THE EMBO JOURNAL

Manuscript EMBO-2014-90878

Regulation of the Rev1-Polζ complex during bypass of a DNA interstrand crosslink

Magda Budzowska, Thomas G. W. Graham, Alexandra Sobeck, Shou Waga and Johannes C. Walter

Corresponding author: Johannes C. Walter, Harvard Medical School

Review timeline:

Submission date:	22 December 2014
Editorial Decision:	23 January 2015
Revision received:	20 April 2015
Editorial Decision:	06 May 2015
Accepted:	06 May 2015

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 23 January 2015

Thank you for submitting your manuscript on ICL replication after unhooking to The EMBO Journal. It has now been reviewed by three expert referees, whose reports are copied below for your information. I am happy to say that all of them consider this work in principle of interest and potential importance, and we would therefore be interested in considering a revised version further for publication. The referees nevertheless raise a number of seemingly well-taken concerns that would need to be satisfactorily answered before eventual acceptance of the paper.

I am thus inviting you to prepare a revised version of this study, addressing the various specific experimental and technical issues of the referees, but also taking into account some of the more conceptual queries. In this respect, I feel that particularly the (overlapping) points 1 and 2 of referee 1, and 2 and 5 of referee 3 should be experimentally addressed. Furthermore, any data to address the concern raised in referee 1's point 3 re. PCNA ubiquitination would clearly be helpful. Regarding the related queries of referee 2 and 3 (point 3) about the polymerases responsible for translesion synthesis in this system, I realize that this may be difficult to clarify within the scope of the present study, but nevertheless wonder if some further insights could be obtained by following the specific suggestions offered by the referees.

Please note that it is our policy to allow only a single round of major revision, and that it will therefore be important to carefully answer to all points raised at this stage. We generally allow three months as standard revision time, and it is our policy that competing manuscripts published during this period will have no negative impact on our final assessment of your revised study; should you foresee a problem in meeting this three-month deadline, please let me know in advance and we could discuss the possibility of an extension. Finally, I should point out that we now require a completed 'author checklist' to be submitted with all revised manuscripts - see below for more detail.

Thank you again for the opportunity to consider this work for The EMBO Journal, and please do not hesitate to contact me should you have any feedback or questions regarding the referee reports or this decision. I look forward to your revision.

REFEREE REPORTS

Referee #1:

The laboratory of Johannes Walter has made outstanding contributions to our understanding of the mechanisms of replication-coupled ICL repair using plasmids bearing a defined ICL and Xenopus egg extracts. With this system, the authors now provide novel insights into how DNA polymerases bypass a cisplatin DNA interstrand crosslink. More specifically, they show that (i) DNA synthesis to within one nucleotide from the ICL is performed by a replicative polymerase; (ii) REV1-pol ζ(REV3-REV7) performs extension beyond the lesion; (iii) The FA core complex recruits REV1-pol ζ to the ICL. (iv) The mutation rate during DNA synthesis to within one nucleotide from the cisplatin ICL and beyond the lesion is about 3%, which corresponds to a 30 fold increase compared to control DNA. The data presented by Budzowska and colleagues bring novel and useful information to understand how crosslinked DNA is replicated. The manuscript is well written and should be of interest to the readership of the EMBO journal.

Main points:

1. Figure 2: FANCA and FANCD2 exhibit an intriguing pattern of association with pICL at the ICL and control loci, is that reproducible? Replicates for FANCA and FANCD2 Chip experiments for the control locus are missing (Figure E1).

The authors suggest that FANCD2 binds to a large region surrounding the ICL, referring to (Klein Douwel et al., 2014). This, however, does not explain the multiphasique mode of FANCD2 binding to the ICL and control loci, including reloading of FANCD2 beginning at around 120 min., well after the ICL has been bypassed. Does the binding of FANCD2 depend on the presence of an ICL? ChIP signals of control locus have often the same shape (but with a lower % input, except for FANCD2). Is it a residual signal from the ICL locus due to low sonication efficiency?
To answer both questions, the authors should use the pCTR plasmid to ChIP proteins in the absence of the ICL. The FANCD2 ChIP on pCTR is essential.

2. Figure 3. Likewise, to better comprehend the role of the FA pathway in REV1-pol ζ recruitment to ICLs, it would be appropriate to probe for FANCA and for FANCD2 by western blotting in pCTR and pICL pull down.

3. Based on the timing of Ub-PCNA association with pICL, which does not correlate with the peak of translesion DNA synthesis and REV1-pol ζ recruitment, the authors conclude that PCNA ubiquitylation is not essential for the bypass of interstrand DNA crosslinks. Yet Ub-PCNA is retained on the plasmid for a longer time in the presence of the ICL. Why? Ub-PCNA may still promote the loading of the "insertion" polymerase that remains to be discovered, in particular if it is a Y family polymerase. The timing of protein loading and reaction products may not coincide exactly. Ub-PCNA could regulate TLS and mutagenesis. It is difficult to draw conclusions before PCNA has been replaced with a non-ubiquitylatable PCNA mutant.

4. Figure 7 and E6: The strategy used for sequencing the plasmid is described in the legend of Fig E6 but not in the main text. Some explanation in the main text would facilitate the reading.

The authors mentioned that many of the mutations were due to errors during PCR before deep - sequencing. Did they try to deep sequence directly the plasmids?

Minor points:

1. Figure E7: I was surprised by the quite high frequency of indels in the control. It would be interesting to determine if it is real or if it is due to PCR step.

2. It should be clearly stated that experiments are performed using cisplatin DNA crosslinks, specifically. Some conclusions could differ in the context of different types of DNA crosslinks.

Referee #2:

Budzowska et al., have used Xenoupus egg extracts to delineate the molecular mechanism of interstrand cross link repair. The pathway is broken into discrete and temporal steps, which includes replicative stalling 20-40 bases before the lesion, followed by approach to the lesion, insertion opposite the unhooked lesion and finally extension. By using a combination of techniques, the authors have eloquently identified key participants in most of the steps. The approach is likely to be performed by a high fidelity replicative DNA polymerase, while extension is dependent upon Rev1 and DNA pol zeta. The authors also demonstrate that ubiquitinated PCNA does not recruit Rev1 or pol zeta to the lesion, but their recruitment is instead dependent upon the Fanconi core complex. Deep DNA sequencing of the fully extended replication products reveals that bypass is remarkably accurate and when mutations occur, they do so in a short 6-7 base pair repair track. The highest level of mutagenesis is observed either 1 bp before the lesion, or more likely, opposite the lesion itself.

This is a well-executed study and the conclusions drawn are convincingly supported by the data.

My only reservation is that the DNA polymerase that performs the actual TLS step was not identified. The authors speculate that it is either DNA polymerase eta or DNA polymerase kappa and I wonder how difficult (or time consuming) it would be to actually test this hypothesis with extracts depleted of either enzyme?

Referee #3:

Budzowska et al.
Regulation of the Rev1-Polzeta complex during bypass of a DNA interstrand crosslink

This manuscript from the Walter group builds on work they have published over the past seven years in which they have elegantly dissected the replication-dependent repair of a DNA interstrand crosslink in Xenopus egg extracts. A key step at the heart of the process is the replication of the unhooked crosslink, a step that requires translesion synthesis dependent on Rev1 and DNA pol zeta. It is this phase of the reaction that is the focus of the current work. The authors show that the approach step, in which one of the blocked forks edges towards the crosslink after removal of the blocked CMG helicase, is likely to be mediated by the replicative polymerases. Surprisingly, though, the insertion step, in which a base is incorporated opposite the unhooked crosslink is not dependent on Rev1, despite the damaged template being a dG. Rather depletion of Rev1 actually results in an accumulation of the insertion product, but a delay in formation of the extension product. Interestingly, the authors provide persuasive evidence that the recruitment of Rev1-Polzeta is temporally separated from the peak of PCNA ubiquitination that is seen during replication in Xenopus egg extracts. Rather they suggest that its recruitment is dependent on the FA core complex. The authors also provide sequencing data to show that mutagenesis during ICL repair is limited to just around the lesion and does not extend much beyond.

This is an interesting and important next step in the story. The paper is also very clearly written and presented. However, I do have a number of questions and suggestions.

1. The key experimental technique used to dissect the recruitment and retention of the various proteins to the ICL-containing and control plasmids is ChIP. These are presented as individual experiments rather than as normalised replicates with error on a single graph. Why is this? In many ways, it is the timing of the peak recruitment that is most important. Is there a way in which this can be estimated, its variability measured and the significance of the differences assessed?

2. The second approach immunoprecipitates the plasmids from the extract at different times. This provides very clear and compelling results (Figure 3). The separation of PCNA ubiquitination from REV1 recruitment is particularly striking. However, it would be good to see this technique extended to FancA, or other components of the FA core complex to provide a second line of evidence to back up the ChIP data.

3. The sequencing experiment is very nice. It provides very little evidence of dC insertion (on either strand), suggesting that the catalytic activity of Rev1 is not deployed. The majority of mutagenic activity by A-rule incorporation opposite the lesion. Since Rev1 depletion also likely depletes Pol zeta (at least the Rev7 part), is it possible that the apparently proficient insertion step is being mediated by pol delta, which is now known to share its pold2 and pold3 subunits with pol zeta, even though during normal replication its principal role is on the lagging strand? Have the authors tried depleting pold3?

4. Is there any change in the spectrum of mutagenesis in Rev1-depleted extracts? I am a bit confused about whether the sequencing has to be carried out on completed, SapI-digestible, products, or whether information could still be obtained from the 'extension' product, which remains abundant in the Rev1-depletion experiments (Figure 4F).

5. The authors suggest that Rev1 binds FancA. Is this direct? If so, which domains are important? Or is it related to the previously described interaction of Rev1 with FAAP20?

Minor points.

6. The authors note that they were unable to interpret the plasmid pull down with pol delta because of problems with high background. Does this not also apply to the ChIP experiment shown in Figure 2E?

7. Is there an explanation for the curious behaviour of FANCD2 in Figure 2K?

8. Is there really a justification for using curvy connecting lines on the ChIP experiments, rather than just straight lines joining the points? [EDITOR NOTE: I agree that the individual data points should be connected by straight lines]

9. Why is there such a large variation in the efficiency of the ICL repair, as assessed by SapI digestion? For instance the lack of repair in FancA depleted extracts (Figure 5C) is similar to the maximum extent of proficient repair in the mock depleted extract in Figure 4E. Some explanation of the variability of this assay, and how the reader is to assess significance of differences on this background variability, would be good.

10. Is there a word missing in the sentence in the abstract 'Deep sequencing of ICL repair products showed that most of approach and extension are error-free.' ?

1st Revision - authors' response 20 April 2015

Referee #1:

The laboratory of Johannes Walter has made outstanding contributions to our understanding of the mechanisms of replication-coupled ICL repair using plasmids bearing a defined ICL and Xenopus egg extracts. With this system, the authors now provide novel insights into how DNA polymerases bypass a cisplatin DNA interstrand crosslink. More specifically, they show that (i) DNA synthesis to within one nucleotide from the ICL is performed by a replicative polymerase; (ii) REV1-pol ζ(REV3-REV7) performs extension beyond the lesion; (iii) The FA core complex recruits REV1-pol ζ to the ICL. (iv) The mutation rate during DNA synthesis to within one nucleotide from the cisplatin ICL and beyond the lesion is about 3%, which corresponds to a 30 fold increase compared to control DNA. The data presented by Budzowska and colleagues bring novel and useful information to understand how crosslinked DNA is replicated. The manuscript is well written and should be of

interest to the readership of the EMBO journal.

Main points:

1. *Figure 2: FANCA and FANCD2 exhibit an intriguing pattern of association with pICL at the ICL and control loci, is that reproducible? Replicates for FANCA and FANCD2 Chip experiments for the control locus are missing (Figure E1).*

The "intriguing" multiple peaks of FancD2 binding at the ICL locus (Fig 2K in the original manuscript) are not reproducible, and we have therefore removed this panel from figure 2. Please see below for a more detailed discussion of FancD2 ChIP data.

The broad peak of FancA binding to the ICL locus is reproducible (see Fig 2J and Fig 5A for FancA profiles, and Fig 5B and E6A for FancD2 profiles). The sharp early peak followed by the shallow late peak at the control locus probably also reflect actual FancA binding. We think the sharp early peak reflects some binding that occurs as a result of DNA replication. Consistent with this, we observe binding of another Fanconi Anemia core complex component, FancG, to replicating pCTR (Fig 3). Since we focus on the role of FancA and FancD2 in ICL repair, and not in unperturbed replication, we do not have another example of FancA and FancD2 ChIP at the control locus.

The authors suggest that FANCD2 binds to a large region surrounding the ICL, referring to (Klein Douwel et al., 2014). This, however, does not explain the multiphasique mode of FANCD2 binding to the ICL and control loci, including reloading of FANCD2 beginning at around 120 min., well after the ICL has been bypassed. Does the binding of FANCD2 depend on the presence of an ICL?

In most ChIP experiments, we do not observe reloading of FancD2 at late time points (see Fig 5B and E6A for examples). We also do not see such a binding pattern in the plasmid pull down assay (Fig 3). Moreover, we previously reported FancD2 binding to the ICL locus by ChIP, and in these experiments FancD2 reloading was not observed (Figs 2E, 3J, 7E and S2N in (Long *et al*, 2014) and Fig S5A in (Klein Douwel *et al*, 2014)). Therefore, it is likely that the decrease in FancD2 recovery seen at 120' in our former Fig 2K is not representative. Given that this FancD2 ChIP is not essential for the main message of the paper, and FANCD2 ChIP has been previously published, we removed this panel (former Fig 2K) and instead refer to FancD2 ChIP in Fig 5B and E6A.

ChIP signals of control locus have often the same shape (but with a lower % input, except for FANCD2). Is it a residual signal from the ICL locus due to low sonication efficiency?
To answer both questions, the authors should use the pCTR plasmid to ChIP proteins in the absence of the ICL. The FANCD2 ChIP on pCTR is essential.

Some signal seen at the control loci may be caused by incomplete sonication, e.g. 40' time points in Rev1 and Rev7 graphs (Fig 2H-I). However, most often the signals represent the actual binding of the proteins to the control loci. We know this because we have preformed the ChIP experiment on replicating pCTR, as requested, and observed significant binding of FancD2 and Rev1 (and of course Mcm7 and PCNA) (Supplementary Fig S2 in the revised manuscript). As expected, in the absence of damage, binding was the same at both loci. This data is consistent with the plasmid pull down experiments, in which we observe replication-dependent binding of ubiquitylated FancD2 to both pCTR and pICL (new Fig 3). We have included the new FANCD2 plasmid pull down data in Figure 3. Moreover, the binding of FancD2 to an undamaged internal control plasmid (pQuant) as detected by ChIP has been reported previously (Fig 5D and Fig S5A in (Klein Douwel *et al*, 2014)).

2. *Figure 3. Likewise, to better comprehend the role of the FA pathway in REV1-pol ζ recruitment to ICLs, it would be appropriate to probe for FANCA and for FANCD2 by western blotting in pCTR and pICL pull down.*

We have now included FancD2 and FancG in the plasmid pull down experiment (Fig 3 in the revised manuscript), and they confirm the ChIP data in Fig 2. Both FancA and FancG are components of the Fanconi Anemia core complex. We used FancG instead of FancA because FancA sticks non-specifically to the pull-down beads in the absence of DNA or DNA replication. This high background prevented detection of any specific recruitment of FancA to replicating pCTR and

pICL. Although there is also a significant background for FANCG, there is a clear signal above background.

3. *Based on the timing of Ub-PCNA association with pICL, which does not correlate with the peak of translesion DNA synthesis and REV1-pol ζ recruitment, the authors conclude that PCNA ubiquitylation is not essential for the bypass of interstrand DNA crosslinks. Yet Ub-PCNA is retained on the plasmid for a longer time in the presence of the ICL. Why? Ub-PCNA may still promote the loading of the "insertion" polymerase that remains to be discovered, in particular if it is a Y family polymerase. The timing of protein loading and reaction products may not coincide exactly. Ub-PCNA could regulate TLS and mutagenesis. It is difficult to draw conclusions before PCNA has been replaced with a non-ubiquitylatable PCNA mutant.*

We agree with the referee that the absence of precise correlation between PCNA ubiquitylation and binding of Rev1-pol ζ complex does not exclude the involvement of PCNA ubiquitylation in regulation of TLS during ICL repair, e.g. in recruitment of the "insertion" polymerase, or stabilizing the Rev1-pol ζ complex. We have now mentioned these possibilities more clearly in the results section (page 11 in the revised manuscript).
The only experiment that could address the role of PCNA ubiquitylation is to replace wild type PCNA with the non-ubiquitylatable PCNA mutant. Unfortunately, due to the extremely high PCNA concentration in the nucleoplasmic extract (~30 μM) we have not succeeded in depleting it.

4. *Figure 7 and E6: The strategy used for sequencing the plasmid is described in the legend of Fig E6 but not in the main text. Some explanation in the main text would facilitate the reading.*

We do say in the main text that we PCR amplified a 115 bp region around the ICL, which was then deep-sequenced. If we want to provide more description, we would have to provide all the technical details, which would really break up the flow. We therefore respectfully suggest that the current solution is best as is.

The authors mentioned that many of the mutations were due to errors during PCR before deep - sequencing. Did they try to deep sequence directly the plasmids?

The experimental setup we used for deep sequencing involves a PCR step to amplify the repair products and to add six-nucleotide barcodes unique to products from each repair reaction. Regarding the reviewer's point, it should be possible to cut out the ICL region in pCTR, and ligate the adaptors for sequencing directly. However, this would require a new deep sequencing reaction, which is quite expensive. Additionally, this control would not eliminate errors introduced during the sequencing reaction itself. Most importantly, it would not alter the results or change the conclusions, and therefore we prefer not to perform this control experiment.

Minor points:

1. *Figure E7: I was surprised by the quite high frequency of indels in the control. It would be interesting to determine if it is real or if it is due to PCR step.*

We agree that the high frequency of indels in the control reaction is unexpected. We intend to investigate this, and the higher level of indels during the ICL repair reaction, in more detail in the future.

2. *It should be clearly stated that experiments are performed using cisplatin DNA crosslinks, specifically. Some conclusions could differ in the context of different types of DNA crosslinks.*

We have now clearly indicated the type of the crosslink in the description of the experiments and included this information in the conclusions.

Referee #2:

Budzowska et al., have used Xenoupus egg extracts to delineate the molecular mechanism of interstrand cross link repair. The pathway is broken into discrete and temporal steps, which includes replicative stalling 20-40 bases before the lesion, followed by approach to the lesion,

insertion opposite the unhooked lesion and finally extension. By using a combination of techniques, the authors have eloquently identified key participants in most of the steps. The approach is likely to be performed by a high fidelity replicative DNA polymerase, while extension is dependent upon Rev1 and DNA pol zeta. The authors also demonstrate that ubiquitinated PCNA does not recruit Rev1 or pol zeta to the lesion, but their recruitment is instead dependent upon the Fanconi core complex. Deep DNA sequencing of the fully extended replication products reveals that bypass is remarkably accurate and when mutations occur, they do so in a short 6-7 base pair repair track. The highest level of mutagenesis is observed either 1 bp before the lesion, or more likely, opposite the lesion itself.

This is a well-executed study and the conclusions drawn are convincingly supported by the data.

My only reservation is that the DNA polymerase that performs the actual TLS step was not identified. The authors speculate that it is either DNA polymerase eta or DNA polymerase kappa and I wonder how difficult (or time consuming) it would be to actually test this hypothesis with extracts depleted of either enzyme?

We have depleted the extracts of pol kappa and have not seen inhibition of the insertion step. Depletion of pol eta resulted in the transient accumulation of -3 and -2 products and no particular enrichment of -1 species, as expected if pol eta were required for insertion (pol kappa/pol eta double depletion also did not cause -1 accumulation). This result suggests that extending the leading strand all the way to the -1 position involves pol eta, implying that the DNA duplex immediately preceding an ICL is difficult to copy by a replicative polymerase. However, the accumulation of extension products (visualized on sequencing gels) and fully replicated supercoiled plasmids was hardly affected in pol eta-depleted extracts. This suggests that while in some cases pol eta may aid approach, its action is not essential for the success of the reaction. In addition to the defect in approach, we observed slightly longer persistence of X-structures in pol eta depleted extracts, which suggests a defect in incisions. Therefore, the participation of pol eta in cisplatin ICL repair appears to be rather complex. Sorting out these various roles will require extensive additional work and thus falls outside the scope of the current paper.

Referee #3:

Budzowska et al.
Regulation of the Rev1-Polzeta complex during bypass of a DNA interstrand crosslink

This manuscript from the Walter group builds on work they have published over the past seven years in which they have elegantly dissected the replication-dependent repair of a DNA interstrand crosslink in Xenopus egg extracts. A key step at the heart of the process is the replication of the unhooked crosslink, a step that requires translesion synthesis dependent on Rev1 and DNA pol zeta. It is this phase of the reaction that is the focus of the current work. The authors show that the approach step, in which one of the blocked forks edges towards the crosslink after removal of the blocked CMG helicase, is likely to be mediated by the replicative polymerases. Surprisingly, though, the insertion step, in which a base is incorporated opposite the unhooked crosslink is not dependent on Rev1, despite the damaged template being a dG. Rather depletion of Rev1 actually results in an accumulation of the insertion product, but a delay in formation of the extension product. Interestingly, the authors provide persuasive evidence that the recruitment of Rev1-Polzeta is temporally separated from the peak of PCNA ubiquitination that is seen during replication in Xenopus egg extracts. Rather they suggest that its recruitment is dependent on the FA core complex. The authors also provide sequencing data to show that mutagenesis during ICL repair is limited to just around the lesion and does not extend much beyond.

This is an interesting and important next step in the story. The paper is also very clearly written and presented. However, I do have a number of questions and suggestions.

***1.** The key experimental technique used to dissect the recruitment and retention of the various proteins to the ICL-containing and control plasmids is ChIP. These are presented as individual experiments rather than as normalised replicates with error on a single graph. Why is this? In many ways, it is the timing of the peak recruitment that is most important. Is there a way in which this can be estimated, its variability measured and the significance of the differences assessed?*

While the shapes of the ChIP graphs and the relative differences between proteins and conditions are consistent between experiments, the absolute values of the peaks and the timing of recruitment are somewhat different. Some of these differences come from using different extracts, e.g. extract used in experiment 2 (Supplementary Fig S1) is slower than extracts in experiments 1 and 3 (Fig 2 and Supplementary Fig S1). Differences in % of recovery for each time point are likely caused by small changes in the reaction kinetics and in the reproducibility of ChIP. Additionally, depletion procedures affect the fitness of the extracts and the kinetics of the replication reactions, and these effects are also slightly different between individual experiments. Given these considerations, we feel that plotting multiple repeats of an experiment on the same graph will overestimate the variability in the actual result, that is when a protein loads relative to another or relative to a different condition. We have therefore opted for showing individual repeats of the same experiment.

2. *The second approach immunoprecipitates the plasmids from the extract at different times. This provides very clear and compelling results (Figure 3). The separation of PCNA ubiquitination from REV1 recruitment is particularly striking. However, it would be good to see this technique extended to FancA, or other components of the FA core complex to provide a second line of evidence to back up the ChIP data.*

We have now included FancD2 and FancG in the plasmid pull down experiment (Fig 3 in the revised manuscript), and they confirm the ChIP data in Fig 2. Both FancA and FancG are components of the Fanconi Anemia core complex. We used FancG instead of FancA because FancA sticks non-specifically to the pull-down beads in the absence of DNA or DNA replication. This high background prevented detection of any specific recruitment of FancA to replicating pCTR and pICL. Although there is also a significant background for FANCG, there is a clear signal above background.

3. *The sequencing experiment is very nice. It provides very little evidence of dC insertion (on either strand), suggesting that the catalytic activity of Rev1 is not deployed. The majority of mutagenic activity by A-rule incorporation opposite the lesion. Since Rev1 depletion also likely depletes Pol zeta (at least the Rev7 part), is it possible that the apparently proficient insertion step is being mediated by pol delta, which is now known to share its pold2 and pold3 subunits with pol zeta, even though during normal replication its principal role is on the lagging strand? Have the authors tried depleting pold3?*

We have tried depleting pol delta with antibodies against p125 and p66 and succeeded in removing ~ 95% of both subunits from egg extract.. However, consistent with a previous report (Fukui *et al*, 2004), depletion of pol delta significantly inhibited DNA replication (data not shown). Since ICL repair is replication-dependent, it is therefore impossible to investigate the effect of pol delta depletion on the TLS reaction.

4. *Is there any change in the spectrum of mutagenesis in Rev1-depleted extracts? I am a bit confused about whether the sequencing has to be carried out on completed, SapI-digestible, products, or whether information could still be obtained from the 'extension' product, which remains abundant in the Rev1-depletion experiments (Fig 4F).*

The referee is correct that sequencing of repair products does not need to be carried out on SapI-digestible products. The extension products, regardless of whether or not they can be digested with SapI can be amplified and sequenced. The data shown in Fig 7B-C and Supplementary Fig S8B-C was obtained using samples that were not digested with SapI. The referee is also correct that, although at a reduced level, the extension products are generated in Rev1-depleted extracts and that these products can be sequenced. We amplified and sequenced the extension products generated in Rev1-depleted extracts. We found that after 99% depletion of Rev1, the mutation frequency dropped significantly. We have included this data in the revised manuscript (Supplementary Fig S8C in the revised manuscript), and briefly describe this data in the results section (page 14).

5. *The authors suggest that Rev1 binds FancA. Is this direct? If so, which domains are important? Or is it related to the previously described interaction of Rev1 with FAAP20?*

Although the reviewer's question is valid, addressing this point would be a large amount of work (express and purify Rev1 and FancA, do pull-downs, followed by deletion analysis), with uncertain outcome, since FancA normally binds Rev1 in the context of the entire core complex, which contains many subunits and is therefore very difficult to express. Indeed, it is quite likely that the interaction is mediated by the core complex-associated protein Faap20, and we have stated this in the manuscript (page 12 in the revised manuscript).

Minor points.

6. *The authors note that they were unable to interpret the plasmid pull down with pol delta because of problems with high background. Does this not also apply to the ChIP experiment shown in Figure 2E?*

ChIP and plasmid pull downs are very differently assays, e.g. they are performed using different beads and different wash conditions. Both of these variables affect the level of background. In plasmid pull downs, we use magnetic beads and very mild wash conditions, since our goal is to preserve the protein complexes bound to DNA at different steps of the reaction. Consequently, for some proteins, e.g. pol δ, we observe high background binding in samples without DNA or replication, and no increased binding during replication/repair, which tells us these signals are non-specific. In ChIP, we crosslink DNA to proteins, and use much harsher washing conditions, including a wash with SDS-containing buffer. As measured by ChIP, pol δ exhibits large, time-dependent changes in binding on pICL (Fig 3D-F, Supplementary Fig S1A-C and E-G) and undamaged plasmid (pQuant; Fig R1A), which mirrors the binding of PCNA and pol ε. Additionally, we performed ChIP in the presence of geminin, which inhibits replication initiation. The recovery of pol ε, pol δ, and MCM7 was greatly reduced in the geminin samples (Fig R1B), further arguing that the pol δ ChIP is specific. Taken together, our data indicates that the ChIP profiles represent real and specific binding of pol δ during pICL replication and repair.

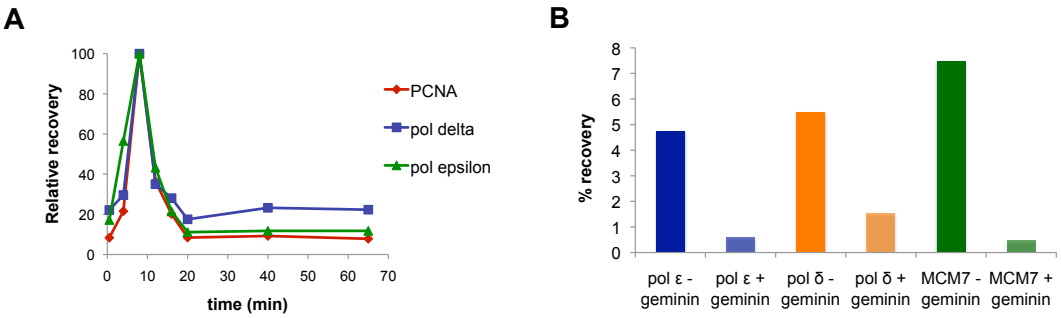

Figure R1. Binding of pol δ, pol ε, PCNA and MCM7 to undamaged plasmids.

A. Recruitment of pol δ, pol ε and PCNA to the undamaged replicating plasmid (pQuant). pQuant was replicated, and samples were withdrawn at the indicated times for ChIP with antibodies against pol δ, pol ε, and PCNA.

B. Undamaged plasmid (pCTR) was licensed in HSS extract -/+ geminin. Replication was initiated by addition of nucleoplasmic extract. Samples for ChIP with anti-MCM7, anti-pol δ and anti-pol ε antibodies were withdrawn at 3 minutes.

7. *Is there an explanation for the curious behavior of FANCD2 in Figure 2K?*

See response to reviewer #1 point 1.

8. *Is there really a justification for using curvy connecting lines on the ChIP experiments, rather than just straight lines joining the points? [EDITOR NOTE: I agree that the individual data points should be connected by straight lines]*

We have changed the graphs, and the data points are now connected by straight lines.

9. *Why is there such a large variation in the efficiency of the ICL repair, as assessed by SapI digestion? For instance the lack of repair in FancA depleted extracts (Figure 5C) is similar to the maximum extent of proficient repair in the mock depleted extract in Figure 4E. Some explanation of the variability of this assay, and how the reader is to assess significance of differences on this background variability, would be good.*

There are several factors that contribute to the variability in the repair assay. First, the amount of uncrosslinked plasmid, responsible for background accumulation of SapI cleavage, is different in each preparation of pICL. Second, individual batches of extract have different repair capacities. This is probably due to many different factors. For example, since regeneration of the SapI site depends on homologous recombination, the level of SapI cleavage products will depend on how efficiently holliday junctions are resolved/dissolved. The best way to look at the results is whether a particular manipulation completely or partially reduces Sap I site regeneration to background levels. We now do a better job of describing the results in these terms, and we have added the following sentence: "The amount of SapI cleavable products can vary between experiments due to differences in the amount of uncrosslinked plasmid, and differences in the repair capacity of individual batches of extract" in the legend to Fig 4E, in which the assay is first shown.

10. *Is there a word missing in the sentence in the abstract 'Deep sequencing of ICL repair products showed that most of approach and extension are error-free.' ?*

No, but the reviewer is correct that the sentence is awkward. We have changed it to the following: 'Deep sequencing of ICL repair products showed that the approach and extension steps are largely error-free.'

References Cited:

Fukui T, Yamauchi K, Muroya T, Akiyama M, Maki H, Sugino A & Waga S (2004) Distinct roles of DNA polymerases delta and epsilon at the replication fork in Xenopus egg extracts. *Genes Cells* **9:** 179–191

Klein Douwel D, Boonen RACM, Long DT, Szypowska AA, Räschle M, Walter JC & Knipscheer P (2014) XPF-ERCC1 acts in Unhooking DNA interstrand crosslinks in cooperation with FANCD2 and FANCP/SLX4. *Mol Cell* **54:** 460–471

Long DT, Joukov V, Budzowska M & Walter JC (2014) BRCA1 Promotes Unloading of the CMG Helicase from a Stalled DNA Replication Fork. *Mol Cell* **56:** 174–185

2nd Editorial Decision 06 May 2015

Thank you for submitting your revised manuscript for our consideration. My colleague Dr. Hartmut Vodermaier is currently away, and I am stepping in to not cause further delays.
Your manuscript has now been seen once more by two of the original referees (see comments below), and I am happy to inform you that they are both satisfied with the revisions and therefore have no further objections towards publication in The EMBO Journal.

Referee #1:

In our view, the authors have addressed the comments well and the paper should now be published.

Referee #3:

The authors have responded carefully to my comments and I would be happy to see this paper published.

