**Structural basis for a novel mechanism of DNA bridging and alignment in eukaryotic DSB DNA repair**

Jérôme Gouge, Sandrine Rosario, Félix Romain, Frédéric Poitevin, Pierre Béguin and Marc Delarue

_Corresponding author: Marc Delarue, Pasteur Institute/ CNRS UMR 3528_

---

### Review timeline:

- **Submission date:** 29 July 2014
- **Editorial Decision:** 06 August 2014
- **Rebuttal:** 07 August 2014
- **Additional correspondence (editor):** 26 August 2014
- **Additional correspondence (editor):** 29 August 2014
- **Additional correspondence (author):** 29 August 2014
- **Editorial Decision:** 05 October 2014
- **Revision received:** 03 December 2014
- **Editorial Decision:** 03 January 2015
- **Revision received:** 08 January 2015
- **Accepted:** 09 January 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.)

**1st Editorial Decision 06 August 2014**

Thank you for submitting your manuscript (EMBOJ-2014-89643) to our editorial office. I have now had a chance to read it carefully and to discuss it with my colleagues, and I am sorry to say that we cannot offer publication in _The EMBO Journal_.

I apologize for the delay in contacting you, due to the unforeseen temporary absence of a colleague.

Unfortunately, we have decided not to send your manuscript to formal peer review, as the priority for publication in a broad interest journal such as this is, in our view, limited.

You present the crystal and solution structures of the polX DNA family member Tdt in complex with a substrate mimicking a DSB. You identify functional domains and generate two point mutations based on the structure which are functionally validated, also extending the same mutations to polu, which leads to your argument that VDJ 'evolved' from NHEJ recently. You conclude that Tdt can bind both ends of the DSB without help of the rest of the NHEJ/V(D)J apparatus (i.e. intrinsic direction of synapsis by the enzyme). You characterize a base stacking mechanism largely in line with the literature.

We note previous related structures in the literature, notably cited ref 7, 8, although they did not reflect a bona fide DSB break substrate. Importantly the biochemical analysis in ref 19 comes to many of the same conclusions and in our view this manuscript largely provides the structural backdrop to this work.
Please note that we publish only a small percentage of the many manuscripts that we receive at the EMBO Journal, and that we can therefore subject to external review only those submissions that have a good chance of timely acceptance. I am sorry to disappoint you on this occasion.

Rebuttal

07 August 2014

Thank you for your e-mail concerning our recent submission to EMBOJ. I have read it carefully, and I am greatly surprised by the reasons you mention to reject it. Since there are not so many of them, I will address them below.

Please understand my purpose: it's OK for me to have one of my papers rejected by EMBOJ. But at least I want to make sure this is for good reasons.

I am sure you feel the same as well.

I feel great difficulty in forwarding your comments as they are now to the post-doc who did all the crystallographic work, because I am sure he would feel injustice in reading them.

So I am counting on you either to reconsider your decision or to give me arguments that he could agree with, especially concerning the state-of-the-art.

First of all, this structure is not a mere addition to our previous EMBO J. 2002 paper on Tdt structure, nor to the known gap-filling complex of pol mu (published in 2007 by T. Kunkel and co-workers). To my knowledge, it is the only paper that gives the structure of a complex showing what is going on when a eukaryotic DNA polymerase acts on double-strand break substrates. As such it is a major breakthrough. And it concerns both the cancer field and immunology. People are seriously considering targeting pol mu for drugs against acute myelomas, testifying of the potential importance of these results.

I am unaware of results like this in the field of DNA polymerases and DNA repair, with a bound templating strand in trans. Actually, I did not think it was possible to get this information before seeing it with Tdt. In fact, the downstream DNA duplex is too short to be stable in solution at RT. This means that the polymerase "selected" its substrate out of the two separate strands in solution. This is a complex that a lot of people would like to see at the atomic level. And the competition in the field is fierce (I could comment on that further if needed). I am pretty sure that once they get hold of it, including proper definition of what the substrate should be (with only one micro-homology base-pair), the co-workers of T. Kunkel in the NIH, USA will immediately start crystallizing pol mu with this substrate. We all know that the real substrate of pol mu is not a gapped-filling one. Loop1 is completely disordered in this case (Moon et al., 2007). I am pretty sure that these crystals, if they get it before us, would make it to Nature, as the apo (!) structure of pol mu made it to NSMB (Moon et al., 2014).

Second of all, the papers by Martin and Blanco in 2012 and 2014, although they showed that pol mu binds both upstream and downstream DNAs, also show that they have no clue as to what the real substrate of pol mu is. In fact their Figure 1 (Martin and Blanco, 2014) indicates that the best substrate is the one with only MH-bp. However, they apparently did not realize that and went on testing their mutants with substrates with either 0 or 3 MH-bp (!), which does not make sense once you know the structure. This is written up - softly - in the Discussion. They even proceed to present an atomic MODEL (based on Tdt structures solved by us in 2002) with a 0 MH-bp substrate, which we also presented in our recent paper Gouge et al., JMB, 2013, and which they don't even care to cite, even though it's obvious they have seen it, considering that the mutants they present concern positions that we highlighted there for the first time. This model with a 0 MH-bp substrate is now clearly obsolete.

I am surprised, therefore, that you qualify our results as mere illustrations of biochemecal data presented by them.

They are not and they go much further that the functional tests of Martin and Blanco. Our results actually explain theirs but certainly not the other way round. I am actually quite concerned that the EMBO J editorial staff seems be inclined to consider new structural results never seen before as mere "illustrations of previous biochemical experiments", without documenting it further. Crystallographic results go a long way deeper than biochemical results, I would tend to think that everyone recognizes that. Actually the structural results are much harder to get, considering the difficulty to get diffracting crystals.
Finally, for the evolutionary points that we make, we never said that V(D)J recombination evolved "recently" from NHEJ. Everybody know that V(D)J recombination and the adaptive immune system evolved 500 or so millions years ago when jawed fish evolved from jawless fish. This is not at all "recent" (about 15% of the time since life appeared on earth). It is clear that it recruited the same machinery than NHEJ, namely Ku 70/80, DNA-PK etc... and also borrowed the Transib transposase for its purpose. The novelty is that it also borrowed pol mu from NHEJ and transformed it into a much more tolerant one, which checks only base stacking, not base-pairing. We actually took the challenge to try to transform Tdt back into a template polymerase acting with an trans substrate, with some success. This whole idea is indeed a simple one, but it has never, to my knowledge, been articulated before. And certainly not by the team of T. Mora and A. Walczak who analysed the "natural sequences" of T-cell receptors by statistical physics methods but did not present any molecular model explaining their data. They did a great job in inferring the "dinucleotide step" rule but not as we see it now.

So, I will stop this letter here and would like to ask you, with all due respect, to consider carefully my arguments. If they make sense to you, I would like to ask you to reconsider your decision. Since they concern mainly the state-of-the-art and the novelty of our results, you should indeed be able to decide for yourself. If you need further explanations or feel I have not made myself clear, I am all to ready to call you on the phone through a pre-arranged call.

Thank you for your letter. We appreciate the opportunity to engage in constructive discussion about editorial decisions. I had read your carefully argued letter with interest and appreciate the arguments put forward. I was happy to consider the study again in light of your response and note that your manuscript is of course on an interesting topic and I would like to assure you that it is expertly written and very well argued. On a general note, please allow me to state that an editorial rejection must not be interpreted as a comment on the quality of the data or the analysis, or indeed any deficiencies with the manuscript. Our decisions at the editorial level are based on an assessment of how interesting a particular manuscript would be to our broad readership. One of our key considerations in this respect is the level of conceptual advance a study provides.

First, I have to agree with you that the decision letter I sent was in this case worded in a rather imprecise manner. This is unfortunate and you were quite right to point this out (one deficiency you did elegantly put aside was to lump the crystallography data and the biochemical analysis together under the heading structure). Regarding the evolutionary argument, of course we are aware that 'recent' is an ambiguous term and not the topic of analysis here - it is more appropriate to state that the polmu and Tdt mechanisms of action are closely related, as you did. In fact, I certainly found your argument in the discussion section interesting that the surprising mechanism for Tdt action could be rather elegantly explained by an evolutionary derivation from the equivalent NHEJ enzyme in connection with a more tolerant polymerase specificity. Secondly, I have to note that it was certainly never my intention to state - as you imply - that biochemical data is superior to structure data. Of course it is not and we continue to publish X-ray structures happily and frequently - usually supported by biochemical analysis as in your dataset. However, we do look at the level of conceptual advance of the findings presented. In this sense we do not ignore biochemical or genetic evidence. In fact neither did you in your fair and detailed analysis of the related literature.

You emphasize the unique aspect of a polymerase structure with a ds DNA substrate and we have no reason to disagree with that statement. However, the papers we pointed out in our letter are just some of the relevant articles here and you discuss the literature in a fairly detailed and authoritative way in your manuscript. It is clear that the data presented allows a much clearer insight into the nature of the substrate and the mechanism of action of both polymerases, but it clearly builds on a significant level of previous insight.

Nevertheless, I decided in mid August to consult with expert advisors to obtain an independent set of views on your manuscript. Unfortunately, the appropriate advisors I selected where themselves
travelling. I returned only today myself (as you saw from the out of office notices) and managed to make contact with the advisor who is indeed reading the manuscript. I am not sure at this time if his/her view will deviate from the assessment we forwarded to you, but the views are entirely independent and I would recommend to wait for them at this point.

If you wish to submit the manuscript elsewhere tomorrow, please inform me as soon as possible and I will ask our advisor to desist from further evaluation.

Additional correspondence (editor) 29 August 2014

Thank you for your patience. We have decided to formally review the manuscript after all after consulting with the outside advisors. I cannot say if this will change our initial decision at the end of the day, but it will provide you with more detailed feedback form experts (which - I would like to re-emphasize - was entirely based on our assessment of the overall conceptual advance, not any technical deficits or issues with the approach taken).

Could you please confirm if you are interested in this option and further if we should review the revised manuscript you sent in?

Additional correspondence (author) 29 August 2014

Thanks for your e-mail.
I confirm that I am interested in this option and that it is best if you review the revised manuscript that I sent this week.

2nd Editorial Decision 05 October 2014

Thank you for submitting your manuscript for consideration by the EMBO Journal. As we discussed, we did sent your revised manuscript out for formal review after your rebuttal. It has now been seen by three referees whose comments are shown below.

Clearly all three referees value this work, but they all recommend that the manuscript be rewritten to focus on TdT.
Importantly, referee 1 also raises a limitation in the scope of the work, stating: 'It remains unclear, however, what the precise biological significance of the synaptic complex in TdT is, apart from serving as a model how Pol mu functions. The authors correctly ask the question why a downstream duplex binding site is maintained, but do not provide data to address this question.'
As the referee notes, the biochemical results confirm earlier work.
Referee 2 on the other hand notes the inherent limitation of presenting the Tdt structure, given the low templates requirement of this enzyme relative to polmu.

Given these limitations and the state of the field, it would be essential to develop the dataset to investigate the biological significance of the synaptic complex of TdT for further consideration at this journal. Please note that this specific point was also fully endorsed by referee 3 in cross-commenting, who stated that the authors should be asked to investigate the relevance of the structure to the TdT mechanism.

Please note that all three referees take issue with the clarity and style of the writing. Referee 1 requests that the overly speculative modelling discussion is removed or strongly de-emphasized. Indeed, referee 3 states 'The major emphasis of this paper should be on TdT and what the structures tells us about the terminal transferase mechanism of these enzymes.' Indeed, referee 1 responded to ref 3's report: 'I generally concur with Referee 3 that a focus on TdT rather than Polmu would be
much more appropriate. The manuscript would also benefit from an improved writing and focus.

Referee 2 commented on referee 3: 'I subscribe to the point made by ref 3, about how the paper reads like a draft and could do with rewriting in a more rigorous, tighter style.'

Given the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. We understand that this revision would not only require a rewrite of the manuscript, but also extensive further reaching experimentation. We would understand if you regarded this as being beyond the scope of the current project, but as emphasized by referees 1 and 3 it would be a requirement for resubmission at this journal.

I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

Thank you for the opportunity to consider your work for publication.

Referee #1:

Polymerases of the Pol X family are involved in variety of DNA processes, in particular base excision repair and non-homologous end joining. Some of these enzymes are well characterized at the structural and biochemical level and the mechanism of gap filling is to a large extent understood. Less understood, and the main point of this paper, is how some Pol X family polymerase can bridge to DNA ends, elongating one end in order to facilitate NHEJ (Pol mu) or junctional diversification in V(D)J recombination.

The main result of this manuscript is the determination of a crystal structure of TdT in complex with a primer, ddCTP and a downstream duplex "DNA end", which visualizes how PolX family enzymes - at least TdT - can bridge two DNA ends. The structural analysis proceeds by testing a variety of DNAs to investigate structural consequences of micro-homologies or lack of thereof. In general, the structures are of good resolution with good refinement statistics. The strong and novel aspects of the manuscript are structures with trans-templates and a microhomology base pair and the observed ordering of loop 1. The model that loop 1 substitutes for the missing cis-template and helps stabilize the MH base-pair is intriguing and an important achievement.

The authors complement the structural studies with a variety of primer extension and DNA binding assays. They find that TdT can bridge two DNA molecules in vitro, albeit with much less efficiency than Pol mu (which functions in NHEJ). Also, while TdT does apparently not check basepairs in the biochemical assays, Pol mu does. These biochemical results confirm earlier work.

In summary, the significance of this work lies in the new conformation of the loop 1 and visualizing a synapsis at a microhomology or mismatched basepair. It remains unclear, however, what the precise biological significance of the synaptic complex in TdT is, apart from serving as a model how Pol mu functions. The authors correctly ask the question why a downstream duplex binding site is maintained, but do not provide data to address this question.

Technical comments:

1) I wonder why the authors observe only one Mg2+ or Mn2+ ion in the active site instead of the typical two ions (according to Figs. 1D and S5)? Please note, Pol mu bound to a gapped substrate has two metal ions, please correct Fig. 1D. At the resolution these structures were determined, two metals should clearly be visible, especially at 50 mM Mg2+. Please comment.

2) P5 2nd paragraph and 5th paragraph/Fig. 2: How are the 50% occupancies defined? Do you refine the structure with alternate conformations and occupancy refinement?
3) Discussion: I find the whole section on modeling rather speculative, especially since modeling long loops in a faithful manner is virtually impossible. I suggest to remove this part and the corresponding figure.

Editorial comments:

1) P3: DNA-PK should probably DNA-PKcs
2) P3: perhaps spell out "coll" as "colleagues"
3) P4 bottom: "surrounded in the minor groove" reads awkwardly.
4) Materials and methods: this part needs substantial editing in terms of capitalization of nouns (very inconsistent), chemicals (sometimes Ac, acetate or CH3-COOH are used), nomenclature (pol mu or pol µ) and proper English. Also, sometimes commas are used instead of decimal points.
5) For instance, on page 15 the sentence "... was annealed by heating during 5 minutes at 90{degree sign}C then allowed to return to room temperature overnight". Please revise, the annealing is done during the slow cool phase, not during heating at 90{degree sign}C.
6) P13: what does convergence of the R-factors mean? R and Rfree converge or both reach a minimum?
7) P14: phosphoImager should probably read phosphorimager
8) P15 bottom: the S_cross formula has some issues with indices in the sum, at least in my PDF copy.
9) Fig 3b: please label the Y-axis.

Referee #2:

1. The main claim of the authors, that their structure captures for the first time a PolX bound to a bona-fide NHEJ intermediate is correct. A corollary of their achievement is that they provide a role for the loop1 sequence in substrate DNA recognition, which has remained elusive so far. In my view, this achievement is at least as significant as what reported recently by Moon et al, NSMB, 2014.

2. Perhaps ironically, the structures described by the authors refer to TdT, the PolX family member with least or no reliance on a template, either cis or trans. The fact that TdT can apparently bind to and be active on a NEHJ substrate, an intermediate that is more relevant to Pol mu, highlights once again the substantial overlap in the biochemical properties in the X-family Pols. A consequence of this is that researchers in the field tend naturally to emphasise molecular determinants for functional differences that are rather subtle.

3. The paper could do with some rewriting, as the meaning is sometimes difficult to grasp, through a usage of English that is obscure or excessively colloquial. Some of the figures could also be improved: The small inset in the bottom right corner of Figure 2 is the only place I could find in the paper that shows details of the interaction between Loop1 and DNA.

Despite its shortcomings - less-than-ideal match of DNA NHEJ substrate and PolX member, long-winded prose - in my view this paper represents an important enough advance to make sure that it will be widely read in the PolX field. Highlights includes capture in a crystal of the first NHEJ intermediate, structural basis for the 1bp-microhomology preference by PolX members and further insight into the role of Loop1 sequence in the end-joining process. Structurally it is at least as interesting as Moon et al, NSMB, 2014 (and a more physiologically relevant structure than the bacterial NHEJ synapsis by Brissett et al, Science, 2007).

Referee #3:

General summary

In this paper, Delarue and colleagues describe the elucidation of a novel complex of Tdt bound to a synapsis of two DNA molecules, held together by a single microhomology-mediated pairing. Although other Pol X structures (Pol l/m) have been elucidated with gapped intermediates, this is the first to potentially show a Pol X member in complex with a NHEJ-like DSB intermediate.
Notably, the complex shows, for the first time, the position of the elusive Loop I and its possible role in binding to the incoming "primer" strand. This is therefore a significant new structural study as it provides insights into the location and likely roles of this key surface loop in Loop 1, proposed by other groups to be important for end-joining but not previously been visualized until now.

- Specific major concerns

Given the potential significance of this story, the functional studies and their description (both figures and text) really let down this paper, particularly in the latter part, which focuses on investigating how Tdt can be made to be more like Pol Mu. The major emphasis of this paper should be on Tdt and what the structures tell us about the terminal transferase mechanism of these enzymes. However, the authors largely ignore this really important central story and the published paradigm of end-synapsis by prokaryotic NHEJ polymerases, to the major detriment of the paper. Instead, they proceed to focus on determining how Tdt and Pol Mu are very similar rather than the role of Loop I in Tdt end-extension. The mutagenesis experiments again leave the reader very confused. The numbering of residues appears to wrong in many places and at one stage they mix up the proteins/residues they are describing by referring to the other by mistake. This paper contains many significant errors or inaccuracies including, the running order of and reference to the figures, referencing key papers and the details described in the text is not visible at all in many of the figures. This manuscript reads like a confused early draft of a paper, rather than a journal ready version, where all the key ideas have not been fully formed and joined up into a cohesive unit. In my opinion, this manuscript needs to be fully rewritten with more focus and emphasis on the insights this study provides on how Tdt performs extension at the termini of DNA, rather than presenting it as a Pol Mu story, which does a disservice to this otherwise important new structural model.

1st Revision - authors' response 03 December 2014

Answers to Referees

Referee #1:

Polymerases of the Pol X family are involved in variety of DNA processes, in particular base excision repair and non-homologous end joining. Some of these enzymes are well characterized at the structural and biochemical level and the mechanism of gap filling is to a large extent understood. Less understood, and the main point of this paper, is how some Pol X family polymerases can bridge to DNA ends, elongating one end in order to facilitate NHEJ (Pol mu) or junctional diversification in V(D)J recombination.

The main result of this manuscript is the determination of a crystal structure of TdT in complex with a primer, ddCTP and a downstream duplex "DNA end", which visualizes how PolX family enzymes - at least TdT - can bridge two DNA ends. The structural analysis proceeds by testing a variety of DNAs to investigate structural consequences of micro-homologies or lack of thereof. In general, the structures are of good resolution with good refinement statistics. The strong and novel aspects of the manuscript are structures with trans-templates and a microhomology base pair and the observed ordering of loop 1. The model that loop 1 substitutes for the missing cis-template and helps stabilize the MH base-pair is intriguing and an important achievement.

The authors complement the structural studies with a variety of primer extension and DNA binding assays. They find that TdT can bridge two DNA molecules in vitro, albeit with much less efficiency than Pol mu (which functions in NHEJ). Also, while TdT does apparently not check basepairs in the biochemical assays, Pol mu does. These biochemical results confirm earlier work.

We agree with these comments but we insist that the biochemical results on the ability of Tdt to bind both an upstream DNA strand and a downstream DNA duplex are completely new, while indeed this fact was known for pol mu.
We have rephrased the opening sentence of the Abstract to make it more clear. A large part of the Introduction has been rewritten as well.

In summary, the significance of this work lies in the new conformation of the loop 1 and visualizing a synapsis at a microhomology or mismatched basepair. It remains unclear, however, what the precise biological significance of the synaptic complex in TdT is, apart from serving as a model how Pol mu functions. The authors correctly ask the question why a downstream duplex binding site is maintained, but do not provide data to address this question.

We indeed ask the question why nature has done this in the Conclusion and Perspectives paragraph of our article. We answer it by a simple evolutionary argument, namely that it would be advantageous to keep this functionality (if indeed Tdt was derived from a proto-pol mu), so that the DNA partners to be rejoined never come apart too far away during NHEJ (or V(D)J recombination). Of course it is just an appealing (and very reasonable) hypothesis, and we have modified the paragraph to make it clear that it remains an hypothesis.

Concerning biological data that address the in vivo function of Tdt, the structural model described here provides for the first time a clear molecular basis for the statistical model that best explains the natural sequences synthesized in vivo by Tdt in V(D)J recombination in T-cell receptors (Murugan, Mora, Walczak and Callan, PNAS, 2012, 109:16161-6). Indeed, in this article we read:

“Summary/Abstract
Our probabilistic model predicts the generation probability of any specific CDR3 sequence by the primitive recombination process, allowing us to quantify the potential diversity of the T-cell repertoire and to understand why some sequences are shared between individuals.”

...-Nucleotide Insertions (Figure 3)
“The nucleotide frequencies in the inserted segments are not uniform and are well explained by a dinucleotide Markov model where the probability of inserting A, C, G, or T depends on the immediately 5’ nucleotide.”

This corresponds very well to our structural model that isolates a Micro-Homology mini-helix (MH-mh) made of just these two base-pairs.

We now highlight this fact in the Summary and fully describe it, hopefully in a better way, in the Discussion.

See also comments by Referee #2 (“in my view this paper represents an important enough advance to make sure that it will be widely read in the PolX field “).

Technical comments:

1) I wonder why the authors observe only one Mg2+ or Mn2+ ion in the active site instead of the typical two ions (according to Figs. 1D and S5)? Please note, Pol mu bound to a gapped substrate has two metal ions, please correct Fig. 1D. At the resolution these structures were determined, two metals should clearly be visible, especially at 50 mM Mg2+. Please comment.

We didn't observe any density in the 2Fo-Fc nor in the Fo-Fc maps that would correspond to a catalytic metal ion. The structure of the ternary complex (Tdt/primer strand/incoming nucleotide) is also devoid of electron density in Metal A binding site despite the presence of 50 mM Mg acetate in the storage buffer (Gouge et al., JMB, 2013). We suspect that the presence of Mg2+ is only temporary and needs a complete coordination sphere to be stably bound (i.e. with a 3’OH that is absent -by design- in our experiments).

Nevertheless we have added the Metal A binding site in Figure 1D.

This phenomenon has also been documented in murine Pol mu's structure (Moon et al., 2007, pdb id 2IHM). Out of the two monomers in the ASU, only one contains an ion in the catalytic site and has been identify as a Na+ in the PDB file.

2) P5 2nd paragraph and 5th paragraph/Fig. 2: How are the 50% occupancies defined? Do you refine the structure with alternate conformations and occupancy refinement?

The quality of the map at 100% occupancy, as well as the B factors, clearly indicated that alternate conformations had to be built. Their occupancy were set either at 75%/25%, 50%/50% or 25%/75% and the resulting maps were calculated. Temperature factors were refined and used as an internal control. Visual inspection of the resulting density maps and the values of the B-factors led us to fix the occupancy at 50%/50% in each case.
3) Discussion: I find the whole section on modeling rather speculative, especially since modeling long loops in a faithful manner is virtually impossible. I suggest to remove this part and the corresponding figure.
OK, we removed the part on modeling Loop3 and the corresponding Supplementary Figure.

Editorial comments:
1) P3: DNA-PK should probably DNA-PKcs
OK, this has been corrected

2) P3: perhaps spell out "coll" as "colleagues"
OK, this has been corrected

3) P4 bottom: "surrounded in the minor groove" reads awkwardly.
OK, "surrounded" has been replaced by “recognized”

4) Materials and methods: this part needs substantial editing in terms of capitalization of nouns (very inconsistent), chemicals (sometimes Ac, acetate or CH3-COOH are used), nomenclature (pol mu or pol μ) and proper English. Also, sometimes commas are used instead of decimal points.
OK

5) For instance, on page 15 the sentence "... was annealed by heating during 5 minutes at 90{degree sign}C then allowed to return to room temperature overnight". Please revise, the annealing is done during the slow cool phase, not during heating at 90{degree sign}C.
OK

6) P13: what does convergence of the R-factors mean? R and Rfree converge or both reach a minimum?
OK, the second solution is the one we meant.

7) P14: phospoImager should probably read phosphorimager
OK

8) P15 bottom: the S_cross formula has some issues with indices in the sum, at least in my PDF copy.
OK, the size of these indices has been increased

9) Fig 3b: please label the Y-axis
OK (cpm)

Referee #2:

1. The main claim of the authors, that their structure captures for the first time a PolX bound to a bona-fide NHEJ intermediate is correct. A corollary of their achievement is that they provide a role for the loop1 sequence in substrate DNA recognition, which has remained elusive so far. In my view, this achievement is at least as significant as what reported recently by Moon et al, NSMB, 2014.
OK

2. Perhaps ironically, the structures described by the authors refer to TdT, the PolX family member with least or no reliance on a template, either cis or trans. The fact that TdT can apparently bind to and be active on a NEHJ substrate, an intermediate that is more relevant to Pol μ, highlights once again the substantial overlap in the biochemical properties in the X-family Pols. A consequence of this is that researchers in the field tend naturally to emphasise molecular determinants for functional differences that are rather subtle.

Yes, we agree with the referee, differences with pol μ are likely to be very subtle. We have completely rewritten the Discussion, and centered it on Tdt anyway, following the suggestions of Referee #3.
3. The paper could do with some rewriting, as the meaning is sometimes difficult to grasp, through a usage of English that is obscure or excessively colloquial.

OK, the entire text has been rewritten and then checked by two English-speaking persons, one senior EMBO member and all co-authors.

Some of the figures could also be improved: The small inset in the bottom right corner of Figure 2 is the only place I could find in the paper that shows details of the interaction between Loop1 and DNA.

OK. Figure 2 has been redrawn and made wider so that this panel is larger.

Supplementary Figure S1 has two additional panels that document this interaction, as well as Figure 6, which focus on the water molecules network in the minor groove of the micro-homology mini-helix (MH-mh) DNA.

Actually, all Figures have been modified and checked, including the ones in the SI section.

Despite its shortcomings - less-than-ideal match of DNA NHEJ substrate and PolX member, long-winded prose - in my view this paper represents an important enough advance to make sure that it will be widely read in the PolX field. Highlights includes capture in a crystal of the first NHEJ intermediate, structural basis for the 1bp-microhomology preference by PolX members and further insight into the role of Loop1 sequence in the end-joining process. Structurally it is at least as interesting as Moon et al, NSMB, 2014 (and a more physiologically relevant structure than the bacterial NHEJ synapsis by Brisset et al, Science, 2007).

OK

Referee #3:

General summary

In this paper, Delarue and colleagues describe the elucidation of a novel complex of Tdt bound to a synapsis of two DNA molecules, held together by a single microhomology-mediated pairing. Although other Pol X structures (Pol l/m) have been elucidated with gapped intermediates, this is the first to potentially show a Pol X member in complex with a NHEJ-like DSB intermediate. Notably, the complex shows, for the first time, the position of the elusive Loop I and it's possible role in binding to the incoming "primer" strand. This is therefore a significant new structural study as it provides insights in the location and likely roles of this key surface loop in Loop 1, proposed by other groups to be important for end-joining but not previously been visualized until now.

Specific major concerns

Given the potential significance of this story, the functional studies and their description (both figures and text) really let down this paper, particularly in the latter part, which focuses on investigating how Tdt can be made to be more like Pol Mu. The major emphasis of this paper should be on Tdt and what the structures tells us about the terminal transferase mechanism of these enzymes.

OK, this has been done.

However, the authors largely ignore this really important central story and the published paradigm of end-synapsis by prokaryotic NHEJ polymerases, to the major detriment of the paper. Instead, they proceed to focus on determining how Tdt and Pol Mu are very similar rather than the role of Loop I in Tdt end-extension.

OK. We have rewritten the paper (as well as the Title and the Abstract) to center the paper on Tdt. We have re-analyzed our structural data and provide new insight into the mechanism of Tdt through two new features and concepts: i) the use of tautomers in the incoming position and ii) the use of a dedicated water molecule network in the minor group of the micro-homology mini-helix (MH-mh) that could get strongly influenced/polarized by nearby divalent transition metal ions.
Concerning the “published paradigm of end-synapsis by prokaryotic NHEJ”, we find that is does not apply for eukaryotic DNA polX, and this is one of the major results of our paper. We just don’t see the same interactions in our crystal structures (see Discussion).

_The mutagenesis experiments again leave the reader very confused._

We have considerably simplified this section. We have regrouped all mutagenesis experiments on Tdt in a single principal Figure (Figure 3), where we have added a control experiment with wild-type Tdt, as well as one Suppl. Figure (Figure S5), and all mutants on (murine) pol mu in one Suppl. Figure (Figure S4). We hope it is now more clear.

_The numbering of residues appears to wrong in many places and at one stage they mix up the proteins/residues they are describing by referring to the other by mistake._

We have systematically checked all residue-numbering again. One possible reason for confusions on numbering of pol mu is that we did our mutations on murine pol mu, while mutations in the literature have focused on human pol mu. We have rewritten this section to be more clear throughout this entire section.

_This paper contains many significant errors or inaccuracies including, the running order of and reference to the figures, referencing key papers_

We have addressed this issue carefully. All calls to Figures have been checked. References have been checked and some additional References have been added (such as the historical ones by F.J. Bollum and colleagues in the Introduction)

... and the details described in the text is not visible at all in many of the figures.

We have re-drawn Figure 2 to make it larger. Many of the Figures have been re-drawn, using better color-coding and all of them carefully checked. The Loop1 and SD1 regions are now colored differently and systematically. The Supplementary Figures have also been re-organized and made more complete.

_This manuscript reads like a confused early draft of a paper, rather than a journal ready version, where all the key ideas have not been fully formed and joined up into a cohesive unit. In my opinion, this manuscript needs to be fully rewritten with more focus and emphasis on the insights this study provides on how Tdt performs extension at the termini of DNA, rather than presenting it as a Pol Mu story, which does a disservice to this otherwise important new structural model._

We have indeed followed the reviewer’s suggestion and we now focus the paper more on Tdt (see in particular the Title, the Abstract, the Introduction and the Discussion). Results on pol mu are regrouped in a single paragraph and a single Figure (Suppl. Figure S4). The molecular mechanism of Tdt is now discussed in detail and the importance of i) the water network and ii) identification of rare tautomers is now carefully analyzed in a new paragraph, and a new Figure (Figure 6). In the Discussion we invoke how this could also help explain the changed activity of both Tdt and pol mu in the presence of divalent transition metal ions.

We believe this is the first time that such an in-depth analysis is presented, opening the door for future more detailed computational studies of the underlying molecular mechanism.

Thank you for submitting your revised manuscript to the EMBO Journal. It has now been seen by one of the original referees (see comments below). As you will see, the referee recommends publication upon textural revision and we are happy to pursue this course upon incorporation of the
following editorial changes. Please ensure that you paper is as accessible as possible to a broad readership not necessarily expert at mechanistic detail of VDJ recombination or NHEJ.

1) After having looked at the three references suggested by referee 3, we agree that all three references (i.e. Cell Reports 5, 1108-1120; Mol. Cell 41, 221-231; Science 318, 456-459) should be included with the appropriate context in the fourth paragraph of the introduction (where ref 27 is currently mentioned) in order to ensure that the relevant literature is discussed. This is in line with our policy to mandate citation of all the directly relevant primary literature in our papers (we have no reference limit for this reason). Instead, as the referee suggests, ref 27 could be removed; if it is not removed, please adapt the context of the discussion of this reference.

2) We suggest a number of textural enhancements to render the work more broadly accessible:
   a) a key advance is to extend the concept to eukaryotes, so we suggest to mention this in the title.
   b) the title term 'DNA polX: terminal deoxynucleotidyltransferase' is not going to be accessible to the more general readership. We would instead recommend to try to focus on the novel mechanism including the bridging. At the end of the introduction you state 'Tdt has the intrinsic capacity to direct DNA synapsis preassembly and alignment' - something along these lines is more informative.
   c) similar considerations apply to the abstract: 'DNA synapsis' should be explained for a general readership at first mention. 'Cognate or non-cognate' for MH basepair will also elude many readers and I would recommend to remove this level of detail from the abstract (or to explain these terms if you consider it essential to retain them). We recommend to revise the fourth, fifth & sixth sentences thus: 'This structure reveals how two domains of Tdt serve as a mould so that a templating base can be provided in trans at a site of DNA synapsis. Two residues in loop 1 of Tdt introduce a wedge in the primer strand that reduces the MH region to only two basepairs. This data is consistent with a recent model that takes account of the sequences synthesized in vivo by Tdt based on this dinucleotide step.' We consider detail including '8kDa domain', specific residue numbers and 'Markov model' as too much specific detail for an abstract. Finally, we would remove the clause 'assist the selection of rare tautomers at the incoming base position'.
   d) in the introduction, please remove 'Historically' (second para) and change 'frustrating since' (third para) to 'is inconsistent with'. Change 'would provide much insight into' to 'is necessary to provide a detailed understanding of'. We would recommend that the 8kDa domain (5th para) would need to be introduced at first mention. Please consider that most readers will have no background knowledge of this domain.
   e) Please remove the subtitle 'Conclusion and perspective' for the 'discussion' section. It would be important to revisit the related literature systematically also in this section (see point a).

Thank you for the opportunity to consider your work for publication. I look forward to your revision incorporating the above points in the next few days in which case we will pursue publication rapidly.

Referee #3:

The previous version of the paper was very badly written, full of errors, unstructured and unfocused. In the main, these major "structural" issues have been addressed, although I still consider that it needs some editing to improve the flow of the text. The story they are trying to get across is certainly much clearer, helped by improvements to both the text and the figures, which together does the work more justice and this manuscript is now much more accessible to the reader.

However, one major issue remains regarding their exclusion of key background literature that preceded this study. Prior to their elucidation of this structure, three major studies were published reporting NHEJ intermediates of direct relevance to their findings and these are not referred to at all. Although they refer to indirectly to these studies in Ref. 27 (Pitcher et al., 2007), I've checked and this is a completely inaccurate reference. They should instead reference and discuss the following papers:


Critically, upon reading these papers it is evident that the prokaryotic NHEJ polymerases also possess a terminal transferase activity. The Doherty group have described an in trans mechanism to explain how the addition of a nucleotide to the 3' end is achieved (Brisse et al 2011) and, notably, they went on to solved the crystal structure of a catalytically-competent polymerase-DNA in trans intermediate (Brisse et al, 2013). I wonder why these studies have not been reference and discussed in detail given how directly relevant they are to the integral mechanistic fabric of their story? This imbalance must be addressed in any revision of this paper.

2nd Revision - authors' response 08 January 2015

Please find attached our point-by-point answers to your suggestions.

1) After having looked at the three references suggested by referee 3, we agree that all three references (i.e. Cell Reports 5, 1108-1120; Mol. Cell 41, 221-231; Science 318, 456-459) should be included with the appropriate context in the fourth paragraph of the introduction (where ref 27 is currently mentioned) in order to ensure that the relevant literature is discussed. This is in line with our policy to mandate citation of all the directly relevant primary literature in our papers (we have no reference limit for this reason). Instead, as the referee suggests, ref 27 could be removed; if it is not removed, please adapt the context of the discussion of this reference.

Answer: The referee is absolutely correct. All three references should be included in the Introduction and the Discussion. This is now done (Ref 27-29).

2) We suggest a number of textural enhancements to render the work more broadly accessible:

a) a key advance is to extend the concept to eukaryotes, so we suggest to mention this in the title.

Answer: We propose the following new title:

Structural basis for DNA synapsis assembly and alignment by Tdt, a eukaryotic DNA polymerase (polX)

Or (alternatively)

Structural basis for a novel mechanism of DNA bridging and alignment in eukaryotic DSB DNA repair

b) the title term 'DNA polX: terminal deoxyribonucleotidyltransferase' is not going to be accessible to the more general readership. We would instead recommend to try to focus on the novel mechanism including the bridging. At the end of the introduction you state 'Tdt has the intrinsic capacity to direct DNA synapsis preassembly and alignment' - something along these lines is more informative.

Answer: See above (perhaps the second proposed title?)

c) similar considerations apply to the abstract: 'DNA synapsis' should be explained for a general readership at first mention. 'Cognate or non-cognate' for MH basepair will also elude many readers
and I would recommend to remove this level of detail from the abstract (or to explain these terms if you consider it essential to retain them). We recommend to revise the fourth, fifth & sixth sentences thus: 'This structure reveals how two domains of Tdt serve as a mould so that a templating base can be provided in trans at a site of DNA synapsis. Two residues in loop 1 of Tdt introduce a wedge in the primer strand that reduces the MH region to only two basepairs. This data is consistent with a recent model that takes account of the sequences synthesized in vivo by Tdt based on this dinucleotide step'. We consider detail including '8kDa domain', specific residue numbers and 'Markov model' as too much specific detail for an abstract. Finally, we would remove the clause 'assist the selection of rare tautomers at the incoming base position'.

Answer: Here is the new Abstract:

Eukaryotic DNA polymerase mu of the polX family can promote the association of the two 3'-protruding ends of a DNA double-strand break (DSB) being repaired (DNA synapsis) even in the absence of the core Non-Homologous End Joining (NHEJ) machinery. Here we show that terminal deoxynucleotidyltransferase (TdT), a closely-related polX involved in V(D)J recombination, has the same property. We solved its crystal structure with an annealed DNA synapsis containing one micro-homology (MH) base-pair and one nascent base-pair. This structure reveals how the N-terminal domain and Loop 1 of Tdt cooperate for bridging the two DNA ends, providing a templating base in trans and limiting the MH-search region to only two base-pairs. A network of ordered water molecules is proposed to assist the incorporation of any nucleotide independently of the in trans templating base. This data is consistent with a recent model that explains the statistics of sequences synthesized in vivo by Tdt based solely on this dinucleotide step. Site-directed mutagenesis and functional tests suggest that this structural model is also valid for pol mu during NHEJ.

d) in the introduction, please remove 'Historically' (second para) and change 'frustrating since' (third para) to 'is inconsistent with'. Change 'would provide much insight into' to 'is necessary to provide a detailed understanding of'. We would recommend that the 8kDa domain (5th para) would need to be introduced at first mention. Please consider that most readers will have no background knowledge of this domain.

Answer: OK, this is now done

e) Please remove the subtitle 'Conclusion and perspective' for the 'discussion' section. It would be important to revisit the related literature systematically also in this section (see point a).

Answer: OK, the subtitle has been removed and the last sentence rephrased. We are unaware of related literature pointing to the evolution of Tdt from pol mu. Or do you suggest to provide the reader with a general review on the evolution of the adaptive immune system (like Flajnik and Kasahara, Nature Reviews Genetics, 2010)? This of course could be done if you think it is necessary.

4th Editorial Decision 09 January 2015

I am very pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

Thank you for taking the time to incorporate the changes into the final version of the manuscript, which in our view renders this interesting paper more broadly accessible. We suggest to choose the second title ('Structural basis for a novel mechanism of DNA bridging and alignment in eukaryotic DSB DNA repair').