# THE
# EMBO
## JOURNAL

Manuscript EMBOJ-2014-89865

# Okazaki fragment maturation involves α-segment error editing by the mammalian FEN1/MutSα functional complex

Songbai Liu, Guojun Lu, Shafat Ali, Wenpeng Liu, Li Zheng, Huifang Dai, Hongzhi Li, Hong Xu, Yuejing Hua, Yajing Zhou, Janice Ortega, Guo-Min Li, Thomas A. Kunkel and Binghui Shen

*Corresponding author: Binghui Shen, City of Hope National Medical Center and Beckman Research Institute*

---

**Review timeline:**

| | |
|---|---|
| Submission date: | 20 August 2014 |
| Editorial Decision: | 10 October 2014 |
| Revision received: | 08 January 2015 |
| Editorial Decision: | 04 February 2015 |
| Revision received: | 02 April 2015 |
| Accepted: | 14 April 2015 |

---

*Editor: Alexander Kohlmaier / Hartmut Vodermaier*

**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                                                          10 October 2014

We have now received reviews from two experts who agreed to evaluate your manuscript "Alpha-segment error editing by the mammalian FEN1/MutS functional complex". Despite repeated inquiries a third referee (#1) has so far not submitted the review of your paper. We have to apologize for the delay caused by this, but in the interest of your time, the editorial team has now decided to come to a decision that is based on two completed reviews (please find them copied below).

The two referees concur that your work is of general interest in principle, but that some major conceptual and technical issues remain to be decisively addressed by experimentation before publication can be offered. Therefore, we invite you to revise the manuscript according to the referees' suggestions, which we consider very constructive.

Please keep in mind that it is our policy to allow only a single round of major revision and that it is therefore important to carefully respond to all points at this stage.

I will not repeat here referee #2's or #3's specific requests concerning mechanistic and conceptual points and technical clarifications (please see the reports). I would just additionally like to communicate to you two additional points that were raised by referee #2 during further consultation, and that should additionally be addressed in detail within your revision: Referee #2 additionally pointed out that the in vitro effect of stimulation of FEN1 activity by MutS, when a mismatch was present, should be re-addressed by quantitation in triplicate with error bars provided to substantiate the magnitude of the effect. We think that this additional point is important considering referee #3's

---

concerns related to this. Moreover, referee #2, when reflecting on referee #3's concerns regarding the in vivo relevance of the proposed alpha-segment-editing function of FEN1, additionally suggests to possibly down-regulate/manipulate a MMR protein in cells to more conclusively exclude the possibility that the cellular defects shown are due to a defect in the traditional mismatch repair (MMR) pathway or that the mutation in FEN1 directly affects the traditional MMR pathway itself. As such, this turns out to be a major concern shared by referees #2 and #3.

We generally allow three months as standard revision time. Should you foresee a problem in meeting this three-month deadline, please let us know in advance. We can in principle extend the due date for revision. Also, any competing manuscripts published here or elsewhere in the meantime will have no negative impact on our final assessment of your revised study.

-----------------------------------------

REFEREE REPORTS:

Referee #2:

In the manuscript, tilted "Alpha-segment error editing by the mammalian FEN1/MutS functional complex", the authors present data suggesting a significant role for FEN1/MutS correcting errors from Polymerase during extension from RNA primers in Okazaki fragment maturation. They show direct protein interaction (IP from Cells and with purified proteins), that FEN1 can remove errors in the -segment (the region polymerized by Pol , and evidence that this particular function of FEN1 may the cause of mutant FEN1 cancer susceptibility. This latter is the most striking, as it was thought that cancer susceptibility was caused by defects in FEN1's role in cutting the RNA primers, not the segments. This seems like work of broad-based and general importance. The authors were able to nicely distinguish between the RNA primer removal and error editing.

The authors should correct, and/or comment on the following points.

1. Given the structure of FEN1/DNA showed the structural importance of the 3' flap binding to the FEN1 active site, it is difficult to consider how the Exo activity of FEN1 could go up to 6 nt as evident in Figure 2. If the 3' flap remains bound, is the ssDNA looping out? How is the activity compared to the "best" FEN1 substrate? Could the authors please quantitate and compare. Could MutS facilitate this looping out structure? This work merits more thought on possible mechanisms. the structures should be noted and discussed at a minimum.
2. In Figure 3, the authors do a nice job showing that FEN1 is active in the AEE and RPR assays. However, the authors should show that their purified nucleases are active on known substrates as a control in supplemental.
3. The authors should include DNaseI in their immunoprecipitation from crude extracts, to reduce contamination through tangential DNA binding. Although they also show interaction with purified proteins, more stringency is needed for the IP in extracts.
4. As the DNA-bound structures of FEN1 and MutS have been determined (Cell. 2011 Apr 15;145(2):198-211; Mol Cell. 2007 May 25;26(4):579-92), the authors should consider these structures and where the interacting interface might be, which side of MutS would make sense for segment editing, and which side of FEN1. A fast look suggests both structures could bind different DNA regions. The interaction, based on the % pulled down in the IP results, seems extremely strong, suggesting a large interface. How is MutS working to facilitate FEN1 activity? Is it simply an enhanced interaction with the DNA or does the bending by MutS facilitate?
5. The buffers used in the assays were referenced from previous work, but should be given in the text or supplemental, so a general reader need not read other papers to understand this one.

Referee #3:

This manuscript addresses the mechanisms by which mismatches introduced by Pol alpha during Okazaki fragment synthesis are repaired. Essentially the authors propose that FEN1 can remove deoxynucleotides in nascent Okazaki fragments if mismatches exist in the DNA segment 3-prime of the RNA primer.

The model of the authors, referred to as alpha-segment error editing (AEE), is interesting, but there is little evidence that this mechanism is relevant in vivo. Other mechanisms can also be envisioned. For example, the mismatches could be removed by traditional mismatch repair. Overall, I am afraid that I am not convinced that the proposed AEE process exists in vivo.

Specific Comments:

1. The authors wish to study alpha-segment editing in vitro (Figs 1 and 2). They refer to the process as RNA primer removal. But, as far as I can understand, the template they study in vitro contains no RNA, only DNA (see legends for Figs 1 and 2). Further, what is the evidence that this template is repaired by the relevant machinery for repair of mismatches at Okazaki fragments?

2. Fig. 3 uses purified proteins, but almost nothing is mentioned regarding how these proteins were expressed and purified. The authors cite some of their earlier papers, but a few lines of text and some images of the gels showing the purified proteins as supplementary figures would have been helpful.

3. Fig. 4 shows that FEN1 mutants do not support repair of the templates bearing DNA flaps. Nuclear extracts prepared from heterozygous FEN1 mutant mice are also shown to have reduced activity as compared to extracts prepared from wild-type cells.

4. Fig. 5E shows that MSH2 stimulates mismatch repair mediated by FEN1. The magnitude of the effect, however, seems to be small and one wonders whether MSH2 and FEN1 act together, as the authors propose, or if MSH2 acts independently of FEN1, in which case the result would be less interesting. According to the model of the authors, a FEN1 mutant that does not interact with MSH2, should not support repair and this defect would be restored by a heterologous dimerization domain that re-establishes the FEN1-MSH2 interaction.

5. Fig. 7 shows that FEN1 mutant heterozygous mice are more prone to mutagenesis and tumor formation. This result is interesting, but not necessarily novel (except in the context of this specific mutant).

In conclusion, I am afraid that this study seems more suitable for a more specialized journal.

---

**1st Revision - authors' response**                                        08 January 2015

*1. Referee #2 additionally pointed out that the in vitro effect of stimulation of FEN1 activity by MutS, when a mismatch was present, should be re-addressed by quantitation in triplicate with error bars provided to substantiate the magnitude of the effect. We think that this additional point is important considering referee #3's concerns related to this.*

We have done as requested. The major effect of MSH2 on FEN1 action is that MSH2 enhances the processivity of the FEN1 exonuclease activity until the mismatch is removed or FEN1 will drop off after cleaving 1 or 2 nucleotides from the 5' end of the nick. Based on the comments from reviewer #3, we have repeated the experiment in Figure 5E and quantified the amount of the products and extended products with error bars. The magnitude of the MSH2-stimulating effect on the extended products is 3-4 folds. The quantification data is presented in the lower panel of Figure 5E and the additional result is included in the Results section (page 12, paragraph 1).

*2. Moreover, referee #2, when reflecting on referee #3's concerns regarding the in vivo relevance of the proposed alpha-segment-editing function of FEN1, additionally suggests to possibly down-regulate/manipulate a MMR protein in cells to more conclusively exclude the possibility that the cellular defects shown are due to a defect in the traditional mismatch repair (MMR) pathway or that*

*the mutation in FEN1 directly affects the traditional MMR pathway itself. As such, this turns out to be a major concern shared by referees #2 and #3.*

We have knocked down four relevant genes: FEN1, MSH2, EXO1 and MLH1 and measured the AEE reaction efficiency using nuclear extracts prepared from each of these mutant cells and the control (scrambled RNAi) cells. We found that the AEE reactions with knock-down of FEN1 or MSH2 were severely impaired while knock-down of EXO1 or MLH1 did not affect the reaction efficiency at all. In addition, we have used cell lines which are defective in MSH2 or MLH1 gene to test both the MMR and AEE reaction efficiency and found that deficiency of MSH2 or MLH1 causes mismatch repair (MMR) impairment but only that of MSH2 but not MLH1 leads to AEE impairment.  Based on these data and the data presented in Figures 5 and 6, we conclude that MSH2 and FEN1 form a unique complex, which together plays an important role in AEE. which is a specialized pathway for repair of mismatches near the RNA primer. These data have been included in the Results section (page 14, paragraph 2) and shown in the Figure 8 and Figure E7.

Referee #2

*3. Given the structure of FEN1/DNA showed the structural importance of the 3' flap binding to the FEN1 active site, it is difficult to consider how the Exo activity of FEN1 could go up to 6 nt as evident in Figure 2. If the 3' flap remains bound, is the ssDNA looping out? How is the activity compared to the "best" FEN1 substrate? Could the authors please quantitate and compare. Could MutS facilitate this looping out structure? This work merits more thought on possible mechanisms. The structures should be noted and discussed at a minimum.*

In order to address these interesting questions, we have designed a set of new substrates including the "best" substrate for the FEN1's exonuclease activity with a 3' flap, a nick, and a mismatch that is 8 nucleotides away from the nick. With the best substrate, FEN1 can make a few initial cuts from the 5' of the nick, consistent with what Bambara's group reported many years ago (Murante et al., 1994, JBC, 269:1191-1196). In the presence of MutSα, FEN1's cleavage can then reach to the mismatch site and remove the mismatch efficiently with the "best" substrate. Using a substrate missing the 3' flap or nick (with a gap) or both, FEN1's cleavage activity dropped below the detectible level. With the presence of MutSα in reactions with one of the other three substrates, FEN1 could then inefficiently remove the mismatch. Based on our original and amended data, which are included in Figure 7A and the Results section (pages 12-13), we believe that MutSα was able to recognize the mismatch, bind to it and interact with FEN1 to facilitate its exonuclease activity while looping out the template strand. Additional data and discussion have also been included in the revised manuscript to further address this point and Point 6 below (pages 13).

*4. In Figure 3, the authors do a nice job showing that FEN1 is active in the AEE and RPR assays. However, the authors should show that their purified nucleases are active on known substrates as a control in supplemental.*

Using the "C" series of substrates in Figure E1, we showed the nuclease activity profiles of FEN1, EXO1, and DNA2 in the original Figure E3 (now Figure E4 in the revised manuscript). All of these three purified nucleases are active. Their activity profiles are consistent with the ones that we have observed previously (e.g., Finger et al., 2009, JBC; Zhang et al., 2005, Cell) and those described elsewhere in the literature. Related to this and in response to the demand from the Reviewer #3 below, we have also included the gel images to show the purity of the all of these nucleases, in Figure E3, with the appropriate references.

*5. The authors should include DNase I in their immunoprecipitation from crude extracts, to reduce contamination through tangential DNA binding. Although they also show interaction with purified proteins, more stringency is needed for the IP in extracts.*

In order to eliminate the possibility of the tangential nucleic acid binding problem, we incubated the nuclear extracts with Benzonase nuclease (EMD Millipore) instead of DNase I. Benzonase nuclease is a genetically engineered nuclease that degrades all forms of DNA and RNA and does not possess

proteolytic activity. This information has been added to the revised manuscript. This procedural note has been added to <u>page 20, paragraph 1</u>.

*6. As the DNA-bound structures of FEN1 and MutS α have been determined (Cell. 2011 Apr 15;145(2):198-211; Mol Cell. 2007 May 25;26(4):579-92), the authors should consider these structures and where the interacting interface might be, which side of MutS would make sense for α segment editing, and which side of FEN1. A fast look suggests both structures could bind different DNA regions. The interaction, based on the % pulled down in the IP results, seems extremely strong, suggesting a large interface. How is MutS α working to facilitate FEN1 activity? Is it simply an enhanced interaction with the DNA or does the bending by MutS facilitate?*

An additional question is how FEN1 can both interact with the 3' flap and remove the nearby nucleotide as well as the mismatched nucleotide as far as in the 8th position downstream of the 3' flap, as shown in Figure 7A. Does MutSα facilitate such an action by looping out the single stranded DNA in the template strand while FEN1 sequentially removes newly synthesized DNA? To answer these questions, we have built a FEN1/MutSα/DNA complex model using the ZDOCK software (Pierce et al, 2014). We used the FEN1 (PDB code 3q8k (Tsutakawa et al, 2011)) and MutSα (PDB code 3thx (Gupta et al, 2012; Warren et al, 2007) X-ray crystallographic structures in complex with DNA molecules for docking. The best model was selected from a total of 40,000 initial complex models with their distinct consensus contact scores (Figure 7B). The consensus contact scores were calculated based on the following two criteria: 1) FEN1's helical region with amino acid residues from 245 to252, which was previously identified to interact with the downstream DNA duplex (Tsutakawa et al, 2011) (now where the mismatch is), may interact with MutSα protein, and 2) the ends of the DNA molecules bound to the two proteins respectively should be as close as possible. In the established model, residues of FEN1 in regions of K244-H253 and V260-Y268 interact directly with the residues in regions of S498-D506 and E529-F539 of MSH2 with contact area of about 377 $Å^2$ (Figure 7C). It is suggested that the strong interaction between MSH2 and FEN1 may stabilize the FEN1 protein to bind with the DNA molecule after the 5'-flap is removed. Due to the strong interaction with FEN1 and energy release in the process of the stabilization of the FEN1/MutSα/mismatch-containing DNA complex, the MutSα protein will pull the FEN1 protein towards the mismatch and be pushed backward. The protein complex could then cleave the nucleotide diester bonds of the Pol α-synthesized error-prone DNA while it loops out the template strand. This information is discussed on <u>pages 13 and 14</u>.

*7. The buffers used in the assays were referenced from previous work, but should be given in the text or supplemental, so a general reader need not read other papers to understand this one.*

The reaction buffer conditions for the AEE assay were referred in the manuscript text as described in our previous paper (Zheng et al., 2007a). They are also the same as the RPR assay, as described in the previous section. This has been clarified in the revised manuscript on the <u>last paragraph of page 20 and the first paragraph of page 21</u>.

<u>Referee #3</u>

*8. The authors wish to study alpha-segment editing in vitro (Figs 1 and 2). They refer to the process as RNA primer removal. But, as far as I can understand, the template they study in vitro contains no RNA, only DNA (see legends for Figs 1 and 2). Further, what is the evidence that this template is repaired by the relevant machinery for repair of mismatches at Okazaki fragments?*

Based on the previously published work from my group and other groups, it has been established that flap endonuclease 1 is a major nuclease responsible for RNA primer removal. However, in the in vitro biochemical reactions, the enzyme does not distinguish DNA or RNA content in the flap. In our previous work, we have compared the biochemical properties of flap endonuclease 1 on the flap substrates with RNA or DNA as the flap. We did not observe any differences in the RPR reactions regardless of whether we used DNA or RNA as the ss flap (e.g. Zheng et al., 2008, Mol. Cell). Therefore DNA flap substrates have been used to mimic the in vivo RNA primer removal processes due to their stability and ease of use. To clarify the reviewer's concern, we have included the substrates with an RNA portion in the flap in the same reaction as the ones in Figure 1 in the

revision. The reaction pattern is the same as the ones with the DNA flap (Figure E2). This point has also been clarified in the Results section (page 7, paragraph 1).

*9. Fig. 3 uses purified proteins, but almost nothing is mentioned regarding how these proteins were expressed and purified. The authors cite some of their earlier papers, but a few lines of text and some images of the gels showing the purified proteins as supplementary figures would have been helpful.*

Based on the comments, we have included Figure E3 in the current revision to show the images of the purified proteins. We have indicated how all these proteins were expressed and purified as well as the references for the detailed protocols in the Materials and Methods section (page 20, paragraph 2).

*10. Fig. 4 shows that FEN1 mutants do not support repair of the templates bearing DNA flaps. Nuclear extracts prepared from heterozygous FEN1 mutant mice are also shown to have reduced activity as compared to extracts prepared from wild-type cells.*

That is right. In addition, in a reconstitution fashion, A159V failed to support both the RPR and AEE reactions while E160D was still competent in RPR but not in the AEE reaction. This partly explains the phenotype difference when they are individually knocked into the mouse genome.

*11. Fig. 5E shows that MSH2 stimulates mismatch repair mediated by FEN1. The magnitude of the effect, however, seems to be small and one wonders whether MSH2 and FEN1 act together, as the authors propose, or if MSH2 acts independently of FEN1, in which case the result would be less interesting. According to the model of the authors, a FEN1 mutant that does not interact with MSH2, should not support repair and this defect would be restored by a heterologous dimerization domain that re-establishes the FEN1-MSH2 interaction.*

The major effect of MSH2 on FEN1 action is that MSH2 enhances the processivity of the FEN1 exonuclease activity until removal of the mismatch or until FEN1 drops off after cleaving 1 or 2 nucleotides from the 5' end of the nick. Based on the comments from this reviewer and the other reviewer, we have repeated the experiment in Figure 5E and quantified the amount of the products with error bars. The magnitude of the MSH2-stimulating effect is 3-4 folds. The quantification data are presented in the lower panel of the Figure 5E and the additional result is included in the Results section (page 12, first paragraph).

Do MSH2 and FEN1 really act together *in vivo*, as we suggested here, or does MSH2 act in traditional MMR, independently of FEN1? An additional experiment was designed to address this point. We have knocked downFEN1, MSH2 and two additional genes (EXO1 and MLH1), which are involved in traditional MMR only (Figure 8A). We have then examined their effects on AEE using the respective NEs. We found that knock down of FEN1 and MSH2 impaired AEE, while down-regulation of the expression of the EXO1 or MLH1 genes did not cause defects in the AEE reaction (Figure 8B). Based on these data and data presented in Figures 5 and 6, we conclude that MSH2 and FEN1 form a unique complex, which together plays an important role in AEE.

*12. Fig. 7 shows that FEN1 mutant heterozygous mice are more prone to mutagenesis and tumor formation. This result is interesting, but not necessarily novel (except in the context of this specific mutant).*

The key point is captured by the reviewer's phrase "except in the context of this specific mutant". The data are the first to indicate that impairment of the AEE mechanism can lead to a unique mutator phenotype and cancer formation in the mouse.

---

**2nd Editorial Decision**                                                                 04 February 2015

---

Thank you for submitting your revised manuscript for our consideration. It has now been assessed once more by one of the original referees, whose comments are copied below. I am happy to say that this referee considers the study significantly improved and now in principle suited for publication, pending addressing of a few specific issues that still remain to be satisfactorily addressed. I am therefore returning the study to you for one additional, final round of revision, in order to address

these points.

I hope you will be able to make the necessary further minor revisions (which I consider justified given the importance of the topic and the potential significance of the conclusions) and resubmit a final version of the manuscript as early as possible. Should you have any further questions in this regard, please do not hesitate to get back to me. I look forward to receiving your final version!
_____

REFEREE REPORTS:


Referee #2:

Liu and co-authors have done additional experiments and modified the paper in response to the reviewer comments. This paper, which suggests a novel interaction of MutS at the replication fork with the machinery for Okazaki fragment maturation, will be of broad interest to EMBO readers. Although there are some concerns that should be addressed before publishing, I think that the conclusions are viable, and the results of substantial interest. The authors should address the following issues before publication.

1. The requested quantitation in Figure 5 for the FEN flap shows that the activities shown are not in a linear range and are poorly reproducible, with error bars being half of the signal. The amount of product formed should be less than 20-30%. Although the overall conclusions are still viable, the authors should improve the precision of their analysis.

2. Unpairing the 3' flap appears to be a major step for optimal FEN1 catalytic activity and would be a good step for MutS to facilitate. The finding in Figure 7A (which I recommend be quantitated) suggests that MutS is working on some other area of DNA binding. FEN1 is diffusion controlled so it is unlikely that MutS is facilitating catalysis. The authors should comment for readers as to the implications of MutS optimally stimulating the 3' unpaired flap substrate.

3. Quantitation of all the nuclease assays would strengthen the conclusions of the paper.

4. For Figure 7 the authors should add a description of the substrates into the figure legend or into the figure itself (as nicely done for the other figures). The only reference to substrates D1-4 is in Supplemental, which took digging.

---

2nd Revision - authors' response                                                    02 April 2015

Reviewer #2:

1. *The requested quantitation in Figure 5 for the FEN flap shows that the activities shown are not in a linear range and are poorly reproducible, with error bars being half of the signal. The amount of product formed should be less than 20-30%. Although the overall conclusions are still viable, the authors should improve the precision of their analysis.* The experiments have been re-done three times and the amount of product has been controlled to be not completely depleted. The experimental conditions were well controlled so that the resulting data were more reproducible. The amount of product was quantified well and error bars were minimized. We feel comfortable now that the results more clearly support our conclusion.

2. *Unpairing the 3' flap appears to be a major step for optimal FEN1 catalytic activity and would be a good step for MutS to facilitate. The finding in Figure 7A (which I recommend be quantitated) suggests that MutS is working on some other area of DNA binding. FEN1 is*

*diffusion controlled so it is unlikely that MutS is facilitating catalysis. The authors should comment for readers as to the implications of MutS optimally stimulating the 3' unpaired flap substrate.*

The data in Figure 7A have been repeated and quantified with error bars. In the Results section, we have now clearly described how MutSα may coordinate the mismatch and 3' flap DNA elements. However, rather than speculate on the implications of MutS optimally stimulating the 3' unpaired flap substrate with partial information, we prefer to incorporate this question into our future experimental work.

3. *Quantitation of all the nuclease assays would strengthen the conclusions of the paper.*
   With addition of the qualification graph for Figure 7A, we have now provided quantitation for all the nuclease assays in the current work.

4. *For Figure 7 the authors should add a description of the substrates into the figure legend or into the figure itself (as nicely done for the other figures). The only reference to substrates D1-4 is in Supplemental, which took digging.*
   We have added schematics of the substrates into Figure 7A, in addition to the  references to substrates D1-4 in the Extended View.

| Acceptance letter | 14 April 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.