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BRCA2 promotes R-loop resolution by DDX5 helicase at DNA breaks to facilitate their repair by homologous recombination

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1st Editorial Decision 27th Jul 2020

Thank you for submitting your manuscript on BRCA2-DDX5 cooperation in R-loop resolution during HR for our consideration. Three expert referees have now reviewed the manuscript, with their comments copied below. As you will see, the referees are currently somewhat ambivalent about the study: while all acknowledge the potential interest of your findings and conclusions, they all remain to certain degrees unconvinced that the present set of data provides strongly supports all key conclusions, but also that it provides sufficiently definitive insights into the underlying physiological rationale/significance of the proposed mechanisms.

Given the general importance of the topic and the encouraging nature of the reports, I would be open to giving you an opportunity to address the concerns raised by the reviewers through a revised version of this manuscript. Key issues in this respect would be the addition of essential controls for the S9.6 IF experiments (ref 1 pt 1); strengthening of the DRIPc-seq and DRIP-qPCR data by showing their reproducibility, (internal) comparability and controls (ref 1 pt 2, ref 2 pt 3, ref 3); and validating the PLA data with complementary methodologies (ref 2 pt 2). In addition to these main (and several other, more specific) technical points, there are however also important conceptual concerns raised by referees 2 and (particularly) referee 3, which in my view deserve attention, in particular regarding the confusion between co-transcriptional R-loops and hybrids formed at DSBs, and the general significance of BRCA2-DDX5 interplay and the models proposed based on the results.

I realize that convincingly addressing these issues to the referees' satisfaction may not be trivial and may require significant further time and effort, possibly also resulting in confounding of some key conclusions. This makes it somewhat difficult for me to predict the outcome of eventual re-review and to commit to EMBO Journal publication already at the present stage; but I do feel that the study could become a much more compelling candidate for an EMBO Journal article if improved along the lines suggested by all three reviewers.

REFEREE REPORTS

Referee #1:

Sessa et al. present here an investigation of the interplay between DDX5 and BRCA2 in regulating DNA:RNA hybrids at DNA double-strand breaks. The authors use a range of techniques and approaches, initially identifying DDX5, a known hybrid helicase, as a BRCA2 interactor. Subsequently, the authors characterise the role of this interaction to maintain DDX5 at DNA damage sites ultimately and to significantly increase its helicase activity, facilitating the removal of hybrids around DSBs. The formation of DNA:RNA hybrids at DSBs is currently of great interest in the field of DNA repair and specifically DNA double-strand break repair and this manuscript does well to bridge other published results while also presenting a significant amount of novel and interesting results.

We believe this manuscript will be suitable for publication in EMBO, however we have several comments we need addressing first. Whereas we found the manuscript to be very well written and their conclusions to be insightful, there are a number of issues we would like to raise, several of which are regarding the presentation of biological replicates. All of these are fully explained below.

Major comments:

1. S9.6 immunofluorescence is used here to show overall DNA:RNA hybrid changes in different conditions. However, I strongly believe these experiments need an RNase-H treated negative control in which prior to antibody hybridisation an RNase-H containing buffer is used to digest any hybrids and therefore show if the immunofluorescent signal is specific. There is already substantial evidence that the S9.6 antibody has specificity issues towards other RNA species, especially in the case of immunofluorescence. This can be seen in Figures 2A, S1B and 6B which appear to show strong extranuclear staining from the antibody.
2. Only two replicates of DRIPc-seq were completed and whereas I understand these experiments can be very expensive, 3 biological replicates is ideal for any experiment to ensure accuracy. However, since the manuscript does not rely heavily on these results, 2 replicates should be acceptable if the authors provide sufficient evidence of these replicates being consistent. The current genome browser plots in Figure 2C are not sufficient to show overall reproducibility and also show differences that raise concerns, e.g. the peak over "DGLUCY" is higher in siDDX5 than siC in replicate 2, but not in replicate 1. I would recommend some form of correlation analysis or something similar between the conditions and replicates.

Minor comments:
1. End of introduction paragraph 1 uses reference 10 (Michelini et al.) as an example of evidence for DNA:RNA hybrids forming at DSBs. This article only looked at lncRNA at DSBs and makes no mention of hybrids.
2. As a matter of quality control, please provide gel images or other visualisation methods that confirm the DNA fragmentation sizes for both the ChIP and DRIP experiments.
3. Figure 3B the bar for siSETX, -RH -OHT at HIST1H2BG appears to have incorrect dots, they are

all very below the top of the bar.
4. As far as I can tell it is not stated how many biological replicates were completed for many of the experiments including mass-spec, IF and PLA. Please state either in the main text or methods how many biological replicates were completed for each experiment.
5. For the mass-spec results in Figures 1 and S1, please provide a western blot to show the IP of the bait and preferably also of DDX5 in the replicates alongside input samples.

Referee #2:

This paper by Sessa et al investigates the function of DDX5 in R-loop biology. In particular, the authors demonstrate that DDX5 interacts with BRAC2, and this interaction is important for DDX5 recruitment to DSBs and stimulation of RNA/DNA helicase activity of DDX5. In cells depleted for DDX5 or with patient cancer mutation in BRCA2 protein, affecting the interaction with DDX5, DDX5 is no longer found at DSBs and interacting with R-loops, RPA1 foci are reduced and a change in Rad51 kinetics is observed upon irradiation. All these information suggests that DDX5 and BRCA2 are both required to remove R-loops to repair DSBs through the process of homologous recombination.

Overall the paper is interesting elucidating a novel mechanistic aspect of DSB repair, involving R-loops, DDX5 and BRAC2 proteins. Having said that, the overall quality of multiple experiments need to be improved. I am seriously concerned about the interpretation of all PLA assays (since the observed differences are less than half a spot/per cell) and alternative, more robust techniques are needed here to validate the results (as discussed below in point 2). With substantial revision this paper may be suitable for publication in EMBO J.

Major comments:
1. The quality of the IP figure 1 should be improved. What is the origin of the bands in the stain-free sample in fig 1A? What do 2 lanes for MBP (BRCA2NT), which look very different on WB, actually represent (what is the difference between the two)? This figure lacks any negative controls (i.e. helicase not interacting with BRCA2). The paper states that the interaction between BRCA2 and DDX5 is enhanced under IR conditions. However, this is not observed (the interaction is actually reduced) when the IP is performed with endogenous proteins in Fig 1B. Can the authors explain such discrepancy?

2. There are about 6 separate figure panels throughout the paper which use Proximity ligation assays (PLA) for various co-localization tests. However, I am concerned about the interpretation of all these results, especially when the average number of spots per nucleus in most of these panels (apart from one in Fig.6E) is lower than 0.5. The authors use high-power statistical tests to get the statistics of these data however, biologically thinking if the differences between 2 samples are less than half of the spot/per cell, I am not sure how can we biologically interpret such data. Therefore, the conclusions from these figures should be validated with alternative more robust approaches.

3. The authors present genome-wide analysis of DRIP-seq in DDX5 KD cells Fig 3. On one hand this could have been interesting, however in my opinion there are a number of issues with these data. First I am concerned about the fact that the siControl samples were taken from another publication - does it mean that the siControl and siDHX9 were not carried out side by side? This may be a problem for carrying out comparative analysis between two samples, which the authors have done here (done with 'different hands' and library and sequencing being prepared separately). Previous

paper (Mersaoui et al EMBO 2019), have already showed that DDX5 KD results in accumulation of R-loops, so in a sense the authors have not really taken this finding forward enough to bring any further new information regarding DDX5 function. Carrying out this DRIP-seq in U2OS DIvA cells would have been much more informative for their story when they want to focus on the role of DDX5 in DSB. Furthermore, presented DIP-seq lacks RNase H control and no statistical analysis is presented to know how significant the data are. The genome-wide data lacks DRIP-qPCR validation for the points discussed on specific genomic loci.
4. Previous work has indicated that DDX5 is involved in regulation of transcription, so what happens with transcription at genes where DDX5-dependent increase of R-loops is observed? Where does DDX5 bind genome-wide - does this binding correspond to regions of R-loop increases? The authors observe an overall increase of R-loops over gene bodies and over-ant-sense transcripts which raises the question of specificity of R-loop-associated function of DDX5.
5. I am slightly surprised that following BRCA2 KD, it seems that there is more recruitment of DDX5 in normal conditions, while it is less to DSBs (Fig.4B). Can the authors confirm if this is the case and provide some interpretation to this result? Overall speaking, there is certainly two DDX5 functions under normal physiological conditions and at DSBs. So how this is all orchestrated? Is BRCA2 required for DDX5 function at physiological R-loops? At least discussion should include these points.
6. The authors need to provide gels to demonstrate the quality of their recombinant preps for in vitro helicase assays. The authors state that the short N-terminal BRAC2 fragment can enhance helicase activity of DDX5 protein (though added at high concentration of 50X fold). Can the activity be still enhanced with slightly longer fragment (LT2, LT3) added at a lower concentrations of BRAC2 proteins?

Minor comments
1. Both AQR and DHX9 helicases with corresponding references should be added in sentence referring to known R-loops helicases (page 3).
2. In Fig2c -DDX5 over-expression results in visible appearance of S9.6 foci in siControl cells - what is the explanation for this?
3. What does statistical number p=0.00039 refer in Fig 3B?
4. Figure 3 C should have statistical analysis comparing siCTRL to si SETX (positive control), siBRCA2 and siDDX5 for all genes presented to be able to make any conclusions from these data presented.

Referee #3:

In this manuscript, Sessa et al identify the helicase DDX5 as a novel BRCA2 interacting partner. They show that BRCA2 increases the hybrid unwinding ability of DDX5 in vitro and facilitates recruitment of DDX5 to DSB sites. They also show that BRCA2-T207A, a mutant found in breast cancer patients, has a weakened interaction with DDX5, and that this further augments the level of DNA-RNA hybrids and interferes with the repair of DNA damage by homologous recombination.

The heart of the story is the interaction of BRCA2 with DDX5 and herein the authors propose an interesting model and function for BRCA2 in regulating DDX5. They also identify a patient mutation that affects this interaction, increasing the general interest in the work and establishing its physiological relevance. However there are a number of weaknesses with this work. Some of the conclusions are not justified by the data shown and need further support. Additionally, the story is confusing at several turns and some point are not clearly explained.

Generally points:
Generally there is confusion about whether the hybrids under investigation and affected by DDX5 and BRCA2 are formed cotranscriptionally throughout the genome, at DSB sites and/or both. Figure 2 focuses on cotranscriptional hybrid formation; Figures 3,4 suggest DNA breaks trigger hybrid formation and BRCA2/DDX5 recruitment, implying that DNA damage is the cause of hybrid formation and BRCA2/DDX5 recruitment is the effect. Then the model in Figure 7 seems a hybrid of the two ideas - showing a cotranscriptional R-loop at a DSB site. Further explanation of the model and the logic behind it (vs other possibilities - i.e. hybrids being de novo synthesized at the DSB site - would helpful).
Specific comments:
Figure 1
- Is BRCA2 1-250 necessary for the interaction of BRCA2 with DDX5 - the authors should test a mutant lacking AA's 1-250 but containing the rest of the BRCA2 protein

Figure 2.
- The genomic analysis is weak. A more complete look at where hybrids are increased and decreased is needed. Also, the authors should validate some of the new sites identified with DRIP-qPCR.
- I don't understand the rationale for examining gammaH2AX in cells with wildtype DDX5 and comparing this to the DRIP peaks in DDX5-depleted cells. The authors should use gammaH2AX data from cells where DDX5 is depleted. Is the overlap observed even significant or above what would be expected from a random sampling of genes? And how much gammaH2AX is really present at baseline in normal cells?

Figure 3.
- GammaH2AX is phosphorylated not only upon DSB formation but following other types of stress so suggesting that gammaH2AX sites are DSBs site is not appropriate. The authors should use other markers of DSBs to say if hybrids colocalize with breaks in DDX5-depleted cells.
- The authors should check more than one site (RBMXL1) in their analysis of DSBs with hybrids affected by BRCA2 and DDX5 (Fig 3b)
- The impact of SETX, BRCA2 and DDX5 depletion on the hybrids at DSB sites seems modest and is only a fraction of the impact of the DSB itself on hybrid formation. This should at least be noted

Figure 4.
- Figure 4a - why is DDX5-GPF decreasing at only 11% of cells. Is this 11% of all cells with laser irradiated sites or just 11% of cells, a subset of which have been irradiated. Presenting this as a fraction of laser irradiated sites seems most appropriate and I assume that is the case here. If so, however, why only 11%. Are these cells in a certain phase of the cell cycle? Generally this is a confusing presentation as in the end they argue that BRCA2 helps to keep DDX5 at these sites, yet they start off talking about the exclusion of DDX5 from damage sites.
- What is the binding/localization of BRCA2 in this assay? Do both BRCA2 WT and T207A bind to the 'stripe' and does the mutant BRCA2 bind the DSB site by ChIP as well as the WT BRCA2? This would help establish if the mutant is functional in other ways and that the effects are on recruitment of DDX5.
- Figure 4b - please examine additional sites of DSB induction and negative controls. Also if DDX5 affects hybrids at HIST1H2BG as the authors state, does DDX5 localize there? It's hard to determine this from the data show as the value of the background is not clear. And why is DDX5 ChIP increased by siBRCA2 if it's localization is BRCA2 dependent. Finally, why does the break impact the recruitment of DDX5 in the siBRCA2 condition. These results need further discussion

Figure 5
- The impact of BRCA2 on DDX5 activity is rather modest. Also, the authors should show the impact of other BRCA2 fragments on DDX5 activity as controls. They suggest the effect does not depend on AA's 250-500 but never test this in the in vitro assay.
- Why are the authors using an R-loop type hybrid as their substrate. Since they are looking at hybrids at DSB ends, it might be more appropriate to use more simple hybrid. Does BRCA also stimulate DDX5 activity at this type of hybrid?
- -The authors conclude that 'BRCA2 stimulates the R-loop unwinding activity of DDX5'. This statement needs more support. Although co-incubation with BRCA2 increases R-loop unwinding ability of DDX5, it is possible that BRCA2 only increases the binding ability of DDX5 to the R-loop structure without changing its catalytic activity. The results in Figure 6 indicate that BRCA2 recruits DDX5 to hybrids, which supports this idea.

Figure 6
- Figure 6 - please show effect of RNaseH on hybrid levels for IF and please test additional genomic sites by DRIP-qPCR to demonstrate the impact of the BRCA2 mutation at T207 on hybrid formation
- Figure 6d - the effects here are extremely small - with average increases of about no PLA foci to about 0.2 PLA foci per nucleus? Is this real? I think labeling of this graph may also be incorrect - are the two right samples the BRCA2 mutant?
- Figure 6e - why does the interaction of DDX5 and S9.6 in the T207 mutant go down with IR from a higher baseline, and also why don't the WT cells show an increase in this interaction. This seems contrary to the predictions of the model.
- The authors should test the interaction of BRCA2 T207A with DDX5 in vitro using purified proteins, as in Figure 1e.
- It is unclear why the BRCA2T1-T207A mutant would inhibit the helicase activity of DDX5 if it interacts with it less effectively. This is not really consistent with the authors model. More development of this mechanism is needed. The authors ultimately suggest that BRCA2 might affect the ATP hydrolysis rate of DDX5 or the binding of DDX5 to RNA. Further work on this question and what the T207A mutant is doing is needed to strengthen this story.
- In Figure 6, it is clear that BRCA2-DDX5 and DDX5-S9.6 PLA are enhanced by IR in DLD1 cells with BRCA2 WT. To conclude that the BRCA2-DDX5 interaction promotes localization of DDX5 to hybrids that are actually at the site of DNA damage, the authors should also analyze the PLA between DDX5 and rH2AX in BRCA2 WT and T207A cells.
- Does overexpression of RNaseH reduce the binding of BRCA2-DDX5 to damage sites?

Figure 7
- The impact of the BRACA2 mutant on Rad51 foci is quite modest as is the effect of RNaseH expression. More data in support of the idea that this mutant is having a biological effect on HR is needed. Counting foci could be challenging and I wonder if the results would be stronger if the chromatin bound Rad51 were measured instead of foci number.

Minor Points
Discussion p17/18. - Parts of the discussion text is very confusing with long sentences. In particular the two sentences starting "On the one hand...." And "On the other hand" need work, as does the one following this....

Referee #1:

Major comments:

1. S9.6 immunofluorescence is used here to show overall DNA:RNA hybrid changes in different conditions. However, I strongly believe these experiments need an RNase-H treated negative control in which prior to antibody hybridisation an RNase-H containing buffer is used to digest any hybrids and therefore show if the immunofluorescent signal is specific. There is already substantial evidence that the S9.6 antibody has specificity issues towards other RNA species, especially in the case of immunofluorescence. This can be seen in Figures 2A, S1B and 6B which appear to show strong extranuclear staining from the antibody.
For data in figs 2A, S1B and 6B, we have pre-extracted the nuclei and selected the nuclear area excluding nucleoli to avoid cytoplasmic S9.6 signal contamination. In any case we are aware of the issue raised by the referee and we have included RNase H in most our experiments. As requested, we have now added the RNase H controls missing in old figs 2A and S1B (see new Figs EV2B and EV2A, respectively). Figure 6B RNase H control was shown in old Fig S3C; so we have moved these data to the main figure (new Fig 6B). These results were also confirmed by DRIP including RNase H controls and we have added DRIP in two more specific loci confirming our results as shown in new Fig 6C. Finally, in the same line we have repeated the experiments of PLA S9.6-anti-DDX5 to include RNase H controls (now shown in new Fig 7A). Thanks

2. Only two replicates of DRIPc-seq were completed and whereas I understand these experiments can be very expensive, 3 biological replicates is ideal for any experiment to ensure accuracy. However, since the manuscript does not rely heavily on these results, 2 replicates should be acceptable if the authors provide sufficient evidence of these replicates being consistent. The current genome browser plots in Figure 2C are not sufficient to show overall reproducibility and also show differences that raise concerns, e.g. the peak over "DGLUCY" is higher in siDDX5 than siC in replicate 2, but not in replicate 1. I would recommend some form of correlation analysis or something similar between the conditions and replicates.
As requested, the correlation between the two analyzed replicas is now provided in new Figure EV3D showing consistency (PCC= 0.91). Moreover, we have performed a third biological replica of the DRIPc-seq experiment in DDX5-depleted cells that is also in agreement with the first 2 replicas as shown in the new Figures EV2A, B and 2C.

Minor comments:

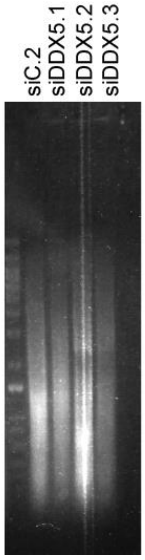
1. End of introduction paragraph 1 uses reference 10 (Michelini et al.) as an example of evidence for DNA:RNA hybrids forming at DSBs. This article only looked at lncRNA at DSBs and makes no mention of hybrids.

We have corrected the mistake as requested. Thanks

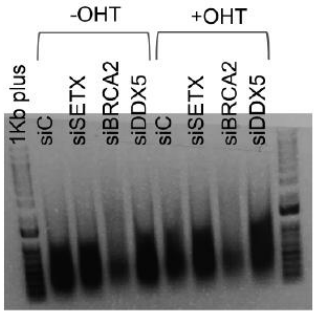
2. As a matter of quality control, please provide gel images or other visualisation methods that confirm the DNA fragmentation sizes for both the ChIP and DRIP experiments.
As requested, we provide here below the gel images showing the DNA fragmentation sizes for DRIPc-seq, DRIP, and ChIP.

Fragmentation controls for:

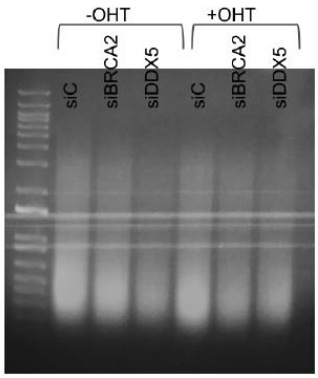
DRIPc-seq in K562 cells



DRIP experiment in DIVA cells



ChIP experiment in DIVA cells



3. Figure 3B the bar for siSETX, -RH -OHT at HIST1H2BG appears to have incorrect dots, they are all very below the top of the bar.
Thanks for pointing this out, this is now corrected in new Figure EV3B.

4. As far as I can tell it is not stated how many biological replicates were completed for many of the experiments including mass-spec, IF and PLA. Please state either in the main text or methods how many biological replicates were completed for each experiment.
This is now indicated as requested.

5. For the mass-spec results in Figures 1 and S1, please provide a western blot to show the IP of the bait and preferably also of DDX5 in the replicates alongside input samples.
Figure 1 shows a confirmation of the MS result using whole cell extracts and cells treated versus non-treated with IR. The input and pull-down bait are shown. For Fig S1 (new Fig EV1A), as requested, we have now included a western blot of the experiment performed for the MS analysis from nuclear cell extracts, showing the input, pull-down elution fraction and beads after elution as detected by MBP antibody.

Referee #2:

Major comments:

1. The quality of the IP figure 1 should be improved. What is the origin of the bands in the stain-free sample in fig 1A?

We think there must be a misunderstanding here. The strong bands in the Stain Free gel (used as loading control) are the ones corresponding to the 2xMBP (tag alone control), which is highly over-expressed compared to $BRCA2_{NT}$. The other bands in the gel show that the loading of the samples is equivalent.

What do 2 lanes for MBP (BRCA2NT), which look very different on WB, actually represent (what is the difference between the two)? This figure lacks any negative controls (i.e. helicase not interacting with BRCA2).

The two lanes represent – and + IR. The negative control is the 2xMBP alone to show that the binding of DDX5 to $BRCA2_{NT}$ is specific and not due to non-specific binding to the amylose beads or to the MBP tag.

As additional controls, we have now included two WB showing two RNA binding proteins, RBMX and DDX21, which although were found in our MS analysis they were not confirmed by WB with the endogenous proteins (Fig EV1C).

The paper states that the interaction between BRCA2 and DDX5 is enhanced under IR conditions. However, this is not observed (the interaction is actually reduced) when the IP is performed with endogenous proteins in Fig 1B. Can the authors explain such discrepancy?

We observe interaction in both non-treated and irradiated conditions as stated. However, we did the experiments in Fig 1B few minutes after irradiation. We have repeated these experiments at 4h post-IR which is the time we see the maximal BRCA2-DDX5 co-localization by PLA and under these conditions we observe a modest but reproducible increase in the DDX5 pull-down in IR conditions compared to non-treated cells. This is now shown in new Fig 1B. However, the increase is more evident by PLA and by amylose pull-down with $BRCA2_{NT}$ (Fig 1A, C and Fig 6D).

2. There are about 6 separate figure panels throughout the paper which use Proximity ligation assays (PLA) for various co-localization tests. However, I am concerned about the interpretation of all these results, especially when the average number of spots per nucleus in most of these panels (apart from one in Fig.6E) is lower than 0.5. The authors use high-power statistical tests to get the statistics of these data however, biologically thinking if the differences between 2 samples are less than half of the spot/per cell, I am not sure how can we biologically interpret such data. Therefore, the conclusions from these figures should be validated with alternative more robust approaches.

BRCA2 protein is in low abundance in the cell (https://pax-db.org/protein/1857163) and based on our IP experiments the pool of BRCA2 that binds DDX5 is also small; thus, we expect low number of PLA spots. Importantly, we use very stringent conditions to minimize nonspecific signal and remove all soluble proteins so that only the co-localization that involves proteins bound to chromatin are counted. We have now stated this in the main text. Although the average number of spots is small due to the number of cells that contain 0 spots, the distribution is clear. Another way to represent it would be using number of cells with more than 2 spots, but we think this represents less accurately the data. Controls with RNase H and cordycepin are also provided to demonstrate the specificity of the signal. Finally, as mentioned by the reviewer, all our PLA experiments have been validated with orthogonal experiments as explained below:

Figure 1C: (BRCA2-DDX5 PLA): this experiment is complementary to the pulldown of Fig. 1A, IP in Fig. 1B, and pulldown with purified proteins of Fig. 1E.
Figure 2B (S9.6-DDX5 PLA): this experiment is complementary to the one performed in DLD1 cells (Fig 7A).
Figure 3A (S9.6-γH2AX): this experiment is complementary to the DRIP experiment in Fig 3B.
Figure 6D (BRCA2-DDX5 PLA in T207A vs WT). In this case the antibody-only controls showed too much background signal so we decided to repeat the experiments. The new results (new Figure 6D) show now low PLA signal in the control but overall the same trend (an increase in PLA signal in IR conditions *vs* non-treated) and a stronger interaction in WT cells compared to T207A.
Figure 7A (S9.6-DDX5 PLA): Although this panel was not problematic on the levels of PLA spots, we have repeated the experiments to include RNase H treatment controls as requested by referee 1.
Figure 7D (S9.6-γH2AX). Following the request from reviewer 3 we have confirmed these results using a different DSB marker, NBS1. This new experiment showing S9.6-anti-NBS1 PLA is now included in Figure EV4A and confirms our results with S9.6-γH2AX.

3. The authors present genome-wide analysis of DRIP-seq in DDX5 KD cells Fig 3. On one hand this could have been interesting, however in my opinion there are a number of issues with these data. First I am concerned about the fact that the siControl samples were taken from another publication - does it mean that the siControl and siDHX9 were not carried out side by side? This may be a problem for carrying out comparative analysis between two samples, which the authors have done here (done with 'different hands' and library and sequencing being prepared separately).
The DRIPc-seq experiments in siDDX5-depleted K562 cells were actually performed in parallel to those published for the siC-treated cells (Pérez-Calero et al GD 2020) since we were working in both projects at the same time and these experiments were performed by the same author (Carmen Pérez-Calero). In contrast, a third replica (Fig EV3B and C) has been now performed and added. However, there is no need to perform these kind of experiments in parallel since they are treated and analyzed separately as independent replicas, and they need to be repetitive regardless of when, where and who performs them. Indeed, the reason to perform these experiments in K562 cells was to be able to compare to genome-wide GEO-deposited data that are available from previously performed experiments in other labs, as we do for γH2AX ChIP-seq data. In any case, to avoid possible misinterpretations, we have now ordered the replicas in a different manner so that the siC data are clustered together versus the siDDX5 data in new Fig 2C and new Figure EV2B,C. Thanks for raising this point.

Previous paper (Mersaoui et al EMBO 2019), have already showed that DDX5 KD results in accumulation of R-loops, so in a sense the authors have not really taken this finding forward enough to bring any further new information regarding DDX5 function. Carrying out this DRIP-seq in U2OS DIvA cells would have been much more informative for their story when they want to focus on the role of DDX5 in DSB. Furthermore, presented DIP-seq lacks RNase H control and no statistical analysis is presented to know how significant the data are. The genome-wide data lacks DRIP-qPCR validation for the points discussed on specific genomic loci.

The mentioned paper analyzed non-strand specific DNA-RNA hybrids in 2-3 specific loci, whereas we have performed a strand-specific analysis genome-wide, which includes much more relevant information on the pattern of R-loops in DDX5 depleted cells compared to WT cells. A report published while we were revising our manuscript by the same lab (Villarreal *et al* 2020.) has shown genome-wide data on non-strand specific DNA-RNA hybrids in siDDX5-treated cells. Our strand-specific data agree with their conclusion that DDX5 depletion causes DNA-RNA hybrid accumulation at the TSS and TTS but find also an increase at the gene body (Figure 2D). Importantly, we have used our genome-wide data to analyze the accumulation of DNA-RNA hybrids specifically at DSB sites by comparing them to the γH2AX ChIPseq data, which are especially relevant for this manuscript and it was not analyzed before. Finally, we have now provided correlation between replicas analyzed in depth (Figure EV2D) showing consistency as requested by referee 1 and, as indicated in the methods section, R loop-gain peaks were established selecting peaks whose DRIPc-seq signal fold-change was higher than 2.5-fold in siDDX5 respect to the siC control cells in both replicates and *vice versa* for R loop-gain peaks in siC cells. Regarding the RNase H control, we have now provided it in new Figure EV2A.

4. Previous work has indicated that DDX5 is involved in regulation of transcription, so what happens with transcription at genes where DDX5-dependent increase of R-loops is observed? Where does DDX5 bind genome-wide - does this binding correspond to regions of R-loop increases? The authors observe an overall increase of R-loops over gene bodies and over-ant-sense transcripts which raises the question of specificity of R-loop-associated function of DDX5.

New Figure EV3C shows that DDX5 depletion impacts gene expression in accordance with its reported role in transcription regulation. As far as we are aware, no DDX5 ChIPseq is available in K562 or any other cell type. We agree that this is an interesting question regarding the role of DDX5 in transcription in general but this is out of the scope of the manuscript. DDX5 a ubiquitous factor that has functions beyond DNA-RNA hybrid processing as it is well documented (reviewed in Xing et al Wiley Interdiscip Rev RNA. 2019). Indeed, DDX5 occupancy on chromatin shown by ChIP (Figure EV3D) does not depend on the presence of hybrids, as we observe similar low levels of DDX5 immunoprecipitation at hybrid-prone and non-prone regions (ex. *HIST1H2BG vs SNRPN*).

5. I am slightly surprised that following BRCA2 KD, it seems that there is more recruitment of DDX5 in normal conditions, while it is less to DSBs (Fig.4B).
Can the authors confirm if this is the case and provide some interpretation to this result?
We have performed a third replica of this ChIP experiment and included analyses of other three genes, one of them also containing an *Asi*SI target site. Altogether, the data (new Figs 4B and EV3D) clearly show that DSB induction by tamoxifen (+OHT) increases DDX5 occupancy at chromatin at different loci and this is dependent on BRCA2. The increase in DDX5 ChIP in BRCA2-depleted cells left untreated (-OHT) is not significant and values are close to background. To show this better, we have now also included DDX5 ChIP data in DDX5-depleted cells as a measurement of the background levels detected, background DDX5 ChIP signal is marked with a green line (Fig EV3D). Thanks

Overall speaking, there is certainly two DDX5 functions under normal physiological conditions and at DSBs. So how this is all orchestrated? Is BRCA2 required for DDX5 function at physiological R-loops? At least discussion should include these points.
We agree. We have now clarified this point in the Discussion, as requested. Thanks

6. The authors need to provide gels to demonstrate the quality of their recombinant preps for in vitro helicase assays.
We have now provided the stain-free SDS-PAGE gels showing the proteins used in the helicase experiments as requested.

The authors state that the short N-terminal BRAC2 fragment can enhance helicase activity of DDX5 protein (though added at high concentration of 50X fold). Can the activity be still enhanced with slightly longer fragment (LT2, LT3) added at a lower concentrations of BRAC2 proteins?
The amount of T1 required to achieve the same stimulation is 25-fold higher than that of BRCA2 (2 nM vs 50 nM). This is not surprising as T1 is a disordered region which might adopt a different conformation within the full-length protein. To address reviewer's suggestion, we have used LT3 (aa 1-750) purified as in von Nicolai et al., 2016, and used it at 10 nM concentration in a similar unwinding experiment with increasing concentrations of DDX5. LT3 at 10 nM was able to achieve the same level of stimulation as full-length BRCA2 at 2 nM confirming that a longer fragment than T1 can stimulate at lower concentration DDX5 unwinding activity. This result is now integrated in new Fig 5.

Minor comments
1. Both AQR and DHX9 helicases with corresponding references should be added in sentence referring to known R-loops helicases (page 3).
Thank you for the suggestion, we have now added this information in the introduction.

2. In Fig2a -DDX5 over-expression results in visible appearance of S9.6 foci in siControl cells - what is the explanation for this?
Sorry for the misunderstanding. These S9.6 "foci" correspond to nucleoli, which are excluded from the analysis as explained in Materials and Methods and now better explained in the text. To address this point, we have revised our images to show more representative examples of cells with similar nucleoli intensity; this is now shown in new Fig 2A.

3. What does statistical number p=0.0309 refer in Fig 3B?
We have placed the statistical values closer to the bars in the graphs to avoid confusion.

4. Figure 3B should have statistical analysis comparing siCTRL to si SETX (positive control), siBRCA2 and siDDX5 for all genes presented to be able to make any conclusions from these data presented.
As stated in the figure legend, only significant statistical values are shown to avoid graphs excessively crowded. We have also included other genes loci as proposed by Referee #3 (New Fig EV3).

Referee #3:

Generally points:
Generally there is confusion about whether the hybrids under investigation and affected by DDX5 and BRCA2 are formed cotranscriptionally throughout the genome, at DSB sites and/or both. Figure 2 focuses on cotranscriptional hybrid formation; Figures 3,4 suggest DNA breaks trigger hybrid formation and BRCA2/DDX5 recruitment, implying that DNA damage is the cause of hybrid formation and BRCA2/DDX5 recruitment is the effect. Then the model in Figure 7 seems a hybrid of the two ideas - showing a cotranscriptional R-loop at a DSB site. Further explanation of the model and the logic behind it (vs other possibilities - i.e. hybrids being de novo synthesized at the DSB site - would helpful).
The function of DDX5 and BRCA2 we observe is dependent on transcription, is sensitive to RNase H and is enhanced at induced DSB so we favor a model in which BRCA2-DDX5 act at DSBs occurring in transcribed regions. However, this is compatible with a role of DDX5 and BRCA2 in R loops accumulated genome-wide regardless of DNA damage, as responded to referee #2. This has now been clarified in the Discussion section.

Specific comments:
Figure 1
- Is BRCA2 1-250 necessary for the interaction of BRCA2 with DDX5 - the authors should test a mutant lacking AA's 1-250 but containing the rest of the BRCA2 protein
We have shown in two types of cells (U2OS and DLD1) by PLA, with the endogenous proteins by IP, and with overexpressed fragments by pulldown that BRCA2 and DDX5 interact and that T1 is sufficient for the interaction. We have also confirmed that T1 is sufficient for the interaction by pulldown using the purified T1 and DDX5 proteins. Moreover, we have now included another set of pull-downs with purified BRCA2-T1 and BRCA2-T207A showing that BRCA2-T1 physically interacts with DDX5 whereas T207A reduces the interaction. These complementary assays should suffice to state that the interaction is located in the first 250 aa of BRCA2.

Figure 2.
- The genomic analysis is weak. A more complete look at where hybrids are increased and decreased is needed. Also, the authors should validate some of the new sites identified with DRIP-qPCR.
As stated above (point 2, referee 1), we have performed a third replica of the DRIPc-seq analysis that validates our results (new Figure EV2B and 2C). We have also added the correlation analysis between the two analyzed replicas (new Figure EV2D) showing consistency. Moreover, a report published while we were revising our manuscript by the same lab (Villarreal et al 2020.) has shown genome-wide data on non-strand specific DNA-RNA hybrids in siDDX5-treated cells. Our strand-specific data agree with their conclusion that DDX5 depletion causes DNA-RNA hybrid accumulation at the TSS and TTS but find also an increase at the gene body (Figure 2D). Importantly, we have used our genome-wide data to analyze the accumulation of DNA-RNA hybrids specifically at DSB sites by comparing them to the γH2AX ChIPseq data, of especial relevance for the conclusions of this manuscript.

- I don't understand the rationale for examining gammaH2AX in cells with wildtype DDX5 and comparing this to the DRIP peaks in DDX5-depleted cells. The authors should use gammaH2AX data from cells where DDX5 is depleted. Is the overlap observed even significant or above what would be expected from a random sampling of genes? And how much gammaH2AX is really present at baseline in normal cells?
The rationale for examining γH2AX in wild-type cells (available online from GEO-deposited data) is to use it as a proxy to interrogate the possible role of DDX5 at spontaneous break-prone sites. The results in Fig 2E-F made us think that DDX5 might be relevant particularly at DSBs. We agree that examining γH2AX in siDDX5 cells might be more informative as these spontaneous DSB-prone sites might be different in these cells. However, we believe that these data are sufficient to suggest a potential role at DSBs that we have further confirmed in DIvA cells by qPCR at particular DSB sites (new Fig 3B and 4B), and by PLA showing DDX5-γH2AX signal in irradiated cells (new Fig 7B).
To address the question of the reviewer regarding the significance of the overlap with γH2AX-positive sites compared to a random sampling of genes, we have done the appropriate calculation. Our results (included in the text, page 8) indicate that the overlap observed in siDDX5-cells is strongly significant ($p<0.0001$, Chi-square test) compared to a situation in which the same number of regions was distributed randomly, which led to almost 4-fold less overlap. Thanks for helping us strengthen the conclusions.

Figure 3.
- GammaH2AX is phosphorylated not only upon DSB formation but following other types of stress so suggesting that gammaH2AX sites are DSBs site is not appropriate. The authors should use other markers of DSBs to say if hybrids colocalize with breaks in DDX5-depleted cells.
γH2AX is a standard DSB marker and has already been used in PLA experiments together with S9.6 to detect DNA-RNA hybrid-associated DSBs. (ex. Stork *et al*., eLife 2016). In any case, to confirm our results, we have added a new set of data showing PLA signal of S9.6-anti-NBS1, a component of the MRN complex and known interacting factor of DSB-flanking chromatin (Bekker-Jensen *et al.*, JCB 2006). The new data are now shown in new Fig EV4A). These results are consistent with our DRIP experiments in DIvA cells showing that DDX5-depleted cells accumulate DNA-RNA hybrid signal where DSBs are induced by *Asi*SI (Fig 3B).
- The authors should check more than one site (RBMXL1) in their analysis of DSBs with hybrids affected by BRCA2 and DDX5 (Fig 3b)
As requested, we have now included the *ASXL1* site as well as another non-DSB sites (*WDR90*) in new Fig EV4. The results are consistent with those of the other loci.
- The impact of SETX, BRCA2 and DDX5 depletion on the hybrids at DSB sites seems modest and is only a fraction of the impact of the DSB itself on hybrid formation. This should at least be noted.
We agree. However, this moderate effect is consistent with previous reports and we have noted it in the text.

Figure 4.
- Figure 4a - why is DDX5-GPF decreasing at only 11% of cells. Is this 11% of all cells with laser irradiated sites or just 11% of cells, a subset of which have been

irradiated. Presenting this as a fraction of laser irradiated sites seems most appropriate and I assume that is the case here. If so, however, why only 11%.
As mentioned by the reviewer, the % of cells quantified in the graph corresponds to all laser irradiated cells that are DDX5-GFP positive. We have now stated this more clearly in the graph and in the Figure legend. 11% means that at time 0, 11% of DDX5-GFP transfected damaged cells show an anti-stripe as compared to 20% in cells depleted of BRCA2. This makes sense because there is a short time required to detect the DNA damage in the cell and the response. Importantly, at time 6 min, the number of cells with GFP-DDX5 exclusion is over 60% in BRCA2-depleted cells as opposed to 20% in the cells with BRCA2.
- Are these cells in a certain phase of the cell cycle?
These are asynchronous cells but since over 60% of them present similar phenotype at a given time, we do not think this phenomenon is cell-cycle dependent but rather a general trend that is not visible in some of the cells due to the detection limit in the assay.
- Generally this is a confusing presentation as in the end they argue that BRCA2 helps to keep DDX5 at these sites, yet they start off talking about the exclusion of DDX5 from damage sites.
The phenomenon we observe is exclusion or anti-stripe, thus we think the best way to represent it is to show the number of cells with this pattern. Our interpretation is indeed that since depleting BRCA2 leads to increase exclusion, the presence of BRCA2 might help retain DDX5. This interpretation is consistent with the reduced DDX5 at induced DSBs in BRCA2-depleted cells (Fig 4B).
- What is the binding/localization of BRCA2 in this assay?
- Do both BRCA2 WT and T207A bind to the 'stripe' and does the mutant BRCA2 bind the DSB site by ChIP as well as the WT BRCA2? This would help establish if the mutant is functional in other ways and that the effects are on recruitment of DDX5.
To address these two questions about the localization of BRCA2 and BRCA2-T207A at DSBs (laser-induced DNA damage or “stripe”) we have performed new experiments expressing GFP-BRCA2 and GFP-BRCA2-T207A in U2OS. As we cannot observe the recruitment in live cells due to the limited detection of BRCA2, we fixed the cells at 5 min and quantified the recruitment of BRCA2 and BRCA2-T207A at the damage using γH2AX to detect irradiated cells and anti-GFP antibody to detect BRCA2 or BRCA2-T207A. The quantification of our results show clearly that both BRCA2 and BRCA2-T207A are recruited at equivalent levels to DSBs as indicated by the intensity of GFP signal at the laser stripes. Thus, we think T207A is perfectly functional in its recruitment to DSBs. Representative images of the recruitment and the quantification of the intensity from three independent experiments is shown in new Fig 7C.
- Figure 4b - please examine additional sites of DSB induction and negative controls. Also if DDX5 affects hybrids at HIST1H2BG as the authors state, does DDX5 localize there? It's hard to determine this from the data show as the value of the background is not clear. And why is DDX5 ChIP increased by siBRCA2 if it's localization is BRCA2 dependent. Finally, why does the break impact the recruitment of DDX5 in the siBRCA2 condition. These results need further discussion
Please see the answer to point 5 of referee 2 which addresses this question.

Figure 5
- The impact of BRCA2 on DDX5 activity is rather modest. Also, the authors should

show the impact of other BRCA2 fragments on DDX5 activity as controls. They suggest the effect does not depend on AA's 250-500 but never test this in the in vitro assay.
We have now included the unwinding activity with $BRCA2_{LT3}$ fragment and showed that it also stimulates the helicase activity of DDX5 (New Fig 5B). We have also performed the experiment with $BRCA2_{T2}$ but in this case we see an inhibition. We think this is due to the DNA binding activity of T2 which may outcompete DDX5 from the substrate. We have included these data in Fig EV5A.
- Why are the authors using an R-loop type hybrid as their substrate. Since they are looking at hybrids at DSB ends, it might be more appropriate to use more simple hybrid. Does BRCA also stimulate DDX5 activity at this type of hybrid?
Our model suggests that the function of BRCA2 and DDX5 at DSBs depends on transcription, so Rloop structures are compatible with this model.
We have nevertheless performed the experiment with DNA-RNA hybrids. For the DNA-RNA hybrid used, DDX5 shows very little activity compared to the Rloops, however, BRCA2 can readily stimulate the activity. These new data are shown in Fig EV5B.
-The authors conclude that 'BRCA2 stimulates the R-loop unwinding activity of DDX5'. This statement needs more support. Although co-incubation with BRCA2 increases R-loop unwinding ability of DDX5, it is possible that BRCA2 only increases the binding ability of DDX5 to the R-loop structure without changing its catalytic activity. The results in Figure 6 indicate that BRCA2 recruits DDX5 to hybrids, which supports this idea.
As suggested by the reviewer and stated in our discussion, the stimulation could arise by stimulating the binding to the substrate or enhancing the catalytic activity of DDX5. We have performed in vitro ATPase with purified $BRCA2_{LT3}$, a fragment that contains the interacting domain of DDX5 and stimulates its unwinding activity (Fig 5A). The results shown now in Fig 5B indicate that sub-stoichiometric concentrations of $BRCA2_{LT3}$ enhance the ATPase activity of DDX5 by almost 3-fold. Thus, we favor the hypothesis that BRCA2 stimulates the unwinding ability of DDX5 by enhancing its catalytic activity.

Figure 6
- Figure 6 - please show effect of RNaseH on hybrid levels for IF and please test additional genomic sites by DRIP-qPCR to demonstrate the impact of the BRCA2 mutation at T207 on hybrid formation
The effect of RNase H was already shown in supplementary data but, as stated above (point 1 referee 1), we have now moved these data to the main figure to substitute the data performed with non-transfected cells (new Fig 6B).
We have now tested two extra sites (*MALAT1* and *RRPH1*) previously shown to accumulate DNA-RNA hybrids in U2OS cells upon DDX5 depletion (Mersaoui et al EMBO 2019) and in both BRCA2-/- versus BRCA2 +/+ and BRCA2-T207A versus WT cells. The results show a similar trend of increased hybrid levels (new Figs 6C and EV5D).
- Figure 6d - the effects here are extremely small - with average increases of about no PLA foci to about 0.2 PLA foci per nucleus? Is this real?
As mentioned above (answer to comment 2 of reviewer 2) the pool of interacting molecules is small and we use stringent conditions to detect only proteins that are bound to the chromatin. All soluble protein pools are washed off in our extraction

method, we have now stated this also in the text rather than only in the Methods section.
Given that the antibody-only controls in this set showed high background signal we decided to repeat these experiments. This is now included in new Figure 6D. The results show now low signal in controls but overall the same trend (increase in PLA signal in IR conditions vs non-treated) and stronger interaction in WT cells compared to T207A.

I think labeling of this graph may also be incorrect - are the two right samples the BRCA2 mutant?

Yes, it is correct, the two right lanes in the graph correspond to - and + IR (non-treated *vs* irradiated cells), and it increases also although to less extent in the T207A cells.

- Figure 6e - why does the interaction of DDX5 and S9.6 in the T207 mutant go down with IR from a higher baseline, and also why don't the WT cells show an increase in this interaction. This seems contrary to the predictions of the model.

We have confirmed these results with a new set of experiments including now the RNase H control that was missing. They are shown in new Fig 7A. The decrease is real both in the case of BRCA2-depleted cells and T207A mutated cells. Our interpretation is that a pool of DDX5 in non-treated conditions is at the DNA-RNA hybrids, consistent with a previous work (Mersaoui et al., EMBO 2019). In irradiated cells, in the absence of BRCA2 (BRCA2-/-) or reduced BRCA2 interaction (T207A), DDX5 is excluded from DSBs (as shown if Fig 4A) due to transcription reduction occurring under these conditions and that results in a decrease in the PLA signal of anti-DDX5-S9.6.
With respect to whether the WT shows an increase in the interaction, indeed it does, even though is not statistically significant probably due to the small number of PLA spots under these stringent conditions (new Fig 7A).

- The authors should test the interaction of BRCA2 T207A with DDX5 in vitro using purified proteins, as in Figure 1e.

As suggested, we have performed these experiments now and they are included in Fig 7E. These in vitro results are in agreement with the pull-down shown in 6A performed with cell lysates and indicating that $BRCA2_{T1}$-T207A reduces the interaction with DDX5 although the effect is overall smaller than in the case of the full-length protein.

- It is unclear why the BRCA2T1-T207A mutant would inhibit the helicase activity of DDX5 if it interacts with it less effectively. This is not really consistent with the authors model. More development of this mechanism is needed. The authors ultimately suggest that BRCA2 might affect the ATP hydrolysis rate of DDX5 or the binding of DDX5 to RNA. Further work on this question and what the T207A mutant is doing is needed to strengthen this story.

The reduction in the pull-down with the purified $BRCA2_{T1}$ and $BRCA2_{T1}$-T207A is more modest than with the full-length protein and yet $BRCA2_{T1}$-T207A strongly inhibits the reaction. In addition, as stated above, we have now performed a series of in vitro ATPase experiments using a fragment comprising BRCA2-T1 (BRCA2-LT3 1-750aa) and found that sub-stoichiometric amount of BRCA2-LT3 enhances the ATPase activity suggesting BRCA2 stimulates the catalytic activity of DDX5. Thus, our interpretation is that the fraction of $BRCA2_{T1}$-T207A that binds DDX5 does it in a non-productive way probably reducing the ATPase activity of DDX5.

- In Figure 6, it is clear that BRCA2-DDX5 and DDX5-S9.6 PLA are enhanced by IR in DLD1 cells with BRCA2 WT. To conclude that the BRCA2-DDX5 interaction

promotes localization of DDX5 to hybrids that are actually at the site of DNA damage, the authors should also analyze the PLA between DDX5 and rH2AX in BRCA2 WT and T207A cells.
As suggested, we have done a new set of PLA experiments to show DDX5-γH2AX co-localization in both WT and T207A mutated cells; our results are shown now in Fig 7B and indicate that the PLA signal for DDX5-γH2AX in cells bearing T207A variant is reduced compared to WT cells. In both cases, the signal is further reduced when incubated with RNase H. These results suggest that the recruitment of DDX5 to DNA damage is reduced in cells bearing BRCA2-T207A and is not due to a reduced recruited BRCA2-T207A as now shown in Fig. 7C.
- Does overexpression of RNaseH reduce the binding of BRCA2-DDX5 to damage sites?
This is a difficult question to address since it would imply PLA in combination with IF for a marker of DSBs which is technically quite challenging. An indirect measure of this is that the co-localization of BRCA2-DDX5 (PLA signal) increases in IR-treated cells and that this signal is sensitive to both transcription inhibition (cordycepin) and RNase H treatment (Fig 1C).

Figure 7
- The impact of the BRCA2 mutant on Rad51 foci is quite modest as is the effect of RNaseH expression. More data in support of the idea that this mutant is having a biological effect on HR is needed. Counting foci could be challenging and I wonder if the results would be stronger if the chromatin bound Rad51 were measured instead of foci number.
As suggested, we have performed chromatin fractionation in cells bearing T207A in irradiated conditions and determined the levels of RAD51 bound to chromatin. Consistent with our results with RAD51 foci (Fig 7D), new Fig 8E shows that the fraction of RAD51 bound to chromatin increases in irradiated T207A cells when overexpressing RNase H1.

Minor Points
Discussion p17/18. - Parts of the discussion text is very confusing with long sentences. In particular the two sentences starting "On the one hand...." And "On the other hand" need work, as does the one following this....
As requested, we have revised the Discussion section to make it clearer. Thanks

1st Revision - Editorial Decision 6th Jan 2021

Thank you for submitting your revised manuscript to The EMBO Journal, and please excuse the delay in getting back to you with the outcome of its re-evaluation. All three original reviewers have now looked at it again, and I am pleased to say that they found the study overall significantly improved. Nevertheless, especially referee 1 still retains some substantive concerns that, after discussing them further with the other referees, I feel would still be important clarify prior to publication, in order to strengthen the conclusions. Given that the study has already addressed a considerable number of issues during the first revision, I would be happy to grant an exceptional second round of experimental revision in this case, to allow you to deal with these remaining presentational and conceptual points. Furthermore, I would also offer to discuss proposals for how to best address the open issues directly with you in the coming weeks.

When re-revising the manuscript, please also pay attention to incorporate the following editorial points in the final version of the study:

REFEREE REPORTS

Referee #1:

I would like to thank the authors for their responses to my previous comments. My previous comments have been appropriately addressed. I believe the manuscript has been improved, but I do have some further comments and suggestions I would like addressed first.

Major comments:

1. I would prefer to see the ChIP of DDX5 with control/BRCA2/DDX5 (Fig. EV4D) knockdown at both RBMXL1 and ASXL1 cut sites should be moved to the main figures (Fig. 4B). This data is far more convincing than the laser track and PLA analysis for the recruitment of DDX5 to DSBs and also shows that this is dependent on BRCA2. Also, I would rescale the y-axis, I understand you want to fit in tiered statistics, however if you limit the stats to only compare -OHT to +OHT for each siRNA (rather than comparing siRNA as well) then the bars could be taller and then I think this could be quite convincing.
2. My biggest issue at the moment is that the final figure (Fig. 8) is unfortunately not as strong as the rest of the manuscript. All the differences to the T207A mutant are very subtle, especially the RPA foci. The authors should try to validate the claims here regarding HR, hybrids and the T207A mutant more. Perhaps with the use of reporter assays, additional IF or laser tracking. A more direct measure of genome instability, such as comet assays or metaphase spreads might also provide a functional interpretation of these results.
3. I also have a suggestion that may be beneficial. A previous publication on Senataxin (Cohen et al., 2018) found that Senataxin depletion had little impact with IR treatment, but showed a significant phenotype with etoposide treatment and with OHT in DIvA cells. This was due to IR being random

and since 95% of the genome is untranscribed and Senataxin specifically acted at transcribed loci, most IR induced sites were Senataxin independent. Since your ChIP in DIvA cells at the highly transcribed RBMXL1 and ASXL1 sites showed a good enrichment of DDX5, I would like to suggest you either repeat some of your PLA or IF experiments either with DIvA cells or etoposide treatment instead of IR? Particularly those with subtle phenotypes, such as those with the T207A mutant. Also, because DNA:RNA-hybrids at damage sites have been shown to be transcriptionally driven (Bader, Bushell, 2020) as well as HR repair in general (Aymard et al., 2014), your phenotype for DDX5/BRCA2 may be stronger with a TOPII inhibitor like etoposide that will specifically damage active transcription sites. (Aymard et al., 2014, Cohen et al., 2018, Bader, Bushell, 2020)

Minor comments:
1. Fig6C, what is the DRIP signal relative to? This is not explained. Why not just do % of input like for other plots?
2. Thanks for the DRIPc correlation, but what is it correlating? Peak location, peak height, coverage at TSS sites? This analysis needs explaining.
3. Figure 3A quantification states S9.6 and DDX5 on the left of the x-axis, however states S9.6 and yH2AX for the rest. From the legend and main text I assumed the DDX5 should be yH2AX?
4. What is the difference between figures 8D and EV5H? They appear to be different plots, but they are labelled the same and described the same in the legends and main text.

References:

Aymard, F., Bugler, B., Schmidt, C.K., Guillou, E., Caron, P., Briois, S., Iacovoni, J.S., Daburon, V., Miller, K.M., Jackson, S.P. & Legube, G. 2014, "Transcriptionally active chromatin recruits homologous recombination at DNA double-strand breaks", Nature structural & molecular biology, vol. 21, no. 4, pp. 366-374.

Bader, A.S. & Bushell, M. 2020, "DNA:RNA hybrids form at DNA double-strand breaks in transcriptionally active loci", Cell Death & Disease, vol. 11, no. 4, pp. 280.

Cohen, S., Puget, N., Lin, Y., Clouaire, T., Aguirrebengoa, M., Rocher, V., Pasero, P., Canitrot, Y. & Legube, G. 2018, "Senataxin resolves RNA:DNA hybrids forming at DNA double-strand breaks to prevent translocations", Nature Communications, vol. 9, no. 1, pp. 533.

Referee #2:

The authors have made a reasonable job revising this manuscript. However I am slightly confused by some of their responses.

Related to my previous comment 4:
The authors say that DHX5 chromatin binding does not depend on the presence of the hybrids, however it does affect gene expression of the genes with hybrids. So my question is how is the DHX5 binding specificity achieved? Currently this is not discussed in the response to reviewers' letter or manuscript, but biologically represents an important point related to the whole paper.

Regarding the qPCR validation of genome-wide data - the increase in DRIP signal in DDX5 KD cells seems small (Fig.EV3A), the authors state that it is significant based on 2 biological repeats. Could

they please indicate p values as compared between siCtrl and siDHX5 conditions.

Referee #3:

The authors have completed a number of additional experiments that address the majority of my concerns and that clarify previously confusing points. I still have minor concerns about the small effect in some experiments (especially PLA) but most points are supported in more than one way. Thus, I think the manuscript is now ready for publication.

2nd Authors' Response to Reviewers 22nd Jan 2021

Referee #1:

I would like to thank the authors for their responses to my previous comments. My previous comments have been appropriately addressed. I believe the manuscript has been improved, but I do have some further comments and suggestions I would like addressed first.
Thank you very much for all the corrections and constructive comments.

Major comments:
1. I would prefer to see the ChIP of DDX5 with control/BRCA2/DDX5 (Fig. EV4D) knockdown at both RBMXL1 and ASXL1 cut sites should be moved to the main figures (Fig. 4B). This data is far more convincing than the laser track and PLA analysis for the recruitment of DDX5 to DSBs and also shows that this is dependent on BRCA2. Also, I would rescale the y-axis, I understand you want to fit in tiered statistics, however if you limit the stats to only compare -

OHT to +OHT for each siRNA (rather than comparing siRNA as well) then the bars could be taller and then I think this could be quite convincing.
Thanks for the suggestion. This is now corrected in the revised manuscript (NEW Fig. 4).

2. My biggest issue at the moment is that the final figure (Fig. 8) is unfortunately not as strong as the rest of the manuscript. All the differences to the T207A mutant are very subtle, especially the RPA foci. The authors should try to validate the claims here regarding HR, hybrids and the T207A mutant more. Perhaps with the use of reporter assays, additional IF or laser tracking. A more direct measure of genome instability, such as comet assays or metaphase spreads might also provide a functional interpretation of these results.
The results from the mutant T207A span figures 6-8. We show that this single amino-acid change in a protein of 3418 aa (BRCA2) reduces the interaction with DDX5 to half (Fig 6A, D), augments significantly the number of R-loops by PLA and DRIP (Fig. 6B, C) especially those associated with DNA breaks (Fig. 7D). Although T207A is correctly recruited to DNA breaks (Fig. 7C), DDX5 association with R-loops in IR conditions is reduced to the levels of BRCA2-deficient cells (PLA S9.6-DDX5) (Fig. 7A). A truncated form containing this single change abolishes the R-loop unwinding activity of DDX5 (Fig. 7E). Cells bearing this single amino-acid change in BRCA2 delay the kinetics of recruitment of RAD51 to DNA damage induced by IR (Fig. 8B), an effect that is not smaller than the one observed with the depletion of DDX5. This phenotype is partially reverted by RNase H treatment showing increased RAD51 foci in irradiated cells (Fig. 8D) and increased chromatin-bound RAD51 under the same conditions (Fig. 8E). We cannot expect the same effect of a single amino acid change that reduces an interaction as a total depletion of a protein, but we think this variant is a unique genetic tool to elucidate directly the function of DDX5-BRCA2 interaction that cannot be inferred from the depletion of either protein. We might have observed a stronger phenotype deleting several amino-acids of the region of interaction, but we deemed that this variant found in breast cancer patients was more physiologically relevant.
The levels of RPA foci are significantly reduced even though the levels of yH2AX foci are slightly higher in the mutant T207A; thus, although we do not claim an effect in resection because we have not investigated further other resection factors, this is a hint that resection might be indeed altered and the statistics suggest the difference is not subtle.
Importantly, as depicted in our model (Fig 8F) and discussed in the text, we do not believe that BRCA2-DDX5 interaction is the only way to deal with DNA-RNA hybrids at DSBs; other RNases and/or helicases have already been shown to operate at DSB-associated DNA-RNA hybrids. Thus, we do not expect that the consequences of reducing BRCA2-DDX5 interaction (as it is the case for T207A) would lead to detectable levels of genome instability in metaphase spreads or in HR reporter assays. Indeed, we have already performed a reporter DSB repair assay using cells expressing this variant (T207A) in a recent paper from the lab and showed that the DSB repair (at the end point) is similar to the one observed in WT cells (Ehlen et al., 2020; Fig. 9f). This is in fact not surprising as the kinetics of a reaction does not necessarily correlate with a difference in the final product, since they measure two different parameters. Our work on the repair of replication-born DNA breaks in yeast cells in which we observe that a DNA-break repair reaction can be delayed with little effect on the final products is a good example of this (González-Barrera et al, Mol Cell 2003; Muñoz-Galván et al, PLoS Genet 2017; Ortega et al Nat Comm 2019, among others).

3. I also have a suggestion that may be beneficial. A previous publication on Senataxin (Cohen et al., 2018) found that Senataxin depletion had little impact with IR treatment, but showed a significant phenotype with etoposide treatment and with OHT in DIvA cells. This was due to IR being random and since 95% of the genome is untranscribed and Senataxin specifically acted at transcribed loci, most IR induced sites were Senataxin independent. Since your ChIP in DIvA cells at the highly transcribed RBMXL1 and ASXL1 sites showed a good enrichment of DDX5, I would like to suggest you either repeat some of your PLA or IF experiments either with DIvA cells or etoposide treatment instead of IR? Particularly those with subtle phenotypes, such as those with the T207A mutant.

Thank you for this suggestion. We are aware of these results and we have cited this reference several times in the text. We do not have the mutation T207A in DIvA cells for which we would need to make a BRCA2-deficient DIvA cell system and, if the cells survive, make stable clones bearing the mutation. We could do this experiment in DIvA cells using transient transfection but this would probably not provide reliable data because even small differences in overexpression could end up masking the effect of the mutation.

Also, because DNA:RNA-hybrids at damage sites have been shown to be transcriptionally driven (Bader, Bushell, 2020) as well as HR repair in general (Aymard et al., 2014), your phenotype for DDX5/BRCA2 may be stronger with a TOPII inhibitor like etoposide that will specifically damage active transcription sites. (Aymard et al., 2014, Cohen et al., 2018, Bader, Bushell, 2020)

In the Cohen et al paper, the authors could not detect any difference in the recruitment of RAD51 under IR conditions upon Senataxin depletion. However, using a single amino acid change in BRCA2 (T207A) we observe clear differences in the kinetics of RAD51 recruitment to DNA damage (Fig. 8B), and on the proximity of DDX5 to DNA-RNA hybrids (Fig 7A) under IR conditions. We chose IR because DDX5 depleted cells have already been shown to be sensitive to this treatment (Nicol et al., 2013). As stated above, we do not expect a defect in HR repair but a less efficient or delayed repair, and we have shown that DSB repair by gene targeting is equivalent in T207A and BRCA2 WT cells (Ehlen et al., 2020). In that paper we also detected a mild sensitivity to MMC as mentioned in the discussion of this manuscript which might be caused by the delayed RAD51 foci formation we observe here. Although the TOPII inhibitor might enhance the sensitivity of T207A mutant, we do not think this experiment is justified at this point as the main message of the manuscript is the role of BRCA2 and DDX5 in resolving DNA-break associated hybrids which we have demonstrated in different ways. In addition to this, this point assumes that the break would originate first and the hybrid formed afterwards (certainly, hybrids have to be transcriptionally-driven, otherwise no RNA would be generated); however, it may well be that, at least in a good proportion of cases, the break occurs after the DNA-RNA hybrid. A related example for this could be the recent reports showing that ligands that bind G4 structures (which are stimulated by DNA-RNA hybrids) cause DNA breaks by a transcription-driven Top2 mediated mechanism (Slazchta et al, NAR 2020; Bruno et al, PNAS 2020; Olivieri et al, Cell 2020), implying that the break comes after the aberrant structure. There is indeed an intense debate on this topic that we and others have reviewed in the past (i.e., Aguilera and Gómez-González, NSMB 2017).

This uncertainty about the transcription-driven action of etoposide together with the arguments exposed above make us believe that, although it is an interesting experiment, it will not be clarifying to this manuscript, as it needs to resolve a question that is out of the scope of this manuscript. We hope that Editor and referee agree with our position, which we are open to discuss further if needed.

Minor comments:

1. Fig6C, what is the DRIP signal relative to? This is not explained. Why not just do % of input like for other plots?

The signal is relative to the WT DRIP levels set to 1 in the graphs where relative units are indicated. We have now stated this in the legends, thanks. This is due to the fact that the efficiency of immunoprecipitation of spontaneous DNA-RNA hybrids fluctuates between experiments and this can mask the relative effects that we are interested in (depleted versus siC cells). This is not the case in Fig 3B (DRIP in DIvA cells) because the induction of the break makes hybrids a much more frequent event allowing us to plot the % input in that case.

2. Thanks for the DRIPc correlation, but what is it correlating? Peak location, peak height, coverage at TSS sites? This analysis needs explaining.

We have now clarified this in the legend. Thank you for pointing this out. We have also revised other aspects of the DRIPc-seq and explained them better in the legends. As a consequence and, to be fully consistent in the parameters used in all the analyses, we have

modified slightly Fig 2 and EV3. We have preferred to separate the meta-analysis of each cell line to avoid confusion.

3. Figure 3A quantification states S9.6 and DDX5 on the left of the x-axis, however states S9.6 and yH2AX for the rest. From the legend and main text I assumed the DDX5 should be yH2AX?
Thanks for pointing out this mistake, it should say yH2AX. We have now corrected this in NEW Fig. 3.
4. What is the difference between figures 8D and EV5H? They appear to be different plots, but they are labelled the same and described the same in the legends and main text.
EV5H shows the number of yH2AX and RAD51 foci in non-irradiated conditions so it is a control of Fig. 8D (+IR) to show that the levels of yH2AX or RAD51 foci are not affected by RNaseH treatment. We have now stated in Fig. EV5H non-treated conditions so that it is more clear for the reader.

References:

Aymard, F., Bugler, B., Schmidt, C.K., Guillou, E., Caron, P., Briois, S., Iacovoni, J.S., Daburon, V., Miller, K.M., Jackson, S.P. & Legube, G. 2014, "Transcriptionally active chromatin recruits homologous recombination at DNA double-strand breaks", Nature structural & molecular biology, vol. 21, no. 4, pp. 366-374.

Bader, A.S. & Bushell, M. 2020, "DNA:RNA hybrids form at DNA double-strand breaks in transcriptionally active loci", Cell Death & Disease, vol. 11, no. 4, pp. 280.

Cohen, S., Puget, N., Lin, Y., Clouaire, T., Aguirrebengoa, M., Rocher, V., Pasero, P., Canitrot, Y. & Legube, G. 2018, "Senataxin resolves RNA:DNA hybrids forming at DNA double-strand breaks to prevent translocations", Nature Communications, vol. 9, no. 1, pp. 533.

Referee #2:

The authors have made a reasonable job revising this manuscript. However I am slightly confused by some of their responses.
Thank you very much for all the corrections and constructive comments.

Related to my previous comment 4:
The authors say that DHX5 chromatin binding does not depend on the presence of the hybrids, however it does affect gene expression of the genes with hybrids. So my question is how is the DHX5 binding specificity achieved? Currently this is not discussed in the response to reviewers' letter or manuscript, but biologically represents an important point related to the whole paper.
DDX5 binding to chromatin is detected in genes both with and without hybrids. Please note in figure EV4D that we detected similar levels of DDX5 ChIP at all the tested loci (*RBMXL1, ASXL1, HIST1H2BG, WDR90* and *SNRPN*) and that these levels are close to background. If DDX5 were specifically recruited to hybrids, we would expect a higher level of DDX5 ChIP in *HIST1H2BG* or *WDR90* than in *SNRPN* in accordance with the hybrid levels shown in figure EV4B, since we used the same primers. In contrast, we think that DDX5 is recruited to all transcribed genes regardless of R-loop formation and, if a DSB occurs, it is retained there by the interaction with BRCA2 to aid in hybrid removal. This is what would cause a major DDX5 occupancy (not necessarily recruitment) after tamoxifen addition as detected by ChIP.

Regarding the qPCR validation of genome-wide data - the increase in DRIP signal in DDX5 KD cells seems small (Fig.EV3A), the authors state that it is significant based on 2 biological

repeats. Could they please indicate p values as compared between siCtrl and siDHX5 conditions?
Throughout the paper, only significant p-values are shown as stated in the legends. We have now also stated this in the *Quantification and statistical analysis* section to be clearer. Nonetheless, we have realized that one of the p values was not significant and still shown. We have now removed this value for consistency. In any case, the differences between siC and siDDX5 are 0.0677, 0.1524 and 0.3390 in the three different genes respectively, and therefore not significant and not shown. We have only used these data to confirm the presence of DNA-RNA hybrids as shown by their sensitivity to RNase H treatment. Consistent with this, we stated in the text: "Importantly, all S9.6 signals were severely reduced after *in vitro* treatment with RNase H1 indicating that S9.6 immunoprecipitation was specific for DNA-RNA hybrids".

Referee #3:

The authors have completed a number of additional experiments that address the majority of my concerns and that clarify previously confusing points. I still have minor concerns about the small effect in some experiments (especially PLA) but most points are supported in more than one way. Thus, I think the manuscript is now ready for publication.
Thank you very much for all the corrections and constructive comments.

Accepted **25th Jan 2021**

Thank you for submitting your final revised manuscript for our consideration. I am pleased to inform you that we have now accepted it for publication in The EMBO Journal.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

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Corresponding Author Name: Andres Aguilera, Aura Carreira
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2020-106018R

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	No sample size calculation was performed.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No data were excluded.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	No they weren't. Randomization is not applicable as the samples are considered independent.
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No blinding was performed but the analysis and quantification of data from microscopy images were automatically performed (metamorph sofware) to avoid any possible bias.
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes
Is there an estimate of variation within each group of data?	Yes, SD or SEM are represented in every graph, as indicated.
Is the variance similar between the groups that are being statistically compared?	Yes, it's similar. In experiments with small sample sizes, equal variances were assumed and t-test applied. In IF assays, with data from big populations, Mann-Whitney tests were applied since the populations did not exhibit Gaussian distribution.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	anti-MBP (R29) (mouse, immunoblot (IB): 1:5000) Thermo Fisher Scientific Cat# MA5-14122 anti-BRCA2 (OP95) (mouse, (co-IP): 1µg , (IB): 1:1000 (PLA); 1:2000) EMD Millipore OP95 anti-DDX5 (mouse, (IB): 1:100 or 1:500 (ChIP): 4 µg (IF): 1:500) Santa Cruz Biotechnology Cat# sc-166167 anti-DDX5 (goat, (PLA)1:3000) Abcam Cat# ab10261 anti-pSer139-γ-H2AX (rabbit, (PLA):1:3000) EMD Millipore Cat# 07-164 anti-pSer139-γH2AX (clone JBW301) (mouse, IF: 1:1000) EMD Millipore Cat #05-636 anti-pSer139-γH2AX (rabbit (IF):1:1000) (ChIP): 4 µg Abcam Cat # ab2893 anti-RAD51 (clone H-92) (rabbit, (IF): 1:100 or 1:1000 clone H-92) Santa Cruz Biotechnology Cat#sc-8349 anti-RPA32 (rat, (IF):1:1000) Ozyme (Cell Signaling) Cat# 2208S anti-DNA-RNA hybrids (S9.6) (mouse, (PLA):1:100000 (IF): 1:500) Protein Expression and Purification Core facility, Institut Curie anti-DNA-RNA hybrids (S9.6) ((IF): 1:2000 Purified from hybridoma cell line HB-8730 anti-nucleolin (rabbit, (IF):1:1000) Abcam Cat#ab50279 anti-nucleolin (rabbit, (IF):1:1000) Abcam Cat#ab22758 anti-GFP (rabbit (IF):1:200) Abcam Cat#ab6556

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http://www.selectagents.gov/	List of Select Agents

6. Continued	anti-biotin (rabbit (IF): 1:3000) Bethyl Laboratories Cat# BETA150-109A anti-rabbit IgG (ChIP): 4 µg SIGMA Cat# I8140 anti-mouse IgG (ChIP): 4 µg SIGMA Cat# I8765 goat anti mouse IgG-HRP: (co-IP): 1µg Santa Cruz Biotechnology Cat# sc-2055 mouse-IgGκ BP-HRP (IB: 1:5000) Santa Cruz Biotechnology Cat#sc-516102 goat anti-mouse IgG-HRP (IB: 1:10 000) Interchim Cat#115-035-003 chicken anti-rat Alexa Flour 647 (IF): 1:1000 Life Technologies Cat#A-21472 donkey anti-mouse Alexa-594 (IF): 1:1000 Thermo Fisher Scientific Cat#A-21203 donkey anti-rabbit Alexa-488 (IF): 1:1000 Thermo Fisher Scientific Cat#A-21206 goat anti-human Alexa-555 (IF: 1:1000) Thermo Fisher Scientific Cat#A-21433 donkey anti-mouse Alexa-488 (IF: 1:1000) Thermo Fisher Scientific Cat#A-21202 chicken anti-mouse Alexa-594 (IF): 1:1000 Thermo Fisher Scientific Cat#A-21201 chicken anti-mouse Alexa-488 (IF): 1:1000 Thermo Fisher Scientific Cat#A-21200 goat anti-mouse Alexa-546 (IF): 1:1000 Thermo Fisher Scientific Cat#A-11030 goat anti-rabbit Alexa-568 (IF): 1:1000 Thermo Fisher Scientific Cat#A-11011 goat anti-mouse Alexa-488 (IF): 1:1000 Thermo Fisher Scientific Cat#A-11029 Where indicated, IgG was used as negative control for antibodies. In the case of anti-DDX5 (Cat# sc 166167), immunofluorescence with DDX5 depleted cells was performed for additional antibody validation. In the case of S9.6, RNase H treatment was performed for additional antibody
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	U2OS (human osteosarcoma epithelial cell line) Kind gift from Mounira Amor-Gueret HEK293T (human embryonic kidney cell line) Kind gift from Mounira Amor-Gueret K562 (Erythromyeloblastoid leukemia cell line) ATCC CCL-243 DLD1 (human colorectal adenocarcinoma cell line) Horizon Discovery HD-PAR-008 DLD1 BRCA2-/- Horizon Discovery (Hucl, T. et al 2008) Horizon Discovery HD 105-007 DLD1 BRCA2-/- GFPMBPBRCA2 WT clone C1 (Ehlen et al.2020) DLD1 BRCA2-/- GFPMBPBRCA2 T207A clone B1 (Ehlen et al.2020) DIvA cells (AsiSI-ER-U2OS) Kind gift from Gaelle Legube (Aymard et al, 2014). All cell lines used have been authenticated by genotyping. All cell lines used in this study have been regularly tested negatively for mycoplasma contamination (MycoAlert, Lonza).

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8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
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E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
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18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Mass Spectrometry source data for Table EV1 and Figure EV1B is accesible at ProteomeXchange Consortium via PRIDE (accesion PXD018979). The DRIPc-seq source data used in figure 2C-F are available in GEO: GSE150163 for siDDX5 samples (this study), GSE127979 for siC samples (Pérez-Calero et al 2020) and GSE104800 for H2AX ChIP-seq data (Kim et al 2018).
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