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Factors determining DNA double strand break repair pathway choice in G2 phase

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1st Editorial Decision 13 September 2010

Thank you for submitting your manuscript on DSB repair pathway choice in G2 for consideration by The EMBO Journal. We have now received the reports from three expert reviewers, which you will find copied below. As you will see, all three reviewers consider the topic and scope of your analysis important, and also acknowledge the potential interest of its results. Nevertheless they all raise a number of specific concerns and questions that would need to be addressed before publication. In this respect, they are mostly concerned about the decisiveness and conclusiveness of the presented dataset, and satisfactory answers to various experimental and methodological queries appear to be required to allow full and definitive assessment of the validity of the current conclusions.

Given that you may well be in a position to address these various points, I am inclined to give you the opportunity to respond to the referees' comments in the form of a revised version of this manuscript. Please keep in mind however that it is EMBO Journal policy to allow a single round of major revision only, and that it will thus be important to diligently answer to all the various major and minor points raised at this stage. When preparing your revision, please also bear in mind that your letter of response will form part of the Peer Review Process File, and will therefore be available online to the community in the case of publication (for more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html). Finally, please make sure to carefully assemble and proofread the final version also from an editorial point of view, especially regarding the format and completeness of both the in-text citations and the bibliography section, and also briefly indicate the individual author's contributions, either in the acknowledgements section or in an adjacent separate section, as we are attempting to adopt this as a common policy now. In any case,

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please do not hesitate to get back to us should you need feedback on any issue regarding your revision.

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

The manuscript entitled "Factors determining DNA double strand break repair pathway choice in G2 phase" by Shibata, A. et al examined factors which influence the choice between NHEJ and HR for repair of DSBs in G2 phase of the cell cycle. Kinetics of DSB repair via γH2AX foci resolution and DSB end resection via RPA and Rad51 foci following exposure to agents which produce DSBs with differing complexities were used to study DNA repair rate and pathway choice in G2. The authors find that the speed of DSB repair as a major component in influencing repair choice, as well as the complexity of the damage and chromatin. The authors identified a novel role for ATM in regulation of DSB end resection by regulating Kap1-mediated chromatin relaxation and activating CtIP via phosphorylation. The observations are interesting and the data provided support the authors' claims. However, there are concerns regarding one specific issue:
1. It has been shown in the literature that phosphorylation dead DNA-PK may stuck at DSB ends and affect HR. In the manuscript, they used 6A vs 6D mutation of DNA-PK at T2609 cluster and monitor IR induced Rad51 foci. This new data supports the published notion that phosphorylation dead DNA-PK may affect HR. However, reduction of Rad51 foci cannot be interpreted that DNA end resection is defective. At this moment, approaches measuring DNA end resection in vivo in mammalian cells is limited; it depends on RPA or BrdU foci formations. While human RPA antibody is not working on hamster cells but it recognize mouse RPA, the author should express DNA-PK mutants (6A and 6D) in mouse DNA-PK knockout cell. Alternatively, it should monitor the BrdU at foci.
2. There is no description or characterization of the DNA-PK 6A and 6D mutants. The expression level and relevant characterization should be presented. In addition, wild type human DNA-PK complemented V3 cells should also been used as control.
3. Therefore, until it is conclusively confirmed the authors should remove the statement that cells expressing phosphorylation dead DNA-PK is defective in DNA end resection. Without RPA or BrdU foci results, the author can only speculate or hypothesize that one of the reasons that case IR-induced Rad51 foci is defective in DNA end resection.

Referee #2 (Remarks to the Author):

This manuscript addresses factors that determine how dsbs are repaired in G2 phase. The authors use IF to quantify the amount and duration of DNA resection taking place in response to different types of DNA damage and use this to infer which types of lesion are repaired by NHEJ and which by HR. They conclude that lesions are initially subjected to repair by NHEJ, which is rapid, but when this is unsuccessful dsbs are resected for repair by HR. Shibata et al identify a role for ATM in the regulation of this process through both KAP-1 dependent chromatin relaxation and through CtIP activation.

This work examines the interesting and topical question of dsb repair pathway choice. The study is presented in a logical and considered manner and makes sense. On the other hand this study is very limited in approach focusing exclusively on the quantification of nuclear foci, and therefore very difficult to independently validate.

Since a lot depends on the quantification of foci it was disappointing that the methods for scoring

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these data were not clear. Were images scored automatically by computer analysis or by eye. Was the data scored blind, if not why not? How many fields were scored and from how many independent experiments? The SEM are very small. The methodology used for handling data here is not obvious in the manuscript but is extremely important because in this type of study, where the primary data is not available, the reader is required to take a lot on trust.

Overall this study is logical and the data are consistent with the conclusions drawn. However, it is also true that most of the conclusions about resection and repair are inferred from the localization of protein marks such as RPA and RAD51. If one accepts that these associations are a true representation of the inferred function then the manuscript has something to say, but it would be enhanced considerably by an attempt at more direct experimentation.

Specific points-

1. How much resection is required for the detection of an RPA focus? How might the conclusions of the work be different if less extensive resection goes undetected.

2. In Fig1B the y-axis should represent the data i.e. co-localised RPA/γH2AX foci, and not an inferred % end resection, which is not being measured.

3. In the last paragraph on p6 it was not clear where the figure of approx 10% colocalization of RAD51 and γH2AX comes from. It is not Fig1C as stated, which refers to RPA.

4. In fig 2A the graph reports no of Etp induced RAD51 foci. Is this per cell? Similarly in other figures.

5. The data in figure 2A suggest that damage caused by higher doses Etp is repaired more slowly than damage caused with lower concentrations. E.g. With 20μM Etp only one quater of the RAD51 foci diminish between 4hrs and 8hrs. while with 5μM dose of Etp three quarters of the RAD51 foci disappear between 4 and 8 hours. Is this because the lesions are qualitatively different at high etp doses?

6. On page 7 the authors suggest that a subset of Etp-induced breaks occur at heterochromatin. Is there any evidence that topoisomerases function in HC?

7 In Fig 2C the colocalization of pKap-1 and γH2AX is difficult to determine. The pan-nuclear staining of pKap1 means that it co-localizes with all γH2AX foci. Admittedly this improves after 8 hours (the important time point) but it reinforces the point made earlier about the difficulties in judging this type of data.

8. On page 8 the authors refer to loss of DNA-PK or DNA-PK + Ku causing enhanced resection (Fig3C. Compared to what, wild type? Knockdown of Ku alone also causes enhanced RPA/53BP1 foci compared to wild type. In fact Ku and DNA-PK appear to have an additive effect. Perhaps I am misunderstanding the experiment, but this was not at all clear. Similarly I was not sure where the figure of 60-70% breaks undergoing resection after Ku + DNA-PK knockdown comes from. This needs to be explained or provide a clear reference to the data.

9. The analysis of dsb repair in this manuscript avoids reference to alternative end-joining / microhomology repair. This is pertinent to the discussion of CtIP function since both CtIP and its yeast relative Sae2 have been implicated in this form of repair.

Minor point
There is no date for the reference Ritter and Durante p7 and in the reference list

Referee #3 (Remarks to the Author):

Shibata el al investigated kinetics of gamma H2AX and RPA/Rad51 foci formation with respect to the DNA damage type and the chromatin status (euchromatin vs. heterochromatin) and proposed that repair pathway choice is dictated by the successful completion of rapid end joining and then

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subsequent end-resection if end joining was failed. The topics are interesting and timely; the experiments were generally well crafted and the outcomes were carefully assessed. Nevertheless, the manuscript and the experiments therein have multiple issues and therefore, the main thesis of the manuscript is not fully supported yet.

1. One of the key hypothesis/conclusion of the manuscript is that type of DNA damage dictates speed of DSB repair and in turn determines pathway choice. The repair speed is deduced by the kinetics of gamma H2AX after challenging cells with different types of DNA damage (C12, X-ray, or Etp)(see Fig. 1). However, how do authors pick particular dose of Etp and comparing the responses with C12 or X-ray? Is it possible that C12 causes more damage than Etp and more damage leads to more complicated and thus slowly repairing lesions?

2. The authors propose that slowly repairing lesions are subjected to end resection since only a subset of gamma H2AX in Etp treatment colocalizes with RPA/Rad51 foci whereas most C12 induced lesions associated with RPA/Rad51 (Fig. 1C). However, the amount of damage was estimated by scoring the number of gamma H2AX foci at 30 min (or 15 min in the figure legend) post radiation/Etp treatment while the resection was measured at 2 h post treatment. Therefore, the measurement is strongly biased for those slowly repairing lesions and the lesions scored in Etp treatment at 30 min post treatment may be already fixed and no longer associated with RPA/Rad51.

3. The authors said that the repair speed dictates which pathway to use. Then the authors also said that the pathway utility also dictates the repair speed. This is circular reasoning.

4. The authors showed only the kinetics of RPA focus formation upon C12 induced DNA damage (Fig. 1d). X-ray and Etp-induced DNA damage also need to be assessed with the same way. In Fig 1d and F, why is the number of RPA foci fewer than that in Fig. 1c? Does it mean that many foci are with Rad51 only?

5.It is not clear how Etp was applied to? In Fig 2B, Etp was applied for 30 min. and then was removed subsequently. How about other experiments? Need to clarify them. I also wonder why did the authors examine % gamma H2AX foci upon Etp treatment in G1 instead of G2 (Fig 2B)?

6. With regard to the KAP1 foci, are they truly representing pre-existing HC? or do they form after persistent DSB? Is there any way to discern these two?

7. It is concerning that the results were drawn from different types of damaging agent treatment (In Fig. 2 Etp, in Fig 3, X-ray).

8. Is the panel on the right in 3A different from the one in Fig. 3C?

9. In DNA-PK 6A mutant, is the lack of Rad51 due to the resection defect or an inability load Rad51? May need to look at resection by other means?

9. Why was the number of Rad51 foci higher in DNA-PK mutant than those transfected with wild type DNA-PK or the SD mutant derivative (Fig. 3E)?

10. Why is there less number of breaks per radiation at G1 (Fig. 4B-D)?

11. The y axis in Fig. 4D should be % remaining gamma H2AX.

12. Fig 5D does not have error bars.

13. Could the effect of DNA-PK mutant be due to the lack of RPA phosphorylation?

14. Why is NHEJ defective in HC even if DNA-PK can still bind?

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1st Revision - authors' response 11 December 2010

Reviewer 1.

1) Reduction of Rad51 foci cannot be interpreted that DNA end resection is defective in DNA-PK autophosphorylation mutant cell line.

We agree with Reviewer 1 that Rad51 foci do not represent DSB end resection. The difficulty was that our previous RPA antibodies did not recognise hamster RPA. We have analysed BrdU foci formation under non-denaturing conditions as an assay for resection in the V3 cell lines. Additionally, we have also carried out RPA foci analysis using a new a-RPA antibody, which cross-reacts with hamster RPA. Consistent with the finding using Rad51 foci analysis, we observed a significant reduction of BrdU foci (Figure 3E), and RPA foci after IR (data not shown). We have not shown the RPA foci data since there is not enough space in Figure 3.

2) There is no description or characterization of the DNA-PK 6A and 6D mutants.

We have now examined DNA-PKcs expression levels in the V3 cell lines sent to us (Figure S5B). DNA-PK is expressed in all lines (except the empty vector control). Also, we have included Dr. Katheryn Meek as a coauthor. Kathy made the V3 DNA-PKcs mutant cell lines and has extensively characterized them. Her detailed characterization of these mutant cell lines has been published previously (Cui X, MCB, 2005, Katheryn Meek, MCB, 2007) and we have now referenced this work.

3) Wild type human DNA-PK complemented V3 cells should also be used as control.

This was actually included but may have been missed. We have amended the text to make this more obvious. Effectively, we have used V3 cells complemented with WT DNA-PK in our analysis of Rad51 and BrdU (Figure 3E-G).

Reviewer 2.

1)

a) *On the other hand, this study is very limited in approach focusing exclusively on the quantification of nuclear foci, and therefore is very difficult to independently validate*

We have developed a “non-Foci” procedure to monitor resection in G2 phase cells by FACS analysis (Figure 3C, 4B and 6A). We focused on examining resection in an alternative way because this is a significant question in our paper. Although this is not as sensitive as foci analysis, it provides a confirmatory (non-foci) procedure.

To further back up foci results, we had performed SCEs and chromosomal aberration assays in some significant experiments in the original submitted version (Figure 3D, 5D and 6C).

Unfortunately we could not carry out either of these analyses following exposure to carbon ions since we do not have beam time of carbon irradiation until next February. However, we have attached PFGE data, which was obtained in our previous work (Figure 1 in this letter, new data and Riballo et al, Mol Cell, 2004). Although we do not have perfectly matched cell lines after Carbon ions, X-rays and Etoposide, the data strongly supports our foci analysis and demonstrates that DNA damage complexity determines the speed of repair. Thus, this significant aspect of our work – i.e. that damage complexity influences the speed of DSB repair is supported by an alternative approach.

b) most of the conclusions about resection and repair are inferred from the localization of protein marks such as RPA and RAD51.....It would be enhanced considerably by an attempt at more direct experimentation..

We have also added BrdU foci analysis, which directly monitors DSB end resection rather than a protein marker, e.g. RPA or Rad51, in DNA-PK mutant V3 cell lines, since a novel aspect of our work is that DNA-PK binds all DSB ends first and the autophosphorylation mutant blocks resection.

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Importantly, consistent with RPA and Rad51 foci data, we found a significant reduction of BrdU foci formation in the DNA-PK A6 mutant.

In summary, our foci analysis is supported by a range of other approaches, FACS analysis, SCE, chromosome aberration analysis and PFGE data. We recognise that, largely for technical reasons, we have not been able to do all approaches in all experiments, but we hope the overall range of approaches reassures the referee.

2) *Since a lot depends on the quantification of foci it was disappointing that the methods for scoring these data were not clear.*

We apologise for this. We address the specific questions raised below – which we believe minimises any bias.

a) Were images scored automatically by computer analysis or by eye?
We score foci by eye. It is difficult to score foci accurately by computer analysis because cells are round, especially G2 cell. Therefore, to measure the number of foci in G2 by computer analysis, we have to take an image by 3 dimensions. This is very time-consuming, and unfortunately, we do not have a 3 dimensional microscope that is suitable for computer foci scoring analysis.

b) Was the data scored blind, if not why not?
We carry out foci scoring blindly. We have now stated this in the Materials and methods. Also, for nearly all experiments two or more persons have performed similar experiments to consolidate the results. As an example, Shibata scored the foci after CtIP siRNA #1 in Figure 4A and B, and Ismail scored the foci in CtIP siRNA #2 in Figure 4A and B. Further, most significant results have been consolidated in the two major collaborating laboratories (Lobrich and Jeggo). Although we try to minimise direct repetition, some overlap is inevitable and has been highly useful for consolidation of findings. On occasions, we have found differences and these have usually had valid and interesting explanations.

c) How many fields were scored and from how many independent experiments? The SEM are very small.
We routinely score >800 foci (~20 cells if a cell has 40 foci) or 30 cells per slide. We basically repeated three independent experiments. We have stated this in the Materials and methods. Since we developed the technique to identify cells in defined cell cycle phases using cell cycle markers, we have been able to dramatically reduce variation between independent experiments, which has significantly contributed to reducing the error bars. Additional control experiments and details of foci analysis have been discussed in our recent review (Lobrich M, Cell Cycle, 2010). Briefly, we previously improved our foci scoring technique with cell cycle markers, e.g. CENPF. This dramatically reduced the variation between individual cells (Deckbar, JCB, 2007, Lobrich M, Cell Cycle, 2010). We show the standard deviation between three independent experiments.. Alternatively, we could show our data using scatter plots with statistical analysis. However, we hesitate to use scatter plots routinely because they occupy extra space in a Figure. An example is attached here (Figure 2 in this letter).

Specific points-
1) How much resection is required for the detection of an RPA focus? How might the conclusions of the work be different if less extensive resection goes undetected.

This is an interesting point and we do not really know the answer. We believe that quite extensive regions of ss DNA are required to detect RPA foci, and BrdU foci and likely for the FACS analysis. However, other than in defined mutants (e.g. BRCA2 cell line), we generally find that the number of RPA foci correlates with Rad51 foci – i.e. if there is a reduction in one, we observe a reduction in the other. Further, when we observe Rad51 foci we also observe SCEs – and their number suggests that there is 50% resolution with cross-overs. We have never observed Rad51 foci without observing RPA foci and similarly not seen SCEs without seeing RPA foci. We have, however, in certain conditions (not described in this work) seen reduced but still substantial numbers of RPA foci (which can appear smaller also), suggesting that resection might be impaired – this is normally accompanied by a greater reduction in Rad51 foci and in SCE formation. Thus, overall, we think the RPA assay is sensitive and detects resection if it is good enough to allow the completion of HR.

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There is possibly however, the opposite effect, that we may score RPA foci that represent inefficient resection – but that should not affect the results here.

2) In Fig1B the y-axis should represent the data i.e. co-localised RPA/gH2AX foci, and not an inferred % end resection, which is not being measured.

We have changed this to RPA or Rad51/gH2AX foci at 15 min. What we actually measure is RPA or RAD51 foci at each time point divided by gH2AX foci at 15 mins. We have stated this clearly in the figure legend and text. Additionally, to address reviewer 3 point 1, we have presented the raw data (i.e. the actual number of gH2AX induced by the different treatments and the number of RPA or Rad51 foci present at 2 h) as a supplementary figure. We believe this makes it clear what we have plotted and the rationale for doing this. (see the response to reviewer 3 point 1 for further discussion of this).

3. In the last paragraph on p6 it was not clear where the figure of approx 10% colocalization of RAD51 and gH2AX comes from. It is not Fig1C as stated, which refers to RPA.

Sorry for this confusion – it should have read 10% colocalization to Rad51, since we looked at Rad51 foci after Etp (Figure 1C). We have clarified this point in the manuscript.

4. In fig 2A the graph reports no of Etp induced RAD51 foci. Is this per cell? Similarly in other figures.

Yes this should represent foci per cell. We have corrected to Etp induced Rad51 foci per cell in Fig 2A as well as other Figures.

5. The data in figure 2A suggest that damage caused by higher doses Etp is repaired more slowly than damage caused with lower concentrations. E.g. With 20uM Etp only one quarter of the RAD51 foci diminish between 4hrs and 8hrs. while with 5uM dose of Etp three quarters of the RAD51 foci disappear between 4 and 8 hours. Is this because the lesions are qualitatively different at high etp doses?

We previously found Rad51 foci formation reaches a saturation value at approximately 40 foci/cell at 2 h although it shows a linear dose response to this level (Beucher et al, 2009). It is possible, therefore, that at later times after high doses, there is a balance between ongoing resection and Rad51 loading and HR repair. Thus, a slower rate of formation of Rad51 foci at high doses may lead to an apparent slow rate of repair. This is an interesting possibility that lies outside the scope of this paper so we have not addressed it. It is important to note, however, that all our analysis assessing the fraction of Etp-induced DSBs which undergo repair by HR (Figure 2 and others) were performed using concentrations below the saturation value.

6. On page 7 the authors suggest that a subset of Etp-induced breaks occur at heterochromatin. Is there any evidence that topoisomerases functions in HC?

We have now included data demonstrating that Etp can induce DSB at HC regions in NIH3T3 cells (Figure S4B). We previously reported the role of ATM in HC-associated DSB repair by using NIH3T3 cell line (Goodarzi et al, Mol Cell, 2008), since cells have readily visualised dense DAPI regions which correspond to pericentromeric and centromeric HC. In Figure S4B, Etp induces HC-associated DSBs (~14% of total) whilst X-ray can induce double this level of HC-associated DSBs. We observed a slightly higher percentage of HC-DSB compared to the expected percentage in human cells. This could be due to differences between species and/or some EC-DSBs might be repaired even within the 30 min Etp treatment, i.e. we might underestimate the induction of DSBs in NIH3T3 cells. We do not know the role of Etp at HC regions nor why DSBs arise there. Nevertheless, our data demonstrate that Etp can induce DSBs at HC regions. It should be mentioned that not all HC-DNA is silent all the time and additionally that a DSB locating on the very periphery of HC-DNA could have gH2AX foci that expand into the HC region – and this could impair the repair of the DSB located very close to HC-DNA.

7 In Fig 2C the colocalization of pKap-1 and gH2AX is difficult to determine. The pan-nuclear staining of pKap1 means that it co-localizes with all gH2AX foci. Admittedly this improves after 8

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hours (the important time point) but it reinforces the point made earlier about the difficulties in judging this type of data.

We have examined if persistent pKAP-1 foci represent HC-DSBs after Etp treatment in Figure S4A. We cannot analyze pKAP-1 foci when cells have more than >20-30 foci because of high pan nuclear KAP1 phosphorylation signal. We have now included data showing that all persistent gH2AX foci do not have pKAP-1 foci, and that the % of pKAP-1 positive gH2AX foci is approximately 20% in XLF cells, which corresponds to the population of HC regions (i.e. we have used XLF cells (which are impaired in NHEJ) to examine a situation where EC and HC DSBs persist. In our previous work, we used imaging software to detect gH2AX foci that have above average levels of pKAP1 (Noon et al, NCB, 2010) at early times post IR. We observed that only 20 % of the gH2AX foci were associated with pKAP1 and that these foci represented those repaired with slow kinetics and hence accumulate at later times. Thus at later times the gH2AX foci remaining predominantly co-localise with pKAP-1. Here, we could not assess the % of HC-associated DSBs at early time points using pKAP-1 foci since a higher concentration of Etp is needed to detect a low population of HC-associated DSB. This leads to a strong pan-nuclear pKAP-1 signal, which interferes with the quantification of individual pKAP-1. Importantly, however, our data strongly suggests that Etp can induce HC-associated DSBs (from the analysis at later times) and that the population is 2-3 fold lower than that generated by X-rays. Importantly, this significantly correlates with the ratio of HR:NHEJ in G2 cells.

8. *On page 8 the authors refer to loss of DNA-PK or DNA-PK + Ku causing enhanced resection (Fig3C. Compared to what, wild type? Knockdown of Ku alone also causes enhanced RPA/53BP1 foci compared to wild type. In fact Ku and DNA-PK appear to have an additive effect. Perhaps I am misunderstanding the experiment, but this was not at all clear. Similarly I was not sure where the figure of 60-70% breaks undergoing resection after Ku + DNA-PK knockdown comes from. This needs to be explained or provide a clear reference to the data.*

We apologise for this lack of clarity. Firstly, because Ku and DNA-PKcs are abundant transcripts, we have not found siRNA to be particularly efficient. Thus, we used combined knockdown to get the greatest impact. In fact, siRNA of either causes some level of enhanced resection and the two are additive. However, since there is not complete loss, this additivity cannot be considered as being non-epistatic – rather just combined effects limit the lack of full knockdown. We had attempted to explain that but have tried to make it clearer in this resubmission.

9. The analysis of dsb repair in this manuscript avoids reference to alternative end-joining / microhomology repair. This is pertinent to the discussion of CtIP function since both CtIP and its yeast relative Sae2 have been implicated in this form of repair.

Alternative NHEJ appears to represent several pathways. Back-up NHEJ appears to be a pathway that functions predominantly in the absence of Ku and involves PARP. Actually, we have some evidence that this can arise but it's a slow process and does not appear to block HR since HR can clearly arise when Ku is absent. Primarily, we think we do not detect this pathway in our work here. Microhomology repair is, arguably more interesting. Our working model is that this process represents the slow component of DSB repair in G1 phase cells whilst in G2 phase cells, HR instead of microhomology mediated end-joining occurs – this is reasonable since in G2 phase, resection occurs avidly whilst in G1 phase, if it occurs at all, it is more restrictive. This certainly fits with data from Kevin Hiom in chicken cells. Thus, in this study, when resection is avid, HR is examined, and little rejoining via microhomology mediated end-joining occurs. This would be consistent with the data but further work is needed to consolidate the model. However, we hope this provides a satisfactory answer to the question. We have not attempted to discuss this in the manuscript.

Minor point
There is no date for the reference Ritter and Durante p7 and in the reference list

We have now added the date of the reference.

Reviewer 3.

1. *One of the key hypothesis/conclusion of the manuscript is that type of DNA damage dictates speed of DSB repair and in turn determines pathway choice. The repair speed is deduced by the kinetics of gamma H2AX after challenging cells with different types of DNA damage (C12, X-ray, or Etp)(see Fig. 1). However, how do the authors pick a particular dose of Etp and comparing the responses with C12 or X-ray? Is it possible that C12 causes more damage than Etp and more damage leads to more complicated and thus slowly repairing lesions?*

The dose we chose for the 3 agents was decided on the basis of several inter-balancing factors. We aimed to have the number of DSBs induced as close as possible but also considered that we wanted the number of RPA/Rad51 foci to be similar in number. We were particularly mindful to avoid the saturation problem discussed in the response to reviewer 2 point 5. Finally, for carbon ions we had to choose a dose that gave a scoreable number of tracks per cell. The doses chosen actually created similar numbers of gH2AX foci at 2 h post treatment, the time when we first monitor RPA/Rad51 foci numbers, which we consider to be important. For the comparison between Etp and X-rays, despite the higher number of DSBs induced for Etp, there is clearly less RPA foci forming. For C12, there could be an underestimation of DSB formation if they arise in close proximity. But given that we observe fewer not more gH2AX foci, it is unlikely that this can explain the magnitude of the increase in Rad51/RPA foci that we observe. Additionally, we have exposed cells to 10 Gy X-rays and observed that by 24 h around 8 foci remain (Figure 3 in this letter) – i.e. even after 10 times as many DSBs (250 assuming 25 DSBs/Gy induction) induced by X-rays, fewer DSBs remain at 24 h compared to C12. Thus, we believe that it is the complex DSBs with lesions in close proximity and the spatial distribution of lesions that causes the slow DSB repair (and that it is this slow repair that allows for more resection). Further, the analysis of BRCA2 and RAD54 mutants consolidates our conclusions since we observe a substantial DSB repair defect after carbon ions which is greater than the number of HC-DSBs. We stress that we used Etp as well as C12 to help overcome any possible limitations in DSB estimation by C12.

To make this analysis more transparent, we have included the actual number of gH2AX and Rad51-RPA foci enumerated as a supplementary figure (see Supplementary Figure S3C and D). Alternatively, we could put the induced DSB numbers in the figure legend if the reviewers/editors prefer this.

2. The authors propose that slowly repairing lesions are subjected to end resection since only a subset of gamma H2AX in Etp treatment colocalizes with RPA/Rad51 foci whereas most C12 induced lesions associated with RPA/Rad51 (Fig. 1C). However, the amount of damage was estimated by scoring the number of gamma H2AX foci at 30 min (or 15 min in the figure legend) post radiation/Etp treatment while the resection was measured at 2 h post treatment. Therefore, the measurement is strongly biased for those slowly repairing lesions and the lesions scored in Etp treatment at 30 min post treatment may be already fixed and no longer associated with RPA/Rad51.

We understand this point and have previously evaluated it carefully. We have carried out a time course analysis on RPA/Rad51 foci formation in a previous study (Beucher et al., 2009) and this work (Figure 1C, S2 and others). We find that they are both maximal in human cells around 2 h, which is why we used that time point here. The question is: does that mean they do not form at earlier times (i.e. all foci have RPA/Rad51 at them but we cannot see it) or is this timing because the more rapidly repaired DSBs are repaired by NHEJ. All our findings, (which are largely argued in the Beucher et al., 2009 paper) argue that the latter is the explanation e.g. NHEJ defective cells fail to repair the rapidly repaired DSBs and HR defective cells fail to repair the subset that is evident at 2 h. Perhaps the strongest argument is that BRCA2 defective cells, which are proficient in RPA foci formation but fail to load Rad51, show a similar level of RPA foci to that shown by control cells at 2 h – they persist however. In other words, if all DSBs were undergoing resection and Rad51 loading, one would anticipate that RPA foci would be much greater in BRCA2 defective cells. Indeed we do find persistent RPA foci at nearly 100 % of the DSBs after C12 irradiation. Given this, we considered that the best way to estimate % resection/Rad51 loading is to estimate DSB numbers at 15-30 min post IR and RPA/Rad51 foci at 2 h, when the numbers are optimal. To make the justification for this clearer, we have referred to our previous kinetic analysis of RPA and Rad51 foci. We have also discussed this argument in Supplementary Figure S2 legend.

Therefore, we examined resection and gH2AX analysis in BRCA2-/- cells, specifically to be sure that we do not underestimate resection. The logic here is that BRCA2-/- cells form RPA foci but do

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not load RAD51. Thus, if we missed resection occurring at early times, we would expect it to be greater in BRCA2-/- cells. As we show in Figure S2, it is very hard to measure RPA/Rad51 foci at < 2 h in human cells. However, importantly, we demonstrate that HR defective BRCA2-/- and Rad54-/- cells clearly show no DSB repair up to 24 hr after C12 irradiation (Figure 1E and S3B). We observed persistent RPA foci in BRCA2-/- and Rad54-/- cells up to 24 hr after C12 irradiation (Figure 1D and 1F). This strongly indicates that most DSBs are repaired by HR after C12 irradiation. On the other hand, <~10% of HC-DSBs are repaired by HR after Etp treatment (Figure 2) and this minor level of HR is not detectable in BRCA2-/- cells (Figure 1D).

3. The authors said that the repair speed dictates which pathway to use. Then the authors also said that the pathway utility also dictates the repair speed. This is circular reasoning.

We struggled to explain this but obviously failed! For each DSB, the speed of repair determines the pathway utilization. Thus rapidly repaired DSBs are repaired by NHEJ; slowly repaired ones by HR. However, slow repair occurs in G1 phase where HR does not take place. Thus, we argue that pathway choice does not regulate this aspect of repair kinetics. Two known factors determine this; lesion complexity and chromatin complexity. However, additionally, there is a difference in repair kinetics when repair occurs via HR or NHEJ with HR being slower than the slow-NHEJ process that occurs in G1 phase. Since only slowly repaired DSBs (determined by damage or lesion complexity) undergo repair by HR, one cannot argue that HR is inherently a slow process. However, our analysis of the slow DSB repair process alone shows that when repair occurs via NHEJ in G1 it occurs more rapidly than when HR is utilized in G2. Thus, the speed of the slow DSB repair process can differ depending on whether HR or NHEJ is used. Since this aspect of repair kinetics only affects the slow DSB repair process and is only be evident in G2 phase (where HR can occur), it is not a factor determining pathway choice but rather a consequence of the choice. Nonetheless, it adds another factor influencing the kinetics of repair. However, it is only relevant when considering the slow DSB repair component.

The argument is difficult to explain but is not circular reasoning. We have rephrased our previous explanation to try to make it clearer but obviously word limitations apply.

4. The authors showed only the kinetics of RPA focus formation upon C12 induced DNA damage (Fig. 1d). X-ray and Etp-induced DNA damage also need to be assessed with the same way. In Fig 1d and F, why is the number of RPA foci fewer than that in Fig. 1c? Does it mean that many foci are with Rad51 only?

a) We previously reported kinetics of RPA foci after X-ray +/- BRCA2 cells (Beucher et al., 2009). We have now referenced this. Here, we have examined % of RPA or Rad51 foci formation (Figure 1C), to consolidate this. Also we have shown dose dependent Rad51 foci formation after Etp since this was not previously examined (Figure 2A).
b) We showed actual numbers of RPA foci in Figure 1D and F whereas % of RPA or Rad51 foci/induced gH2AX foci is used in Figure 1C.

5. It is not clear how Etp was applied to? In Fig 2B, Etp was applied for 30 min. and then was removed subsequently. How about other experiments? Need to clarify them. I also wonder why did the authors examine % gamma H2AX foci upon Etp treatment in G1 instead of G2 (Fig 2B)?

a) In our revised manuscript, we have stated more clearly the details of Etp in the materials & methods and Figure legends. Basically, we used 15 (Figure 1 data) or 30 min (elsewhere) in all experiments although the concentration differs.
b) We showed the repair kinetics up to 8 hr in G1 and G2 after Etp in Figure 1A and B, respectively. It is, however, very difficult to arrest cells in G2 phase because the G2/M checkpoint arrest is insensitive (Deckbar, JCB, 2007), i.e. since most of Etp induced DSBs are repaired with very fast kinetics, the remaining DSBs are not sufficient to sustain cells in G2 phase during analysis. Therefore, to quantitatively measure DSB repair kinetics with a longer time course after Etp treatment, we examined gH2AX foci analysis in G0/G1 (confluency) arrested cells. Additionally, G0/G1 phase cells were used to avoid any impact of the use of HR on DSB repair kinetics. We have now stated that G0/G1 phase cells were used in our revised manuscript.

6. With regard to the KAP1 foci, are they truly representing pre-existing HC? or do they form after persistent DSB? Is there any way to discern these two?

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As mentioned in comment 6 & 7 from reviewer #2, we have now verified that only a subset of gH2AX foci (~20%) contain pKAP-1 foci in XLF cells after Etp, suggesting that pKAP-1 foci do not form at all persistent DSBs after Etp. i.e. in XLF deficient cells, the DSBs remaining at later times can be EC- or HC-DSBs and, even though there is some enrichment for HC-DSBs due to their slow repair, clearly not all DSBs have pKAP1. We previously demonstrated that pKAP-1 represent HC-associated DSBs after IR (Noon et. al., NCB, 2010).

7. It is concerning that the results were drawn from different types of damaging agent treatment (In Fig. 2 Etp, in Fig 3, X-ray).

We are using three different damaging agents to induce different types of DNA damage in this paper. In Figure 1, we compared the repair kinetics and levels of resection between three damages. We analyzed the reason why DSBs undergo resection after Etp in Figure 2. We further examined if NHEJ factors are involved in DSB repair pathway choice after IR in Figure 3. Thus, we used three different agents for each purpose.

Since we believe that the speed of repair influences pathway choice, we had to find ways to change the speed of DSB repair. The easiest way to achieve this was by changing the damaging agent. The other approach we used was to use cells that lack NHEJ proteins where here also DSB repair occurs slowly. However, obviously this logic is limited since NHEJ mutants cannot undergo NHEJ. Notwithstanding this limitation, this aspect of the work serves to consolidate the idea using X-rays. Currently, we do not know of another way to change the speed of DSB repair.

8. Is the panel on the right in 3A different from the one in Fig. 3C?

We used two different blotting analysis between Figure 3A and 3C. We have moved the blotting and images in Figure 3A to Figure S5A since there is not enough space in Figure 3.

9. In DNA-PK 6A mutant, is the lack of Rad51 due to the resection defect or an inability load Rad51? May need to look at resection by other means?

We have now monitored levels of resection by BrdU foci, which directly monitor DSB end resection (Figure 3E). Consistent with the Rad51 result, DNA-PK 6A mutant shows a dramatic reduction of resection after IR. We also carried out this analysis using a new antibody that recognizes hamster RPA and obtained results similar to those for Rad51. We have mentioned this as data not shown.

9. Why was the number of Rad51 foci higher in DNA-PK mutant than those transfected with wild type DNA-PK or the SD mutant derivative (Fig. 3E)?

As shown in Figure 3B and C (revised version), we found enhanced resection in DNA-PKcs siRNA treated cells. Consistent with this data, DNA-PK null V3 cells show greater Rad51 and BrdU foci than WT and 6D mutant cell lines. This is consistent with the siRNA analysis in human cells in Figure 3B.

10. Why is there less number of breaks per radiation at G1 (Fig. 4B-D)?

G2 cell contains double amount of DNA compare to G1 cell. This consequently causes double the number of DSBs induced.

11. The y axis in Fig. 4D should be % remaining gamma H2AX.

We have now corrected this.

12. Fig 5D does not have error bars.

We have added the error bars in Figure 5D.

13. Could the effect of DNA-PK mutant be due to the lack of RPA phosphorylation?

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We have examined BrdU foci analysis, which directly monitors ssDNA formation, in DNA-PK cs mutant cell lines. After resection, RPA binds ssDNA regions and is phosphorylated by ATM and/or DNA-PK. Therefore, since we observed a substantial reduction of IR-induced BrdU foci formation in the DNA-PKcs autophosphorylation mutant, our data demonstrate that the effect of the mutation is to block resection rather than reducing or affecting RPA phosphorylation.

14. Why is NHEJ defective in HC even if DNA-PK can still bind?

We do not fully understand what the reviewer means by this comment. We assume that this reviewer is asking why NHEJ in G2 doesn't undergo completion at HC-DSBs since DNA-PK can bind. Our results would suggest that the ligation step of NHEJ or a processing step is restricted as a consequence of the chromatin complexity. Further, DNA-PK must be bound dynamically at the DNA end and able to be released without the completion of NHEJ either by an active process or by dynamic on/off binding. This allows the possibility of resection in G2 phase. Once resection occurs, NHEJ cannot be utilized. So one answer is that resection precludes the ability to utilize NHEJ. But NHEJ can occur at HC-DSBs if resection is prevented.

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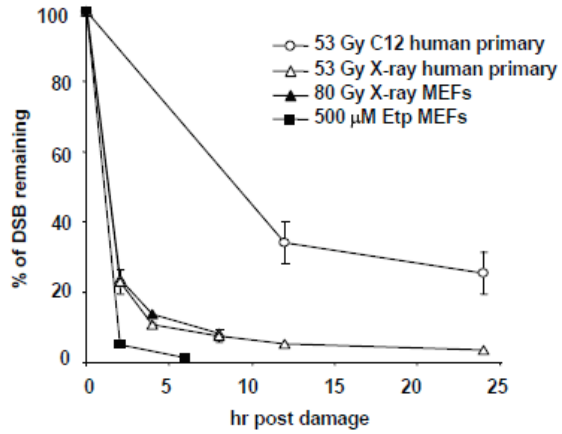

Figure1.
The kinetics of DSB repair following exposure to 53 Gy carbon ions, 53 or 80 Gy X-rays and 500 μM Etp in G1 cells, was monitored by PFGE in HSF1 primary cells and MEFs. The error bars represent SE from two or three experiments in C12, and X-ray MEFs and Etp MEFs. The data after X-ray in human cells is from a single experiment.

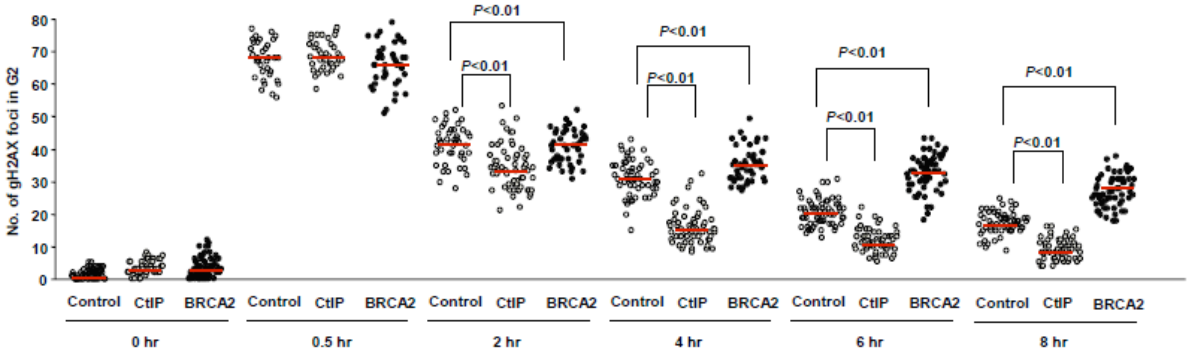

Figure2.
The kinetics of DSB repair in G2 cells. The foci scatter plots are combined from three independent experiments in the Figure 4C. Student t-test was carried out between control and knockdown cells. The red bars represent median.

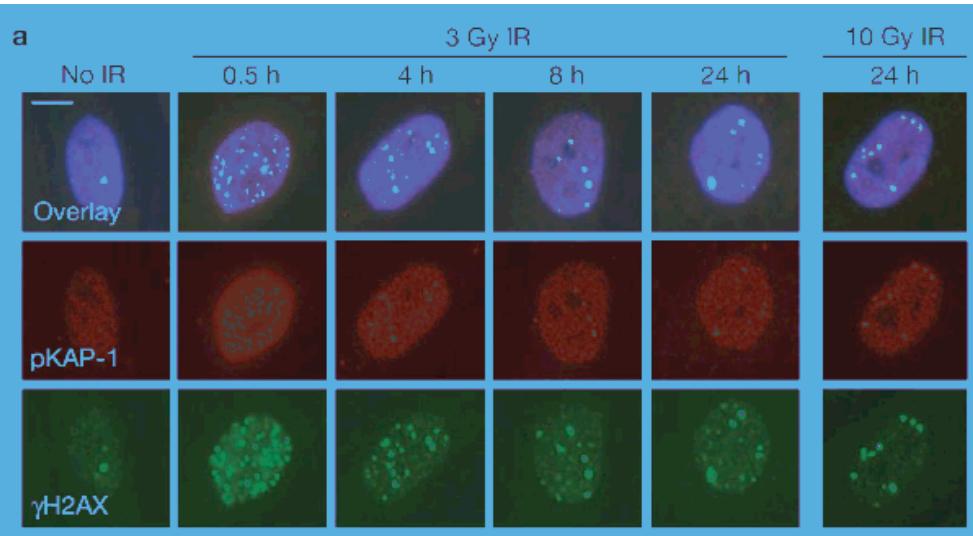

Figure 3.
1BR3 primary human fibroblasts were irradiated with 3 or 10 Gy IR. 10 Gy X-rays induces 250 gH2AX foci assuming 25 DSBs/Gy in G1 cells. ~8 foci, which is approximately 1% of induction, remain at 24 hr after 10 Gy X-ray whereas ~40% DSBs remain after C12 irradiation. (Noon et al, NCB, 2010)

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2nd Editorial Decision 10 January 2011

Thank you for submitting your revised manuscript for our consideration. It has now been assessed once more by two of the original reviewers, who consider the manuscript significantly improved in response to the initial comments and thus in principle now suited for The EMBO Journal. Nevertheless referee 3 retains one specific concern regarding the role of specific DNA-PK subunits (DNA-PKcs or Ku) in the inhibition of resection, the clarification of which would clearly benefit the paper and its conclusiveness. The referee offers two straightforward experimental suggestions to obtain such insight, and I have therefore decided to return the study to you once more for a final round of revision, kindly inviting you to address this issue ideally by attempting these experiments.

When sending us the final version, please make sure to also include the following, in order to avoid any unnecessary further delays with the processing of the manuscript:
- an 'Author Contribution' statement in the text, to be included adjacent to the 'Acknowledgements' section
- a 'Conflict of Interest' statement in the text, following the 'Acknowledgement' section
- one single 'Supplementary Information' file (in PDF format) combining all supplementary figures and text.

Once we will have received your re-revised manuscript, we should then hopefully be able to swiftly proceed with its acceptance and publication.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #2 (Remarks to the Author):

Shibata et al have made a genuine attempt to improve the manuscript. They have now used FACS analysis to measure localisation of RPA at DNA ends. Although this is a different method for scoring RPA this cannot really be considered a new assay since it measuring the same outcome as before. On the other hand it does support the RPA foci scored by eye, which is positive. The authors have improved the reporting of their methodologies which was essential and also clarified a few issues where misunderstanding was possible. Given that there was nothing fundamentally wrong with the manuscript first time round the current submission is definitely an improvement and in this referees opinion there is nothing substantial to block this work from publication.

Referee #3 (Remarks to the Author):

The revision is done well, addressing most of the concerns raised by the reviewers, giving further credence to the integrity of the hypothesis. The main thesis of the proposal that two distinct parameters dictate repair speed and end resection, thereby affecting the pathway usage at G2 seems logical and convincing. The role of ATM in modulating heterochromatin at DNA breaks and end resection (by phosphorylating CtIP) is nicely presented. Having said that, the most controversial interpretation to this reviewer are those of DNA-PKcs null or 5A mutant that Ku can be removed from or vacates from the DSB end to allow resection when NHEJ does not progress. This point also raises the question that not Ku but DNA-PKcs is inhibitory to end resection and challenges the results found in other model organisms. This point needs additional validation as I am not aware of any data suggesting that Ku is readily released from DNA in these mutants. Alternatively, in DNA-PKcs 5A mutant, Ku may persist at DSBs along with DNA-PKcs and such may interfere end resection. To test this idea, I suggest testing the effect of expressing DNA-PKcs 5A mutant in Ku deleted cells on RPA foci formation following DNA damage. I also suggest to check retention of Ku in DNA-PKcs 5A mutant using nuclear insoluble fractionation experiments as done previously in Cui et al.

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2nd Revision - authors' response 17 January 2011

We thank reviewer 3 for appreciating our efforts for the revision.

This reviewer still has concern, however – which is stated as:

The most controversial interpretation to this reviewer are those of DNA-PKcs null or 5A mutant that Ku can be removed from or vacates from the DNA end to allow resection when NHEJ does not progression. This point also raises the question that not Ku but DNA-PKcs is inhibitory to end resection and challenges the results found in model organisms. …Alternatively, in 5A mutant, Ku may persist at DSBs along with DNA-Pkcs and such may interfere end resection

The reviewer's concern is not entirely clear since our model is that indeed in the 6A mutant, Ku (and DNA-PKcs) persists at the DSBs and delays resection. Thus, its unclear why the reviewer prefaces the last line above with *Alternatively.*

Additionally, we state that that DNA-PK **holoenzyme** (ie DNA-PKcs + Ku) is inhibitory to resection. The reviewer above says *that not Ku but DNA-PKcs is inhibitory.* Maybe the reviewer has misread our statement as DNA-PKcs rather than DNA-PK holoenzyme. We do not propose that DNA-PKcs is inhibitory unless Ku is present – ie loss of either Ku or DNA-PKcs allows greater resection. Indeed, there is enhanced resection when Ku is absent. It is, therefore, unclear how examining expression of the 6A mutant in the absence of Ku would be informative. There is strong and abundant evidence that DNA-PKcs requires Ku for end binding and for activation of its kinase activity. Thus, in the absence of Ku, DNA-PKcs will not bind and be activated (whether 6A or WT) – thus it is to be expected that the 6A mutant will not inhibit the increased resection. Thus, there will be nothing gained from this experiment. To make this clearer, we have reclarified our statement in the Results section to read: 'loss of either Ku or DNA-PKcs can enhance resection demonstrating that the DNA-PK holoenzyme (Ku + DNA-PKcs) functions as a complex to ensure the appropriate regulation of resection at DNA ends'

Below, we make the assumption that the referee's concern is that the presence of Ku alone should be sufficient to inhibit resection in mammalian cells and make the following points:
a) this conclusion does not stem from the analysis of the 6A mutant but rather from situations resulting in loss of DNA-PKcs – ie we see enhanced resection in the V3 mutant and following DNA-PKcs siRNA. This conclusion is therefore consolidated with two distinct analyses. Therefore, analysis of the 6A DNA-PKcs mutant in the absence of Ku will not be informative in considering the main basis underlying this conclusion.
b) the suggestion that Ku alone in the absence of DNA-PKcs does not block resection is consistent with the results of Kathy Meek and others (eg Jac Nickloff) that loss of DNA-PKcs (eg the V3 mutants) show elevated HR. Although this does not represent a monitor of resection per se, resection must have occurred for HR to ensue. Thus Ku cannot be a complete barrier to resection.
c) FRAP studies from Dik van Gent's laboratory have suggested that Ku binding is dynamic at DSB ends (Mari et al, 2006; Uematsu et al, 2007). Thus, there is at least some evidence that there could be competition with resection. Ku mobility might be affected by the status of DNA-PKcs phosphorylation, i.e. slower mobility might reduce resection in the 6A mutant. However, to examine the role of DNA-PKcs autophosphorylation on Ku mobility is a follow up study and outside the scope of this paper.
d) We do not know what evidence in model systems argues against out data. In yeast, Ku is present yet HR occurs avidly – thus HR must occur despite the presence of Ku. Loss of Ku enhances resection but that is not inconsistent with our data but the role of DNA-PKcs cannot be assessed because there is not a DNA-PKcs homologue. The situation in DT40 chicken cells is perhaps the one most distinct to our findings: loss of DNA-PKcs does not enhance HR whereas loss of Ku does. However, HR occurs avidly in chicken cells despite the presence of Ku – thus the lack of any increase after loss of DNA-PKcs may be because the DNA-PK holoenzyme itself has only a small inhibitory effect. We consider that in chicken cells, avid resection can out compete DNA-PK end binding.

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e) Studies in yeast have suggested that MRX or Sae2 can function to prevent Ku's inhibition of resection (Mimitou et al, EMBO 2010) – thus models and some evidence is available to argue that Ku does not entirely block resection.

2nd point:
I also suggest to check retention of Ku in 5A mutant using nuclear insoluble fraction experiments as done in Cui et al.

There are two difficulties with this suggestion.
Firstly, we propose that NHEJ makes a first attempt at repairing DSBs. If rejoining does not ensue, then, we suggest that resection occurs allow HR to pursue. We believe this represents an important new concept that has encompassed a lot of work to consolidate. This raises the question of how failed NHEJ allow resection to ensue. One possibility is that Ku (and DNA-PKcs) are released to allow resection. However, its also possible that the internal translocation of Ku allows resection to ensue. Thus, vacating the DNA end does not mean it has to come off the DNA. We took care not to propose a defined mechanism although we suggested in the discussion that it might involve loss of Ku from the end. The phraseology was:
Thus, resection can occur in the presence of Ku. FRAP studies have suggested that Ku binding is dynamic at DSB ends and it is possible that Ku/DNA-PKcs is dynamically bound and released at the DNA end allowing a "competition" with resection (Mari et al, 2006; Uematsu et al, 2007).
However, defining the mechanism is really a follow up study and outside the scope of this paper. As discussed above, the experiment proposed would not consolidate or negate the conclusions in this paper since the arguments substantially rest on the analysis of cells lacking DNA-PKcs. We cannot predict what would happen to Ku in the absence of DNA-PKcs, it may be more readily released or it may translocate inwards.

The second difficulty with the experiment proposed is the issue of cell cycle specificity. To carry out the experiment planned requires the specific examination of G2 phase cells. However, the synchronization methods can not be readily achieved as we mentioned in our m/s (p5, lane 10-12). Further, Ku is a highly abundant protein and binds avidly to DNA ends created during analysis. Thus, this will represent a difficult experiment and likely will require many pitfalls to be overcome.

In summary, we do not feel that the proposed experiments will address the issue of how the DNA-PK complex, which certainly has the ability to block resection, can be regulated to allow resection to occur. We recognise that this is important but feel it represents a follow up study. We have, however, modified our statement to make it clear that it is the DNA-PK holoenzyme under discussion.

Additional Correspondence 18 January 2011

Thank you for submitting your revised manuscript and your detailed response to the remaining referee concerns. I have now had a chance to look through it and to assess your responses, and I am happy to inform you that there are no further objections towards publication in The EMBO Journal.

You shall receive a formal letter of acceptance shortly.

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