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A BRCA1 interacting lncRNA regulates homologous recombination

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Editor: Esther Schnapp

1st Editorial Decision 24 April 2015

Thank you for the submission of your research manuscript to EMBO reports. I apologize for my late reply, I was not in the office this week. We have now received the enclosed referee reports on your study.

As you will see, all referees note that the effects of DDSR1 depletion are rather minor, and that the biological significance of this lncRNA in the DNA damage response remains somewhat unclear. Referees 2 and 3 remark that it should be tested whether DDSR1 knockdown affects DNA end resection, and referee 1 suggests that a role for hnRNPUL1 in DDSR1-mediated BRCA1 and RAP80 recruitment should be analyzed. All referees also pinpoint several missing controls and statistical analyses.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as mentioned above and in their reports) must be fully addressed and their suggestions taken on board. I think that in this case, all referee concerns should be addressed, as they are all either technical or relate to the question whether DDSR1 has a functionally relevant role in the DNA damage response, which needs to be demonstrated for publication of the work by EMBO reports.

Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is

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EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript. Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the issue further.

I suggest that we publish your manuscript as a normal article (in case of favorable referee reports) and not as a short report. The difference is that you have no length restrictions, the results and discussion sections are separate and all methods are included in the main manuscript file. Please note that the reference style needs to be changed to the numbered EMBO reports style.

Regarding data quantification, can you please specify the number "n" for how many experiments were performed, along with the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends? This information is currently incomplete and must be provided in the figure legends.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS:

Referee #1:

Sharma and colleagues identify lncRNAs induced by DNA damage, and characterize a lncRNA they named DDSR1. DDSR1 is inducible by ATM, NF-kB, and p53, although p53 is not required for DNA damage inducibility. The novel findings are that DDSR1 depletion affects homologous recombination efficiency and recruitment of BRCA1 and RAP80 to sites of DNA damage. Nonetheless, several important controls are needed to interpret these results, and the mechanism by which a lncRNA can affect DNA repair, either directly or indirectly, are still unclear. As such, this work is not ready to be published in the present form.

1. Level of DDSR1. The authors document a ~2-5 fold induction of this lncRNA. What is the absolute copy number in cells? Is the number sufficient to mediate the proposed effects at sites of DNA damage directly?

2. A single siRNA was used to deplete DDSR1, followed by analysis of gene expression effects and all downstream analyses. Multiple independent siRNAs knockoing down DDSR1 should be analyzed to exclude off target effects. This is the standard applied to coding and noncoding genes for RNAi experiments.

3. Effect of DDSR1 on DDR signaling. The authors report a 30% decrement of several phosphor-proteins upon DDSR1 depletion. This is a quite modest effect that may not have any biological significance. Moreover, to make such a modest difference convincing, multiple replicates and error bars should be shown.

4. MS2-TRAP RNA affinity purification was used to identify DDSR1 interacting proteins. Does the MS2-DDSR1 fusion complement DDSR1 depletion? Without knowing that the fusion RNA is functional, the relevance of the resulting complex is unclear.

5. It is unclear whether the effects of DDSCR1 on BRCA1 and RAP80 recruitment to DNA damage sites are direct or indirect. Do they depend on hnrnpUL1, or other effects on gene expression documented above?

Referee #2:

In this manuscript the authors identify the lnRNA DDSR1 as a new player in DDR and Homologous

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Recombination. Upon treatment with several DSB-inducing agents, DDSR1 is expressed in an ATM-NFkB dependent way and represses the expression of p53 target genes. Furthermore, DDSR1 binds to the RNA binding protein hnRNPUL1 and regulates the level of the BRCA1-RAP80 complex at DSB.
The first part of the ms describing the induction of DDSR1 and its involvement in the ATM-NF-KB and p53 pathways is convincing. The second part probing into the mechanisms of action is less so, with differences reported not being very dramatic and, at times, questioning their biological relevance.

The major concerns of this referee are the following ones:
1. In general, it is not shown whether the effect of DDSR1 knockdown on DNA end resection and HR is direct or due to altered expression of HR proteins. The level of the main proteins involved in DNA-end resection and homologous recombination (or, to the least, their mRNA levels) should be checked in control and DDSR1 knockdown samples. The fact that DDSR1 is not recruited to laser induced DSB may support an indirect role of DDSR1 in this process.
2. The authors also report an interaction between DDSR1 and hnRNP1 and claim that this interaction may contribute to DNA end resection activity. As also mentioned by the authors, Polo et al. in 2012 reported an RNA-independent recruitment of hnRNPUL1 to DNA damage sites, where it favors DNA end resection. Although it is possible, as the authors suggest, that DDSR1 modulates the activity but not the localization at DSB of hnRNP1, this is, at this stage, amply speculative.
3. Since kd of DDSR1 affects cell proliferation, the authors should demonstrate that the reduced DDR activation is not the consequence of reduced cell proliferation. Furthermore, the reduced RPA, H2AX and Chk1 activation shown by western blots would be more convincing if the total level of the proteins was shown.
4. The authors show an enhanced recruitment of the BRCA1-RAP80 complex upon DDSR1 knockdown. The ability of this complex to inhibit DNA end resection could explain why RPA and Chk1 phosphorylation is reduced upon DDSR1 knockdown. Considering the different roles of BRCA1 at DSB, it would be interesting (and experimentally easy) to test whether DNA end resection is actually affected by monitoring ssDNA (by BrdU staining under non denaturing conditions) and RPA levels.

Referee #3:

This m/s reports identification of a lncRNA, named by the authors "DNA Damage-Sensitive RNA 1" (DDSR1) that is induced 2- to 3-fold in response to DNA damage. This induction is noted only several hours following DNA damage, which raises doubts about its specific relevance to the DDR. Inhibitors of Atm and NF-kappa B signaling suppress induction of DDSR1 following DNA damage. Induction of DDSR1 expression is not abolished in p53 mutant cells, but overexpression of p53 in a non-physiological context was found to increase expression. The significance of this finding is unclear. siRNA-mediated depletion of DDSR1 results in induction of expression of a variety of genes, some of which are validated. However, specificity controls for the use of siDDSR1 are not provided. The list of genes that show modified expression following DDSR1 depletion is presented without a biological context. The impact of siDDSR1 on cell proliferation is minimal and the evidence that it significantly modulates DDR signaling (Fig 4b) is unpersuasive. The authors use the I-SceI HR reporter to show that HR is reduced following siRNA-mediated depletion of DDSR1. Specificity controls for siDDSR1 are missing in these HR experiments, as they are throughout the m/s. The impact of siDDSR1 on PARP inhibitor sensitivity is modest and no other DNA damaging agents are tested. No statistical analysis is performed on the impact of siDDSR1 on BRCA1 recruitment kinetics (Fig 6). This observation is not readily reconciled with the claim that DDSR1 affects HR. If DNA end resection is impaired in this setting, as the authors speculate, this should be shown experimentally.

1st Revision - authors' response 22 July 2015

Referee #1

1. Level of DDSR1. The authors document a ~2-5 fold induction of this lncRNA. What is the

absolute copy number in cells? Is the number sufficient to mediate the proposed effects at sites of DNA damage directly?

As requested, we have now used qPCR to compare the relative copy number of DDSR1 to that of a housekeeping mRNA, TBP. We find DDSR1 to be about 12-fold less abundant than TBP. This estimate is in line with our observation of a relatively weak, but significant, signal in our FISH experiments (Fig. EV 1C). The finding is also in line with the notion that most lncRNA are expressed at relatively low levels in the cells, which does not appear to preclude them from exerting key biological functions including sequestration of proteins (Rinn JL and Chang H, Annu Rev Biochem 2012 ; Geisler S and Coller J, Nat Rev Mol Cell Biol 2013; Dodd et al., Nucleic Acid Ther 2013). It is important to note that accurate estimation of low copy number RNAs per cell is technically very difficult due to significant variation in total transcripts levels per cell (Marinov GK et al., Genome Res 2014), differential expression of transcripts in sub population of cells, and limited efficiency of the reverse transcriptase step (Sanders et al., PLoS One 2013). We therefore mention estimate of the cellular abundance of DDSR1 relative to TBP on page 7 of the manuscript. It is correct that DDSR1 is gradually induced over several hours after DNA damage (Fig. 1). However, we also find that loss of DDSR1 alters recruitment of BRCA1 and RAP80 within 10 mins of inducing DNA damage (Figure 6 A and B). These observations suggest that basal levels of DDSR1 are important for DNA repair.

2. A single siRNA was used to deplete DDSR1, followed by analysis of gene expression effects and all downstream analyses. Multiple independent siRNAs knocking down DDSR1 should be analyzed to exclude off target effects. This is the standard applied to coding and noncoding genes for RNAi experiments.

As requested, we have now used two independent means of knockdown to confirm our results. We use an additional siRNA of the same chemistry but different target sequence as used before, and in addition, we use a locked nucleic acid (LNAs) antisense oligonucleotide against DDSR1. We show similar results on gene expression (Figure EV2) and HR efficiency (Figure 5A) using these two approaches, suggesting that the observed phenotypes are not due to off target effects.

3. Effect of DDSR1 on DDR signaling. The authors report a 30% decrement of several phosphor-proteins upon DDSR1 depletion. This is a quite modest effect that may not have any biological significance. Moreover, to make such a modest difference convincing, multiple replicates and error bars should be shown.

As requested, we now provide quantification of our western blots from three independent experiments and also show total protein levels of these phophoproteins (Figure 4B and Figure EV 3B). All reductions are statistically significant.

4. MS2-TRAP RNA affinity purification was used to identify DDSR1 interacting proteins. Does the MS2-DDSR1 fusion complement DDSR1 depletion? Without knowing that the fusion RNA is functional, the relevance of the resulting complex is unclear.

We appreciate this point. Since DDSR1 is an ncRNA and does not have a 3'UTR, it is not possible to design siRNAs towards the 3'UTR and perform complementation assays. The experiment could be performed by CRISPR but this would require establishment of the method from scratch, which is beyond the scope of this manuscript. To address this point, at least for BRCA1, we now include new RNA-IP data, showing that that DDSR1 interacts with BRCA1 (Figure 7D). This observation is in line with our previous biochemical data.

5. It is unclear whether the effects of DDSR1 on BRCA1 and RAP80 recruitment to DNA damage sites are direct or indirect. Do they depend on hnrnpUL1, or other effects on gene expression documented above?

We now provide new data to strengthen the evidence for a direct role of DDSR1 in regulating BRCA1 recruitment. We show, in Figure 7D, interaction of BRCA1 with the lncRNA DDSR1 as detected by a formaldehyde cross-linked-RIP analysis. These data complement our findings that DDSR1 knockdown increases BRCA1 recruitment to DSBs (Figure 6). Additionally, as requested, we further explored the role of the DDSR1 interacting partner hnRNPUL1. We now show that, similar to DDSR1 knockdown, depletion of hnRNPUL1 results in increased recruitment of BRCA1 and RAP80 to laser-induced DSBs (Figure 7 A and B). The knockdown of hnRNPUL1 does not result in changes in expression of DDSR1 and vice versa (Figure 7 C and Figure EV 4B). Taken together these observations suggest a functional interaction between DDSR1 and hnRNPUL1

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in regulating access of BRCA1 and RAP80 to DSBs and indicates that altered BRCA1 and RAP80 recruitment upon DDSR1 depletion is not due to changes in gene expression caused by DDSR1 depletion.

Referee #2:

1. In general, it is not shown whether the effect of DDSR1 knockdown on DNA end resection and HR is direct or due to altered expression of HR proteins. The level of the main proteins involved in DNA-end resection and homologous recombination (or, to the least, their mRNA levels) should be checked in control and DDSR1 knockdown samples. The fact that DDSR1 is not recruited to laser induced DSB may support an indirect role of DDSR1 in this process.

As requested, we now show that depletion of DDSR1 does not alter mRNA levels of genes involved in DNA repair (Figure EV 4B). These observations complement our gene expression analysis upon DDSR1 depletion (Table EV3) where we did not observe any change in expression of DNA end resection and HR related transcripts. Another line of evidence against indirect effects is our newly added data showing an interaction between DDSR1 and BRCA1 in RIP experiments further pointing to a direct role for DDSR1 in regulating HR. Finally, similar effects on HR efficiency (Figure 5A) were obtained upon DDSR1 depletion using an additional siRNA and an antisense locked nucleic acid against DDSR1, suggesting these affects are not due to off target effects, such as altered expression of HR proteins which would not be expected when multiple siRNAs are used.

2. The authors also report an interaction between DDSR1 and hnRNPUL1 and claim that this interaction may contribute to DNA end resection activity. As also mentioned by the authors, Polo et al. in 2012 reported an RNA-independent recruitment of hnRNPUL1 to DNA damage sites, where it favors DNA end resection. Although it is possible, as the authors suggest, that DDSR1 modulates the activity but not the localization at DSB of hnRNP1, this is, at this stage, amply speculative.

We appreciate the reviewer for highlighting this important point. We have now directly tested the effect of DDSR1 depletion on DNA end resection. We now show that DNA end resection is impaired upon DDSR1 depletion as measured by GFP-RPA accumulation at laser induced DSBs in S-phase cells and by native BrDU staining of ssDNA upon IR in cyclin A positive cells (Figure 5 E and F). We now also present additional data to show that knock down of the DDSR1 interacting partner hnRNPUL1 results in increased BRCA1 and RAP80 recruitment to laser induced DSBs, similar to DDSR1 depletion. These observations point to a functional interaction between DDSR1 and hnRNPUL1 in regulating access of BRCA1 and RAP80 to DSBs. We mention these points in the discussion.

3. Since kd of DDSR1 affects cell proliferation, the authors should demonstrate that the reduced DDR activation is not the consequence of reduced cell proliferation. Furthermore, the reduced RPA, H2AX and Chk1 activation shown by western blots would be more convincing if the total level of the proteins was shown.

We now show that DDSR1 depletion does not percentage of cells in S/G2 phase as assessed by Cyclin A staining (Figure EV 4D). Similarly, DDSR1 depletion does not perturb DNA replication as measured by BRDU incorporation (Figure EV 4E). This result indicates that reduced DDR activation upon DDSR1 depletion is not a consequence of reduced cell proliferation. We now show total protein levels in the Western blots for RPA, Chk1 and H2A-X and no changes were found upon DDSR1 knockdown as now shown in Figure 4B.

4. The authors show an enhanced recruitment of the BRCA1-RAP80 complex upon DDSR1 knockdown. The ability of this complex to inhibit DNA end resection could explain why RPA and Chk1 phosphorylation is reduced upon DDSR1 knockdown. Considering the different roles of BRCA1 at DSB, it would be interesting (and experimentally easy) to test whether DNA end resection is actually affected by monitoring ssDNA (by BrdU staining under non denaturing conditions) and RPA levels.

We thank the reviewer for suggesting these important experiments. As requested, we now show that DNA end resection is impaired upon DDSR1 depletion as measured by GFP-RPA accumulation at laser induced DSBs and by native BrDU staining of ssDNA formation upon IR (Figure 5E and F).

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Referee #3:

This induction is noted only several hours following DNA damage, which raises doubts about its specific relevance to the DDR.

It is correct that DDSR1 is gradually induced over several hours after DNA damage (Fig. 1). However, we also find that loss of DDSR1 alters recruitment of BRCA1 and RAP80 within 10 mins of inducing DNA damage (Figure 6 A and B). This observation suggests that basal levels of DDSR1 are important for DNA repair. While the induction of DDSR1 upon damage made it a hit in our unbiased screen, the lncRNA clearly also plays a housekeeping role. This interpretation is supported by our observations that in the absence of DNA damage DDSR1 binds and sequesters BRCA1 as demonstrated in new RNA-IP data (Figure 7D) and that upon DNA damage the binding of DDSR1 and BRCA1 is reduced, possibly facilitating the recruitment of BRCA1 to sites of damage. One plausible interpretation is that DDSR1, along with hnRNPUL1 as we show in Figures 6 and 7, fine tunes BRCA1 and RAP80 access to DNA repair sites upon DNA damage to regulate DNA repair by HR.

Inhibitors of Atm and NF-kappa B signaling suppress induction of DDSR1 following DNA damage. Induction of DDSR1 expression is not abolished in p53 mutant cells, but overexpression of p53 in a non-physiological context was found to increase expression. The significance of this finding is unclear.

We agree that the relationship of p53 and DDSR1 requires more detailed analysis, which goes beyond the scope of this initial study on DDSR1 identification and its role in HR. We felt it was important to show these data, even in the absence of a full mechanistic explanation, since p53 is a prominent, well -documented regulator of DR induced lncRNAs as we discuss in our Introduction. Our experiments, shown in Figure 3C, indicate that DDSR1 induction upon DNA damage (for 6 h) is not dependent upon p53. This is consistent with the fact that the DDSR1 promoter does not have a p53 binding site. The ability of p53 to induce DDSR1 without DNA damage (upon expression for 30 h) likely depends on activation of other p53 targets associated with transcription. We now mention these results in this context on page 7 in the results section on page 7 and in the Discussion.

siRNA-mediated depletion of DDSR1 results in induction of expression of a variety of genes, some of which are validated. However, specificity controls for the use of siDDSR1 are not provided.

We have now validated these results with an additional siRNA as well as an antisense locked nucleic acid (LNA) against DDSR1. Similar results on gene expression (Figure EV2) were obtained upon DDSR1 depletion using these two independent knockdown strategies, suggesting these affects are not due to off target effects.

The list of genes that show modified expression following DDSR1 depletion is presented without a biological context.

We did not mean to imply a particular biological function or pathway based on these genes. There is no reason to think that the target genes belong to a particular pathway. We are acutely weary of over-interpreting gene expression data and refrain from attempting to derive pathway information. The presented data were merely meant as a list of affected genes for informational purpose and we simply provide the GO annotations.

The impact of siDDSR1 on cell proliferation is minimal and the evidence that it significantly modulates DDR signaling (Fig 4b) is unpersuasive.

We have now expanded the data in Figure 4B. We show quantification of blots in Figure EV 3B, and we have added western blots for total RPA, Chk1 and H2A-X in Figure 4B. The effects on phosphorylation of p53, pRPA, and pChk1 as well as proliferation (Fig. 4A) are all statistically significant.

The authors use the I-SceI HR reporter to show that HR is reduced following siRNA-mediated depletion of DDSR1. Specificity controls for siDDSR1 are missing in these HR experiments, as they are throughout the m/s.

We now provide the necessary specificity controls. In addition to a non-targeting siRNA, we now show the same effect of DDSR1 depletion on HR efficiency using an additional siRNA of the same chemistry but distinct target sequence and an independent antisense oligonucleotide based on locked nucleic acids (LNA) chemistry (Figure 5A), ruling out off-target effects. We also use

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these oligos in experiments assessing the effect of DDSR1 knockdown on gene expression (Figure EV 2).

The impact of siDDSR1 on PARP inhibitor sensitivity is modest and no other DNA damaging agents are tested.

We now also show a colony formation assay upon DDSR1 depletion with Neocarzinostatin in Figure EV 4F. Both the effect of NCS and PARP inhibitor are shown to be statistically significant.

No statistical analysis is performed on the impact of siDDSR1 on BRCA1 recruitment kinetics (Fig 6).

Increased BRCA1 recruitment upon DDSR1 depletion was found to be statistically significant as determined by Student's two-tailed t test at each imaging time point. In the original version of the manuscript we indicated the p-values as a color-coded heat map in Figure 6 A and B, on top of the time course panels and this was also indicated the respective figure legend. This might be a somewhat unusual representation but one that we have found to be useful as the heat-map overcomes the difficulty of presenting in a visually appealing and clear fashion the error bars and statistical significance for all of the several hundred data points in this graph. This method has been previously used in Khurana et al., Cell Reports, 2014.

This observation is not readily reconciled with the claim that DDSR1 affects HR. If DNA end resection is impaired in this setting, as the authors speculate, this should be shown experimentally.

As requested, we now provide direct experimental evidence for impairment resection. We show that DNA end resection is impaired upon DDSR1 depletion as measured by GFP-RPA accumulation at laser induced DSBs and by native BrDU staining upon IR (Figure 5E and F).

2nd Editorial Decision 17 August 2015

Thank you for the submission of your revised manuscript to our journal. We have now received the enclosed reports from the referees that were asked to assess it, and both support publication of your study now. I am happy to tell you that we can therefore accept it upon a few minor modifications and additions.

Please add the statistical tests used to calculate p-values in all respective figure legends, including EV figure legends. The legends and figures need to be self-explanatory. Please also specify "n" and the bars and error bars for figure panels 4A and 5C. Panel 5E has error bars, but n=2, so no statistics can be calculated. Please show all data points along with the mean instead, or repeat the experiment one more time. In EV1 the definition of the scale bars is not visible, please amend.

I look forward to seeing a final version of your manuscript as soon as possible, and to see your work published. I agree that your study is indeed a good fit for our journal, as referee 1 notes.

REFEREE REPORTS:

Referee #1:

The authors have satisfactorily addressed my concerns and improved this work. The paper is a good fit for EMBO Reports.

Referee #2:

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In the manuscript, Sharma et al. identify a lnRNA DDSR1 that is induced upon DNA damage and has a role in homologous recombination. Together with its interacting partner hnRNPUL1, DDSR1 regulates BRCA1-RAP80 recruitment to DSB, where they inhibit DNA end resection. Consequently, DDSR1-hnRNPPUL1 controls
homologous recombination by fine-tuning DNA end resection.

In their present form, the data are quite solid and they nicely support the model proposed.

2nd Revision - authors' response 21 August 2015

Thank you again for the positive decision on our manuscript *"A BRCA1 interacting lncRNA regulates homologous recombination" (*EMBOR-2015-40437V2). We appreciate the referees' endorsement of our work and as per your invitation, are now submitting our final files.

As outlined by you we have added the statistical tests used to calculate p-values in all respective figure legends and also modified figure panels and legends for Figures 4A, 5C and 5E.

3rd Editorial Decision 25 August 2015

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

Thank you for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

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