cells expressing wild type BRCA1 compared to parental cells expressing only the vector (New Figure 7A). These data are consistent with a role for BRCA1 in TNF-alpha responsiveness.
We further examined a recently published in vivo datasets (Proia et al., Cell Stem Cell, 2011). In this paper, the authors examine the role of BRCA1 in lineage differentiation using human cells derived from prophylactic mastectomies of individuals carrying BRCA1 mutations (-/+). As a control (+/+), they use tissues from reduction mammoplasty of healthy individuals. Cells are infected with a cocktail of activated oncogenes and injected into 'humanized' fat pads of mice, the originated tumors are subjected to transcriptional profiling. The authors of the study find a large number of genes differentially regulated in BRCA1 mutants (-/+) compared to the control (+/+). Our analysis on this group of regulated genes retrieved NFkB as a major pathway affected. We conducted this analysis using the Ingenuity Pathway Analysis suite. Specifically, the Upstream Regulator Analysis uses a proprietary algorithm to identify upstream regulators on a dataset of genes and predict whether they are activated or inhibited given the expression changes of the dataset. This is based on a literature compiled list of expected causal effects between an upstream regulator (be it a transcription factor or a molecule) and its target. Strikingly, TNF and NFkB scored 2nd and 6th in the list of the most significant regulators (z-score ranked). We comment on these results in the revised version of the manuscript and we include the full analysis as an additional table (Table S6).

(5) Inclusion of some more negative controls would be useful. For example, Fig. 4D shows effects of p65 depletion on BRCA1/PALB2 recruitment at specific target genes. Some genes where p65 did not affect BRCA1/PALB2 recruitment would demonstrate the specificity of these effects (e.g. RA regulated genes).

We agree with the reviewer that this is an important control, the new Figure 5E, includes the constitutively expressed growth regulator FOS and the RA-responsive HOXA1 as negative controls.

Other point

(6) The authors should cite the recent paper by Harte et al (Oncogene 2013) that also links NF-kB and BRCA1 function.
This has been cited in the revised version of the manuscript.

Response to Reviewer 2:
We would like to thank the reviewer for finding our study novel and that it will provide further sights into the role of BRCA1 and PALB2 as guardian of the genome.

1. Although the overall pattern of Pol II occupancy on chromatin seems similar to those of BRCA1 and PALB2, it is much closer to BRCA1 than PALB2 (Fig. 2A). Thus, in Fig. 2C the authors should also compare the occupancy pattern of P-Ser2 with that of BRCA1, findings from which may provide certain sights into the observation that PALB2 plays a role to a smaller extent than BRCA1 in response to stimuli such as TNFα (Fig. 5D).

The reviewer raised an important point and we now show Ser2 average profiles and heatmap analysis along with BRCA1, PALB2 and RNAPII in New Figure 3C and in Supplementary Figure 3.

Indeed, BRCA1 profile is closer to total RNAPII and its effect on transcription is more profound, as compared to PALB2, both at the RNA level (Figure 6) and in terms of RNAPII recruitment (New Figure 7B). Even though BRCA1 and PALB2 are known to be part of the same multi-protein complex, PALB2 appears to be a modular component, which is recruited to the TSS in a stimulus dependent manner like BRCA1 but tends to be more associated to the elongating form of RNAPII and accumulates at the 3' end of genes, suggesting a more prominent role in 3' termination or mRNA maturation.

2. The authors demonstrated that depletion of p65/RelA abolished the binding of BRCA1, PALB2 and Pol II at NFκB response promoters examined. In contrast, they found that depletion of BRCA1 or PALB2 did not affect the occupancy of p65. They concluded that p65 mediates recruitment of BRCA1 and PALB2. These are very interesting findings. However, a number of outstanding issues remain. First, how does p65 recruit BRCA1 and PALB2 into their target loci and what are the underlying mechanisms? Second, what's the role for BRCA1 in p65 mediated gene activation in response to TNFα stimulation? At least the authors should provide information regarding which functional domain(s) of BRCA1 or PALB2 are required for p65-mediated transactivation.

Our data demonstrate the requirement of BRCA1 and PALB2 at several NFkB-regulated genes. We performed a set of ChIP-seq and RNA-seq experiments whose unbiased analysis reported an impairment of the NFkB response. Such finding extends and corroborate a previous report by the Licht laboratory (Benezra et al., JBC 2003), which described an association between NFkB and BRCA1. In the original paper, the authors showed enhanced activation of a reporter plasmid when BRCA1 was co-transfected along with p65 in 293T cell. They further characterized the association between p65 and BRCA1 with in vitro GST pull down and transient co-trasfection experiments. They mapped the interaction to the Rel homology domain (RHD) of p65 and to a broad N-terminal region of BRCA1 encompassing approximately 300 amino acids. Interestingly, Benezra et al. also report that BRCA1 fails to enhance p65 transactivation of a phosphorylation mutant (S276A). This mutation of p65 does not affect its physical interaction with BRCA1. Since phosphorylation of S276 is important to recruit CBP, the authors hypothesize that BRCA1 helps stabilizing the interaction with CBP.
Our data extend these findings and indicate that BRCA1 is critical for the recruitment of RNAPII at p65 target sites (new Figure 7B). Moreover, depletion of BRCA1 also diminishes the Ser2 and Ser5 phosphorylated RNAPII in vivo, suggesting an important role for BRCA1 in recruitment of RNAPII and its phosphorylated forms to its transcriptionally responsive genes.
On the other hand, PALB2 is likely to be recruited to NFkB targets through BRCA1 itself. While PALB2 was originally identified as a BRCA2 interacting protein (Xia et al. Mol Cell 2006), later studies (Zhang et al, Curr Biol 2009; Sy et al. PNAS 2009; unpublished data from our laboratory) placed this protein at the core of BRCA1/BRCA2 complexes.
We are now discussing this point more extensively in the revised version of the manuscript.

3. Does BRCA1 or PALB2 utilize similar mechanisms to mediate gene activation in response to retinoic acid as to TNFα? If so, can BRCA1 or PALB2 be recruited by retinoic acid receptor into target loci and how?

We have performed a knock-down experiment in MCF7 cells prior to stimulation with Retinoic Acid (New Figure 8d). We show that, upon depletion of RARalpha, BRCA1 and PALB2 reduce proportionally their occupancy of HOXA1 and HOXA2 proximal promoters at an early time point of stimulation (6hr), suggesting a direct recruitment of the BRCA proteins at RA responsive genes.

4. In Fig. 6E, the authors showed that depletion of BRCA1 or PALB2 diminished RA-induced inhibition of cell growth. However, it is unclear if this is a RA specific effect or simply caused by the general effect of BRCA1 or PALB2 depletion on cell cycle progression and cell proliferation.

The growth curve we presented in Figure 6 (New Figure 9C) plot cell counts under RA stimulation that are normalized to unstimulated cells, to minimize non-RA dependent growth effects due to BRCA1 and PALB2 knock down. We know include an additional panel (Supplementary Figure 7B) showing the growth curve of unstimulated cells with BRCA1, PALB2 and CTRL shRNAs. Despite a detrimental effect on basal cell growth, BRCA1 depletion remarkably delays the proliferative arrest induced by retinoic acid in MCF7 cells.

Minor concerns:
1. IgG ChIP should be included in Figs. 4D and 5C.
We have included IgG control bars in New Figure 5d and, due to space constraints, we have added IgG controls relevant to New Figure 6c as a separate panel (Supplementary Figure. 5A).

2. The authors stated that depletion of BRCA1 or PALB2 resulted in inhibition of transcription of 101 and 92 class I genes, respectively, including 56 co-regulated genes. In this case, the numbers in the Venn diagram in Fig. 3C should be corrected.
We have made the requested amendment in New Figure 4C.

Response to Reviewer 3:
We wish to thank the referee for finding our work interesting; we have responded to his/her comments below.

1. Antibody specificity. The anti-BRCA1 and anti-PALB2 Abs used may cross-react with other cellular proteins. The data reported using other anti-BRCA1 Abs (Suppl Fig 2) is not adequate validation. Full ChIP-seq localization of BRCA1 should be repeated with at least one different BRCA1 antibody and with a one different PALB2 Ab. An alternative is to use epitope-tagged BRCA1 and PALB2 proteins, and to demonstrate that the localization by ChIP-seq (not just by a small number of selected ChIP experiemnts, as in Suppl Fig 2) is similar to that identified for the current set of ChIP-seq experiments.

To address the reviewers concerns, we have performed an additional ChIP-seq experiment in MCF10A cells using a monoclonal antibody against BRCA1 (the same antibody that is being used in several qChIP experiments throughout the paper). We present a comparative analysis of the previously used polyclonal and the monoclonal anti-BRCA1 in New Figure 2. There is a compelling overlap between both antibodies genome-wide as evidenced by a heatmap analysis of read intensity across all human RefSeq genes (Figure 2A). We also present a few snapshots to compare the occupancy of both antibodies at different chromosome locations (Figure 2B and C). We have tested other commercially available antibodies against PALB2 and we were not able to use them successfully in either CoIP or ChIP, while the polyclonal antibody used throughout this study pulls down efficiently PALB2 and its partner BRCA2 (Figure 4A). We have performed additional validation with a Flag-PALB2 stably transfected cell line (Supplementary Figure 2F), the Flagged-protein however was not suitable for a genome-wide analysis due to known cross-reactivity of anti-FLAG antibodies with the histone methyltransferase PRMT5 and its partner MEP50. This is noticeable in the control 293T cell line, where the recovery of chromatin is significantly lower than Flag-PALB2 but constantly above IgG levels.

2. Biological specificity. The findings suggest that BRCA1 and PALB2 co-localize with elongating RNA PolII as well as at the TSS of PolII and PolIII -regulated genes. The manipulation of TNFalpha, NFkappaB and RA responses suggest that BRCA1 and PALB2 support gene activation in response to these signals, but the experiments presented do not speak to the specificity of this effect. In particular, the RA signaling experiments seem to have a weak rationale. In the absence of convincing negative controls, one could conclude that BRCA1 and PALB2 support transcriptional activation in a non-specific fashion-this is indeed suggested by the close co-localization with PolII by ChIP-seq and by pre-existing publications on BRCA1. The concept that BRCA1 and PALB2 support transcriptional activation of a specific subset of genes would be strengthened if the authors could report detailed negative control examples of signal-induced transcription activation responses that are not dependent upon
BRCA1 or PALB2.

The reviewer has raised an important point regarding how specific and general are the effects of the BRCA proteins on gene stimulation. To address this question we analyzed the transcriptional response to EGF and to the p53 activator Nutlin, two widely studied signaling pathways with enormous implications on epithelial cell growth, cell cycle control and genomic stability. EGF stimulation was carried out in HeLa cells while Nutlin treatment was performed in HCT116 cells. In both conditions, BRCA1 and PALB2 shRNAs do not significantly alter the transcriptional response

compared to the non-targeting control, arguing for a specificity of their effect on NF-kB and Retinoic acid signaling (New Supplementary Figure 6).
The specificity of BRCA1/PALB2 effect on NF-kB is further corroborated by our analysis on a gene expression dataset of defective BRCA1 epithelial cells (Table S6) which we now discuss in the manuscript.

3. Interaction with the DNA damage response. One interpretation of the flavopiridol experiments is that the drug induces a DNA damage response that recruits BRCA1 and PALB2 away from their basal locations at highly expressed genes. It would be desirable to study the effect of other transcription inhibitors (e.g., DRB). It would also be valuable to determine whether the localization of BRCA1/PALB2 ChIP-seq signal is perturbed by acute extrinsic DNA damage. Repeat of the ChIP-seq experiments following exposure of cells to ionizing radiation would be one way to elicit such a response.

We understand the reviewer's concern about DNA damage response being the indirect cause of BRCA1 and PALB2 decrease at highly transcribed genes. Flavopiridol induces DNA damage and stabilization of p53, although this generally happens at longer time points (cf. Bible et al., Cancer Res 2000). To further address this point, we used Mitomycin C (MMC) a potent DNA interstrand crosslinker that leads to the formation double strand breaks. We treated exponentially growing MCF10A with MMC for 6h and analyzed the chromatin occupancy of BRCA1, PALB2, the elongating RNAPII as well as the steady state of mRNA at four NFkB target genes previously examined with flavopiridol (Figure 3D).
Mitomycin C treatment significantly reduced the levels of BRCA1, and elongating form of RNAPII to a similar extent (Supplementary Figure 4). Corresponding mRNA levels are also decreased by 30-40% (with the exception of SOD2, which appears to have a longer half life). While depletion of BRCA1, PALB2 and RNA Polymerase (as well as the decay of mRNA levels) is far more pronounced after 2h of transcriptional elongation block following Favopiridol treatment, the decrease in BRCA occupancy and inhibition of transcription following MMC treatment suggests that a component of Flavopiridol responsiveness may be the result of the drug inducing a DNA damage response. This is now included in the revised manuscript.

2nd Editorial Decision 16 January 2014

Thank you for submitting your revised manuscript on BRCA1/PALB2 transcription roles for our considerations, and my apologies for the slight delay in obtaining the re-reviews from the referees at this time of the year. As you will see from the enclosed comments of referees 1 and 3, they consider several important parts of the study now substantiated. However, one of the points emphasized earlier by my colleague Anke Sparmann in her original decision letter remains problematic, as expanded on by referee 3: the issue of biological specificity of BRCA1/PALB2 roles in TNF/NF-kB and RA/RAR signaling responses. I realize that this is to some extent addressed by the new negative control data on EGF and Nutlin expression responses, but referee 3 nevertheless makes some well-taken points regarding the presentation and interpretation of the data, which appear to be indicative of a more general transcription supporting role rather then a fully pathway-specific effect. Moreover, the referee still remains unconvinced by the stated rationale for focussing on NF-kB and RA signaling responses. In this light, it would appear that

In my view, addressing these remaining issues will primarily require re-analyses of the data you already obtained, including the more complete statistical analyses, induction ratio calculation and other quantifications, and alternative data plotting and presentation, to allow for the more direct assessment and facilitated interpretation requested by referee 3. Depending on the outcome of this re-analysis, please then carefully consider whether the respective conclusions (including those in title and abstract) would need further modification/qualification. In any case, I would certainly appreciate your detailed thoughts in response to the reviewer's arguments.

At this stage, I am therefore returning the study to you once more for an ultimate round of revision, hoping that the referee comments will help to improve the study to one with ultimately firmly supported conclusions. When sending you final version, I would like ask you to include (with the

resubmission cover letter) a list of 2-5 one-sentence 'bullet points' (complementary to the abstract) that summarize key aspects of the paper - they will accompany the online version of the article as part of a 'synopsis'. Please see the latest research articles on our renewed website (emboj.embopress.org) for examples.

Should you have any further question regarding the referee reports and the final revision, please do not hesitate to contact me and I'd be happy to discuss them.

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REFEREE REPORTS:

Referee #1:

The authors have addressed my original concerns.

I have one minor comment which is that the formatting of the pdf for new Table S6 seemed to be off, such that the different columns did not fit in the width of one page.

Referee #3:

The resubmitted paper shows some improvements. For example, the repeated ChIP-seq using a second BRCA1 Ab is good evidence of an accurate signal. The association of BRCA1 and PALB2 with TSS and gene bodies is convincing. The connection to NFkB remains less compelling and the rationale for studying RA signaling is still unclear. Some of the data (e.g., in Suppl Tables) is incompletely processed and inadequately described, making it difficult to know how to interpret the data, or what the significance of the data is in some cases. For example, in Table 4, a series of log values is presented for a selected set of genes, but this is not a complete picture, since the number of genes shown is small and the degree of enrichment of each gene following TNF stimulation is not calculated directly. A waterfall plot would present this in an unbiased fashion. The data presented here does not give the reader adequate information with which to interpret and critique the full data set. For much of the individual NFkB pathway gene analysis, P values are provided only in selected cases. For example, in Figure 5 P values are provided for the positive results but not for the negative controls. In general, P values should be calculated for each comparison that the authors report, with adequate discussion of discrepant results. The justification for focusing on the NFkB pathway as a specific physiological target is similarly opaque. The Table 3 list of "over-represented matrices in the promoter regions of BRCA1/PALB2 target genes" includes apparently some non-mammalian transcription factor motifs. The authors present no information that might help the reader to understand what the DNA sequence of these enriched elements is, nor do they discuss how they came to ignore some of these elements and home in on NFkB alone. The lack of reasoned discussion of this underscores the impression that NFkB has been "cherry-picked" for analysis. Despite previous requests for a more compelling and data-based reasons to study the RA signaling pathway, the authors justify the study of this pathway as follows:

"Since these proteins occupy a large number of transcriptionally active genes, we envisage that they may play a broader role in stimulus-dependent transcriptional activation. To test this contention, we turned our attention to the retinoic acid (RA) signaling, which was previously shown to have anti-proliferative effects in breast cancer cells".

This vague and subjective rationale does not inspire confidence in the subsequent analysis, and suggests that the effects of BRCA1 and PALB2 on gene transcription may be less targeted and specific than the authors claim. Analysis of the impact of BRCA1/PALB2 depletion on TNF alpha-responsive genes (Figure 6) shows that, while the activated signal is reduced by loss of BRCA1/PALB2, the basal transcription level is also often reduced proportionately, or even to a greater extent, by depletion of BRCA1 or PALB2. The authors do not calculate the TNF alpha inducibility directly (e.g., by the ratio of TNF-induced:uninduced signal), but this ratio, if quantified, might well not show a consistent role for BRCA1 or PALB2. Overall, the data suggests that BRCA1 and PALB2 are playing largely supportive roles in TNF-alpha-dependent transcription. The incomplete statistical analysis, apparent "cherry-picking" of NFkB and RA signaling and incomplete

analysis of the data make the claim of specific roles for BRCA1 and PALB2 in regulation of these genes less compelling. In general, the use of unselected methods of summarizing the complete set of transcription data (e.g. waterfall plots) would be more authoritative than the current methods of presentation.

I think the authors have documented an association of BRCA1 and PALB2 with TSS and gene bodies and have good evidence in support of a role in general transcription. This is interesting data in its own right. The notion that BRCA1 and PALB2 specifically regulate NFkB and RA signaling is less convincing.

2nd Revision - authors' response 29 January 2014

Response to Referee 1

The formatting of Table S6 has now been corrected.

Response to Referee 3

We like to thank the reviewer for finding our genome-wide analysis important and able to provide important information with regards to the role of BRCA1 and PALB2. We have performed additional data analysis and quantification to provide a comprehensive picture of BRCA1 and PALB2 function in NF-kB responsiveness. Moreover, we have provided additional panels to Table 3 to fully illustrate the bioinformatics analysis of motif searches. Below you will find a point-by-point discussion of our response.

1-The association of BRCA1 and PALB2 with TSS and gene bodies is convincing. The connection to NFkB remains less compelling

As indicated in the manuscript, we analyzed the gene expression changes following depletion of BRCA1 and PALB2 in genes highly occupied by these two proteins. This analysis revealed a significant number of genes commonly down regulated by BRCA1 and PALB2. We unbiasedly searched for upstream regulators of these repressed genes and found NF-kB as the most significant transcription factor according to the Ingenuity Pathway Analysis tools ($p<4.31E-12$). We validated this finding by depleting p65/RelA and measuring the responsiveness of a candidate set of genes (Figure 5C).

Moreover, we performed a motif search in promoters of genes with the highest occupancy for BRCA1 and PALB2. These results indicated that from the top 10 motifs recovered, 4 corresponded to human DNA motifs (6 motifs corresponded to DNA sequences in other species) and 3 of the 4 human motifs represented NF-kB binding sites. This is now included in Supplementary Table 3 and discussed in the revised manuscript.

2-Some of the data (e.g., in Suppl Tables) is incompletely processed and inadequately described, making it difficult to know how to interpret the data, or what the significance of the data is in some cases. For example, in Table 4, a series of log values is presented for a selected set of genes, but this is not a complete picture, since the number of genes shown is small and the degree of enrichment of each gene following TNF stimulation is not calculated directly.

We found 37 genes (33 from the microarray and 4 genes discovered from initial RNA-sequencing) that display significant ($log2(foldChange)>0.4$, $p<0.01$) activation following TNF-alpha stimulation in MCF7 cells. As shown in Figure 6D and E and commented by the reviewer, depletion of BRCA1 and PALB2 diminished the basal or ongoing levels of transcription of a large number of these genes prior to their stimulation with TNF-alpha (this had been initially observed and documented in Figure 4 for basal activity). Therefore, while following TNF-alpha induction we observed a diminished responsiveness for 18 out of 37 genes depleted of BRCA1 and 10 out of 37 genes depleted of PALB2, the basal to stimulated ratio or the fold change of most genes did not change. This was predominantly due to the robust decrease in basal activity observed with depletion of BRCA1 and PALB2.

While this was amply documented for 12 genes using real-time PCR in Figures 6D and E, we now have included the heat map representation of the results for all 33 genes on the array following BRCA1 and PALB2 depletion prior to and following stimulation with TNF-alpha (Supplementary Figure 6). Moreover, we show the fold change for all 33 genes in Supplementary figure 7, which indicates that depletion of BRCA1 and PALB2 does not diminish the fold change of most TNF-alpha responsive genes. Indeed, a number of genes display an increased fold change since their basal activity is greatly reduced. This is now added to the re-revised manuscript.

3- For much of the individual NFkB pathway gene analysis, P values are provided only in selected cases. For example, in Figure 5 P values are provided for the positive results but not for the negative controls.

p values of FOS and HOXA1 in figure 5 were not indicated because not significant, this is now specified in the legend.

4- Reasons for studying retinoic acid pathway:

The retinoic acid pathway was chosen predominantly due to its prominence in causing cellular differentiation and its anti-proliferative effects in many cancer cell lines including breast cancer lines. Moreover, our initial analysis of candidate RA-responsive genes, HOXA1 and HOXA2, following depletion of BRCA1 and PALB2 demonstrated a significant diminution in RA-responsiveness, which motivated us to perform additional genome-wide analysis on this class of genes.

5- Overall, the data suggests that BRCA1 and PALB2 are playing largely supportive roles in TNF-alpha-dependent transcription.

We agree with the referee and the assertion that BRCA1 and PALB2 play a modulatory role in TNF-alpha responsiveness and this effect is most pronounced when one measures the ongoing (basal) transcriptional activity of these genes and is not reflected in the fold activation. We have revised the manuscript to reflect this point and further highlight that the transcriptional effects of BRCA1 and PALB2 may not be specific to any one signaling pathway and these proteins similar to other transcriptional co-activators may have a broad range of transcriptional regulatory effects.

