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Ubiquitin-like protein UBL5 promotes the functionality of the Fanconi anemia pathway

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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.)

1st Editorial Decision 14 November 2014

Thank you for submitting your manuscript on UBL5 in the Fanconi Anemia pathway to The EMBO Journal. We have now received the reports from four expert referees, which you will find copied below. All four referees find the study interesting and potentially important, and we would therefore be interested in considering a revised version further for publication. As you will see, the reviewers do however raise a number of overlapping, well-taken concerns with the conclusiveness of some of the data, which would have to be satisfactorily addressed before eventual acceptance of the paper. Since these issues are clearly explained in the referee reports, I will not repeat them in detail here but would be happy to discuss specific points further with you if needed.

I would like to remind you 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, please note 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. I look forward to your revision!

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REFEREE REPORTS:

Referee #1:

This is a beautiful paper that describes in detail how UBL5 functions by interacting with and stabilizing FANCI which results in full FA pathway activation. The study is technically superb and the authors are able to make a separation of function mutant to show that UBL5 specifically acts as FANCI interactor. The arrive at the model in which they propose that UBL5 stabilizes FANCI and promotes ubiquitylation of FANCI ultimately allowing for efficient FANCD2 ubiquitylation and FA pathway function.

Couple of minor comments:

1. They might want to consider changing the title to
"Ubiquitin-like protein UBL5 participates in Fanconi anemia pathway of DNA repair."
or
"Ubiquitin-like protein UBL5 promotes Fanconi anemia pathway activation."

2. I find the data in Figure 1D interesting and maybe underexplored. It looks like the interaction with the UB-FANCI is specific for 0 and 2 hours after release from Thymidine, even though Ub FANCI is also seen in the cycling cells and 6 and 12 hours after the release. Is this a reproducible finding? What do the authors think is going on? Thymidine block is of course by itself DNA damaging so this experiment would be better/different if there was a mitotic shake-off. Cells then can be followed through the cell cycle in a truly unperturbed way. I wonder if the results would be different.

3. Figure 2F, it would be nice to have depletion of FANCD2 and FANCI as controls. I would like to know the extent of sensitivity of UBL5 depleted cells.

4. I would mention the author's inability to identify UBL5 localization in foci earlier in the text, before the discussion. I think this is interesting and helps in thinking about the function.

Referee #2:

"Ubiquitin-like protein UBL5 promotes the functionality of the Fanconi anemia pathway" by Oka et al is an interesting paper describing an Ubiquitin-like protein UBL5 as new FANCI interacting partner. They also provide evidence that this UBL5-FANCI interaction is required for the D2-I hetero-dimer to function in the FA-DNA damage response pathway. Given the fact that FANCI and FANCD2 levels are reduced in UBL5 depleted cells via shRNA, UBL5 seems to control the stability of FANCI (also FANCD2) but most of the conclusions drawn from these experiments as presented are very weak, and as such more experiments are required to strengthen their claims.

Comments:
Figure 1
-Since no silver stained gels was shown, I have no idea about the purity of the UBL5-complex isolated.
-no interaction is shown between both endogenous proteins.
- lot of confusion about the two forms of FANCI, sometime the upper band is indicated as nonspecific, sometime not, and no UB-FANCI band at all in some blots(it is all over the entire paper, even the mobility/separation between the two forms are not consistent from one blot to the other)
Figure 2:
-clearly the stability of FANCD2 is affected more(2D)
- Also,why not include some other DNA repair proteins as control?
-MMC sensitivity of UBL5 KD cells is very modest, any comparison with either D2 ot I KD cells in the same experimental set up?
Figure 3:
-FANCD2 blot should be included in 3A, also there is a clear indication that other proteins also affect the FANCI stability. Authors should treat the cells with proteasome inhibitor to rule out other mechanism.

-Given the fact that D64A mutant pull-down lane is under-loaded(3C), authors can't claim that D64A is defective in FANCI binding.
- Why only the ub-FANCD2 is affected (3D) in contrast to the earlier results (2D etc)?
-
Figure 4:
Again EH-AA mutant IP lane is under loaded(2F) making it more difficult to conclude. And there is no UB-FANCI band with this mutant.

Figure 5&6:
Authors extensively used EH-AA FANCI mutant that weakly binds to UBL5 to show the functional significance of FANCI-UBL5 interaction in the FA-DNA damage response pathway. The biggest flaw in this approach is the lack of ubiquitination of this EH-AA mutant , this defective mono-ubiquitination could very well be due to the global structural collapse of the EF-AA FANCI mutant rather than the result of its inability to bind to UBL5. Authors should show convincingly with well-designed experiments using additional mutants that over-all global protein structure is not perturbed. This is very important since non-Ubiquitinated FANCI will fail to function in the FA-DNA damage response pathway irrespective of its ability to bind other proteins such as UBL5 or not.

Referee #3:

In this manuscript Oka et al. aimed to identify new functional roles for the ubiquitin-like modifier UBL5, which has recently been implicated in pre-mRNA splicing. In a mass spectrometry/proteomics approach they discovered and further characterised a specific interaction between UBL5 and FANCI. UBL5 depletion destabilised the cellular pool of FANCI resulting in a defective DNA damage response and hypersensitivity to the ICL-inducing genotoxin MMC. The identification of separation-of-function mutations targeting the binding interface of UBL5/FANCI but not UBL5's function in the spliceosom complex, allowed them to further show that impaired UBL5/FANCI interaction compromises the integrity of the FA repair pathway in particular the formation and activation of the FANCI/FANCD2 complex.

This is a very interesting piece of work that identifies a novel function of UBL5 in protein stability and its role in maintaining the integrity of a major DNA repair pathway. It should be of great interest to a wide audience in the ubiquitin biology field as well as the DNA repair community. However, the authors should address a few points to further validate their findings and to strengthen their claims.

Major points:

1) Fig. 1: Authors need to validate the association between UBL5 and FANCI by showing the presence of an endogenous UBL5/FANCI complex. What is the stoichiometry of the UBL5/FANCI complex? How much of the cellular FANCI pool is bound to UBL5? Expression levels of UBL5 do impact on FANCI stability (as shown in Fig. 2) and hence overexpression of UBL5 might mask small changes in the UBL5/FANCI complex formation and abundance. In fact they cannot rule out that the endogenous UBL5/FANCI complex might be regulated by DNA damage and/or cell cycle.

2) Fig. 2F: siUBL5 knock down clearly sensitizes cells for MMC, but how does it compare to FANCI deficient cells? In case UBL5 mainly impacts on FANCI stability, UBL5 depletion should mimic a FANCI depletion. Hence it would be informative to add a kill curve of siFANCI. Given the variations seen with the two different siUBL5s, a "rescue" experiment is critical. Authors should complement the experiment with sensitivity data from siUBL5-depleted HA-UBL5(siR) cells.

3) Fig. 4: Whereas the evidence for a functional role of UBL5 in FANCI stability is overall sound, I have a few concerns regarding the claimed role of UBL5 in FANCI/FANCD2 complex formation. I am not convinced that the EH-AA mutation exclusively disrupts UBL5 (but not FANCD2 binding). If the EH-AA mutation compromises FANCD2 binding, it will have a major impact on the validity of the conclusion drawn from figure 5-6. These "EH" sites are very close to the FANCI/FANCD2 interface. Can authors exclude that EH-AA mutations do not impact on the overall structural integrity of FANCI? When authors analysed the FANCI truncations/deletions (Fig. 4B-D), have they in parallel to UBL5 binding also assessed FANCD2 binding? To further validate the UBL5 binding site, authors should aim to test the FANCI mutants in an in vitro binding assay (such as

described in Suppl. Fig1C). Furthermore, FANCI (EH-AA) binding to FANCD2 should be tested.

4) Fig. 5: On page 10 authors described the generation of a cell line (which is used in all consecutive experiments described in Fig. 5-6) expressing the mutant FANCI-(EH-AA) version to similar levels as a wild type FANCI expressing cell line. How is the overexpressed mutant FANCI stabilized in this cell line? Is there an increase in UBL5 to compensate for the weaker affinity of UBL5 to FANCI-(EH-AA)? There is a possibility that this cell line might have uncoupled FANCI stability from UBL5 or alternatively, regained binding ability to UBL5. At least to rule out the latter, IPs in Fig. 5D should be tested for the co-precipitation of endogenous UBA5.

5) The authors provide evidence that UBL5 is required for the integrity and functionality of the FA pathway but I am less convinced of a direct involvement of UBL5 in the activation of the pathway. As shown in figure 1, UBL5/FANCI interaction and consequently FANCI stability is not regulated by DNA damage. Hence, UBL5 is unlikely to be part of an activation mechanism. Throughout the manuscript, authors seem not to discriminate between "functional integrity" and "activation" of the FA pathway. The authors should aim to be at least more consistent in their interpretations.

Minor points:

1) p3, line 18: please correct format for the reference "8,9".
2) p5, Fig. S1C: Authors show a direct binding between FANCI and UBL5 in an in vitro assay using recombinant purified proteins. This is an important piece of data and should not be buried in the supplementary data.
3) Figure legend Fig1 E: details of the MMC treatment are not given (concentration, time)
4) Fig. 2E: FANCI laser stripes can hardly be seen (at least in the current manuscript).
5) Fig5. 3D: Why did authors expose cells to MMC for the presented experiment? MMC did apparently not affect UBL5/FANCI interaction. Authors should also show an immunoblot visualizing both FANCI and FANCI-Ub. Reduced levels of FANCI might be due to a shift of FANCI to FANCI-Ub.
6) Fig. 5E and 6A: Please add scale bars.

Referee #4:

UBL5 (Hub1 in yeasts) is an ubiquitin-like protein that is roughly the size of ubiquitin but lacks a C-terminal glycine motif that would normally be expected for attachment to other proteins. Indeed, it is not covalently ligated to target proteins but can nevertheless form tight complexes with specific partners. From a previous SILAC analysis of UBL5 interacting proteins, Oka et al. identified FANCI, a key factor in the Fanconi Anemia (FA) pathway for the repair of interstrand DNA crosslinks in mammalian cells. In this manuscript, the authors verify the interaction by co-IP analysis in tissue culture cells and show that the interaction is not cell cycle regulated, dependent on FANCD2 or changed by exposure to mitomycin C (MMC), an inducer of interstrand crosslinks. Reduction of UBL5 by siRNA, however, causes a strong decrease in FANCI levels and that of its heterodimerization partner, FANCD2. Loss of UBL5 also causes a modest increase in sensitivity of cells to MMC. Importantly, the function of UBL5 in the FA pathway can be at least partially separated from a previously established function of UBL5 in pre-mRNA splicing. An UBL5 point mutant, D64A, that is impaired in FANCI binding also shows a modest defect in FANCD2 mono-ubiquitination and formation of FANCD2 nuclei foci, but the mutant can still rescue the splicing defect of UBL5 knockdown cells. A key determinant of UBL5 binding was mapped to FANCI residues 261-280. Mutating two conserved residues in this element caused FANCI to become metabolically destabilized and impaired FANCI mono-ubiquitination and FANCI-FANCD2 binding. Finally, reduced UBL5-FANCI binding impaired FANCI oligimerization, localization to nuclei foci after MMC treatment, and prevention of chromatid breaks.

This is an interesting set of results, and there is a great deal of interest in the FA pathway of DNA repair. The mechanism of a ubiquitin-like protein regulating cellular processes is also very intriguing, so I think this manuscript would in principle be appropriate for the EMBO Journal. The experimental data are generally strong and well controlled My main concerns, which I do not consider insuperable, are 1) that the effects on MMC sensitivity of loss of UBL5 or UBL5-FANCI interaction are quite modest and 2) control experiments are missing in a few places or there is a lack

of quantification in places where it is essential. These are elaborated below.

Specific comments and questions:

1. Fig. S1C. SUMO2 seems to bind to FANCI too. Was this known? Is it thought to have any biological significance?

2. p. 6. "Concomitant with the reduced levels of FANCI and FANCD2, UBL5 knockdown impaired their MMC-induced monoubiquitylation and recruitment of FANCI to ICLs generated by UVA laser-activated psoralen (Fig. 2D,E)." I would be careful here: I'm not convinced there is a ubiquitination defect or ICL recruitment defect: the ENTIRE effect might be reduced I/D2 levels so that one cannot detect the protein-ubiquitin on Westerns or FANCI at UVA lesions as easily (I still DO see faint FANCI-Ub in MMC in Fig. 2D, lane 4).

3. p. 7. "We found that cells co-depleted of UBL5 and WAPL displayed a marked sensitivity to MMC...." (Fig. 2F,G). I have two issues here. First, "marked sensitivity" is a little too strong; I would say this is at most a 2-5 fold effect at the highest MMC concentration (I don't know how this compares to FANCI depletion, which would be worth showing or at least noting). The values should be stated in the text (rather than saying "marked"). Also, it would be important to see the siUBL5 alone experiment, in case the mild sensitivity to MMC is in fact due to a combination of the two factors (WAPL and UBL5) and not evident with either alone.

4. p. 7. "This suggests that UBL5 provides an additional, spliceosome-unrelated contribution to FANCI stability." At this point there is no evidence that UBL5 is affecting FANCI stability as opposed to translational efficiency since only steady state levels of FANCI are evaluated.

5. p. 8. and Fig. 3C. D64A is a little underexpressed, so I worry that the effect might be overestimated here.

6. p. 8. "Interestingly, although D22 is required for interaction between Hub1 and Snu66 in S. cerevisiae, mutating this residue did not affect the interaction between UBL5 and FANCI in human cells (Fig. S3B)." Does D22A mutation affect UBL5-SART1 interaction?

7. p. 8. "In contrast, induction of UBL5-D64A did not restore FANCI and FANCD2 expression and only weakly stimulated MMC-induced FANCD2 monoubiquitylation (Fig. 3D, lanes 5-8). FANCD2-Ub stimulation looks almost the same as with WT UBL5 addition. I would want this quantified. Also, the siRNA-resistant transgene expression still looks to be siRNA-sensitive (compare lanes 3 to 4, and 7 to 8, "Strep-HA-UBL5"); can the authors comment on this?

8. p. 9. "Inspection of this region showed that it was well conserved among vertebrate FANCI orthologues (Fig. 4E), and that it displayed weak similarity with the Hub1-binding HIND sequence in budding yeast Snu66 (Fig. S4A)." This homology suggests that similar or same region of UBL5 binds to SART1 and FANCI even though D64A seems to separate effects. The authors should comment on this in the text.

9. p. 15. "Despite considerable efforts, we have not observed stable recruitment of UBL5 to chromosomal ICLs, hence the association between UBL5 and the FANCD2-FANCI heterodimer is likely to be of a transient nature or may not occur directly in the context of the damaged DNA." Is there any evidence in this paper for association of UBL5 with the FANCD2-FANCI heterodimer? The data in Fig. 5D could be interpreted this way, but I don't know if it is certain that the FANCI-EH-AA mutant does not have a direct defect in FANCD2 binding? Maybe I just missed the control for this.

1st Revision - authors' response 23 February 2015

(see next page)

Reply to the Reviewers

We would like to thank the referees for their constructive and insightful comments on our manuscript. We were delighted to see that all reviewers found our study interesting and worthy, in principle, of publication in The EMBO Journal. In the revised version of the manuscript, we have included the results of a range of new experiments performed on the basis of the reviewers' helpful suggestions, which we believe address their concerns and further strengthen our original conclusions about an important role of UBL5 in underpinning the functionality of the Fanconi anemia (FA) pathway, through its interaction with and stabilization of the FANCI protein.

Below, we provide a detailed point-by-point response to the issues raised by the reviewers and explain how the new additions and revisions address their concerns.

Point-by-point reply to the Reviewers' comments:

Referee #1:

This is a beautiful paper that describes in detail how UBL5 functions by interacting with and stabilizing FANCI which results in full FA pathway activation. The study is technically superb and the authors are able to make a separation of function mutant to show that UBL5 specifically acts as FANCI interactor. The arrive at the model in which they propose that UBL5 stabilizes FANCI and promotes ubiquitylation of FANCI ultimately allowing for efficient FANCD2 ubiquitylation and FA pathway function.

Couple of minor comments:

1. They might want to consider changing the title to
"Ubiquitin-like protein UBL5 participates in Fanconi anemia pathway of DNA repair."
or
"Ubiquitin-like protein UBL5 promotes Fanconi anemia pathway activation."
We thank the reviewer for this suggestion. Other referees pointed out the importance of discriminating between a role of UBL5 in promoting the functional integrity and activation, respectively, of the FA pathway. While our data clearly suggest that UBL5 is needed for the proper functioning of the FA pathway, we do not have evidence to show that UBL5 directly mediates its activation *per se*, hence we have decided to change the title of the manuscript to: *"Ubiquitin-like protein UBL5 promotes the functional integrity of the Fanconi anemia pathway"*.

2. I find the data in Figure 1D interesting and maybe underexplored. It looks like the interaction with the UB-FANCI is specific for 0 and 2 hours after release from Thymidine, even though Ub FANCI is also seen in the cycling cells and 6 and 12 hours after the release. Is this a reproducible finding? What do the authors think is going on? Thymidine block is of course by itself DNA damaging so this experiment would be better/different if there was a mitotic shake-off. Cells then can be followed through the cell cycle in a truly unperturbed way. I wonder if the results would be different.
We apologize for not labeling this figure properly, which unfortunately led to a misunderstanding of this data: In FANCI blots of whole cell extracts, a band that is insensitive to FANCI siRNA and therefore non-specific (now marked by an asterisk in Fig. 1D and elsewhere in the revised

manuscript to avoid confusion) appears just above the real band corresponding to FANCI-Ub. As can be seen from this blot, the ubiquitylated form of FANCI is mainly observed at 0 and 2 h after release from the thymidine block, and the same pattern is evident for the pool of FANCI co-purifying with UBL5 (the non-specific band is not seen in this blot). We agree with the notion that a thymidine block is genotoxic and so is not an ideal protocol to use when assaying whether the FANCI-UBL5 interaction is regulated by DNA damage. Fortunately, we managed to detect complex formation between the endogenous FANCI and UBL5 proteins (new Fig. 1C, described on p.5) and were thus able to also establish that this interaction is not regulated by exposure of otherwise unsynchronzied cells to Mitomycin C (new Fig. 1C). Together, these data suggest that the ubiquitylation state of FANCI does not affect its ability to interact with UBL5.

3. Figure 2F, it would be nice to have depletion of FANCD2 and FANCI as controls. I would like to know the extent of sensitivity of UBL5 depleted cells.
This is a helpful suggestion, and we have now included data on the extent of MMC hypersensitivity caused by depletion of FANCD2 or FANCI in this figure (Fig. 2F, described on p.7) for comparison. This revealed that cells lacking UBL5 exhibit a milder degree of MMC sensitivity than FANCD2- or FANCI-depleted cells, in line with the finding that knockdown of UBL5 markedly reduces, but does not completely eliminate expression and DNA damage-induced monoubiquitylation of FANCD2 and FANCI.

4. I would mention the author's inability to identify UBL5 localization in foci earlier in the text, before the discussion. I think this is interesting and helps in thinking about the function.
This is a good point, and we now mention these findings already in the Results section. The new text reads as follows (p.9): *"Unlike FANCI, however, we did not observe detectable accumulation of UBL5 at damaged DNA (data not shown), hence UBL5 is likely to mainly facilitate FANCI function prior to its direct engagement at DNA lesions."*

Referee #2:

"Ubiquitin-like protein UBL5 promotes the functionality of the Fanconi anemia pathway" by Oka et al is an interesting paper describing an Ubiquitin-like protein UBL5 as new FANCI interacting partner. They also provide evidence that this UBL5-FANCI interaction is required for the D2-I hetero-dimer to function in the FA-DNA damage response pathway. Given the fact that FANCI and FANCD2 levels are reduced in UBL5 depleted cells via shRNA, UBL5 seems to control the stability of FANCI (also FANCD2) but most of the conclusions drawn from these experiments as presented are very weak, and as such more experiments are required to strengthen their claims.

Comments:
Figure 1
-Since no silver stained gels was shown, I have no idea about the purity of the UBL5-complex isolated.
While it is our experience that pull-downs of Strep-tagged proteins generally enable isolation of complexes that are relatively pure, we use SILAC labeling, a thoroughly established method in which the proteins from individual cell populations are differentially labeled with heavy or light isotopes, as a powerful means to distinguish factors showing specific enrichment in Strep-UBL5 pull-downs from the factors that non-specifically interact with the Strep affinity resin. Because of the sensitivity of current mass spectrometers, we normally use an amount of input cell lysate that is

too low to allow detectable silver staining of the bait protein. Since many of the prominent candidate UBL5 interactors (mainly spliceosomal proteins) identified by this approach had been already validated by our previous work (Oka et al., EMBO Rep. 2014), we are confident about its suitability for faithful detection of *bona fide* UBL5-interacting proteins.

-no interaction is shown between both endogenous proteins.
We fully agree that showing interaction between the endogenous UBL5 and FANCI is important for validating a physiological relevance of this association. We now include new data showing that a complex between endogenous UBL5 and endogenous FANCI can indeed be detected by co-immunoprecipitation analysis (new Fig. 1C, described on p.5), further supporting the validity of our SILAC- and mass spectrometry-based strategy for mapping cellular UBL5-binding proteins described above.

- lot of confusion about the two forms of FANCI, sometime the upper band is indicated as nonspecific, sometime not, and no UB-FANCI band at all in some blots(it is all over the entire paper, even the mobility/separation between the two forms are not consistent from one blot to the other)
We apologize for any confusion arising due to a lack of consistent labeling of the non-specific band migrating just above the bands corresponding to unmodified and monoubiquitylated FANCI in blots of whole cell extracts. We believe this upper band is indeed non-specific, as it is insensitive to FANCI siRNA. In some cases, we cropped away this band, and it is never detected in FANCI blots of pull-downs or immunoprecipitates. Wherever relevant (Fig. 1F; Fig. 2A), we have now indicated this cross-reactive band with an asterisk to avoid confusion. We also replaced previous FANCI blots (particularly in Fig. 3D) with new ones in which FANCI monoubiquitylation can be clearly appreciated.

Figure 2:
-clearly the stability of FANCD2 is affected more(2D)
The data in Fig. 2 (e.g. Fig. 2A and Fig. 2D) collectively indicate that the expression levels of FANCI and FANCD2 are affected to a similar extent following UBL5 knockdown. Because FANCD2 is destabilized by knockdown of FANCI but not *vice versa* (Fig. S5A) and we have no evidence to suggest that UBL5 interacts directly with FANCD2, we believe the reduced levels of FANCD2 expression in UBL5-depleted cells most likely result from the destabilization of FANCI.

- Also,why not include some other DNA repair proteins as control?
As suggested by the referee, we have included immunoblot data showing expression levels of two other components of the FA pathway, FANCC and FANCE (new Fig. S2A, described on p.6). We find that unlike FANCI and FANCD2, the expression levels of these proteins are not affected by UBL5 knockdown, supporting the notion that UBL5 has a direct role in interacting with and stabilizing FANCI (and, as a consequence, FANCD2) that is separable from its impact on global pre-mRNA splicing.

-MMC sensitivity of UBL5 KD cells is very modest, any comparison with either D2 ot I KD cells in the same experimental set up?
This is a very useful suggestion, which was also raised by the other referees. We have now included data showing the effect of depleting FANCI or FANCD2 on cellular sensitivity to MMC in this figure (Fig. 2F, described on p.7) for comparison. This revealed that cells lacking UBL5 exhibit a milder degree of MMC sensitivity than FANCD2- or FANCI-depleted cells, in line with the finding

that knockdown of UBL5 markedly reduces, but does not completely eliminate expression and DNA damage-induced monoubiquitylation of FANCD2 and FANCI.

Figure 3:
-FANCD2 blot should be included in 3A, also there is a clear indication that other proteins also affect the FANCI stability. Authors should treat the cells with proteasome inhibitor to rule out other mechanism.
We have included a FANCD2 blot in Fig. 3A; as can be seen, the expression level of FANCD2 mirrors that of FANCI. The reviewer is correct in pointing out that knockdown of SART1 and EFTUD2 also affect FANCI (and FANCD2) stability (Fig. 3A), although not to the same extent as UBL5 knockdown. As described in the manuscript (p.3 and p.8), this is because depleting any of these factors compromise global pre-mRNA splicing efficiency. Importantly, however, expression of an intron-less (and thus splicing-insensitive) HA-FANCI cDNA is only impaired in cells depleted of UBL5, but not SART1 or EFTUD2 (Fig. 3B), demonstrating that UBL5 has an additional, direct role in stabilizing FANCI that is separable from its involvement in pre-mRNA splicing.

As suggested by the referee, we also performed new experiments in which we analyzed the impact of the proteasome inhibitor MG132 on GFP-FANCI protein levels in cells depleted of UBL5. We found that the reduced expression levels of GFP-FANCI after UBL5 knockdown are substantially restored in the presence of MG132 (new Fig. S3B, described on p.8), suggesting that the decreased level of FANCI in UBL5-depleted cells is largely a consequence of its turnover via the proteasome.

-Given the fact that D64A mutant pull-down lane is under-loaded(3C), authors can't claim that D64A is defective in FANCI binding.
This is a valid point, which was also raised by other referees. We have repeated this experiment a number of times to confirm that the UBL5 D64A mutant is indeed impaired for binding to FANCI. We have now exchanged the previous Fig. 3C with data from a new and representative experiment showing reduced binding of UBL5 D64A to FANCI under conditions where its expression levels are similar to that of UBL5 WT (new Fig. 3C).

- Why only the ub-FANCD2 is affected (3D) in contrast to the earlier results (2D etc)?
We apologize for not including a blot in which FANCI monoubiquitylation could be seen in the original version of this figure (Fig. 3D). To better address this, we performed new experiments in which the effect of manipulating UBL5 status and levels on FANCI monoubiquitylation can be clearly appreciated. We found that ectopically expressed UBL5 WT was more efficient than the D64A mutant in rescuing the MMC-induced FANCD2 and FANCI monoubiquitylation defect arising from depletion of endogenous UBL5 (new Fig. S3D).

-Figure 4:
Again EH-AA mutant IP lane is under loaded(2F) making it more difficult to conclude. And there is no UB-FANCI band with this mutant.
The referee is correct in pointing out that the mutant FANCI EH-AA mutant (which we renamed FANCI ΔUBL5 in the revised manuscript) is monoubiquitylation-deficient. Based on results shown in Fig. 5, we strongly believe this is due to the compromised ability of this mutant to interact with FANCD2 in cells. We reproducibly observe reduced binding of the FANCI ΔUBL5 mutant to UBL5, and we are confident that this is not simply explained by the slight underloading of this mutant relative to FANCI WT that is occasionally observed in these experiments (due to its reduced protein stability (Fig. 5)).

Figure 5&6:
Authors extensively used EH-AA FANCI mutant that weakly binds to UBL5 to show the functional significance of FANCI-UBL5 interaction in the FA-DNA damage response pathway. The biggest flaw in this approach is the lack of ubiquitination of this EH-AA mutant , this defective mono-ubiquitination could very well be due to the global structural collapse of the EF-AA FANCI mutant rather than the result of its inability to bind to UBL5. Authors should show convincingly with well-designed experiments using additional mutants that over-all global protein structure is not perturbed. This is very important since non-Ubiquitinated FANCI will fail to function in the FA-DNA damage response pathway irrespective of its ability to bind other proteins such as UBL5 or not.
We agree that this is a very important point to consider. To address this, we performed *in vitro* binding assays using purified, recombinant FANCD2 and Strep-GFP-FANCI proteins expressed in cells and then purified on Strep-Tactin resin under stringent conditions. We found that under these conditions, the FANCI ΔUBL5 mutant is fully competent to interact with FANCD2 (new Fig. 5G, described on p.12), suggesting that the point mutations introduced to generate this mutant do not simply prevent the interaction with FANCD2 and that the overall structural integrity of FANCI is preserved. We also found that the reduced protein stability of the FANCI ΔUBL5 mutant was restored by forced dimerization (Fig. S6C). This further indicates that the tertiary structure and protein folding of the FANCI ΔUBL5 mutant is intact. Finally, we found that unlike FANCI WT, the FANCI ΔUBL5 mutant is unable to interact with UBL5 *in vitro* (new Fig. 4G, described on p.10). Together, these data show that the ΔUBL5 mutations in FANCI selectively impair binding to UBL5 but not FANCD2.

Referee #3:

In this manuscript Oka et al. aimed to identify new functional roles for the ubiquitin-like modifier UBL5, which has recently been implicated in pre-mRNA splicing. In a mass spectrometry/proteomics approach they discovered and further characterised a specific interaction between UBL5 and FANCI. UBL5 depletion destabilised the cellular pool of FANCI resulting in a defective DNA damage response and hypersensitivity to the ICL-inducing genotoxin MMC. The identification of separation-of-function mutations targeting the binding interface of UBL5/FANCI but not UBL5's function in the spliceosom complex, allowed them to further show that impaired UBL5/FANCI interaction compromises the integrity of the FA repair pathway in particular the formation and activation of the FANCI/FANCD2 complex.

This is a very interesting piece of work that identifies a novel function of UBL5 in protein stability and its role in maintaining the integrity of a major DNA repair pathway. It should be of great interest to a wide audience in the ubiquitin biology field as well as the DNA repair community. However, the authors should address a few points to further validate their findings and to strengthen their claims.

Major points:

1) Fig. 1: Authors need to validate the association between UBL5 and FANCI by showing the presence of an endogenous UBL5/FANCI complex.

We fully agree that showing interaction between the endogenous UBL5 and FANCI is important for validating a physiological relevance of this association. We now include new data showing that a complex between endogenous UBL5 and endogenous FANCI is indeed detectable by co-immunoprecipitation analysis (new Fig. 1C, described on p.5).

What is the stoichiometry of the UBL5/FANCI complex? How much of the cellular FANCI pool is bound to UBL5?
Judging from our co-immunoprecipitation data, only a low proportion of FANCI molecules are bound by UBL5 at any one time. It is possible that once FANCI engages in homodimerization or heterodimer formation with FANCD2 (both of which may be actively facilitated by UBL5 in cells), the association with UBL5 may be dispensable for its stability.

Expression levels of UBL5 do impact on FANCI stability (as shown in Fig. 2) and hence overexpression of UBL5 might mask small changes in the UBL5/FANCI complex formation and abundance. In fact they cannot rule out that the endogenous UBL5/FANCI complex might be regulated by DNA damage and/or cell cycle.
This is a valid point. We addressed this concern by using our set-up to detect complex formation between the endogenous UBL5 and FANCI proteins. As shown in Fig. 1C, we observe no difference in endogenous UBL5-FANCI interaction upon DNA damage resulting from exposure of cells to MMC. This observation corroborates our previous finding that the ubiquitylation state of FANCI does not affect its ability to bind UBL5 (Fig. 1F; Fig. S1C).

2) Fig. 2F: siUBL5 knock down clearly sensitizes cells for MMC, but how does it compare to FANCI deficient cells? In case UBL5 mainly impacts on FANCI stability, UBL5 depletion should mimic a FANCI depletion. Hence it would be informative to add a kill curve of siFANCI.
This is a very useful suggestion, which was also raised by the other referees. We have now included data showing the effect of depleting FANCI or FANCD2 on cellular sensitivity to MMC in this figure (Fig. 2F, described on p.7) for comparison. This revealed that cells lacking UBL5 a milder degree of MMC sensitivity than FANCD2- or FANCI-depleted cells, in line with the finding that knockdown of UBL5 markedly reduces, but does not completely eliminate expression and DNA damage-induced monoubiquitylation of FANCD2 and FANCI.

Given the variations seen with the two different siUBL5s, a "rescue" experiment is critical. Authors should complement the experiment with sensitivity data from siUBL5-depleted HA-UBL5(siR) cells.
We agree with the referee that this is an important control experiment, which we performed using U2OS cells stably expressing an siRNA-resistant form of Strep-HA-UBL5. We found that sensitization of cells to MMC resulting from knockdown of endogenous UBL5 could be fully rescued by expression of ectopic, siRNA-resistant UBL5 WT in these cells (new Fig. 2G, described on p.7). These data strengthen the notion that the sensitivity to MMC resulting from depletion of UBL5 (Fig. 2F) is not due to off-target effects of the siRNAs.

3) Fig. 4: Whereas the evidence for a functional role of UBL5 in FANCI stability is overall sound, I have a few concerns regarding the claimed role of UBL5 in FANCI/FANCD2 complex formation. I am not convinced that the EH-AA mutation exclusively disrupts UBL5 (but not FANCD2 binding). If the EH-AA mutation compromises FANCD2 binding, it will have a major impact on the validity of the conclusion drawn from figure 5-6. These "EH" sites are very close to the FANCI/FANCD2 interface. Can authors exclude that EH-AA mutations do not impact on the overall structural integrity of FANCI?

We fully agree that this is an important point to consider. To address this, we performed in vitro binding assays between purified, recombinant FANCD2 and Strep-GFP-FANCI proteins expressed in cells and then purified on Strep-Tactin resin under stringent conditions. We found that under these conditions, the FANCI ΔUBL5 (EH-AA) mutant is fully competent to interact with FANCD2 (new Fig. 5G, described on p.12), suggesting that the point mutations introduced to generate this mutant do not simply prevent the interaction with FANCD2 and that the structural integrity of FANCI is conserved. We also found that the reduced protein stability of FANCI ΔUBL5 mutant was reversed by forced dimerization (Fig. S6C), further indicating that the tertiary structure and protein folding of the FANCI ΔUBL5 mutant is not perturbed.

When authors analysed the FANCI truncations/deletions (Fig. 4B-D), have they in parallel to UBL5 binding also assessed FANCD2 binding?

Following the reviewer's suggestion, we have performed immunoprecipitation experiments to analyze interactions between GFP-tagged FANCI fragments and endogenous FANCD2 (included as new Fig. S4). We found that fragments F1 (aa 1-400), F3 (aa 601-1000) and F4 (aa 901-1329) interact with FANCD2 even though these interactions are weaker than that observed with full-length GFP-FANCI. We also noticed that these GFP-tagged FANCI fragments mainly interact with the non-ubiquitylated form of FANCD2, indicating that they are likely unable to support DNA damage-dependent monoubiquitylation of FANCD2.

To further validate the UBL5 binding site, authors should aim to test the FANCI mutants in an in vitro binding assay (such as described in Suppl. Fig1C). Furthermore, FANCI (EH-AA) binding to FANCD2 should be tested.

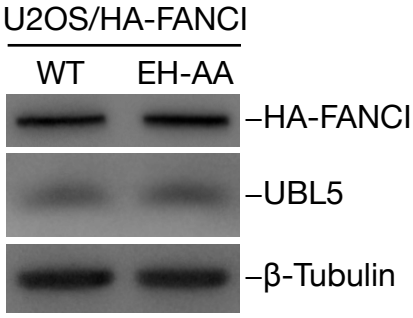
As described above, we tested the binding between FANCD2 and WT and mutant forms of FANCI in an *in vitro* binding assay. We found that the FANCI ΔUBL5 mutant is fully competent to interact with FANCD2 under these conditions (new Fig. 5G, described on p.12). We also found that unlike FANCI WT, the FANCI ΔUBL5 mutant is unable to interact with UBL5 *in vitro* (new Fig. 4G, described on p.10). Together, these data show that the ΔUBL5 mutations in FANCI selectively impair binding to UBL5 but not FANCD2.

4) Fig. 5: On page 10 authors described the generation of a cell line (which is used in all consecutive experiments described in Fig. 5-6) expressing the mutant FANCI-(EH-AA) version to similar levels as a wild type FANCI expressing cell line. How is the overexpressed mutant FANCI stabilized in this cell line? Is there an increase in UBL5 to compensate for the weaker affinity of UBL5 to FANCI-(EH-AA)? There is a possibility that this cell line might have uncoupled FANCI stability from UBL5 or alternatively, regained binding ability to UBL5. At least to rule out the latter, IPs in Fig. 5D should be tested for the co-precipitation of endogenous UBA5.

While it is formally possible that there may be compensatory effects acting to stabilize the FANCI ΔUBL5 mutant in the stable cell line, we have no evidence for this to be the case. Firstly, the levels of endogenous UBL5 are similar in the stable cell lines expressing FANCI WT or ΔUBL5 (please see Fig. R1 below). Secondly, we do not think that the FANCI mutant is stabilized in the U2OS/Strep-HA-FANCI ΔUBL5 cell line. On the contrary, cycloheximide (CHX) chase experiments show that the FANCI ΔUBL5 mutant is less stable than FANCI WT (please see Fig. R2 below). Moreover, we have performed immunoblot analyses to determine the extent to which UBL5 regulates the stability of HA-FANCI WT and ΔUBL5 in the stable cell lines. We find that while the abundance of HA-FANCI ΔUBL5 is not strongly affected by knockdown of UBL5, levels of the HA-FANCI WT protein drop precipitously (please see Fig. R3 below). Together, these

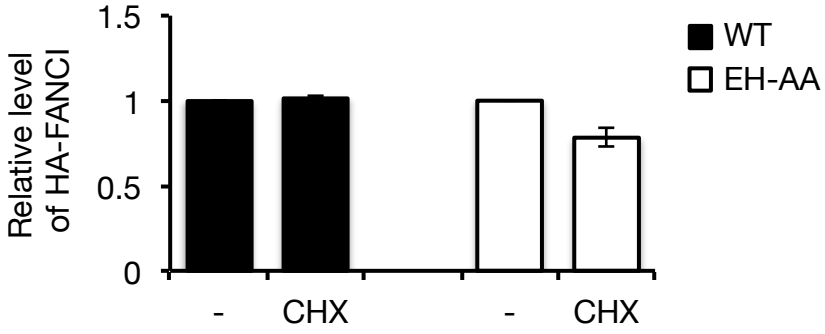
results suggest that the HA-FANCI ΔUBL5 mutant has reduced stability at the protein level due to its impaired interaction with UBL5. We consider it likely that a higher gene expression level of the FANCI ΔUBL5 mutant may account for the similar abundance of the WT and mutant FANCI proteins in these cell lines.

Fig. R1



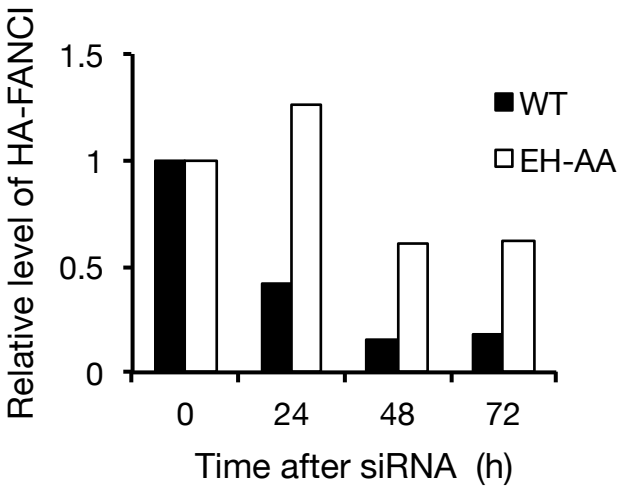
Extracts of U2OS cells stably expressing HA-tagged FANCI WT or EH-AA were analyzed by immunoblotting with indicated antibodies.

Fig. R2



U2OS cells stably expressing HA-FANCI WT or EH-AA were harvested at 2 h after addition of cycloheximide (CHX). Cell lysates were immunoblotted with HA and β-Tubulin antibodies. Quantification of HA-FANCI levels by image analysis, normalized to β-Tubulin levels. Results (mean ± S.D) of three independent experiments are shown.

Fig. R3



U2OS cells stably expressing HA-FANCI WT or EH-AA were harvested at indicated times following transfection of siRNA against UBL5. Cell extracts were analyzed by immunoblot with HA and β-Actin antibodies. HA-FANCI levels were quantified by image analysis, normalized by β-Actin levels.

5) The authors provide evidence that UBL5 is required for the integrity and functionality of the FA pathway but I am less convinced of a direct involvement of UBL5 in the activation of the pathway. As shown in figure 1, UBL5/FANCI interaction and consequently FANCI stability is not regulated by DNA damage. Hence, UBL5 is unlikely to be part of an activation mechanism. Throughout the manuscript, authors seem not to discriminate between "functional integrity" and "activation" of the FA pathway. The authors should aim to be at least more consistent in their interpretations.

We concur with this criticism, as we indeed do not have evidence that UBL5 is directly involved in activation of the FA pathway but clearly supports its ability to undergo activation (i.e. its functional integrity). To avoid unintentional overstatements, we have therefore carefully formatted the revised manuscript text so that we consistently only refer to UBL5 as being important for the functional integrity of the FA pathway and not its activation *per se*.

Minor points:

1) p3, line 18: please correct format for the reference "8,9".

This has been corrected accordingly.

2) p5, Fig. S1C: Authors show a direct binding between FANCI and UBL5 in an in vitro assay using recombinant purified proteins. This is an important piece of data and should not be buried in the supplementary data.

As suggested by the Reviewer, we have moved this data to Fig. 1 (Fig. 1D in the revised paper).

3) Figure legend Fig1 E: details of the MMC treatment are not given (concentration, time)

These details have now been included in the legend for Fig. 1E (Fig. S1C in the revised paper).

4) Fig. 2E: FANCI laser stripes can hardly be seen (at least in the current manuscript).
We agree that the intensity of the signal corresponding to endogenous FANCI at laser-induced DNA damage shown in this figure is relatively weak. We tested a range of FANCI antibodies in this assay, but unfortunately we were not able to obtain a stronger signal than that shown in the figure.

To further address this issue, we analyzed recruitment of overexpressed HA-FANCI (which is easier to detect by immunofluorescence than endogenous FANCI) to MMC-induced foci. As shown in Fig. S2C, overexpressed HA-FANCI showed strongly impaired accumulation at sites of DNA damage after UBL5 knockdown, similar to endogenous FANCI (Fig. 2E).

5) Fig5. 3D: Why did authors expose cells to MMC for the presented experiment? MMC did apparently not affect UBL5/FANCI interaction. Authors should also show an immunoblot visualizing both FANCI and FANCI-Ub. Reduced levels of FANCI might be due to a shift of FANCI to FANCI-Ub.
The purpose of this experiment was to monitor the extent to which the UBL5 WT and D64A proteins restore monoubiquitylation of FANCI and FANCD2 in cells depleted of endogenous UBL5. To better address this point, we performed new experiments in which we compared the effect of MMC treatment to a mock control. We also included blots in which both the unmodified and monoubiquitylated forms of FANCI can be clearly appreciated. As can be seen from these data (new Fig. S3D), expression of Strep-HA-UBL5 WT in UBL5-depleted cells more efficiently rescued MMC-induced monoubiquitylation of FANCI and FANCD2 than did Strep-HA-UBL5 D64A.

6) Fig. 5E and 6A: Please add scale bars.
Scale bars have now been added to these figures.

Referee #4:

UBL5 (Hub1 in yeasts) is an ubiquitin-like protein that is roughly the size of ubiquitin but lacks a C-terminal glycine motif that would normally be expected for attachment to other proteins. Indeed, it is not covalently ligated to target proteins but can nevertheless form tight complexes with specific partners. From a previous SILAC analysis of UBL5 interacting proteins, Oka et al. identified FANCI, a key factor in the Fanconi Anemia (FA) pathway for the repair of interstrand DNA crosslinks in mammalian cells. In this manuscript, the authors verify the interaction by co-IP analysis in tissue culture cells and show that the interaction is not cell cycle regulated, dependent on FANCD2 or changed by exposure to mitomycin C (MMC), an inducer of interstrand crosslinks. Reduction of UBL5 by siRNA, however, causes a strong decrease in FANCI levels and that of its heterodimerization partner, FANCD2. Loss of UBL5 also causes a modest increase in sensitivity of cells to MMC. Importantly, the function of UBL5 in the FA pathway can be at least partially separated from a previously established function of UBL5 in pre-mRNA splicing. An UBL5 point mutant, D64A, that is impaired in FANCI binding also shows a modest defect in FANCD2 mono-ubiquitination and formation of FANCD2 nuclei foci, but the mutant can still rescue the splicing defect of UBL5 knockdown cells. A key determinant of UBL5 binding was mapped to FANCI residues 261-280. Mutating two conserved residues in this element caused FANCI to become metabolically destabilized and impaired FANCI mono-ubiquitination and FANCI-FANCD2 binding. Finally, reduced UBL5-FANCI binding impaired

FANCI oligimerization, localization to nuclei foci after MMC treatment, and prevention of chromatid breaks.

This is an interesting set of results, and there is a great deal of interest in the FA pathway of DNA repair. The mechanism of a ubiquitin-like protein regulating cellular processes is also very intriguing, so I think this manuscript would in principle be appropriate for the EMBO Journal. The experimental data are generally strong and well controlled My main concerns, which I do not consider insuperable, are 1) that the effects on MMC sensitivity of loss of UBL5 or UBL5-FANCI interaction are quite modest and 2) control experiments are missing in a few places or there is a lack of quantification in places where it is essential. These are elaborated below.

Specific comments and questions:

1. Fig. S1C. SUMO2 seems to bind to FANCI too. Was this known? Is it thought to have any biological significance?
To the best of our knowledge, there is currently no evidence that FANCI interacts non-covalently with SUMO in cells. A previous study showed that human FANCI has a SUMO-interacting motif (SIM), which interacts with SUMO-like domains (SLDs) but does not bind to SUMO2 (Yang et al., Genes Dev 2011). Chicken FANCI (which was the source of purified, recombinant FANCI used in our *in vitro* binding experiments) contains a potential SIM (residues 684-687) that is equivalent to this SLD-binding motif, and also harbors an additional, potential SIM (residues 520-523), which is not conserved in human FANCI. It is possible that this latter SIM in chicken FANCI might be mediating the observed association with recombinant SUMO2 *in vitro*. Whether this has any biological significance remains to be established.

In the revised manuscript, we extended the UBL5-FANCI *in vitro* binding studies by showing that unlike FANCI WT, the FANCI ΔUBL5 mutant is unable to interact with UBL5 (new Fig. 4G, described on p.10).

2. p. 6. "Concomitant with the reduced levels of FANCI and FANCD2, UBL5 knockdown impaired their MMC-induced monoubiquitylation and recruitment of FANCI to ICLs generated by UVA laser-activated psoralen (Fig. 2D,E)." I would be careful here: I'm not convinced there is a ubiquitination defect or ICL recruitment defect: the ENTIRE effect might be reduced I/D2 levels so that one cannot detect the protein-ubiquitin on Westerns or FANCI at UVA lesions as easily (I still DO see faint FANCI-Ub in MMC in Fig. 2D, lane 4).
This is a valid point. We repeated the experiment shown in Fig. 2D several times to carefully assess whether FANCI and FANCD2 monoubiquitylation is indeed impaired or whether the effect might be explained simply by the reduced levels of these proteins in UBL5-depleted cells, as suggested by the referee. From the fully representative new data now shown in Fig. 2D, we believe that at least for FANCD2 it can be clearly seen that its monoubiquitylation is impaired in UBL5 siRNA-treated cells (compare the ratio between unmodified and monoubiquitylated FANCD2 in lanes 2 and 4). We also analyzed recruitment of overexpressed HA-FANCI to MMC-induced foci. As shown in Fig. S2C, also overexpressed HA-FANCI showed strongly impaired accumulation at sites of DNA damage after UBL5 knockdown, ruling out that this is simply a result of reduced FANCI expression in UBL5-depleted cells. In light of the referees' concern and these data, we have rephrased the sentence highlighted by him/her, so that it now reads as follows (p.7): *"Consistent with the reduced abundance of FANCI and FANCD2, no detectable accumulation of FANCI at ICLs induced by UVA laser-activated psoralen or MMC was evident in UBL5-depleted cells, and FANCD2 monoubiquitylation in response to MMC treatment was diminished (Fig. 2D,E; Fig. S2C)."*

3. p. 7. "We found that cells co-depleted of UBL5 and WAPL displayed a marked sensitivity to MMC...." (Fig. 2F,G). I have two issues here. First, "marked sensitivity" is a little too strong; I would say this is at most a 2-5 fold effect at the highest MMC concentration (I don't know how this compares to FANCI depletion, which would be worth showing or at least noting). The values should be stated in the text (rather than saying "marked").

This is a valid point that echoes criticism from referees #1, #2 and #3. We have now included data on the extent of MMC hypersensitivity caused by depletion of FANCI or FANCD2 in this figure (Fig. 2F) for comparison. This revealed that cells lacking UBL5 exhibit a milder degree of MMC sensitivity than FANCD2- or FANCI-depleted cells, in line with the finding that knockdown of UBL5 markedly reduces, but does not completely eliminate expression and DNA damage-induced monoubiquitylation of FANCD2 and FANCI. These observations are now described as follows (p.7): *"We found that co-depletion of UBL5 and WAPL significantly sensitized cells to MMC, although not to the same extent as knockdown of FANCI or FANCD2 (Fig. 2F; Fig. S2D), consistent with the substantial reduction, but not complete loss, of FANCI and FANCD2 expression in cells lacking UBL5."*

Also, it would be important to see the siUBL5 alone experiment, in case the mild sensitivity to MMC is in fact due to a combination of the two factors (WAPL and UBL5) and not evident with either alone.

We fully agree that this would, in principle, be an important control experiment. However, we are consistently unable to obtain colonies after knockdown of UBL5 alone. As we have shown recently, this is due to the strong sister chromatid cohesion defect arising from UBL5 knockdown, and which can be fully relieved by co-depletion of the cohesion dissociation factor WAPL (Oka et al., EMBO Reports 2014). This unfortunately precludes us from analyzing the effect of knocking down UBL5 alone on MMC sensitivity.

4. p. 7. "This suggests that UBL5 provides an additional, spliceosome-unrelated contribution to FANCI stability." At this point there is no evidence that UBL5 is affecting FANCI stability as opposed to translational efficiency since only steady state levels of FANCI are evaluated.

We concur with this notion, and we have therefore changed the wording of this sentence to: *"This suggests that UBL5 has an additional, spliceosome-unrelated role in maintaining FANCI expression"* (p.8).

5. p. 8. and Fig. 3C. D64A is a little underexpressed, so I worry that the effect might be overestimated here.

This is a valid point that was also raised by Referee #2. We have repeated this experiment a number of times to confirm that the UBL5 D64A mutant is indeed impaired for binding to FANCI. We have now exchanged the previous Fig. 3C with data from a new and representative experiment showing reduced binding of UBL5 D64A to FANCI under conditions where its expression levels are similar to that of UBL5 WT.

6. p. 8. "Interestingly, although D22 is required for interaction between Hub1 and Snu66 in S. cerevisiae, mutating this residue did not affect the interaction between UBL5 and FANCI in human cells (Fig. S3B)." Does D22A mutation affect UBL5-SART1 interaction?

No; we have previously shown that a D22A mutation in UBL5 does not detectably affect its interaction with SART1 (Oka et al., EMBO Rep 2014). We have removed the sentence highlighted by the referee from the revised manuscript, as we felt it detracted somewhat from the main focus on the D64A mutation in this section.

7. p. 8. "In contrast, induction of UBL5-D64A did not restore FANCI and FANCD2 expression and only weakly stimulated MMC-induced FANCD2 monoubiquitylation (Fig. 3D, lanes 5-8). FANCD2-Ub stimulation looks almost the same as with WT UBL5 addition. I would want this quantified.

As requested by the reviewer, we have quantified the relative level of FANCD2 monoubiquitylation in this blot (Fig. S3D). This showed a pronounced difference in the ability of the UBL5 WT and D64A alleles to restore FANCD2 monoubiquitylation in cells depleted of endogenous UBL5.

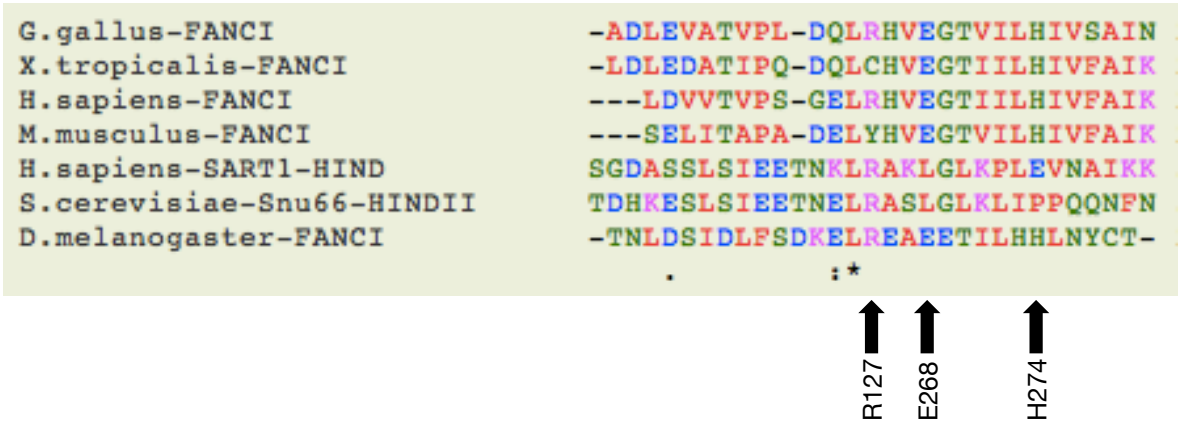
Also, the siRNA-resistant transgene expression still looks to be siRNA-sensitive (compare lanes 3 to 4, and 7 to 8, "Strep-HA-UBL5"); can the authors comment on this?

We agree with the Reviewer that the siRNA-resistant UBL5 cDNAs are still partially sensitive to UBL5 siRNA, despite several silent mutations were introduced in order to render the constructs siRNA-insensitive. Importantly, while we do not know the exact reason for this, we would like to point out that the expression levels of both ectopic UBL5 WT and D64A in Doxycycline-treated cells transfected with UBL5 siRNA are fully sufficient to rescue the strong cohesion defect caused by UBL5 knockdown (Fig. 3F), therefore the functionality of UBL5 in terms of its role in pre-mRNA splicing appears to be restored in both cases. Likewise, we find that the sensitivity to MMC caused by siRNA-mediated depletion of UBL5 is restored by stable expression of this (partially) siRNA-resistant Strep-HA-UBL5 WT allele (new Fig. 2G), suggesting that it is fully functional in the response to DNA interstrand crosslinks.

8. p. 9. "Inspection of this region showed that it was well conserved among vertebrate FANCI orthologues (Fig. 4E), and that it displayed weak similarity with the Hub1-binding HIND sequence in budding yeast Snu66 (Fig. S4A)." This homology suggests that similar or same region of UBL5 binds to SART1 and FANCI even though D64A seems to separate effects. The authors should comment on this in the text.

This is a valid point. Although we did indeed mention in the original manuscript that the region of FANCI interacting with UBL5 shares weak similarity with the Hub1-binding HIND domain, it is also clear that both the E268 and H274 residues in FANCI that are important for interaction with UBL5 are not conserved in the HIND (please see alignment in Fig. R4 below). Moreover, R127, a residue within the HIND domain that is critically required for interaction with Snu66 (Mishra et al., Nature 2011) is not conserved in the mouse and frog FANCI homologues. This may explain why a D64A mutation in UBL5 selectively impairs binding to FANCI but not SART1. Accordingly, despite the weak similarity that the HIND and the UBL5-binding region in FANCI do share, it is not clear that these motifs would be functionally equivalent, and we have therefore removed the statement about their similarity from the revised manuscript.

Fig. R4



Sequence alignment of amino acids in the UBL5-interacting region of FANCI among various species and hub1-binding HIND sequence from human SART1 and *S. cerevisiae* Snu66. Glu268 and His274 residues of human FANCI and Arg127 of human SART1 are indicated by arrows.

9. p. 15. "Despite considerable efforts, we have not observed stable recruitment of UBL5 to chromosomal ICLs, hence the association between UBL5 and the FANCD2-FANCI heterodimer is likely to be of a transient nature or may not occur directly in the context of the damaged DNA." Is there any evidence in this paper for association of UBL5 with the FANCD2-FANCI heterodimer? The data in Fig. 5D could be interpreted this way, but I don't know if it is certain that the FANCI-EH-AA mutant does not have a direct defect in FANCD2 binding? Maybe I just missed the control for this.

We thank the reviewer for bringing this to our attention. The referee is right in pointing out that we have no clear evidence for an interaction between UBL5 and the FANCD2-FANCI heterodimer. We therefore removed the highlighted statement from the manuscript. As suggested by Referee #1, we now mention our inability to observe UBL5 accumulation at DNA damage sites already in the results section. The text reads as follows (p.9): *"Unlike FANCI, however, we did not observe detectable accumulation of UBL5 at damaged DNA, hence UBL5 is likely to mainly facilitate FANCI function prior to its direct engagement at DNA lesions."*

We also include new data showing that while the FANCI EH-AA mutant (now called FANCI ΔUBL5 in the revised manuscript) shows impaired binding to FANCD2 in cells, it remains fully competent to interact with FANCD2 *in vitro* (new Fig. 5G, described on p.12).

Pre-acceptance letter 16 March 2015

Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by three of the original referees (see comments below), and I am happy to inform you that they are satisfied with the revisions and thus supportive of publication in The EMBO Journal.

Referees 3 and 4 still mention a few minor points regarding more cautios interpretations, which I would like to ask you to incorporate into the manuscript text (and possibly into the final model figure).

After these final modifications, we should then be able to swiftly proceed with formal acceptance and production of the manuscript!

Referee #1

The authors did a terrific job addressing the points raised by the referees

Referee #3

The authors have made the relevant changes to the manuscript in order to address all the critical issues raised in the first round of review. However, I am still puzzled how a small molecule such as UBL5 can directly stabilize the cellular pool of FANCI (as well as FANCI/FANCI homo-dimer and FANCI/FANCD2 hetero-dimer) despite UBL5's sub-stoichiometric abundance, and that only a small fraction of FANCI is actually bound by UBL5 at any one time. As suggested by the authors in response to point 1), UBL5 might stabilize FANCI by promoting FANCI's assembly into stable FANCI/FANCI and FANCI/FANCD2 complexes. Once formed, these complex stabilities are independent of UBL5 association. I suggest to consider amending their model in figure 7E (or add an additional model) to implement this alternative mechanism. Overall, I support publication in EMBO J.

Referee #4

Most of my original criticisms were relatively easy to address, and I think the authors have done an adequate job of doing so in the revised manuscript. The effect of reduced UBL5 on ICL repair is still substantially below what is seen with reduced FANCI or FANCD2, so I would at least ask the authors to replace "underpin" in the abstract with "promotes" or "enhances."

