Manuscript EMBO-2014-89184

USP45 deubiquitylase controls ERCC1-XPF endonuclease-mediated DNA damage responses

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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.)

Pre-decision letter   24 June 2014

Thank you for again for submitting your manuscript EMBOJ-2014-89184, "USP45 deubiquitylase controls ERCC1-XPF endonuclease mediated DNA damage responses" for our consideration. We have now received a complete set of reviews from all referees, which you will find enclosed below for your information.

As you will see, the referees appreciate you identification and careful characterization of USP45 as a novel interactor and regulator of the ERCC1-XPF1 endonuclease, but they at the same time all remain unsatisfied by the follow-up work on the functional significance of ERCC1 regulation by ubiquitination and degradation. Given the unanimous nature of these concerns, I am afraid we do not find the manuscript suitable for EMBO Journal publlication in its present form, but realize that it may become a much more compelling candidate if extended along the lines suggested by our three referees.

In this situation, it would be very helpful for us to determine whether you may be able to provide such an extension (to reasonable limits) during the regular time frame of a major revision, before finalizing an editorial decision in this case. I would therefore appreciate if you could, following careful consideration and discussion of the referees' comments with your coworkers, provide me with a tentative point-by-point response letter detailing what you may (and may not) be able to do during revision to address the concerns and strengthen the manuscript. This tentative response (parts of which we may choose to share and discuss with some of the referees) would be taken into account when making our final decision on this manuscript, and may of course also form the basis for further, direct discussions. I would therefore appreciate if you could send us such a response at your earliest convenience, ideally by early next week.

Referee #1

Perez-Oliva and colleagues report on the interaction of the USP45 DUB with the ERCC1 subunit of the ERCC1-XPF structure-selective nuclease. To date, we know some details of this nuclease complex is controlled in NER (XPA-interaction/recruitment) and ICL repair (SLX4). Given the dire potential consequences of uncontrolled activity, stability and localisation it is clear that these aspects must be closely regulated, which makes this an important topic of investigation. The description of the interaction between ERCC1 and USP45 is adequate, whereas the functional characterization of USP45 loss/disruption of its ERCC1 interaction not so well developed.

Major point:

The functional studies of the damage response and repair defects offer some initial insights, but need to be developed further, and could be done so in some relatively straightforward experiments. Does loss of USP45 produce the hallmarks of an NER defect, ICL repair defect or both? Looking at RCC1 nuclear foci is not likely to answer these questions, since factors undertaking NER do not typically form observable foci during this process, unless irradiation is applied locally in patches through filters. Determining the kinetics of UV-CPD and 6-4 removal is a reasonably simple task, and would report on any overt NER defect. Likewise, cells that have lost ERCC1 activity ccumulate broken chromosomes after MMC treatment, as shown by Niedernhofer and others. Revisiting Suppl. Fig. 5 could address this point, since previous analysis shows that gH2AX accumulates due to fork collapse in ERCC1-null cells. However, to see the types of defect observed in previous work the analysis would need to be extended to longer times to see if the foci are resolved in USP45 KO/mutant cells, and if any defects in resolution mirror those seen in ERCC1-defective cells. Also, a dose that does not saturate the method of quantification employed (greater than 10 foci per cell) should be avoided as no dynamics can be inferred in such experiments. Taken together, such assays could provide essential initial information on the nature of DDR defect resulting from lack of ERCC1 control by USP45 (I appreciate that a full DDR-repair characterization is well beyond the scope of the current MS). A potential issue here is that KO or mutation of USP45 reduces ERCC1 levels. However, this is only perhaps two-fold looking at the gels presented, and it is therefore unlikely that ERCC1 concentrations would be limiting in these assays, if sensible doses of damage were selected.

Technical points:

Fig. 2 - for the gel filtration blots we need to see the Mw calibration to give an indication of the mass of the complexes.

Fig. 2F, left hand panel, is poor and the change in USP45 behaviour not convincing.

Fig. 4 A. What is the lower band in the IB:XPF lanes? it is not seen in any of the other blots. For that matter, which of the two band is XPF in this blot? in the other blots we only see one band but they might have been differently cropped.

Fig. 4B and C. These blots are not so consistent. The high Mw/Ub-modified ERCC1 runs in a atypical fashion in 4B and the reduction in ERCC1 levels in KO cells in 4C difficult to fully credit without further quantification.

Fig. 6. Important to see this data repeated as a time-course. Are the USP45 loss/mutation-associated defects in ERCC1 recruitment to foci due to delayed recruitment or shorter occupancy time at lesions?

Referee #2

Using a Mass spectrometry analysis the authors have identified ERCC1, XPF and SLX4 as interactors of endogenous purified USP45. Follow-up studies showed that the USP45-ERCC1 interaction is direct and is mediated via the N-terminal domain of USP45. Additional experiments

showed that ERCC1 levels are increased upon proteasome inhibition which is accompanied by slower migrating forms of the ERCC1 protein. In 2 different cell lines deficient for USP45, ERCC1 levels are reduced, while the slower migrating ERCC1 forms are increased, suggesting that USP45 is the DUB of ERCC1. In addition the USP45 KO cells are somewhat more sensitive than wild type cells, to both UV and MMC. In addition, MMC-induced focal accumulation of ERCC1 is strongly reduced in USP45 KO cells. This reduction can however be rescued by the expression of WT USP45 but not with a catalytic mutant. Finally USP45 is recruited in an ERCC1-independent manner to sites of DNA damage.

Altogether this paper describes a novel ubiquitin-based regulation of ERCC1 by the DUB USP45. Although the initial observation of ubiquitin-dependent regulation of the ERCC1 function is interesting and the first part of the paper describes the development of solid experimental tools (antibodies and even defined knock-out cells) and sound experimental approaches have been used (e.g. proteomics, yeast two-hybrid), the second half of the paper analyzing the functional ignificance of the USP45-dependent role on ERCC1 function, is much less developed (see specific comments below).

I have the impression that the paper is finished in a rush. The first part describes an elaborate, time consuming strategy to first raise antibodies, which are used to immune-precipitate endogenous USP45 to identify possible interactors and in parallel USP45 knock-out cells were generated. They next provide a good body of evidence that ERCC1 level or function are regulated by ubiquitylation and that USP45 plays an important role in preventing ERCC1 degradation, although part of this evidence remains circumstantial as no direct ubiquitylation of ERCC1 is shown, nor the mechanism of ERCC1 ubiquitylation is described. The second half, describing the biological role of USP45, focused on its role on ERCC1 functioning, is however much less developed and would strongly benefit from more solid and convincing experimental evidence. It is strange that an observation that is apparently important enough to highlight at the end of the abstract: "Finally, we observed that USP45 localises to sites of DNA damage ...., but independent of its ability to bind ERCC1-XPF.", where the ERCC1-independent damage loading which is only shown in a single cell (without any statistical information or on how frequent this observation is made) ends up in a supplementary figure of poor image quality.

Major specific comments:
• The claim that the SLX4-USP45 interaction is dependent on ERCC1 cannot be drawn from figure Fig.2D since no USP45 is co-IPed with SLX4 in ERCC1
proficient cells.
• It has been shown that in the absence (or in cases that mutations affect the stability) of one of the complex partners ERCC1 or XPF, the steady-state levels of the other polypeptide of this heteroduplex is also severely reduced (e.g. Niederhofer, 2007, Nature; Ahmad, 2010, PLoS Genet), suggesting that the 2 proteins stabilize each other. However the XPF proteins levels are not significantly (except in Figure 4B where a reduction is visible) reduced upon USP45 KD, authors should comment on this discrepancy with the previous literature.
• Figure 4D: USP45 is indeed able to reduce the slower migrating ERCC1 bands, though no other DUBs were tried, thus this does not show specificity of USP45.
• Using pulldown procedure (e.g. TUBEs, His-ubiquitin) it should be shown that the slower migrating bands are actually ubiquitylated forms of ERCC1 in the experiments in Fig2A-D.
• The USP45 KO cells were only slightly sensitive to MMC and UV. Since no other information is provided on the phenotype of the KO cells in terms of cell cycle distribution and growth characteristics, sensitivity to genotoxic agents should be treated with care as secondary effects, rather than only compromised DNA repair could contribute to reduced cellular survival. The authors should show more features of the KO cells.
• The reduced focal accumulation of ERCC1 after DNA damage in USP45 KO appeared clear. The authors conclude form this that loading of ERCC1 to DNA damage is compromised in the absence of functional USP45. However, loading kinetics of ERCC1 to laser-induced DNA damage stripes should be measured.
• Surprisingly, the authors claim to observe a focal pattern for XPA and ERCC1 upon UV damage (although the focal pattern of XPA in Sup Figure 6 is not visible from the provided images). While the localization of NER factors, including XPA en ERCC1, upon UV damage has been extensively studied over the last decades, UV induced focal patterns were never observed. Therefore the effect of USP45 on the function of ERCC1 during the UV induced damage response should be performed either by applying local UV damage or FRAP studies on GFP-tagged ERCC1 and the claim of a

focal pattern of NER factors should be substantiated and explained.
• The effect of USP45 happens also in undamaged cells, however is there a difference in DUB activity towards ERCC1 upon damage? This might be expected since USP45 is recruited to the site of damage. Are there differences in ERCC1 half-life between unperturbed and damaged cells?
• What is the effect of USP45 on ERCC1, is this only stabilization, or is the de-ubiquitylation by USP45 needed somewhere during the repair reaction? Can overexpression of ERCC1 rescue the phenotype of USP45 KO? Rescue of USP45 KO cells with ERCC1 should be included.
• How is USP45 recruited to the site of damage?
• The claim that USP45 accumulation to DNA damage is independent of ERCC1 is not convincingly shown in Supplemental figure 7B, only one cell is shown (with poor image quality). What is frequency of localization to laser stripes, does it occur in all cell, is it cell cycle dependent? Further statics should be provided. This holds also for Figure 8 and supplemental figure 7A.

Minor comments:

• Figure 1, page 5: limited information is provided on the Mass spec data, e.g. how many proteins were identified?; specificity of the identified proteins.
• USP45 is a pleiotropic DUB, as shown in earlier studies and clear from the other identified interactors in this study. Any information is lacking on how the specific interaction with ERCC1 is controlled.
• The D25A,E26A and D27A mutants all lack interaction, or show reduced interaction with ERCC1, however also the other mutations show a strong reduction in ERCC1 binding, this should be discussed as well in the text.
• The effect of proteasome inhibition on the ERCC1 levels in USP45 KO cells should be shown in Figure 2B and C.
• Is USP45, next to prosalen or angelicin induced DNA damage, also recruited to UV-C induced DNA damage (NER lesions)?
• Can proteasome inhibition rescue the loss of ERCC1 foci formation upon DNA damage in USP45 KD cells?
• A complete overview of the results of the MS analysis should be represented, including the identified proteins (aspecific interactors) in gel lane from USP45 IP in the USP45 KO cells.
• What is the phenotype of USP45-/- in combination with ERCC1-/- does this sensitize cells further than the ERCC1-/- or USP45-/- cells to UV or MMC?

Referee #3

Manuscript by Alessi and colleagues describe a previously uncharacterized deubiquitylase USP45 as a critical regulator of DNA repair via association and stabilization of the endonuclease XPF-ERCC1. While the reader will certainly appreciate adding another new DUB to the ever-growing list of ubiquitin regulators of DNA repair, in its current form, it fails to show what is the importance of ubiquitinated XPF-ERCC1. This should be the fundamental question that the authors need to address both mechanistically and functionally. Before identifying novel regulators of ubiquitinated XPF-ERCC1, I would think it makes more sense to understand the role of ubiquitinated XPF-ERCC1 in DNA repair. For example, what is the role of ubiquitinated XPF-ERCC1? Is this an inducible post-translational modification that is stimulated by DNA damage or recovery? Is it to target XPF-ERCC1 for proteasomal degradation? If so, how does a non-degradable form of XPF-ERCC1 affect DNA repair of UV or crosslink-induced DNA damage? Does this alter cell cycle checkpoint activation (G2/M accumulation or G1 arrest)? However, if ubiquitinated XPF-ERCC1 also possess a non-proteolytic function in protein localization, it would be critical to understand what is the nature of this localization (or lackthereof) that causes DNA repair defect. In summary, simply characterizing a new DUB in DNA repair without sufficient biological context and mechanism temper the reader's enthusiasm for this study.

Major points:
Figure 1, what is the role of USP45 in the SLX4 complex? Does it alter SLX4-dependent function in DNA crosslink repair? SLX4 is not required for UV-induced gap
repair, but ERCC1-XPF is. Is USP45 functioning via the SLX4 complex (which also contains ERCC1-XPF) or does it also have a SLX4-independent function during UV
damage via ERCC1-XPF in NER. How do you reconcile this discrepancy?

Figure 4, Is degradation of ERCC1 regulated in a DNA damage or cell cycle-dependent manner? This would give more insight into when USP45 may be critical to suppress ERCC1 degradation, otherwise it appears to be performing a housekeeping function in keeping ERCC1 levels elevated.

Figure 6, how does USP45 promote ERCC1 recruitment to sites of DNA damage? Is it through simple stabilization of the protein or is it through modulating the ubiquitinated environment surrounding the DNA lesion site? The authors need to show whether a hyperstable ERCC1 mutant remains permissive for recruitment to sites of DNA damage and repair in the absence of USP45.

Additional correspondence (authors)   01 July 2014

Thank you for sending us the 3 Reviewers comments for our manuscript in such a timely manner.

Reviewers 1 and 2 have made some very insightful and constructive suggestions on how to improve our manuscript. As outlined below we feel that with the exception of point 10 made by Reviewer 2, we should be able to undertake all the other additional experiments they requested within a 3-4 month period. Reviewer 3 is obviously more critical and the points they make are mostly beyond the scope of what we can do in this project. Nevertheless we can attempt to address point 3 and 4 of what they have requested.

As requested, we have listed all of the reviewers' comments below and put in green the points we plan to address and those that we will be unable to address are in red. The one point is in orange and depends on us being able to locate specialized apparatus to undertake this experiment.

Referee 1
Major point:

1.The functional studies of the damage response and repair defects offer some initial insights, but need to be developed further, and could be done so in some relatively straightforward experiments. Does loss of USP45 produce the hallmarks of an NER defect, ICL repair defect or both? Looking at ERCC1 nuclear foci is not likely to answer these questions, since factors undertaking NER do not typically form observable foci during this process, unless irradiation is applied locally in patches through filters. Determining the kinetics of UV-CPD and 6-4 removal is a reasonably simple task, and would report on any overt NER defect.

 We plan to undertake this straightforward experiment

2. Likewise, cells that have lost ERCC1 activity accumulate broken chromosomes after MMC treatment, as shown by Niedernhofer and others. Revisiting Suppl. Fig. 5 could address this point, since previous analysis shows that gH2AX accumulates due to fork collapse in ERCC1-null cells. However, to see the types of defect observed in previous work the analysis would need to be extended to longer times to see if the foci are resolved in USP45 KO/mutant cells, and if any defects in resolution mirror those seen in ERCC1-defective cells.

We plan to undertake this straightforward experiment

3. Also, a dose that does not saturate the method of quantification employed (greater than 10 foci per cell) should be avoided as no dynamics can be inferred in such experiments. Taken together, such assays could provide essential initial information on the nature of DDR defect resulting from lack of ERCC1 control by USP45 (I appreciate that a full DDR-repair characterization is well beyond the scope of the current MS). A potential issue here is that KO or mutation of USP45 reduces ERCC1 levels. However, this is only perhaps two-fold looking at the gels presented, and it is therefore unlikely that ERCC1 concentrations would be limiting in these assays, if sensible doses of damage were selected.

We plan to reanalyze our data in accordance with these comments

Technical points:

4. Fig. 2 - for the gel filtration blots we need to see the Mw calibration to give an indication of the mass of the complexes.
We plan to add this to figure

5. Fig. 2F, left hand panel, is poor and the change in USP45 behavior not convincing.
We plan to repeat this experiment to improve quality of data

6. Fig. 4 A. What is the lower band in the IB:XPF lanes? it is not seen in any of the other blots. For that matter, which of the two band is XPF in this blot? in the other blots we only see one band but they might have been differently cropped.
We plan to address this point

7. Fig. 4B and C. These blots are not so consistent. The high Mw/Ub-modified ERCC1 runs in a atypical fashion in 4B and the reduction in ERCC1 levels in KO cells in 4C difficult to fully credit without further quantification.
We plan to perform the additional quantitation requested and immunoblot with the different ubiquitin chain antibodies as suggested

8. Fig. 6. Important to see this data repeated as a time-course. Are the USP45 loss/mutation-associated defects in ERCC1 recruitment to foci due to delayed recruitment or shorter occupancy time at lesions?
We plan to undertake the time course requested

Referee #2

Major Points:

1. The claim that the SLX4-USP45 interaction is dependent on ERCC1 cannot be drawn from figure Fig.2D since no USP45 is co-IPd with SLX4 in ERCC1 proficient cells.
We plan to address this by repeating the experiment with more protein

2. It has been shown that in the absence (or in cases that mutations affect the stability) of one of the complex partners ERCC1 or XPF, the steady-state levels of the other polypeptide of this heteroduplex is also severely reduced (e.g. Niederhofer, 2007, Nature; Ahmad, 2010, PLoS Genet), suggesting that the 2 proteins stabilize each other. However the XPF proteins levels are not significantly (except in Figure 4B where a reduction is visible) reduced upon USP45 KD, authors should comment on this discrepancy with the previous literature.

 It is controversial whether ERCC1 stabilizes XPF. Another group has published similar results to ours that XPF is still present in ERCC1 knock-down (PMID 22323595) cells. Moreover, in ERCC1 knock-out cells we have obtained from the David Melton lab, we find that XPF levels are expressed at similar levels-this data was not included in our manuscript but we plan to add this data to the revised manuscript.

3. Figure 4D: USP45 is indeed able to reduce the slower migrating ERCC1 bands, though no other DUBs were tried, thus this does not show specificity of USP45.
We plan to undertake this experiment

4. Using pull-down procedure (e.g. TUBEs, His-ubiquitin) it should be shown that the slower migrating bands are actually ubiquitylated forms of ERCC1 in the experiments in Fig2A-D.
We plan to undertake this experiment

5. The USP45 KO cells were only slightly sensitive to MMC and UV. Since no other information is

provided on the phenotype of the KO cells in terms of cell cycle distribution and growth characteristics, sensitivity to genotoxic agents should be treated with care as secondary effects, rather than only compromised DNA repair could contribute to reduced cellular survival. The authors should show more features of the KO cells.

We can show growth of WT vs KO USP45 cell lines. The KO cells grow more slowly. No difference in FACS cell cycle profiles was found in KBM7 between WT and KO. We will undertake FACS cell cycling analysis of U2OS WT vs KO USP45 cells to address the reviewers concerns.

6. The reduced focal accumulation of ERCC1 after DNA damage in USP45 KO appeared clear. The authors conclude form this that loading of ERCC1 to DNA damage is compromised in the absence of functional USP45. However, loading kinetics of ERCC1 to laser-induced DNA damage stripes should be measured.
Referee 1 has requested this point and we will undertake the additional experiments to address this. To our knowledge ERCC1 stripe formation has never been reported in the literature and we have not been able to detect this in numerous experiments.

7. Surprisingly, the authors claim to observe a focal pattern for XPA and ERCC1 upon UV damage (although the focal pattern of XPA in Sup Figure 6 is not visible from the provided images). While the localization of NER factors, including XPA en ERCC1, upon UV damage has been extensively studied over the last decades, UV induced focal patterns were never observed. Therefore the effect of USP45 on the function of ERCC1 during the UV induced damage response should be performed either by applying local UV damage or FRAP studies on GFP-tagged ERCC1 and the claim of a focal pattern of NER factors should be substantiated and explained.

Other authors have published that XPA can form Foci after UV treatment for example see Fig 5 in publish about XPA FOCI, for example Fig 5 in PMID 16862173). We can also amplify our figures to make much clearer the foci formation. Nevertheless the CPD experiment also asked by Reviewer 1 will also be undertaken.

8. The effect of USP45 happens also in undamaged cells, however is there a difference in DUB activity towards ERCC1 upon damage? This might be expected since USP45 is recruited to the site of damage. Are there differences in ERCC1 half-life between unperturbed and damaged cells?

We plan to undertake half life measurements requested ± DNA damage

9. What is the effect of USP45 on ERCC1, is this only stabilization, or is the de-ubiquitylation by USP45 needed somewhere during the repair reaction? Can overexpression of ERCC1 rescue the phenotype of USP45 KO? Rescue of USP45 KO cells with ERCC1 should be included. **We plan to undertake this experiment**

10. How is USP45 recruited to the site of damage?
 This is a good point, but in our opinion is beyond the scope of this study and would represent a new project and it is not realistic to achieve this in a 3-4 month time period.

11. The claim that USP45 accumulation to DNA damage is independent of ERCC1 is not convincingly shown in Supplemental figure 7B, only one cell is shown (with poor image quality). What is frequency of localization to laser stripes, does it occur in all cell, is it cell cycle dependent? Further statics should be provided. This holds also for Figure 8 and supplemental figure 7A.
 We plan to repeat the experiment to address this point and show more cells per panel and re-quantify Fig 8

Minor comments:

12. Figure 1, page 5: limited information is provided on the Mass spec data, e.g. how many proteins were identified?; specificity of the identified proteins. **We plan to include this data as a supplementary Excel file**

13 USP45 is a pleiotropic DUB, as shown in earlier studies and clear from the other identified interactors in this study. Any information is lacking on how the specific interaction with ERCC1 is controlled.
Figure 3D shows that USP45 is the only DUB studied that binds to ERCC1. As requested by reviewer 1 we plan to test a panel of DUBs for their ability to deubiquitylate ERCC1 in vitro.

14 The D25A,E26A and D27A mutants all lack interaction, or show reduced interaction with ERCC1, however also the other mutations show a strong reduction in ERCC1 binding, this should be discussed as well in the text.
 We plan to improve the presentation and discussion of this data.

15 The effect of proteasome inhibition on the ERCC1 levels in USP45 KO cells should be shown in Figure 2B and C.
 We plan to undertake the proteasome inhibition study for Fig 2B (Fig 2C may be a mistake as this is a two hybrid screen and would not make sense to inhibit proteasome).

16 Is USP45, next to prosalen or angelicin induced DNA damage, also recruited to UV-C induced DNA damage (NER lesions)?
We do not have apparatus to preform UV-C treatment. We can explore possibility to collaborate with another group at another University if this experiment is deemed critical

17 Can proteasome inhibition rescue the loss of ERCC1 foci formation upon DNA damage in USP45 KD cells?
We plan to undertake this experiment

18 A complete overview of the results of the MS analysis should be represented, including the identified proteins (aspecific interactors) in gel lane from USP45 IP in the USP45 KO cells.
We plan to include this data as a supplementary Excel file

• What is the phenotype of USP45-/- in combination with ERCC1-/- does this sensitize cells further than the ERCC1-/- or USP45-/- cells to UV or MMC?
 This is a good point but making a double knock-out will not be possible within a 3 month period.

Referee #3

1. Manuscript by Alessi and colleagues describe a previously ncharacterized deubiquitylase USP45 as a critical regulator of DNA repair via association and stabilization of the endonuclease XPF-ERCC1. While the reader will certainly appreciate adding another new DUB to the ever-growing list of ubiquitin regulators of DNA repair, in its current form, it fails to show what is the importance of ubiquitinated XPF-ERCC1. This should be the fundamental question that the authors need to address both mechanistically and functionally. Before identifying novel regulators of ubiquitinated XPF-ERCC1, I would think it makes more sense to understand the role of ubiquitinated XPF-ERCC1 in DNA repair. For example, what is the role of ubiquitinated XPF-ERCC1? Is this an inducible post-translational modification that is stimulated by DNA damage or recovery? .

This is a good point but this is an entirely new project. The aim of our study was to characterize USP45 as it was overesxpressed in a significant number of cancers and there have been no previous papers on this enzyme. Our game-plan was to find what USP45 works on and then take it from there, not to work out how ERRC1 is regulated by ubiquitylation.

2. Is it to target XPF-ERCC1 for proteasomal degradation? If so, how does a non-degradable form of XPF-ERCC1 affect DNA repair of UV or crosslink-induced DNA damage? Does this alter cell cycle checkpoint activation (G2/M accumulation or G1 arrest)? However, if ubiquitinated XPF-ERCC1 also possess a non-proteolytic function in protein localization, it would be critical to understand what is the nature of this localization (or lackthereof) that causes DNA repair defect. In summary, simply characterizing a new DUB in DNA repair without sufficient biological context and

mechanism temper the reader's enthusiasm for this study.
This is a good point but this is an entirely new project.

3. Figure 1, what is the role of USP45 in the SLX4 complex? Does it alter SLX4-dependent function in DNA crosslink repair? SLX4 is not required for UV-induced gap repair, but ERCC1-XPF is. Is USP45 functioning via the SLX4 complex (which also contains ERCC1-XPF) or does it also have a SLX4-independent function during UV damage via ERCC1-XPF in NER. How do you reconcile this discrepancy?.
We plan to discuss this point in the revised manuscript

4. Figure 4, Is degradation of ERCC1 regulated in a DNA damage or cell cycle-dependent manner? This would give more insight into when USP45 may be critical to suppress ERCC1 degradation, otherwise it appears to be performing a housekeeping function in keeping ERCC1 levels elevated.
As requested by reviewer 2 we plan to undertake half life measurements of ERCC1 ± DNA damage in wt and USP45 KO cells-that might help address this point

Figure 6, how does USP45 promote ERCC1 recruitment to sites of DNA damage? Is it through simple stabilization of the protein or is it through modulating the ubiquitinated environment surrounding the DNA lesion site? The authors need to show whether a hyperstable ERCC1 mutant remains permissive for recruitment to sites of DNA damage and repair in the absence of USP45.
This is a good point but represents an entirely new project.

1st Editorial Decision   01 July 2014

Thank you for your response to the referee comments on your manuscript, EMBOJ-2014-89184.

I am pleased to read that you appear to be in a position to address the majority of the key points of referees 1 and 2, which in my view should also help to ameliorate some of the further-reaching conceptual issues raised by referee 3. With regard to point 10 of referee 3 (mechanism of USP45 damage site recruitment), I agree that this may require substantial further work beyond the scope of the present study; I also realize that generation of double knock-out cells would not be a realistic revision request. On the other hand, I feel that attempts to demonstrate USP45 recruitment to UV-C lesions (ref 2 point 16) would indeed be helpful to strengthen the conclusions of the present study. With regard to the criticisms of referee 3, I appreciate that the general focus on USP45 roles and targets appears to make a full analysis of ERCC1 regulation by ubiquitination the subject of future studies, but given the relevance of ERCC1 as an apparent key USP45 target, I would encourage you to nevertheless carefully discuss referee 3's comments and suggestions; especially in light of any new insights the other planned revision experiments should bring.

I would therefore like to invite you to address the referees' comments as discussed, and resubmit a revised study using the link below. Please keep in mind that it is our policy to allow only a single round of major revision, making it important to carefully answer to all points during this round - we shall be happy to grant some additional revision time in case this should be helpful for accomplishing the required experiments during this period. As always, our 'scooping protection' policy means that any competing manuscripts published during the revision period will have no negative impact on our final assessment of your study.

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

Referee 1
Major point:

1. The functional studies of the damage response and repair defects offer some initial insights, but need to be developed further, and could be done so in some relatively straightforward experiments. Does loss of USP45 produce the hallmarks of an NER defect, ICL repair defect or both? Looking at ERCC1 nuclear foci is not likely to answer these questions, since factors undertaking NER do not typically form observable foci during this process, unless irradiation is applied locally in patches through filters. Determining the kinetics of UV- and 6-4 removal is a reasonably simple task, and would report on any overt NER defect.

We have now undertaken the experiment requested and examined the impact of USP45 on the repair of UV-induced cyclobutane pyrimidine dimers (CPD). As shown in a new Fig 8, we observed slower repair of CPDs in the USP45 knockout compared with the wild type U2OS. These data are consistent with USP45 regulating ERCC1-XPF function. We also found, using porous filters, that USP45 is recruited to sites of CPD lesion following UV-C irradiation.

2. Likewise, cells that have lost ERCC1 activity accumulate broken chromosomes after MMC treatment, as shown by Niedernhofer and others. Revisiting Suppl. Fig. 5 could address this point, since previous analysis shows that gH2AX accumulates due to fork collapse in ERCC1-null cells. However, to see the types of defect observed in previous work the analysis would need to be extended to longer times to see if the foci are resolved in USP45 KO/mutant cells, and if any defects in resolution mirror those seen in ERCC1-defective cells.

To address the question, we have compared chromosomal abnormalities in wild type and USP45 knock-out U2OS cells. These data is now presented in a new Figure (SFig 7A &B). They demonstrates that there is a significant increase in chromosomal abnormalities in USP45 knock-out cells compared to wild type cells. In addition, as suggested by the Reviewer, we have performed the kinetic analysis of the γ-H2AX recruitment to the foci lesion for longer periods of times. This revealed that disappearance of γ-H2AX foci during recovery of USP45 KO cells from exposure to ICL-inducing agent is much slower than in wild-type cells (Fig 6D and SFig 14).

3. Also, a dose that does not saturate the method of quantification employed (greater than 10 foci per cell) should be avoided as no dynamics can be inferred in such experiments. Taken together, such assays could provide essential initial information on the nature of DDR defect resulting from lack of ERCC1 control by USP45 (I appreciate that a full DDR-repair characterization is well beyond the scope of the current MS). A potential issue here is that KO or mutation of USP45 reduces ERCC1 levels. However, this is only perhaps two-fold looking at the gels presented, and it is therefore unlikely that ERCC1 concentrations would be limiting in these assays, if sensible doses of damage were selected.

As requested, we have repeated this experiment and studied the number of ERCC1 foci per cell foci over a much longer time period. We have also performed more stringent quantification analysis using ImageJ with plug-in PzFOCI. These experiments revealed that in wild type cells, the number of ERCC1 containing foci were markedly elevated at 24 and 48h post MMC treatment, and declined to near basal levels within 96h (new data see Fig 6C & SFig 13). In contrast, in USP45 knock-out cells, the number of ERCC1 containing foci remained low at all time points analysed (see new data see Fig 6C & SFig 13). Related to the point that the Reviewer makes on whether USP45 might exert its effect by ERCC1 levels, we have undertaken additional studies monitoring ERCC1 levels in wild type and USP45 knock-out cells treated with the protein synthesis inhibitor cycloheximide. This revealed that 8h cycloheximide treatment did not markedly affect ERCC1 stability in either the wild type or USP45 knock-out cells treated in the presence or absence of MMC (new data shown in SFig 8). Moreover, we also performed an additional experiment where we overexpressed ERCC1 in USP45 knock-out U2OS cells and found that this failed to rescue the hypersensitivity of USP45 knock-out cells to MMC or UV-C (new data shown in SFig 8). These results indicate that USP45 is not exerting its effects on DNA damage responses by simply modulating ERCC1 levels. It is likely that the ubiquitylation of ERCC1, which is reversed by USP45, does not simply control ERCC1

protein levels, but controls ERCC1 function in a more direct way. In this light we go on to show that ERCC1 recruitment to sites of DNA damage is blocked in cells lacking USP45. It is of course possible that there are other substrates of USP45 relevant to DNA repair that have as yet not been identified. We have modified the Results and Discussion section to bring out these points.

Technical points:

4. Fig. 2 - for the gel filtration blots we need to see the Mw calibration to give an indication of the mass of the complexes.

The Molecular weight calibration is now included in Figure 2.

5. Fig. 2F, left hand panel, is poor and the change in USP45 behavior not convincing.

In light of the Reviewers comments, we have repeated this experiment and obtained a more convincing immunoblot that demonstrates that in gel filtration analysis of SLX4 wild type cell extracts that USP45 co-elutes with SLX4 in fractions 1-6. However in SLX4 knock-out cells no USP45 was detected in Fractions 1-6 even at long exposures of the immunoblot.

6. Fig. 4 A. What is the lower band in the IB:XPF lanes? It is not seen in any of the other blots. For that matter, which of the two band is XPF in this blot? in the other blots we only see one band but they might have been differently cropped.

We believe that the lower band is a non-specific band that the XPF antibody recognizes in immunoblot analysis of cell extracts, as this band is not seen in ERCC1-XPF immunoprecipitates (see Fig 2B). The differences in the XPF immunoblot analysis observed in the original version of the manuscript were a result of different cropping of the immunoblots. In the revised manuscript all the XPF immunoblots are now cropped in the same manner and show the lower band that is now marked "non-specific" on the immunoblot to avoid confusion.

7. Fig. 4B and C. These blots are not so consistent. The high Mw/Ub-modified ERCC1 runs in a atypical fashion in 4B and the reduction in ERCC1 levels in KO cells in 4C difficult to fully credit without further quantification.

To address the point that the Reviewer makes relating to the impact that USP45 might have on modulating stability ERCC1 levels, we have undertaken additional half-life studies to address this point. We monitored ERCC1 levels in wild type and USP45 knock-out cells treated with the protein synthesis inhibitor cycloheximide. This revealed that 8h cycloheximide treatment did not markedly affect ERCC1 levels in either the wild type or USP45 knock-out cells treated in the presence or absence of MMC (new data shown in SFig 8). The reduction in levels of unmodified 37 kDa ERCC1 in knock-out U2OS cells is likely to be due increased levels of ubiquitylated ERCC1 that migrates at a higher molecular weight. Consistent with this we find that treatment of ubiquitylated Flag-ERCC1 with USP45 results in deubiquitylation of ERCC1 and is accompanied by a commensurate increase in levels of unmodified 37 kDa ERCC1 (see new data in Fig 4C). We have modified the Results and Discussion sections to state explicitly that we do not think that USP45-mediated deubiquitylation has a major impact on ERCC1 stability.

8. Fig. 6. Important to see this data repeated as a time-course. Are the USP45 loss/mutation-associated defects in ERCC1 recruitment to foci due to delayed recruitment or shorter occupancy time at lesions?

As mentioned above, we have now studied ERCC1 foci at longer times and improved quantitation of these data by calculating the total ERCC1 focus number per cell, over a 96 h time course. This revealed that in wild type cells, the number of ERCC1 containing foci were markedly elevated at 24 and 48h post MMC treatment, and declined to near basal levels within 96h (new data see Fig 6C & SFig 13). In contrast, in USP45 knock-out cells, the number of ERCC1 containing foci remained low at all time points analysed (new data see Fig 6C & SFig 13). This data provides firmer evidence

that USP45 regulates the recruitment of ERCC1 to sites of DNA damage

Referee #2

Major Points:
1. The claim that the SLX4-USP45 interaction is dependent on ERCC1 cannot be drawn from figure Fig.2D since no USP45 is co-IPd with SLX4 in ERCC1 proficient cells.

In light of this Reviewers comments, we have repeated this experiment using a higher amount of extract and the new immunoblot in Fig 2D demonstrates more convincingly that USP45 is only co-immunoprecipitated with ERCC1 or SLX4 in wild type MEFs but not in ERCC1 knock-out MEFs.

2. It has been shown that in the absence (or in cases that mutations affect the stability) of one of the complex partners ERCC1 or XPF, the steady-state levels of the other polypeptide of this heteroduplex is also severely reduced (e.g. Niederhofer, 2007, Nature; Ahmad, 2010, PLoS Genet), suggesting that the 2 proteins stabilize each other. However the XPF proteins levels are not significantly (except in Figure 4B where a reduction is visible) reduced upon USP45 KD, authors should comment on this discrepancy with the previous literature.

As outlined in response to reviewer 1 point 3, we feel that the data points towards USP45 acting to regulate the level of ERCC1 ubiquitylation in a manner that is not impacting on ERCC1 stability/half life. Our data suggests in USP45 knock-out cells the levels of ubiquitylated ERCC1 is enhanced which is accompanied by a commensurate decrease in levels of non-ubiquitylated ERCC1 migrating at 37 kDa. If ERCC1 did indeed control XPF stability, the finding that XPF levels are similar in wild type and USP45 knock-out U2OS cells would be consistent with our conclusion that USP45 knock-out is not impacting on total levels of ERCC1 in cells. Our understanding is that it is controversial whether ERCC1 stabilizes XPF. For example the group of George Garinis has reported that siRNA knock-down of ERCC1 did not effect XPF levels (Proc Natl Acad Sci U S A. 2012 109:2995-3000). As shown below we have also found that immunoblot analysis suggests that XPF levels are similar in wild type and ERCC1 knock-out MEFs provided to us by David Melton (University of Edinburgh).
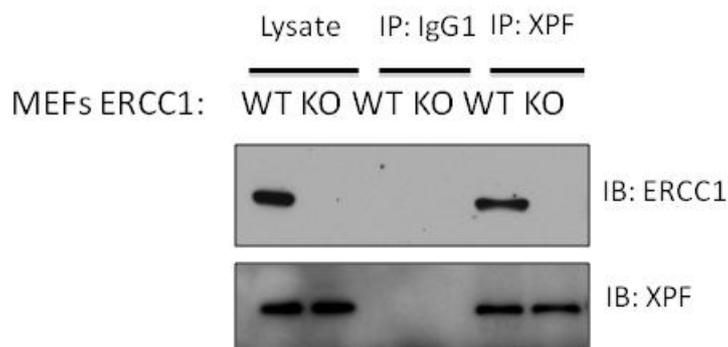
3. Figure 4D: USP45 is indeed able to reduce the slower migrating ERCC1 bands, though no other DUBs were tried, thus this does not show specificity of USP45.

To further demonstrate that the specific interaction of the two proteins is required for USP45 to deubiquitylate ERCC1, we repeated the deubiquitylation assays using a mutant USP45 [Asp25Ala, Glu26Ala] that cannot bind to ERCC1. Importantly, this mutant displayed similar specific activity towards the non-selective ubiquitin-rhodamine substrate as wild type USP45 (new SFig 5A). However, we found that the USP45 [Asp25Ala, Glu26Ala] mutant failed to deubiquitylate ERCC1 in contrast to wild type USP45 (new Fig 4C). Similar results were observed when experiment was repeated with ubiquitylated GFP-ERCC1 (new SFig 5B). We also tested a panel of 5 other DUBs and found that none of these were as effective as USP45 in catalysing the deubiquitylation of GFP-ERCC1 (new SFig 5C).

4. Using pull-down procedure (e.g. TUBEs, His-ubiquitin) it should be shown that the slower

migrating bands are actually ubiquitylated forms of ERCC1 in the experiments in Fig2A-D.

We have attempted this experiment but found that major ERCC1 immuno-reactive species observed in immunoblot analysis of wild type and USP45 knock-out cell extracts were not absorbed by the TUBE-resins, so we are unable to undertake this experiment. Nevertheless, the finding in Fig 4C that deubiquitylation of Flag-ERCC1 with wild type USP45 (but not the USP45 mutant that does not bind ERCC1 or the catalytically inactive USP45) removed the slower migrating bands resulting in a commensurate increase in the level of unmodified ERCC1 species migrating at 37 kDa, does provides further evidence that the slower migrating species of USP45 do indeed represent ubiquitylated forms of the enzyme.

5. The USP45 KO cells were only slightly sensitive to MMC and UV. Since no other information is provided on the phenotype of the KO cells in terms of cell cycle distribution and growth characteristics, sensitivity to genotoxic agents should be treated with care as secondary effects, rather than only compromised DNA repair could contribute to reduced cellular survival. The authors should show more features of the KO cells.

As requested, we have provided some additional information on the phenotype of the cells. Cell cycle analysis indicated that in asynchronous cells populations, knock-out of USP45 had no major effect on the proportion of cells in each phase of the cell cycle (new data in SFig 6B). However, in SFig 6A we show that the USP45 knock-out cells proliferate more slowly that the wild type cells and this reduced growth rate is rescued by overexpression of wild type but not catalytically inactive USP45. It has been argued in the literature that cells with a higher proliferation rate are more sensitive to MMC, and therefore it might be that the moderate sensitivity of USP45 knock-out cells is due to the slow proliferation of these cells. Even though the USP45 cells grow more slowly than normal, the hypersensitivity of these cells to ICLs and UV is unlikely to be a result of general sickness since the cells are not sensitive to a range of other genotoxins, including hydroxyurea. These points are now brought out in the Discussion of the manuscript.

6. The reduced focal accumulation of ERCC1 after DNA damage in USP45 KO appeared clear. The authors conclude form this that loading of ERCC1 to DNA damage is compromised in the absence of functional USP45. However, loading kinetics of ERCC1 to laser-induced DNA damage stripes should be measured.

As requested, we have studied and quantified the total number of ERCC1 foci per cell foci at longer times points post-MMC treatment. This revealed that in wild type cells, the number of ERCC1 containing foci were markedly elevated at 24 and 48h post MMC treatment, and declined to near basal levels within 96h (new data see Fig 6C & SFig 13). In contrast, in USP45 knock-out cells, the number of ERCC1 containing foci remained low at all time points analysed (new data see Fig 6C & SFig 13). In our University and Medical School, we only have a UV-A laser which we can use in combination with psoralen or angelicin, but we do not have a UV-C laser for micro-irradiation. To our knowledge ERCC1 stripe formation requested by the Reviewer has never been reported in the literature. As a result of the Reviewers comments we have attempted this experiment on numerous occasions but have not been able to detect such stripes on overexpressed GFP tagged ERCC1.

7. Surprisingly, the authors claim to observe a focal pattern for XPA and ERCC1 upon UV damage (although the focal pattern of XPA in Sup Figure 6 is not visible from the provided images). While the localization of NER factors, including XPA en ERCC1, upon UV damage has been extensively studied over the last decades, UV induced focal patterns were never observed. Therefore the effect of USP45 on the function of ERCC1 during the UV induced damage response should be performed either by applying local UV damage or FRAP studies on GFP-tagged ERCC1 and the claim of a focal pattern of NER factors should be substantiated and explained.

Zou and colleagues, and others, have reported that XPA can form Foci after UV treatment (Oncogene. 2007 26:757-64 see Figure 5). We have adjusted the brightness settings and included a zoom of the XPA after treatment in the relevant figure (SFig 11) to make XPA foci clearer. We have also now undertaken an additional as requested, and used porous filters to apply local UV-induced DNA damage. This data is shown in a new Fig 8. Our results reveal that USP45 is recruited to sites of localized UV-C-induced damage. We also conclude that USP45 controls repair of CPD damage as after UV-C irradiation as we observed a markedly slower recovery CPD damage in the USP45

knock-out U2OS cells compared with the wild type cells. This new data provides further evidence that that USP45 plays a role in NER pathway (Fig 8).

8. The effect of USP45 happens also in undamaged cells, however is there a difference in DUB activity towards ERCC1 upon damage? This might be expected since USP45 is recruited to the site of damage. Are there differences in ERCC1 half-life between unperturbed and damaged cells?

As requested, we have investigated the impact that USP45 might has on modulating ERCC1 levels/stability. We undertook additional half-life studies to address this point. We monitored ERCC1 levels in wild type and USP45 knock-out U2OS cells treated with and without the protein synthesis inhibitor cycloheximide. This revealed that 8h cycloheximide treatment did not markedly affect ERCC1 stability in either the wild type or USP45 knock-out cells treated in the presence or absence of MMC (new data shown in SFig 8).

9. What is the effect of USP45 on ERCC1, is this only stabilization, or is the de-ubiquitylation by USP45 needed somewhere during the repair reaction? Can overexpression of ERCC1 rescue the phenotype of USP45 KO? Rescue of USP45 KO cells with ERCC1 should be included.

As requested, we have performed additional experiments where we overexpressed ERCC1 in USP45 knock-out U2OS cells and found that this failed to rescue the hypersensitivity of USP45 knock-out cells to MMC or UV-C (new data shown in SFig 9). These results also provide further evidence that USP45 is not exerting its effects on DNA damage responses by simply modulating ERCC1 levels. It is likely that the ubiquitylation of ERCC1, which is reversed by USP45, does not simply control ERCC1 protein levels, but controls ERCC1 function in a more direct way. In this light we go on to show that ERCC1 recruitment to sites of DNA damage is blocked in cells lacking USP45. It is of course possible that there are other substrates of USP45 relevant to DNA repair that have as yet not been identified. We have modified the Results and Discussion section to bring out these points.

10. How is USP45 recruited to the site of damage?

This is an excellent question, but we have unfortunately not been able to address this question within the 3 month time period given to us by the EMBO J Editor to revise our manuscript. This clearly represents a major question for future investigation, and we mention this point in the Discussion.

11. The claim that USP45 accumulation to DNA damage is independent of ERCC1 is not convincingly shown in Supplemental figure 7B, only one cell is shown (with poor image quality). What is frequency of localization to laser stripes, does it occur in all cell, is it cell cycle dependent? Further statics should be provided. This holds also for Figure 8 and supplemental figure 7A.

As requested, we have undertaken further quantification of the data shown in Fig 7B and this is now shown in a New Figure (SFig 15B&C) in which more cells are shown per field. The data shown in old Figure 8 and SF7 (now Fig 9 and SFig 15) are in vivo experiments and all of these were undertaken at least 3 times with similar results obtained. This is now emphasized in the Legends to Fig 9 and SFig 15.

Minor comments:

12. Figure 1, page 5: limited information is provided on the Mass spec data, e.g. how many proteins were identified?; specificity of the identified proteins.

As requested, we have now included in the supplementary section of our paper excel files of the complete set of mass spectrometry data of our experiments undertaken in KBM7 (Excel File 1) and U2OS cells (Excel file 2) for readers to peruse.

13 USP45 is a pleiotropic DUB, as shown in earlier studies and clear from the other identified interactors in this study. Any information is lacking on how the specific interaction with ERCC1 is controlled.

In Figure 3E, we have investigated whether a panel of 7 DUBs that are most closely related to

USP45 that all contain an N-terminal ZNF-UBP domain are able to interact with ERCC1. This revealed that only USP45 is capable of binding to ERCC1. This is explained by the finding that binding to USP45 is mediated by a short motif at the N-terminus of USP45 that not present in any other USP isoform. Consistent with this we demonstrate that mutation of two residues within this motif (Asp25Ala, Glu26Ala) not only prevents USP45 from binding to ERCC1 (Fig 3D) but also prevents USP45 from deubiquitylating ERCC1 (Fig 4C). The USP45 [Asp25Ala, Glu26Ala] mutant displayed normal catalytic activity towards the generic DUB substrate rhodamine-ubiquitin substrate however (SFig 5A). In future work it would be interesting to investigate whether interaction of USP45 with ERCC1 is constitutive or triggered in response to DNA damage

14 The D25A,E26A and D27A mutants all lack interaction, or show reduced interaction with ERCC1, however also the other mutations show a strong reduction in ERCC1 binding, this should be discussed as well in the text.

To address this question, we have performed more quantitative LICOR Odyssey analysis of the binding data, which is shown in a new Figure (SFig 4). This demonstrates that only mutation of Asp25, Glu26 or Asp27 produced a clear-cut disruption of the binding of the N-terminal fragment of USP45 to ERCC1. Loss of binding seen in the Thr37Ala mutation results from a loss of stability of the N-terminal USP45 domain (SFig 4). We also demonstrate that a double USP45 mutant [Asp25Ala, Glu26Ala] is unable to bind ERCC1 (Fig 3D). We also demonstrate that the USP45 [Asp25Ala, Glu26Ala] mutant is unable to deubiquitylate ERCC1 (Fig 4C) despite being catalytically active as judged by its ability to hydrolyse the generic DUB substrate rhodamine-ubiquitin with similar efficiency to wild type USP45 (SFig 5A).

15 The effect of proteasome inhibition on the ERCC1 levels in USP45 KO cells should be shown in Figure 2B and C.

We have now analysed the effects of lactacystin and bortezomib on ERCC1 levels in HEK293 cells, which were the ones used in Fig 2B requested by the Reviewer. In Fig 2C was a yeast two-hybrid experiment and yeast cells are unlikely to respond to lactacystin and bortezomib treatment.

16 Is USP45, next to prosalen or angelicin induced DNA damage, also recruited to UV-C induced DNA damage (NER lesions)?

To obtain further evidence that USP45 is required for NER lesions we have treated cells with UV-C to generate cyclobutane pyrimidine dimers (CPD) lesions. This new data is shown in a new Fig 8. Our results reveal that USP45 is recruited to sites of CPD damage following UV-C irradiation. We also observed a markedly slower recovery of CPD damage in the USP45 knock-out (KO) compared with the wild type U2OS, consistent with a role for USP45 in repairing this type of lesion.

17 Can proteasome inhibition rescue the loss of ERCC1 foci formation upon DNA damage in USP45 KD cells?

As requested, we have performed this additional experiment and the new data is shown in SFig 12. The results show that proteasome inhibition fails to rescue the loss of ERCC1 foci formation upon DNA damage in USP45 knock-out cells. It is likely therefore that the ubiquitylation of ERCC1, which is reversed by USP45, does not simply control ERCC1 protein levels, but controls ERCC1 function in a more direct way. In this light we go on to show that ERCC1 recruitment to sites of DNA damage is blocked in cells lacking USP45. It is of course possible that there are other substrates of USP45 relevant to DNA repair that have as yet not been identified. We have modified the Results and Discussion section to bring out these points.

18 A complete overview of the results of the MS analysis should be represented, including the identified proteins (aspecific interactors) in gel lane from USP45 IP in the USP45 KO cells.

As mentioned above (Reviewer 2, point 12), we have now included in the supplementary section of our paper an excel files of the complete set of mass spectrometry data of our experiments undertaken in KBM7 (Excel File 1) and U2OS cells (Excel file 2).

• What is the phenotype of USP45-/- in combination with ERCC1-/- does this sensitize cells further

than the ERCC1-/- or USP45-/- cells to UV or MMC?

This is a good suggestion, but we have unfortunately not been able to generate and characterize the double knock-out cells within the 3 month time period given to us by the EMBO J Editor to revise our manuscript.

Referee #3

1. Manuscript by Alessi and colleagues describe a previously uncharacterized deubiquitylase USP45 as a critical regulator of DNA repair via association and stabilization of the endonuclease XPF-ERCC1. While the reader will certainly appreciate adding another new DUB to the ever-growing list of ubiquitin regulators of DNA repair, in its current form, it fails to show what is the importance of ubiquitylated XPF-ERCC1. This should be the fundamental question that the authors need
to address both mechanistically and functionally. Before identifying novel regulators of ubiquitylated XPF-ERCC1, I would think it makes more sense to understand the role of ubiquitylated XPF-ERCC1 in DNA repair. For example, what is the role of ubiquitylated XPF-ERCC1? Is this an inducible post-translational modification that is stimulated by DNA damage or recovery? .

This is a good point, but to address it experimentally would require identification of sites of ERCC1 ubiquitylation, analysis of the types of ubiquitin linkage and the molecular basis for the modulation of the many aspects of ERCC1 function by ubiquitylation. Unfortunately it would not be possible to carry out the necessary experiments within the 3 month time period given to us by the EMBO J Editor to revise our manuscript. The aim of our study was to characterize USP45 as it was reported to be over-expressed in a significant number of cancers and there have been no previous papers on this enzyme. Our game plan was to find what USP45 works on and then take it from there. In future work it will of course be fascinating to study how ERCC1 is regulated by ubiquitylation. We hope the finding presented in this manuscript will stimulate this endeavor. We have also mentioned in the Discussion of our paper that it would be important in future work to investigate how ubiquitylation of ERCC1 controls its function.

2. Is it to target XPF-ERCC1 for proteasomal degradation? If so, how does a non-degradable form of XPF-ERCC1 affect DNA repair of UV or crosslink-induced DNA damage? Does this alter cell cycle checkpoint activation (G2/M accumulation or G1 arrest)? However, if ubiquitylated XPF-ERCC1 also possess a non-proteolytic function in protein localization, it would be critical to understand what is the nature of this localization (or lack thereof) that causes DNA repair defect. In summary, simply characterizing a new DUB in DNA repair without sufficient biological context and mechanism temper the reader's enthusiasm for this study.
This is an interesting question, which relates to the above point aimed at understanding more about how ubiquitylation would control the function of ERCC1 and XPF. We were unfortunately unable to address this point within the 3 month time period given to us by the EMBO J Editor to revise our manuscript.

3. Figure 1, what is the role of USP45 in the SLX4 complex? Does it alter SLX4-dependent function in DNA crosslink repair? SLX4 is not required for UV-induced gap repair, but ERCC1-XPF is. Is USP45 functioning via the SLX4 complex (which also contains ERCC1-XPF) or does it also have a SLX4-independent function during UV damage via ERCC1-XPF in NER. How do you reconcile this discrepancy?.

We don't believe there's a discrepancy. The data in Figure 2 argue that USP45 binds to XPF-ERCC1 directly, and the presence of SLX4 in USP45 precipitates requires ERCC1. We previously estimated that around half of the total cellular complement of ERCC1-XPF binds to SLX4, and this pool of XPF-ERCC1 is likely to be involved in ICL repair. The remaining pool of ERCC1-XPF is free of SLX4 (Stoepker et al, Nat Genet. 2011 43:138-41.) and is thought to be the pool involved in NER since SLX4 defective cells are not sensitive to UV. USP45 knockout cells are sensitive to both ICLs and UV, and so USP45 must control an aspect of ERCC1 function that is independent of ERCC1-XPF binding to SLX4.

4. Figure 4, Is degradation of ERCC1 regulated in a DNA damage or cell cycle-dependent manner? This would give more insight into when USP45 may be critical to suppress ERCC1 degradation, otherwise it appears to be performing a housekeeping function in keeping ERCC1 levels elevated.

As requested, we have investigated the impact that USP45 has on modulating ERCC1 levels/stability. We undertook additional half-life studies to address this point. We monitored ERCC1 levels in wild type and USP45 knock-out U2OS cells treated with and without the protein synthesis inhibitor cycloheximide. This revealed that 8h cycloheximide treatment did not markedly affect ERCC1 stability in either the wild type or USP45 knock-out cells treated in the presence or absence of MMC (new data shown in SFig 8). It is likely that the ubiquitylation of ERCC1, which is reversed by USP45, does not simply control ERCC1 protein levels, but controls ERCC1 function in a more direct way. In this light we go on to show that ERCC1 recruitment to sites of DNA damage is blocked in cells lacking USP45. It is of course possible that there are other substrates of USP45 relevant to DNA repair that have as yet not been identified. We have modified the Results and Discussion section to bring out these points.

Figure 6, how does USP45 promote ERCC1 recruitment to sites of DNA damage? Is it through simple stabilization of the protein or is it through modulating the ubiquitylated environment surrounding the DNA lesion site? The authors need to show whether a hyperstable ERCC1 mutant remains permissive for recruitment to sites of DNA damage and repair in the absence of USP45.

Working out how USP45 works to promote ERCC1 recruitment to sites of DNA damage is an important question. Unfortunately we were not able to address this point within the 3 month time period given to us by the EMBO J Editor to revise our manuscript. We have modified the Discussion of our paper to emphasize that this would be an important topic for future research. As mentioned above we have performed additional experiments where we overexpressed ERCC1 in USP45 knock-out U2OS cells and found that this failed to rescue the hypersensitivity of USP45 knock-out cells to MMC or UV-C (new data shown in SFig 8). This also suggests that overexpression of ERCC1 is insufficient to bypass the requirement of USP45 in regulating DNA damage and that the role of USP45 in this process is not to simply regulate *ERCC1* stability and/or levels.

2nd Editorial Decision   27 October 2014

Thank you again for submitting your revised manuscript on USP45-ERCC1 interplay for our consideration. We have now finally heard back from the two referees (1 & 2) who had raised the most constructive criticisms on the original submission. As you will see from their comments copied below, both of them consider the manuscript generally improved, but also retain some significant reservations regarding publication of this work in The EMBO Journal. As discussed before, I would at this stage not insist on further mechanistic insight as requested by referee 2; however, I do feel that it would be essential to address the remaining concerns regarding data quality and experimental support for key conclusions, given that these issues could potentially undermine the significance of the results.

In this light, I would like to return the study to you for an exceptional second round of revision, to give you an opportunity to address the remaining concerns raised by referee 1. When resubmitting a re-revised manuscript, please make sure to include in your cover letter a direct response to the referee comments, as well as a short list of 2-5 one-sentence 'bullet points' (containing brief factual statements that summarize key aspects of the paper), which would form the basis for an online 'synopsis' that would accompany an eventual publication of the study.

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REFEREE REPORTS:

Referee #1:

The authors have addressed a number of concerns previously raised. I am however skeptical about the new data on UV damage repair in Figure 8. Fig. 8B is not of particulary high quality (cDNA loading in 0 lanes very variable for instance) and the kinetics extraordinarily fast, which concerns me. Typical CPD half life in human cells is about 6-12 hours in nearly all studies, not 1 hour. As this is the only real evidence of a DNA repair defect, and a number of the major conclusions in the abstract rely on this data, it is crucial that this part of the work is solid.

Minor point: the minor, XPF cross reacting bands are not marked on Fig. 4A.

Referee #2:

For the resubmission the authors have added several important experiments and have improved a number of data presentations/experiments that overcome several of our main concerns. Together this has significantly approved the quality of the manuscript. However, despite these efforts, still important mechanistically insights concerning how USP45 is recruited to sites of DNA damage and how the De-ubiquitylating activity of USP45 affects the DNA repair capacity and ERCC1 function in particular (not just by stabilization of ERCC1) remain unanswered. The remaining lack of mechanistic insight dampens our enthusiasm about the manuscript and leave it up to editor, whether she/he finds these findings of sufficient novelty/impact to warrant publication in EMBO.

2nd Revision - authors' response   18 November 2014

Referee #1:

The authors have addressed a number of concerns previously raised. I am however skeptical about the new data on UV damage repair in Figure 8. Fig. 8B is not of particularly high quality (cDNA loading in 0 lanes very variable for instance) and the kinetics extraordinarily fast, which concerns me. Typical CPD half life in human cells is about 6-12 hours in nearly all studies, not 1 hour. As this is the only real evidence of a DNA repair defect, and a number of the major conclusions in the abstract rely on this data, it is crucial that this part of the work is solid.

Minor point: the minor, XPF cross reacting bands are not marked on Fig. 4A.

As requested we have repeated the experiments that show the dot blot analysis of CPD removal, and the DNA loading control dot blot. The new data is significantly improved, with all three replicates showing consistent results (Fig 8 B & SFig 15). We agree that the kinetics of repair seems particularly fast in the U2OS cells, and to be certain that the assay we used was a true reflection of CPD removal, we decided to confirm our results by utilising a completely different antibody (TDM2, which has been used by many labs), and a different assay format – ELISA rather than dot blot. This approach yielded similar data which is now shown on a new Fig. 8C. Repair of CPDs in U2OS cells, at least at the UV dose and assay conditions we used, appears to be rapid and is obvious within 3 hours (but not 1 hour). Most importantly however, repair is delayed significantly in cells lacking USP45 (Fig 8B, 8C and SFig 15).

The XPF cross reacting band is marked now in Fig 4A.