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A non-proteolytic release mechanism for HMCES-DNA-protein crosslinks

Maximilian Donsbach, Sophie Dürauer, Florian Grünert, Kha Nguyen, Richa Nigam, Denitsa Yaneva, Pedro Weickert Vivancos, Rachel Bezalel-Buch, Daniel Semlow, and Julian Stingele
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Prof. Julian Stingele
Ludwig-Maximilians-University Munich
Gene Center and Department of Biochemistry
Feodor-Lynen-Str. 25
Munich, Bavaria 81377
Germany

13th Feb 2023

Re: EMBOJ-2022-113360
An auto-release mechanism for HMCES-DNA-protein crosslinks

Dear Julian,

Thank you again for submitting your manuscript on HMCES-DPC release to The EMBO Journal. It has now been assessed by three expert referees, whose comments are copied below for your information. As you will see, all referees consider the work timely and important, as well as technically well-done. We would therefore be happy to pursue a revised version of the study further for publication, following addressing of a limited number of specific concerns noted in the three reports - in particular to strengthen the physiological significance of some of the findings, as asked by referees 1 and 3.

Since it is our policy to allow only a single round of (major) revision, it would be important to adequately clarify these issues by the time of resubmission, and I would therefore encourage you to contact me with a tentative response letter and revision plan already during the early stages of the revision. On the basis of this, I would be happy to discuss the revision further with you, as well as the possibility of an extended resubmission deadline if needed. Our 'scooping protection' (meaning that competing work appearing elsewhere in the meantime will not affect our considerations of your study) would of course remain valid even during extended revision.

Detailed information on preparing, formatting and uploading a revised manuscript can be found below and in our Guide to Authors. Thank you again for the opportunity to consider this work for The EMBO Journal, and I look forward to your revision!

With kind regards,

Hartmut

Hartmut Vodermaier, PhD
Senior Editor, The EMBO Journal
h.vodermaier@embojournal.org

*** PLEASE NOTE: All revised manuscript are subject to initial checks for completeness and adherence to our formatting guidelines. Revisions may be returned to the authors and delayed in their editorial re-evaluation if they fail to comply to the following requirements (see also our Guide to Authors for further information):

1) Every manuscript requires a Data Availability section (even if only stating that no deposited datasets are included). Primary datasets or computer code produced in the current study have to be deposited in appropriate public repositories prior to resubmission, and reviewer access details provided in case that public access is not yet allowed. Further information: embopress.org/page/journal/14602075/authorguide#dataavailability

2) Each figure legend must specify
- size of the scale bars that are mandatory for all micrograph panels
- the statistical test used to generate error bars and P-values
- the type error bars (e.g., S.E.M., S.D.)
- the number (n) and nature (biological or technical replicate) of independent experiments underlying each data point
- Figures may not include error bars for experiments with n<3; scatter plots showing individual data points should be used instead.

3) Revised manuscript text (including main tables, and figure legends for main and EV figures) has to be submitted as editable text file (e.g., .docx format). We encourage highlighting of changes (e.g., via text color) for the referees' reference.

4) Each main and each Expanded View (EV) figure should be uploaded as individual production-quality files (preferably in .eps,

.tif, .jpg formats). For suggestions on figure preparation/layout, please refer to our Figure Preparation Guidelines: http://bit.ly/EMBOPressFigurePreparationGuideline

5) Point-by-point response letters should include the original referee comments in full together with your detailed responses to them (and to specific editor requests if applicable), and also be uploaded as editable (e.g., .docx) text files.

6) Please complete our Author Checklist, and make sure that information entered into the checklist is also reflected in the manuscript; the checklist will be available to readers as part of the Review Process File. A download link is found at the top of our Guide to Authors: embopress.org/page/journal/14602075/authorguide

7) All authors listed as (co-)corresponding need to deposit, in their respective author profiles in our submission system, a unique ORCiD identifier linked to their name. Please see our Guide to Authors for detailed instructions.

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In the interest of ensuring the conceptual advance provided by the work, we recommend submitting a revision within 3 months (14th May 2023). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions. Use the link below to submit your revision:

Link Not Available

Referee #1:

In this manuscript, Donsbach et al. study the auto-release mechanism for HMCES-DNA-protein crosslinks (DPCs), which form through an enzymatic reaction at AP sites to prevent DNA breaks. While proteolytic mechanisms (mediated by SPRTN or the proteasome) have been reported to remove HMCES-DPCs, this study demonstrates that HMCES itself is equipped with enzymatic activities to reverse the crosslink with AP sites to regenerate intact HMCES enzymes. The finding is significant because it suggests that HMCES might be like other DNA enzymes, such as topoisomerases, that release intact enzymes after forming DNA-protein crosslinks.

While direct reversal of HMCES-DPCs was also reported recently (Ref. #), the current study extends this observation further and investigates its relevance to physiological contexts. The authors find that the active site of HMCES cycles between a crosslinked and non-crosslinked state. Furthermore, the authors show that, while in the non-crosslinked state, HMCES could be re-crosslinked or released from DNA depending on the affinity with the surrounding DNA structures. This is important given the recent report (by the some of the authors of this manuscript) that demonstrated that translesion DNA synthesis past intact HMCES-DPCs could occur. The preferential HMCES-DPC release from dsDNA might explain how release of HMCES-DPCs could be triggered by DNA synthesis across HMCES DPCs in physiological conditions (see Major point #1). As the authors point out, the auto-release mechanism could resolve HMCES-DPCs in dsDNA that SPRTN would not be able to act on. Therefore, the auto-release mechanism from dsDNA could serve as a fail-safe mechanism that ensures HMCES-DPC resolution even if proteolytic mechanisms fail to remove them.

The presented data are high quality and interpreted carefully. Findings are discussed intelligently in the context of prior literature. The study provides novel insight into cellular mechanisms that protect the genome integrity and will be of great interest

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Major point
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In an elegant study, Donsbach and others demonstrate a mechanism by which HMCES is enzymatically released from abasic sites present in dsDNA but not ssDNA. This is a beautifully written manuscript. The experiments are clearly described and presented, and discussed in the context of previous findings. The results explain how HMCES is likely to be catalytically released during DNA replication, by a mechanism that does not require expensive proteolytic degradation of the enzyme (as was previously proposed). The work significantly advances our understanding of abasic site protection and repair.
The authors identify several mutant forms of HMCES that support their hypothesis of enzymatic enzyme turnover, which are analysed in beautifully conducted in vitro experiments (Figs 1-3) and sophisticated biochemical reconstitutions of DNA replication (Fig 4).
The data as presented convincingly support the mechanism of HMCES release, and I only propose two additions to the manuscript:
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Donsbach et al. report in this manuscript a novel mechanism on how HMCES DPCs are removed from DNA. They provide evidence via biochemical in vitro studies that HMCES-DPCs can revert themselves, implying a non-proteolytic mechanism of resolution of the crosslink. Although HMCES DPCs were shown to reverse themself by a different study last year (Paulin et al. JBC, 2022), Donsbach et al. now provides a molecular understanding of how this reaction is stimulated, which depends on 2 factors: the DNA context and the HMCES/DNA affinity. First they show that reversal is greatly stimulated when the lesion is located on dsDNA in contrast to ssDNA and ssDNA/dsDNA junctions. Physiologically, this makes a lot of sense since it would allow HMCES to quickly reverse once the lesion has been bypassed during DNA replication allowing the downstream repair of the crosslink via APE1. Mechanistically, they show that a conserved glutamate (E127) residue within HMCES'active site catalyzes reversal of the crosslink demonstrating that this is an enzymatic process. Then, via a mutant that is compromised in DNA binding (R98E), they show that the affinity of the underyling DNA structure determines whether HMCES recrosslinks or dissociates. Using a model system that recapitulates replication-coupled HCMES crosslinking (Xenopus egg extracts) they further show that the E127A mutant remains permanently crosslinked to their DNA substrate, suggesting that this process also occurs in a whole proteome environment.

I believe this manuscript is very important in our understanding of how HMCES DPCs are resolved/resolve themselves. The

biochemistry is elegant and very well performed and I have no major criticism to it. I was perhaps a bit disappointed with the experiments in egg extracts, which have the potential to really improve the manuscript by providing a biological relevance of this mechanisms, which I think would be required for an EMBO publication.

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Minor questions

1. Figure 1E- I suppose that the incised DNA is caused by spontaneous hydrolysis of the AP site? If so, maybe add a sentence in the legend to clarify this.

2. P3- the authors state that HMCES DPCs "reversed quickly in dsDNA". This is an overstatement since the reaction occurs over several hours.

Reviewer Comments (reproduced in their entirety, our responses in black)

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We thank the reviewer for their supportive comments.

Major point

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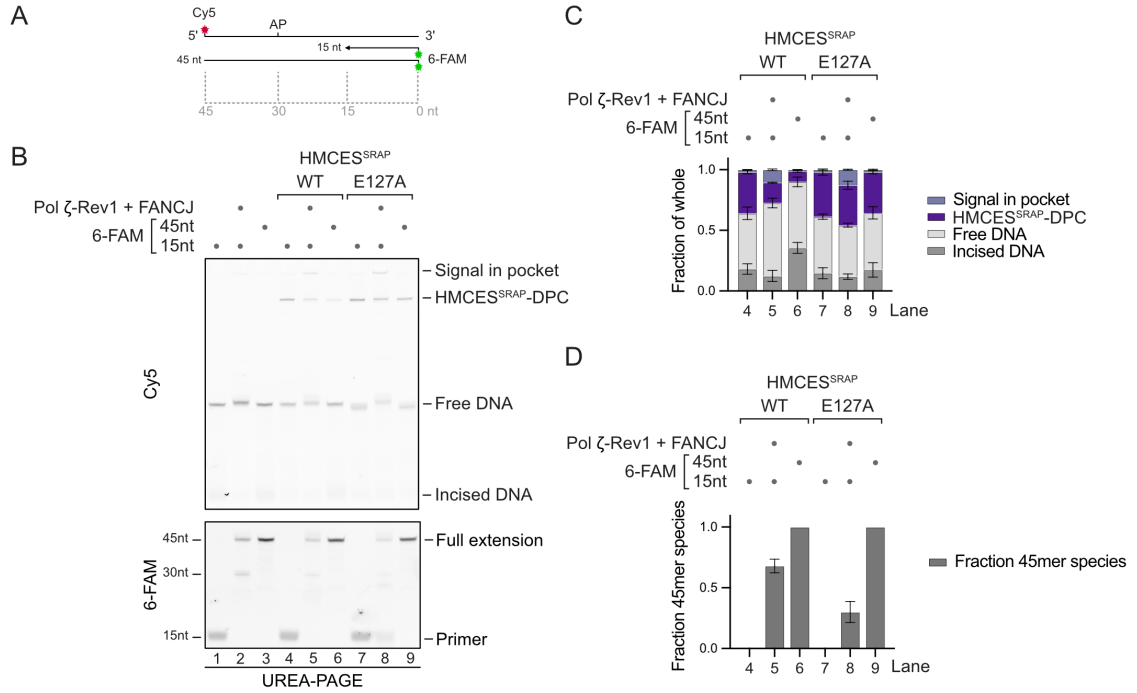
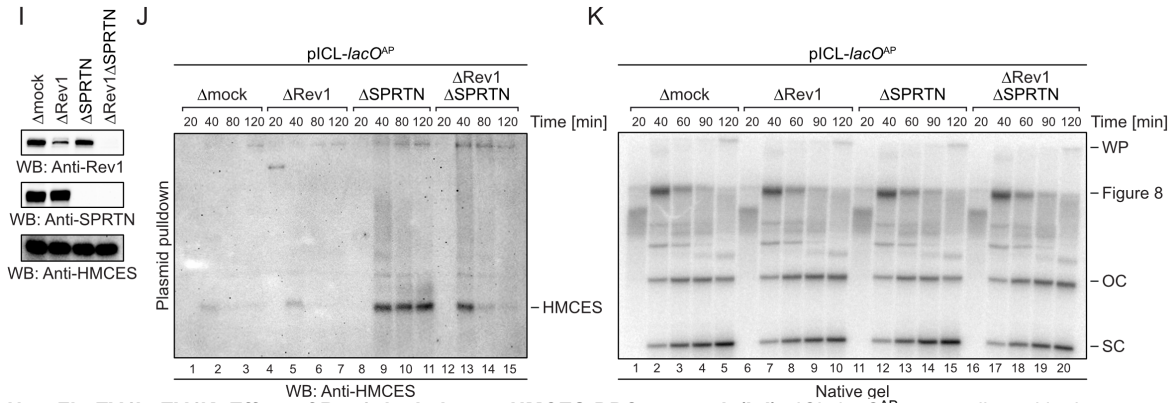
First, we asked whether reversal can be triggered by TLS past the HMCES-DPC by reconstituting the reaction *in vitro*. We placed a HMCES^{SRAP}-WT and -E127A-DPC in template DNA downstream of a primer (Fig 6A) and added recombinant Pol ζ-Rev1 and FANCJ, which is required for TLS across intact DPCs by unfolding the crosslinked protein adduct (Yaneva *et al.*, 2023). Consistent with our other *in vitro* experiments, extension of the primer by Pol ζ-Rev1 appeared to trigger release of HMCES^{SRAP}-DPCs, as evidenced by a loss of WT-DPCs but not of E127A-DPCs (Fig 6B, compare lanes 4 and 5 and lanes 7 and 8, and 6C for quantification). We noted, however, that a fraction of the Cy5 signal remained in the pocket of the gel upon addition of Pol ζ-Rev1-FANCJ which made the quantification of DPC release difficult. DPC release was more pronounced when a complementary 45nt reverse oligo was annealed to the template (Fig 6B, lane 6), which is in line with the fact that TLS did not extend all primers across the DPC (Fig 6B, 6-FAM scan, and 6D for quantification). In addition,

we observed that extension of the primer was less efficient in templates containing a HMCES^{SRAP}-E127A-DPC (Fig 6B, compare lanes 5 and 8 and 6C for quantification). Thus, we cannot exclude that reversal of some DPCs occurs prior to TLS-dependent extension.



New Fig 6: Primer extension assay using Pol ζ-Rev1. (A-B) Primer extension assay using Pol ζ-Rev1. Fluorescently-labelled primer template substrates containing an AP-site at the indicated position were incubated alone or in the presence of HMCES^{SRAP}-WT or -E127A, recombinant human FANCJ and Pol ζ-Rev1 as indicated for 2 h at 37°C prior to separation by denaturing UREA-PAGE. **(A)** Model of oligonucleotides. **(B)** Cy5 scan and 6-FAM scan of denaturing UREA-PAGE. **(C)** Quantification of Cy5 scan in (B). **(D)** Quantification of 6-FAM scan in (B). Bar graphs show the mean of three independent experiments ± SD

Moreover, we have performed the experiment requested by the reviewer and tested whether REV1 depletion blocks release of HMCES-DPCs in SPRTN-depleted extracts. In these experiments, SPRTN depletion alone had a strong stabilizing effect on HMCES-DPCs, while REV1 depletion destabilized HMCES-DPCs specifically in SPRTN-depleted extracts (Fig EV4I and EV4J). Consistent with a TLS defect upon Rev1 depletion, we observed accumulation of gapped-circular plasmids in replication gels done in parallel with the plasmid pulldowns (Fig EV4K). Therefore, we assume that when DPCs persist at the junction, residual SPRTN or another activity can resolve the DPCs, which would be blocked once TLS transfers the DPCs in dsDNA.



New Fig EV4I - EV4K: Effect of Rev1 depletion on HMCES-DPC reversal. (I-J) pICL-*lacO*^{AP} was replicated in the indicated egg extracts. To detect HMCES-DPCs, chromatin was recovered under stringent conditions and the DNA was digested. Samples were boiled and released proteins were separated by SDS-PAGE. **(K)** In parallel with the reactions shown in (C), pICL-*lacO*^{AP} was replicated in the indicated egg extracts supplemented with [α-32P]dCTP. Replication intermediates were separated on a native agarose gel and visualized by autoradiography. SC, supercoiled. OC, open circular

We conclude that TLS across an intact HMCES-DPC can trigger reversal *in vitro* but that this mechanism does not seem to quantitively contribute to the resolution of HMCES-DPCs in *Xenopus* egg extracts during ICL repair under the conditions tested. One explanation for this discrepancy could be that the replication reactions in egg extracts must be performed at 20°C, while the *in vitro* reactions were all performed at 37°C. Indeed, autocatalytic reversal was significantly delayed at 20°C (Fig EV4G and EV4H, compare lanes 5-8 and 9-12).

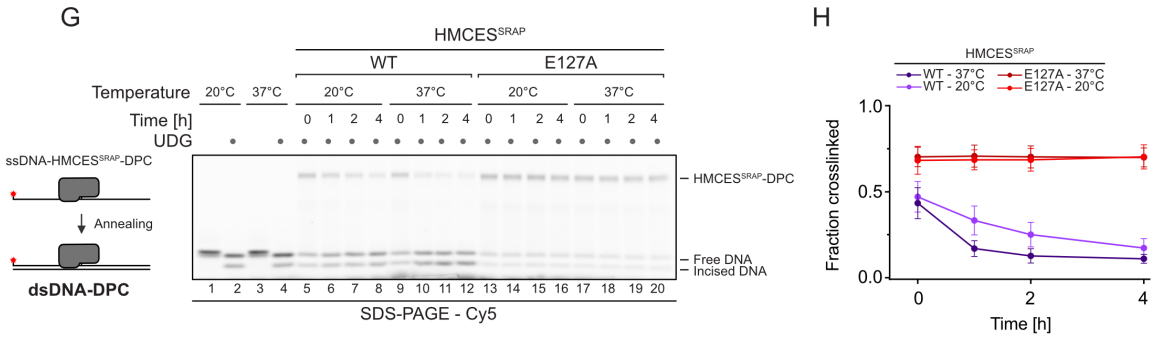


Fig EV4G and EV4H: Reversal of HMCES^{SRAP}-DPCs at different temperatures. (I) Reversal of HMCES^{SRAP}-WT or -E127A-DPCs in dsDNA was assessed at the indicated temperature for the indicated amount of time before analysis by denaturing SDS-PAGE. **(J)** Quantification of DPC reversal of HMCES^{SRAP}-WT and -E127A shown in (A): data represent the mean of three individual experiments ± SD.

Minor points

1. Page 4, line 24: The sentence "Thus, indicating that failure to auto-release results in inhibition of AP site repair in dsDNA" seems incomplete.

We rephrased this sentence.

2. Page 4, line 27: Is it accurate to call the R98E variant hyper-reversal? Hyper-reversal sounds like the R98E mutant exhibits higher crosslink reversal activity, which was not shown. Would hyper-release be more appropriate?

We agree and changed the text accordingly.

3. Page 4, the second last lane: Is Figure S3C supposed to be S3E?

Yes, correct. Please note that this panel was omitted in the revised version.

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We included the data in the revised version of the manuscript (Fig EV5).

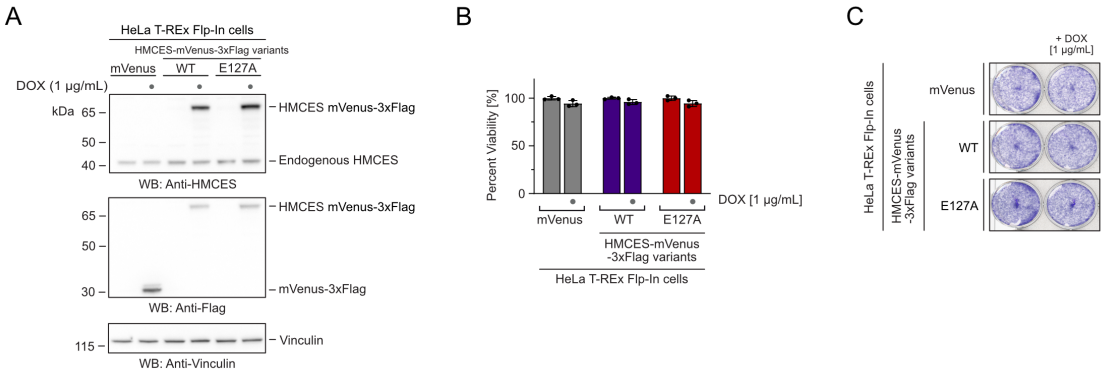

Fig EV5: Cell viability is not affected upon overexpression of reversal-defective HMCES-variants. (A-C) HeLa T-REx Flp-In cells expressing the indicated doxycycline-inducible HMCES variants with a C-terminal mVenus-3xFlag-tag were grown in the presence of 1 µg/mL doxycycline, as indicated. Expression levels were analysed by Western blotting (A). Cell viability was determined using AlamarBlue cell viability assay (B), or crystal violet staining (C).

2. Perform the experiment of Figure 2A with a DNA substrate that contains a nick (and/or gap) opposite the abasic site in otherwise dsDNA. This DNA substrate would more closely resemble the post-TLS or post-converging fork model that would be present when HMCES is proposed to be released (as per the second paragraph of the discussion). Such an experiment would greatly support the model, and give greater insight into the point at which HMCES is normally released from DNA.

We have analysed DPC formation in substrates that contain a nick or a 4nt-gap opposite the AP site as suggested. Despite the largely dsDNA context, DPC formation occurred efficiently (Fig 2C and 2D). This indicates that HMCES can form DPCs efficiently in the absence of long stretches of ssDNA and, thus, in DNA structures that arise after forks have converged.

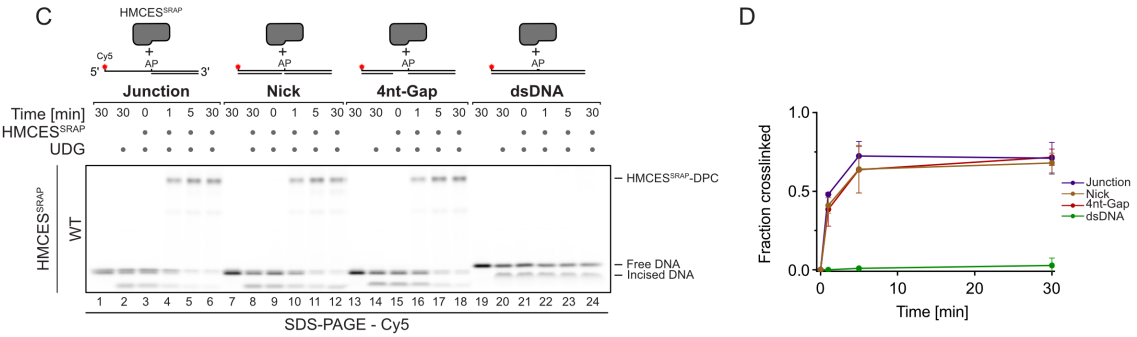

Fig 2C and 2D: Formation of HMCES-DPCs in different DNA structures. (C) Kinetics of DPC formation by HMCES^{SRAP}-WT to junction DNA and dsDNA containing a nick or a 4nt-gap. Corresponding reverse oligonucleotides were annealed to create junction DNA and dsDNA containing a nick or a 4nt-gap prior to adding HMCES^{SRAP}-WT. HMCES^{SRAP}-WT was incubated with the indicated DNA structures for the indicated amount of time at 37ºC prior to separation by denaturing SDS-PAGE. **(D)** Quantification of DPC formation assays shown in (C): data represent the mean of three individual experiments ± SD.

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Abasic sites are known as one of the most abundant lesions in the genome as they occur from spontaneous hydrolysis of DNA bases. While repaired very efficiently via BER when located on dsDNA, AP sites are particularly dangerous when formed on ssDNA or ssDNA/dsDNA junctions (e.g., during DNA replication) as they can lead to cytotoxic DNA double strand breaks (DSBs). To protect AP sites on ssDNA, the protein HMCES was recently described to covalently link to the open form of AP sites and thereby protect the lesion from inadvertent nucleolytic processing (Mohni et al. 2019, Cell). Removal of HMCES DPCs were shown to either dependent on the ubiquitin/proteasome system (Mohni et al. 2019, Cell) or via the DPC protease SPRTN (Semlow et al. 2022, NSMB).

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While setting up the experiments proposed by the reviewer, we noted unusual fluctuations between different experiments when analysing the behaviour of the rHMCES-3xFlag-E129A variant. Given that this variant binds to DNA much tighter (as described in our manuscript and in previous work by the Arrowsmith lab), we were concerned that the strong DNA binding of this variant may confound some of our results. Therefore, we titrated up the salt in the wash buffer of the plasmid pulldown assays.

Even up to 1.5 M NaCl did not decrease the efficiency of plasmid recovery with LacI coated beads. However, the pronounced accumulation of rHMCES-3xFlag-E129A seen with standard wash conditions was absent when using stringent high-salt wash conditions (Fig R1, right).

These data indicate that our previous conclusion that the accumulation of HMCES-E129A during ICL repair in frog extracts (previous Fig 4C) stems from defective reversal was confounded by tight binding of HMCES-E129A to ssDNA on the gap downstream of the AP site.

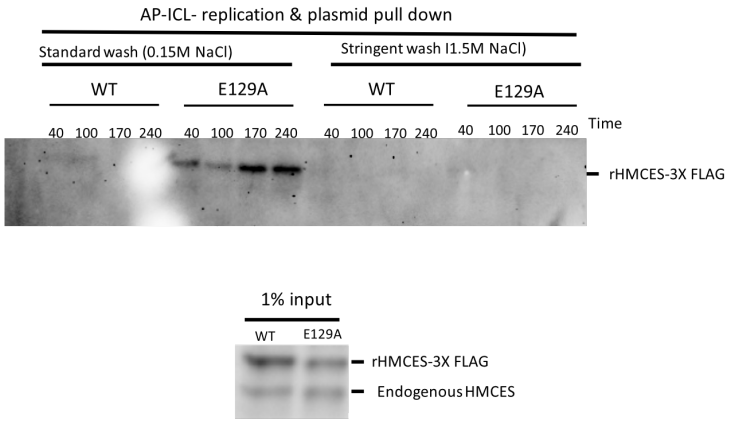

Fig R1: Effect of stringent wash on HMCES-E129A signal. pICL-*lacO*^{AP} was replicated in the indicated egg extracts. To detect HMCES-DPCs, chromatin was recovered and washed with either 0.15 M NaCl or 1.5 M NaCl before the DNA was digested. Samples were boiled and released proteins were separated by SDS-PAGE.

Using the optimized stringent wash conditions, we attempted to determine the impact of the E129A mutation on HMCES-DPC stability in mock- and SPRTN-depleted extract supplemented with rHMCES-3xFlag (Fig EV4C-F). The HMCES-DPC was detected using an antibody raised against SRAP domain that permits simultaneous monitoring of endogenous HMCES protein and the recombinant 3xFlag-tagged HMCES (which migrates slower during SDS-PAGE due to the tag). In this experimental setup, the endogenous protein serves as a control for the effects of SPRTN-depletion and auto-release. Like the endogenous HMCES, both WT and E129A-mutated rHMCES-3xFlag seemed to become stabilized by SPRTN-depletion. However, it was challenging to assess the behaviour of WT vs mutant protein because the recombinant E129A flag-tagged protein crosslinked less efficient than endogenous HMCES (even when present in large excess, Fig EV4F, lower right panel). While depletion of endogenous HMCES could be used to enhance crosslinking of the recombinant proteins, as suggested by the reviewer, we are finding it technically challenging to efficiently co-deplete SPRTN and HMCES with our available reagents.

We conclude that SPRTN-dependent proteolysis is the dominant mechanism for the repair of HMCES-DPCs that occur during ICL repair in *Xenopus* egg extracts under the conditions tested. Unfortunately, we were not able to determine whether reversal contributes in addition to the resolution of HMCES-DPCs due to the technical challenges described above. Of note, template switching does not occur under these conditions in *Xenopus* egg extracts (Semlow *et al.*, 2022) but would be expected to transfer the DPC into dsDNA, thereby leading to reversal. Thus, it seems likely that HMCES-DPC reversal occurs downstream of template switching in mammalian cells. We will carefully discuss these models in the discussion of the revised manuscript. However, testing these ideas directly will be very difficult due to the lack of reliable methods to track the fate of HMCES-DPCs in mammalian cells.

Additionally, we agree that it will be interesting to determine whether the E129A mutated influences the rate (and mutagenicity) of TLS past the AP site during ICL repair in both mock- and SPRTN-

depleted egg extracts. However, this is not straight forward since the effects of HMCES depletion on the efficiency of TLS are relatively subtle, the WT and E129A rHMCES proteins form DPCs with different rates, and SPRTN-depletion itself influences the rate of TLS. For these reasons we feel the suggested experiment lies beyond the scope of the current manuscript and we will instead pursue these lines of inquiry in future work.

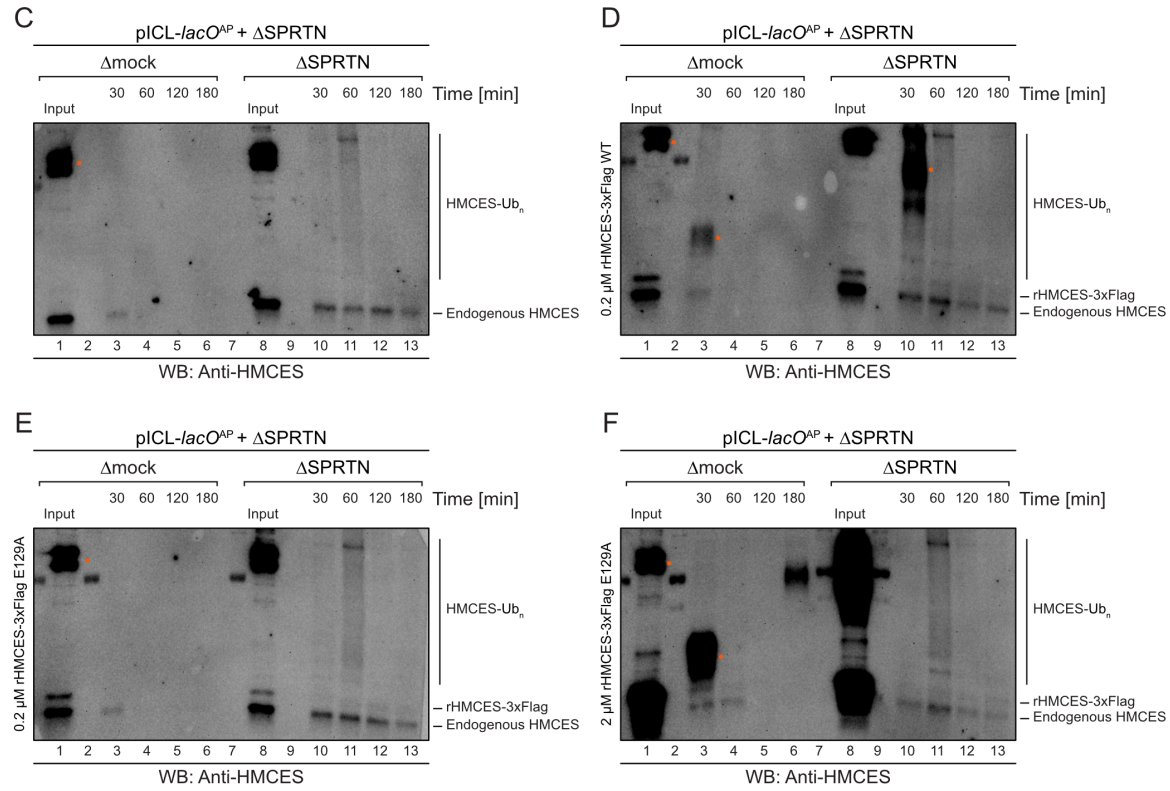

Fig EV4C-F: Effect of SPRTN depletion on stability of HMCES-DPCs. (C-F) pICL-lacOAP was replicated in the indicated egg extracts supplemented with p97i, MG262, and recombinant xl-HMCES-3xFlag, as indicated. Chromatin was recovered and washed with 1.5 M NaCl, the DNA was digested, and released proteins were separated by SDS-PAGE.

Minor questions

1. Figure 1E- I suppose that the incised DNA is caused by spontaneous hydrolysis of the AP site? If so, maybe add a sentence in the legend to clarify this.

That is correct, we clarified this point in the revised version.

2. P3- the authors state that HMCES DPCs "reversed quickly in dsDNA". This is an overstatement since the reaction occurs over several hours.

We rephrased and now only state that HMCES-DPCs reversed in dsDNA.

In addition to addressing reviewer's suggestions, we strengthened the manuscript by including additional data indicating that the capacity of SRAP domain-DPCs to auto-release is conserved across different species. We analysed the stability of DPCs formed by the prokaryotic HMCES-orthologs YedK or *Xenopus laevis* HMCES (xl-HMCES) (Fig EV3C and EV3D) and observed results comparable to human HMCES (with slight variations). YedK-DPCs were stable at ssDNA-dsDNA junctions, prohibiting cleavage of the AP site by APE1 (Fig EV3C, lanes 7-8). In dsDNA, APE1 was able to cleave the AP site, indicating release of the DPC (Fig EV3D, lanes 7-8). AP site cleavage in dsDNA was not observed upon replacing Glu105 (corresponding to Glu127 in human HMCES) with alanine (Fig EV3D, lanes 11-12). Of note, in contrast to human HMCES^{SRAP}, release of the DPC was barely detectable in the absence of APE1 (Fig EV3D, lanes 5-6). While the protection of AP sites at ssDNA-dsDNA junctions against APE1 cleavage by xl-HMCES-DPCs was less strong than observed for the human or prokaryotic protein (Fig EV3C, lanes 15-16), replacement of Glu129 (corresponding to Glu127 in human HMCES), entirely blocked reversal at ssDNA-dsDNA junctions and in dsDNA (Fig EV3C, lanes 19-20, and Fig EV3D, lane 19-20, respectively).

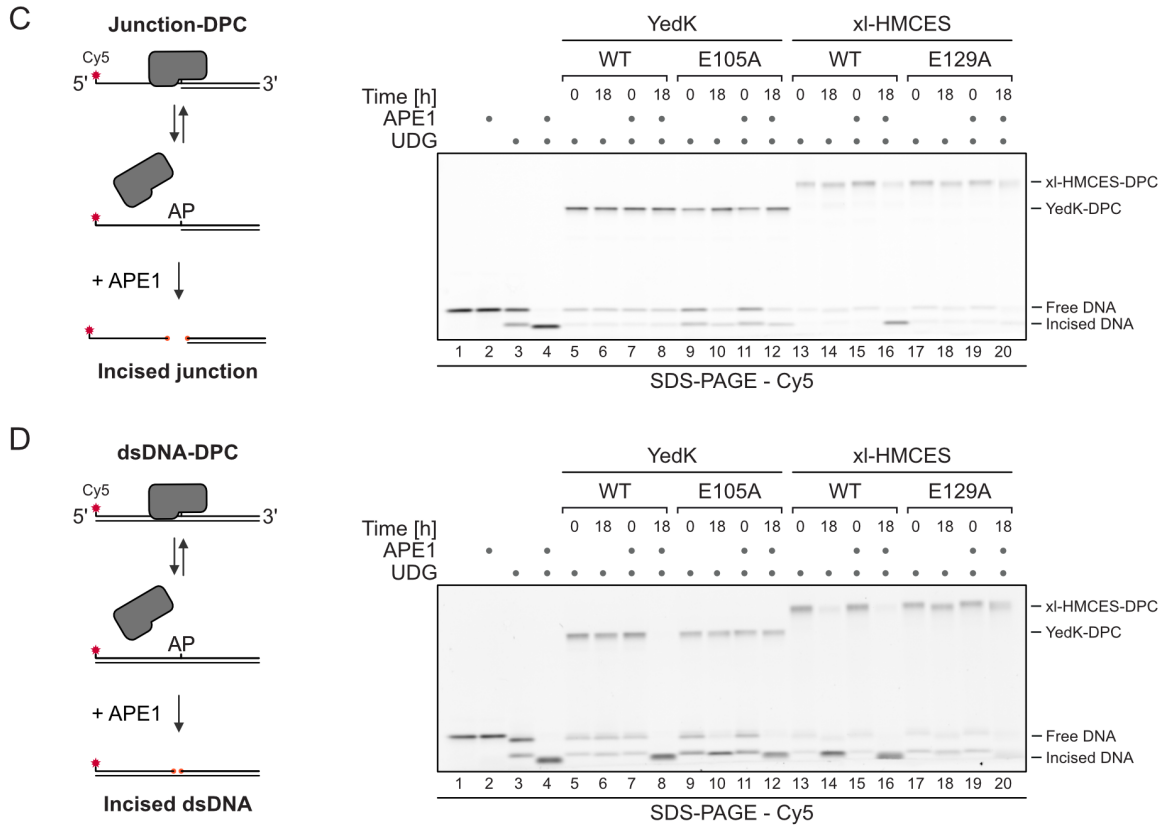

Fig EV3C-D: SRAP domain-DPC auto release is conserved across species. (C-D) APE1 incision of an AP site protected by the indicated YedK-WT-DPC or YedK-E105A-DPC and xl-HMCES-WT-DPC or xl-HMCES-E129A-DPC at ssDNA-dsDNA junctions (C) or within dsDNA (D). Free dU-containing DNA was incubated alone or in the presence of UDG and YedK/xl-HMCES for 1 h at 37ºC. Next, corresponding reverse oligonucleotides were annealed to generate a ssDNA-dsDNA junction (C) or dsDNA (D), and reactions were incubated alone or with APE1 for the indicated amount of time at 37°C prior to separation by denaturing SDS-PAGE.

Prof. Julian Stingele
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Feodor-Lynen-Str. 25
Munich, Bavaria 81377
Germany

6th Jul 2023

Re: EMBOJ-2022-113360R
A non-proteolytic release mechanism for HMCES-DNA-protein crosslinks

Dear Julian,

Thank you again for submitting your revised manuscript for our consideration. Referees 1 and 3 have now assessed it once more, and are mostly satisfied with the revisions and improvements of the manuscript. As you will see from the comments below, referee 3 still has some specific questions and suggestions for specific modifications, which I would invite you to incorporate and respond to in a final round of minor revision.

Once we will have received this, we expect we should be able to swiftly proceed with formal acceptance and production of the manuscript.

With kind regards,

Hartmut

Hartmut Vodermaier, PhD
Senior Editor, The EMBO Journal
h.vodermaier@embojournal.org

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2) Each figure legend must specify
- size of the scale bars that are mandatory for all micrograph panels
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- the type error bars (e.g., S.E.M., S.D.)
- the number (n) and nature (biological or technical replicate) of independent experiments underlying each data point
- Figures may not include error bars for experiments with n<3; scatter plots showing individual data points should be used instead.

3) Revised manuscript text (including main tables, and figure legends for main and EV figures) has to be submitted as editable text file (e.g., .docx format). We encourage highlighting of changes (e.g., via text color) for the referees' reference.

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In the interest of ensuring the conceptual advance provided by the work, we recommend submitting a revision within 3 months (4th Oct 2023). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions. Use the link below to submit your revision:

Link Not Available

Referee #1:

In their revised manuscript, the authors have addressed the reviewer's concerns regarding the physiological relevance of the auto-release mechanism of HMCES-DPCs from dsDNA. They responded by conducting an in vitro reconstitution experiment (Fig. 6), which demonstrated that TLS across HMCES-DPC triggered resolution of HMCES-DPCs. The HMCES-DPC resolution was found to occur in a Glu127-dependent manner, a residue proposed by the authors to be crucial for the crosslink reverse reaction. This finding confirms that the HMCES-DPCs are indeed resolved through the auto-release mechanism in this experiment, clearly demonstrating the significance of this mechanism in a physiologically relevant setting.

The authors also conducted experiments to test whether depletion of a TLS factor, Rev1, prevents the release of HMCES-DPCs in Xenopus extracts, as requested by the reviewer (Fig. EV4G-H). The results of this experiment suggest that SPRTN plays a dominant role in resolving HMCES-DPCs. Depleting Rev1 together with SPRTN did not lead to further accumulation of HMCES-DPC, but rather led to enhanced resolution of the initial accumulation of HMCES-DPCs. This result highlights the difficulties of dissecting DNA repair pathways due to the involvement of multiple pathways. The authors conclude that it is challenging to determine whether auto-release contributes to HMCES-DPC resolution when proteolysis is blocked.

The revision greatly enhanced the manuscript by incorporating the biological context in which the HMCES auto-release mechanism could be advantageous. Furthermore, the revised manuscript provides a well-balanced view of the pathway choices cells could make to dissolve HMCES-DPCs after completion of its function. This study should be of great interest to wide readers from the DNA repair field.

Referee #3:

The manuscript from Donsbach et al. has been revised and novel data has been incorporated to the manuscript to respond to reviewers comments. They have added:

-an in vitro extension assay that suggests that TLS extension past the HMCES-DPC can trigger HMCES crosslink reversal.

-data in Xenopus egg extract that indicate that the process of HMCES' reversal does not appear to operate in this system where

SPRTN and/or additional proteases prevail.

-data in human cells that show that the auto-release deficient mutant is not toxic.

Although the new results added were a bit disappointing because they failed to show any physiological relevance for the process of HMCES self-release, I still consider this study an important step forward for our understanding of how this enzyme functions and potentially protects the genome from abasic sites during DNA replication.

For clarity in the discussion I would correct as follow:
"Notably, in either case, extension of the nascent strand **past the protein adduct** would prevent DPC proteolysis by SPRTN, which requires the presence of a ssDNA-dsDNA junction in close proximity to the protein adduct to become activated (Larsen et al., 2019; Reinking et al, 2020)."

Last suggestion/question: have the authors looked at the effect of the E127A cell line under abasic site lesion inducer (e.g. KBromate)? This would be an easy way to show some physiological relevance for this mechanism.

Reviewer Comments (reproduced in their entirety, our responses in black)

Referee #1 (Report for Author)

In their revised manuscript, the authors have addressed the reviewer's concerns regarding the physiological relevance of the auto-release mechanism of HMCES-DPCs from dsDNA. They responded by conducting an in vitro reconstitution experiment (Fig. 6), which demonstrated that TLS across HMCES-DPC triggered resolution of HMCES-DPCs. The HMCES-DPC resolution was found to occur in a Glu127-dependent manner, a residue proposed by the authors to be crucial for the crosslink reverse reaction. This finding confirms that the HMCES-DPCs are indeed resolved through the auto-release mechanism in this experiment, clearly demonstrating the significance of this mechanism in a physiologically relevant setting.

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The revision greatly enhanced the manuscript by incorporating the biological context in which the HMCES auto-release mechanism could be advantageous. Furthermore, the revised manuscript provides a well-balanced view of the pathway choices cells could make to dissolve HMCES-DPCs after completion of its function. This study should be of great interest to wide readers from the DNA repair field.

We thank the reviewer for their supportive comments.

Referee #3 (Report for Author)

The manuscript from Donsbach et al. has been revised and novel data has been incorporated to the manuscript to respond to reviewers comments. They have added:

-an in vitro extension assay that suggests that TLS extension past the HMCES-DPC can trigger HMCES crosslink reversal.

-data in Xenopus egg extract that indicate that the process of HMCES' reversal does not appear to operate in this system where SPRTN and/or additional proteases prevail.

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Although the new results added were a bit disappointing because they failed to show any physiological relevance for the process of HMCES self-release, I still consider this study an important step forward for our understanding of how this enzyme functions and potentially protects the genome from abasic sites during DNA replication.

We thank the reviewer for their support.

For clarity in the discussion I would correct as follow:
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We changed the text accordingly.

Last suggestion/question: have the authors looked at the effect of the E127A cell line under abasic site lesion inducer (e.g. KBromate)? This would be an easy way to show some physiological relevance for this mechanism.

We did test whether overexpression of HMCES-E127A causes sensitivity to KBr4 in various set-ups but did not observe any hypersensitivity. However, these negative data are of preliminary nature and were thus not included in the manuscript.

Prof. Julian Stingele
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19th Jul 2023

Re: EMBOJ-2022-113360R1
A non-proteolytic release mechanism for HMCES-DNA-protein crosslinks

Dear Julian,

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

Your article will be processed for publication in The EMBO Journal by EMBO Press and Wiley, who will contact you with further information regarding production/publication procedures and license requirements. You will also be provided with page proofs after copy-editing and typesetting of main manuscript and expanded view figure files.

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

Yours sincerely,

Hartmut

Hartmut Vodermaier, PhD
Senior Editor, The EMBO Journal
h.vodermaier@embojournal.org

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Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

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Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Figure legends
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Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Yes	Figure legends
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Not Applicable	

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Studies involving experimental **animals**: State details of **authority granting ethics approva**l (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Yes	Materials and Methods
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For **phase II and III randomized controlled trials**, please refer to the **CONSORT** flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under ‘Reporting Guidelines’. Please confirm you have submitted this list.	Not Applicable	

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Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	