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Peer Review Information

Journal: Nature Structural & Molecular Biology
Manuscript Title: Catalytic and non-catalytic functions of DNA polymerase kappa in translesion DNA synthesis
Corresponding author name(s): Julien Duxin

Reviewer Comments & Decisions:

Decision Letter, initial version:

Message: 5th Oct 2023

Dear Dr. Duxin,

Thank you again for submitting your manuscript "Catalytic and non-catalytic functions of DNA polymerase kappa in translesion DNA synthesis". I apologise for the delay in responding, which resulted from the difficulty in timely obtaining suitable referee reports. Nevertheless, we now have comments (below) from the 3 reviewers who evaluated your paper. In light of these reports, we remain interested in your study and would like to see your response to the comments of the referees, in the form of a revised manuscript.

You will see that all referees find the novel mechanistic findings interesting and appreciate the clarity of the presentation of such results. However, the experts raise important concerns that need to be addressed in a revised manuscript. With respect to the mechanistic insight, reviewer #3 poses several relevant questions and provides ample experimental guidance about (i) validating the purported (lack of) interplay between Polkappa with Rev1, depending on the context, an issue raised by all reviewers (end of point 8 of reviewer #1 and point 4 of reviewer #2), (ii) expanding the UV data to more relevant damage, such as DPC or abasic site lesion-containing plasmids, to demonstrate the interdependency between PolKappa and PolZeta, and (iii) either presenting more evidence that validate the mutually exclusive nature of the presented models in figure 7 or amending the models.

Furthermore, we editorially agree that expanding the data to human cells by using Polkappa mutants and relevant DNA damage, as recommended in point 8 of reviewer #1, would significantly boost the functional value of the manuscript. You will also note that reviewer #1, and to a lesser extent reviewer #2, bring up technical issues that require addressing. Amongst these we highlight the need to convincingly respond to the concern about CHROMASS. Finally, all experts request at certain points toning down statements and occasional clarifications to improve the flow of the story.

Please be sure to address/respond to all concerns of the referees in full in a point-by-point response and highlight all changes in the revised manuscript text file.

We are committed to providing a fair and constructive peer-review process. Do not hesitate to contact us if there are specific requests from the reviewers that you believe are technically impossible or unlikely to yield a meaningful outcome.

We appreciate the requested revisions are extensive. We thus expect to see your revised manuscript within 6 months. If you cannot send it within this time, please let us know. We will be happy to consider your revision as long as nothing similar has been accepted for publication at NSMB or published elsewhere. Should your manuscript be substantially delayed without notifying us in advance and your article is eventually published, the received date would be that of the revised, not the original, version.

As you already know, we put great emphasis on ensuring that the methods and statistics reported in our papers are correct and accurate. As such, if there are any changes that should be reported, please submit an updated version of the Reporting Summary along with your revision.

Reporting Summary:
https://www.nature.com/documents/nr-reporting-summary.pdf

Please note that the form is a dynamic 'smart pdf' and must therefore be downloaded and completed in Adobe Reader.

When submitting the revised version of your manuscript, please pay close attention to our href="https://www.nature.com/nature-portfolio/editorial-policies/image-integrity">Digital Image Integrity Guidelines. and to the following points below:

-- that unprocessed scans are clearly labelled and match the gels and western blots presented in figures.
-- that control panels for gels and western blots are appropriately described as loading on sample processing controls
-- all images in the paper are checked for duplication of panels and for splicing of gel lanes.

Finally, please ensure that you retain unprocessed data and metadata files after publication, ideally archiving data in perpetuity, as these may be requested during the peer review and production process or after publication if any issues arise.

SOURCE DATA: we urge authors to provide, in tabular form, the data underlying the graphical representations used in figures. This is to further increase transparency in data reporting, as detailed in this editorial (http://www.nature.com/nsmb/journal/v22/n10/full/nsmb.3110.html). Spreadsheets can be submitted in excel format. Only one (1) file per figure is permitted; thus, for multi-paneled figures, the source data for each panel should be clearly labeled in the Excel file; alternately the data can be provided as multiple, clearly labeled sheets in an Excel file. When submitting files, the title field should indicate which figure the source data pertains to. We encourage our authors to provide source data at the revision stage, so that they are part of the peer-review process.

While we encourage the use of color in preparing figures, please note that this will incur a charge to partially defray the cost of printing. Information about color charges can be found at http://www.nature.com/nsmb/authors/submit/index.html#costs

We require deposition of coordinates (and, in the case of crystal structures, structure factors) into the Protein Data Bank with the designation of immediate release upon publication (HPUB). Electron microscopy-derived density maps and coordinate data must be deposited in EMDB and released upon publication. Deposition and immediate release of NMR chemical shift assignments are highly encouraged. Deposition of deep sequencing and microarray data is mandatory, and the datasets must be released prior to or upon publication. To avoid delays in publication, dataset accession numbers must be supplied with the final accepted manuscript and appropriate release dates must be indicated at the galley proof stage. Please find the complete NRG policies on data availability at http://www.nature.com/authors/policies/availability.html.

Nature Structural & Molecular Biology is committed to improving transparency in authorship. As part of our efforts in this direction, we are now requesting that all authors identified as 'corresponding author' on published papers create and link their Open Researcher and Contributor Identifier (ORCID) with their account on the Manuscript Tracking System (MTS), prior to acceptance. This applies to primary research papers only. ORCID helps the scientific community achieve unambiguous attribution of all scholarly contributions. You can create and link your ORCID from the home page of the MTS by clicking on 'Modify my Springer Nature account'. For more information please visit please visit www.springernature.com/orcid.

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Note: This URL links to your confidential home page and associated information about manuscripts you may have submitted, or that you are reviewing for us. If you wish to forward this email to co-authors, please delete the link to your homepage.

We look forward to seeing the revised manuscript and thank you for the opportunity to review your work.

Sincerely,

Dimitris Typas
Associate Editor
Nature Structural & Molecular Biology
ORCID: 0000-0002-8737-1319

Referee expertise:

Referee #1: DNA damage tolerance, TLS mechanisms

Referee #2: DNA damage tolerance and TLS mechanisms

Referee #3: Xenopus egg extracts and DNA damage

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
This manuscript describes findings related to the function of the translesion DNA synthesis (TLS) polymerase k (Polk) in the bypass of minor and major grooves DNA lesions. Data have been obtained first in mammalian cells by measuring their sensitivity to a specific minor groove DNA lesion generated by exposing cells to Illudin S, and mostly using protein extracts derived from eggs of the frog Xenopus laevis that are able to recapitulate TLS processes in vitro on a plasmid DNA template bearing a single DNA lesion, as well as on UV-C irradiated chromatin. The authors describe catalytic and non-catalytic functions of Polk using a variety of Polk mutants and define that the Polk non-catalytic role is involved in the bypass of major groove DNA lesions, such as DNA-Protein crosslinks (DPC). These findings shed light on previous observations showing a non-catalytic function of Polk in mediating bypass of certain DNA lesions, such as those generated by hydrogen peroxide or menadione (Kanemaru et al., 2015). This latest finding constitutes the more novel result of the manuscript, since it was already known that Polk is involved in the bypass of minor groove DNA lesions. However, authors characterize in more detail this process, in particular in Xenopus extracts, and highlight the importance of Polk as a probable scaffolding factor mediating interaction with two other TLS pols, Rev1 and Pol zeta, corroborating previous in vitro findings (Xie et al., 2012). Overall the work is well executed and well presented, although more data are required to strengthen the major claims of the manuscript, as well as addition of several important controls. Unfortunately, the detection of a possible PolK-Rev1-Polzeta complex is not addressed. One major technical concern is related to the experiments involving detection of chromatin-bound proteins by mass spectrometry (also known as CHROMASS approach, see comments appended below) and the conclusions drawn from this approach.

Specific comments
1. The CRISP/CAS9 tailing screen in mammalian cells does not fit in fig 1 since there is no follow up of these findings in mammalian cells. This could be moved to Fig. 6, to corroborate findings observed in Xenopus (see also point 7).
2. Specificity of the antibodies raised against Polk, Rev3, HMCES and FancA must be shown. Authors must show western blot performed with sera before immunization and after immunization and as such demonstrate the specificity of the polypeptide recognized by each antibody and identify the non-specific polypeptides recognized by the serum. This is particularly important for HMCES and Polk antibodies. HMCES antibodies are poorly described in the reference provided by the authors (Semlow et al., 2022). Regarding western blots with Polk antibody, these do not show always the same pattern (compare Fig. 1F with Fig. 6D-E and Fig. S2C for example). It is known that Polk is monoubiqutinated. Can the allegedly non-specific polypeptide recognized by this antibody in Fig. 1F (marked with a star) corresponds to this Polk form, which by the way is not depleted by the antibody? Authors must clarify this point which could be critical in order to interpret the results, since one my argue that the Pol k antibody raised by the authors does not remove the ubiquitinated form of Polk.
3. Further, authors must show the purity of the recombinant proteins they have used to reconstitute depleted extracts.
4. Regarding the western blots showing depletion with antibodies, they miss basic loading controls to ascertain that equal amounts of proteins were present in each lane.

5. Fig. 1G shows that Polk depletion produces a similar outcome than E1 inhibition. But I do not see how one can conclude that this is TLS. Although the authors show that this process depends upon Polk catalytic activity, this could well be DNA repair. Indeed, Polk has been previously involved in DNA repair (Ogi et al., 2006). I reckon that in order to be formal here, sequencing of the reaction products is required, so to demonstrate a real TLS event.
6. Second section of results, although the authors show that the Polk activity required to rescue replication stalling is dependent upon one of its ubiquitin binding domains (UBZ2), thus concluding that it depends upon PCNA monoubiquitnation, here a formal demonstration is required showing that addition of a non-ubiquitinatable PCNA (K164R mutant) to the reaction generates the same result. Further, a formal demonstration that Polk wild-type and not Polk mutated in the Rev1 binding domain (RIR) physically interacts with Rev1 is required.
7. Regarding the chromass experiment (Fig. 6), I have several concerns. First, authors performed this experiment on UV-damaged chromatin, while so far they described Polk functions in minor groove DNA lesions and DPC bypass. Why they now switch to UV-damaged chromatin in order to test a possible interaction with Rev1 and Pol zeta is unclear. It was expected that authors would test this possibility using chromatin damaged with agents that generate DPC, or better, following observations by Kanemaru et al., 2015, with chromatin exposed to hydrogen peroxide or menadione. Second, I doubt that the way the chromass experiment has been performed represents a physiological TLS process occurring on chromatin damaged by UV-Cs. Authors have exposed sperm chromatin to a UV-C dose of 2000 J/m2 which is excessively high. First, in these conditions, mostly DSBs are formed. This may probably explain why several proteins involved in DSBs repair show up in the mass spectrometry results. Second, I am not sure that the sperm chromatin will be able to decondense at this UV dose. In the original work of Raschle et al., 2015 Science, mentioned in the manuscript, this UV-C dose was used to identify a whole bunch of proteins involved in DNA repair, which is not the aim here. Further, by looking at the mass spectrometry data filed in Table S2, it appears that there are several proteins involved in the initiation and elongation steps of DNA synthesis (ORC, Cdc6, MCM2-7, DNA polymerase epsilon catalytic subunit for instance) that are underrepresented in the mock-depleted +UV compared to the mock-depleted extracts conditions (and apart from PCNA which is involved in several DNA repair pathways, which Is indeed well enriched), which is not what expected, since the UV treatment should not affect loading of these proteins, on the contrary, it is often observed their increase. In particular (and worrying) I could not find neither Cdc45 nor MCM10, two key proteins involved in setting up replication forks. These observations put doubts on the validity of this approach to monitor replication-dependent TLS functions. Therefore, I reckon that in order to draw significant and physiological conclusions, this experiment must be performed at much lower doses of UV-C, not higher than 800 J/m2, unless authors can show that sperm chromatin decondensation, nuclear formation and replisome assembly is fine at the UV dose they used. Further, at least one marker of DNA damage responding to UV irradiation and DSBs signaling must be show (γH2AX and/or PChk1; PChk2, pATM, p53BP1). Further, the observation of Rev1 dependency for Polk chromatin binding, does not demonstrate Rev1-Polk complex formation on chromatin. An immunoprecipitation experiment from mock-treated or Rev1-treated nuclear or chromatin protein extracts is required. It would be also important to show here the level of TLS pol iota, so to formally demonstrate that only Polk is required to rescue stalled replication forks. There are antibodies available in the market that recognize Xenopus pol iota.
8. Concerning the most important message of the manuscript, related to the finding of a non-catalytic function of Polk in DNA lesion bypass, this finding should be substantiated in

mammalian cells, following the CRISPR/CAS9 tiling screen which can be moved into this section. Hence, similar mutants than those used in Xenopus must be used to complement Polk-invalidated cells treated with DPC-forming agents and measuring their viability. It would be also interesting to test the effect of these mutants upon treatment with hydrogen peroxide, so to explain previous observations (Kanemaru et al., 20015).

Minor points
1. Abstract, first sentence, TLS is not only "an essential process that allows bypass lesions encountered during DNA replication", since TLS includes TLS DNA polymerases that bypass DNA lesions outside the context of DNA replication, while those TLS involved in rescuing stalled replication forks are mainly Y-family TLS pols. Consider making this point clear. Further, TLS pols are generally non-essential proteins, (with some exceptions such as Pol zeta for instance), hence, the term "essential" should probably be removed.
2. Introduction, first sentence, not all DNA lesions impede DNA synthesis. Consider rephrasing. Second paragraph, apart from TLS DNA, repriming is also involved in rescuing replication forks stalled by DNA lesions, this should be mentioned. Page 4, first sentence, please explain what M.HpaII-based-DNA-protein crosslinks is. Second paragraph, I am not sure one can say that the role of Polk in TLS remains poorly understood. Much is already known. Consider rephrasing. Last paragraph, the observation that Polk deficient cells are sensitive to UV-C and Cisplatin, does not suggest a non-catalytic Polk function. This was proposed by Kanemaru et al., 2015, as stated just after in the paragraph. Consider rephrasing. End of the paragraph, unlike what stated, there are a bunch of publication showing an important role of Polk during DNA replication that authors may want to cite.
3. Page 7, last sentence, at this stage it is a speculation to speak about TLS inhibition. All that can be said is that inhibition of E1 stalls replication forks. Stalling could be also a consequence of ongoing DNA repair or other processes. Consider rephrasing.
4. Discussion, line 531, reference to original work of how TLS are recruited to PCNAmUb in mammalian cells must be credited (Kannouche et al., 2004 Mol Cell; Watanabe et al., 2004 EMBO J). Line 565, also a function for Pol eta in replication through heterochromatin has been recently reported (Twayana et al., 2021 PNAS; Lo Furno et al., 2022 NAR).
5. Fig. S1F is not of a sufficient standard and needs improving.

Reviewer #2:
Remarks to the Author:
In the present article, Sellés-Baiget et al. investigate the role of DNA polymerase kappa in the bypass of different type of DNA lesions. This is a very nice study that evidences a dual role for Pol kappa in TLS: one catalytic role required for the bypass of the minor groove DNA lesion p3d-Phen-A. One structural role involved in the bypass of major groove DNA lesion an abasic site, through Pol kappa interaction with Rev1-Pol zeta.

They first validated previous biochemical observation using their Xenopus egg extract system that recapitulate DNA replication and TLS: they showed that Pol kappa is required to bypass the minor groove lesion p3d-Phen-A. They showed that indeed, the catalytic activity of Pol kappa was required for the bypass of this lesion, as well as its interaction with PCNA and ubiquitin. In contrast, the interaction with Rev1 was not required for this bypass.
They then looked at major groove DNA Protein Crosslinks and abasic sites, and showed that Pol kappa was also required for the bypass of such lesions, but that its catalytic activity was not required. Its structural role occurs through the interaction with Rev1 as

well as PCNA.

The strength of their system is that it can monitor the bypass of specific DNA lesion at a high resolution. They are able to see block at the nucleotide resolution and therefore to attribute the role of inserter and extender to the different polymerases.
The base editor tiling screen is a nice way to enter into the subject, and the data arising from it is confirmed by their replication assay.

The article is very clear and the story reads really well.
In addition to the two main roles of Pol kappa that are shown, the study reveals several interesting aspects of the regulation of TLS. This includes the role of the two UBZ domains as well as the two PIP domains that were not clearly understood. Overall, it gives a new look on the recruitment and interplay of DNA polymerases that will stimulate further investigation in the future.
The study also suggests an interesting model where Pol kappa, through its different interacting domains participate to the control of TLS at different types of lesions.

The discussion is interesting and the speculation about the arrangement of Rev1-Pol zeta-Pol kappa as a safeguarding mechanism will stimulate new lines of research. It also replaces the presented results in the context of human cells giving even more relevance to their findings.

I feel this paper is greatly suitable for NSMB as it reports interesting and, in my opinion, important and novel findings. The data are clear and support the conclusion.
I only have a few questions or suggestions:

-The author used the p3d-Phen-A lesion as a minor groove DNA lesion. While they clearly show the requirement of Pol kappa to bypass this lesion, I am not sure they can generalize this role of Pol kappa for ALL the minor groove DNA lesions solely based on this data. I would recommend they discuss this point, by suggesting from their p3d-Phen-A lesion, their Illudin S screening and previous in vitro data that Pol kappa is a minor groove specialized TLS polymerase.

-line 104: isn't AAF generally considered as a major groove DNA adduct? The 2 references given here (Choi 2006 and Ogi 2002) relate to BaP, and not to AAF.

-line 173-: "…the screen containing several sgRNAs that were predicted to introduce missense mutations in each of these domains." I understand that mutations were predicted in these domains, but did the authors checked if mutations actually arose at these particular sites? If for some reason the base editor did not work at these sites, this could explain why the cells were not sensitized to Illudin S.

-The authors propose that the role of Pol kappa is to stabilize the Rev1-Pol zeta complex. While this is presented like a hypothesis in the manuscript (line 505), it appears like a statement in the abstract (line 40) and in the introduction (line 137).

-Fig6A: in the absence of Pol eta, Rev1-Pol zeta can achieve both the insertion AND the extension steps. This insertion activity also requires Pol kappa as in the absence of Pol kappa, no insertion is achieved by Rev1-Pol zeta. The authors only mention the requirement of Pol kappa for the extension capacity of Pol zeta, not the insertion. This

might be worth mentioning.

-In the discussion (lines 475-), I am not sure to fully understand the reinterpretation of the previous study from Williams et al. . Maybe some more details on these previous findings should be given to improve the clarity of this paragraph.

Minor points
- It would facilitate the reading of the manuscript if the explanation of the assay (Fig S1E) were in the main text.
-line 82: M.HpaII-based DPC should be further describe here.
-Figure 1D: the resolution of the p3d-Phen-A structure should be improved
-It would made the manuscript easier to read if the name of the lesion studied were displayed on the figures. This is the case in some occurrences (Fig 1E or Fig 3C for instance), but in most of the cases the reader has to go get the information in the legend.
-line 339: to facilitate the reading, AP-ICL should be described as it is not a well-known type of lesion.

Reviewer #3:
Remarks to the Author:
This manuscript describes important new mechanistic insights into the role of the translesion synthesis (TLS) polymerase Pol kappa (PolKappa) in the bypass of DNA lesions in the major or minor groove of the DNA. Different functions, catalytic and non-catalytic, are attributed specifically to minor and major groove lesions respectively. Based on previous reports showing that PolKappa specifically bypasses lesions present in the minor groove in vitro, but that PolKappa deficient cells are also sensitive to agents inducing major groove DNA lesions, the authors suggest a non-catalytic role in the bypass of major groove lesions. In a series of elegant experiments the authors define this mechanism. First, using a CRISPR-Cas9 base editor tiling screen the authors identify regions in PolKappa required to confer resistance to Illudin S, a compound that induces minor groove lesions. Next, using Xenopus egg extracts the DNA replication-coupled bypass of another minor groove lesion (p3d-Phen-A, an Illudin S derivative) is recapitulated and confirms the requirement of PolKappa's catalytic activity and the importance of PCNA and ubiquitin interaction domains. Subsequently, other DNA lesions, those that form in the major groove of the DNA (DNA protein crosslinks), and/or require PolZeta mediated extension (abasic sites) are studied. PolKappa is also required for the bypass of these lesions but this is independent of its catalytic activity. Moreover, lesion bypass requires interaction with REV1 through PolKappa's REV1 interaction domain (RIR) that was not required for bypass of the minor groove lesions. The non-catalytic function of PolKappa seems to act by stabilization of the REV1-PolZeta complex on chromatin through a direct interaction. A model indicates that the catalytic role of polymerase kappa in the bypass of minor groove lesions is independent and maybe even mutually exclusive with the non-catalytic role of PolKappa together with REV1-PolZeta.

Translesion synthesis is an important mechanism in the response to DNA damage. Several specialized polymerases act in this response and among these PolKappa is one of the least well-studied. This work contributes important new mechanistic details into several functions of PolKappa and its interplay with other factors involved in TLS which will be of great value to the field. The manuscript is clearly written and the experiments are well explained and beautifully executed. To my opinion this work is suited for NSMB but I

would like to see a few points addressed before I would recommend publication.

Major points:

The model in Fig. 7 describes two modes of action of PolKappa on different lesions. According to this model bypass of minor groove lesions such as p3d-Phen-A require PolKappa's catalytic activity and this is independent of REV1. This is based on experiments (mainly Fig. 1 and 2), and modeling (Fig. S7). However, the presence of REV1 at the lesion is not experimentally examined. While the interaction between PolKappa and REV1 through the RIR domain does not seem to be involved (Fig. 2B) it can be envisioned that REV1 is recruited to the lesion by another interaction site (possibly through PCNA-Ub). Through this interaction REV1 could be important to recruit PolZeta for extension. While the authors show that REV1 depletion (Fig. 2C) does not lead to a defect in lesion bypass this could be caused by a redundant function of PolKappa that has been shown to be able to perform this extension step. While the authors implicate that the bypass of minor groove lesions in general depend on polKappa's catalytic activity and do not require REV1/PolZeta, it is important to note that there are examples in the literature that show involvement of REV1/PolZeta in such lesions (Hodskinson et al. 2020). The potential involvement or presence of REV1 is an important point to address since it can have implications for the model (potentially the complexes formed on minor and major groove lesion are in fact more similar but the division of labor differs). Therefore, I would strongly suggest an experiment that shows whether REV1 is recruited to the p3d-Phen-A containing plasmid during bypass or not. And if it is recruited, how does PolKappa depletion affect this recruitment? Moreover, a double depletion of PolKappa and REV1 would be highly interesting (if experimentally possible) since the single PolKappa depletion does not fully block lesion bypass (extension products of the damaged strand still increase over time). If this further blocks lesion bypass it indicates REV1 can act redundantly.

To determine the mechanism of the non-catalytic function of PolKappa in the bypass of DPC's and abasic sites the authors switch to using UV-treated chromatin. This is surprising since it is not clear whether UV damage is restricted to the major groove, and more importantly, whether the bypass of UV lesions requires the non-catalytic function of PolKappa. While the authors nicely show that PolKappa and PolZeta form a partially interdependent complex on chromatin after UV treatment the functional relevance of this data for the non-catalytic role of PolKappa is not clear. To show that this interdependent complex is also formed on the relevant lesions the authors need to show this in the context of a DPC or abasic site lesion.

While two compelling models are drawn in Fig. 7 showing the catalytic and non-catalytic roles of PolKappa, not all aspects of these models are supported by data. First of all, as mentioned above, it is not shown experimentally that REV1 and PolZeta are not recruited to minor groove lesions. Second, the authors base the mutually exclusive nature of these models on the fact that binding of the PolKappa's RIR domain to REV1 in this model prohibits interaction of PIP1 with PCNA. However, the data showing the relative roles of the PIP domains in the non-catalytic function are not so clear. While it seems that PIP2 might be slightly more important (Fig. S4F), there is very little difference with PIP1 mutants and the data definitely does not support the claim 'PolKappa non-catalytic function requires PIP2 and is independent of PIP1 (line 501)'. Based on the current data I would suggest a third model in which REV1 and PolZeta are also recruited to minor groove lesions where PolKappa's catalytic activity is important and REV1/PolZeta may act

redundantly (depending on the outcome of the experiment suggested above). Moreover, instead of the very complicated structural models I would prefer to see this in an easier to grasp cartoon form (structural models can be moved to the supplement).

Other points:

1) Throughout the manuscript it is mentioned that PolKappa is unique in bypassing minor groove lesions. While the references indeed show PolKappa can do this, I don't see the evidence that this is unique among all TLS polymerases. Moreover, this has only be studied in reconstitution assays. I think this claim could be toned down a little bit (or proper references should be supplied).

2) Title of the second paragraph of the results 'PolKappa-mediated bypass of minor groove lesions depends on PCNA ubiquitylation but is independent of REV1'. The dependence on the PIPs is shown but it is not shown that PCNA is ubiquitylated. The authors should at least show a blot that bypass of this specific lesion induces PCNA ubiquitylation.

3) Fig. S2C and D. No protein add back is shown in the blot for the double PIP mutant. A defect in this mutant could thus be caused by low or absent protein. Protein levels are crucial to draw any meaningful conclusion. Same point for S2E and F and the double UBZ mutant.

4) Line 245: While UBZ2 seems to be important it is definitely not critical, there is still much more bypass compared to the buffer control (Fig. 2E). Tone down statement.

5) Fig. S4G, a sequencing gel of this experiment is would be helpful to properly assess the rescue of the UBZ1* mutant.

6) Line 415: PolKappa is reduced but no abolished (Fig. 6D, band is still visible). Tone down conclusion.

7) Line 418: REV1/PolZeta is only slightly reduced (Fig. 6E), definitely not lost. Tone down conclusion.

8) It would be useful to include Coomassie stained gels of the purified proteins to assess their purity. To confirm that the catalytic site mutant is inactive the authors use PolKappa expressed in rabbit reticulocytes (Fig. S1F, methods for this experiment seem to be missing) while all other experiments are performed with protein purified from E. Coli. Did the authors do the activity test also on the protein from E. Coli?

9) Line 478: The lesion studied in the Williams et al. paper is a trimethylene ICL, how is this reminiscent to a MMC induced ICL? The structure is very different.

Author Rebuttal to Initial comments

Specific Response to Reviewers Comments

We greatly appreciate the time and expertise of the reviewers in evaluating our manuscript. Their insightful comments and suggestions have provided valuable perspectives that have enhanced the quality of our work. In this rebuttal letter, we address each of the raised points, highlighting the modifications made to strengthen the manuscript. As a result, we are confident that the revised version of our manuscript is greatly improved.

All reviewer comments are shown in their entirety with our response in ***bold italics***. Additional data not shown in the manuscript is presented below in Figures R1-6.

Reviewer #1:
Remarks to the Author:
This manuscript describes findings related to the function of the translesion DNA synthesis (TLS) polymerase k (Polk) in the bypass of minor and major grooves DNA lesions. Data have been obtained first in mammalian cells by measuring their sensitivity to a specific minor groove DNA lesion generated by exposing cells to Illudin S, and mostly using protein extracts derived from eggs of the frog Xenopus laevis that are able to recapitulate TLS processes in vitro on a plasmid DNA template bearing a single DNA lesion, as well as on UV-C irradiated chromatin. The authors describe catalytic and non-catalytic functions of Polk using a variety of Polk mutants and define that the Polk non-catalytic role is involved in the bypass of major groove DNA lesions, such as DNA-Protein crosslinks (DPC). These findings shed light on previous observations showing a non-catalytic function of Polk in mediating bypass of certain DNA lesions, such as those generated by hydrogen peroxide or menadione (Kanemaru et al., 2015). This latest finding constitutes the more novel result of the manuscript, since it was already known that Polk is involved in the bypass of minor groove DNA lesions. However, authors characterize in more detail this process, in particular in Xenopus extracts, and highlight the importance of Polk as a probable scaffolding factor mediating interaction with two other TLS pols, Rev1 and Pol zeta, corroborating previous in vitro findings (Xie et al., 2012). Overall the work is well executed and well presented, although more data are required to strengthen the major claims of the manuscript, as well as addition of several important controls. Unfortunately, the detection of a possible PolK-Rev1-Polzeta complex is not addressed. One major technical concern is related to the experiments involving detection of chromatin-bound proteins by mass spectrometry (also known as CHROMASS approach, see comments appended below) and the conclusions drawn from this approach.

Specific comments
1. The CRISP/CAS9 tailing screen in mammalian cells does not fit in fig 1 since there is no follow up of these findings in mammalian cells. This could be moved to Fig. 6, to corroborate findings observed in Xenopus (see also point 7).

We considered moving the CRISPR/Cas9 gene editor experiment to Figure 6. However, we agree with reviewer #2 that this experiment represents a nice introduction to Polκ's catalytic regulations before we transit and characterize this via biochemical reactions in Xenopus egg extracts. We have therefore opted to leave the screen in its initial location (Figure 1).

2. Specificity of the antibodies raised against Polk, Rev3, HMCES and FancA must be shown. Authors must show western blot performed with sera before immunization and after immunization and as such demonstrate the specificity of the polypeptide recognized by each antibody and identify the

non-specific polypeptides recognized by the serum. This is particularly important for HMCES and Polk antibodies. HMCES antibodies are poorly described in the reference provided by the authors (Semlow et al., 2022). Regarding western blots with Polk antibody, these do not show always the same pattern (compare Fig. 1F with Fig. 6D-E and Fig. S2C for example). It is known that Polk is monoubiqutinated. Can the allegedly non-specific polypeptide recognized by this antibody in Fig. 1F (marked with a star) corresponds to this Polk form, which by the way is not depleted by the antibody? Authors must clarify this point which could be critical in order to interpret the results, since one my argue that the Pol k antibody raised by the authors does not remove the ubiquitinated form of Polk.

Our antibodies generated against Polκ, Rev3, HMCES, and FancA were all affinity purified using the peptide used for immunization and thus recognize and bind to the targeted peptide. Once the antibodies are purified, we test them for their ability to immunoprecipitate and recognize the targeted protein and via Western blotting (compared to an IgG control purified from the pre-immune serum). We have now added this characterization for the Polκ (new Figure S1F), HMCES (new Figure S5B), and FancA (new Figure S6E) antibodies. Rev3 antibody was previously characterized in (Schubert et al., 2022, Figure 4B, REV3-522)

Note that it is common that antibodies generated against peptides recognize additional proteins than the one they are targeted against and even if the band is specifically recognized by the post-immune serum, this is not a proof of antibody specificity. The best way to demonstrate specificity of a depletion is to have a functional effect, which is then rescued by reintroducing the recombinant version of the protein. Functional rescue experiments were achieved following either Polκ (in this manuscript) and HMCES depletions (Semlow et al., 2022 for HMCES). Thus, even if the antibody recognizes or depletes additional proteins, we can be certain that the effects observed are caused by the depletion of the targeted protein.

In our Polκ immunoblots we indicate the band running above Polκ (~135 kDa) as non-specific because this band is not depleted from the extracts with the Polκ antibody. We do not know the nature of this band/protein but since it is not depleted from the extracts, it does not affect the conclusions of the manuscript.

For the reviewer's discretion, we have blotted immunoprecipitation experiments with either the pre-immune or post-immune serums as suggested. As can be seen in Figure R1A, the ~135 kDa band detected in Polκ immunoblots is detected by both pre-immune and post-immune serums (compare lanes 1-3 from left and right immunoblots), which further suggests that this band is non-specific and does not correspond to ubiquitylated Polκ. We have also blotted HMCES immunoprecipitates with the pre-immune and post-immune serums. As can be seen in Figure R1B, the post immune specifically recognizes the ~40 kDa bands corresponding to Xenopus HMCES.

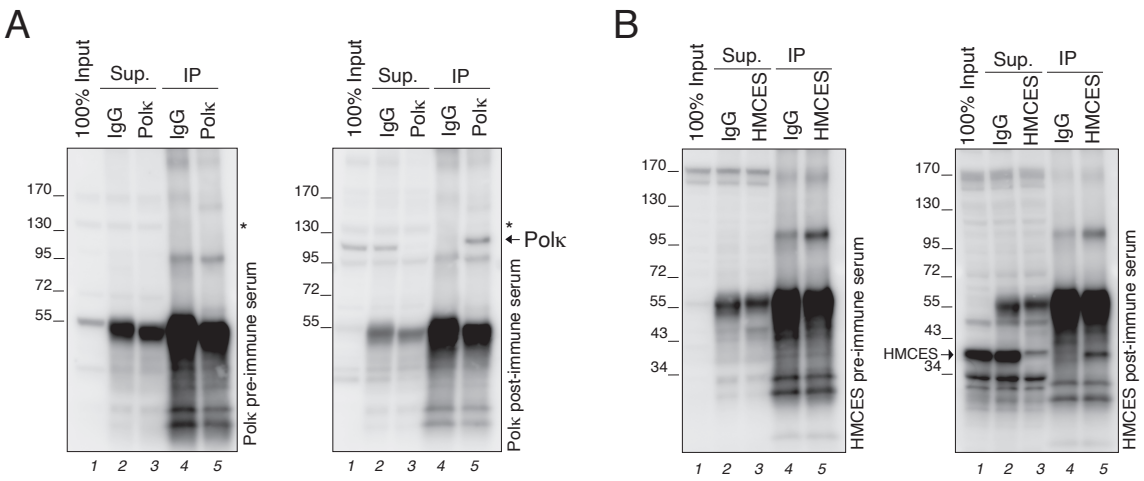

Figure R1. ***A****. Xenopus egg extracts (NPE) were either immunoprecipitated with an IgG or a Polκ antibody. The supernatant (Sup.) and immunoprecipitate (IP) were loaded next to a 100% input control (lane 1). Samples were immunoblotted with either the pre-immune (left immunoblot) or post-immune Polκ (right immune blot) serum. Note that the band running above 130 kDa is recognized by both pre- and post-immune serums (lanes 1-3, indicated with an *).* ***B****. Xenopus egg extracts (NPE) were either immunoprecipitated with an IgG or HMCES antibody. The supernantant (Sup.) and immunoprecipitates (IP) were loaded next to an input control (lane 1). Samples were immunoblotted with either the pre-immune (left immunoblot) or post-immune HMCES serum (right immunoblot).*

3. Further, authors must show the purity of the recombinant proteins they have used to reconstitute depleted extracts.

We have added a Coomassie blue stained gel of our recombinant Polκ preparations (new Figure S1G).

4. Regarding the western blots showing depletion with antibodies, they miss basic loading controls to ascertain that equal amounts of proteins were present in each lane.

We have added the corresponding loading controls (new Figure 1F, S2A, S2C, S2E, and S5C).

5. Fig. 1G shows that Polk depletion produces a similar outcome than E1 inhibition. But I do not see how one can conclude that this is TLS. Although the authors show that this process depends upon Polk catalytic activity, this could well be DNA repair. Indeed, Polk has been previously involved in DNA repair (Ogi et al., 2006). I reckon that in order to be formal here, sequencing of the reaction products is required, so to demonstrate a real TLS event.

We respectfully disagree with the reviewer's comment. When we monitor DNA replication across the minor groove adduct, we observe a -1 arrest of the nascent DNA strand, which indicates a DNA synthesis block one nucleotide upstream of the damaged based. The adduct is then bypassed in a ubiquitin dependent process,

which is consistent (but we agree that it is not a proof) with the requirement of a TLS polymerase (Gallina et al., 2021). We demonstrate that this is indeed the case since the bypass of the lesion depends on Polκ's catalytic activity (Figure 1H-I).

If Polκ function in NER was involved in repairing the lesion (as shown in Ogi & Lehmann, 2006) there should be no adduct left on the template DNA following nucleotide excision and removal of the adducted strand by the NER machinery and therefore no -1 arrest when Polκ fills in the DNA gap.

To further show that the bypass is dependent on TLS and not DNA repair, we have incubated our plasmid in the presence or absence of DNA replication (since NER is a process that is uncoupled from DNA replication). As can be observed, the -1 arrest is specific to the replicating sample (Figure R2, lanes 1-5) and therefore caused by the stall of a DNA polymerase at the lesion during DNA replication.

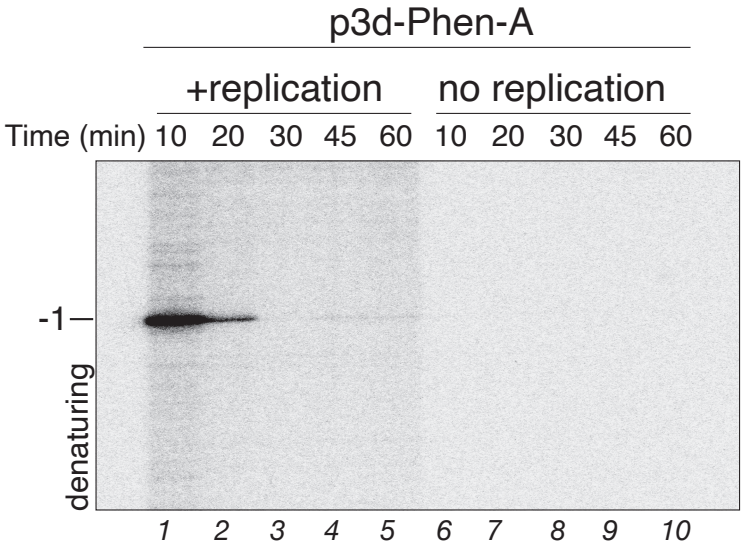

Figure R2. *p3d-Phen-A was incubated in egg extracts in the absence (+replication) or presence of Cdc7i (no replication). Samples were analized on a denaturing acrylamide gel following HaeII and HindIII digest as in Figure 1E. An overexposure of the radiolabeled gel is shown on purpose to illustrate that the -1 signal is specific to the replicating sample (compare lanes 1-2 to lanes 6-7).*

6. Second section of results, although the authors show that the Polk activity required to rescue replication stalling is dependent upon one of its ubiquitin binding domains (UBZ2), thus concluding that it depends upon PCNA monoubiquitnation, here a formal demonstration is required showing that addition of a non-ubiquitinatable PCNA (K164R mutant) to the reaction generates the same result. Further, a formal demonstration that Polk wild-type and not Polk mutated in the Rev1 binding domain (RIR) physically interacts with Rev1 is required.

We thank the reviewer for the suggestion. The depletion of PCNA in NPE has unfortunately been a long and difficult task, which we have not been able to solve. The main issue is that PCNA is one of the most concentrated proteins present in NPE (50 μM concentration) and once we have efficiently depleted PCNA (it takes 5 rounds of depletion to get it below 5%), the extract is inactivated likely due to co-depletion of essential replication components that interact with PCNA (e.g. DNA polymerases).

To get around this technical hurdle we have attempted to deplete PCNA partially with 4 rounds of depletion, which brings PCNA down to around 10% (Figure R3A compare lane 8 to lanes 1-5). Even in this condition, the extract was inactivated and did not support DNA replication of a plasmid (following add back of PCNA wildtype). We therefore moved to a replication-independent process with a plasmid containing a lesion on a DNA gap, which undergoes gap filling synthesis and TLS in the absence of DNA replication (Figure R3B; and Larsen et al. 2019). Under this setting we were able restore TLS across the adduct via the addition of PCNA wildtype but not to the same extent with the K164R mutant (evidenced by the generation of SC products, compare lanes 7-12 to 17-18). Although these results are promising, they are technically extremely challenging because they require "the perfect amount" of PCNA depletion so that we can partially inhibit its function without inactivating the extract. Thus, we have been unable to consistently reproduce this effect and are unfortunately unable to provide a clean system where PCNA K164R function can be assessed with confidence.

The Polκ-Rev1 interaction via Polκ RIR is a very well accepted concept in the field that has been reported in many studies (Guo et al., 2003; Ohashi et al., 2009; Sale et al., 2012; Williams et al., 2012). Therefore, due to time and resources contrains, we opted to focus our review on the other more relevant points.

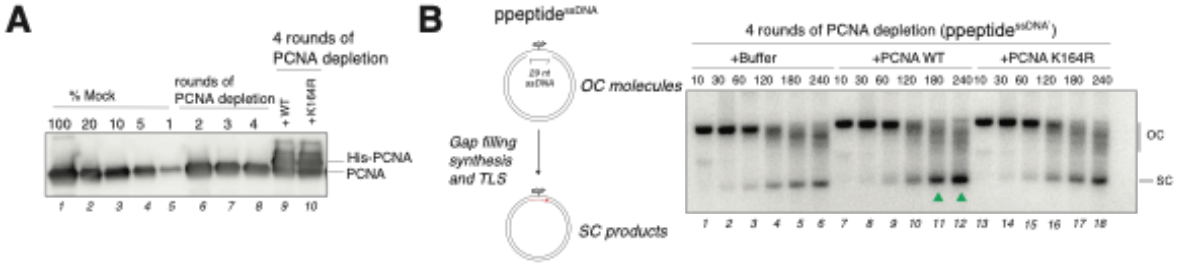

Figure R3 ***A.*** *Egg extracts were depleted of PCNA for either 2 rounds (lane 6), 3 rounds (lane 7), or 4 rounds (lane 8), and compared to a serial dilution of a mock depleted extract. Following 4 rounds of depletion the extracts were supplemented with either wildtype (WT) or K164R His-PCNA.* ***B.*** *ppeptide^{ssDNA} was incubated in extracts from (A) in the presence of radiolabeled nucleotides. Reactions were analyzed on a native agarose gel. Note the generation of SC products when the extract is reconstituted with the WT PCNA, which suggests rescue of TLS across the peptide adduct.*

7. Regarding the chromass experiment (Fig. 6), I have several concerns. First, authors performed this experiment on UV-damaged chromatin, while so far they described Polk functions in minor groove DNA lesions and DPC bypass. Why they now switch to UV-damaged chromatin in order to test a possible interaction with Rev1 and Pol zeta is unclear. It was expected that authors would test this possibility using chromatin damaged with agents that generate DPC, or better, following observations by Kanemaru et al., 2015, with chromatin exposed to hydrogen peroxide or menadione. Second, I doubt that the way the chromass experiment has been performed represents a physiological TLS process occurring on chromatin damaged by UV-Cs. Authors have exposed sperm chromatin to a UV-C dose of 2000 J/m2 which is excessively high. First, in these conditions, mostly DSBs are formed. This may probably explain why several proteins involved in DSBs repair show up in the mass spectrometry results. Second, I am not sure that the sperm chromatin will be able to decondense at this UV dose. In the original work of Raschle et al., 2015 Science, mentioned in the manuscript, this UV-C dose was used to identify a whole bunch of proteins involved in DNA repair, which is not the aim here. Further, by looking at the mass spectrometry data filed in Table S2, it appears that there are several proteins involved in the initiation and elongation steps of DNA synthesis (ORC, Cdc6, MCM2-7, DNA polymerase epsilon catalytic subunit for instance) that are underrepresented in the mock-depleted +UV compared to the mock-depleted extracts conditions (and apart from PCNA which is involved in several DNA repair pathways, which Is indeed well enriched), which is not what expected, since the UV treatment should not affect loading of these proteins, on the contrary, it is often observed their increase. In particular (and worrying) I could not find neither Cdc45 nor MCM10, two key proteins involved in setting up replication forks. These observations put doubts on the validity of this approach to monitor replication-dependent TLS functions. Therefore, I reckon that in order to draw significant and physiological conclusions, this experiment must be performed at much lower doses of UV-C, not higher than 800 J/m2, unless authors can show that sperm chromatin decondensation, nuclear formation and replisome assembly is fine at the UV dose they used. Further, at least one marker of DNA damage responding to UV irradiation and DSBs signaling must be show (γH2AX and/or PChk1; PChk2, pATM, p53BP1). Further, the observation of Rev1 dependency for Polk chromatin binding, does not demonstrate Rev1-Polk complex formation on chromatin. An immunoprecipitation experiment from mock-treated or Rev1-treated nuclear or chromatin protein extracts is required. It would be also important to show here the level of TLS pol iota, so to formally demonstrate that only Polk is required to rescue stalled replication forks. There are antibodies available in the market that recognize Xenopus pol iota.

We thank the reviewer for the suggestion. We agree that it would be interesting to damage chromatin with a DPC inducing agent. Unfortunately, we are not aware of any DNA damaging agent that specifically generates DPCs. Topoisomerase poisons such as Camptothecin and Etoposide generate breaks on the DNA (in addition to DPCs), which lead to replication stress and DPC indirect effects. Formaldehyde, which is often used as a DPC inducer in cells, induces a plethora of other lesions (e.g. protein to protein and DNA interstrand crosslinks). Moreover, we show below that our POLK KO cells are not sensitive to a peroxide analog. Therefore, we do not think that there is "an ideal" DNA damaging agent to perform a CHROMASS experiment. Because we had previously observed the recruitment of TLS polymerases to UV-C damaged chromatin via CHROMASS (Gallina et al., 2021; Schubert et al., 2022), we used the same approach to investigate the co-dependency between Rev1-Polζ and Polκ.

As noted by the reviewer, we use a very high dose of UV-C (the same that we had used in our previous studies (Gallina et al., 2021; Schubert et al., 2022). We purposely use this very high dose to monitor replication-independent TLS recruitment (Sertic et al., 2018). At high UV-C dose, both strands of the DNA are expected to be damaged in close proximity so that a lesion remains on the DNA template following one round of

nucleotide excision. In this scenario, the subsequent gap filling DNA synthesis now depends on TLS to close up the gap (Sertic et al., 2018). In Schubert et al., 2022, we show how despite the very high dose, all the proteins enriched are the expected DNA repair and TLS factors and thus very specific to UV-C lesions. We have duplicated the panels from our orginal publication here below for the reviewer's convenience (Figure R4A-E, Schubert et al., 2022).

While the dose of UV-C is very high, we do not think that we mainly induce DNA double strand breaks (but do not exclude that we might generate some). This is evident from the GO Term analysis and the volcano plot shown below, which highlights that the expected predominant enrichment of NER, TLS, and MMR factors (Figure R4 D-E).

Importantly, the main proteins that are enriched on chromatin in the absence of either Rev1 or Polκ are 53bp1, Polη, Wrnip1, and Rnf168 (Figure 6C). While 53bp1 and Rnf168 are involved in DSB response it is important to note that 53bp1 has also been recently proposed to regulate the balance between TLS and TS (Chen et al., 2022) and Rnf168 has been proposed to contribute to PCNA ubiquitylation (Yang et al., 2021). Conversely, the main proteins de-enriched are Rev3l and Rev7. Thus, out of the entire proteome, the very few proteins that modulate their concentration on chromatin in the absence of Polκ or Rev1 are all expected factors, which really highlights the specificity of our assay.

As mentioned above our CHROMASS experiment was performed in the absence of DNA replication. We apologize for the confusion. Although this was indicated in the material and methods, we now clarify this in the text of the result section (p14). This is the reason why CMG components such as Cdc45, Gins, and Mcm10 are not detected on chromatin.

We also wanted to share our reasoning for purposely performing this experiment with a high dose UV-C in the absence of DNA replication instead of a low dose UV-C during DNA replication. The rationale is that the absence of TLS during DNA replication leads to profound changes to DNA replication fork structures caused by the uncoupling of the replisome, which would lead to indirect changes in protein recruitment to chromatin (e.g. replication fork remodelers and DNA nucleases). In the absence of replication, the expectation is that we are only stabilizing the gaps and therefore minimizing the impact of abnormal DNA structures on protein recruitment. We can therefore more directly assess the proteins that are directly dependent on Polκ or Rev1 and in fact we only detect a subset of proteins that are dependent on both. We have now also clarified our reasoning for performing the CHROMASS experiment in the absence of DNA replication in the result section (p14).

Having clarified our rationale, we still agree with the reviewer that it is also important to show the co-dependency between Polκ and Rev1-Polζ on replicating UV-C chromatin at a low dose. To this end we replicated sperm chromatin or chromatin treated with a 20 J/m2 of UV-C and monitored the recruitment of Polκ and Rev1-Polζ (new Figure S6G). Importantly, both in the presence of high dose UV-C (in the absence of replication; Figure 6D) or low dose UV-C during DNA replication (Figure S6G), we observe the same co-dependency between Rev1 and Polκ. We further show that this co-dependency is not restricted to UV-C damage, but it can also be observed on our replicating pDPC and p3d-Phen-A plasmids (new Figure S6H-I). This highlights

that the Rev1-Polζ-Polκ complex is formed during TLS irrespectively of the lesions and whether the complex is actually required to bypass the lesion or not.

Lastly, we agree that Pol iota is an interesting TLS polymerase with poorly defined functions. Unfortunately, we have never detected it in any of our CHROMASS nor plasmid pulldown mass spectrometry experiments (see primary MS data in Gallina et al., 2021; Larsen et al., 2019; Schubert et al., 2022, and this manuscript). We therefore do not think that monitoring its recruitment is relevant to this study. Instead, we clearly show that Polη, which is clearly enriched on UV-C treated chromatin operates independently of the Rev1-Polζ-Polκ complex.

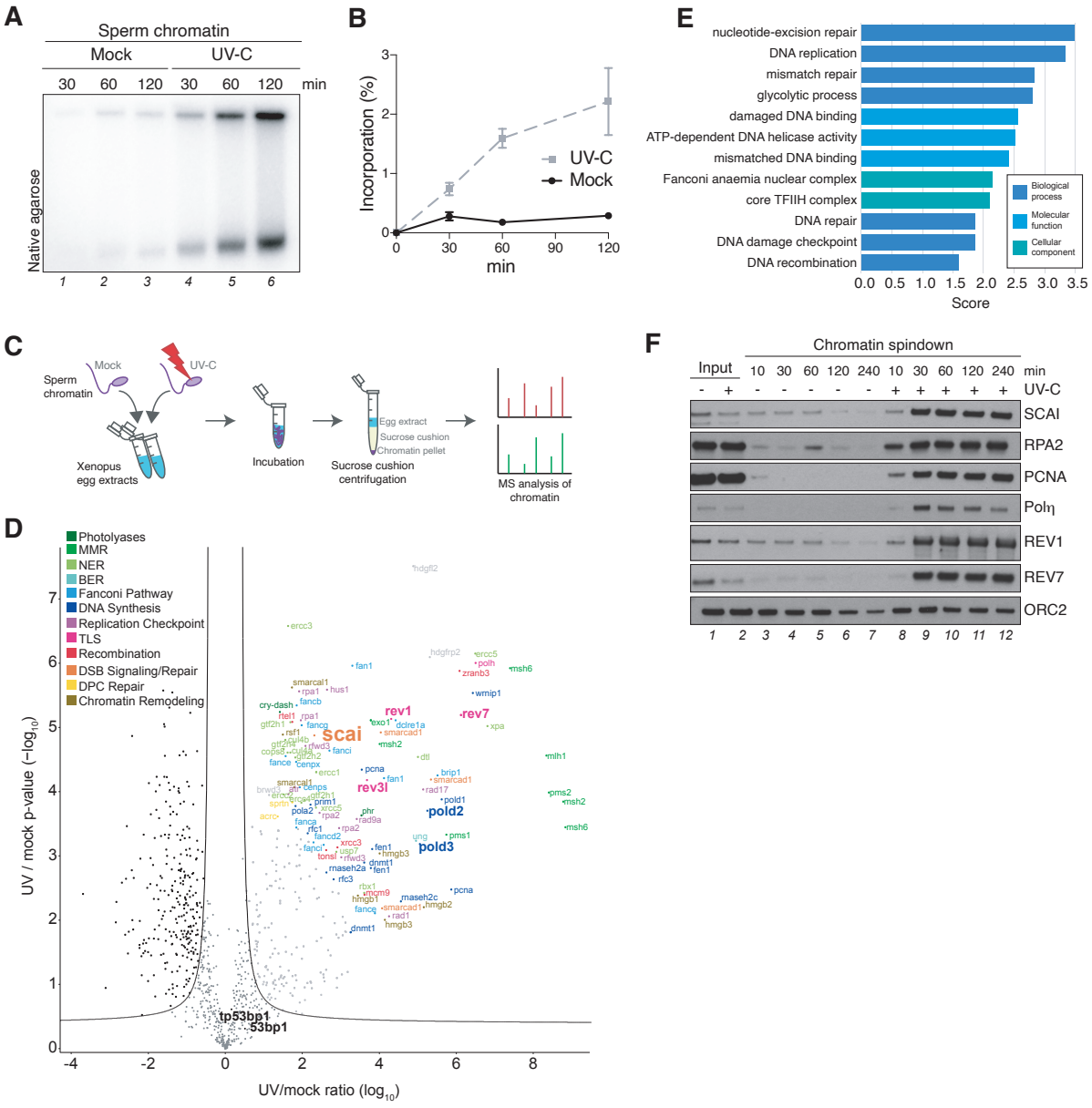

Figure R4 (duplicated from *Schubert et al., 2022*, Figure EV4). *A.* *Sperm chromatin was left untreated or exposed to UV-C (2,000 J/m), incubated in HSS/NPE mix in the presence*

of [α- P]dATP and nucleotide incorporation into chromatin was analyzed at the indicated time points via native agarose gel electrophoresis. Note that we are monitoring here replication-independent DNA synthesis, which is likely dependent on NER. ***B.*** *Quantification of data in (A) (mean +/- SEM; n = 3 independent experiments).* ***C.*** *Schematic outline of CHROMASS workflow to analyze protein recruitment to UV-damaged chromatin. Sperm chromatin left untreated or irradiated with UV-C (2,000 J/m) was incubated in Xenopus egg extract for 30 min, isolated by sucrose cushion centrifugation, and analyzed by label-free mass spectrometry (MS).* ***D.*** *Protein recruitment to UV-damaged chromatin compared to an undamaged control. Volcano plot shows enrichment of individual proteins (UV/mock ratio) plotted against the P-value (n = 4 independent experiments; FDR < 0.05, s0 = 0.5).* ***E.*** *Term enrichment analysis showing GO terms corresponding to proteins significantly recruited to UV-C-damaged chromatin. All displayed terms were significant with P < 0.02, as determined through Fisher exact testing with Benjamini–Hochberg correction.* ***F.*** *Sperm chromatin left untreated or irradiated with UV-C (2,000 J/m) was isolated at the indicated time points and analyzed by immunoblotting.*

8. Concerning the most important message of the manuscript, related to the finding of a non-catalytic function of Polk in DNA lesion bypass, this finding should be substantiated in mammalian cells, following the CRISPR/CAS9 tiling screen which can be moved into this section. Hence, similar mutants than those used in Xenopus must be used to complement Polk-invalidated cells treated with DPC-forming agents and measuring their viability. It would be also interesting to test the effect of these mutants upon treatment with hydrogen peroxide, so to explain previous observations (Kanemaru et al., 20015).

We thank the reviewer for this suggestion. To address the relevant functions of Polκ in human cells we first generated U2OS POLK knockout cells, which we tested for Illudin S (minor groove lesion), cisplatin (major groove lesion), and tert-butyl hydroperoxide (a hydrogen peroxide analog) sensitivity. We found out that while POLK KO cells were sensitive to Illudin S and cisplatin (new Figure 7A-D), these cells were not sensitive to tert-butyl hydroperoxide (Figure R5). We therefore proceeded to rescue Illudin S and cisplatin sensitivity with either the wildtype or the catalytic dead version of Polκ. In new Figure 7, we show how Illudin S sensitivity of POLK-KO cells can be largely rescued by the wildtype Polκ but not by its catalytic dead version (new Figure 7A-B). In contrast, cisplatin sensitivity could be rescued to the same extent with either the WT or the catalytic dead (new Figure 7C-D). These results agree with our biochemical work in Xenopus egg extracts, which show that the catalytic activity is needed to bypass minor groove DNA adducts (such as the ones that would be generated by Illudin S), while the non-catalytic function is needed for the tolerance to major groove lesion (such as the ones that would be generated by cisplatin). We hope that the reviewer can appreciate our effort in validating the non-catalytic function of Polκ in human cells and is now satisfied with this important addition to the manuscript.

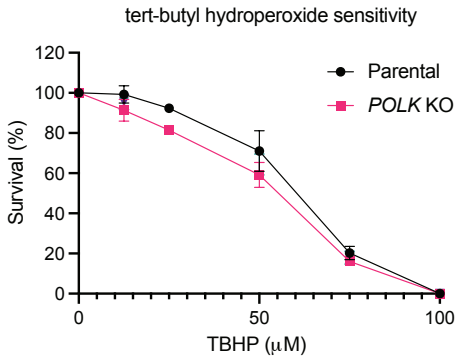

Figure R5. *CellTiter assay with tert-butyl hydroperoxide (TBHP). U2OS parental cells or POLK knockout (KO) cells were seeded in 96 well plates, and 24 hours after seeding, cells were left untreated or treated with increasing concentration of TBHP for an additional 3 days before a CellTiter 96 AQ_{ueous} One solution cell proliferation assay (Promega) was used to estimate cell viability. Means and standard deviations of normalized viability from a technical triplicate are plotted.*

Minor points

1. Abstract, first sentence, TLS is not only "an essential process that allows bypass lesions encountered during DNA replication", since TLS includes TLS DNA polymerases that bypass DNA lesions outside the context of DNA replication, while those TLS involved in rescuing stalled replication forks are mainly Y-family TLS pols. Consider making this point clear. Further, TLS pols are generally non-essential proteins, (with some exceptions such as Pol zeta for instance), hence, the term "essential" should probably be removed.

We have removed the term essential from the abstract.

2. Introduction, first sentence, not all DNA lesions impede DNA synthesis. Consider rephrasing. Second paragraph, apart from TLS DNA, repriming is also involved in rescuing replication forks stalled by DNA lesions, this should be mentioned. Page 4, first sentence, please explain what M.HpaII-based-DNA-protein crosslinks is. Second paragraph, I am not sure one can say that the role of Polk in TLS remains poorly understood. Much is already known. Consider rephrasing. Last paragraph, the observation that Polk deficient cells are sensitive to UV-C and Cisplatin, does not suggest a non-catalytic Polk function. This was proposed by Kanemaru et al., 2015, as stated just after in the paragraph. Consider rephrasing. End of the paragraph, unlike what stated, there are a bunch of publication showing an important role of Polk during DNA replication that authors may want to cite.

We have modified the first sentence which now reads "which generates lesions that <u>can</u> impede DNA synthesis".

We have also included PrimPol-mediated repriming in our introduction as a pathway to resume DNA replication.

We have also clarified what a M.HpaII DPC is: "This process is observed during the bypass of UV-induced 6-4 photoproducts, cisplatin-induced intrastrand crosslinks, and DNA-protein crosslinks (DPCs) <u>generated by the covalent linking of the M.HpaII DNA methyl transferase</u>".

We have added missing references on Polκ in the introduction (Bétous et al., 2013; Kannouche et al., 2004; Ogi et al., 2010; Dall'Osto et al., 2014; Watanabe et al., 2004, Tonzi et al., 2018)

3. Page 7, last sentence, at this stage it is a speculation to speak about TLS inhibition. All that can be said is that inhibition of E1 stalls replication forks. Stalling could be also a consequence of ongoing DNA repair or other processes. Consider rephrasing.

See our response to specific comment #5. We have also modified the sentence which now reads: "In contrast, in the presence of ubiquitin E1 inhibitor, nascent strand synthesis also stalled at -1 but now persisted up to two hours suggesting severe TLS inhibition (Figure 1E, lanes 7-12). "

4. Discussion, line 531, reference to original work of how TLS are recruited to PCNAmUb in mammalian cells must be credited (Kannouche et al., 2004 Mol Cell; Watanabe et al., 2004 EMBO J). Line 565, also a function for Pol eta in replication through heterochromatin has been recently reported (Twayana et al., 2021 PNAS; Lo Furno et al., 2022 NAR).

Thank you for poiting this out. We have added the missing references (Kannouche et al., 2004; Watanabe et al., 2004).

The references related to Polη and heterochromatin replication, although interesting, were not added as in this paragraph we compare Rev3 to Polκ and refering to Polη would be out of context.

5. Fig. S1F is not of a sufficient standard and needs improving.

We have replaced old Figure S1F with a new experiment, which more clearly shows the polymerase activity of Polκ wildtype, which is absent in the catalytic dead version of the protein (new Figure S1H).

Reviewer #2:
Remarks to the Author:
In the present article, Sellés-Baiget et al. investigate the role of DNA polymerase kappa in the bypass of different type of DNA lesions. This is a very nice study that evidences a dual role for Pol kappa in TLS: one catalytic role required for the bypass of the minor groove DNA lesion p3d-Phen-A. One structural role involved in the bypass of major groove DNA lesion an abasic site, through Pol kappa interaction with Rev1-Pol zeta.

They first validated previous biochemical observation using their Xenopus egg extract system that recapitulate DNA replication and TLS: they showed that Pol kappa is required to bypass the minor groove lesion p3d-Phen-A. They showed that indeed, the catalytic activity of Pol kappa was required for the bypass of this lesion, as well as its interaction with PCNA and ubiquitin. In contrast, the interaction with Rev1 was not required for this bypass.
They then looked at major groove DNA Protein Crosslinks and abasic sites, and showed that Pol kappa was also required for the bypass of such lesions, but that its catalytic activity was not required. Its structural role occurs through the interaction with Rev1 as well as PCNA.

The strength of their system is that it can monitor the bypass of specific DNA lesion at a high

resolution. They are able to see block at the nucleotide resolution and therefore to attribute the role of inserter and extender to the different polymerases.
The base editor tiling screen is a nice way to enter into the subject, and the data arising from it is confirmed by their replication assay.

The article is very clear and the story reads really well.
In addition to the two main roles of Pol kappa that are shown, the study reveals several interesting aspects of the regulation of TLS. This includes the role of the two UBZ domains as well as the two PIP domains that were not clearly understood. Overall, it gives a new look on the recruitment and interplay of DNA polymerases that will stimulate further investigation in the future.
The study also suggests an interesting model where Pol kappa, through its different interacting domains participate to the control of TLS at different types of lesions.

The discussion is interesting and the speculation about the arrangement of Rev1-Pol zeta-Pol kappa as a safeguarding mechanism will stimulate new lines of research. It also replaces the presented results in the context of human cells giving even more relevance to their findings.

I feel this paper is greatly suitable for NSMB as it reports interesting and, in my opinion, important and novel findings. The data are clear and support the conclusion.
I only have a few questions or suggestions:

-The author used the p3d-Phen-A lesion as a minor groove DNA lesion. While they clearly show the requirement of Pol kappa to bypass this lesion, I am not sure they can generalize this role of Pol kappa for ALL the minor groove DNA lesions solely based on this data. I would recommend they discuss this point, by suggesting from their p3d-Phen-A lesion, their Illudin S screening and previous in vitro data that Pol kappa is a minor groove specialized TLS polymerase.

We agree with the reviewer's comment. We have modified the abstract so that we do not generalize Polκ catalytic activity across all minor groove lesions:

"Strikingly, we show that Polκ has two main functions during TLS, which are differentially regulated via Rev1 binding. On the one hand, Polκ is essential to replicate across a minor groove DNA lesion in a process that depends on PCNA ubiquitylation but is independent of Rev1."

Similarly, we have modified the text throughout the manuscript to avoid generalization across all minor groove adducts. In the discussion, we now propose that Polκ is likely to be a TLS polymerase specialized in minor groove bypass based on previous in vitro work, our tilling screen with Illudin S, and our replication reaction across the minor groove adduct.

-line 104: isn't AAF generally considered as a major groove DNA adduct? The 2 references given here (Choi 2006 and Ogi 2002) relate to BaP, and not to AAF.

Thank you for pointing this out. We have corrected the mistake and now refer to benzo[a]pyrene (BP) and acylfuvenes (AF).

-line 173-: "…the screen containing several sgRNAs that were predicted to introduce missense mutations in each of these domains." I understand that mutations were predicted in these domains,

but did the authors checked if mutations actually arose at these particular sites? If for some reason the base editor did not work at these sites, this could explain why the cells were not sensitized to Illudin S.

We have not checked whether the base editing occurred at these sites and the reviewer is correct that in principle the absence of sensitivity could be due to the absence of editing. We have now clarified this point in the results section (p7).

-The authors propose that the role of Pol kappa is to stabilize the Rev1-Pol zeta complex. While this is presented like a hypothesis in the manuscript (line 505), it appears like a statement in the abstract (line 40) and in the introduction (line 137).

We agree with the reviewer's comment. Since this is a model based on our mutant analysis, we have toned down the statements in the abstract and line 137 to clarify that this is a model that best explain our biochemical evidence.

-Fig6A: in the absence of Pol eta, Rev1-Pol zeta can achieve both the insertion AND the extension steps. This insertion activity also requires Pol kappa as in the absence of Pol kappa, no insertion is achieved by Rev1-Pol zeta. The authors only mention the requirement of Pol kappa for the extension capacity of Pol zeta, not the insertion. This might be worth mentioning.

Thank you for pointing this out. We have clarified this point in the result section (p13).

-In the discussion (lines 475-), I am not sure to fully understand the reinterpretation of the previous study from Williams et al. . Maybe some more details on these previous findings should be given to improve the clarity of this paragraph.

We have clarified our thoughts with regards to Polκ function during NER. See new section of the discussion (p19 and 20).

Minor points
- It would facilitate the reading of the manuscript if the explanation of the assay (Fig S1E) were in the main text.

We have added the scheme present in Figure S1E to the main figure (new Figure 1G).

-line 82: M.HpaII-based DPC should be further describe here.

We have clarified what a M.HpaII-based DPC is.

-Figure 1D: the resolution of the p3d-Phen-A structure should be improved

We now provide a high-resolution image of the p3d-Phen-A structure (new Figure 1D).

-It would made the manuscript easier to read if the name of the lesion studied were displayed on the figures. This is the case in some occurrences (Fig 1E or Fig 3C for instance), but in most of the cases the reader has to go get the information in the legend.

We have added the name of the plasmid above all replication gels.

-line 339: to facilitate the reading, AP-ICL should be described as it is not a well-known type of lesion.

We have clarified what an AP-ICL is (p12).

Reviewer #3:
Remarks to the Author:
This manuscript describes important new mechanistic insights into the role of the translesion synthesis (TLS) polymerase Pol kappa (PolKappa) in the bypass of DNA lesions in the major or minor groove of the DNA. Different functions, catalytic and non-catalytic, are attributed specifically to minor and major groove lesions respectively. Based on previous reports showing that PolKappa specifically bypasses lesions present in the minor groove in vitro, but that PolKappa deficient cells are also sensitive to agents inducing major groove DNA lesions, the authors suggest a non-catalytic role in the bypass of major groove lesions. In a series of elegant experiments the authors define this mechanism.
First, using a CRISPR-Cas9 base editor tiling screen the authors identify regions in PolKappa required to confer resistance to Illudin S, a compound that induces minor groove lesions. Next, using Xenopus egg extracts the DNA replication-coupled bypass of another minor groove lesion (p3d-Phen-A, an Illudin S derivative) is recapitulated and confirms the requirement of PolKappa's catalytic activity and the importance of PCNA and ubiquitin interaction domains. Subsequently, other DNA lesions, those that form in the major groove of the DNA (DNA protein crosslinks), and/or require PolZeta mediated extension (abasic sites) are studied. PolKappa is also required for the bypass of these lesions but this is independent of its catalytic activity. Moreover, lesion bypass requires interaction with REV1 through PolKappa's REV1 interaction domain (RIR) that was not required for bypass of the minor groove lesions. The non-catalytic function of PolKappa seems to act by stabilization of the REV1-PolZeta complex on chromatin through a direct interaction. A model indicates that the catalytic role of polymerase kappa in the bypass of minor groove lesions is independent and maybe even mutually exclusive with the non-catalytic role of PolKappa together with REV1-PolZeta.

Translesion synthesis is an important mechanism in the response to DNA damage. Several specialized polymerases act in this response and among these PolKappa is one of the least well-studied. This work contributes important new mechanistic details into several functions of PolKappa and its interplay with other factors involved in TLS which will be of great value to the field. The manuscript is clearly written and the experiments are well explained and beautifully executed. To my opinion this work is suited for NSMB but I would like to see a few points addressed before I would recommend publication.

Major points:

The model in Fig. 7 describes two modes of action of PolKappa on different lesions. According to this model bypass of minor groove lesions such as p3d-Phen-A require PolKappa's catalytic activity and this is independent of REV1. This is based on experiments (mainly Fig. 1 and 2), and modeling (Fig. S7). However, the presence of REV1 at the lesion is not experimentally examined. While the interaction between PolKappa and REV1 through the RIR domain does not seem to be involved (Fig. 2B) it can be envisioned that REV1 is recruited to the lesion by another interaction site (possibly through PCNA-Ub). Through this interaction REV1 could be important to recruit PolZeta for extension. While the authors show that REV1 depletion (Fig. 2C) does not lead to a defect in lesion bypass this could be caused by a redundant function of PolKappa that has been shown to be able to perform this extension step. While the authors implicate that the bypass of minor groove lesions in

general depend on polKappa's catalytic activity and do not require REV1/PolZeta, it is important to note that there are examples in the literature that show involvement of REV1/PolZeta in such lesions (Hodskinson et al. 2020). The potential involvement or presence of REV1 is an important point to address since it can have implications for the model (potentially the complexes formed on minor and major groove lesion are in fact more similar but the division of labor differs). Therefore, I would strongly suggest an experiment that shows whether REV1 is recruited to the p3d-Phen-A containing plasmid during bypass or not. And if it is recruited, how does PolKappa depletion affect this recruitment? Moreover, a double depletion of PolKappa and REV1 would be highly interesting (if experimentally possible) since the single PolKappa depletion does not fully block lesion bypass (extension products of the damaged strand still increase over time). If this further blocks lesion bypass it indicates REV1 can act redundantly.

We thank the reviewer for pointing out this relevant point.

To address whether Rev1-Polζ could act independently of Polκ for the bypass of the minor groove DNA adduct, we performed the experiment suggested by the reviewer and compared a Polκ–depleted reaction to a Polκ-Rev1 double depleted one (Figure R6A). Although some minor bypass can be observed in the absence of Polκ (Figure R6C, lanes 1-3), this bypass does not appear to be dependent on Rev1 (compare lanes 1-4 to 9-12). To address whether Polκ could stimulate Rev1-Polζ to bypass the minor groove adduct via its non-catalytic function, we also supplemented the depleted extracts with the catalytic dead version of Polκ. However, we did not observe any significant impact of Polκ CD in either condition suggesting that Rev1-Polζ is not involved in the bypass of p3d-Phen-A (Figue R6C, lanes 5-8 and 13-16).

Although Rev1-Polζ is not needed for bypass of the minor groove adduct, Rev1-Polζ complex could still be recruited to the plasmid as suggested by the reviewer. We have now investigated this possibility during the replication of p3d-Phen-A. In new Figure S6I we show how Rev1 and Rev7 are also recruited to of p3d-Phen-A, and that this recruitment is also dependent on Polκ. These data suggest that different TLS polymerase complexes are formed regardless of the nature of the DNA lesion but that the TLS employed for bypass is dictated by the lesion itself. We now added this conclusion in the results and discussion sections (p15 and p20).

As pointed by the reviewer, Rev1-Polζ has been recently implicated in the bypass of the acetaldehyde ICL, which is expected to result in a minor groove DNA adduct following crosslink reversal of the ICL. Because the exact nature of the lesion left behind following reversal of the ICL remains unknown, it is difficult for us to speculate on why would the bypass be dependent on Rev1-Polζ (note that Polκ was not tested in that study). As mentioned by the reviewer, this acetaldehyde ICL substrate could be an example where Polκ is not able to fully bypass the lesion despite the lesion being located in the minor groove. Polκ might require Rev1-Polζ for extension and full bypass. In the discussion, we speculate on the advantage of having two TLS polymerases with extension activity being part of the same complex (i.e. if one polymerase cannot extend the other one might), although our works only reports the stimulation by Polκ of Rev1-Polζ.

Note that we have also toned down the conclusions of the manuscript regarding the minor groove activity of Polκ according to reviewer #2's comment #1.

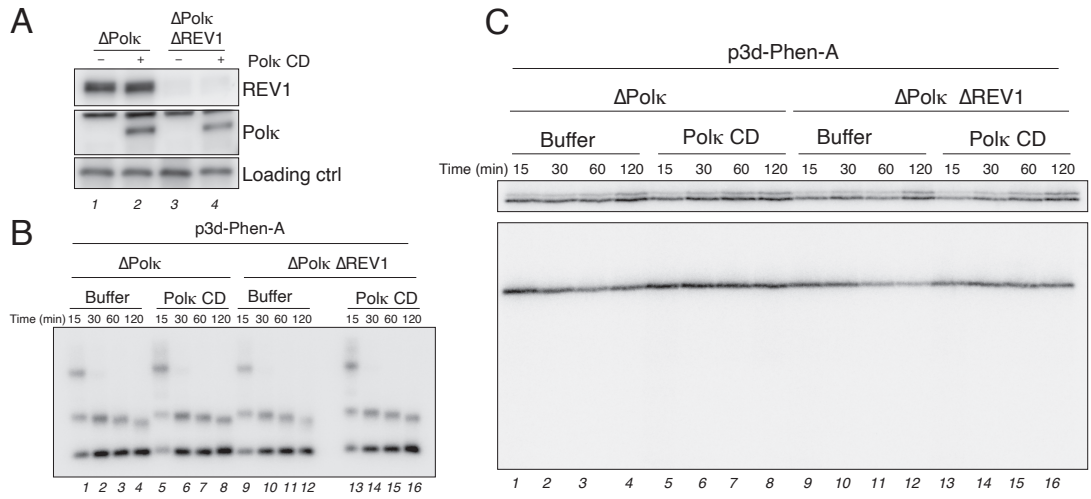

Figure R6. A*. Polκ or Polκ and Rev1-depleted extracts were supplemented with either buffer or Polκ CD.* ***B****. Extracts from (A) were used to replicate 3d-Phen-A plasmid in the presence of [α-^{32}P]dATP. Reaction samples were analyzed by native agarose gel electrophoresis.* ***C****. Samples from (B) were digested with HaeII and HindIII followed by separation on a denaturing polyacrylamide gel as in Figure 1E.*

To determine the mechanism of the non-catalytic function of PolKappa in the bypass of DPC's and abasic sites the authors switch to using UV-treated chromatin. This is surprising since it is not clear whether UV damage is restricted to the major groove, and more importantly, whether the bypass of UV lesions requires the non-catalytic function of PolKappa. While the authors nicely show that PolKappa and PolZeta form a partially interdependent complex on chromatin after UV treatment the functional relevance of this data for the non-catalytic role of PolKappa is not clear. To show that this interdependent complex is also formed on the relevant lesions the authors need to show this in the context of a DPC or abasic site lesion.

Because we had previously observed the recruitment of TLS polymerases to UV-C damaged chromatin (Gallina et al., 2021; Schubert et al., 2022), we used the same approach to investigate the co-dependency between Rev1-Polζ and Polκ. Importantly, we knew from the literature that POLK KO cells are sensitive to UV treatment despite Polκ being unable to synthesize across UV lesions in vitro (Ogi & Lehmann, 2006; Olivieri et al., 2020). Moreover, Polζ is required for the bypass of UV induced 6-4 photoproducts (Gibbs et al., 2005; Yoon et al., 2010). Thus, we thought that Polκ could also assist Rev1-Polζ in bypassing UV lesions (i.e. 6-4 photo products). Based on our results using UV-treated sperm chromatin, we think that this might indeed be the case, although as pointed by the reviewer, we do not directly show the functional relevance of the complex in this context as we do not monitor replication across 6-4 photoproducts (we have not been able to synthesize such a plasmid).

Another reason for using damaged induced sperm chromatin is that it is technically easier to monitor TLS polymerase recruitment to sperm chromatin (which involves a quick spindown of the chromatin via a sucrose cushion), than via plasmid pulldown (which is a 30 min process during which the polymerases can fall off the plasmid). In fact, although we do detect a very nice recruitment of Polη to UV-C treated chromatin,

we have never been able to detect its recruitment to the DPC plasmid via plasmid pulldown despite its activity being needed for insertion.

Having clarified our reasoning for using UV-C treated sperm chromatin, we agree with the reviewer that we also needed to show the interdependency between Polκ and Rev1-Polζ on our relevant pDPC plasmid. We have now added this experiment (new Figure S6H; and see also our response to reviewer #1, point #7 for more details related to our UV-C CHROMASS).

While two compelling models are drawn in Fig. 7 showing the catalytic and non-catalytic roles of PolKappa, not all aspects of these models are supported by data. First of all, as mentioned above, it is not shown experimentally that REV1 and PolZeta are not recruited to minor groove lesions. Second, the authors base the mutually exclusive nature of these models on the fact that binding of the PolKappa's RIR domain to REV1 in this model prohibits interaction of PIP1 with PCNA. However, the data showing the relative roles of the PIP domains in the non-catalytic function are not so clear. While it seems that PIP2 might be slightly more important (Fig. S4F), there is very little difference with PIP1 mutants and the data definitely does not support the claim 'PolKappa non-catalytic function requires PIP2 and is independent of PIP1 (line 501)'. Based on the current data I would suggest a third model in which REV1 and PolZeta are also recruited to minor groove lesions where PolKappa's catalytic activity is important and REV1/PolZeta may act redundantly (depending on the outcome of the experiment suggested above). Moreover, instead of the very complicated structural models I would prefer to see this in an easier to grasp cartoon form (structural models can be moved to the supplement).

We now provide a new experiment that more clearly shows the difference between PIP1* and PIP2* mutants in the bypass of the DPC lesion (new Figure S4F).

As mentioned above, we also show that Rev1-Polζ is indeed recruited to the minor groove adduct plasmid despite not being required for bypass (new Figure S6I). We think that the different TLS complexes are formed and recruited to DNA lesion sites irrespectfully of the nature of the lesion. Yet, the nature of the lesion is what dictates the polymerase employed for bypass. Thus, our models are solely based on the TLS activities observed and we now clarified in the result and discussion that these complexes might form on DNA irrespectively of the lesion type.

Other points:

1) Throughout the manuscript it is mentioned that PolKappa is unique in bypassing minor groove lesions. While the references indeed show PolKappa can do this, I don't see the evidence that this is unique among all TLS polymerases. Moreover, this has only be studied in reconstitution assays. I think this claim could be toned down a little bit (or proper references should be supplied).

We agree with the reviewer's comment. This point was also raised by reviewer #2 and we have toned down this claim throughout the manuscript (see our response to reviewer #2, point #1).

2) Title of the second paragraph of the results 'PolKappa-mediated bypass of minor groove lesions depends on PCNA ubiquitylation but is independent of REV1'. The dependence on the PIPs is shown

but it is not shown that PCNA is ubiquitylated. The authors should at least show a blot that bypass of this specific lesion induces PCNA ubiquitylation.

We have now added a plasmid pulldown experiment where we detect PCNA ubiquitylation during replication of pMinor groove (Figure S6I).

3) Fig. S2C and D. No protein add back is shown in the blot for the double PIP mutant. A defect in this mutant could thus be caused by low or absent protein. Protein levels are crucial to draw any meaningful conclusion. Same point for S2E and F and the double UBZ mutant.

We have added the corresponding controls where we compare protein addback across the different PIP (new Figure S2C) and UBZ mutants (new Figure S2E). Note that the Polκ antibody was generated against the C-terminus of Polκ, which contains PIP2. As a result, Polκ mutated in PIP2 is not efficiently recognized via the Polκ antibody. This is the reason a His blot is also provided for the PIP mutants to demonstrate that we are indeed adding similar amounts of proteins across the mutants.

4) Line 245: While UBZ2 seems to be important it is definitely not critical, there is still much more bypass compared to the buffer control (Fig. 2E). Tone down statement.

We have toned down the statement which now reads:

"Lastly, analysis of Polk UBZ domains further agreed with our base editor screen and revealed that UBZ2 but not UBZ1 is important for Polκ function (Figure 2E and S2E-F)."

5) Fig. S4G, a sequencing gel of this experiment is would be helpful to properly assess the rescue of the UBZ1* mutant.

We have added the requested sequencing gel (new Figure S4G).

6) Line 415: PolKappa is reduced but no abolished (Fig. 6D, band is still visible). Tone down conclusion.

We have modified the text which now reads:

"Similarly, the recruitment of Polκ was reduced in the absence of Rev1 (Figure 6D, compare lanes 4 to 7 and 10 to 13)."

7) Line 418: REV1/PolZeta is only slightly reduced (Fig. 6E), definitely not lost. Tone down conclusion.

We have toned down the text which now reads:

"The reduction of Rev1-Polζ recruitment observed upon Polκ depletion could be reverted by addition of either Polκ WT or Polκ CD (Figure 6E, compare lanes 5 to 6-7)."

8) It would be useful to include Coomassie stained gels of the purified proteins to assess their purity. To confirm that the catalytic site mutant is inactive the authors use PolKappa expressed in rabbit

reticulocytes (Fig. S1F, methods for this experiment seem to be missing) while all other experiments are performed with protein purified from E. Coli. Did the authors do the activity test also on the protein from E. Coli?

At the start of this project, we used Polκ expressed from rabbit reticulocytes to rescue Polκ depletion in egg extracts. We then moved to a bacterial expression system because the yield was much higher. The experiment shown in old Figure S1F was performed with the old reticulocyte preparations. We have now performed the same experiment with the bacterial Polκ preparation (new Figure S1H). As a result, all experiments that are now shown in this manuscript were performed using recombinant Polκ purified from bacteria. We have therefore removed the rabbit reticulocyte methodology from material and methods and have also added a Coomassie stained gel of our Polκ preparations (new Figure S1G).

9) Line 478: The lesion studied in the Williams et al. paper is a trimethylene ICL, how is this reminiscent to a MMC induced ICL? The structure is very different.

Both lesions are formed via crosslinks at the N2 position of guanosine, which is located on the minor groove. However, we agree that these are structurally very different and now refer to the ICL generated in the Williams et al. manuscript as a trimethylene ICL.

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Decision Letter, first revision:

Message: 22nd Apr 2024

Dear Dr. Duxin,

Thank you again for submitting your manuscript "Catalytic and non-catalytic functions of DNA polymerase kappa in translesion DNA synthesis". I apologise for the delay in responding, which resulted from the difficulty in obtaining suitable referee reports. Nevertheless, we now have comments (below) from the 3 reviewers who evaluated your paper. In light of these reports, we remain interested in your study and would like to see your response to the comments of the referees, in the form of a revised manuscript.

You will see that Reviewers #2 and #3 deem that the manuscript is ready for publication, pending toning down and caveating in some areas in accordance to the guidelines for reviewer #3 which we kindly ask you to do at this stage. On the other side, reviewer #1 retains some technical concerns which we deem require addressing. Particularly, we think that the requested controls and clarifications in points 2,3,7 are important and should be provided or highlighted. We additionally note that points 1 and 6 require textual addressing and that though experimentally addressing points 4 and 5 would further strengethen the manuscript, we do not deem them as essential for the publication of the story.

Please be sure to address/respond to all concerns of the referees in full in a point-by-point response and highlight all changes in the revised manuscript text file. If you have comments that are intended for editors only, please include those in a separate cover letter.

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We require deposition of coordinates (and, in the case of crystal structures, structure factors) into the Protein Data Bank with the designation of immediate release upon publication (HPUB). Electron microscopy-derived density maps and coordinate data must be deposited in EMDB and released upon publication. Deposition and immediate release of NMR chemical shift assignments are highly encouraged. Deposition of deep sequencing and microarray data is mandatory, and the datasets must be released prior to or upon publication. To avoid delays in publication, dataset accession numbers must be supplied with the final accepted manuscript and appropriate release dates must be indicated at the galley proof stage.

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We look forward to seeing the revised manuscript and thank you for the opportunity to review your work.

Sincerely,

Dimitris Typas
Associate Editor
Nature Structural & Molecular Biology
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Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
The additional data and clarifications provided have greatly improved the manuscript. The experiments clearly show that Polk has a non-catalytic role in the by pass of major groove DNA lesions, probably in a complex with Rev1 and Pol zeta. An important point to clarify is that although Polk depletion leads to transient replication forks stalling on circular DNA templates, this is not a permanent arrest since the plasmid is replicated by the extract in the absence of Polk (Fig. 1H; 3D; 4A, D as an example). Authors must speculate how this can happen.

In my opinion, there are still few points listed below that need to be addressed to support the stated claims.

1_The authors have now provided more satisfactory data supporting the specificity of the antibodies they have used in this study. Notwithstanding, while authors claim that they have affinity-purified the antibodies, it is unclear why the affinity-purified antibodies still recognizes two non-specific polypeptides (apparent electrophoretic mobility 130 and 95 kDa visible in Fig. R1). It may suggest that the affinity purification was not very productive. At any rate, since the specificity of the antibody is convincing, for the sake of

clarity, the authors could crop the Polk blots to show only the Polk signal?

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3_Fig. S2C shows that the level of Polk PIP1 and 2 added back is lower than Polk wild-type. This is a critical point since these mutants only partially rescue the phenotype compared to Polk wild-type. Either the authors have to repeat the experiment using a higher amount of these mutants comparable to wild-type, or state that they cannot exclude that failure to rescue is due to lower concentration of these mutants.

4_Regarding the effect of a non-ubiquitynatable PCNA (PCNAK164R mutant), due to difficulty to completely deplete PCNA from NPE, one alternative would have been to add excess recombinant PCNAK164R to the extract to mimic a dominant negative effect. Have the authors tried this strategy?

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Polk is not visible in the cell extract, when compared to the knock-out clone by western blot. Where is Polk? What is the polypeptide recognized by the antibody marked with a star? This is not described.

Reviewer #2:
Remarks to the Author:
The authors have responded to all of my inquiries and have modified the manuscript accordingly, providing clarification on points that were previously unclear to me. Notably, the role of PolK in NER is now much clearer (p19).

They have also addressed all major issues raised by the other two reviewers, primarily by incorporating a significant amount of additional data to meet their requests or by revising the text and offering constructive responses to the reviewers' comments.

In particular, they have presented a new set of data using U2OS cells that validates their findings and underscores the most important message of the manuscript in mammalian cells.

As previously stated in my earlier report, I believe this paper is highly suitable for NSMB.

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A minor observation: the abbreviation 'CSOL' (closely spaced opposing lesions) is only utilized twice throughout the paper (p14 and p19), suggesting that it may not need to be abbreviated.

Vincent Pagès

Reviewer #3:
Remarks to the Author:
To address the reviewers comments the authors performed a number of new experiments and made many textual changes. As described below most of my questions have been anwered and I recommend publication of this work in NSMB after some additional textual changes as indicated below.

I suggested to analyse the recruitment of REV1/PolZeta to minor and major groove lesions on plasmids in the presence and absence of PolKappa. The authors now show that REV1/polZeta is also recruited to minor groove (p3d-Phen-A) and major groove lesions (pDPC), and that this recruitment is reduced upon polKappa depletion. This is consistent with what they observed for UV-treated sperm. This is a great experiment and fits with their hypothesis, however, the claim that recruitment of REV1/PolZeta is dependent on PolKappa needs to be toned down. REV1/PolZeta is reduced but not absent from the blots in S6H, G, and I. Therefore, PolKappa is important for efficient REV1/PolZeta recruitment, but this recruitment is not fully dependent on PolKappa.

As I requested, double depletion experiments of PolKappa and REV1 have been performed. These showed no further reduction of TLS compared to PolKappa depletion only. While this experiment has some limitations since it does not contain a positive control (mock or WT add back), and it can therefore not be formally excluded that the defect is caused by inactivation of the extract, I realize these experiments are challenging to perform with many conditions. I consider this experiment sufficient since the data is not presented in the manuscript.

The catalytic activity of the polKappa protein (mutant and WT) purified from bacteria is now properly tested and coomassie gels of the purified proteins are presented.

A new experiment with the PIP box mutants is now presented in Figure S4F. This shows more clearly the difference between those mutants and supports the claim that PIP2 is more important than PIP1.

I do still have some issues with the description of the roles of the PIP/UBZ domains in the minor groove lesions. Especially the title of the paragraph: Polk-mediated bypass of minor groove lesions depends on PCNA ubiquitylation but is independent of Rev1. To my opinion, it has not been experimentally shown in this manuscript that polKappa binds to ubiquitylated PCNA, or that bypass depends on PCNA ubiquitylation. It has only been shown that the PIP domain and the UBZ domain are important for bypass. Bypass does not fully depend on PIPs or UBZs as none of the mutants (especially not the single mutants, but also not the double mutants) are as defective as the depleted condition. Therefore, the title should be rephrased accordingly.

Finally, I suggested a minor groove lesion of which bypass required REV1 as described in Hodskinson et al. 2020. The authors indicate that: 'Because the exact nature of the lesion left behind following reversal of the ICL remains unknown, it is difficult for us to speculate on why would the bypass be dependent'. However, in Extended Data Figure 6E of that paper a N2-propanoguanine monoadduct is described which is a highly defined lesion and REV1 is required for efficient lesion bypass. However, based on our and the other reviews the authors toned down the claim that this REV1 independent lesion bypass is used by all minor groove lesions, therefore this is no longer relevant.

Author Rebuttal, first revision:

Point-by-Point response to reviewers

General remarks
We appreciate the time of the reviewers in evaluating the revisions of our manuscript. See below our response to their remaining comments.

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
The additional data and clarifications provided have greatly improved the manuscript. The experiments clearly show that Polk has a non-catalytic role in the by pass of major groove DNA lesions, probably in a complex with Rev1 and Pol zeta.

We thank the reviewer for acknowledging our efforts in improving the manuscript.

An important point to clarify is that although Polk depletion leads to transient replication forks stalling on circular DNA templates, this is not a permanent arrest since the plasmid is replicated by the extract in the absence of Polk (Fig. 1H; 3D; 4A, D as an example). Authors must speculate how this can happen.

We are unsure what the reviewer is questioning. When bi-directional DNA replication is initiated on a plasmid, two DNA replication forks originating from the same replication origin move in opposite directions and eventually meet. The presence of a minor groove adduct does not directly inhibit CMG (Cdc45-MCM-GINS) translocation (although CMG is likely to slow down once it becomes uncoupled from nascent strand synthesis). Once the two opposite CMGs encounter each (after one of them has bypassed the lesion), DNA replication terminates. This termination occurs regardless of whether DNA synthesis is completed (i.e., whether the polymerase has bypassed the DNA lesion or not). Thus, the appearance of monomeric plasmids occurs with almost identical kinetics in Mock or Polκ depleted reactions (Figure 1H, OC and SC molecules). However, in the absence of Polκ, one of the daughter molecules accumulates with a DNA gap because of the polymerase block (Figure 1H and illustration in Figure 1G). These gap molecule intermediates smear down on the gel and lose intensity because of 5' to 3' DNA resection (see Duxin et al., 2014, Cell, for more details).

Since we had already moved the scheme of plasmid replication to the main figure (Figure 1G), we do not feel that we need to explain this any further.

In my opinion, there are still few points listed below that need to be addressed to support the stated claims.

1_The authors have now provided more satisfactory data supporting the specificity of the antibodies they have used in this study. Notwithstanding, while authors claim that they have affinity-purified the antibodies, it is unclear why the affinity-purified antibodies still recognizes two non-specific polypeptides (apparent electrophoretic mobility 130 and 95 kDa visible in Fig. R1). It may suggest that the affinity purification was not very productive. At any rate, since the specificity of the antibody is convincing, for the sake of clarity, the authors could crop the Polk

blots to show only the Polk signal?

We are pleased that the reviewer is now satisfied with the antibodies' specificity. Rather than cropping the blots, we have now indicated the bands as none-specific (in the Figure panels and legends; new Figure 1F, S2A, S2C, S2E, S6G).

2_Fig. S1G only displays recombinant Polk wild-type and catalytic-dead (CD), but no other recombinant Polk mutant proteins used in the manuscript (RIR, PIP1, PIP2, Polk C-ter etc.). These must be shown. Also, the Coomassie gel shows the presence of two polypeptides, the slower migrating one supposed to be Polk. What about the faster migrating one? Have the authors performed a western blot to ascertain the identity of this latter polypeptide (degradation product or contaminant?).

We do not know the identity of the lower band but since it is not recognized by the HIS antibody, it is unlikely to be a Polκ degradation product purified via the nickel beads (Figure R1A-B below). Consequently, we have now designated the lower band as a contaminant in the Polκ preparation (new Figure S1G).

Regrettably, we lack Coomassie stains for the remaining mutants, and many of our stocks are now depleted, which would require re-purification of the Polκ mutants. However, we have included Western blots for the addback of all the mutants in the manuscript. These blots clearly illustrate the amount of recombinant protein reintroduced into the extracts. Notably, the key mutants exhibit distinct behavior depending on the substrate, demonstrating their functionality. For instance, the RIR mutant rescues p3d-PhenA but not pDPC TLS. Below, we further present a Western blot where all the mutants were carefully titrated so that we added (to the best of our capacity) the same amount of Polκ protein back into the extracts (Figure R1C).*

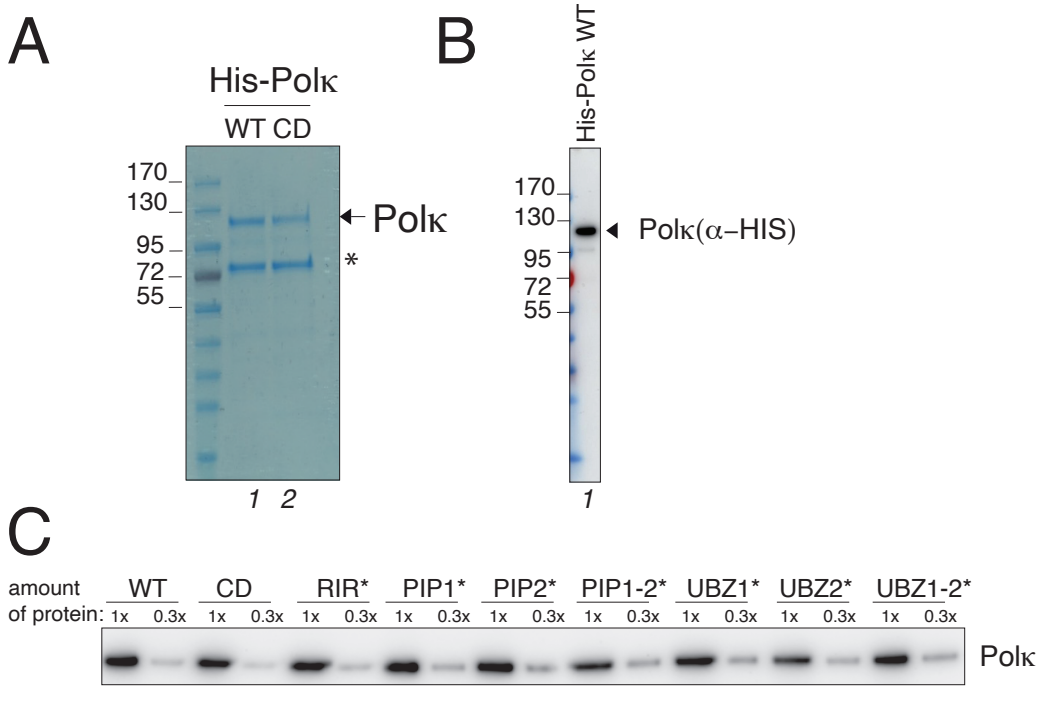

Figure R1: *A) Coomassie blue staining of recombinant Polk WT and Polk CD purified from E.coli. (duplicated panel from Figure S1G). B) Immunoblot (with anti-HIS) of the Polκ WT preparation. Note that the band observed around 75 kDa in the Coomassie stain (A), is not recognized with the HIS antibody. C) The different Polκ preparations were titrated, and equal amounts were confirmed via immunoblotting (via HIS antibody). 1x corresponds to the amount needed to add back roughly endogenous levels of Polκ (when added to 10% F.C in NPE).*

3_Fig. S2C shows that the level of Polk PIP1 and 2 added back is lower than Polk wild-type. This is a critical point since these mutants only partially rescue the phenotype compared to Polk wild-type. Either the authors have to repeat the experiment using a higher amount of these mutants comparable to wild-type, or state that they cannot exclude that failure to rescue is due to lower concentration of these mutants.

The small difference alluded by the reviewer on the HIS blot (e.g. PIP1 signal is very slightly less than WT) is not observed when the same sample is blotted with the Polκ antibody (if anything, PIP1* signal is now slightly more than WT; Figure S2C). We attribute these minor variations to intrinsic variability in Western Blotting. Given our careful protein titration (as shown in Figure R1C), we wish to maintain the conclusions of our manuscript.*

4_Regarding the effect of a non-ubiquitynatable PCNA (PCNAK164R mutant), due to difficulty to completely deplete PCNA from NPE, one alternative would have been to add excess recombinant PCNAK164R to the extract to mimic a dominant negative effect. Have the authors tried this strategy?

In our original response to point #6, we attempted to address this issue. However, due to PCNA's high concentration in NPE (~50 μM; one of the most abundant proteins in egg extracts), conducting this experiment without prior partial depletion of endogenous PCNA is impossible. In Figure R3 of our original response, we demonstrated that after four rounds of depletion, we reduced PCNA concentration to 10% (approximately 5 μM). Under these conditions, we successfully restored TLS across the adduct by adding back PCNA wildtype. In contrast, the same extent of restoration was not achieved with the K164R mutant (as evidenced by the generation of SC products; compare lanes 7-12 to 17-18). While these results suggest that TLS depends on PCNA K164 in egg extracts, the technical challenges lie in achieving the precise level of PCNA depletion—partially inhibiting its function without fully inactivating the extract. Unfortunately, we have been unable to consistently reproduce this effect, and we cannot provide a clean system for assessing PCNA K164R function with confidence.

The sole effective method we have for inhibiting PCNA ubiquitylation is by depleting RFWD3 from egg extracts (as demonstrated in Gallina et al., 2021, Molecular Cell). Our data below indicate that RFWD3 depletion hinders the bypass of both pDPC and pMG lesions, consistent with PCNA ubiquitylation playing a regulatory role in translesion synthesis (TLS) activity across these DNA lesions (Figure R2A-B). However, we do not feel that adding this data to the manuscript would benefit our story.

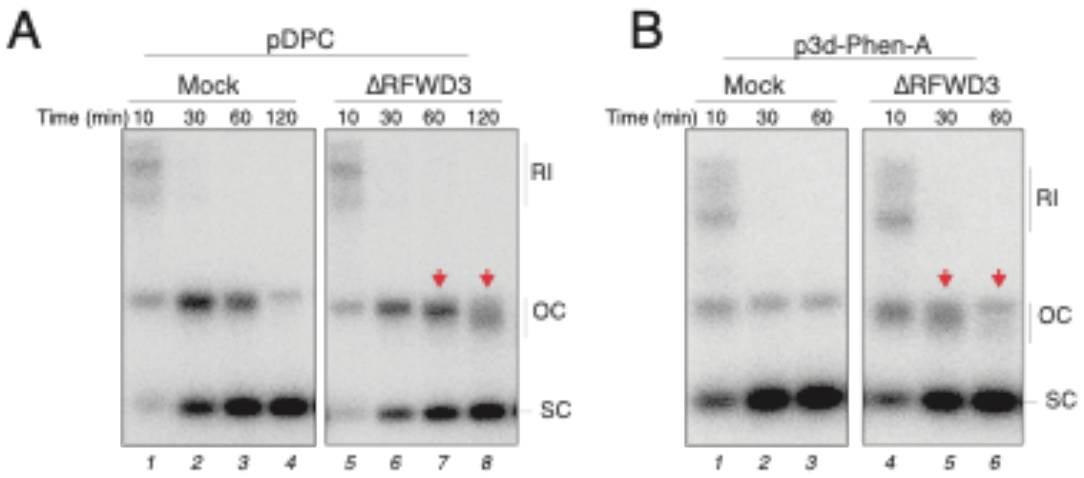

Figure R2: A) pDPC was replicated in Mock or RFWD3 depleted extracts. pDPC replication in the absence of RFWD3 leads to stabilized OC molecules due to TLS inhibition. B) Samples from (A) were used to replicate p3d-Phen-A. Note that RFWD3 depletion also leads to the accumulation of OC molecules consistent with TLS inhibition.

5_Regarding the Polk Rev1 binding domain (RIR), although it is established that this is required for interaction in cellular systems, it is important to show that this is also the case in the Xenopus system. This is in principle an easy experiment, since it simply involves adding the two recombinant proteins (Polk wild-type and delta RIR) in the egg extract and then perform an immunoprecipitation to see whether Rev1 co-immunoprecipitates. Otherwise, authors must withdraw this claim and rephrase it is as " Polk lacking the RIR failed to stimulate the bypass of major groove DNA lesions and abasic sites".

We conducted the experiment suggested by the reviewer. We immobilized either Polκ WT or Polκ RIR on nickel resin and incubated them in egg extracts (NPE). While Polκ WT efficiently recovered Rev1, Polκ RIR* exhibited reduced Rev1 binding. Because Polκ-Rev1 interaction via Polκ's RIR has been extensively reported in the past, we wish to maintain our claim. However, we have rephrased our statement as follow:*

"Thus, in contrast to Polκ-mediated TLS of the minor groove adduct, the Polκ-Rev1 interaction via Polκ's RIR domain appears to be critical for the bypass of a major groove DPC."

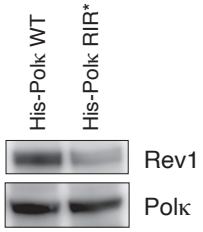

Figure R3: Equal amount of His-Polκ WT and His-Polκ RIR was immobilized on Ni-NTA resin and incubated in 10 μL of NPE for 2 hours. The precipitate was then washed and analyzed by immunoblot via the indicated antibody.*

6_Regarding the CHROMASS experiment, as a comment to the fact that "at high UV-C dose, both strands of the DNA are expected to be damaged in close proximity…", this generates a DNA double strand break. Hence, in the conditions of high UV-C doses this type of DNA damage is supposed to be predominant.
Authors now clearly state that this experiment was performed in the absence of DNA replication, (as suggested in the materials and methods). Can the authors clearly state what they mean by "non-licensing extracts" for non-specialist and clealry define at which step of DNA replication the process is blocked? It is somehow confusing though, to switch from a replicating system (NPE supplemented with a circular template molecule containing a single DNA lesion) to a no-replicating system supplemented with sperm chromatin damaged by a high UV-C dose, in order to obtain evidence for a Polk-Rev1-Polzeta complex on damaged chromatin. Yet, these experiments, and the new ones provided by the authors (Fig. S6H-I) do not formally demonstrate a Polk-Rev1-Polzeta complex formation, but dependency for chromatin binding. Authors can rephrase the claim in this direction, stating that "this result suggests complex formation". Of note, I believe that authors can somehow claim complex formation since Figure S6G shows that Polk depletion also partially co-depletes Rev1 and Rev7 (quantification could prove this point). Have the authors tried to blot Polk immunnoprecipitates with Rev1 and rev7 antibodies? This result would strengthen the conclusions of the manuscript.

The primary damage caused by UV-C radiation is the formation of pyrimidine dimers. We posit that closely spaced lesions do not predominantly result in DNA double-stranded breaks; instead, they lead to pyrimidine dimers on each of the DNA strands. This scenario necessitates translesion synthesis (TLS) following a first round of nucleotide excision repair (NER), as detailed in our response to the original point #7 raised by the reviewer.

To replicate DNA, we initially license the DNA in HSS, which facilitates MCM2-7 licensing. Subsequently, we add NPE, which triggers DNA replication. Notably, due to the high concentration of geminin in NPE, this extract does not support licensing. Consequently, by mixing HSS and NPE before adding DNA (rather than the sequential incubation in HSS followed by NPE), one can effectively block licensing, preventing MCMs from loading onto the DNA.

Because the Polκ-Rev1-Polζ complex complex has previously been reported (Xie et al., 2012), we do not think that we need to tone down our claims any further.

7_Fig. S7B, form the Materials and methods section and the figure legend it is not clear what the parental cell line is. If this is supposed to be a wild-type cell line, the endogenous Polk is not visible in the cell extract, when compared to the knock-out clone by western blot. Where is Polk? What is the polypeptide recognized by the antibody marked with a star? This is not described.

Thanks for pointing this out. We have not been able to detect endogenous human Polκ with available antibodies. This is probably because of the low levels of Polκ in U2OS cells and the presence of non-specific bands running at the same size than Polκ. This is the reason we had

to validate the knockout by RT-PCR (Figure S7A). Further experiments demonstrating the sensitivity of the KO cells to Illudin S validated that these cells are indeed depleted of Polκ. We have now added a sentence in the legend of Figure S7B to clarify the fact that we were unable to detect endogenous Polκ.

Reviewer #2:
Remarks to the Author:
The authors have responded to all of my inquiries and have modified the manuscript accordingly, providing clarification on points that were previously unclear to me. Notably, the role of PolK in NER is now much clearer (p19).

They have also addressed all major issues raised by the other two reviewers, primarily by incorporating a significant amount of additional data to meet their requests or by revising the text and offering constructive responses to the reviewers' comments.

In particular, they have presented a new set of data using U2OS cells that validates their findings and underscores the most important message of the manuscript in mammalian cells.

As previously stated in my earlier report, I believe this paper is highly suitable for NSMB.

We appreciate the reviewer's constructive critique of our manuscript and noting that we have addressed all the reviewers' comments, and that the manuscript is suitable for publication at NSMB.

I have only one remaining question regarding the new figure S1G, which shows purified PolK and displays two bands on the Coomassie gel: I could not find in the text or legend an explanation for the lower band. Additionally, 'Coomassie' is misspelled in the legend.

We unfortunately do not know the identity of the lower band, but it is not recognized by our Polκ antibody (Figure R1). We have now marked the lower band as non-specific. We have corrected the misspell.

A minor observation: the abbreviation 'CSOL' (closely spaced opposing lesions) is only utilized twice throughout the paper (p14 and p19), suggesting that it may not need to be abbreviated.

We have removed the abbreviation in the text.

Vincent Pagès

Reviewer #3:
Remarks to the Author:
To address the reviewers comments the authors performed a number of new experiments and made many textual changes. As described below most of my questions have been anwered and I recommend publication of this work in NSMB after some additional textual changes as indicated below.

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We have toned down our conclusions that now reads: "Moreover, although Rev1-Polζ is not involved in the bypass of the minor groove adduct (Figure 2C), we could still detect its recruitment to the plasmid, which was also partially dependent on Polκ (Figure S6I)."

As I requested, double depletion experiments of PolKappa and REV1 have been performed. These showed no further reduction of TLS compared to PolKappa depletion only. While this experiment has some limitations since it does not contain a positive control (mock or WT add back), and it can therefore not be formally excluded that the defect is caused by inactivation of the extract, I realize these experiments are challenging to perform with many conditions. I consider this experiment sufficient since the data is not presented in the manuscript.

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We have modified the tittle of the paragraph which now reads: "Polκ-mediated bypass of a minor groove DNA lesion is independent of Rev1"

Finally, I suggested a minor groove lesion of which bypass required REV1 as described in Hodskinson et al. 2020. The authors indicate that: 'Because the exact nature of the lesion left behind following reversal of the ICL remains unknown, it is difficult for us to speculate on why would the bypass be dependent'. However, in Extended Data Figure 6E of that paper a N2-propanoguanine monoadduct is described which is a highly defined lesion and REV1 is required for efficient lesion bypass. However, based on our and the other reviews the authors toned

down the claim that this REV1 independent lesion bypass is used by all minor groove lesions, therefore this is no longer relevant.

Thank you for pointing this out.

Decision Letter, second revision:

Message: Our ref: NSMB-A48174B

31st May 2024

Dear Dr. Duxin,

Thank you for submitting your revised manuscript "Catalytic and non-catalytic functions of DNA polymerase kappa in translesion DNA synthesis" (NSMB-A48174B). It has now been seen by the remaining referee and their comments are below. Given that in the previous round two reviewers had found the manuscript ready for publication and the remaining reviewer has assessed that the paper has improved in revision, raising concerns which we deem are textually addressable, we can accept in principle the work in Nature Structural & Molecular Biology, pending minor revisions to satisfy the referees' final requests and to comply with our editorial and formatting guidelines.

We are now performing detailed checks on your paper and will send you a checklist detailing our editorial and formatting requirements in about two weeks. Please do not upload the final materials and make any revisions until you receive this additional information from us.

To facilitate our work at this stage, it is important that we have a copy of the main text as a word file. If you could please send along a word version of this file as soon as possible, we would greatly appreciate it; please make sure to copy the NSMB account (cc'ed above).

Thank you again for your interest in Nature Structural & Molecular Biology Please do not hesitate to contact me if you have any questions.

Sincerely,

Dimitris Typas
Senior Editor
Nature Structural & Molecular Biology
ORCID: 0000-0002-8737-1319

Reviewer #1 (Remarks to the Author):

The manuscript has further improved, but few points that remain unclear.

Point 2, I do not understand why authors cannot provide Coomassie staining of the purified recombinant proteins. At a certain stage, they have had to check the purity of these proteins before carrying out the rescue experiments. Evaluating the purity of the purified proteins is the essential basis of a biochemical approach. Why they do not have the raw data corresponding to these experiments? A western blot does not assure the purity of the proteins, but the presence of a protein recognized by the antibody.

Point 6, I believe that it cannot be denied that DSBs are formed on sperm DNA template at high UV-C doses as those used by the authors in the manuscript. Processing of UV-C lesions in egg extracts is very rapid as well as DSBs repair.

Regarding the "non-licensing" extracts", I did not mean that authors have to explain it to me, but rather in the text, to be clear to a non-specialist audience.

I still believe that authors must demonstrate Polk-Rev1-Polzeta complex formation in their experimental system.

Point 7, Polk has been detected in a number of mammalian cell lines. Have the authors tried to use different antibodies to detect Polk in U2OS cells?

Author Rebuttal, second revision:

point-by-point response

Reviewer #1 (Remarks to the Author):

The manuscript has further improved, but few points that remain unclear.

Point 2, I do not understand why authors cannot provide Coomassie staining of the purified recombinant proteins. At a certain stage, they have had to check the purity of these proteins before carrying out the rescue experiments. Evaluating the purity of the purified proteins is the essential basis of a biochemical approach. Why they do not have the raw data corresponding to these experiments? A western blot does not assure the purity of the proteins, but the presence of a protein recognized by the antibody.

All the proteins were originally checked by Coomassie but unfortunately, we do not have record of these gels and we apologize for this. Nevertheless, protein concentrations and titrations of the different Polκ preparations were carefully carried to ensure equal protein addback (now presented in Extended Data 2A).

Point 6, I believe that it cannot be denied that DSBs are formed on sperm DNA template at high UV-C doses as those used by the authors in the manuscript. Processing of UV-C lesions in egg extracts is very rapid as well as DSBs repair.

We do not deny that DSBs can form in our conditions with high UV-C. However, in our previous CHROMASS experiments (Schubert et al. 2022) and the CHROMASS performed in this manuscript (both performed with similar UV-C treatment), we observe that most proteins recruited to DNA upon high UV-C treatment are DNA repair proteins involved in NER, BER, MMR, TLS, and the UV photolyases. Thus, we do not think that we majority of the lesions formed are DSBs but do not exclude that we generate some, particularly once TLS proteins are depleted.

We copy below our original response to this point:

While the dose of UV-C is very high, we do not think that we mainly induce DNA double strand breaks (but do not exclude that we might generate some). This is evident from the GO Term analysis and the volcano plot shown below, which highlights that the expected predominant enrichment of NER, TLS, and MMR factors (Figure R1 D-E).

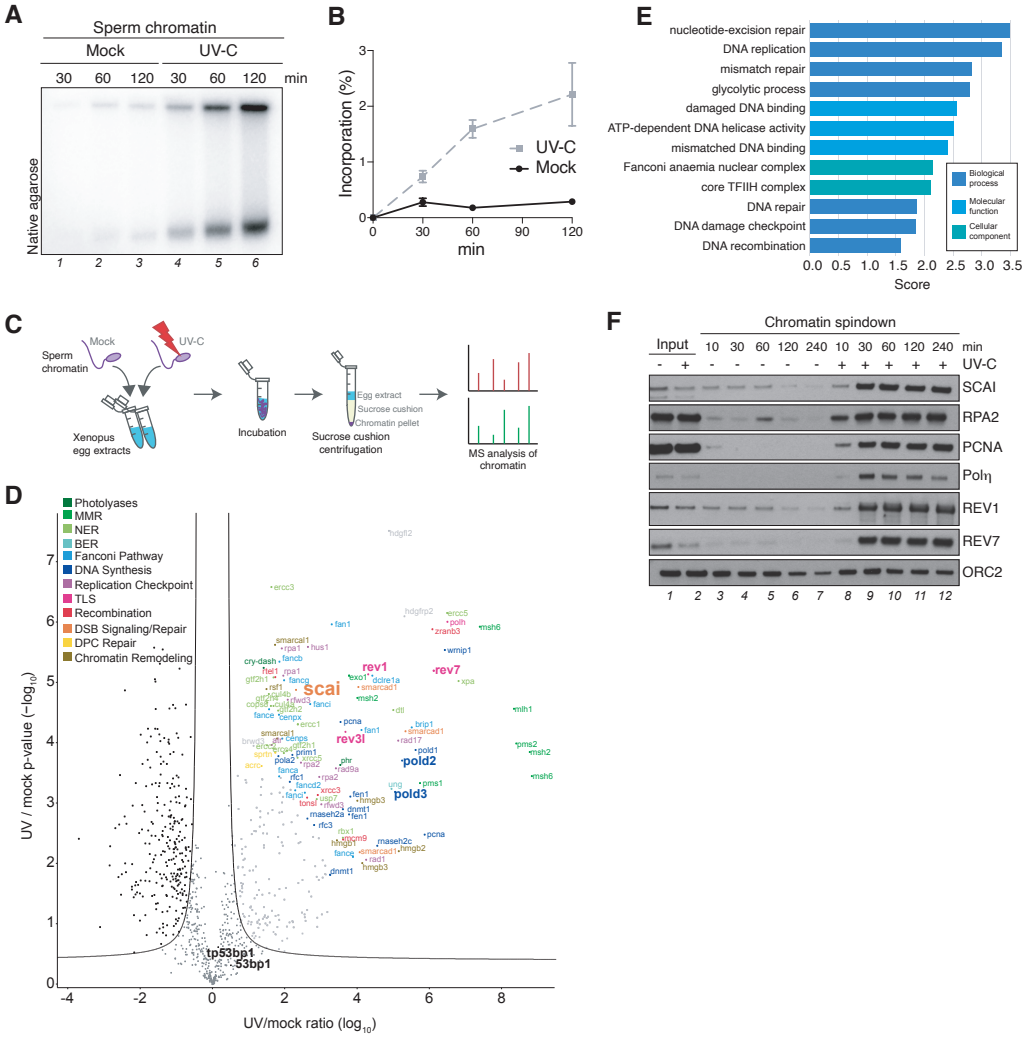

Figure R1 (duplicated from Schubert et al., 2022, Figure EV4). A. *Sperm chromatin was left untreated or exposed to UV-C (2,000 J/m), incubated in HSS/NPE mix in the presence of [α-P]dATP and nucleotide incorporation into chromatin was analyzed at the indicated time points via native agarose gel electrophoresis. Note that we are monitoring here replication-independent DNA synthesis, which is likely dependent on NER.* ***B.*** *Quantification of data in (A) (mean +/- SEM; n = 3 independent experiments).* ***C.*** *Schematic outline of CHROMASS workflow to analyze protein recruitment to UV-damaged chromatin. Sperm chromatin left untreated or irradiated with UV-C (2,000 J/m) was incubated in Xenopus egg extract for 30 min, isolated by sucrose cushion centrifugation, and analyzed by label-free mass spectrometry (MS).* ***D.*** *Protein recruitment to UV-damaged chromatin compared to an undamaged control. Volcano plot shows enrichment of individual proteins (UV/mock ratio) plotted against the P-value (n = 4 independent experiments; FDR < 0.05, s0 = 0.5).* ***E.*** *Term enrichment analysis showing GO terms corresponding to proteins significantly recruited to UV-C-damaged chromatin. All displayed terms were significant with P < 0.02, as determined through Fisher exact testing with Benjamini–Hochberg correction.* ***F.*** *Sperm chromatin left untreated or irradiated with UV-C (2,000 J/m) was isolated at the indicated time points and analyzed by immunoblotting.*

Regarding the “non-licensing” extracts”, I did not mean that authors have to explain it to me, but rather in the text, to be clear to a non-specialist audience.

We now specify in the Methods section that non-licensing extracts correspond to extracts that do not support MCM2-7 licensing.

I still believe that authors must demonstrate Polk-Rev1-Polzeta complex formation in their experimental system.

The reviewer had a very long list of “must do” for the revision of this manuscript. Because the Polκ-Rev1-Polζ complex has been very well characterized in the past, we opted to focus on more relevant experiments that would improve the manuscript (e.g. providing evidence of the non-catalytic function in human cells, which was a long and time-consuming endeavor).

Point 7, Polk has been detected in a number of mammalian cell lines. Have the authors tried to use different antibodies to detect Polk in U2OS cells?

We have tested 3 different antibodies to detect endogenous Polκ (listed now in Extended Data 7B). Unfortunately, none detected endogenous Polk. Therefore, we confirmed our KO cells via RT-PCR.

Final Decision Letter:

Message: 28th Aug 2024

Dear Dr. Duxin,

We are now happy to accept your revised paper "Catalytic and non-catalytic functions of DNA polymerase kappa in translesion DNA synthesis" for publication as an Article in Nature Structural & Molecular Biology.

Acceptance is conditional on the manuscript's not being published elsewhere and on there being no announcement of this work to the newspapers, magazines, radio or television until the publication date in Nature Structural & Molecular Biology.

Over the next few weeks, your paper will be copyedited to ensure that it conforms to Nature Structural & Molecular Biology style. Once your paper is typeset, you will receive an email with a link to choose the appropriate publishing options for your paper and our Author Services team will be in touch regarding any additional information that may be required.

After the grant of rights is completed, you will receive a link to your electronic proof via email with a request to make any corrections within 48 hours. If, when you receive your proof, you cannot meet this deadline, please inform us at rjsproduction@springernature.com immediately.

You will not receive your proofs until the publishing agreement has been received through our system.

Due to the importance of these deadlines, we ask that you please let us know now whether you will be difficult to contact over the next month. If this is the case, we ask you provide us with the contact information (email, phone and fax) of someone who will be able to check the proofs on your behalf, and who will be available to address any last-minute problems.

To assist our authors in disseminating their research to the broader community, our SharedIt initiative provides all co-authors with the ability to generate a unique shareable link that will allow anyone (with or without a subscription) to read the published article. Recipients of the link with a subscription will also be able to download and print the PDF.

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