Polynucleotide kinase-phosphatase enables neurogenesis via multiple DNA repair pathways to maintain genome stability

Mikio Shimada, Lavinia C. Dumitrache, Helen R. Russell and Peter J. McKinnon

Corresponding author: Peter J. McKinnon, St. Jude Children's Research Hospital

Review timeline:

Submission date: 23 February 2015
Editorial Decision: 26 March 2015
Revision received: 19 June 2015
Editorial Decision: 09 July 2015
Accepted: 09 July 2015

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.)

Editor: Hartmut Vodermaier

1st Editorial Decision 26 March 2015

Thank you for submitting your manuscript on PNKP mutant mouse models for consideration by The EMBO Journal. Three expert referees have now reviewed it and I am pleased to inform you that all of them consider this study in principle interesting and important. Following satisfactory revision of a number of specific issues and comments raised in the reports, we should therefore be happy to consider this study further for eventual publication in our journal.

I would therefore like to invite you to prepare a revised manuscripts along the lines suggested by the referees. Please keep in mind that we allow only a single round of major revision, making it important to carefully respond to all points raised at this stage. With regard to the main concern of referee 3, I feel that some additional experimental data should be provided, maybe by demonstrating epistasis through the use of a DNA-PK inhibitor.

We generally grant 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; should you foresee a problem in meeting this three-month deadline, please let me know in advance and we could discuss the possibility of an extension. Finally, I should point out that we now require a completed 'author checklist' to be submitted with all revised manuscripts - see below for more detail. Please do not hesitate to contact me should you have any further questions regarding the reports or your revision.
Thank you again for the opportunity to consider this work for The EMBO Journal. I look forward to your revision.

REFEREE COMMENTS

Referee #1:

The study here has engineered novel mouse models to investigate the consequence of a genetic defect in the BER/SSBR enzyme, PNKP, both during and after neurogenesis. The models include conditional deletion (whole embryo and tissue-specific) of exons 4-7 early in development; a frameshift mutant found in a MCSZ patient that results in expression of a truncated protein; a Neo cassette insertion that leads to attenuated protein production (which best recapitulates the human MCSZ disorder); and tamoxifen-inducible deletion after neurogenesis. The models collectively provide important insights into the biology of PNKP, indicating that the protein is essential for neural development and contributes to the preservation of postnatal brain integrity, including of the glial cell population. The different neuropathologies are shown to result from a defect in DNA repair, DNA damage accumulation and p53-driven apoptosis. Moreover, since PNKP deletion had a more profound effect than deletion of XRCC1 or LIG4 alone, the results indicate a function of PNKP in both BER/SSBR and DSBR; additional analysis revealed a specific role of PNKP in NHEJ, and not HR. Overall, the work is extensive, and the results important, yet there are several points that need to be addressed:

1. It would be useful to briefly mention the rationale for deletion of exons 4 thru 7. What are the functional consequences with regards to the repair activities/domains of PNKP?
2. I agree that the rapid death observed using the PNKP mouse (5 days) is more dramatic than seen for other BER/SSBR knockouts, but it's not totally accurate to imply that conditional neural deletion of XRCC1 does not have a pronounced effect (as the senior author has reported). Please consider modifying the sentence in the Results on p. 5-6 to better represent these data.
3. It's not necessarily a problem, but it's odd that the H2AX staining images are not shown for the LIG4 mutant mouse (Fig. 1B).
4. The authors are encouraged to show the "more developed cortex" of the PNKP/P53 mouse (p. 7), at least as Supplementary Data.
5. If PNKP functions in both BER/SSBR and DSBR, shouldn't deletion of the gene result in a more dramatic defect in the repair of IR-induced DNA damage than the single mutants of XRCC1 (SSBR/BER) or LIG4 (DSBR)? This does not appear to be the case, based on the results of Fig. 4A. The authors should address.
6. While I agree that the results of Fig. 4B indicate a minor role, at best, of XRCC1 in DSBR, is there not a defect at "Bleo R30"? Statistical analysis of the bar graphs should be included. More extensive statistical analysis could be considered throughout.
7. Regarding the T424GfsX48 model, is there sufficient material to determine whether a truncated protein is being produced? One could presume based on the more dramatic phenotype than the knockout mice, but confirmation (if possible) would be ideal.
8. Regarding the PNKP-Neo mice, the authors should quantify the relative percentage of remaining protein in comparison to wild-type mice (Fig. 6B western). Related to the results of Fig. 6B, the brain images that are presented are not really mentioned in the text.
9. In the repair assays presented in Figure 6D, the initial level of damage appears to be higher in the PNKP mutant cells relative to wild-type, for both IR and MMS. Do the authors have an explanation? And how does that affect the comparison of the repair rates (from time 0 to 30 min) for PNKP and wild-type cells? Related to the MMS experiments, do the authors have an idea of what substrate PNKP might be responsible for processing in this scenario?
10. In the Discussion (p. 19), I'm not sure that I would argue that the neurodegenerative phenotypes of AOA2 and SCAN1, for example, arise from "genome instability". Genome (or genomic) instability often has a very specific meaning and implies chromosome integrity defects, and I'm not sure chromosome rearrangements or other abnormalities have been seen in these neurological disorders. Since these inherited diseases do not develop cancer, which often has a hallmark of genome instability, perhaps another terminology can be used, such as a more general phrase like "genomic damage".

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Minor
1. Abstract. Remove the comma after "Directed postnatal neural inactivation of PNKP".
2. Introduction, p. 3. Sentence beginning "Amongst these" is awkward. Perhaps change to "Amongst these, PARP is a signaling protein that activates..." In the following sentence, change to "Other central components of BER/SSBR include..."
3. Introduction, p. 4. "clearly a differential in" should perhaps read "clearly a difference in".
4. Results, p. 5. The statement referencing germline deletion of XRCC1 should include a reference.
5. Results, p. 7. "required" is repeated twice.
6. Results, p. 7. Figure 4C and 4D are not correctly cited in the text.
7. Results, p. 15. "that, that" is repeated.
8. Discussion, p. 19. "links...warrant", not "warrants".
9. Discussion, p. 19. "disease progression may also involve", not "involves".
10. Discussion, p. 19. "compromise genome stability", not "instability".
11. Watch the use of the SSB, DSB, SSBR and DSBR abbreviations. Identify when first mentioned, and then use throughout. Please check.

Referee #2:

Mutations in polynucleotide kinase-phosphatase (PNKP) give rise to microcephaly with early-onset with seizures syndrome (MCSZ). The mutations involved reduce the stability of the protein and reduce its phosphatase activity. PNKP interacts with XRCC1 to play a role in BER of oxidative and alkylation lesions on DNA and crystal structure data suggest that this enzyme also plays a role in repair of DNA double strand breaks (DSB). The present submission employs a series of mouse models with different Pnkp mutants to investigate the role of this enzyme in neural development. It is a well designed study that moves through a series of Pnkp mutants disrupted at various stages of neural development, restriction to the nervous system resulted in lethality. Marked effects on corticogenesis were observed depending on timing of disruption of the gene. Cell death observed in the cortex was due to apoptosis and p53-dependent. Interneuron loss in the cerebellum could be recovered by disruption of p53. A more severe neurogenesis phenotype compared to e.g XRCC1 mutants suggested that a repair defect in addition to BER was occurring in these mutants. In support of this they showed that primary cortical astrocytes were defective in DSB repair, comet assay, γH2AX and chromosomal lesions. They go further in showing that this is due to a defect in NHEJ rather than in HR. This confirms what was predicted from earlier structural studies. Furthermore they were able to attenuate expression of PNPK in a / Pnpk Neo/ Neo mutant similar to what is seen in MCSZ in humans exhibiting neurodevelopmental defects. The similarity in aspects of the phenotype of these animals to the earlier described mutants argues in favour of the relevance of this model. Finally they go on to show that PNKP plays a role not only in neurogenesis but also in preserving neuronal homeostasis, especially that of oligodendrocytes.

So in summary this study is significant in that it provides evidence for the importance of PNKP activity in BER in the nervous system but also for a role in repair of DNA DSB as was predicted previously from structural analysis. It employs a series of PNKP mutants to support this and provides greater insight into the role of a repair protein in preserving genome integrity in the nervous system to avoid neuropathology.

Specific comments

1. In addition to demonstrating that DNA DSB was defective in primary cortical astrocytes it should be shown that this is also the case in interneurons.
2. Evidence is provided in Fig4A for defective repair of IR damage in PNKP neo/neo astrocytes. Data should also be shown for h2O2 or camptothecin. Did these agents cause DNA DSB under these conditions?
3. The levels of PNKP are reduced in the Pnkpneo/neo mutant. Does this protein have kinase and phosphatase activities?
4. Why are reduced levels of PNKP more critical in the mouse?
5. Do they have any evidence that transcription is disrupted in their mutants?
Referee #3:

Shimada et al use mouse knockout and knockin models to determine the consequences of loss/mutation of PNKP on nervous system development. Overall the paper is well written and the data presented is excellent and of a very high standard. To be honest it is a breath of fresh air to review a manuscript of such quality.

My one minor criticism is that the authors suggest that PNKP is critical for NHEJ-dependent repair of DSBs. Whilst the defect in the resolution of DSBs induced by IR in PNKP null cells is comparable to a cell lacking LigIV, the authors have not definitively demonstrated that there is a defect in NHEJ. To demonstrate this the authors would have to either make a double LigIV/PNKP null cell line or treat the PNKP cells with a DNA-PK inhibitor and show that the DSB repair defect is epistatic. Lastly, the authors would also have to carry out a modified plasmid-based NHEJ assay in the PNKP null cells (since the standard I-SceI/GFP-based NHEJ assays will not create a DSB with the relevant termini that requires processing by PNKP). However, since this is a relative minor criticism, my suggestion is for the authors just to tone down references in the text to the direct involvement of PNKP in NHEJ.

This paper provides some well needed insight into the role of PNKP in development and maintenance of the nervous system and also the impact that patient-derived mutations have on this. I would recommend this paper for publication.

1st Revision - authors' response 19 June 2015

We thank the reviewers for their thorough evaluation of our manuscript. We have now generated a revised version that incorporates all suggestion of the reviewers. Below we provide a point-by-point discussion of all concerns raised.

Referee #1:

Overall, the work is extensive, and the results important, yet there are several points that need to be addressed:

1. It would be useful to briefly mention the rationale for deletion of exons 4 thru 7. What are the functional consequences with regards to the repair activities/domains of PNKP?

Our rationale for deletion of exons 4 through 7 is based both on the suitability of this region for manipulation to generate a targeting construct and the intention to generate an allele that is non-functional. By deleting these exons, we generate an out-of-frame message producing a truncation/termination potentially generating only a small amino-terminal PNKP peptide lacking the kinase and phosphatase domains. We wanted to generate a null-allele as were also simultaneously creating the specific frame-shift allele (the T424GfsX48 mutation). The idea behind a complete null was to determine what happens in the absence of PNKP as a comparison to the milder phenotype we were initially anticipating with the hypomorphic frame-shift.

We have now indicated more details regarding the rationale of our cKO design: Results section (page 5), lines 5-7.

2. I agree that the rapid death observed using the PNKP mouse (5 days) is more dramatic than seen for other BER/SSBR knockouts, but it's not totally accurate to imply that conditional neural deletion of XRCC1 does not have a pronounced effect (as the senior author has reported). Please consider modifying the sentence in the Results on p. 5-6 to better represent these data.

We have now modified our text to read (Page 6, lines 5-7): “These data highlight the essential role for PNKP during neural development and contrast the viability of mice after germ-line inactivation of other DNA end-processing factors such as APTX or TDP1 (Katyal, 2007). Even conditional
neural deletion of XRCC1, while having a substantial impact on the brain, is viable for many months (Lee et al, 2009).”

3. It's not necessarily a problem, but it's odd that the H2AX staining images are not shown for the LIG4 mutant mouse (Fig. 1B). Consider including.

We have now included γH2AX immunostaining for Lig4 in Figure 2B. We omitted the PCNA staining and just included the γH2AX and a merged field for uniformity and space reasons. We initially didn’t include the γH2AX panels in lieu of illustrating the different types of immunostaining for the Pnk^{Neo-cve} cells (DNA damage vs. apoptotic γH2AX foci).

4. The authors are encouraged to show the "more developed cortex" of the PNKP/P53 mouse (p. 7), at least as Supplementary Data.

We have used comparative immunostaining for the cortical marker Satb2 to indicate that loss of p53 facilitates much enhanced development of the cortex (see Fig. 3B; red immunostain). We have now also added a white asterisk to the figure to highlight the rescue after p53 deletion.

In Suppl. Fig. 2 we have shown the (Pnkp;p53)^{Neo-cve} cortex, and include dashed lines on this figure indicate the relative degree of cortical rescue. This now referred to in the text (Page 7, line 14).

5. If PNKP functions in both BER/SSBR and DSBR, shouldn't deletion of the gene result in a more dramatic defect in the repair of IR-induced DNA damage than the single mutants of XRCC1 (SSBR/BER) or LIG4 (DSBR)? This does not appear to be the case, based on the results of Fig. 4A. The authors should address.

This is a good point. As these results are very reproducible, I would interpret this to indicate that, at least as far as IR-induced damage is concerned, the core NHEJ factors facilitate repair (although less efficiently than when PNKP is functional). PNKP is likely required for processing a sub-fraction of DNA produced after IR as in addition to 3'phosphoglycolate ends, other types of complex DNA lesions are also produced that may utilize other processing enzymes for generating compatible end for ligation. We have added text to the discussion on Page 17, last sentence and page 18 lines 1-3.

Additionally, to address this more directly, we are also planning to further understand the relative contribution of PNKP to DSB repair by generating (Xrcc1:Lig4)^{Neo-cve} double-mutant mice (in which we directly reduce SSBR and disable NHEJ). We think that this will be a more direct way to assess the real biologic consequence of dual pathway disruption and thereby allow us to understand more about relative contribution of PNKP to NHEJ. However, these mice are not yet available (we are only at the first round of breeding).

6. While I agree that the results of Fig. 4B indicate a minor role, at best, of XRCC1 in DSBR, is there not a defect at "Bleo R30"? Statistical analysis of the bar graphs should be included. More extensive statistical analysis could be considered throughout.

We agree that the Bleo R30 point does seem to show a defect in repair after XRCC1 loss at 30 mins (although not at 60mins). Part of the issue is the y-axis scale is smaller than those of the alkaline comets to the left of this panel. Neutral comets are less sensitive and so don't show the larger differences seen in alkaline conditions. Statistical analysis of the data indicates non-significance in the data between control and Xrcc1 (and a significant different between either PNKP or Lig4 and controls). We have modified the graphs in Figure 4B to indicate significance of the data.

7. Regarding the T424GfsX48 model, is there sufficient material to determine whether a truncated protein is being produced? One could presume based on the more dramatic phenotype than the knockout mouse, but confirmation (if possible) would be ideal.

There is little material to isolate, and what we have salvaged so far are malformed embryos. Because the yield of useful tissue for protein extraction was so minimal, we used the material for generating cDNA and establishing the relative expression of the T424GfsX48 allele. So, unfortunately we did not secure suitable amounts of tissue for Western blot studies. Frankly, given the lethality of this mouse, together with the marked phenotype of the Pnkp-Neo mouse (in which
PNKP is already quite reduced) we expect very little stable mutant PNKP is generated from the T424GfsX48 allele.

8. Regarding the PNKP-Neo mice, the authors should quantify the relative percentage of remaining protein in comparison to wild-type mice (Fig. 6B western). Related to the results of Fig. 6B, the brain images that are presented are not really mentioned in the text.

We have now quantified the relative PNKP levels in the Pnkp<sup>Neo/Neo</sup> mice, and the ratio of PNKP compared to control are indicated in Fig. 6B. We have also now included text referring to Figure 6B (see page 12, line 9).

9. In the repair assays presented in Figure 6D, the initial level of damage appears to be higher in the PNKP mutant cells relative to wild-type, for both IR and MMS. Do the authors have an explanation? And how does that affect the comparison of the repair rates (from time 0 to 30 min) for PNKP and wild-type cells? Related to the MMS experiments, do the authors have an idea of what substrate PNKP might be responsible for processing in this scenario?

We agree that there is increased damage in the Pnkp<sup>Neo/Neo</sup> samples (this is also the case for the Lig4<sup>−/−</sup> after IR and the Tdp1<sup>−/−</sup> after MMS). We think that this may be a reflection of relative repair deficiencies and possibly variations in the handling/processing time of samples. Regarding the question about the type of lesion generated by MMS treatment that may be the substrate for PNKP; given that alkylation damage requires BER (Xrcc1) for efficient repair, we would speculate that PNKP participates to modify DNA end intermediates after alkyltransferase removal of the alkylated residue and Xrcc1-mediated processes (BER) complete repair.

10. In the Discussion (p. 19), I'm not sure that I would argue that the neurodegenerative phenotypes of AOA2 and SCAN1, for example, arise from "genome instability". Genome (or genomic) instability often has a very specific meaning and implies chromosome integrity defects, and I'm not sure chromosome rearrangements or other abnormalities have been seen in these neurological disorders. Since these inherited diseases do not develop cancer, which often has a hallmark of genome instability, perhaps another terminology can be used, such as a more general phrase like "genomic damage".

We take the term genome instability to mean changes in the genome in addition to gross structural changes i.e. the inability to maintain DNA strand integrity would be considered unstable in this regard. However we appreciate the reviewers concern, and have modified our statement to incorporate the suggested phrase “genomic damage” (this is now page 20 of the revised manuscript).

Minor
1. Abstract. Remove the comma after "Directed postnatal neural inactivation of PNKP”.
2. Introduction, p. 3. Sentence beginning "Amongst these" is awkward. Perhaps change to "Amongst these, PARP is a signaling protein that activates..." In the following sentence, change to "Other central components of BER/SSBR include...
3. Introduction, p. 4. "clearly a differential in" should perhaps read "clearly a difference in".
4. Results, p. 5. The statement referencing germline deletion of XRCC1 should include a reference.
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10. Discussion, p. 19, "compromise genome stability", not "instability".
11. Watch the use of the SSB, DSB, SSBR and DSBR abbreviations. Identify when first mentioned, and then use throughout. Please check.

All changes listed above have been made to the text (thanks for alerting us to these errors).
Referee #2:

... this study is significant in that it provides evidence for the importance of PNKP activity in BER in the nervous system but also for a role in repair of DNA DSB as was predicted previously from structural analysis. It employs a series of PNKP mutants to support this and provides greater insight into the role of a repair protein in preserving genome integrity in the nervous system to avoid neuropathology.

Specific comments

1. In addition to demonstrating that DNA DSB was defective in primary cortical astrocytes it should be shown that this is also the case in interneurons.

We find that γH2AX is increased in the interneurons of the cerebellum, however we didn't include because of limited space (and it would potentially reduce readability) in the figure. However, we have now added text to the manuscript (page 8, Line 4) indicating that γH2AX immunoreactivity occurs in this neuronal population prior to apoptosis.

2. Evidence is provided in Fig4A for defective repair of IR damage in PNKP neo/neo astrocytes. Data should also be shown for h2O2 or camptothecin. Did these agents cause DNA DSB under these conditions?

Figure 4A shows DNA repair in astrocytes derived from Pnkp\textsuperscript{Neo-cre} (not the Neo/neo mouse). We have indicated repair studies using H\textsubscript{2}O\textsubscript{2} and CPT with cells from the neo/neo mouse in Figure 6D. Thus, collectively, we have presented DNA repair studies involving a collection of different DNA damaging agents, but spread over two figures. Space limitation in each figure precluded using all genotoxins. However, to address the concern about not showing repair activity of Pnkp\textsuperscript{Neo/Neo} and H2O2, we have now included this data in Suppl. Fig. 4. The conditions we use for H2O2 is treatment on ice to reduce DSBs and for CPT, we use quiescent cells to avoid generating replication associated DSBs.

3. The levels of PNKP are reduced in the Pnkp\textsuperscript{neo/neo} mutant. Does this protein have kinase and phosphatase activities?

We don't formally know if the protein contains these enzymatic activities, but given the effect of the neo cassette is to dampen expression and there is full-length protein present in Pnkp\textsuperscript{Neo/Neo} tissue as determined by Western blot analysis, we would expect that both activities are intact (but as there is so little protein, normal PNKP function is compromised).

4. Why are reduced levels of PNKP more critical in the mouse?

This is an interesting point and based on our study there are clear differences between the consequences of PNKP disruption in the mouse vs. Human. Although we don't know why there is a greater dependency of the mouse for PNKP, we have presented speculation on possible scenarios that might be relevant (see Discussion, top of page 18).

5. Do they have any evidence that transcription is disrupted in their mutants?

We don't have any data addressing this question. We decided not to do gene expression analysis using the Pnkp\textsuperscript{Neo-cre} model because of the pronounced developmental effects. However, work is in progress to undertake gene expression analysis of some recently developed newer Pnkp models we have established in which PNKP deletion is driven by cre expression in the mature brain. These mice develop a neurologic impairment without the associated cell loss seen when PNKP is deleted during development. We hope that these mutants will be a suitable source for a careful analysis of the effects of PNKP disruption (i.e. BER defects and DNA damage induction) towards transcription.

Referee #3:

Shimada et al use mouse knockout and knockin models to determine the consequences of
loss/mutation of PNKP on nervous system development. Overall the paper is well written and the data presented is excellent and of a very high standard. To be honest it is a breath of fresh air to review a manuscript of such quality.

My one minor criticism is that the authors suggest that PNKP is critical for NHEJ-dependent repair of DSBs. Whilst the defect in the resolution of DSBs induced by IR in PNKP null cells is comparable to a cell lacking LigIV, the authors have not definitely demonstrated that there is a defect in NHEJ. To demonstrate this the authors would have to either make a double LigIV/PNKP null cell line or treat the PNKP cells with a DNA-PK inhibitor and show that the DSB repair defect is epistatic. Lastly, the authors would also have to carry out a modified plasmid-based NHEJ assay in the PNKP null cells (since the standard I-SceI/GFP-based NHEJ assays will not create a DSB with the relevant termini that requires processing by PNKP). However, since this is a relative minor criticism, my suggestion is for the authors just to tone down references in the text to the direct involvement of PNKP in NHEJ.

Thanks for this suggestion. We have now undertaken these experiments by using the DNA-PKcs inhibitor, NU7026. As shown in new Suppl. Figure 3, DNA-PKcs inhibition is epistatic to PNKP loss suggesting that PNKP functions in NHEJ. To preserve the flow of the original figures we have included this data in new Suppl. Figure 3, and text on page 9 (Lines 11-16) reports this new data.

This paper provides some well needed insight into the role of PNKP in development and maintenance of the nervous system and also the impact that patient-derived mutations have on this. I would recommend this paper for publication.

Thanks for the positive comments; they’re always appreciated!

2nd Editorial Decision 09 July 2015

Thank you for submitting your final revised manuscript for our consideration. I am pleased to inform you that we have now accepted it for publication in The EMBO Journal.

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

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REFEREE COMMENTS

Referee #1:
The authors have addressed my concerns adequately, and the study represents an important step in understanding the biological contributions of PNKP during and after the process of neurogenesis.

Referee #2:
The authors have adequately addressed all the issues that I raised in their re-submission. It is also evident that they have carried out the additional experiments requested in the extensive comments from reviewer 1. I am pleased to recommend acceptance of this latest version of the manuscript.