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Replication through an Abasic DNA Lesion: Structural Basis for Adenine Selectivity

Samra Obeid, Nina Blatter, Ramon Kranaster, Andreas Schnur, Kay Diederichs, Wolfram Welte and Andreas Marx

Corresponding author: Andreas Marx, Universitaet Konstanz

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(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 22 January 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal. First, I would like to apologise for the delay in getting back to you with a decision. This was caused by difficulties in finding suitable and willing referees for this manuscript during the past Christmas holiday season.

Your manuscript has now finally been seen by three referees whose comments are shown below. As you will see all three referees are supportive regarding publication of the study here in principle. Still, referee 1 raises certain concerns that should be addressed or responded to in an adequate manner. Taking together all issues raised we will be able to consider a suitably revised manuscript that addresses the referees' comments. I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

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

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor

The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

The manuscript by Obeid et al reports kinetic and structural analyses of how KlenTaq DNA polymerase extends a primer opposite an abasic site or without a template at all. The kinetic measurements of WT and mutant proteins suggest of the importance of a conserved Tyr (Y671) in stabilizing the incoming adenine in addition to stabilizing the replicating base pair and preventing template slippage. Overall the manuscript contains several interesting and important observations, particularly the preference of pyrimidines instead of purines in the template-independent primer extension by the Y671W mutant protein. If the authors can avoid over-stating and over-interpreting their experimental data and tone down the overarching paradigm shifting conclusion, this is potentially suitable for publication in EMBO. The following two points, which the authors strongly advocate, don't mesh well with the vast amount of published data. This is not to say that the authors are wrong, but they need additional experimental data to support such overarching statements.

1. The authors suggest that the preference for incorporating dA (the A-rule) is because of the conserved Tyr (Y671) and not because of the better base stacking property of the adenine base as the polymerase field believes. This might be true. But their data only indicate that in addition to the A-rule, Y671 may strengthen the preference for A rather than replacing the A-rule. There is a vast collection of data supporting the A-rule in the last 20 plus years. For instance, RNA polymerase II obeys the A-rule without a conserved Tyr (Damsma et al, NSMB, 2007, 14, p1127). The same is true for the RB69 DNA polymerase. A Tyr is conserved in B family polymerases, but it doesn't form a hydrogen bond with an incoming purine opposite an abasic templating nucleotide (Freisinger E et al., EMBO 2004). In contrast to the authors's suggestion that several X and Y family polymerases don't obey the A-rule, the published data cited by the authors indicate the opposite. Most X and Y family polymerases do obey the A-rule, but they have additional mechanisms to bypass lesions. Sometimes the alternative mechanisms may be more efficient than the A-rule. Y671 is indeed conserved through out the A family. But as the authors' own data show replacing it by Ala reduces the catalytic efficiency by 5000 folds, the primary reason for its conservation is likely for normal replication rather than bypassing an abasic lesion.

2. The authors also strongly argue that the incoming nucleotide ddATP doesn't base stack with the primer strand. To support such a statement, experiments are needed. One is to use a primer one nucleotide shorter at the 3' end, which cannot form base stack with dNTP, and see if the incoming ddATP remains bound to the polymerase. Alternatively, an adenine analog that has strong stacking propensity but no hydrogen bonding potential, e.g. 4-fluoroindole, wouldn't be incorporated if the authors' conclusion is correct.

Technically, the following point should be clarified.
3. The structure reported in this manuscript appears to be more similar to the "open" state rather than being "more open than the closed state" as shown in Fig. 2c. In fact, Y671 in the ternary complex structure with an abasic lesion appears to be located at a very similar position as Y671 in the binary open state. The authors should make a comparison with the binary complex structure (PDB: 4ktq). If Y671 is indeed located at the similar position, the observed conformation of ddATP opposite the abasic site may be specific to KlenTaq rather than universal.

Referee #2 (Remarks to the Author):

This article presents 2 new structures of Taq polymerase co-crystallized with a DNA duplex whose template strand contains an abasic site in the instructing position.
The authors are able to explain in structural terms the preference for incorporating dATP (the so-called "A-rule") and highlight the role of Tyr671 in this process. They verify their prediction by studying the functional properties of mutant Y671W.

I have the following comments to improve the manuscript:

Abstract (p. 3)
-mention that Y671 is in the well-known motif B
-mention the results of the Y->W mutation here

Introduction (p. 4)
-Need to define what family A and B and X are (Ito and Braithwaite, 1991 or 1993 for the original definition); see also Patel and Loeb, 2001 for a good review.

Discussion (p. 14) and Fig. 5D
-Why are there so few sequences displayed? What is the criterion used for selecting just these ones? Why is there no numbering?
-Why not mention that this is actually Motif B, known since 1990 (Delarue et al., Prot. Engng. 1990)?
-There is a large body of knowledge on structure-function relationship for Taq polymerase and other pol I that is completely ignored here, especially all the mutants that have been characterized and discussed in a structural perspective by L. Loeb and coll.
See for instance Patel et al., J. Mol. Biol., 2001 and Loh et al., JBC 2007. This should be mentioned and discussed in the Discussion section.

Minor Comments

-Abstract (p. 3) Despite these lesions -> Although these lesions
-Materials and Methods (p. 19) "The coordination spheres verified the presence of Mg++ ion..." What does this mean?
-Why is there only one Mg++ ion in the active site (Table II) instead of the expected two? Please discuss.

Referee #3 (Remarks to the Author):

Obeid et al. are presenting data that solve the longstanding issue why DNA polymerases from the A and B families follow the so-called A-rule when they encounter an abasic site on the DNA. This is a very important discovery, which is of great interest to the scientific community working on the topics of DNA repair as well as those who try to understand hypermutations of immunoglobulin genes. To achieve this the authors did a marvelous combination of structural work combined with biochemistry (product analysis after DNA synthesis, transient kinetic analysis pre-steady state kinetic analysis). This is the first ternary structure of a DNA polymerase that is (i) complexed with an abasic site and (ii) an incoming A opposite the lesion. Structural and biochemical analysis strongly suggested that the amino-acid Tyrosine-671 is placed opposite the in coming dideoxy ATP, thus being placed at the where the templating base would be, suggesting "amino-acid templating". This Tyrosine is conserved in many DNA polymerase form the A and B-Family that follow the A-rule when encountering an abasic site. Moreover, the presentated data with Typtophan in place of Tyrosine 671, which then can perform "amino-acid templating" as well and mimicking a purine. Finally, the fact that all DNA polymerases theta have this Tyrosine might be an explanation for the involvement in hypermutation of Ig genes (see suggestion below). Overall this is a convincing study, which is of great general interest.

Suggestions from this referee are:

1. The introduction in the Abstract the term "amino-acid templating" would make the finding more appealing.
2. In the Discussion I miss the Tryptophan results and a more generalized statement about the two amino-acids that can mimick purins and pyrimidines, respectively.
3. Also in the discussion DNA polymerase theta should be mentioned first as a DNA polymerase having the A-rule (Seki et al EMBO, 23, 4484, 2004) and that this A-rule property might be of advantage for physiological mutation in somatic hypermutation of Ig genes (Masuda et al. J. Biol Chem 282, 17387, 2007).
4. Table II could be transferred to Supplementary Information

5. Figure 5 B and C: add wt and Y671W, respectively
6. Figure 5 D: Transfer Thermus aquation after C. elegans, in order to keep the evolution. Suggestion: A: Eukaroytes; B: Arechaea; C: Bacteria. The D. melanogaster mus308 protein is homologous to DNA polymerase theta: Does this enzyme also has a this conserved Tyrosine? If yes I would put it into the list between Gallus gallus and C.elegans
7. Page 20, Figure 6: Typo: difference instead of different

1st Revision - authors' response 25 February 2010

We gratefully acknowledge the constructive comments by the referees. We have addressed the referee comments as follows.

Referee #1:

"...If the authors can avoid over-stating and over-interpreting their experimental data and tone down the overarching paradigm shifting conclusion, this is potentially suitable for publication in EMBO..."

We apologize for any over-stating which was certainly unintended. We have made numerous changes that hopefully meet the referee's suggestions.

"1. ...Most X and Y family polymerases do obey the A-rule, but they have additional mechanisms to bypass lesions. Sometimes the alternative mechanisms may be more efficient than the A-rule..."

DNA polymerases from families X and Y use different, sequence depending mechanisms that are at least as important than the A-rule when bypassing abasic sites, for example some quotations of references cited in the manuscript:

a) Ling H et al: Mol Cell 2004, 13: 751-762, written in the abstract: "We have determined crystal structures of Dpo4 with five different abasic site-containing DNA substrates and find that translesion synthesis is template directed with the abasic site looped out and the incoming nucleotide is opposite the base 5' to the lesion"

b) Nair DT et al. Structure 2009, 17: 530-537: "Human DNA polymerase iota (Pol iota) is one of the few DNA Pols that does not obey the A-rule for inserting nucleotides opposite the abasic site"

c) Beard WA, et al. J Biol Chem 2009, 284: 31680-31689, page 31681, left column: "When filling a 5-nucleotide DNA gap with a central (position 3) tetrahydrofuran lesion, pol beta does not obey the A-rule and inserts a nucleotide complementary to the first downstream templating base";

d) see as well the title of Efrati E, et al. J Biol Chem 1997, 272: 2559-2569: "Abasic translesion synthesis by DNA polymerase beta violates the "A-rule".

In order to meet the referee's suggestion we now state that the A-rule is sometimes followed by X and Y DNA polymerases depending on the sequence context. The revised text is as follows:

Page 5, last chapter we now state: "DNA polymerases from these families use different, sequence depending mechanisms that might compete with the A-rule when bypassing abasic sites. However, DNA polymerases from family A and B, which are involved in the majority of DNA synthesis in DNA replication and repair, follow the A-rule when bypassing abasic sites."

Page 14:, first chapter: "On the other hand, DNA polymerases from other sequence families like X and Y use different, sequence depending mechanisms that might compete with the A-rule."

"Y671 is indeed conserved through out the A family. But as the authors' own data show replacing it by Ala reduces the catalytic efficiency by 5000 folds, the primary reason for its conservation is likely for normal replication rather than bypassing an abasic lesion."

In the manuscript we state that Y671 is conserved throughout evolution in the A family of DNA polymerases. We agree with the referee that this does not imply that this residue is exclusively responsible for abasic site bypass. However, we never made this point in the manuscript; we just referred to its role in lesion bypass. In fact, we stated and state: "Previous studies on Tyr671 of Taq DNA polymerase and the corresponding Tyr766 residue in DNA polymerase I from E. coli have shown the involvement of this tyrosine residue in discriminating ribonucleotides and non-canonical nucleotides (Suzuki et al, 1996; Minnick et al, 1999; Bell et al, 1997)." This text is now in the "Discussion" section (page 15 of the revised manuscript). Since we have extended the discussion on this topic (see comment of referee #2) we are convinced that it is now clearer that Y671 has a broad role in enzyme function.

2. The authors also strongly argue that the incoming nucleotide ddATP doesn't base stack with the primer strand. To support such a statement, experiments are needed.
One is to use a primer one nucleotide shorter at the 3' end, which cannot form base stack with dNTP, and see if the incoming ddATP remains bound to the polymerase.

Although we appreciate the referee's view, due to the following reasons we are convinced that the results of the suggested experiment will not yield any further insight along the depicted lines:

a) We clearly show and discuss in the manuscript the interactions between the nucleobase at the 3' primer terminus and the incoming nucleotide by a hydrogen bond network (see Figure 3A, B). Removal of this nucleobase would delete these interactions and thus, would not allow drawing a simple conclusion whether an anticipated decline of incorporation efficiency would stem from loss of base stacking or the altered hydrogen bond network.
b) If we shorten the primer by one entire nucleotide, as suggested by the referee, the DNA polymerase would bind the DNA substrate in a different manner: The shortened 3' primer terminus would bind next to the active site and an undamaged nucleotide in the template would direct for nucleotide incorporation. The following scheme should hopefully illustrate this point:

```
Template    3'---G T F ----5'
Primer      5'---C A `ddATP

Template          3'---G T F ----5'
Shortened Primer  5'---C `ddATP     (templating nucleotide in bold)
```

Thus, one would rather investigate incorporation opposite an undamaged site than opposite the abasic site.

Alternatively, an adenine analog that has strong stacking propensity but no hydrogen bonding potential, e.g. 4-fluoroindole, wouldn't be incorporated if the authors' conclusion is correct.

This kind of experiment has been done many times by various groups (e.g. E.T. Kool). It has been shown numerous times that analogues with strong stacking abilities will be incorporated by DNA polymerases opposite abasic sites and sometimes even more efficiently than A.
We doubt that this is a proof that stacking is the sole driving force for nucleotide selection opposite the abasic sites. The situation might not be as "black and white" as the referee implies and instead stacking ability might overrule other mechanisms.
In the manuscript we describe results that do not show any evidence for the participation of stacking in nucleotide selection opposite abasic sites. This is a direct experimental result that the referee may have overlooked.

To address these concerns of the referee we have made the following changes:

a) In order to further collect experimental evidence for the importance of the interaction between the hydroxyl group of Tyr671 and N3 of adenine in the incoming nucleotide in incorporation opposite an abasic site, we have conducted additional experiments using an adenine nucleotide analogue, namely 3-deaza-2'-deoxyadenosine-5'-triphosphate (d3DATP) in primer extension studies (data in Table I, Supplementary Figure S4, discussion of the results on page 11). We found that incorporation efficiency of the analogue was decreased by 54-fold compared to the natural substrate

(Table I). Interestingly, incorporation efficiency of d3DAMP opposite the abasic site was decreased even further (more than 90-fold) compared to dAMP incorporation opposite the lesion. These studies further highlight the importance of the interaction of the hydroxyl group of Tyr671 and N3-adenine in the incoming nucleotide. The discussion of this topic is included in the manuscript (see page 11: "In order to further investigate the interaction between the hydroxyl group of Tyr671 and N3 of adenine in the incoming nucleotide an adenine analogue in which the N3 is substituted by a non-polar CH (namely 3-deaza-2'-deoxyadenosine-5'-triphosphate (d3DATP)) was employed in primer extension studies (Supplementary Figure S4). These kinds of purine analogues were intensively studied in order to access the role of N3-hydrogen bond acceptors in DNA polymerase function (Beckman et al, 2007; Cavanaugh et al, 2009; McCain et al, 2005; Meyer et al, 2004; Moore et al. 2004; Spratt 2001; Trostler et al, 2009; Washington et al, 2003; Wolfle et al, 2005). In accordance with earlier reports (Trostler et al, 2009) we found that incorporation efficiency of the analogue was decreased by 54-fold compared to the natural substrate (Table I). Interestingly, incorporation efficiency of d3DAMP opposite the abasic site was decreased by more than 90-fold compared to dAMP incorporation opposite the lesion. These studies further highlight the importance of the interaction of the hydroxyl group of Tyr671 and N3-adenine in the incoming nucleotide."

b) Based on the present data we do not see any evidence for the participation of stacking in A selection. However, as the referee suggests, we cannot exclude the participation of base stacking in processes that are not disclosed by our structures. We discuss this topic in the revised manuscript (see page 16 -17: "However, our structural data presented here do not provide any evidence for stacking interactions of the incoming ddATP with the primer template terminus and rather indicate that amino acid templating applies for selection of adenine for DNA polymerases from sequence family A. This conclusion is corroborated by the findings that a) no significant stacking of the incoming ddATP to the -system of the DNA duplexes is observed in the structures and b) mutation of the templating tyrosine to tryptophan switches the selectivity for purines to pyrimidines. Nevertheless, it can not be excluded that stacking interactions might play a role in processes that are unresolved by the structural data e.g. at states prior or later on the reaction coordinate than the one resolved here.")

3. The structure reported in this manuscript appears to be more similar to the "open" state rather than being "more open than the closed state" as shown in Fig. 2c. In fact, Y671 in the ternary complex structure with an abasic lesion appears to be located at a very similar position as Y671 in the binary open state. The authors should make a comparison with the binary complex structure (PDB: 4ktq). If Y671 is indeed located at the similar position, the observed conformation of ddATP opposite the abasic site may be specific to KlenTaq rather than universal.

We are grateful for this comment and have done the requested comparison. However, the structure reported here is not in "the "open" state rather than being "more open than the closed state"" as suggested by this referee. The crucial Y671 is located differently in the herein reported structures as in 4KTQ. We mention this in the text of the revised manuscript (pages 8) and added a figure in the supporting information (Figure S2) that corroborates this conclusion.

Referee #2:

Abstract (p. 3)
-mention that Y671 is in the well-known motif B
-mention the results of the Y->W mutation here

The suggested changes are included in the revised manuscript

Introduction (p. 4)
-Need to define what family A and B and X are (Ito and Braithwaite, 1991 or 1993 for the original definition); see also Patel and Loeb, 2001 for a good review.

The suggested changes are included in the revised manuscript as well as the references cited.

Discussion (p. 14) and Fig. 5D

-Why are there so few sequences displayed? What is the criterion used for selecting just these ones? Why is there no numbering?

We have now significantly extended the list that now exceeds the one published by Patel and Loeb, 2001 (see Figure 5D).

-Why not mention that this is actually Motif B, known since 1990 (Delarue et al., Prot. Engng. 1990)?

We now mention this fact (see page 15).

-There is a large body of knowledge on structure-function relationship for Taq polymerase and other pol I that is completely ignored here, especially all the mutants that have been characterized and discussed in a structural perspective by L. Loeb and coll. See for instance Patel et al., J. Mol. Biol., 2001 and Loh et al., JBC 2007. This should be mentioned and discussed in the Discussion section.

A brief discussion was already included in the original manuscript in the "Results" section. The discussion is now extended and embedded in the "Discussion" section. All references quoted by the referee are now cited. For the revised text see page 15, starting with: "Sequence family A members Taq DNA polymerase and E. coli DNA polymerase I have been studied extensively..."

-Abstract (p. 3) Despite these lesions -> Although these lesions

Changed in the revised manuscript

-Materials and Methods (p. 19) "The coordination spheres verified the presence of Mg++ ion..." What does this mean?

We have changed the sentence and hope this point is now clearer (see page 21: "The Mg2+ ion in the enzyme active sites of both structures was octahedrally coordinated characteristic for a bivalent metal ion.")

-Why is there only one Mg++ ion in the active site (Table II) instead of the expected two? Please discuss.

We are very grateful for mentioning this point. A discussion is now included on page 17 (Moreover the location of the -phosphate of the incoming ddATP in KlenTaqAP obviates tight complexation of a Mg2+ ion by coordination together with D785 as found in the KlenTaq structure (Figure 3C, D) and, as a consequence, only one Mg2+ ion was found in the active site.).

Referee #3:

1. The introduction in the Abstract the term "amino-acid templating" would make the finding more appealing.

We changed the manuscript accordingly.

2. In the Discussion I miss the Tryptophan results and a more generalized statement about the two amino-acids that can mimick purins and pyrimidines, respectively.

We have added this discussion (see page 15: "That amino acid templating of Tyr761 is involved in selecting a purine nucleotide is further corroborated by the finding that the respective tryptophan mutant lost its purine specificity and instead incorporates pyrimidines more selectively. The result can be rationalized by assuming that the bicyclic indole consisting of a six-membered ring fused to a five-membered ring will mimic the approximate size and shape of a purine, and as a consequence, will direct for pyrimidine incorporation due to enhanced geometric fit to the enzyme active site. These findings corroborate the model of amino acid templating for abasic site bypass and highlight

the importance of geometric fit of the substrates to the active site of the enzyme for DNA polymerase activity. In agreement with these observations, it has been shown that DNA polymerases are able to process non-natural nucleobase surrogates placed in template strands that mimic the shape and size of the natural nucleobase but have decreased hydrogen bonding capabilities (Kool 2002).)".

3. Also in the discussion DNA polymerase theta should be mentioned first as a DNA polymerase having the A-rule (Seki et al EMBO, 23, 4484, 2004) and that this A-rule property might be of advantage for physiological mutation in somatic hypermutation of Ig genes (Masuda et al. J. Biol Chem 282, 17387, 2007).

We are particularly grateful for this suggestion and added a respective discussion in the revised manuscript (see page 17: "However, recently it was reported that the A-family member human DNA polymerase is able to bypass abasic sites with high activity and thereby incorporates dAMP opposite the lesion preferentially (Seki et al, 2004). DNA polymerase is involved in the diversification of immunoglobulin (Ig) genes during somatic hypermutation (Masuda et al, 2007). Hence, the efficient but intrinsically error-prone bypass of abasic sites by DNA polymerase following the A-rule might be of advantage for physiological mutation in somatic hypermutation of Ig genes.").

4. Table II could be transferred to Supplementary Information

We changed the manuscript accordingly.

5. Figure 5 B and C: add wt and Y671W, respectively

We changed the figure accordingly.

6. Figure 5 D: Transfer Thermus aquation after C. elegans, in order to keep the evolution. Suggestion: A: Eukaroytes; B: Arechaea; C: Bacteria. The D. melanogaster mus308 protein is homologous to DNA polymerase theta: Does this enzyme also has a this conserved Tyrosine? If yes I would put it into the list between Gallus gallus and C.elegans

We changed the manuscript accordingly.

7. Page 20, Figure 6: Typo: difference instead of different

We corrected the typo.

2nd Editorial Decision 13 March 2010

Thank you for sending us your revised manuscript. Our original referee 1 has now seen it again, and you will be pleased to learn that in his/her view you have addressed the criticisms in a satisfactory manner, and that the paper will therefore be publishable in The EMBO Journal. Before this will happen, however, I was wondering whether you would like to consider addressing the minor issues suggested by referee 1 (see below). Furthermore, I need to ask you to include the PDB accession details for the novel structures into the main body of the text of your paper.

Please let us have a suitably amended manuscript as soon as possible. I will then formally accept the manuscript.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

In the revised version, the authors have addressed most of the concerns raised. In conclusion, the authors have shown that, in addition to the A-rule, the A-family polymerases use the conserved Tyr (Y671 in Taq DNA pol) to selectively incorporate dATP in template-independent primer extension. These data do not discredit the A rule, and the preference of inserting dA over the other three dNTP by Y671F mutant indicates that the A-rule still works. Furthermore, none of their mutations result in more efficient template-independent nucleotide incorporation than WT with dA. Secondly, the primary role of Y671 is for normal high-fidelity DNA synthesis as their mutagenic and kinetic data and the previously published data clearly indicate. Their new results show that Y671 has a secondary role, which is to facilitate dA incorporation in the absence of a template base. The manuscript is suitable for publication in EMBO. With attention to the above two points and the writing style changes listed below, the authors can enhance its scholarly quality.

1. The first sentence in Abstract, "Abasic sites ... lead to mutations commonly found in human cancers" needs clinical data to support, if it is true.
2. The third sentence in Abstract, "we present X-ray structures of DNA polymerases ..." is inaccurate. There is one polymerase, TaqKlen only. It is helpful to clarify which polymerase it is here.
3. In the Abstract, the authors should clarify the primary and secondary role of the conserved Tyr instead of stating the secondary role only. Y671 in TaqKlen, and its counterpart in E. coli Pol I and T7 DNA Pol have been extensively studied and shown to greatly affect the efficiency and accuracy of DNA synthesis.

2nd Revision - authors' response 15 March 2010

We have addressed the editorial and referee comments as follows.

Editor:

...Furthermore, I need to ask you to include the PDB accession details for the novel structures into the main body of the text of your paper...
The PDB entry codes are added to the Materials and method section (see under: Accession numbers: The coordinates and structure factors have been deposited in the Protein Data Bank with the accession numbers 3LWL and 3LWM.

Referee #1:

1. The first sentence in Abstract, "Abasic sites ... lead to mutations commonly found in human cancers" needs clinical data to support, if it is true.
EMBO J does not allow citations within the abstract. In the manuscript text body we cite a review that connects mutations and cancer (see Hoeijmakers, 2001). Since there is a large body of literature that connects the onset of cancer to mutations we are convinced that citing a review is best for the purpose.

2. The third sentence in Abstract, "we present X-ray structures of DNA polymerases ..." is inaccurate. There is one polymerase, TaqKlen only. It is helpful to clarify which polymerase it is here.
We have changed this accordingly by writing "a DNA polymerase". There is no space to state more details and stay within the 175 words limit of the abstract.

3. In the Abstract, the authors should clarify the primary and secondary role of the conserved Tyr instead of stating the secondary role only. Y671 in TaqKlen, and its counterpart in E. coli Pol I and T7 DNA Pol have been extensively studied and shown to greatly affect the efficiency and accuracy of DNA synthesis.

We have added the term "functionally-important tyrosine" in the abstract. Within the abstract only 175 words are allowed. Thus, there is simply no space to cover more about the roles of the Tyr that have been described already. We simply focus on that what is new and has not been described before.

