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Ku prevents Exo1 and Sgs1-dependent resection of DNA ends in the absence of a functional MRX complex or Sae2

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Transaction Report:

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

1st Editorial Decision 07 May 2010

Thank you again for submitting your manuscript for consideration by The EMBO Journal. We have now received the reports of three experts, which you will find copied below.

Their evaluations are somewhat mixed: Referee 1 is not convinced that the study contains sufficient novel insight, and also criticizes the missing incorporation of previously reported findings into your model. Referees 2 and 3 are generally more positive about the paper but raise a number of specific concerns that would need to be adequately addressed before publication may be warranted. Given that neither of them seems to share the major novelty concerns of referee 1, I have come to the conclusion that we should be happy to consider a revised version of the manuscript further for publication. For such a revised study to be ultimately accepted, it will however be important to not only adequately respond to/address the various presentational and interpretational issues (including those of referee 1 I just mentioned), but also the major points of referee 2 regarding the kinetic/time point analysis (point 1) and the NHEJ relationship (for which the referee provides an experimental suggestion in point 3). Finally, I am aware that this is a genetic study, but keeping our broad general readership in mind, I would nevertheless appreciate any efforts to keep the presentation of the genetic arguments and evidence as broadly accessible as possible when revising the manuscript text.

I should remind you 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 experimental and editorial points raised at this stage. When preparing your letter of response, please also bear in mind that this 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). In any case, 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):

This manuscript shows that in the absence of Ku, the requirement for the MRX complex in DSB resection is bypassed and resection is executed by Exo1. Both Exo1 and Sgs1 contribute to DSB processing in the absence of Ku or Sae2. By contrast, loss of Ku does not suppress IR sensitivity of exo1 sgs1 double mutant.

There are substantial precedents for the findings, limiting the novelty and contribution to the field. In fact, it has already been shown in both S. pombe and S. cerevisiae that Ku loss suppresses mre11D and sae2 hypersensitivity (Tomita et al., 2003; Limbo et al., 2007; Wasko et al., 2009). In S. pombe, it has been shown that these suppressions require EXO1 (Limbo et al., 2007; Tomita et al., 2003). Thus, it is not surprising that loss of Ku restores resection in the above mutants.
The novel finding is that Sgs1 becomes important for DSB resection in the absence of Mre11 nuclease activity, but I do not think that this information is sufficient to warrant publication in The EMBO J.

Major criticisms:
Based on the finding that deletion of DNL4 does not suppress mre11 hypersensitivity, the authors conclude that loss of end protection by Ku allows increased 5'-3' end processing (pag. 6). This conclusion is contrast with the previous finding that loss of DNL4 allows 5'-3' resection in G1 (Zierhut et al., 2008).

The authors propose a model in which Ku limits Exo1 action. This is not in agreement with a previous finding that DSB resection in ku70 null mutant is primarily MRX-dependent (Clerici et al., 2008). The authors' conclusion is based on the finding that resection in mre11 ku70 is dependent on Exo1 (Fig. 1). However, the authors fail to analyze ku70 single mutant in which resection is known to be more efficiently than in wt and to be dependent on MRX. Thus, if Ku protects from Exo1 in the absence of Mre11, it protects also from MRX (of course when MRX is present).

The suppression of the synthetic lethality of rad27 sae2 and sgs1 sae2 by yku70 is very interesting and suggests that Ku might have some role in DNA replication. However, the authors fail to investigate further this point.

Referee #2 (Remarks to the Author):

Review of manuscript: "Ku prevents Exo1 and Sgs1-dependent resection of DNA ends in the absence of a functional MRX complex or Sae2" by Eleni P. Mimitou and Lorraine S. Symington.

In this manuscript the authors study the interactions between the MRX complex, Sae2 and the Ku heterodimer, with the Sgs1 and Exo1 resection pathways. They show that requirement of the MRX complex can be bypassed by deleting the Ku complex; in that situation resection is carried out by the Exo1 nuclease. In contrast, if Sae2 is absent, deletion of Ku suppresses IR sensitivity, but requires both Sgs1 and Exo1. In an mre11 mutant defective for nuclease activity, deletion of Ku suppresses the IR sensitivity, in a way that is dependent mainly on Sgs1. The authors present evidence for an early role for Ku, at the initiation of resection, as an sgs1 exo1 (in which little resection takes place) cannot be suppressed by deleting the YKU70 gene. The authors present a model explaining their results in wt cells and in various mutant situations.

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:

1) Figure 1:
a. In the third panel of Figure 1C (mre11 yku70 exo1) the kinetics of appearance of the DSB band are different: It takes 2 hs to reach maximal levels. The interpretation of this finding is not clear, and the authors should address the point.

b. The authors claim that figure 1D shows that deletion of Ku suppresses the resection defect of a mre11 strain (bottom of page 6). This, however, is based on a single time point that shows a relatively slight effect. Already the next time points show similar results for all strains. To be convincing, the experiment should be repeated with at least two time points showing significant differences.

2) By far the weakest data in the paper is in the telomeric gels. Contrary to what is reported in the text, expression of YKU70 from a galactose-inducible promoter fails to show a clear elongation in the yku70 control, as well as in the sgs1 sae1 yku70 strain. Even after 30 hours only a very slight level of elongation can be seen in the first strain. This is to be expected, as telomere elongation back to wild type levels requires a large number of generations (usually a number of re-streaks, 5 or more) and is unlikely to be observed in a cell population after such a small number of generations. I therefore suggest that these experiments be repeated under the right conditions to test the hypothesis proposed, or should be plainly taken out.

3) The relationship between NHEJ and the proteins studied is left inconclusive, although the authors suggest (based on data not shown) that NHEJ may play some role. The authors suggest in the Discussion the possibility that the differences between sae2D and mre11-nd may be due to a role of the MRX complex in directing repair to HR or NHEJ. Specifically, binding of Sae2 to Xrs2 may compete with binding by Lif1, similar to what has been observed in mammals. This can be easily tested by adding lif1D mutations to the mre11-nd strains and comparing them. Such an experiment should confirm or rule out a role for NHEJ in the decision process.

Minor corrections:

Page 3, line 14: "predominant"
Page 4, line 3: The sentence reads better as: "Sae2 is directly phosphorylated by CDK activating the initiation of end processing; in addition, nuclear entry of Dna2 during S-phase is regulated by CDK",
Page 8, first line of second paragraph: The sentence reads better as: "Given that the suppression of the sensitivity to IR of mre11-nd by yku70 requires Sgs1 (Figure 2A), we..."
Figure 6: All the text quotations of this figure are misplaced.
Page 19 and References, Manfrini et al., has no date
p. 21, 5 from end: correct "would show be expected".

Referee #3 (Remarks to the Author):

Review on Mimitou and Symington

The manuscript describes the relationship between MRX complex, Ku and other end processing nucleases. The authors show that requirement for MRX complex in the absence of Ku protein is bypassed by Exo1. Furthermore, this suppression is specific to the initiation of resection and Ku aggravates end-processing defects responsible for sae2 sgs1 lethality.

It is a very nice and well-written paper that describes the role of end-resection machinery in processing of both clean and dirty ends. Interestingly, in the presence of functionally but not structural competent MRX complex the rescue becomes dependent not only on Exo1 but also Sgs1. This dependence is more pronounce when processing of dirty ends is required or the end-processing

machinery is saturated with DNA damage.

Fig. 5, how do the authors explain the lethal phenotype of rad27 mre11 ku70 in contrast to mre11 nuclease mutants? Use same labeling of panels as in other figure legends.

Fig.6 Please correct the labeling for the whole figure. No description of panel B is included in the figure legends. The legend for panel D is under panel C, etc.
The overexpression of yKu70 in sae2 sgs1 yku70 background seems to cause some small initial shortening and then elongation possible to the starting level?

The protective role of RMX might be more discussed. In the absence of RMX complex, the bridging of the ends is disrupted and the Ku proteins can then bind these ends, thus deletion of Ku show great suppression. On the other hand the rad50 and mre11 point mutants (that are still supposedly able to bind the DNA ends) are only slightly suppressed by yKu70 deletion, which could reflect high dosage of DNA damage.

1st Revision - authors' response 15 July 2010

We thank the referees for their positive and insightful comments. The additional experiments suggested by Referee 2 have been performed and are described in the detailed response below.

Referee 1:
Novelty: We agree that some of the results presented have been published before. However, since our discovery of the Sgs1-dependent resection pathway it was of interest to determine whether the Exo1- and Sgs1-dependent pathways are both suppressed by Ku in the absence of the MRX complex or when the complex is compromised by sae2 or mre11-nd mutations. Here we present a complete analysis of all combinations of mutations and a model for the interplay of the different factors at DSBs. Furthermore, the strong suppression of sae2 by yku70 raises the interesting possibility that one function of Sae2 is to inhibit NHEJ in addition, to the clipping activity. The other novel findings are the synergism between mre11-nd and sgs1 for IR sensitivity and processing HO-induced DSBs, and the suppression of the rad27 sae2 and sae2 sgs1 lethalities by elimination of Ku.

1. *'Based on the finding that deletion of DNL4 does not suppress mre11 hypersensitivity, the authors conclude that loss of end protection by Ku allows increased 5'-3' end processing (p. 6). This conclusion is contrast with the previous finding that loss of DNL4 allows 5'-3' resection in G1 (Zierhut et al., 2008).'*

In the papers cited, it was shown that loss of Dnl4 suppresses the G1 resection defect of Mre11+ cells, but to a lesser extent than yku mutations. Our findings indicate that loss of Ku but not Dnl4, suppresses the mre11 IR hypersensitivity. This suggests that in the absence of the MRX complex loss of end protection by Ku allows increased 5'-3' end processing. However, as was already mentioned in the text, loss of Dnl4 in sae2 cells slightly suppressed the IR sensitivity, suggesting that indeed loss of Dnl4 can allow some 5'-3' resection when the MRX complex is present. Our results therefore are in agreement with previous findings (Clerici et al., 2008; Zierhut et al., 2008). To help clarify this point we have included the differential suppression of the IR sensitivity by loss of DNL4 in the mre11 , mre11-nd and sae2 backgrounds in Figures 1, 2A and 3A respectively.

2. *'The authors propose a model in which Ku limits Exo1 action. This is not in agreement with a previous finding that DSB resection in ku70 null mutant is primarily MRX-dependent (Clerici et al., 2008). The authors' conclusion is based on the finding that resection in mre11 ku70 is dependent on Exo1 (Fig. 1). However, the authors fail to analyze ku70 single mutant in which resection is known to be more efficiently than in wt and to be dependent on MRX. Thus, if Ku protects from Exo1 in the absence of Mre11, it protects also from MRX (of course when MRX is present).'*

Loss of Ku suppresses the mre11 IR sensitivity due to increased end resection, which by definition is MRX independent but, as we and others have shown, is Exo1 dependent. For this reason we suggested that Ku protects ends in the mre11 mutant from Exo1. Since both Ku and MRX can bind to DSB ends independently we did not want to exclude the possibility that Ku-only bound ends that block Exo1 access can be present in MRX+ cells.
We attribute the failure to observe MRX dependency of increased end resection in yku mutants to cell cycle differences. The reviewer extrapolates information from experiments performed in G1 arrested cells, where loss of Ku indeed increases end resection in an Mre11 dependent manner. In the same paper though it was found that in G2 arrested cells, single yku mutants do not exhibit increased end resection. Our experiments are performed in cycling cells, where resection follows patterns more similar to G2 arrested cells. In agreement with Clerici et al., 2008, we find that physiological levels of Ku do not interfere with end processing in cycling cells. Moreover we show that this can be attributed to the presence of Sae2, which is known to be cell cycle regulated so that it is active in G2.

3. The suppression of the synthetic lethality of rad27 sae2 and sgs1 sae2 by yku70 is very interesting and suggests that Ku might have some role in DNA replication. However, the authors fail to investigate further this point.

We interpret the suppression of the rad27 sae2 lethality by yku70 to be due to increased efficiency of end processing of replication-induced DSBs. This result suggests Ku can bind to replication associated breaks and prevent their normal repair by HR. Interestingly, two recent papers in Science and Molecular Cell report suppression of the ICL sensitivity of FA cell lines by elimination of Ku, suggesting cells use specialized factors to antagonize Ku during replication. This is one of the important findings presented in our manuscript. We previously showed HO-induced DSBs are resected in the sae2 sgs1 double mutant (using a tet-regulated SAE2 allele to overcome the lethality of the double mutant). Thus, we favor the hypothesis that the sae2 sgs1 suppression by yku70 is due to increased resection at telomeres, consistent with recent studies showing absence of telomere resection in the sae2 sgs1 double mutant (Bonetti et al., 2009). The presence of Ku and Rap1-Rif1-Rif2 at telomeres may represent a stronger barrier to resection at telomeres than at internal DSBs (Bonetti et al., 2010).

Referee 2:
1. -In the third panel of Figure 1C (mre11 yku70 exo1) the kinetics of appearance of the DSB band are different: It takes 2 hs to reach maximal levels. The interpretation of this finding is not clear, and the authors should address the point.'

The mre11 ku70 exo1 strain grows more slowly than the other strains and switching HO on and off by addition of galactose and glucose respectively takes longer. We therefore attribute the differences in the initial accumulation of the DSB band in this mutant to its growth defect.

2. -The authors claim that figure 1D shows that deletion of Ku suppresses the resection defect of a mre11Δ strain (bottom of page 6). This, however, is based on a single time point that shows a relatively slight effect. Already the next time points show similar results for all strains. To be convincing, the experiment should be repeated with at least two time points showing significant differences.'

Two new inductions were performed using a shorter (30 min) induction of HO and analysis of DNA extracted at 30 min time intervals after HO shut-off. Again, we only found a significant difference between the mre11 and mre11 yku70 strains at one time point. Because the suppression of the mre11 resection defect at the HO-induced DSB appears to be subtler than for IR-induced DSBs we decided to remove this part of Fig. 1.

3. -Contrary to what is reported in the text, expression of YKU70 from a galactose-inducible promoter fails to show a clear elongation in the yku70Δ control, as well as in the sgs1 sae1 yku70 strain. Even after 30 hours only a very slight level of elongation can be seen in the first strain.

This is to be expected, as telomere elongation back to wild type levels requires a large number of generations (usually a number of re-streaks, 5 or more) and is unlikely to be observed in a cell population after such a small number of generations. I therefore suggest that these experiments be repeated under the right conditions to test the hypothesis proposed, or should be plainly taken out.'

The referee's point is correct; it does require a larger number of generations to detect telomere elongation. We originally focused on earlier time points, because we were interested in detecting telomere-telomere fusions, which we hypothesized would happen shortly after induction of Ku. Since no telomere fusions could be detected, we decided to repeat the experiment as indicated by the reviewer, with longer time points, but the lethality of the sae2 sgs1 mutant over-expressing KU prevented an extensive time course. The experiment clearly indicates that telomere elongation is taking place in the control cells (yku70), but is much slower in the sae2 sgs1 mutant after KU over-expression. This result is now presented in Figure 6, panel C.

4. -*The relationship between NHEJ and the proteins studied is left inconclusive, although the authors suggest (based on data not shown) that NHEJ may play some role. The authors suggest in the Discussion the possibility that the differences between sae2D and mre11-nd may be due to a role of the MRX complex in directing repair to HR or NHEJ. Specifically, binding of Sae2 to Xrs2 may compete with binding by Lif1, similar to what has been observed in mammals. This can be easily tested by adding lif1 mutations to the mre11-nd strains and comparing them. Such an experiment should confirm or rule out a role for NHEJ in the decision process*.

We considered the reviewer's hypothesis and sought to test it by comparing the IR sensitivity of mre11-nd single and mre11-nd lif1 double mutants. No suppression was detected, so removed this suggestion from the discussion.
Finally all the minor text corrections have been made.

Referee 3:

1. -How do the authors explain the lethal phenotype of rad27 mre11 ku70 in contrast to mre11 nuclease mutants?'

Even though loss of Ku allows some resection in mre11 mutants, mre11 yku70 cells are still very sensitive to IR and resection defective. On the other hand, mre11-nd and mre11-nd yku70 mutants are resection proficient, markedly more than mre11 yku70 mutants. We suggest this is because MRX recruits Sgs1, and possibly Exo1, to break sites. Presumably the resection that can take place in mre11 yku70 mutants is not enough to survive the large number of lesions generated in the absence of Rad27, in contrast, the increased level of resection in the mre11-nd yku70 mutant (as shown by the high IR resistance) must be sufficient to deal with the large number of DSBs produced by loss of Rad27. We have added to sentences to the Results section to explain this.

2. -The over-expression of yKu70 in sae2 sgs1 yku70 background seems to cause some small initial shortening and then elongation possible to the starting level?

The shortening of the telomeres was an artifact of the way the samples were analyzed by electrophoresis. To clarify our results and make the telomere elongation more obvious, the experiment was repeated after allowing the cells to grow for more generations, as indicated in the new Figure 6, panel C.
All the figure corrections have been made.

Acceptance letter 19 July 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 2 (comments to authors):

The authors have successfully addressed all my criticisms; in my opinion the paper should be accepted for publication now.

