THE EMBO JOURNAL

Manuscript EMBO-2010-74332

Saccharomyces cerevisiae Mre11/Rad50/Xrs2 and Ku proteins regulate association of Exo1 and Dna2 with DNA breaks

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Review timeline:

Submission date:	29 March 2010
Editorial Decision:	07 May 2010
Revision received:	05 August 2010
Editorial Decision:	18 August 2010
Accepted:	18 August 2010

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 07 May 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal. I apologize for the delay in getting back to you with a decision - finding appropriate referees and getting their reports back had taken somewhat longer than usual, and the mixed recommendations and evaluations we received had also necessitated some further consultations in order to arrive at a balanced and fair decision.

As you will see from the three enclosed referee reports below, there is interest in your potentially important findings on the opposing roles of MRX and Ku proteins in regulating recruitment of Exo1 and Dna2 resection enzymes to double strand breaks. However, all referees at the same time remain unconvinced that your present set of data has provided sufficiently strong and definitive support for these interpretations, and indicate that various alternative explanations and interpretations cannot be decisively ruled out based on the current evidence - especially regarding the distinct possibility of Dna2 being recruited by ssDNA in an RPA-dependent manner. In light of this major conceptual concern, but also given the substantial number of other major issues raised by all referees, I am afraid I have to conclude that the paper is not ready for publication in The EMBO Journal, at least not in its present form, and that it is also not clear whether further work in response to the referees' criticisms would bring the required substantiation, or rather confound the current conclusions.

Because of the overall interest in the topic and your findings, I would nevertheless remain open to the possibility of considering a revised version of the manuscript further. However, given the mentioned concerns and reservations, I hope you understand that I will not be able to make any

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commitments regarding the eventual acceptance of such a revised manuscript at this stage - and since you indicated being in a competitive situation with other labs, you may therefore be best advised to seek rapid publication of the study without significant changes in another journal. Should you nevertheless feel confident that you might be able to satisfactorily and decisively address the main issues, including the major caveat regarding ssDNA-mediated Dna2 recruitment, then please use the link below to resubmit your revision as soon as it is ready. I should however mention that we allow only one round of major revision, so please make sure to diligently answer to all the points raised at this stage if you wish for the paper ultimately to be accepted. Finally, when preparing a letter of response, please bear in mind that this will form part of the 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). In any case, please do not hesitate to get back to us should you need feedback on any issue regarding this decision .

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

This manuscript shows that MRX facilitates association of Exo1 and Dna2 to DSB ends. Efficient loading of Dna2 and Exo1 requires neither Sae2 nor MRX nuclease activity. Furthermore, they show that Dna2 substitutes Mre11 nuclease activity for processing 5' strands.

Overall I do not support publication of the current manuscript.
General criticism: The major finding in this paper is that MRX recruits Dna2 and Exo1 to DSB ends. Dna2 was shown to interact with RPA and to be recruited in a RPA-dependent manner during DNA replication (Bae et al., 2001, 2003). This finding implies that recruitment of Dna2 requires ssDNA. The lack of MRX (but not its nuclease defective variants) severely affect DSB resection and so ssDNA generation. Thus, the inefficient loading of Dna2 (and may be of Exo1) in mrxdelta mutants (Fig. 1) can be simply due to the low amount of ssDNA. The mre11 nuclease defective mutant does not affect DSB resection and so the loading of Exo1 and Dna2 is not compromised. The authors MUST exclude that the reduced loading of Dna2 and Exo1 is due to low amount of ssDNA to assess that MRX has a role in loading these proteins.

Other criticisms
Based on the finding that resection in sae2 mre11-H125 is indistinguishable from sae2 single mutant, the authors assess that "Mre11 and Sae2 do not perform redundant roles in DSB resection". Since mre11-H125 mutant has no resection defect, it is not possible to perform epistasis analysis.

The data shown in figure 2A has been already published. Based on the finding that resection is defective in dna2D mre11-H125N (Fig. 2B), the authors assess that Dna2 substitutes for MRX nuclease activity. The authors should show resection also in the single mutants to assess that resection is more defective in the double mutant. If Dna2 substitutes Mre11 nuclease activity, the resection defect in sgs1 exo1 dna2 and sgs1 exo1 mre11H125N should be similar. The authors should test it.

Figure 3 shows that the absence of Rad50 or Mre11 increases the binding of Ku to the DSB ends. Since Ku dissociation can be merely the result of resection initiation (that is severely affected in mrex null mutant), again the authors must distinguish between a direct role of MRX in inhibiting Ku loading from a resection effect.

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Referee #2 (Remarks to the Author):

Review of the manuscript "Saccharomyces cerevisiae Mre11/Rad50/Xrs2 and Ku proteins regulate association of Exo1 and Dna2 with DNA breaks" by Shim et al.

In this manuscript the authors analyze the interactions between different proteins that play a role in resection of DNA after DSB formation. They analyze the roles of the MRX complex, Ku heterodimer and Sae2 protein, which are implicated in the early stages of resection, and the Sgs1, Exo1 and Dna2 proteins involved in long, processive resection. They show that MRX recruits Dna2 and Exo1 to the breaks, and acts to prevent excess binding of Ku to the DSB ends. Loading of the Dna2 and Exo1 nucleases does not require the nuclease activity of Sae2 or Mre11, although the latter plays a role when the processive nucleases are absent. The authors carry out an in vitro assay using purified components, that fits their conclusions from the genetic data.

The results presented here are of high importance. In general, this manuscript is very well-written, and most of the data presented (with some exceptions, see below) are solid and convincing. The conclusions are logic and the model presented fits most of the data. There are a small number of exceptions (presented by order of appearance):

1) At the beginning of the results (p. 6, line 8) the authors make the important claim that recruitment of Dna2-myc and Exo1-myc to the HO-induced DNA break is severely repressed in mre11Δ or rad50Δ mutants. This is a central result that has to be shown. The recruitment of Dna2 in the mre11Δ strain should be moved from the Suppl. Materials to Figure 1. The authors should also show the recruitment of Exo1 in a mre11Δ strain, which is not present in the paper.
Figure 1 suffers of problems between triangles and circles: The second panel of 1C has only triangles, and in the third panel there might be a switch between them.

2) On page 8 we are introduced to a dna2 pif1-m2 strain, but the rationale of using a pif strain and the fact that the mutation does not affect results is explained only in page 11.

3) Figure 6C: This is an important figure that shows that the observed effects in resection affect the efficiency of DSB repair. The authors should show, using this assay, that the effects seen in all the other mutants (sae2, dna2 and sgs1 in combinations with mutations in MRX or KU) correlate with DSB repair.

4) Figure 6 and relating text: The authors conclude that MRX's role in Exo1 recruitment to a DSB is by preventing the inhibition of Exo1 by Ku. This would be consistent with the left-most panel of Figure 1C. However, a demonstration of that point would need a similar experiment like the one shown in Fig. 1C, with a rad50 ku70 strain. This would allow to compare recruitment of Exo1 (as it stands now, the results are presented by two different assays, which makes it harder to test the hypothesis).

5) In vitro assay (Figure 7):
First and foremost, this figure lacks controls. So in case no signal is observed (e.g., lane 3), it is not possible to know whether there has been no resection or total degradation.
In addition, the dsDNA was generated by linearization with SphI, which leaves protruding overhangs. The authors should show that this feature does not affect the results, by using blunt-end and 5'overhang enzymes.

6) Discussion: How do the authors explain that they see no Exo1 recruitment in rad50 mutants (Figure 1c left most panel) yet Tsubouchi&Ogawa 2000 found mre11 exo1 to have a much more dramatic effect on resection than do any single mutant?

7) Figure 7B and discussion: The authors have indeed demonstrated that MRX is needed for Exo1 recruitment to DSB by preventing Ku70 inhibition of Exo1 recruitment. However they did not show that the MRX facilitates the binding of Exo1 to DSBs in any additional way. Therefore, I find figure 7B and the last sentence of the first paragraph of the discussion to be misleading. (There may still be three distinct manners by which MRX and Sae2 facilitate end resection because Dna2 recruitment is independent of Ku70 inhibition, BUT the authors should make clear that this does not apply to Exo1).

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Referee #3 (Remarks to the Author):

The paper by Shim et al focuses on the molecular events of the initial stage of end resection. They show effect of RMX on loading of Dna2 and Exo1 nuclease and antagonizing/protecting the binding of DNA ends by Ku. In addition, they show that Mre11 nuclease activity is essential for resection in the absence of resection enzyme.

However, the authors do not show evidences that RMX removes high affinity end binding Ku proteins as stated in the discussion. It could be possible that it just prevents its binding to the ends. Only in the absence of RMX complex, the bridging of the ends is disrupted and the Ku proteins can bind these ends. This is in agreement with the observation that the rad50 and mre11 point mutants (that are still structurally competent to hold DNA ends) are dispensable for Ku suppression.

Since the mechanism of end resection is still unclear, this study offers a new insight in the role of Ku in this process. However, detail analysis of the Ku-dependent regulation mechanisms as well as roles of Exo1 and Dna2 nucleases should be provided.

Fig. 2, the authors should move the explanation why they use pif1-m2 background to place where it is first used in the text. It would be useful to show the effect of dna2 pif1-m2 on the resection.
Perhaps mre11-3 sgs1 strain could have been used as an example of Sgs2/Dna2 pathway.
What is the explanation for the product of limited resection depicted by arrows in part B?
In the summary of the paragraph describing this figure, the inability of Sae2 to substitute Mre11 should be included.
No data for sae2Δ are shown in figure 2B in order to claim the both Mre11 and Sae2 are needed to generate short ssDNA. The sgs1 exo1 sae2 triple mutant should be also included.
The exo1 mre11-nd sgs1 triple mutant was reported inviable (Mimitou and Symington 2008)!

Fig. 3, the label for yKu-9Myc should have been added to the figure for clarity, also different scale for sae2Δ and mre11-3 mutant would be better.
These data might also indicate that the Ku proteins accumulate to DSBs only when their structural integrity is affected as mre11-3 or sae2Δ mutants do not seem to have any effect on Ku recruitment.
Are other NHEJ factors also loaded on the DSB in the absence of Rad50 or Mre11?

Fig. 4, there seems to be some increase in Dna2 recruitment in rad50Δ ku70Δ strain. Different scale for rad50Δ and rad50Δ ku70Δ mutant should be used to assess this. The "dramatic" elevation of Exo1 binding in Ku deletion is not obvious from the graph. Why the symbols differ in panel A and B?

Fig. 5, based on the presentation of the data it is hard to interpret the differences between effect of Exo1 and Dna2 on elevated resection in yku70Δ strain. The gels do not show significant difference between initial resection of exo1Δ yku70Δ and dna2Δ yku70Δ pif1-m2 mutant (compare lanes 4 to 24, compare also yku70Δ at 3kb). Similarly, the long resection seems to be more impaired in the dna2Δ yku70Δ pif1-m2 then in yku70Δ exo1Δ. Therefore, inclusion of dna2Δ strain in combination with rad50Δ and rad50Δ yku70Δ should be included (Fig. 6).

Fig. 7, the effect of Ku on MRX activity alone should be addressed to exclude the possibility that the Ku suppression is not due to nuclease activity of RMX complex. Also the effect of Ku proteins on the activity of Dna2 should be tested to confirm the role of Exo1 as a primary nuclease in the absence of Ku proteins. It is also not clear when the RMX complex was added to the reaction mixture: before, after or together with Ku proteins?

Why no sensitivities of individual strains to DNA damaging agents (MMS, CPT etc.) have been studied?

Wasko et al 2009 should have been cited.

Bressan et al have shown that mre11Δ IR sensitivity is suppressed by deletion of yKu70 and this

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should be stated.

In general, legends for the graphs should be used through out the figures. Also similar symbols should be used in the graphs. In one panel of the figure 5 the rad50Δ is marked with an open symbol, in another it is solid and even changed from diamond to circle symbol (Fig. 5).

1st Revision - authors' response 05 August 2010

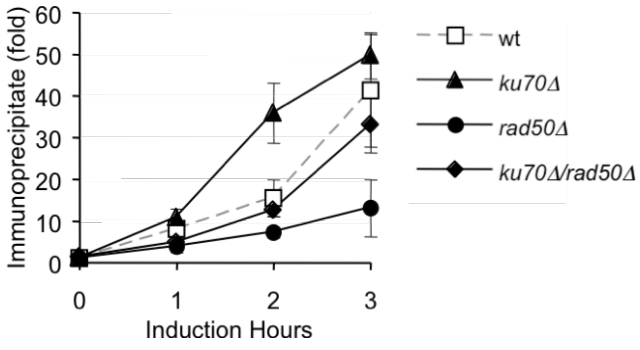
We are very grateful to the reviewers for reading our manuscript and making insightful suggestions. In response to the criticisms of the reviewers, we now extensively revised the manuscript by adding several important controls and experimental evidence to emphasize a few key points of the manuscript. Overall, we believe that the manuscript now becomes much more thorough and should be suitable for publication at EMBO Journal. Outlined below we will describe the point-by-point responses to the reviewers' comments.

Reviewer #1.

The authors MUST exclude that the reduced loading of Dna2 and Exo1 is due to low amount of ssDNA to assess that MRX has a role in loading these proteins.

The reviewer raises here a very important point that we carefully addressed in the revised manuscript (discussed in **page 16**). We think that reduced Dna2 and Exo1 recruitment at DNA breaks observed in mrx mutants cannot be explained solely by a simple decrease of ssDNA for the following reasons:

We found that deletion of YKU70 considerably improves the level of resection and the amount of ssDNA in the rad50D mutant (please see **Fig. 6**). However, the increased ssDNA did not result in equivalent increase in Dna2 recruitment in yku70D rad50D (see **Figure 4A**). If Dna2 recruitment strictly depends on the amount of ssDNA, the level of Dna2 at DSB should be substantially higher in the yku70D rad50D than in the rad50D mutant, which is not the case. These results do not support the model that MRX indirectly regulates Dna2 recruitment by creating RPA-coated ssDNA. Alternatively, we considered the possibility that the increased ssDNA in yku70D rad50D does not increase RPA binding, and thus Dna2 recruitment remains defective in this mutant. Nevertheless, our chromatin immunoprecipitation experiment shown below demonstrates that the level of RPA closely reflects the amount of ssDNA in yku70D rad50D, and becomes almost as high as that in wild type cells. The results are again incongruent with a model that the level of RPA simply dictates Dna2 recruitment at DNA ends and instead supports the role of the Mre11 complex in the recruitment of Dna2 independently of its ability to create RPA coated ssDNA. The model also explains why the increased RPA binding in yku70D has no effect on Dna2 binding at the DSB.



The enrichment of Rpa1 at HO induced DSB in wild type (dotted lines, open symbols), *ku70*D (solid lines, filled triangles), *rad50*D (solid lines, filled circles), or *ku70*D *rad50*D (solid lines, filled diamonds) were shown at indicated times post HO induction. Fold immunoprecipitate was calculated as described in Figure 1. The mean values ± s.d. from three independent experiments were shown.

Additionally, the defect in ssDNA formation and DSB repair in mrx mutants is typically~50% of the wild type whereas the Dna2 binding in mrx mutants is far more severe, suggesting that MRX likely plays a more direct role in recruiting Dna2.

Unlike Dna2, recruitment of Exo1 at DNA ends depends on MRX only when Ku is active. MRX is nearly dispensable for Exo1 recruitment at DNA ends in strains deleted for YKU70 (**Fig. 4B**). Given

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an over 20-fold accumulation of Ku proteins in mrx-deleted strains, we propose that at least one function of Mre11 in Exo1 recruitment is to neutralize the inhibitory effect of Ku on Exo1 recruitment.

Lastly, we would like to point out that the recruitment of Dna2 and Exo1 are not affected by the SAE2 gene deletion that causes a mild resection defect. In sum, the results are consistent with the model that MRX facilitates recruitment of Exo1 and Dna2 not simply by generating ssDNA.

Based on the finding that resection in sae2 mre11-H125 is indistinguishable from sae2 single mutant, the authors assess that "Mre11 and Sae2 do not perform redundant roles in DSB resection". Since mre11-H125 mutant has no resection defect, it is not possible to perform epistasis analysis. The data shown in figure 2A has been already published.

We acknowledged that analysis of single mutants in resection was previously published. Nevertheless, the inclusion of single gene deletion mutant analysis serves here to highlight the effect of double gene deletion mutations on end resection and possible defect in long-range resection that was not done for all of these mutants. Furthermore, for the first time, we compare the resection side by side between these mutants. Published manuscripts analyzed individual single mutants using different assays with or without G2/M arrest, making it difficult to evaluate the degree of resection defects among these mutants. We propose two important points :(i) Sae2 and Mre11 nucleases cannot substitute for each other as the double mutant sae2D mre11-H125N is not more defective in resection than single sae2D mutant. (ii) MRX plays more important role in resection than Sae2. Since the subject of the manuscript centers on the role of MRX, Sae2 and Mre11 nuclease activity in recruiting Dna2 and Exo1, it is important to compare resection defects side by side in these mutants.

Based on the finding that resection is defective in dna2D mre11-H125N (Fig. 2B), the authors assess that Dna2 substitutes for MRX nuclease activity. The authors should show resection also in the single mutants to assess that resection is more defective in the double mutant.

In response to the reviewer #1 and #3's comments, resection in mre11-H125N and that in pif1-m2 dna2 are shown on **Figure 2A** and **Figure 2B** (and in more detail in **Fig. 5C**), respectively, to highlight severe resection deficiency in dna2D pif1-m2 mre11-H125N.

If Dna2 substitutes Mre11 nuclease activity, the resection defect in sgs1 exo1 dna2 and sgs1 exo1 mre11H125N should be similar. The authors should test it.

In the revised version, we showed that resection in sgs1D mre11-H125N is as defective as that in dna2D mre11-H125N, while resection in exo1D mre11-H125N is almost identical to that in exo1D (see new **Fig. 2B**). We also found that sgs1D exo1D dna2D (where the Mre11 nuclease is still active) is not as defective as sgs1D exo1D mre11-H125N (where both Sgs1/Dna2 and Mre11 nucleases are inactivated) in end resection. The results support the model that Sgs1/Dna2 is needed for initial resection in the absence of mre11 nuclease activity. Unfortunately, sgs1D exo1D dna2D pif1-m2 is not viable and we were unable to assess its resection profile. However, every combination of double mutants was tested previously and showed initial resection close to the DSB was dependent on MRX (Zhu et., 2008; Mimitou et al., 2008) and specifically on Mre11 nuclease activity (shown here at **Figure 2C**). This strongly indicates that MRX does not need Sgs1, Exo1 or Dna2 to cleave the 5' strand close to the DSB.

Figure 3 shows that the absence of Rad50 or Mre11 increases the binding of Ku to the DSB ends. Since Ku dissociation can be merely the result of resection initiation (that is severely affected in mrx null mutant), again the authors must distinguish between a direct role of MRX in inhibiting Ku loading from a resection effect.

End resection is greatly attenuated at G1 and any residual resection becomes independent of the Mre11 complex (Ira et al., Nature. 2004 431:1011-7). We thus measured the level of Ku proteins at the DNA break from G1 arrested cells to assess if end resection is responsible for dissociating Ku from DNA breaks. We found that the level of Ku proteins at the DSB is increased only modestly at G1 but markedly increased by the deletion of MRE11 or RAD50 (**Supplementary Figure S5**). Therefore, MRX suppresses accumulation of Ku proteins at the DNA break, which is distinct from its ability to promote end resection.

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Reviewer 2.

The recruitment of Dna2 in the mre11D strain should be moved from the Suppl. Materials to Figure 1. The authors should also show the recruitment of Exo1 in a mre11Dstrain, which is not present in the paper.

We relocated the results demonstrating the recruitment of Dna2 in mre11D, previously included in Supplementary Figure 1 to a new **Figure 1B**. We also include a graph showing the recruitment of Exo1 in mre11D in **Figure 1C**.

Figure 1 suffers of problems between triangles and circles: The second panel of 1C has only triangles, and in the third panel there might be a switch between them.

We corrected the labels in a new **Figure 1C**. I apologize for these mistakes.

On page 8 we are introduced to a dna2 pif1-m2 strain, but the rationale of using a pif strain and the fact that the mutation does not affect results is explained only in page 11.

The pif1-m2 mutation was used to overcome the lethal effect of deleting DNA2, and the effect of the pif1-m2 mutation on end resection was discussed in **page 8** of the revision.

Figure 6C: This is an important figure that shows that the observed effects in resection affect the efficiency of DSB repair. The authors should show, using this assay, that the effects seen in all the other mutants (sae2, dna2 and sgs1 in combinations with mutations in MRX or KU) correlate with DSB repair.

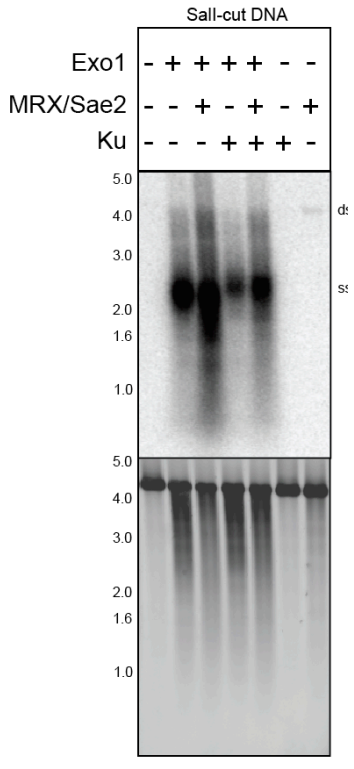
To address this important question, we constructed sgs1D, sgs1D yku70D, sae2D, sae2D yku70D. The sgs1D mre11D mutant was tested and published previously (Zhu et al., 2008). Consistent with only a modest defect in resection close to the break, repair in sgs1D and sae2D mutants is close to wild type level and the deletion of the YKU70 gene does not change the repair frequency (**new Figure 6C**). We do not include here the ectopic recombination frequency in dna2D pif1-m2 because Dna2 has additional roles in DSB repair unrelated to resection. These results will be published elsewhere with a detailed explanation of the additional role for Dna2.

Figure 6 and relating text: The authors conclude that MRX's role in Exo1 recruitment to a DSB is by preventing the inhibition of Exo1 by Ku. This would be consistent with the left-most panel of Figure 1C. However, a demonstration of that point would need a similar experiment like the one shown in Fig. 1C, with a rad50 ku70 strain. This would allow to compare recruitment of Exo1 (as it stands now, the results are presented by two different assays, which makes it harder to test the hypothesis).

The recruitment of Exo1-9Myc in rad50D ku70D is shown in **Fig. 4B**.

In vitro assay (Figure 7):First and foremost, this figure lacks controls. So in case no signal is observed (e.g., lane 3), it is not possible to know whether there has been no resection or total degradation.
In addition, the dsDNA was generated by linearization with SphI, which leaves protruding overhangs. The authors should show that this feature does not affect the results, by using blunt-end and 5'overhang enzymes.

A new experiment was performed for **Fig. 7A** that includes a DNA stain of the gel. This result shows that MRX and Sae2 stimulate the activity of Exo1, that Ku inhibits the activity of Exo1, and MRX and Sae2 partially



DNA resection assays were performed with DNA as a substrate (cut with SalI) and analyzed by native agarose gel electrophoresis and SYBR green staining for the double-stranded DNA (bottom panel) followed by non-denaturing Southern hybridization with a strand-specific RNA probe for the 3' strand, as previously described (Hopkins and Paull, 2008)(top panel). Reactions contained 10 ng (0.35 nM) pNO1 DNA, 5 nM yeast wt Exo1, MRX (25 nM), Sae2 (26 nM) and 10 nM yKu heterodimer as indicated. The position of single-stranded DNA in the gel was marked as "ss", and that of the unresected plasmid is marked as double-stranded, "ds". Migration of molecular weight markers (kb) are shown in the lane marked "M"

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recover the activity of Exo1 when Ku is present, as seen by the increased appearance of single-stranded DNA product in the panel probed for the 3' strand. We also performed a similar experiment with linear DNA containing 5' overhangs generated by SalI, as shown on the above. The stimulation of Exo1 by MRX/Sae2 is not as efficient with 5' overhangs compared to the 3' overhang substrate used in the experiment shown in **Fig. 7A**, but the effect of MRX/Sae2 in promoting Exo1 activity in the presence of Ku is still seen. We did not perform the same experiment with DNA containing blunt ends because of a lack of an appropriate restriction site in a region adjacent to the probe region, but in our experience blunt-ended substrates behave very similarly to that of 3' overhang substrates in this assay.

How do the authors explain that they see no Exo1 recruitment in rad50 mutants (Figure 1c left most panel) yet Tsubouchi & Ogawa 2000 found mre11 exo1 to have a much more dramatic effect on resection than do any single mutant?

Even if MRX stimulates Exo1 recruitment at DNA ends, residual Exo1 recruitment still occurs in mre11D and may account for a less severe resection defect in mre11D than in mre11D exo1D. Additionally, mre11D exo1D cells are very sick and keep accumulating in G2/M. The more severe end resection defect in mre11D exo1D could be partly due to a more important role that the MRX complex plays in resecting DNA ends at G2/M as we previously published (Ira et al., Nature. 2004 431:1011-7).

Figure 7B and discussion: The authors have indeed demonstrated that MRX is needed for Exo1 recruitment to DSB by preventing Ku70 inhibition of Exo1 recruitment. However they did not show that the MRX facilitates the binding of Exo1 to DSBs in any additional way. Therefore, I find figure 7B and the last sentence of the first paragraph of the discussion to be misleading. (There may still be three distinct manners by which MRX and Sae2 facilitate end resection because Dna2 recruitment is independent of Ku70 inhibition, BUT the authors should make clear that this does not apply to Exo1).

If MRX regulates Exo1 recruitment solely by neutralizing an inhibitory action of Ku, we expect that the level of Exo1 in ku70D mre11D should be identical to that in ku70D. However, we found that deletion of Ku70 in mre11D did not fully rescue the level of Exo1 to the level seen in Ku70 deleted strain (see **Fig. 4B**). This prompted us to propose that Mre11 has yet another role in the recruitment of Exo1 at DNA break besides neutralizing Ku-mediated inhibition on the binding of Exo1 to DNA ends.

Reviewer 3.

However, the authors do not show evidences that RMX removes high affinity end binding Ku proteins as stated in the discussion. It could be possible that it just prevents its binding to the ends.

We revised the discussion as the reviewer suggested.

Fig. 2, the authors should move the explanation why they use pif1-m2 background to place where it is first used in the text.

The reason why pif1-m2 mutation was used is now discussed in **page 8**.

It would be useful to show the effect of dna2 pif1-m2 on the resection.

It is now shown in new **Figure 2B and 5C**.

Perhaps mre11-3 sgs1 strain could have been used as an example of Sgs2/Dna2 pathway.

The end resection profile of mre11-H125N sgs1D (mre11-3 and mre11-H125N are equivalent mutants) is shown in **Fig. 2B**.

What is the explanation for the product of limited resection depicted by arrows in part B?

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Those highlighted by arrows represent resection products generated in the absence of Sgs1 and Exo1 and correspond to short (100-500 bp) resected DNA ends as documented previously (Zhu et al., 2008). In new **Figure 2C**, we show that the formation of these residual resection products in the absence of Sgs1 and Exo1 depend on the nuclease activity of Mre11 and Sae2.

In the summary of the paragraph describing this figure, the inability of Sae2 to substitute Mre11 should be included.

We revised the paragraph to indicate this finding.

No data for sae2D are shown in figure 2B in order to claim the both Mre11 and Sae2 are needed to generate short ssDNA. The sgs1 exo1 sae2 triple mutant should be also included.

In sae2D (but not sgs1D exo1D), we did not detect short ssDNA because processive resection enzymes Dna2 and Exo1 quickly convert it to long ssDNA. The role of SAE2 in ssDNA in the absence of SGS1 and EXO1 was reported previously (Mimitou and Symington. Nature. 2008 455:770-4 and Zhu et al., 2008, Cell, 134:981). However, we include the result in new **Figure 2C** of the revision to address the reviewer's comment.

The exo1 mre11-nd sgs1 triple mutant was reported inviable (Mimitou and Symington 2008)!

The triple mutant is sick but viable in our strain background.

Fig. 3, the label for yKu-9Myc should have been added to the figure for clarity, also different scale for sae2D and mre11-3 mutant would be better.

The revised **Figure 3** with the different scale in the vertical axis is included in the supplementary information (**Supplementary Figure S2**) according to the reviewer's suggestions.

These data might also indicate that the Ku proteins accumulate to DSBs only when their structural integrity is affected as mre11-3 or sae2D mutants do not seem to have any effect on Ku recruitment.

We describe this possibility in the discussion.

Are other NHEJ factors also loaded on the DSB in the absence of Rad50 or Mre11?

We showed previously (Zhang et al, Nat Struct Mol Biol 14:639-646.) that Lif1/Dnl4 recruitment is also elevated like Ku in the mre11 deletion mutant.

Fig. 4, there seems to be some increase in Dna2 recruitment in rad50D ku70D strain. Different scale for rad50D and rad50D ku70D mutant should be used to assess this.

We performed this experiment multiple times, and the deletion of YKU70 causes only a modest increase in Dna2-myc recruitment at the DSB in rad50D yku70D. Given the size of the error bars, we are uncertain if the modest increase in Dna2-Myc is statistically significant. Alternatively, the increase in Dna2-Myc recruitment in rad50D yku70D may be attributed to the enhanced resection in this mutant that has certain but limited impact on the amount of Dna2-Myc at DSB ends. In response to the reviewer's suggestion, a figure showing Dna2 recruitment in rad50D and rad50D yku70D with different scales and error bars is included in the Supplementary Information (**Supplementary Figure S3**).

The "dramatic" elevation of Exo1 binding in Ku deletion is not obvious from the graph. Why the symbols differ in panel A and B?

To highlight the difference in Exo1 recruitment among mutants, the filled squares were used to highlight the Exo1-myc recruitment at 1 h post HO expression in the original submission. In response to the reviewers' comments, we replaced them to open squares but marked them with "*".

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Fig. 5, based on the presentation of the data it is hard to interpret the differences between effect of Exo1 and Dna2 on elevated resection in yku70D strain. The gels do not show significant difference between initial resection of exo1D yku70D and dna2D yku70D pif1-m2 mutant (compare lanes 4 to 24, compare also yku70D at 3kb). Similarly, the long resection seems to be more impaired in the dna2D yku70D pif1-m2 then in yku70D exo1D. Therefore, inclusion of dna2D strain in combination with rad50D and rad50D yku70D should be included (Fig. 6).

We would like to emphasize that the graphs are more accurately reflecting the resection profile of a given mutant as they represent the outcomes from at least three independent experiments than the included Southern blot results. We would also point out that assessing Southern blot results should give careful considerations on the variatios of the amount of sample loaded on each gel lane shown in loading controls. In **Figure 5**, we show that deletion of YKU70 makes resection faster and that this accelerated resection requires Exo1. Surprisingly we found that faster resection in the absence of Ku70 depends primarily on Exo1 but partially on Dna2 as well. One possible explanation for these results is that there is some synergy between Exo1 and Dna2 dependent resection pathways. Alternatively, Dna2 is uniquely capable of cleaving 5' strands forming secondary structures that are resistant from cleavage by Exo1.

The observation that dna2D yku70D pif1-m2 is more deficient in resection than yku70D exo1D is not unexpected nor against our model because Dna2 and Sgs1 dependent pathway plays more important role in long range resection than Exo1 at two different loci (Zhu et al., 2008). We would also like to emphasize that Ku effectively blocks resection close to the break but the effect of YKU70 deletion 28 kb away from the break is not pronounced as Ku exerts its effect preferentially on end resection close to the break. Finally as requested by the reviewer we included resection profiles of dna2D rad50D pif1-m2 and dna2D rad50D yku70D pif1-m2 in the **Supplementary Figure S4**. In the figure, we showed that absence of both Dna2 and MRX causes a severe defect in initiation of end resection even when Ku70 is absent. This result confirms our conclusion in Figure 5 that both Exo1 and Dna2 are required for faster resection in absence of Ku70.
We thank reviewer for pointing out this important control experiment.

Fig. 7, the effect of Ku on MRX activity alone should be addressed to exclude the possibility that the Ku suppression is not due to nuclease activity of RMX complex. Also the effect of Ku proteins on the activity of Dna2 should be tested to confirm the role of Exo1 as a primary nuclease in the absence of Ku proteins. It is also not clear when the RMX complex was added to the reaction mixture: before, after or together with Ku proteins?

A previous study showed that the ability of MRX to suppress the binding of Ku in vivo is independent of Mre11 nuclease activity (Zhang et al, Nat Struct Mol Biol 14: 639-646). Additionally, expression of the nuclease deficient mre11 largely restored Exo1 recruitment to DNA in mre11D. The effect of Ku proteins on Dna2 activity was not assessed as it requires several additional recombinant proteins and is outside of the scope of this manuscript. The DNA substrate (in the reaction mix) was added to all of the proteins simultaneously.

Why no sensitivities of individual strains to DNA damaging agents (MMS, CPT etc.) have been studied?

In response to the reviewer's comments, drug sensitivity profiles of various mutants were included in the **Supplementary Figure S1**.

Wasko et al 2009 should have been cited.
Bressan et al have shown that mre11D IR sensitivity is suppressed by deletion of yKu70 and this should be stated.

We revised the manuscript to cite both references

In general, legends for the graphs should be used through out the figures. Also similar symbols should be used in the graphs. In one panel of the figure 5 the rad50D is marked with an open symbol, in another it is solid and even changed from diamond to circle symbol (Fig. 5).

We revised **Figure 5** to use the same labels throughout.

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2nd Editorial Decision 18 August 2010

Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by one of the original referees (see comments below), 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.

Yours sincerely,

Editor
The EMBO Journal

Referee 3 (comments to authors):

The authors addressed all the comments and I recommend the manuscript for publication in EMBO Journal.

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