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# ATM and Artemis promote homologous recombination of radiation-induced DNA double strand breaks in G2

Andrea Beucher, Julie Birraux, Leopoldine Tchouandong, Olivia Barton, Atsushi Shibata, Sandro Conrad, Aaron A. Goodarzi, Andrea Krempler, Penny A. Jeggo

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**Review timeline:**

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## Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 16 March 2009

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are enclosed below, as you will see from their comments the referees provide mixed recommendations, and unfortunately the majority of referees recommend against publication in the EMBO Journal.

As you will see from their reports, while referee #1 is more positive about the manuscript, both referee #2 and #3 raise a number of important technical and conceptual issues. Importantly, these two referees find that while the genetic link of Artemis to HR in G2 could be potentially interesting the current study does not provide sufficient molecular and genetic evidence for Artemis in this context and unfortunately as a consequence, they recommend against publication in EMBO J. Faced with these clear recommendations in the referee reports and in the overall recommendations sent directly to our editorial office and the fact that we can only afford to continue handling of papers that receive enthusiastic support from at least a majority of referees upon initial review, I am afraid, I see little choice but to come to the conclusion that we cannot offer to publish this study.

Thank you for the opportunity to consider your manuscript. I am sorry we cannot be more positive on this occasion, but we hope nevertheless that you will find our referees' comments helpful. We also hope that this negative decision does not prevent you from considering our journal for publication of your future studies.

Yours sincerely,

 

Editor
The EMBO Journal

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REFEREE COMMENTS

Referee #1 (Remarks to the Author):

In this manuscript the authors uncover a role for Artemis in homologous recombination (HR) - mediated in repair of ionising radiation-induced DNA double-strand breaks (DSBs) in G2 cells. The manuscript contains a very extensive set of experiment that are in general very well done and convincing. The work described in the manuscript is highly significant and of great interest to the genome stability field.

The authors may want to pay attention to the following issues:

The authors might reconsider the title. I think I understand what the authors want to say, but that is not what is expressed by the title. The title states that the DSBs studied in this manuscript are ATM and Artemis dependent. Of course they are not; their formation is ionising radiation dependent.

Legend to Figure 2, panel A. The text is mostly redundant with the Results section.

Figure 2. I recommend the use symbols of different more discriminatory symbols for the different cell lines. In certain cases is too hard to see the difference and this is essential to keep the story straight in the mind of the reader.

Page 7. The term ATMi is used for the first time without explanation in the first full paragraph on this page. I assume it means addition of an ATM inhibitor. My assumption is based in part on what follows further down the page 'We also applied RNAi technology to exclude the possibility that KU55933 has off-target ...'. I must also assume that the 'i' in the abbreviation ATMi means something different in the abbreviation RNAi. Alternatively the abbreviation ATMi, implies the use of RNAi against ATM? In any, case the abbreviation ATMi should be abandoned and if it indeed means the addition of inhibitor this should be spelled out instead, throughout the manuscript, with the mention of the specific inhibitor used

Page 9. Modify the last sentence of the first full paragraph to read: 'Moreover, our findings show that HR is only required for the repair of a sub-fraction (~15%) of ionising radiation-induced DSBs in G2 phase.' The modifier is to reflect the fact that HR is an essential DSB repair pathway for the cell, while NHEJ is not.

Page 9. DNA-PKi; see remark above abour ATMi.

Figure 9. I suggest to add primary data to this Figure. Visualizing RPA and BrdU foci is not trivial, let alone quantifying them, so the reader should be given a chance to judge the quality of the primary data.

The experiments shown in Figure 10 are essential to make conclusions about the involvement of the nuclease activity of artemis in ionising radiation-induced HR in G2 cells. A good control would be to test whether the D37N protein is expressed.

In Figure 10 the authors use gH2AX foci as a read out for the effect of the D37N artemis protein. To more strongly implicate the nuclease activity of artemis they may want to consider to check RPA, RAD51 and BrdU foci as was done in Figure 9.

Referee #2 (Remarks to the Author):

 

The manuscript by Beucher et al. addresses a possible role for ATM and Artemis in homologous recombination repair of IR-induced DSBs. Their previous work implicated Artemis, ATM and 53BP1 in a sub-pathway of NHEJ that could repair a defined subset of IR-induced DSBs during the G1 phase of the cell cycle. In this study, the authors show that in G2, as in G1, NHEJ represents the major DSB repair pathway and HR only functions to repair ~15% of IR-induced DSBs. Strikingly, in addition to requiring the known HR proteins, HR in G2 involves Artemis, ATM and the ATM-signaling mediator proteins BRCA1, MDC1, and 53BP1. They also show that these factors function in NHEJ during G1 but promote HR during G2. Finally, they show that Artemis is required for IR-induced HR but is dispensable for I-SceI-induced HR. Based on their data, they propose that Artemis endonuclease is involved in removing lesions or secondary structures which otherwise inhibit end resection and preclude the completion of HR and NHEJ. The authors use immunofluorescence to detect foci of H2AX, RAD54 and MDC1, and they use comet (FAR) assays following irradiation. They do a small amount of biochemical analysis of Artemis. The authors induce damage using IR (and one set of experiments with I-SceI), and in some cases they arrest the cells with polymerase inhibitors or cell cycle cycle blockers.

The issue of relative contributions of HR and NHEJ to DSB repair is of importance, particularly in S-G2 phases of the cell cycle, and I agree that the simple classification of DSB repair pathways (HR/NHEJ) is open to additional levels of complexity. I have several fundamental questions about this study, however. The assays used are not sufficient to draw the conclusion that Artemis is involved in HR. The genetic and immuostaining approaches alone are not sufficient to make this conclusion. Therefore, the model should be removed, and the title and abstract and text should be changed to reflect this insufficiency.

1. In this and previous studies, the authors determined the contribution of each factor or each pathway to IR-induced repair on the basis of PFGE analysis and g-H2AX focus analysis. In the PFGE analysis, non-physiological doses (80 Gy !) is employed, which would be inducing >3000 DSBs per cell. Can such cells really repair the damage? I think not. More importantly, recent evidence clearly indicates that g-H2AX foci can be observed independent of DSBs at G2 phase. Moreover, g-H2AX formation is also induced by chemically synchronizing the cells. Thus, the way of semi-quantitative evaluation would not make sense. Another problem of these analyses, proper contribution of each repair factor or each repair pathway may be masked by other pathways, such as a back-up end joining enzymes, which are reported to rejoin DSBs when both HR and NHEJ are absent or dysfunctional. The authors should discuss their data in light of these.

2. The authors claim that they can count up to 80 foci per nucleus. The authors must provide in the Supplement several high resolution photos demonstrating this. Our experience is that after 30 per nucleus, it becomes very difficult to distinguish foci.

3. The authors' error bars on the foci assays are remarkably tight. This is not our experience. The authors must document that they read these slides in a 'blinded' manner. They need to provide the standard info such as number of cells counted. They also must provide in the supplement a standard curve for how their standard deviation varies with the number of cells counted.

4. The authors must repeat their assays with synchronized cells rather than aphidocholin treated cells. Also, better statistical analysis is needed here.

5. Aphidicolin itself should induce DNA damage, and thus cannot be used to synchronize cells in G2.

6. If Artemis were involved in HR, then Artemis KO (or knockdown) cells would show a reduction in gene targeting efficiency (and perhaps SCE). Is that the case?

 

Referee #3 (Remarks to the Author):

In this report the authors address the question of DSB repair pathway choice and the role of DNA repair/checkpoint proteins in DSB repair in G2 phase mammalian cells exposed to ionizing radiation.

In Fig 1 the authors characterize the G2 system but the authors do not show an example of "Mitotic cells exhibiting distinct centromeric CENP-F staining and condensed chromatin" that were excluded from the analysis. Also, the legend does not specify for Fig. 1B what the control is: no IR + aph? No IR and no aph?

In Fig. 1C, it is clear that neither aph nor BrdU affects focus formation after IR in either G1 or G2 cells, but it is not clear why G2 cells show 3-fold more foci than G1 - does this reflect greater DNA content and if so, wouldn't the difference be 2-fold? In the fig. 1D legend it is stated that the difference is "slightly higher than 2-fold but in Fig. 1C it appears to be more than "slightly" higher. It is stated that a possible explanation for this is in the discussion but I could not find it.

Figs 2 and 3: The basic argument put forth is that if foci removal curves match in distinct mutants, the wild-type proteins act in the same pathway (epistatic). If the distinct repair is seen in slow vs. fast components, then the pathway is designated as HR or NHEJ, respectively. While this second part is supported by a number of studies from this and other labs, the first part is an over-interpretation or worse, a possible mis-interpretation. The "epistasis" analysis described here does not follow the paradigm in which epistasis is defined with double mutants. The fact that two mutants show the same curves may reflect epistasis, but it may just as easily reflect co-incidence. This is the most problematic aspect of the report. The use of inhibitors provides one means to get at this, but the study falls short since there are no BRCA, RAD54, or Artemis inhibitors, nor any double mutants available, and double siRNA or siRNA + inhibitor is generally not tested.

A general criticism: some of the data lacks statistical analysis. While the error bars seem convincing, the authors must report the statistical analysis to back their claims.

Fig. 4: As in Figs 2/3, epistasis is invoked here with single siRNAs (and minor differences). The interpretations of these data are not convincing. It is also concerning that rad51 knockdown does not seem to affect G2 repair very much. Is the fast repair component (2 hr) the same +/- rad51?

p. 8: the authors note in the introduction that high doses needed for PFGE are problematic, then they proceed to use 80 Gy doses, which have questionable biological relevance since they are supra-lethal. It is possible that entirely new mechanisms are brought to bear, or entirely new problems arise, in cells challenged with such extreme doses. Dose rates also become important since long exposures may be needed to achieve high doses (depending on the source) and repair may therefore occur during exposure.

Fig. 10B/p. 15: the minor differences in foci with Artemis complementation or D37N are not convincing. While the general trend supports the conclusion that Artemis nuclease is important for HR after IR, the differences are very small and while statistics are said to have been done, no values are given.

p. 16: "Thus, although currently difficult to substantiate, our findings suggest that the DSBs that are located within or close to heterochromatin are those that undergo repair by HR in G2 phase." I found no data to support this claim, so this is speculation.

p. 19: "Our finding that factors exist which have defined roles in both major DSB repair pathways represents a major conceptual advance and suggests that the previously made distinction between NHEJ and HR may need re-consideration." This is a vast overstatement that ignores publications from several labs that implicate NHEJ proteins in HR regulation: Nucleic Acids Res 30, 3454; Proc Natl Acad Sci USA 99, 3758; Mol Cancer Res 1, 913; Genes Dev 15, 3237.

 

p. 19: "End-processing has been extensively studied in the context of NHEJ (van Gent and van der Burg, 2007; Lieber, 2008) but has up to now not been considered for any models of HR repair (West, 2003; Thorslund and West, 2007). We consider this the second major conceptual advance of our study." This is very misleading as all HR models include resection as a DNA end-processing step. There is a large and growing literature implicating MRN/MRX and other factors in end-resection that varies depending on the structure at the ends. For example, MRE11 has a minimal role in HR induced by HO but is required for meiotic HR initiated by SPO11.

Minor points:

p. 3: The first demonstrations that I-SceI DSBs are repaired by HR in mammalian chromosomes are Liang, 1996 Proc. Natl. Acad. Sci. USA 93:8929 and Taghian, 1997: Mol. Cell. Biol. 17, 6386.

p. 5: "Aphidicolin caused pronounced pan-nuclear H2AX phosphorylation in S phase cells likely due to the activation of ATR following replication arrest (Figure 1A)." This should be clarified to indicate that ATR is activated by fork collapse resulting in DSBs.

p. 7: "ATM and Artemis deficient cells exhibit repair kinetics indistinguishable to HSC62 cells in G2 (Figure 2A) raising the intriguing possibility that HR repairs ATM and Artemis dependent DSBs in G2." This statement is unclear: I don't believe the DSBs are dependent on ATM/Artemis, but what is probably meant is that the HR repair is dependent on these proteins.

p. 16: The I-SceI assay differs from our G2 specific H2AX assay in several ways. Firstly, I-SceI induced DSBs need resection for repair by HR but do not need end processing, which may not reflect the repair of endogenous lesions.....this is confusing: do the authors mean IR induced lesions rather than endogenous?

Rebuttal 05 April 2009

The authors appealed the original editorial decision, and after communication an agreement was reached that a revised manuscript would be considered if they were to provide additional independent data that could address all the major concerns of the referees as outlined in the reponse to referees (see 1st Revision – authors' response)

Invitation to resubmit 13 May 2009

I apologise for the delay in responding to your letter but this correspondence must be handled on top of the daily manuscript workload so sometimes it can be difficult to find a sufficient stretch of time to read such appeals in detail.

I have read your manuscript and the referee reports once more and also your correspondence. I appreciate that your study is of interest to the field and that part of the main concern is if the referees are convinced about the sensitivity and accuracy of the approach used to measure HR in G2. This also seems to have been an issue with previous submissions of the manuscript.

Given the interest in the manuscript and that you are able to add more data such as the SCE assay to strengthen the paper, I am willing to reconsider the original decision and ask other people to look at the manuscript. However, I would like to stress a few things, referee #3 raises concerns about the overall novelty of the study and advance therefore, you should be careful about removing data and thinning out the paper, we would be able to accommodate a longer manuscript if the appeal is successful. I also appreciate that some of the comments raised by referee #2 seem to correspond to an earlier version of the manuscript, however, referee #2 is

 

someone who is highly respected by and has reviewed multiple times for the Journal, therefore, I am not willing to completely ignore her/his report and the overall evaluation of the manuscript, the issues raised should be addressed in detail in a point-by-point response. Finally, I would like to stress that once the manuscript is rewritten and resubmitted I would seek the opinion of referee #3 and also at least one more new referee, who would look at the manuscript afresh and therefore, would be in his/her right to be critical and ask for additional experiments if required. I will then make a decision based on all the reports that I have available to me.

Sincerely,

Editor

EMBO Journal

1st Revision - authors' response 22 June 2009

Response to the reviewers' comments:

General comments:

1) One concern for reviewers 2 and 3 is the small difference in foci numbers in G2 phase cells. We would like to make the following points:

a) Although the numbers appear small, this is a highly sensitive technique (if carried out carefully) and our findings have been reproduced in both the Jeggo and Löbrich laboratories, and by several independent workers in both labs. In many instances, foci counting was performed in a blinded manner.

b) The foci numbers in G2 phase are double that in G1 phase (as expected from the doubling of DNA content - this itself attests to the sensitivity of the technique). The defect in ATM and Artemis defective G2 cells is identical to that observed in G1 phase. We agree that it looks somewhat less convincing because the foci numbers in control cells are higher in G2 than in G1 phase (see below for an explanation)and because we cannot readily maintain cells in G2 phase for more than ~ 8 h. Thus, we would argue that we would not expect a larger defect. We had difficulty having our original report of this difference in G1 phase cells accepted but several laboratories have now reproduced our findings and the ATM/Artemis defect in G1 phase is fully accepted.

c) Factors that help us to achieve tight error bars are:

i) A consideration of cell cycle phase since even 5 % G2 phase cells in a population (with twice the number of foci) enlarges the error bars.
ii) In some instances, we count the foci from digital images to obtain more reliable data. Representative high-quality images are now shown in Suppl. Figure 1C.
iii) We have taken time to choose suitable cell lines for this analysis. Where we can we use primary cells, which have a low background number of γH2AX foci. For siRNA analysis, we have to use transformed cell lines but try to exploit hTERT lines which also have a low background number of foci. Where we use cell lines for siRNA, they can have a higher background but we feel comfortable with the results since they are consistent with those obtained using the primary lines.

Thus, there are valid explanations for our accurate analysis. We stress that we have included primary image data (e.g. high-resolution primary images of H2AX foci induction in Suppl. Figure 1C, and primary images for γH2AX foci repair in different mutants in Suppl. Figure 2) and are happy to provide more if the reviewers wish. Further, the results have been repeated in both our labs and by several workers in each lab.

 

2) The reviewers 2 and 3 seem unhappy about the reliance on the epistasis analysis.

We agree that this data alone has limitations (primarily the possibility of incomplete knock down) and have therefore sought further evidence for our finding that ATM and Artemis defective lines show HR defects. We have performed further experiments with the BrdU incorporation assay to establish this technique as an assay for IR-induced HR events in G2 (mainly showing that BrdU incorporation is not affected by Ku80 or Lig4 depletion and that G1 cells do not show BrdU incorporation, demonstrating that any DNA synthesis by NHEJ is not detected by this technique). Importantly, we also have analysis now showing that SCEs can be observed in metaphases arising from G2 phase cells and that these are diminished in the absence of ATM or Artemis. This is a further, more accepted assay for HR, and was suggested by reviewer 2 as a more decisive approach to demonstrate the role of Artemis and ATM in HR. Thus, our conclusions do not rest on the epistasis data alone. We now use a number of distinct assays to monitor the process of HR in Artemis and ATM defective cells (BrdU incorporation data; SCE data; RPA foci, Rad51 foci, and BrdU resection foci) which collectively provide strong evidence that ATM and Artemis promote HR of IR-induced DSBs in G2. We have rewritten the manuscript to place more emphasis on the new HR assays and present the epistasis analysis in a confirmatory way.

3) In this revision we present new data and address an issue which represents a significant new contribution to our paper. As justly criticized by reviewer 3, we claimed in the original version that the breaks undergoing HR might be those which localize to heterochromatic regions but provide no evidence for this claim. The claim was based on our recent paper showing that in G0/G1 cells ATM is required for the repair of DSBs in heterochromatic regions and that the requirement for ATM for repair is relieved by depleting KAP-1, a heterochromatic building factor whose depletion relaxes heterochromatic regions (Goodarzi et al., 2008, Mol Cell, 31:167). We now provide data showing that the same is true for G2 cells, i.e. depleting KAP-1 in G2 cells relieves the requirement for ATM. Hence, this data shows that the DSBs repaired in G2 by HR are those localizing to heterochromatin. This finding explains what might be special about the 15-20% of breaks which undergo HR in contrast to the majority of breaks which are repaired by NHEJ in G2. Significantly, this adds an important aspect to the current debate about regulatory mechanisms between HR and NHEJ.

Specific comments:

Referee #1:

*In this manuscript the authors uncover a role for Artemis in homologous recombination (HR) - mediated in repair of ionising radiation-induced DNA double-strand breaks (DSBs) in G2 cells. The manuscript contains a very extensive set of experiment that are in general very well done and convincing. The work described in the manuscript is highly significant and of great interest to the genome stability field.*

*The authors may want to pay attention to the following issues:*

*The authors might reconsider the title. I think I understand what the authors want to say, but that is not what is expressed by the title. The title states that the DSBs studied in this manuscript are ATM and Artemis dependent. Of course they are not; their formation is ionising radiation dependent.*

We have changed the title of the paper which is now:
"ATM and Artemis promote homologous recombination of radiation-induced DNA double strand breaks in G2".

*Legend to Figure 2, panel A. The text is mostly redundant with the Results section.*

The redundant parts in the legend to Figure 2 have been removed.

*Figure 2. I recommend the use symbols of different more discriminatory symbols for the different cell lines. In certain cases is too hard to see the difference and this is essential to keep the story straight in the mind of the reader.*

 

We have redrawn this and most other figures in color since we found this the best way to visualize the effects.

*Page 7. The term ATMi is used for the first time without explanation in the first full paragraph on this page. I assume it means addition of an ATM inhibitor. My assumption is based in part on what follows further down the page 'We also applied RNAi technology to exclude the possibility that KU55933 has off-target ...'. I must also assume that the 'i' in the abbreviation ATMi means something different in the abbreviation RNAi. Alternatively the abbreviation ATMi, implies the use of RNAi against ATM? In any, case the abbreviation ATMi should be abandoned and if it indeed means the addition of inhibitor this should be spelled out instead, throughout the manuscript, with the mention of the specific inhibitor used.*

We have abandoned the terms ATMi and DNP-PKi from the text and instead say "the ATM inhibitor KU55933" and "the DNA-PK inhibitor NU7026". The only places where we use ATMi and DNA-PKi is in the figures but clearly say in the figure legends that this is used as the abbreviation for "the ATM inhibitor KU55933" and "the DNA-PK inhibitor NU7026".

*Page 9. Modify the last sentence of the first full paragraph to read: 'Moreover, our findings show that HR is only required for the repair of a sub-fraction (~15%) of ionising radiation-induced DSBs in G2 phase.' The modifier is to reflect the fact that HR is an essential DSB repair pathway for the cell, while NHEJ is not.*

This has been changed.

*Page 9. DNA-PKi; see remark above about ATMi.*

This has been changed.

*Figure 9. I suggest to add primary data to this Figure. Visualizing RPA and BrdU foci is not trivial, let alone quantifying them, so the reader should be given a chance to judge the quality of the primary data.*

We now show primary data for RPA and Rad51 foci in the supplement (Suppl. Figure 5) and for the BrdU incorporation assay in the main manuscript (Figure 3).

*The experiments shown in Figure 10 are essential to make conclusions about the involvement of the nuclease activity of artemis in ionising radiation-induced HR in G2 cells. A good control would be to test whether the D37N protein is expressed.*

We have analysed the expression level of c-myc tagged Artemis by co-staining of H2AX and c-myc and only evaluate cells which show pronounced Artemis expression. We have included original images of cells with and without Artemis expression to this figure (which is now Figure 8) to demonstrate this point. We have also analysed the expression level by Western blotting (Figure 8).

*In Figure 10 the authors use gH2AX foci as a read out for the effect of the D37N artemis protein. To more strongly implicate the nuclease activity of artemis they may want to consider to check RPA, RAD51 and BrdU foci as was done in Figure 9.*

We have done additional experiments with the D37N Artemis protein using Rad51 foci formation and show that the level of Rad51 foci inversely follows that of -H2AX foci, i.e. expression of WT Artemis but not D37N mutant Artemis reduces the γ-H2AX and increases the Rad51 foci level of Artemis deficient cells. Additionally, we show that over-expression of D37N mutant Artemis but not WT Artemis leads to an increase in γ-H2AX and a decrease in Rad51 foci in control cells.

Referee #2:

*The manuscript by Beucher et al. addresses a possible role for ATM and Artemis in homologous recombination repair of IR-induced DSBs. Their previous work implicated Artemis, ATM and*

*53BP1 in a sub-pathway of NHEJ that could repair a defined subset of IR-induced DSBs during the G1 phase of the cell cycle. In this study, the authors show that in G2, as in G1, NHEJ represents the major DSB repair pathway and HR only functions to repair ~15% of IR-induced DSBs. Strikingly, in addition to requiring the known HR proteins, HR in G2 involves Artemis, ATM and the ATM-signaling mediator proteins BRCA1, MDC1, and 53BP1. They also show that these factors function in NHEJ during G1 but promote HR during G2. Finally, they show that Artemis is required for IR-induced HR but is dispensable for I-SceI-induced HR. Based on their data, they propose that Artemis endonuclease is involved in removing lesions or secondary structures which otherwise inhibit end resection and preclude the completion of HR and NHEJ. The authors use immunofluorescence to detect foci of H2AX, RAD54 and MDC1, and they use comet (FAR) assays following irradiation. They do a small amount of biochemical analysis of Artemis. The authors induce damage using IR (and one set of experiments with I-SceI), and in some cases they arrest the cells with polymerase inhibitors or cell cycle cycle blockers.*

We do not use foci to detect Rad54 or MDC1 - we use foci analysis to monitor DSB repair. We do not include any biochemical analysis - this was included in a previous version that this reviewer also reviewed.

*The issue of relative contributions of HR and NHEJ to DSB repair is of importance, particularly in S-G2 phases of the cell cycle, and I agree that the simple classification of DSB repair pathways (HR/NHEJ) is open to additional levels of complexity. I have several fundamental questions about this study, however. The assays used are not sufficient to draw the conclusion that Artemis is involved in HR. The genetic and immunostaining approaches alone are not sufficient to make this conclusion. Therefore, the model should be removed, and the title and abstract and text should be changed to reflect this insufficiency.*

We are monitoring the repair of two ended DSB generated in G2 phase and have avoided the analysis of HR induced by one ended DSBs in S phase. This means that some conventional assays for HR cannot be utilized. As we explained, the I-Sce1 assay is predominantly dependent upon ATR and not ATM - thus, we believe that it might be detecting HR events in S phase more than G2 phase. Thus, even though we do not see a defect in this assay (as presented) it does not limit our conclusions. We do not see why the use of DSB repair assays, and other assays that are now widely accepted as monitoring HR (e.g. Rad51 foci formation) cannot be used. Further, we developed a novel assay for HR in G2 phase and include control data in this revision to justify that it monitors HR. Most importantly, we have consolidated our findings with SCE measurements which are well established markers for HR events.

*1. In this and previous studies, the authors determined the contribution of each factor or each pathway to IR-induced repair on the basis of PFGE analysis and H2AX focus analysis. In the PFGE analysis, non-physiological doses (80 Gy !) is employed, which would be inducing >3000 DSBs per cell. Can such cells really repair the damage? I think not.*

This is unjust - this method was used to show that our results are actually reproduced even using the PFGE assay - they do not rely on this. In fact, we believe that DSBs are repaired after these high doses. The PFGE technique has been used by us and many other laboratories for more than 20 years for numerous publications. It is correct that the assay requires non-physiological doses and this is where the newer foci technology has its greatest advantage. However, referees of our last three publications (including the latest Mol Cell paper showing that ATM dependent DSBs localize to heterochromatic regions) requested that the foci data is reproduced with the older PFGE assay - this is why we included this analysis in the present paper. Further, we also exploit PCC analysis, which uses lower doses. We do not feel that it is reasonable to criticize this experiment, which was simply included as an additional control, in this way. We have however removed the PFGE data without any impact.

*More importantly, recent evidence clearly indicates that γH2AX foci can be observed independent of DSBs at G2 phase.*

The reviewer is correct that we have a slightly higher background number of foci in G2 compared to G1 phase - but this is small compared to the foci induced by IR. We do not know what evidence the reviewer is referring to saying that DSB independent foci are present in G2 phase cells - we do not

 

see that. It is certainly true that H2AX forms in an ATR-dependent manner at certain lesions. However, there is substantial evidence that after IR, the base damage and single strand breaks in G1 and, we show here, in G2 do not activate ATR. Moreover, our foci are lost at a rate that correlates with DSB repair monitored by other techniques and in a manner largely dependent upon NHEJ or HR proteins. We have included further control experiments showing that IR induced foci are identical between control and ATR deficient cells but are absent after the joint inactivation of ATM and DNA-PK, demonstrating that single stranded DNA lesions which active ATR do not contribute to foci formation. We have also performed control experiments showing that H2O2 at a concentration that induces massive amounts of single stranded breaks (>1000 SSBs) but only a few DSBs does not cause substantial foci formation. Thus, under the conditions used (IR of G2 but not S phase cells) it is beyond reasonable doubt that the foci analyzed represent DSBs. We are happy to include also the H2O2 control data if this reviewer wishes.

*Moreover, γH2AX formation is also induced by chemically synchronizing the cells. Thus, the way of semi-quantitative evaluation would not make sense. Another problem of these analyses, proper contribution of each repair factor or each repair pathway may be masked by other pathways, such as a back-up end joining enzymes, which are reported to rejoin DSBs when both HR and NHEJ are absent or dysfunctional. The authors should discuss their data in light of these.*

We do NOT synchronize the cells using chemical inhibitors - we merely exclude the analysis of S phase cells in this way. Our procedure was particularly used to avoid damage that we find is induced by all synchronization approaches that we have tried. The "back up" pathways are minor pathways that only function at later times (and in fact are only seen following the use of PARP inhibitors). Clearly, they are not majorly functioning under these conditions otherwise we would not see unrepaired DSBS. They also cannot explain why we see reduced BrdU incorporation, reduced IR-induced SCE levels and reduced resection and Rad51 foci formation in the absence of ATM, Artemis and other known HR factors.

*2. The authors claim that they can count up to 80 foci per nucleus. The authors must provide in the Supplement several high resolution photos demonstrating this. Our experience is that after 30 per nucleus, it becomes very difficult to distinguish foci.*

We have included controls to show that we can count up to 80 foci in a linear dose response way. We have included several high-resolution images (Suppl. Figure 1C) visually demonstrating that - H2AX foci formation is not saturated until about 100 foci.

*3. The authors' error bars on the foci assays are remarkably tight. This is not our experience. The authors must document that they read these slides in a 'blinded' manner. They need to provide the standard info such as number of cells counted. They also must provide in the supplement a standard curve for how their standard deviation varies with the number of cells counted.*

We have included in the methods section information about the number of cells counted etc. We also state there that we have calculated the standard error of the mean (SEM) either from the number of all analysed cells or from the variation between the different experiments (whichever is bigger). The SEM is a measure for the accuracy of the mean while the standard deviation between foci numbers of different cells only reflects the scatter between the cells. Thus, the standard deviation between individual cells can easily be around 20 foci (for a 60 foci data point) and is independent of the number of cells analysed whereas the SEM (reflecting the accuracy of the 60 foci data point) decreases with increasing cell numbers and can reach much lower values if a sufficient cell population is analysed. We could present this in a graph (and actually have done it for ourselves) but don't think that it is useful for the manuscript. Additionally, we have discussed at the beginning of this letter factors that help to keep our foci numbers tight. These include:
a) Considering G1 versus G2 cells in the population.
b) Use of good cell lines.
c) Counting foci from computerized images.

*4. The authors must repeat their assays with synchronized cells rather than aphidocholin treated cells. Also, better statistical analysis is needed here.*

All the synchronization procedures we have tried introduce DSBs - we specifically used our

 

procedure to avoid this. We do NOT synchronize the cells using aphidicolin but rather use it to exclude S phase cells.

*5. Aphidicolin itself should induce DNA damage, and thus cannot be used to synchronize cells in G2.*

We observe indeed that aphidicolin does induce DNA damage. This is, as might be expected, restricted to S phase cells - we do not count foci in these cells.

*6. If Artemis were involved in HR, then Artemis KO (or knockdown) cells would show a reduction in gene targeting efficiency (and perhaps SCE). Is that the case?*

It is important to appreciate that we are monitoring HR of radiation-induced two-ended DSBs in G2 phase and not all HR. We have tried hard to make this distinction clear in this resubmission. Thus, many assays which reflect HR events at the replication fork cannot be used. We do not know if gene targeting would be reduced in Artemis cells (actually we suspect not). This is an almost impossible experiment and would not be worth it given that even if negative it would not affect our conclusions.

Importantly, we have designed an SCE assay that shows the role of Artemis and ATM in IR-induced HR in G2 by using a more accepted approach. The results completely confirm our findings with the other approaches (foci and PCC technology in combination with epistasis analysis and the newly developed BrdU incorporation assay).

Referee #3:

*Beucher et al. Homologous recombination repairs ATM and Artemis dependent DNA double strand breaks in G2*

*In this report the authors address the question of DSB repair pathway choice and the role of DNA repair/checkpoint proteins in DSB repair in G2 phase mammalian cells exposed to ionizing radiation.*

*In Fig 1 the authors characterize the G2 system but the authors do not show an example of "Mitotic cells exhibiting distinct centromeric CENP-F staining and condensed chromatin" that were excluded from the analysis. Also, the legend does not specify for Fig. 1B what the control is: no IR + aph? No IR and no aph?*

We have clarified that the control in Figure 1B meant IR + aphidicolin. We meant to say that mitotic cells exhibited CENP-F staining and condensed chromatin and were excluded from the analysis. We have previously shown images of mitotic cells (Deckbar et al., 2007, JCB, 176:749) and refer here to this reference.

*In Fig. 1C, it is clear that neither aph nor BrdU affects focus formation after IR in either G1 or G2 cells, but it is not clear why G2 cells show 3-fold more foci than G1 - does this reflect greater DNA content and if so, wouldn't the difference be 2-fold? In the fig. 1D legend it is stated that the difference is "slightly higher than 2-fold but in Fig. 1C it appears to be more than "slightly" higher. It is stated that a possible explanation for this is in the discussion but I could not find it.*

The reason why the overall foci numbers are more than 2 fold higher in G2 than in G1 (at later times) is because repair by HR (in G2) proceeds more slowly than repair by NHEJ (in G1). Thus, this difference is entirely consistent with our finding that the slow repair component represents HR in G2 but NHEJ in G1. It is now stated in the legend to Figure 1 that "The number of DSBs observed in G2 phase at later times (e.g. 8 h) is routinely higher than twice the number in G1 phase since the slow component of DSB repair in G2 is slower than in G1". This issue is complex and we have not wished to address it in detail. The major contribution to the slow kinetics of repair of the slow component is the fact that these represent heterochromatic DSBs (as now stated). However, additionally, HR is slower than NHEJ - hence the slow component in G2 is slower than the slow component in G1. We have not aimed to focus on this latter difference here due to its small magnitude. Nonetheless it is reproducible and attests to the sensitivity of the foci technique. We

 

included the sentence above solely to provide an explanation for your valid comment.

*Figs 2 and 3: The basic argument put forth is that if foci removal curves match in distinct mutants, the wild-type proteins act in the same pathway (epistatic). If the distinct repair is seen in slow vs. fast components, then the pathway is designated as HR or NHEJ, respectively. While this second part is supported by a number of studies from this and other labs, the first part is an over-interpretation or worse, a possible mis-interpretation. The "epistasis" analysis described here does not follow the paradigm in which epistasis is defined with double mutants. The fact that two mutants show the same curves may reflect epistasis, but it may just as easily reflect co-incidence. This is the most problematic aspect of the report. The use of inhibitors provides one means to get at this, but the study falls short since there are no BRCA, RAD54, or Artemis inhibitors, nor any double mutants available, and double siRNA or siRNA + inhibitor is generally not tested.*

We agree with the reviewer that there is a limitation using inhibitors or siRNA to perform epistasis analysis. Indeed, epistasis analysis is best done when null mutants are utilized. However, as stated above, our analysis does not depend solely on the epistasis analysis but is shown equally (and more strongly) by the analysis of BrdU incorporation, the formation of SCEs as well as by resection studies. We have therefore changed the order of our presentation to place less emphasis on the epistasis analysis and now present it as confirmatory data. We actually argue that Artemis and ATM act in HR because we see reduced BrdU incorporation, reduced SCE levels and lower RPA, Rad51, and BrdU resection foci. However, we would like to point out that double siRNA for Artemis and Brca2 and for Artemis and Rad51 was performed (previously shown in Figure 4, now in Figure 6C) and provided the expected result (i.e. Artemis was found to be epistatic with Brca2 and Rad51).

*A general criticism: some of the data lacks statistical analysis. While the error bars seem convincing, the authors must report the statistical analysis to back their claims.*

We now provide statistical analysis for all epistasis studies and data with small differences.

*Fig. 4: As in Figs 2/3, epistasis is invoked here with single siRNAs (and minor differences). The interpretations of these data are not convincing. It is also concerning that rad51 knockdown does not seem to affect G2 repair very much. Is the fast repair component (2 hr) the same +/- rad51?*

We have done earlier times with Rad51 siRNA - and it is normal at 2 h, i.e. the fast repair component in G2 (NHEJ) is unaffected by depleting Rad51, as expected. This new data has been included (Figure 2C). We actually did double siRNAs approaches confirming that Artemis, ATM, Rad51 and Brca2 function in the same repair pathway in G2 (Figure 6C).

*p. 8: the authors note in the introduction that high doses needed for PFGE are problematic, then they proceed to use 80 Gy doses, which have questionable biological relevance since they are supra-lethal. It is possible that entirely new mechanisms are brought to bear, or entirely new problems arise, in cells challenged with such extreme doses. Dose rates also become important since long exposures may be needed to achieve high doses (depending on the source) and repair may therefore occur during exposure.*

This is just an experiment to show that we obtain the same results with a different technique due to concerns some reviewers have with foci counting. We have also included PCC analysis so the PFGE analysis could be omitted without any loss of strength.

*Fig. 10B/p. 15: the minor differences in foci with Artemis complementation or D37N are not convincing. While the general trend supports the conclusion that Artemis nuclease is important for HR after IR, the differences are very small and while statistics are said to have been done, no values are given.*

The differences are entirely as expected. Although the numbers are small, they are entirely consistent with the rest of the data. We have also performed the complementation analysis in G1 cells and obtained the same result as for G2 cells and the same result reported in our previous publication on the repair defect of Artemis deficient cells in G0 (Mol Cell, 2004). This (small) repair defect in G0 Artemis deficient cells has been reproduced by others and has received quite some attention. We do not understand why the same magnitude of the repair defect in G2 is not

 

convincing. We have done statistics which prove the differences are significant - sorry we didn't do this before.

However, as stated above, we have performed further experiments with control and Artemis deficient cells transfected with WT and D37N mutant Artemis constructs. We measured -H2AX and Rad51 foci and show that the level of Rad51 foci inversely follows that of γ-H2AX foci, i.e. expression of WT Artemis but not D37N mutant Artemis reduces the -H2AX and increases the Rad51 foci level of Artemis deficient cells. Additionally, we show that over-expression of D37N mutant Artemis but not WT Artemis leads to an increase in γ-H2AX and a decrease in Rad51 foci in control cells. This data consolidates our finding that Artemis endonuclease is required for efficient DSB repair and further shows that the endonuclease activity promotes end-resection. Since the complementation studies in the original submission were only done with γ-H2AX foci analysis, we consider this more direct link between Artemis endonuclease function and HR an important control experiment.

*p. 16: "Thus, although currently difficult to substantiate, our findings suggest that the DSBs that are located within or close to heterochromatin are those that undergo repair by HR in G2 phase." I found no data to support this claim, so this is speculation.*

As stated above, we are now presenting data showing that siRNA of KAP-1 relieves the ATM repair defect in G2 phase cells as in G1 phase. We believe the addition of this data represents a significant new contribution to the paper.

*p. 19: "Our finding that factors exist which have defined roles in both major DSB repair pathways represents a major conceptual advance and suggests that the previously made distinction between NHEJ and HR may need re-consideration." This is a vast overstatement that ignores publications from several labs that implicate NHEJ proteins in HR regulation: Nucleic Acids Res 30, 3454; Proc Natl Acad Sci USA 99, 3758; Mol Cancer Res 1, 913; Genes Dev 15, 3237.*

This statement has been removed.

*p. 19: "End-processing has been extensively studied in the context of NHEJ (van Gent and van der Burg, 2007; Lieber, 2008) but has up to now not been considered for any models of HR repair (West, 2003; Thorslund and West, 2007). We consider this the second major conceptual advance of our study." This is very misleading as all HR models include resection as a DNA end-processing step. There is a large and growing literature implicating MRN/MRX and other factors in end-resection that varies depending on the structure at the ends. For example, MRE11 has a minimal role in HR induced by HO but is required for meiotic HR initiated by SPO11.*

As above, this has been omitted. What we meant by end-processing were DSB modifying steps which are distinct to end-resection but we agree that this might be confusing and best omitted.

Minor points:

*p. 3: The first demonstrations that I-SceI DSBs are repaired by HR in mammalian chromosomes are Liang, 1996 Proc. Natl. Acad. Sci. USA 93:8929 and Taghian, 1997: Mol. Cell. Biol. 17, 6386.*

We have now quoted these references.

*p. 5: "Aphidicolin caused pronounced pan-nuclear H2AX phosphorylation in S phase cells likely due to the activation of ATR following replication arrest (Figure 1A)." This should be clarified to indicate that ATR is activated by fork collapse resulting in DSBs.*

This has been clarified.

*p. 7: "ATM and Artemis deficient cells exhibit repair kinetics indistinguishable to HSC62 cells in G2 (Figure 2A) raising the intriguing possibility that HR repairs ATM and Artemis dependent DSBs in G2." This statement is unclear: I don't believe the DSBs are dependent on ATM/Artemis, but what is probably meant is that the HR repair is dependent on these proteins.*

 

This and similar statements throughout the manuscript (such as the title) have been clarified.

*p. 16: The I-SceI assay differs from our G2 specific γH2AX assay in several ways. Firstly, I-SceI induced DSBs need resection for repair by HR but do not need end processing, which may not reflect the repair of endogenous lesions.....this is confusing: do the authors mean IR induced lesions rather than endogenous?*

We agree that this was confusing and have removed "…, which may not reflect the repair of endogenous lesions".

Finally, below we summarize the major changes that we made to the manuscript to address the criticisms:

1) We re-structured the manuscript to place more emphasis on the HR techniques and reduce the significance of the epistasis analysis - i.e. we present the epistasis analysis as being confirmatory of our other findings rather than the basis for them.

2) We included the analysis of SCEs arising solely from G2 phase cells as a commonly used monitor of HR (Figure 4).

3) We established the BrdU incorporation technique as a new assay to measure HR of IR-induced DSBs in G2 (Figure 3).

4) We included siRNA of Kap1 showing that the ATM dependent DSBs undergoing HR in G2 represent those in heterochromatic regions (Figure 9).

5) We have included primary images (Suppl. Figures 1C and 2) and performed further control experiments (with H2O2; see comment #1 of reviewer #2) to substantiate that the differences in H2AX foci numbers in G2 cells represent a DSB repair defect.

6)We have consolidated that Artemis endonuclease activity promotes HR (Figure 8).

We have omitted the analysis of the mediator proteins - this part of the paper was not central to the argument and even perhaps side-tracking from the ATM and Artemis data. We have also omitted one control experiment showing that aphidicolin does not affect repair in G1 and G2 (previous Figure 1C) because this experiment was complex and the effect is better shown in Figure 1B. Although we feel that the revision benefits from the omission of this data, we would be happy to add it back if the reviewers think it was important.

We would like to thank to reviewers for the time they have taken to help to improve our manuscript - this is highly appreciated.

2nd Editorial Decision 17 July 2009

Thank you for submitting your new manuscript for consideration by the EMBO Journal. After the previous review process we made the decsion to send the manuscript to two of the original referees and a new referee to assess the new version of the manuscript, I specifically asked each of the referees to look cloelsy at all the comments previously made and if they felt that they had all been satisfactorily addressed. They find that the SCE assay and the demonstration of a role for Artemis nuclease activity significantly strengthen the study and are overall willing to recommend publication after a number of minor revisions have been incorporated (please see below). Please note that the referee numbers are not the same as in the original manuscript.

When you send us your revision, please include a cover letter with an itemised list of all changes made, or your rebuttal, in response to comments from review.

 

Thank you for the opportunity to consider your work for publication. I look forward to reading the revised manuscript.

Yours sincerely,

Editor
The EMBO Journal

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REFEREE COMMENTS

Referee #1 (Remarks to the Author):

p. 4: "We show that the DSBs repaired by HR in G2 require ATM and Artemis endonuclease function" is required an overstatement?

p. 4: "Importantly, Artemis is not a specific NHEJ factor and Artemis and ATM processing can lead to DSB repair by HR or NHEJ depending on cell cycle phase". First part is correct, but the second part implies that these proteins control which pathway is used. While it appears that Artemis and ATM processing can lead to DSB repair by either pathway, there is no data showing that they control which pathway is used in each cell cycle phase. Instead the data suggest that they are involved in both pathways during both cell cycle phases.

Fig. 4: What is the difference between mock and control? Both include irradiation so mock does not refer to mock irradiated. The same question applies to Fig. 6.

p. 10: "Rad51 foci formation is significantly compromised in primary human A-T fibroblasts (Figure 5A), consistent with previous observations (Morrison et al, 2000; Yuan et al, 2003)." Shrivastav (DNA Repair 2009, epub ahead of print) also showed that RAD51 foci are largely eliminated when ATM is inhibited. This should also be cited in the first paragraph of the Discussion.

p. 13: "Given its involvement in HR and its independency of XLF and Lig4 in G2..." Do the authors mean "its interdependency with XLF..."?

Fig 9a: Are these images of Kap1? It is impossible to evaluate the conclusion that Kap1 is reduced in densely staining DAPI regions without the corresponding DAPI images. The same problem exists for the g-H2AX foci as no g-H2AX images are shown. The next conclusion, that knock-down of KAP-1 in G2 phase cells relieves the ATM dependent DSB repair defect in G2 is well supported by the data (although the ATM effect is relatively minor). It is also not possible to evaluate the net conclusion from these expts "that HR occurs at KAP-1 dependent heterochromatin regions."

Referee #2 (Remarks to the Author):

In the revised version of ms EMBOJ-2009-71705 the authors have addressed my comments satisfactorily. Specifically they dealt appropriately with my most important issue which was to show that the effect depends on the nuclease activity of Artemis (Figure 8)

Referee #3 (Remarks to the Author):

I have read carefully the reviewers comments for the previous version of the manuscript, the response to the comments and the revised manuscript. I agree with most of the previous reviewers' comments and I believe that the authors have made a great effort in address these issues.
The question about interplay between HR and NHEJ is a tough one to address because the technical

 

issues (i.e., what is the adequate endpoints to be used ?) compounds with cell cycle specificity. In this manuscript, the authors intend to address the issue of repair of IR-induced DSBs in G2 phase of the cell cycle in relations to the role of Artemis and ATM. While the difference is relative small but it is significant and consistently reproducible by a variety end points, which most likely reflect DSB repair and HR. I appreciate the effort of this group in identifying G2 cells in situ which I believe that it is the best we can do in the field so far until future new break through can be achieved. Further more, this group has also made an effort to compare all the available approaches in monitoring HR in somatic cells.
The conclusion that IR-induced DSBs require Artemis endonuclease activity and ATM in HR repair of heterochromatin-associated DSBs is adequately supported by the experimental data in the revised manuscript. While the molecular mechanism is not clear, the hypothesis is novel.

Revision - authors' response 04 August 2009

List of all changes made in response to reviewer 1:

*p. 4: "We show that the DSBs repaired by HR in G2 require ATM and Artemis endonuclease function" is required an overstatement?*

We have rephrased this sentence to "We show that DSB repair by HR in G2 involves ATM and Artemis endonuclease function". We have also changed the word "requires" to "involves" in line 7 of the abstract.

*p. 4: "Importantly, Artemis is not a specific NHEJ factor and Artemis and ATM processing can lead to DSB repair by HR or NHEJ depending on cell cycle phase". First part is correct, but the second part implies that these proteins control which pathway is used. While it appears that Artemis and ATM processing can lead to DSB repair by either pathway, there is no data showing that they control which pathway is used in each cell cycle phase. Instead the data suggest that they are involved in both pathways during both cell cycle phases.*

We have rephrased this sentence to "Importantly, Artemis is not a specific NHEJ factor and Artemis and ATM are involved in HR or NHEJ depending on cell cycle phase" as suggested by this reviewer.

*Fig. 4: What is the difference between mock and control? Both include irradiation so mock does not refer to mock irradiated. The same question applies to Fig. 6.*

Samples designated "mock" were treated with transfection reagents but no siRNA was added. Samples designated "control" were treated with control siRNA. This has been made clear in the legend to figures 4 and 6.

*p. 10: "Rad51 foci formation is significantly compromised in primary human A-T fibroblasts (Figure 5A), consistent with previous observations (Morrison et al, 2000; Yuan et al, 2003)." Shrivastav (DNA Repair 2009, epub ahead of print) also showed that RAD51 foci are largely eliminated when ATM is inhibited. This should also be cited in the first paragraph of the Discussion.*

The work by Shrivastav et al (2009) has been quoted on page 10 as well as in the first paragraph of the discussion.

*p. 13: "Given its involvement in HR and its independency of XLF and Lig4 in G2..." Do the authors mean "its interdependency with XLF..."?*

We meant to say "Given its involvement in HR and the finding that it functions independently of XLF and Lig4 in G2, we next tested..." and have rephrased the sentence accordingly.

*Fig 9a: Are these images of Kap1? It is impossible to evaluate the conclusion that Kap1 is reduced in densely staining DAPI regions without the corresponding DAPI images. The same problem exists*

 

*for the g-H2AX foci as no g-H2AX images are shown. The next conclusion, that knock-down of KAP-1 in G2 phase cells relieves the ATM dependent DSB repair defect in G2 is well supported by the data (although the ATM effect is relatively minor). It is also not possible to evaluate the net conclusion from these expts "that HR occurs at KAP-1 dependent heterochromatin regions."*

We now provide images of KAP-1 signal together with the corresponding DAPI staining in supplementary figure 8. In this new figure, we also show representative images of H2AX and RPA foci localization in comparison to densely staining DAPI regions. The images support the quantitative evaluation in the table of figure 9A. We agree that the random localization of RPA foci and ATM-dependent DSBs does not itself provide evidence for heterochromatic localization. However, the striking change in localization between G1 and G2 phase, which correlates with the different KAP-1 localization, together with the result that knock-down of KAP-1 in G2 relieves the ATM-dependent DSB repair defect is strongly supportive of the notion that HR occurs at KAP-1 dependent heterochromatic regions.

 