THE EMBO JOURNAL

Manuscript EMBO-2012-82545

# APLF Promotes the Assembly and Activity of Nonhomologous End-Joining Protein Complexes

Gabrielle J Grundy, Stuart L Rulten, Zhihong Zeng, Raquel Arribas-Bosacoma, Natasha Iles, Katie Manley, Antony Oliver, and Keith W Caldecott

*Corresponding author: Keith W. Caldecott, University of Sussex*

**Review timeline:**

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| Submission date: | 04 July 2012 |
| Editorial Decision: | 02 August 2012 |
| Revision received: | 24 October 2012 |
| Accepted: | 29 October 2012 |

## 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 02 August 2012

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been assessed by three expert referees, whose comments are copied below. The referees consider your results describing the role of APLF in assembly of NHEJ repair complexes both interesting and important in principle, yet they also raise a number of specific experimental issues as well as some interpretational concerns, as you will see. Should you be able to satisfactorily address these various points, then we should be happy to consider the manuscript further for publication. I would therefore like to invite you to revise the manuscript in response to the referees' comments and criticisms, making sure to thoroughly and comprehensively answer to all the points raised at this stage, as it is our policy to allow only a single round of major revision. When submitting your revised version, please make sure to include brief Author Contribution and Conflict of Interest statements at the end of the text, and please combine supplementary text, figures and tables into a single Supplementary Information PDF.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time, and it is our policy that competing manuscripts published during this period will have no negative impact on our final assessment of your revised study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

 

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 paper describes domains within APLF (also known as PALF) and Ku that interact with one another, and the authors propose an interesting model based on these refined observations. Though it was known that APLF and Ku interact, it was not known what portion of Ku is responsible for this, and the authors do an outstanding job of defining this using multiple methods. The authors also provide insightful data on Ku-APLF recruitment of XRCC4:ligase IV to DNA ends, and the primary data for this in Figure 5 is quite convincing. Previous data on this point did not use gel shifts, and this addition to the NHEJ field for this aspect is very important.

I have given this paper relatively high marks. The only thing that I would ask of the authors is to provide junctional sequences for example in Figure 7A, lane 3 (upper band). This would confirm that the authors are observing what they think.

Minor.

a. UVA in Figure 3A is used to make DSBs. Can the authors mention why UVA is used here rather than IR, and can they remind readers of the ratio of SSB to DSB using this method? Also, is there a chance that some of the repair factor recruitment using UVA is to sites of repair by other repair pathways.

b. Parts of Figure 5 use Ku80deltaC. Can the authors assure in the text (or provide in the Supplement) that full-length Ku80 here would yield a similar result.

c. The effects in Figure 5 are so strong and striking that I kept having to remind myself that the genetics for APLF KO are not as strong (not like the SCID phenotype of XLF KO). The authors may wish to remind reseaders in the Discussion of this.

Overall, this is a very nice study, and I congratulate the authors.

Referee #2 (Remarks to the Author):

In this manuscript, Caldecott and colleagues provide a biochemical and functional characterization of the interaction between APLF and Ku80. They first refine a conserved Ku80-interaction motif in APLF that is both necessary and sufficient for the interaction with Ku80. The APLF-Ku80 interaction is important for APLF nuclear localization and its stable accumulation at laser micro-irradiation sites. The authors then identify the region in Ku80 involved in APLF binding, namely the vWA domain. They then build a model whereby APLF acts as a platform linking Ku binding to DNA ends to Ligase IV-XRCC4-XLF recruitment at DSBs. This activity of APLF is important for the speed of NHEJ.

Technically, the paper is solid apart from a few issues (detailed below). The strength of this paper is that it provides a compelling model for the function of APLF during NHEJ.

Specific points.

 

1-My main concerns are centered on the laser micro-irradiation experiments and the observation that APLF nuclear localization is in part dependent on its interaction with Ku.

a-The difference between the data in figure 3 and S2 is dramatic. Since S2 is the better-controlled experiment, I suggest that it is moved to the main manuscript. Furthermore, I am somewhat concerned that varying concentrations of APLF in the nucleus might alter the outcome of the laser-micro irradiation experiments. Indeed, one means to produce the curves shown in S2 would be if there were less APLF at the initial time point. The authors need to report the initial intensity of GFP-APLF as a safeguard against the above trivial explanation. Also, the data would be stronger if they were able to fit the curves to extract quantitative information about the accumulation kinetics.
b-Since laser micro-irradiation generates lesions other than DSBs (and since APLF interacts with XRCC1), it would be important to validate the key conclusions of their cell biological experiments using an orthogonal approach such as ChIP at a defined DSB e.g..

c-Are the reconstitution experiments in Figure 4D made with APLF constructs containing an NLS? If not, these need to be done in order to exclude the possibility that the increase in IR sensitivity is due to decreased nuclear accumulation. Similarly, are the experiments shown in Figure 6 done with constitutively nuclear APLF proteins? If not, the experiments should be repeated with APLF constructs containing an NLS.

2-Figure 1B: is the APLF W189G mutant expressed in yeast cells?

3-On p14, I find the rationale for using DT40 cells a little contrived - what is the evidence that cells cycling rapidly have a heightened requirement for NHEJ? As far as I know, budding yeast cells divide every 90 min and rely primarily on HR for radiation resistance. That being said, I am not disputing the fact that the studies in the DT40 cells were informative.

Referee #3 (Remarks to the Author):

EMBOJ-2012-82545
APLF Promotes the Assembly and Activity of Nonhomologous End-Joining Protein Complexes
Authors: Gabrielle J Grundy, Stuart L Rulten, Zhihong Zeng, Raquel Arribas-Bosacoma, Natasha Iles, Katie Manley, Antony Oliver, and Keith W Caldecott
Corresponding Author: Prof. K. Caldecott

In this study, Grundy et al. present a detailed series of experiments describing the role of APLF in the Non Homologous End Joining pathway for DNA double strand break repair. More specifically, this role was already suggested by the same group and by others, but here they demonstrate that APLF operates as a scaffold protein which interacts both with Ku, through the vWA domain of Ku80, and with components of the NHEJ ligation complex, thus promoting the stability of multi-protein Ku complexes and accelerating the repair of DSBs.

Overall the experiments, especially the biochemical ones, are well designed and well conducted, and most of the required controls are present. Indisputably, the manuscript extends our current knowledge of the NHEJ mechanism at the molecular level. The main strength of the manuscript relies on the delineation of the APLF/Ku interface which is reported to involve a small conserved peptide motif from APLF MID domain (~10-12 amino acids) and a small hydrophobic region on the surface of the Ku80 vWA amino-terminal domain. In both protein partners, critical residues have been identifed whose substitution abolishes APLF/Ku interaction in experimental conditions used in the study.

However, I have a couple of reservations regarding some issues.
The main concern relates to the general tendency of the manuscript to overstate the importance of APLF in the NHEJ complex assembly at damage sites, and consequently in the NHEJ reaction as a whole. Indeed, although admittedly the authors ackowledge APLF as "an accessory factor" for NHEJ "rather than an essential component", and point out that they "have failed to detect hypersensitivity to DNA damage in APLF-depleted human cells or APLF-/- MEFs", they repeatedly make use of misleading forms and turns of phrase that tend to overemphasize the role of APLF in the NHEJ reaction.

 

Some examples:
- In the penultimate sentence of the abstract the authors mention an "increased cellular sensitivity to DSBs" (omitting to specify that this result refers to the very peculiar DT40 chicken cell line); then the last sentence extends their observations to "NHEJ in mammalian cells" (omitting to specify that no sensitivity to DNA damage was detected in mammalian cells).
- APLF is repeatedly described as a "core" component of NHEJ which is also confusing. In my opinion, if the term "core" defines the central or most important part of something, it should be devoted to really essential NHEJ factors. Even the specified expression "core structural component" is still misleading since as APLF is not essential for NHEJ to take place, one have to admit that, although less stable, NHEJ complexes assemble at damage sites even in the absence of APLF.
- A similar comment would apply to the term "key": "... APLF is a novel "key" component ..." (page 19).
- In the final model (Fig. 8), the picture suggest that the XRCC4-Lig4 complex does not contact Ku and that it is fully dependent on XLF and APLF to be recruited at damage sites. Instead, it is well established that Ku interacts with both XRCC4 (Mari et al, 2006) and Lig4 (Hsu et al, 2002; Costantini et al, 2007). The model should then be edited to more closely reflect experimental evidence and to illustrate the fact that APLF has no essential role in NHEJ but is part of an intricate network of protein-protein interactions which reinforces NHEJ complex cohesion.

Consequently while APLF is clearly an accessory NHEJ factor which facilitates and stabilizes NHEJ complex assembly, it hardly proves to be a key or a core (even structural) component of NHEJ. The manuscript would then be more in accordance to the experimental reality should the authors edit the text and attenuate some expressions.

Related to the previous comment it is striking that in EMSA-based experiments (Fig. 5) no interaction between Ku and XRCC4-Lig4 is observed in the absence of APLF, in contrast to the well established direct interaction between both core NHEJ factors, even from experiments using similar approach (Nick McElhinny et al, 2000). Can the authors provide some explanation concerning this discrepancy ?

Other comments:
(1) Page 3 (Introduction section): "DNA-PKcs promotes ... possibly also the dissociation of Ku from DNA at the end of the repair reaction".
Which experimental evidence supporting such a role for DNA-PKcs do the authors refer to ? Recent papers rather suggest a polyubiquitin-dependent removal of Ku (Postow et al, 2008; Feng and Chen, 2012).

(2) Page 7 and Fig. 2C: EMSAs presented in Figure 2 were carried out with a 30-mer dsDNA probe (i.e. longer than the 19-mer probe used in the subsequent EMSAs in Fig. 5) which may allow a Ku-independent interaction with the various peptides tested, especially as all of these peptides contain a cluster of basic amino acids. It then appears that this experiment should have been performed with a shorter DNA probe, or, more conveniently, would require additional controls, i.e. peptides and probe in the absence of Ku.
In this respect, it is also surprising that both XLF-pep1 and WRN-pep2 lack the "invariant" tryptophan residue (corresponding to W189 in APLF) from the putative Ku binding motif. Instead, both peptides bear a Glycine, whereas the W189G substitution in APLF completely ablated its interaction with Ku-DNA (Fig. 2B). The authors should clarify this point.

(3) Page 11 and Fig. 5: in connection with comment (5) relative to the role of APLF FHA domain, it would be useful to test the ability of APLF to recruit XRCC4-Lig4 to Ku-DNA complexes after dephosphorylation of XRCC4-Lig4.

(4) Page 12 and Fig. 6: To assess or confirm the contribution of PAR-dependent APLF mobilization on GFP-XRCC4 and GFP-XLF recruitment, it would be mostly interesting to repeat micro-irradiation experiments in the presence of PARP inhibitors. This would connect the present work to previous results from the same lab (Rulten et al, 2011), and would certainly strengthen the model presented in Fig. 8. Similarly, in connection with the model proposed and the comment (3) above, it would be useful to extend the study to the second APLF domain interacting with a NHEJ player and therefore to check the contribution of the FHA domain of APLF to GFP-XRCC4 and GFP-XLF

 

recruitment.

(5) Pages 13-14 and Fig. 7: In Fig. 7B, expression of an FHA-mutated variant of APLF in A549 cells delays gamma-H2AX foci reduction to the same extent as what is observed with PAR-binding or Ku-binding mutant expression. However, in Fig. 7D, while PBZ and Ku-binding mutant expression in DT40 cells do not complement survival to IR, it seems that expression of the FHA mutated APLF has only a modest effect, if any, compared to the complementation with wild-type APLF. These conflicting results apparently challenge the role of the FHA domain in APLF function in NHEJ. Also it brings into question the right way to accurately assess DSBR. Indeed, survival to IR might be sometimes too indirect and not sensitive enough, whereas gamma-H2AX foci formation/reduction is also indirect and has its own drawbacks. In this context, it seems useful to repeat the plasmid rejoining assay (as in Fig. 7C) by testing the different DT40 clones tested in Fig. 7D.
In complement to the previous comment, a more reliable interpretation of Fig. 7D would benefit from the incorporation of error bars in the figure.
Less importantly, to make reading easier, I suggest harmonizing color codes of the various APLF variants in Fig. 7B and 7D.

(6) Page 16 (Discussion section): "To our knowledge, APLF is the first identified partner of this domain, thereby revealing a molecular function for this domain".
At least in S. cerevisiae, the vWA domain of Ku80 ortholog proved to be important specifically for the telomeric function of Ku, while Ku70 vWA domain seemed to be devoted to NHEJ (Ribes-Zamora et al, 2007). In this regard, mutations of ScKu80 vWA domain have also been shown to impede Ku interaction with Tlc1, the RNA component of yeast telomerase (Stellwagen et al, 2003).

Minor typos to amend:
- Page 14, line 8: read "Fig.7B" instead of "Fig.7A".
- Page 14, line 20: read "Supplementary Fig.4" instead of " Supplementary Fig.5".
- Page 35, line 2: "(A)" is needless.
- Figure 4B, right panel: read "L169R" instead of "I169R".

1st Revision - authors' response 24 October 2012

**Referee 1**
This paper describes domains within APLF (also known as PALF) and Ku that interact with one another, and the authors propose an interesting model based on these refined observations. Though it was known that APLF and Ku interact, it was not known what portion of Ku is responsible for this, and the authors do an outstanding job of defining this using multiple methods. The authors also provide insightful data on Ku-APLF recruitment of XRCC4:ligase IV to DNA ends, and the primary data for this in Figure 5 is quite convincing. Previous data on this point did not use gel shifts, and this addition to the NHEJ field for this aspect is very important.

I have given this paper relatively high marks. The only thing that I would ask of the authors is to provide junctional sequences for example in Figure 7A, lane 3 (upper band). This would confirm that the authors are observing what they think.

*We have now PCR-amplified the joined region of head-to-tail ligation products. 9 out of 10 sequenced junctions were the predicted precise join of 2 substrates, with one containing a 1-bp deletion. This data is now added as Supp Fig.8A.*

Minor.

a. UVA in Figure 3A is used to make DSBs. Can the authors mention why UVA is used here rather

than IR, and can they remind readers of the ratio of SSB to DSB using this method? Also, is there a chance that some of the repair factor recruitment using UVA is to sites of repair by other repair pathways.

*Although IR induces foci that co-stain with gH2AX, many NHEJ factors do not detectably accumulate at gH2AX foci (reflecting single DSBs), most likely because the enzymatic steps of DSB repair are rapid (and thus their retention-time is short) and/or because the number of individual enzyme molecules required at each DSB is too low to detect. In contrast, UVA induces a high concentration of DSB damage that allows measurable accumulation of NHEJ proteins such as Ku and XRCC4-Lig4. Like IR, however, UVA introduces far more SSBs than DSBs (for IR this ratio is >2000:1), and since APLF is a component of both pathways it is likely that APLF recruitment at sites of UVA laser damage reflects both SSBs (which involves the FHA and PBZ domains) and DSBs (which involves the FHA, PBZ, and Ku binding domains). Using 20-fold lower levels of UVA damage, however, we have established conditions in which DSBs are not induced in sufficient numbers to induce recruitment of core NHEJ factors such as XRCC4 (or Ku). Under these conditions, APLF is still recruited (consistent with the presence of SSBs) but it is now independent of the Ku-binding domain. This, we believe, confirms that the W189G mutation impacts on APLF recruitment specifically at DSBs. This data is added as Fig.3B and is described on Pages 8 & 9.*

b. Parts of Figure 5 use Ku80deltaC. Can the authors assure in the text (or provide in the Supplement) that full-length Ku80 here would yield a similar result.

*We have now repeated the critical experiments in Fig.5A&B with full length Ku, with the same result, and included this data in Supp Fig.5A.*

c. The effects in Figure 5 are so strong and striking that I kept having to remind myself that the genetics for APLF KO are not as strong (not like the SCID phenotype of XLF KO). The authors may wish to remind readers in the Discussion of this.

*We agree, the impact of APLF on NHEJ is more pronounced at the biochemical level than in cells. We believe this is because we have employed short oligonucleotides (19-bp) that enable us to assess the impact of protein-protein interactions on complex stability, without a confounding impact of the DNA binding function of XRCC4-Lig4. We believe that the ability of XRCC4-Lig4 to bind directly to longer DNA substrates might account for why APLF is an accessory factor for DSB repair in vivo, rather than an essential factor. We have thus also now added EMSA data for longer oligonucleotides (60-bp), which additionally allow DNA binding by XRCC4-Lig4. Consistent with our model, whilst APLF still promotes the assembly of XRCC4-Lig4 into Ku complexes on this longer substrate, XRCC4-Lig4 can engage, to a lesser extent, in Ku-DNA complexes in the absence of APLF. We have added the new data in Fig.5C and clarified the issue in the discussion (Page 17).*

Overall, this is a very nice study, and I congratulate the authors.

*Thank you – this comment is refreshing and very much appreciated.*

**Referee #2 (Remarks to the Author):**

In this manuscript, Caldecott and colleagues provide a biochemical and functional characterization of the interaction between APLF and Ku80. They first refine a conserved Ku80-interaction motif in APLF that is both necessary and sufficient for the interaction with Ku80. The APLF-Ku80 interaction is important for APLF nuclear localization and its stable accumulation at laser micro-irradiation sites. The authors then identify the region in Ku80 involved in APLF binding, namely the vWA domain. They then build a model whereby APLF acts as a platform linking Ku binding to DNA ends to Ligase IV-XRCC4-XLF recruitment at DSBs. This activity of APLF is important for the speed of NHEJ.

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Specific points.

1-My main concerns are centered on the laser micro-irradiation experiments and the observation that APLF nuclear localization is in part dependent on its interaction with Ku.

a-The difference between the data in figure 3 and S2 is dramatic. Since S2 is the better-controlled experiment, I suggest that it is moved to the main manuscript. Furthermore, I am somewhat concerned that varying concentrations of APLF in the nucleus might alter the outcome of the laser-micro irradiation experiments. Indeed, one means to produce the curves shown in S2 would be if there were less APLF at the initial time point. The authors need to report the initial intensity of GFP-APLF as a safeguard against the above trivial explanation. Also, the data would be stronger if they were able to fit the curves to extract quantitative information about the accumulation kinetics.

*We have now repeated the experiments with NLS-tagged WT APLF and APLF$^{W189G}$ and included quantification of initial APLF fluorescence intensity from the raw data, which shows that the average APLF level is similar for WT and APLF$^{W189G}$ across the population. We included the new data in Supplementary Fig.3B, rather than the main text, because the other mutant APLF proteins presented in Fig.3 are not NLS-tagged. These data rule out that the impact of the W189G mutation is via loss of nuclear import/retention or differences in expression level.*

b-Since laser micro-irradiation generates lesions other than DSBs (and since APLF interacts with XRCC1), it would be important to validate the key conclusions of their cell biological experiments using an orthogonal approach such as ChIP at a defined DSB e.g..

*We agree ChIP assays at a site-specific DSB would be ideal, and we are currently attempting to set up such assays. However, these are beyond the time frame of the current manuscript. Nevertheless, we have added experiments in which we employ 20-fold lower levels of UVA damage. These are levels that we use routinely for measuring XRCC1-dependent SSB repair responses, but which induce too few DSBs to trigger measurable accumulation of NHEJ proteins such as XRCC4 or Ku. We show that whilst the PBZ domain is required for APLF accumulation under these conditions (as expected and as shown previously at SSBs), the Ku-binding motif is dispensable. This, we believe, confirms that the W189G mutation impacts on APLF recruitment specifically at DSBs. This data is added as Fig.3B and is described on Pages 8 & 9.*

c-Are the reconstitution experiments in Figure 4D made with APLF constructs containing an NLS? If not, these need to be done in order to exclude the possibility that the increase in IR sensitivity is due to decreased nuclear accumulation. Similarly, are the experiments shown in Figure 6 done with constitutively nuclear APLF proteins? If not, the experiments should be repeated with APLF constructs containing an NLS.

*We have repeated the experiments in Fig.6 using NLS-tagged WT APLF and APLF$^{W189G}$ and observe the same result i.e. APLF$^{W189G}$ is unable to support normal levels of GFP-XRCC4 recruitment at sites of UVA laser damage. These data are now added in Supplementary Fig.7 and described on Page 14. We have also repeated the complementation experiments in Fig.7B (gH2AX repair assay) and Fig.7D (the IR survival exp) using NLS-tagged WT APLF and APLF$^{W189G}$ and observe the same result. These data are now included in Supplementary Fig.8B and in Fig.7D respectively.*

2-Figure 1B: is the APLF W189G mutant expressed in yeast cells?

*Expression of the GAL-AD APLF W189G fusion protein was similar to that of WT APLF, as detected by immunoblotting, and is now included in Supplementary Fig.2A.*

3-On p14, I find the rationale for using DT40 cells a little contrived - what is the evidence that cells cycling rapidly have a heightened requirement for NHEJ? As far as I know, budding yeast cells divide every 90 min and rely primarily on HR for radiation resistance. That being said, I am not disputing the fact that the studies in the DT40 cells were informative.

*Whilst higher eukaryotes are known to be more reliant than yeast on NHEJ, for resistance to IR-induced DSBs, I agree our argument for using DT40 appears a little contrived. We have thus toned this down, accordingly.*

 

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However, I have a couple of reservations regarding some issues.
The main concern relates to the general tendency of the manuscript to overstate the importance of APLF in the NHEJ complex assembly at damage sites, and consequently in the NHEJ reaction as a whole. Indeed, although admittedly the authors ackowledge APLF as "an accessory factor" for NHEJ "rather than an essential component", and point out that they "have failed to detect hypersensitivity to DNA damage in APLF-depleted human cells or APLF-/- MEFs", they repeatedly make use of misleading forms and turns of phrase that tend to overemphasize the role of APLF in the NHEJ reaction.

Some examples:
- In the penultimate sentence of the abstract the authors mention an "increased cellular sensitivity to DSBs" (omitting to specify that this result refers to the very peculiar DT40 chicken cell line); then the last sentence extends their observations to "NHEJ in mammalian cells" (omitting to specify that no sensitivity to DNA damage was detected in mammalian cells).

*We have now clarified the abstract to state which result relates to which organism*

- APLF is repeatedly described as a "core" component of NHEJ which is also confusing. In my opinion, if the term "core" defines the central or most important part of something, it should be devoted to really essential NHEJ factors. Even the specified expression "core structural component" is still misleading since as APLF is not essential for NHEJ to take place, one have to admit that, although less stable, NHEJ complexes assemble at damage sites even in the absence of APLF.

*The referee is correct in that APLF accelerates the reaction, rather than being indispensible for the reaction. We have removed the term ‘core’, and retained the use ‘accessory’, throughout.*

- A similar comment would apply to the term "key": "... APLF is a novel "key" component ..." (page 19).

*Ditto, “key” removed*

- In the final model (Fig. 8), the picture suggest that the XRCC4-Lig4 complex does not contact Ku and that it is fully dependent on XLF and APLF to be recruited at damage sites. Instead, it is well established that Ku interacts with both XRCC4 (Mari et al, 2006) and Lig4 (Hsu et al, 2002; Costantini et al, 2007). The model should then be edited to more closely reflect experimental evidence and to illustrate the fact that APLF has no essential role in NHEJ but is part of an intricate

 

network of protein-protein interactions which reinforces NHEJ complex cohesion.

*Indeed, this is a good point and we have modified the diagram and legend to Fig.8, accordingly.*

Consequently while APLF is clearly an accessory NHEJ factor which facilitates and stabilizes NHEJ complex assembly, it hardly proves to be a key or a core (even structural) component of NHEJ. The manuscript would then be more in accordance to the experimental reality should the authors edit the text and attenuate some expressions.

*Done, as indicated above.*

Related to the previous comment it is striking that in EMSA-based experiments (Fig. 5) no interaction between Ku and XRCC4-Lig4 is observed in the absence of APLF, in contrast to the well established direct interaction between both core NHEJ factors, even from experiments using similar approach (Nick McElhinny et al, 2000). Can the authors provide some explanation concerning this discrepancy?

*We believe this discrepancy is because the DNA duplexes in our experiments are 19-bp (approximately the footprint of a single Ku heterodimer) whereas those employed by McElhinny et al were longer (60-bp). We chose a 19-bp duplex specifically to allow us to assess the impact of protein-protein interactions on the assembly/stability of Ku complexes without the confounding impact of DNA binding by XRCC4-Lig4. However, we have now added data for 60-bp duplexes, which are consistent with the results of McElhinny et al and show that XRCC4-Lig4 can super-shift Ku-DNA complexes in the absence of APLF. However, APLF clearly promotes the assembly of the Ku complexes, even on this longer substrate. These data are now described in the text (Pages 11 & 12), added as Fig.5C, and are discussed on Page 17.*

Other comments:
(1) Page 3 (Introduction section): "DNA-PKcs promotes ... possibly also the dissociation of Ku from DNA at the end of the repair reaction".
Which experimental evidence supporting such a role for DNA-PKcs do the authors refer to ? Recent papers rather suggest a polyubiquitin-dependent removal of Ku (Postow et al, 2008; Feng and Chen, 2012).

I thank the reviewer for picking this up (it was supposed to indicate DNA-PKcs autophosphorylation and dissociation). I have removed this inaccuracy.

(2) Page 7 and Fig. 2C: EMSAs presented in Figure 2 were carried out with a 30-mer dsDNA probe (i.e. longer than the 19-mer probe used in the subsequent EMSAs in Fig. 5) which may allow a Ku-independent interaction with the various peptides tested, especially as all of these peptides contain a cluster of basic amino acids. It then appears that this experiment should have been performed with a shorter DNA probe, or, more conveniently, would require additional controls, i.e. peptides and probe in the absence of Ku.

*The GST-peptide fusion proteins also bind the shorter 19-bp substrate in the presence of Ku, but not in its absence. This data is shown for the APLF peptides in Supp Fig.2B and is described in the text (Page 7).*

In this respect, it is also surprising that both XLF-pep1 and WRN-pep2 lack the "invariant" tryptophan residue (corresponding to W189 in APLF) from the putative Ku binding motif. Instead, both peptides bear a Glycine, whereas the W189G substitution in APLF completely ablated its interaction with Ku-DNA (Fig. 2B). The authors should clarify this point.

*XLF-pep1 and WRN-pep2 have similar properties to the Ku-binding motifs in APLF and WRN-pep1, in that a basic region is followed by non-polar residues including an aromatic residue. We thus initially thought that the W (in APLF/WRN-pep1) and F (in XLF-pep1 & WRN-pep2) may be equivalent, even though the alignment and spacing between the basic and aromatic residues was not an exact match. Supporting this notion, we have now found that a F298A mutation in XLF ablates Ku binding (data not shown). However, since APLF and XLF can bind the Ku heterodimer at the*

*same time it is more likely that XLF and APLF bind at independent sites on the Ku heterodimer, despite their shared chemistry. We have clarified this in the discussion (Page 18).*

(3) Page 11 and Fig. 5: in connection with comment (5) relative to the role of APLF FHA domain, it would be useful to test the ability of APLF to recruit XRCC4-Lig4 to Ku-DNA complexes after dephosphorylation of XRCC4-Lig4.

*Indeed, we have now added this experiment. As predicted, treatment of XRCC4-Lig4 with lambda phosphatase prevents assembly of the ligase complex into Ku-APLF complexes. This data is added in Supp Fig.6C and is discussed in the text (Page 13).*

(4) Page 12 and Fig. 6: To assess or confirm the contribution of PAR-dependent APLF mobilization on GFP-XRCC4 and GFP-XLF recruitment, it would be mostly interesting to repeat micro-irradiation experiments in the presence of PARP inhibitors. This would connect the present work to previous results from the same lab (Rulten et al, 2011), and would certainly strengthen the model presented in Fig. 8. Similarly, in connection with the model proposed and the comment (3) above, it would be useful to extend the study to the second APLF domain interacting with a NHEJ player and therefore to check the contribution of the FHA domain of APLF to GFP-XRCC4 and GFP-XLF recruitment.

*We have added data demonstrating that APLF R27A (harbouring a mutated FHA domain) is unable to support normal recruitment/accumulation of either XRCC4 or XLF at laser tracks (new Fig.6). In terms of the role of PARP activity in XRCC4/XLF recruitment, we have shown previously that mutation of the APLF ZnF (PBZ) domain that binds PAR has only a mild impact on XRCC4 recruitment in laser-tracking experiments (Rulten et al Mol Cell 2011). This is most likely because high levels of over-expression of APLF can circumvent the dependency on PARP3 activity/PAR binding for normal DSB repair rates, and thus XRCC4-Lig 4 recruitment (Rulten et al Mol Cell 2011). This is not the case for the XRCC4-binding (FHA domain) and Ku-binding (W189) functions of APLF, however, which cannot be bypassed by high levels of over-expression of the mutant protein (as indicated in the new Fig.6). This makes sense in a model in which PAR binding increases the localised concentration of APLF at the DSB (which can be circumvented by global over-expression of APLF), with Ku and XRCC4 binding then 'locking' APLF into the complex (which cannot be circumvented by simply elevating APLF levels).*

(5) Pages 13-14 and Fig. 7: In Fig. 7B, expression of an FHA-mutated variant of APLF in A549 cells delays gamma-H2AX foci reduction to the same extent as what is observed with PAR-binding or Ku-binding mutant expression. However, in Fig. 7D, while PBZ and Ku-binding mutant expression in DT40 cells do not complement survival to IR, it seems that expression of the FHA mutated APLF has only a modest effect, if any, compared to the complementation with wild-type APLF. These conflicting results apparently challenge the role of the FHA domain in APLF function in NHEJ. Also it brings into question the right way to accurately assess DSBR. Indeed, survival to IR might be sometimes too indirect and not sensitive enough, whereas gamma-H2AX foci formation/reduction is also indirect and has its own drawbacks. In this context, it seems useful to repeat the plasmid rejoining assay (as in Fig. 7C) by testing the different DT40 clones tested in Fig. 7D.
In complement to the previous comment, a more reliable interpretation of Fig. 7D would benefit from the incorporation of error bars in the figure.
Less importantly, to make reading easier, I suggest harmonizing color codes of the various APLF variants in Fig. 7B and 7D.

*We agree with the referee, that the lack of impact of R27A mutation on IR survival is surprising (Fig.7D now contains a new data set, using NLS-tagged APLF derivatives to rule out any impact of W189G on nuclear localization). We think the most likely explanation for this is not that XRCC4-Lig4 interaction is not important for NHEJ rates (our biochemistry experiments and both current and previous gH2AX assays indicate it is), but rather that that the hypersensitivity observed in DT40 reflects an additional role that is independent of interaction with XRCC4-Lig4. We have discussed this in the text (Page 17). What this role is, given that to date it only manifests in reduced survival in DT40, is unclear. One possibility is the reported histone chaperone function of APLF. We also tried to employ plasmid ligation assays as another measure of DSB repair, but encountered too much*

 

*inter-experimental variation in GFP signal for this assay to be useful, in the complemented clones. We are currently trying to set up a site-specific DSB system in human cells (e.g. I-SCE I), but unfortunately this is beyond the time frame of this revision. We have now harmonized the colour codes in Fig.7.*

(6) Page 16 (Discussion section): "To our knowledge, APLF is the first identified partner of this domain, thereby revealing a molecular function for this domain". At least in S. cerevisiae, the vWA domain of Ku80 ortholog proved to be important specifically for the telomeric function of Ku, while Ku70 vWA domain seemed to be devoted to NHEJ (Ribes-Zamora et al, 2007). In this regard, mutations of ScKu80 vWA domain have also been shown to impede Ku interaction with Tlc1, the RNA component of yeast telomerase (Stellwagen et al, 2003).

*We thank the referee for pointing out these references. We are aware of the yeast work, but since APLF is missing from yeast (as far as we can tell) we didn't think the yeast functions were likely to be relevant to one described in our manuscript. However, we have now specified in the discussion that our data refer to human Ku80 (Page 18).*

Minor typos to amend:
- Page 14, line 8: read "Fig.7B" instead of "Fig.7A".
- Page 14, line 20: read "Supplementary Fig.4" instead of " Supplementary Fig.5".
- Page 35, line 2: "(A)" is needless.
- Figure 4B, right panel: read "L169R" instead of "I169R".

*I thank the referee for his/her care and attention. All of the above have been corrected.*

Acceptance letter 29 October 2012

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.

Thank you again for your contribution to The EMBO Journal and congratulations on a successful publication. Please consider us again in the future for submission of your most exciting work!

Yours sincerely,
Editor
The EMBO Journal

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Referee #3

(Remarks to the Author)
The authors have satisfactorily answered our concerns and from our opininon have sufficiently addressed the concerns of the other two reviewers. We think that the paper is now suitable for publication in The EMBO Journal

 