nature portfolio

Peer Review File

The Fanconi anemia core complex promotes CtIP-dependent end resection to drive homologous recombination at DNA double-strand breaks

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REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

This manuscript reports the results of a CRISPR screen using a DSB repair assay that identifies genes that both promote and inhibit homologous recombination. From this screen, the authors focus on the Fanconi anemia core complex, including Ube2T and FANCL. Using rigorous and well-controlled experiments with KO and complemented cell lines, the authors demonstrate that these factors promote homologous recombination by several measurements including gene targeting and PARPi sensitivity. Mechanistically, a defect in CtIP recruitment to DSBs is observed in cells deficient for the FA core complex. Importantly, overexpression of CtIP can suppress the HR defect that is observed in Ube2t and FANCL KO cells. Overall, this work helps resolve an outstanding question in the field for the role of the FA pathway in HR. The experiments are well-controlled and rigorous, with data being of high quality. There are only a few issues that should be addressed prior to publication. Perhaps most importantly, the authors completed a very interesting screen for HR factors but only focus on the FA pathway. Providing more details on the screen and the hits identified would increase the impact of the work. While there is no doubt that the screen has been validated by the development of the FA pathway investigation, it is disappointing as a reader to not see what else the screen identified. Overall, this work will be of wide interest to the field, as well as a valuable resource for factors involved in HR repair.

Main issues

1. This screen will be of great value to the field as it provides another set of HR promoting and suppressive factors. However, it would be nice to see an analysis of the positive hits. While the authors focus on the FA pathway, it would be valuable and more impactful if they would also analyze the other hits and provide these in either main or supplemental figures.
2. FANCD2 appears to be less sensitive to PARPi than Ube2T or FANCL. Does this suggest another target in HR for these proteins? Also, the significance of the PARPi sensitivity would be more obvious if a positive control, like BRCA1 or BRCA2, was included for comparison. This would help support the role of these new factors in HR as core factors or accessory factors in HR repair.
3. The identification of CtIP and DNA end resection as pathways requiring Ube2T and FANCL is very interesting. Although the authors provide references that MRN is not regulating CtIP, there is other evidence that LEDGF promotes CtIP recruitment to DSBs. The model in Figure 6 G suggests this complex promotes DNA/Chromatin binding of CtIP but some more mechanistic details of this step would help to solidify this model. It would be interesting to put Ube2T and FANCL into this reported pathway. For example, do these proteins promote LEDGF recruitment to DSBs? In addition, what is upstream of Ube2T and FANCL? To provide some additional function data for these observation, it would be nice to see if overexpression of CtIP also suppressed IR/PARPi sensitivity of Ube2T and FANCL KO cells.

Minor issues

1. In sup fig 2, it is stated that some experiments were from a single experiment while the N given is greater than 1. This is confusing and the authors should edit. Clearly experiments done only once would need to be repeated prior to publication. This seems like a misunderstanding, which needs clarifying.
2. Is there an explanation for why Ube2T exhibits two bands by western blotting? There is a bit of uncertainty between the two KO clones for Ube2T. While one exhibits defects in SSA, the other doesn't. It would be better if another KO clone was used that doesn't still express Ube2T by western blot. As is, these results are difficult to interpret.
3. It is nice to see complementation for FANCL KO cells but it would similarly be appropriate to perform the same complementation with the Ube2T KO to rule out off target effects for data in Fig 2.
4. For the sequencing data for FANCL KO lines, only one allele is shown for its mutational analysis. Shouldn't multiple alleles be shown to describe the KO of all alleles? To be certain, one could also check by RT-qPCR to ensure that the mRNA is also degraded once mutated by the nonsense mediated decay pathway. While this doesn't always occur, it could be another way to ensure the KO of FANCL in these cell lines. The authors should give more information on the characterization of the KO status of these cell lines.
5. Ube2T localization to FokI DSBs could be placed in the main figure?

6. Figure 4H is confusing. Do the authors mean 2nd Ab with no FANCD2 primary (but primary and 2nd Ab for MDC1). A better explanation for the experiment should be provided for clarity.

Reviewer #2 (Remarks to the Author):

In this manuscript, van de Kooij et al. demonstrate a role for the Fanconi Anemia (FA) pathway in promoting homologous recombination (HR) through facilitating CtIP-dependent DNA end resection upon DNA double strand breaks (DSB) formation. They notably show that several FA proteins (UBE2T, FANCL, FANCD2) are recruited to DSBs where they facilitate in turn CtIP recruitment to promote DNA end resection.

Importantly, these findings arise from an unbiased screen using the DSB-Spectrum reporter the authors previously published in Nature Communications. Even if a role of the Fanconi Anemia pathway in promoting HR at DSB was already shown/suggested by previous works, as the authors mention, this remained quite controversial with different articles showing contradictory results. I think this work clarifies the question and nicely shows that the FA acts at the level of end resection to promote HR-dependent DSB repair.

Overall, the evidence presented here are strong and convincing. The use of multiple cell lines and targeted genes in the FA pathway as well as various read-outs reinforces the message.
However, I have a few major comments as well as suggestions for the authors (see below).
Moreover, although I think it is a very nice study, I should mention that the interaction between FANCD2 and CtIP was already known and that FANCD2 was already shown to promote CtIP-dependent DNA end resection in the context of interstrand crosslink (ICL) repair. Moreover, a recent study (cited by the authors) also showed that the FA pathway promotes CtIP-dependent resection at DSBs (Cai et al. Cell Reports 2020). My main concern about this work would thus be the novelty of the findings but this study still provides a more detailed characterization of the regulation of CtIP by the FA pathway in response to DSBs.

Major comments:

_ The work from the Sobeck lab should also be cited and discussed:
Yeo et al. HMG 2014. CtIP mediates replication fork recovery in a FANCD2-regulated manner
Raghunandan et al. HMG 2020. Functional crosstalk between the Fanconi anemia and ATRX/DAXX histone chaperone pathways promotes replication fork recovery
(it is also shown in the latter that FANCD2 promotes HR-dependent DSB repair)

_ Fig 5C and more dramatically S3E: why the FANCL Ligase-dead (LD) mutant partially (or largely) complements pRPA foci ? This is very surprising given all the other results presented in this study (for instance, FANCL LD does not rescue at all Olaparib hypersensitivity of FANCL KO cells).
This is problematic too me as no structural, non catalytic, role has been proposed for FANCL so far to the best of my knowledge.
Does FANCL LD also restore the end resection defect (directly measured by qPCR) in FANCL KO cells ?

_ Fig 3 C vs D vs I : it seems that FANCD2 KO cells are less sensitive to Olaparib compared to FANCL and UBE2T KO (at 10-1 for instance).
Is is statistically significant ?
Could this suggest that a potential other substrate ubiquitinated by the FA core complex may be required for Olaparib resistance ?
Or that the total lack of FANCD2 has less detrimental consequences in this context compared to the sole loss of its ubiquitination ?
In line with this, I would suggest to complement FANCD2 KO cells with FANCD2 WT versus K561R mutant and check Olaparib resistance as well as DNA end resection.

_ Fig 5H: the difference in total Rad51 foci intensity between FANCL KO+EV versus FANCL KO+WT is not large, is it statistically significant ? (but the representative biological repeat in S3G is convincing)

_ Fig 6F: the fact that CtIP overexpression partially restores HR in UBE2T or FANCL KO cells is a nice and clear demonstration that FANCL ligase activity significantly regulates CtIP during DSB

repair by HR.
Question: Does CtIP overexpression also partially complement the Olaparib sensitivity of these cells ?

_ Lane 218: "the knock-out status of the selected FANCL KO clones was confirmed by sequence analysis of the genomic target site, which identified out-of-frame mutations in all alleles (Fig. 2C)
◊ I do not agree, it is highly unlikely that both alleles present the same mutation, as suggested here. I think that only one mutation on one allele could have been detected. Moreover, HEK 293T cells are hypotriploid so… 2 or 3 alleles. However, I believe in the true KO of FANCL given the lack of FANCD2 monoubiquitination and the decreased HR, it is just a matter of detection of the other mutation(s)
◊ The same comment applies for the U2OS FANCL KO (Fig S2B) and lane 243-244

Abstract: "Here, we identified the FA core complex members FANCL and Ube2T"
Lane 95-96: "Ube2T and FANCL are both part of the multi-member Fanconi anemia (FA) core complex" and in the discussion as well.
◊ I don't think that UBE2T is considered as a member of the FA core complex. The FA core complex is a 8-subunit E3 ligase that interacts with the E2 UBE2T.

Minor comments:

_ Fig 5A, B, C, H, I: why looking only in EdU+ cells ? Are we focusing on HR only during S phase ? I would understand more to look at Cyclin A-positive cells to enrich for both S and G2 phase where HR also occurs ? Would the result be different ?

Lane 252: "clonogenic outgrowth was measured to asses HR activity"
◊ this is a shortcut to me, Olaparib sensitivity correlates with HR deficiency but does not assess HR activity per se.

Lane 284: "a scaffolding function for FANCL and an enzymatic function for Ube2T"
◊ I think that being an E3 ligase, FANCL may also be considered as a type of enzyme ? (even if it is not an HECT-type E3, a "true enzyme")
Idem for lane 317: "in agreement with their proposed enzymatic and structural roles"

Throughout the manuscript (and in figure legends), there are some english problems (to me, a non-native english):
e.g. Fig2: "cell-lines" should be "cell lines"
Everywhere in the legends :"U-2 OS", I know this can be used but "U2OS" is generally preferred (which is also used in Fig 4E legend).

Globally, they are too much hyphen "-" everywhere in the manuscript
Title: "end-resection" should be "end resection"
Abstract: "DSB-repair" should be "DSB repair"
Lane 60, 61: "DNA-repair" should be "DNA repair"
Lane 62: "strand-removal" should be "strand removal"
Lane 68: "HR-initiation" should be "HR initiation"
Lane 69, 71: "DNA-end" should be "DNA end"
Lane 89: "HR-genes" should be "HR genes"
Etc.

Lane 150: "The targeted genes encoded kinases and phosphatases, ubiquitin and SUMO modifiers, and factors that read, write or remodel chromatin."
◊ Please, precise that the targeted genes also include bona fide DNA repair genes (such as the FANC genes)

Lane 355: "We reasoned that inhibition of canonical end-joining would require maximum end-resection capacity".
◊ "would allow " seems more appropriate ?

Lane 421 (discussion): "In the absence of FANCL, Ube2T or FANCD2, DSB end-resection is impaired and Rad51 loading is reduced."
◊ Rad51 IR-induced foci and recruitment to laser-induced DNA damage are only shown for FANCL

KO cells

◊ I still have difficulties to understand why the end resection defect in FA cells due a reduced CtIP activity does not also impact SSA. However, this is quite nicely discussed by the authors.

Reviewer #3 (Remarks to the Author):

The manuscript by Kooij et al reports a role of the Fanconi anemia core complex in homologous recombination repair of DNA double strand breaks by promoting CtIP-dependent end resection. Overall, it is a very interesting study with quite some convincing data. The manuscript deserves further consideration after some major concerns are adequately addressed. Among all concerns, all data seem to suggest that Ube2T and FANCL promote CtIP recruitment for end resection by monoubiquitinating FANCD2. Some key experiments (such as including a FANCD2 L561 mutant) are needed to test whether this is the case or not.

Major concerns:
1. Fig. 1D: the authors should provide further information about the volcano plot and why Ubet2T and FANCL are selected among many other candidates with higher fold change and better p-value. The selection of FANCL is particularly not well justified considering its relatively low fold change and average p-value.
2. Lines 171—173: This could also very well be because of the overwhelmingly frequent mutagenic repair events instead of “re-balancing repair”.
3. Lines 216-218: Although genome sequencing confirms mutations in the FANCL gene, Western blot should still be done for FANCL KO in HEK293T (Fig. 2) and U2OS (Fig. 3) background. It is not well justified for not doing a Western. Functional FANCL antibodies are commercially available.
4. Fig. 2: Ube2T and FANCL knockout HEK293T cells should be examined for FANCD2 monoubiquitination like what the authors have done for U2OS cells.
5. FANCD2 KO cells (Fig. 3H-3I) should also be tested for their HR or mutagenic repair defects in the reporter system like what the authors have done for Ube2T and FANCL (Fig. 2)
6. Fig. 4 results indicate that FANCD2 monoubiquitination by Ube2T and FANCL is the key for its recruitment to damage sites. The data would be stronger if the authors test a FANCD2 L561 mutant that cannot be monoubiquitinated.

REVIEWER COMMENTS

We thank the reviewers for their constructive comments and useful suggestions. Based on these we have adapted the manuscript textually, performed several additional experiments, and included multiple new figure panels. These changes have solidified the main conclusions. We have addressed all comments point-by-point below.

Reviewer #1 (Remarks to the Author):

This manuscript reports the results of a CRISPR screen using a DSB repair assay that identifies genes that both promote and inhibit homologous recombination. From this screen, the authors focus on the Fanconi anemia core complex, including Ube2T and FANCL. Using rigorous and well-controlled experiments with KO and complemented cell lines, the authors demonstrate that these factors promote homologous recombination by several measurements including gene targeting and PARPi sensitivity. Mechanistically, a defect in CtIP recruitment to DSBs is observed in cells deficient for the FA core complex. Importantly, overexpression of CtIP can suppress the HR defect that is observed in Ube2t and FANCL KO cells. Overall, this work helps resolve an outstanding question in the field for the role of the FA pathway in HR. The experiments are well-controlled and rigorous, with data being of high quality. There are only a few issues that should be addressed prior to publication. Perhaps most importantly, the authors completed a very interesting screen for HR factors but only focus on the FA pathway. Providing more details on the screen and the hits identified would increase the impact of the work. While there is no doubt that the screen has been validated by the development of the FA pathway investigation, it is disappointing as a reader to not see what else the screen identified. Overall, this work will be of wide interest to the field, as well as a valuable resource for factors involved in HR repair.

Main issues

1. This screen will be of great value to the field as it provides another set of HR promoting and suppressive factors. However, it would be nice to see an analysis of the positive hits. While the authors focus on the FA pathway, it would be valuable and more impactful if they would also analyze the other hits and provide these in either main or supplemental figures.

> **Reply:** We thank the reviewer for acknowledging the value of our screen. To more comprehensively present the results, we now discuss the hits from the screen in more detail in the revised manuscript (lines 179-184), and show a list of hits in new supplementary figure 1c. We would also like to point out that in the original manuscript, as well as in the revised manuscript, all results from the screen are included as table S2.

2. FANCD2 appears to be less sensitive to PARPi than Ube2T or FANCL. Does this suggest another target in HR for these proteins?

> **Reply:** This is indeed an interesting observation that might be explained, as the reviewer suggests, by Ube2T/FANCL targeting other substrates for ubiquitination than FANCD2. There may, however,

also be a technical explanation. We noticed that generation of FANCD2 KO clones was generally less efficient than of FANCL/Ube2T KO clones. Hence, FANCD2 loss might be more toxic to U2OS cells than FANCL/Ube2T loss, and the FANCD2 KO cells that were eventually selected might have activated compensatory mechanism to cope with FANCD2 loss. Currently, we cannot exclude either of these hypotheses. We have included a new section in the discussion (lines 506-513) elaborating on the differences between FANCD2 and Ube2T/FANCL phenotypes.

3. Also, the significance of the PARPi sensitivity would be more obvious if a positive control, like BRCA1 or BRCA2, was included for comparison. This would help support the role of these new factors in HR as core factors or accessory factors in HR repair.

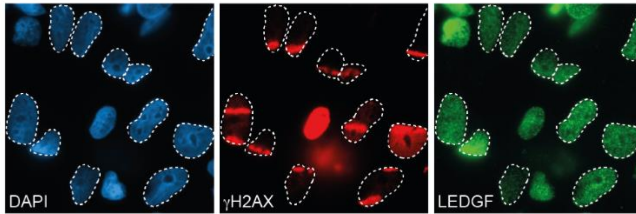
Reply: As suggested by the reviewer, we have now included a side-by-side PARPi sensitivity comparison of siBRCA2-treated cells with FANCL KO cells (new figure S3b). This showed that siBRCA2-treated U2OS cells are hypersensitive to PARPi, consistent with our previous findings in U2OS cells using siPALB2 (Fig. 2H of Luijsterburg et al., eLife, 2017), siBRCA1 (Suppl. Fig. 14B of Singh et al., Nat. Commun 2021), and siBRCA2 (Suppl. Fig. 7E of Rother et al., Nat. Commun. 2020). FANCL KO cells were also sensitive to PARPi, consistent with our previous findings in the manuscript, but less sensitive when compared to siBRCA2-treated cells (new figure S3b), consistent with the stronger HR-defect upon BRCA2 depletion (see *e.g.* van de Kooij et al., Nature comm 2023) compared to FANCL loss.

4. The identification of CtIP and DNA end resection as pathways requiring Ube2T and FANCL is very interesting. Although the authors provide references that MRN is not regulating CtIP, there is other evidence that LEDGF promotes CtIP recruitment to DSBs. The model in Figure 6 G suggests this complex promotes DNA/Chromatin binding of CtIP but some more mechanistic details of this step would help to solidify this model. It would be interesting to put Ube2T and FANCL into this reported pathway. For example, do these proteins promote LEDGF recruitment to DSBs?

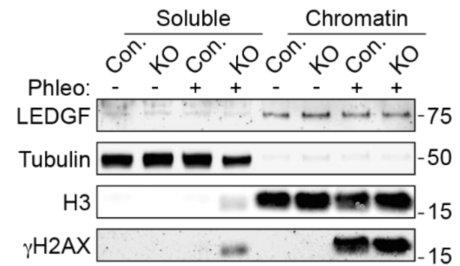
Reply: We thank the reviewer for this interesting suggestion. To address this point, we first performed micro-irradiation experiments to examine the recruitment of endogenous LEDGF to UV-A laser-induced DNA damage. We did not observe a clear recruitment of LEDGF to sites of DNA damage (Reviewer Figure 1a). This corroborates published ChIP-qPCR experiments showing that LEDGF is not recruited to a site-specific DNA break induced by the I-SceI nuclease (Pfister et al., Cell Rep., 2014).
LEGDF was shown to promote DSB repair via HR by binding tri-methylated H3K36 at damaged chromatin (Aymard et al., Nat. Struct. Mol. Biol., 2012; Daugaard et al., Nat. Struct. Mol. Biol., 2012; Pfister et al., Cell Rep., 2014). Given the lack of LEDGF recruitment to DSBs, we next asked if Ube2T and FANCL affect chromatin binding of LEDGF. To this end, we performed chromatin fractionation experiments using control and FANCL KO U2OS cells that were left untreated or treated with the radiomimetic agent phleomycin, which induces DNA breaks. LEGDF was found to be chromatin-associated both in untreated and phleomycin treated cells (Reviewer Figure 1b). Importantly, LEDGF chromatin association was not affected by FANCL loss. Collectively, these findings suggest that FANCL is neither actively involved in the recruitment of LEDGF to chromatin in absence of DNA breaks, nor in the presence of these DNA lesions.

Reviewer figure 1

a



b



Reviewer Figure 1. LEDGF chromatin binding is unaffected by FANCL loss (a) U2OS cells were subjected to UV-A laser micro-irradiation, fixed, immunostained for LEDGF and analyzed by immunofluorescence microscopy. Sites of DNA damage were identified by γH2AX staining. **(b)** U2OS control (Con.) and FANCL KO (KO) cells were treated with phleomycin (Phleo), followed by subcellular fractionation to separate the soluble and chromatin bound fractions. These fractions were analyzed by western blot. H3=histone H3.

5. In addition, what is upstream of Ube2T and FANCL?

Reply: To address this, we studied the recruitment of GFP-FANCL to FokI nuclease-induced DSBs following depletion of Mre11 or FANCM (described in lines 360-370 of the revised manuscript). These factors were chosen because Mre11 is one of the earliest DSB-repair proteins recruited (as part of the MRN complex), and FANCM has been described to recruit the FA core complex to ICLs. We found that while Mre11 depletion had no impact on FANCL recruitment, FANCM depletion almost completely abrogated FANCL's association with DSBs (new Fig. 4j, k). This suggests that FANCM is required for recruitment of the FA core complex members to DSBs to promote their repair via HR. Importantly, FANCM was also a top hit in our CRISPR-based HR screen (Figure 1d, Fig. S1c and table S2). We originally attributed this to the described HR function of FANCM in dissolving double Holliday junctions (Gari et al., Mol Cell 2008). We now added a second HR function of FANCM to our model, which is its role in recruiting the FA core complex to DSBs (Fig. 6g).

6. To provide some additional function data for these observation, it would be nice to see if overexpression of CtIP also suppressed IR/PARPi sensitivity of Ube2T and FANCL KO cells.

Reply: We agree with the reviewer that this would be good additional evidence to our model. To address this point, we performed two experiments of which the results are shown in new supplementary figure 6 in our revised manuscript. First, we transfected U2OS control or FANCL KO cells with GFP-tagged CtIP or GFP alone and sorted for GFP-positive cells by FACS. Directly hereafter, cells were plated for a clonogenic survival assay in absence or presence of olaparib (0.2 μM). As compared to control cells, PARPi treatment significantly reduced colony outgrowth of FANCL KO cells expressing GFP alone, consistent with our data shown in figure 3d and reviewer figure 2. Expression of GFP-CtIP increased clonogenic outgrowth of the FANCL KO cells, albeit modestly, indicating that overexpression of CtIP partly rescues PARPi sensitivity of FANCL KO cells (Fig. S6a).

Next, we repeated the experiment but assessed proliferative capacity rather than viability. To this end, we again expressed GFP-tagged CtIP or GFP alone in control and FANCL KO U2OS cells, exposed the cells to PARPi, and 4 days later determined the number of live GFP-positive cells by flow cytometry. As observed previously (Fig. 3d and reviewer figure 2), we found that FANCL KO cells expressing GFP only were hypersensitive to PARPi. Interestingly, this sensitivity was suppressed when GFP-CtIP was expressed (Fig. S6c). Collectively, these data suggest that CtIP overexpression rescues the HR defect of FANCL KO cells as well as the PARPi sensitivity of these cells.

Minor issues

7. In sup fig 2, it is stated that some experiments were from a single experiment while the N given is greater than 1. This is confusing and the authors should edit. Clearly experiments done only once would need to be repeated prior to publication. This seems like a misunderstanding, which needs clarifying.

Reply: This is indeed a misunderstanding. All experiments to study FA protein accumulation at FokI-induced DSBs were done at least twice. We intended to describe that the GFP-NLS, GFP-FANCL and GFP-Ube2T accumulation was analysed simultaneously in side-by-side experiments. We have now moved the GFP-Ube2T accumulation data to new figure 4f of the revised manuscript, in response to comment 11 of the reviewer, and have adapted the figure legends to resolve this misunderstanding.

How can an image of a representative experiment be shown with statistics and indication n=2 (in C and D)? Same for E and F. This could/should indeed be clarified better.

Reply: Thank you for catching this oversight and misunderstanding. We have clarified the legends to more clearly indicate the number of biological replicates and the number of nuclei analyzed in the depicted experiments

8. Is there an explanation for why Ube2T exhibits two bands by western blotting? There is a bit of uncertainty between the two KO clones for Ube2T. While one exhibits defects in SSA, the other doesn't. It would be better if another KO clone was used that doesn't still express Ube2T by western blot. As is, these results are difficult to interpret.

Reply: With regards to the second point concerning the higher migrating Ube2T species, the most likely explanation is that it represents ubiquitin-loaded Ube2T, which would be consistent with its mass. Although western blot samples were generated under reducing conditions, these may not have been stringent enough to break all Ube2T-ubiquitin thioester bonds.

With regards to the use of another KO clone, we generated two additional Ube2T KO clones using CRISPR/Cas9-based genome engineering in our DSB-Spectrum reporter-containing HEK 293T cells. Western blot analysis revealed a complete lack of Ube2T expression and MMC-induced FANCD2 ubiquitylation, so we now have a total of three complete KO clones (new Figure 2a). Importantly, HR was significantly reduced in these cells, while repair through mut-EJ and SSA were unaffected by Ube2T loss (new Figure 2b). We can thus conclude that the increased SSA in clone 3.3 (the

incomplete KO) was an effect unique to this specific clone. We have now excluded this clone from the manuscript and instead show the results obtained with the three clones showing complete KO.

9. It is nice to see complementation for FANCL KO cells but it would similarly be appropriate to perform the same complementation with the Ube2T KO to rule out off target effects for data in Fig 2.

Reply: As suggested by the reviewer, we re-expressed Ube2T (or an EV control) by transient transfection in HEK 293T DSB-Spectrum_V3-containing control cells and 3 different Ube2T KO clones, and performed reporter assays to assess HR frequencies. While HR was reduced in Ube2T KO cells expressing EV, agreeing with our previous observations (Figure 2b), we found that ectopic expression of Ube2T rescued the HR defect in these KO cells (new Figure S2b, c). This indicates that the defect in HR in all three KO clones is a consequence of Ube2T loss and is not an off-target effect (e.g. of CRISPR/Cas9-based genome editing).

10. For the sequencing data for FANCL KO lines, only one allele is shown for its mutational analysis. Shouldn't multiple alleles be shown to describe the KO of all alleles? To be certain, one could also check by RT-qPCR to ensure that the mRNA is also degraded once mutated by the nonsense mediated decay pathway. While this doesn't always occur, it could be another way to ensure the KO of FANCL in these cell lines. The authors should give more information on the characterization of the KO status of these cell lines.

Reply: We have changed the section on the FANCL sequencing to more clearly describe the methods and results (lines 227-238 of the revised manuscript). Briefly, we PCR amplified the FANCL sgRNA target locus from the FANCL KO cells, and directly sequenced the PCR product. If a WT FANCL allele would be present in addition to the +1 insertion, this would have also been amplified and would therefore have resulted in a mixture of chromatograms reflecting both sequences. However, no trace of the WT sequence was detected in the chromatograms of the FANCL KO cells shown in figure 2c. In the revised manuscript we now also included TIDE analysis of the sequencing chromatograms (Brinkman *et al.*, Nucleic Acids Res., 2014), which confirmed both the absence of WT alleles, as well as the +1 insertion being the dominant editing outcome (shared with a -8 deletion in clone 1.3; new Fig. S2d). Finally, we also analyzed FANCD2 ubiquitination status after MMC treatment, which confirmed the absence of FANCL function in the FANCL KO clones (new Fig. S2e). We have consistently done this analysis for all FANCL KO clones shown in the manuscript.

11. Ube2T localization to FokI DSBs could be placed in the main figure?

Reply: As suggested by the reviewer, we now present the Ube2T localization to FokI DSBs in main figure 2.

12. Figure 4H is confusing. Do the authors mean 2nd Ab with no FANCD2 primary (but primary and 2nd Ab for MDC1). A better explanation for the experiment should be provided for clarity.

Reply: We have clarified this in the figure and figure legend (new figure 4h, i).

Reviewer #2 (Remarks to the Author):

In this manuscript, van de Kooij et al. demonstrate a role for the Fanconi Anemia (FA) pathway in promoting homologous recombination (HR) through facilitating CtIP-dependent DNA end resection upon DNA double strand breaks (DSB) formation. They notably show that several FA proteins (UBE2T, FANCL, FANCD2) are recruited to DSBs where they facilitate in turn CtIP recruitment to promote DNA end resection.

Importantly, these findings arise from an unbiased screen using the DSB-Spectrum reporter the authors previously published in Nature Communications. Even if a role of the Fanconi Anemia pathway in promoting HR at DSB was already shown/suggested by previous works, as the authors mention, this remained quite controversial with different articles showing contradictory results. I think this work clarifies the question and nicely shows that the FA acts at the level of end resection to promote HR-dependent DSB repair.

Overall, the evidence presented here are strong and convincing. The use of multiple cell lines and targeted genes in the FA pathway as well as various read-outs reinforces the message. However, I have a few major comments as well as suggestions for the authors (see below). Moreover, although I think it is a very nice study, I should mention that the interaction between FANCD2 and CtIP was already known and that FANCD2 was already shown to promote CtIP-dependent DNA end resection in the context of interstrand crosslink (ICL) repair. Moreover, a recent study (cited by the authors) also showed that the FA pathway promotes CtIP-dependent resection at DSBs (Cai et al. Cell Reports 2020). My main concern about this work would thus be the novelty of the findings but this study still provides a more detailed characterization of the regulation of CtIP by the FA pathway in response to DSBs.

Major comments:

1 The work from the Sobeck lab should also be cited and discussed:
Yeo et al. HMG 2014. CtIP mediates replication fork recovery in a FANCD2-regulated manner
Raghunandan et al. HMG 2020. Functional crosstalk between the Fanconi anemia and ATRX/DAXX histone chaperone pathways promotes replication fork recovery
(it is also shown in the latter that FANCD2 promotes HR-dependent DSB repair).

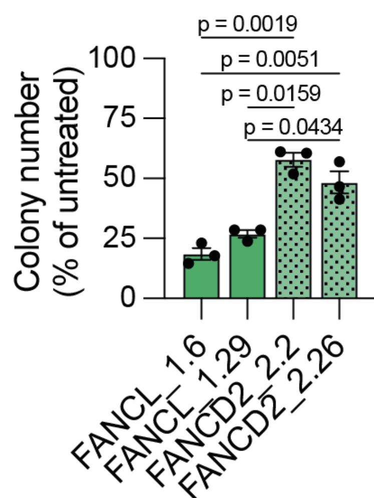
Reply: We thank the reviewer for the relevant literature suggestions, and apologize for omitting these studies from our initial manuscript. We have now cited and discussed the work in the revised manuscript (lines 566-573 of the revised manuscript).

2 Fig 5C and more dramatically S3E: why the FANCL Ligase-dead (LD) mutant partially (or largely) complements pRPA foci ? This is very surprising given all the other results presented in this study (for instance, FANCL LD does not rescue at all olaparib hypersensitivity of FANCL KO cells). This is problematic too me as no structural, non catalytic, role has been proposed for FANCL so far to the best of my knowledge. Does FANCL LD also restore the end resection defect (directly measured by qPCR) in FANCL KO cells ?

Reply: We thank the reviewer for raising this important point. We performed the experiment as suggested and generated AsiSI-nuclease expressing FANCL KO cells that re-express either FANCL WT or FANCL LD. Next, we quantitatively measured end-resection at an AsiSI-induced DSB in the presence of DNA-PK inhibitor, similar to the assay described in figure 5g of the original manuscript. While end-resection was enhanced in the presence of DNA-PK inhibitor, as expected, we observed a dramatic reduction in ssDNA levels in the FANCL KO cells (new Figure 5h), agreeing with our previous findings (Figure 5g). Importantly, we found that the end-resection levels were restored following re-expression of FANCL WT, but not following re-expression of FANCL LD (new Figure 5h). This is consistent with our findings that re-expression of FANCL WT, but not FANCL LD, rescued the HR phenotype and PARPi sensitivity of FANCL KO cells (Figure 2f and 3g). Hence, despite the partial rescue of the pRPA phenotype by re-expression of FANC LD, the combined data strongly argue that it is FANCL's E3 ubiquitin ligase activity that drives end-resection and HR.

3 Fig 3 C vs D vs I : it seems that FANCD2 KO cells are less sensitive to olaparib compared to FANCL and UBE2T KO (at 10-1 for instance). Is is statistically significant ?

Reply: As suggested by the reviewer, we did a statistical comparison between the FANCL and FANCD2 KO cell lines for the 10^{-1} μM olaparib concentration (Reviewer figure 2). All comparisons show that the difference in olaparib sensitivity between the FANCL and FANCD2 KO cell lines is statistically significant.



Reviewer Figure 2. The olaparib sensitivity data for the 10^{-1} μM concentration shown in figure 3d, i were plotted again to allow statistical comparison (paired t-test).

4 Could this suggest that a potential other substrate ubiquitinated by the FA core complex may be required for olaparib resistance ? Or that the total lack of FANCD2 has less detrimental consequences in this context compared to the sole loss of its ubiquitination ?

Reply: It is indeed very well possible that Ube2T/FANCL have FANCD2-independent mechanisms to promote olaparib resistance, as the reviewer suggested, and was also suggested by reviewer 1 (comment 2). We can formally not exclude the second hypothesis, that the presence of non-ubiquitinated FANCD2 is more inhibitory to HR than the absence of FANCD2, but we consider this a less likely explanation as FANCD2 recruitment to DSBs is dependent on its ubiquitination (see

figures 4c, d and S4a-d). Alternatively, there might be a technical explanation for our results. We noticed that generation of FANCD2 KO clones was generally less efficient than of FANCL/Ube2T KO clones. Hence, FANCD2 loss might be more toxic to U2OS cells than FANCL/Ube2T loss, and the FANCD2 KO cells that were eventually selected might have activated compensatory mechanism to cope with FANCD2 loss. We now explicitly discuss the differences between FANCD2 and Ube2T/FANCL KO phenotypes in the discussion of the revised manuscript (lines 506-513 of the revised manuscript).

5 In line with this, I would suggest to complement FANCD2 KO cells with FANCD2 WT versus K561R mutant and check olaparib resistance as well as DNA end resection.

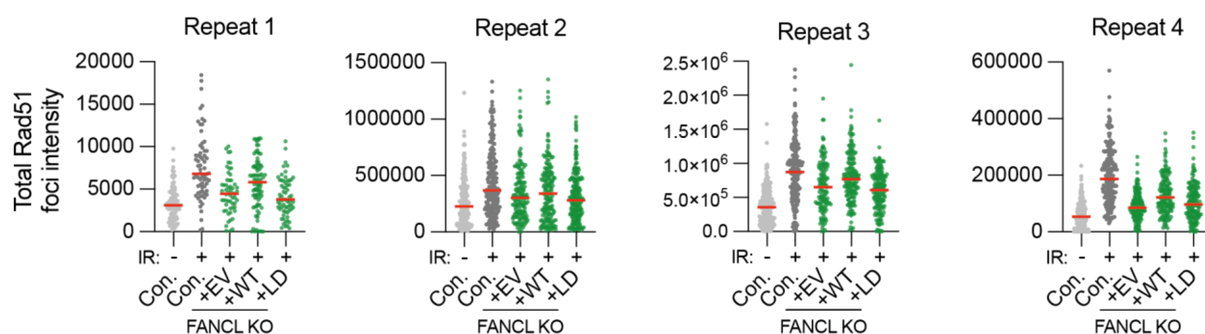
Reply: We thank the reviewer for this suggestion. We generated FANCD2 KO cells re-expressing GFP-FAND2 or GFP-FANCD2 K561R, as well as a FANCD2 KO control cell line expressing GFP-NLS (new figure S3c). Next, we took advantage of the GFP expression of those cells, by performing a competition assay in presence of olaparib. We mixed these three cell lines 1:1 with the parental FANCD2 KO cells, exposed the cells to 1 μM PARPi and quantified the fraction of GFP-positive cells by flow cytometry for up to 12 days as a measure for their proliferation capacity. We found that compared to untreated cells, the fraction of GFP-FANCD2 WT cells increased during olaparib treatment (new figure S3d). Hence, GFP-FANCD2 expressing cells are more tolerant towards olaparib than their FANCD2 KO parental cells. As a control, GFP-NLS expression did not offer any proliferation advantage in presence of olaparib (new figure S3d). GFP-FANCD2 K561R expressing cells also had a growth advantage compared to FANCD2 KO cells in presence of olaparib, but significantly less so than the GFP-FANCD2 WT expressing cells, indicating that cells expressing the FANCD2 K561R mutant are more sensitive to PARPi than cells expressing FANCD2 WT (new Figure S3d). These results are described in lines 284-298 of the revised text.

Secondly, we assessed DNA end-resection in FANCD2 KO cells re-expressing GFP-FAND2 or GFP-FAND2 K561R by measuring IR-induced pRPA foci levels. We found that total pRPA foci intensity was reduced in FANCD2 KO cells (new figure S5f), agreeing with our previous results (Figure S5e in the revised manuscript). Importantly, pRPA foci levels were largely restored in FANCD2 KO cells expressing GFP-FAND2, but not GFP-FAND2 K561R, suggesting that FANCD2 ubiquitylation at K561 is critical to promote end-resection during HR. These results are described in lines 381-386 of the revised text.

6_ Fig 5H: the difference in total Rad51 foci intensity between FANCL KO+EV versus FANCL KO+WT is not large, is it statistically significant ? (but the representative biological repeat in S3G is convincing)

Reply: Although the difference in Rad51 foci intensity between the FANCL KO+EV and FANCL KO+WT is not large, it is highly reproducible, with a p-value of 0.0059 (paired t-test). For the reviewer's appreciation of the reproducibility, we show here the results of all individual experiments (Reviewer figure 3).

Reviewer figure 3



Reviewer Figure 3. Results of the individual biological repeats of the Rad51 foci analysis shown in figure 5i, j of the main manuscript.

7_ Fig 6F: the fact that CtIP overexpression partially restores HR in UBE2T or FANCL KO cells is a nice and clear demonstration that FANCL ligase activity significantly regulates CtIP during DSB repair by HR. Question: Does CtIP overexpression also partially complement the olaparib sensitivity of these cells?

Reply: see also our reply to comment 6 of reviewer 1, which we copied here for the reviewer's convenience.

Reply: We agree with the reviewer that this would be good additional evidence to our model. To address this point, we performed two experiments of which the results are shown in new supplementary figure 6 in our revised manuscript. First, we transfected U2OS control or FANCL KO cells with GFP-tagged CtIP or GFP alone and sorted for GFP-positive cells by FACS. Directly hereafter, cells were plated for a clonogenic survival assay in absence or presence of olaparib (0.2 μM). As compared to control cells, PARPi treatment significantly reduced colony outgrowth of FANCL KO cells expressing GFP alone, consistent with our data shown in figure 3d and reviewer figure 2. Expression of GFP-CtIP increased clonogenic outgrowth of the FANCL KO cells, albeit modestly, indicating that overexpression of CtIP partially rescues PARPi sensitivity of FANCL KO cells (Fig. S6a).

Next, we repeated the experiment but assessed proliferative capacity rather than viability. To this end, we again expressed GFP-tagged CtIP or GFP alone in control and FANCL KO U2OS cells, exposed the cells to PARPi, and 4 days later determined the number of live GFP-positive cells by flow cytometry. As observed previously (Fig. 3d and reviewer figure 2), we found that FANCL KO cells expressing GFP only were hypersensitive to PARPi. Interestingly, this sensitivity was suppressed when GFP-CtIP was expressed (Fig. S6c). Collectively, these data suggest that CtIP overexpression rescues the HR defect of FANCL KO cells as well as the PARPi sensitivity of these cells.

8_ Lane 218: "the knock-out status of the selected FANCL KO clones was confirmed by sequence analysis of the genomic target site, which identified out-of-frame mutations in all alleles (Fig. 2C) ◊ I do not agree, it is highly unlikely that both alleles present the same mutation, as suggested here. I think that only one mutation on one allele could have been detected. Moreover, HEK 293T cells are hypotriploid so… 2 or 3 alleles. However, I believe in the true KO of FANCL given the lack of FANCD2

monoubiquitination and the decreased HR, it is just a matter of detection of the other mutation(s) ◊ The same comment applies for the U2OS FANCL KO (Fig S2B) and lane 243-244

Reply: We thank the reviewer for pointing this out, as we now realize that our description of the genomic analysis was not clear. We have adapted it in the revised manuscript (lines 227-238). Briefly, we PCR amplified the FANCL sgRNA target locus from each FANCL KO cell line. This PCR product would be a mixture of all amplified alleles, and we directly sequenced the total PCR product, without any subcloning. Thus, if multiple, differentially edited alleles would be present in the clonal population, this would result in a mixture of chromatograms reflecting both (or all three) sequences. However, the chromatograms of 293T clone 1.3, and clone 1.4 in particular, are very clean, showing no trace of the WT sequence, or of sequences with other InDels (figure 2c). To study this more thoroughly in the revised manuscript, we now also included TIDE analysis of the sequencing chromatograms (Brinkman *et al*., Nucleic Acids Res., 2014), which confirmed both the absence of WT alleles, as well as the +1 insertion being the single editing outcome in clone 1.4 and the dominant editing outcome in clone 1.3 (shared with a -8 deletion; new Fig. S2d). We cannot exclude that editing would have resulted in deletions sufficiently large to remove the binding site for the primers used to amplify the target region. These edits would have been missed by our analysis, but such large deletions would likely have generated a non-functional allele. Of note, single editing outcomes shared by both (or all three) alleles are not unlikely, given that editing is not completely random but sequence specific (see for example Shen et al., Nature, 2018).

We appreciate that the reviewer acknowledges that the FANCD2-ubiquitination analysis provides additional evidence for the FANCL KO status. The revised manuscript contains this analysis for all presented FANCL KO and reconstituted cell lines (Fig. 2a, e, 3b, e, 5e, S2e, and S5h of the revised manuscript).

9_ Abstract: “Here, we identified the FA core complex members FANCL and Ube2T” Lane 95-96: “Ube2T and FANCL are both part of the multi-member Fanconi anemia (FA) core complex” and in the discussion as well.

◊ I don’t think that UBE2T is considered as a member of the FA core complex. The FA core complex is a 8-subunit E3 ligase that interacts with the E2 UBE2T.

Reply: We agree with the reviewer that in published literature Ube2T is at times discussed as an interaction partner, rather than a member of the FA core complex. However, we also encountered recent reviews from experts in the field listing it as a *bona fide* core complex member (“The Fanconi Anemia Pathway in Cancer” from the d’Andrea lab, Annu Rev Cancer Biol 2019, and “Mechanisms of Vertebrate DNA Interstrand Cross-Link Repair” from the Walter lab, Annu Rev Biochem 2021). Clearly, this is still a matter for debate. We do not aim to take a specific position in this discussion, but would prefer to leave Ube2T discussed as core complex member in our manuscript for simplicity purposes.

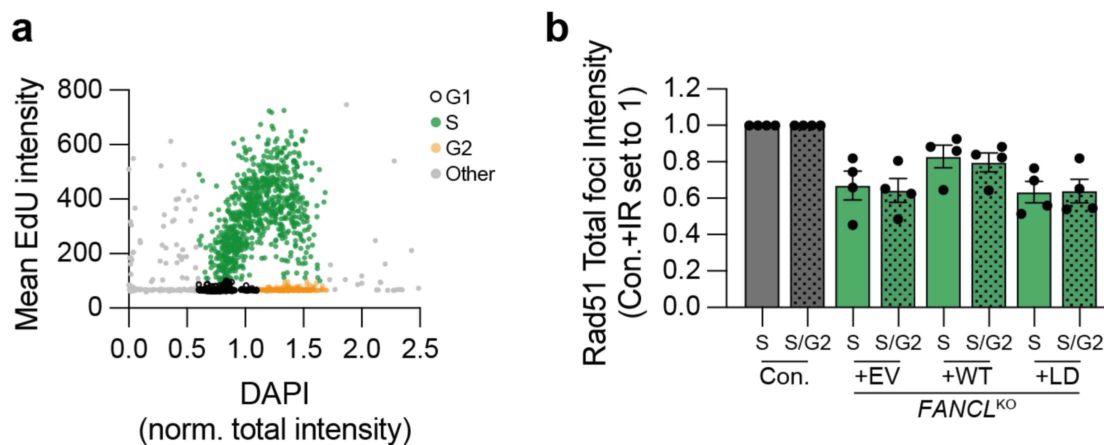
Minor comments:

10_ Fig 5A, B, C, H, I: why looking only in EdU+ cells ? Are we focusing on HR only during S phase ? I

would understand more to look at Cyclin A-positive cells to enrich for both S and G2 phase where HR also occurs ? Would the result be different ?

Reply: EdU pulse labelling is commonly used as an alternative to Cyclin A or geminin staining to distinguish G0/G1 cells from replicating cells in S/G2 phase (see for example Belan *et al.*, Mol Cel 82, 2022). As HR has been shown to peak during mid S-phase (Karanam et al., Mol Cell **47**, 2012), we consider quantification of HR in S-phase a relevant read-out for HR activity. Nevertheless, we re-analyzed the Rad51 foci data (Fig. 5j of the revised manuscript) to compare the results between selecting S-phase only cells versus S- and G2-phase cells combined. Selection of S- and G2-phase cells could easily be done based on the DAPI and EdU staining (Reviewer figure 4a). The Rad51 foci data are identical when comparing S-phase only cells to S- and G2-phase cells combined (Reviewer figure 4b).

Reviewer figure 4



Reviewer Figure 4. (a) EdU and median-normalized DAPI intensities were plotted for all nuclei analyzed for the Rad51 foci experiments described in figure 5i, j of the revised manuscript. G2 phase cells can be identified based on 4n DAPI signal and EdU-negativity. **(b)** As in panel 5j of the revised manuscript, but now comparing analysis of S-phase only cells to S- and G2-phase cells combined. All samples shown were irradiated (10 Gy IR).

11_Lane 252: “clonogenic outgrowth was measured to asses HR activity” ◊ this is a shortcut to me, olaparib sensitivity correlates with HR deficiency but does not assess HR activity per se.

Reply: We agree with the reviewer and have changed the text accordingly.

12_Lane 284: “a scaffolding function for FANCL and an enzymatic function for Ube2T” ◊ I think that being an E3 ligase, FANCL may also be considered as a type of enzyme ? (even if it is not an HECT-type E3, a “true enzyme”) Idem for lane 317: “in agreement with their proposed enzymatic and structural roles”

Reply: We agree with the reviewer that FANCL is also an enzyme, and removed the indicated, perhaps somewhat speculative, sentences from the manuscript.

Throughout the manuscript (and in figure legends), there are some english problems (to me, a non-native english): e.g. Fig2: "cell-lines" should be "cell lines" Everywhere in the legends :"U-2 OS", I know this can be used but "U2OS" is generally preferred (which is also used in Fig 4E legend).

Globally, they are too much hyphen "-" everywhere in the manuscript:

Title: "end-resection" should be "end resection"

Abstract: "DSB-repair" should be "DSB repair"

Lane 60, 61: "DNA-repair" should be "DNA repair"

Lane 62: "strand-removal" should be "strand removal"

Lane 68: "HR-initiation" should be "HR initiation"

Lane 69, 71: "DNA-end" should be "DNA end"

Lane 89: "HR-genes" should be "HR genes"

Etc.

Reply: We have removed the hyphens and replaced U-2 OS by U2OS throughout the manuscript.

Lane 150: "The targeted genes encoded kinases and phosphatases, ubiquitin and SUMO modifiers, and factors that read, write or remodel chromatin."

◊ Please, precise that the targeted genes also include bona fide DNA repair genes (such as the FANC genes)

Reply: We have added this to the revised manuscript.

Lane 355: "We reasoned that inhibition of canonical end-joining would require maximum end-resection capacity".

◊ "would allow " seems more appropriate ?

Reply: We agree and have changed this in the revised manuscript

13_Lane 421 (discussion): "In the absence of FANCL, Ube2T or FANCD2, DSB end-resection is impaired and Rad51 loading is reduced."

◊ Rad51 IR-induced foci and recruitment to laser-induced DNA damage are only shown for FANCL KO cells

Reply: We have changed the conclusion to more correctly represent the data shown in the manuscript.

14_◊ I still have difficulties to understand why the end resection defect in FA cells due a reduced CtIP activity does not also impact SSA. However, this is quite nicely discussed by the authors.

Reply: We thank the reviewer for appreciating our discussion of this puzzling observation.

Reviewer #3 (Remarks to the Author):

The manuscript by Kooij et al reports a role of the Fanconi anemia core complex in homologous recombination repair of DNA double strand breaks by promoting CtIP-dependent end resection. Overall, it is a very interesting study with quite some convincing data. The manuscript deserves further consideration after some major concerns are adequately addressed. Among all concerns, all data seem to suggest that Ube2T and FANCL promote CtIP recruitment for end resection by monoubiquitinating FANCD2. Some key experiments (such as including a FANCD2 L561 mutant) are needed to test whether this is the case or not.

Major concerns:

1. Fig. 1D: the authors should provide further information about the volcano plot and why Ubet2T and FANCL are selected among many other candidates with higher fold change and better p-value. The selection of FANCL is particularly not well justified considering its relatively low fold change and average p-value.

Reply: We thank the reviewer for pointing this out. We now provide a more detailed description of the results of the screen, including a clarification on why we selected Ube2T and FANCL for follow-up (lines 179-194 of the revised manuscript and new figure S1c). In short, this selection was mostly driven by (1) the comparison between results from our screen and the screen by Wienert *et al*. (Nat. Commun., 2020) (Fig. S1d), which showed that FANCM, Ube2T and FANCL were the only genes strongly depleted (*i.e.* their cognate sgRNAs) in both HR screens (apart from BRCA1/BARD1), and (2) the fact that all three FA genes targeted by our custom sgRNA library were depleted from the HR population. Whereas depletion of FANCL sgRNA was not significant, the results taken together suggested an HR function for the FA core complex components.

2. Lines 171—173: This could also very well be because of the overwhelmingly frequent mutagenic repair events instead of “re-balancing repair”.

Reply: We agree with the reviewer that alternative explanations are possible, and have changed the text to provide a more inclusive explanation for the unsuccessful mutagenic repair branch of the screen (lines 174-177).

3. Lines 216-218: Although genome sequencing confirms mutations in the FANCL gene, Western blot should still be done for FANCL KO in HEK293T (Fig. 2) and U2OS (Fig. 3) background. It is not well justified for not doing a Western. Functional FANCL antibodies are commercially available.

Reply: We agree with the reviewer that Western blot is the preferred method to validate KO status. Therefore, we extensively tried to get two antibodies against human FANCL to work: Santa Cruz sc-137067 and Proteintech 66639-1-lg. We initially followed the manufacturer’s instructions, but after obtaining negative results we also varied blocking and probing conditions, membranes, and the read-out (Odyssey versus Chemidoc). Nevertheless, we were not able to detect any band specific for FANCL, neither in HEK293T cells, nor in U2OS cells.

Rather than spending any more resources on FANCL antibodies that might or might not work, we decided to validate loss of FANCL in two alternative ways: by genomic analysis, and by analysis of FANCD2 ubiquitination upon DNA crosslink-inducing treatment with Mitomycin C (MMC) (Fig. 2a, e, 3b, e, 5e, S2e, and S5h of the revised manuscript). Furthermore, we use two independent FANCL KO clones for all our cell systems (HEK 293T, U2OS and U2OS AsiSI), and can rescue all reported phenotypes by re-expressing FANCL (Fig. 2f, 3g, 5c, h, 6b of the revised manuscript). Taken together, using multiple assays, we are confident that our data provides evidence of FANCL loss in our KO clones.

4. Fig. 2: Ube2T and FANCL knockout HEK293T cells should be examined for FANCD2 monoubiquitination like what the authors have done for U2OS cells.

Reply: As suggested by the reviewer, we have performed these validation experiments of which the results are now included as figure 2a (note that we generated two new Ube2T KO clones as suggested by reviewer 1) and figure S2e of the revised manuscript. All Ube2T clones showed impaired FAND2 ubiquitylation following treatment with DNA crosslink-inducing agent Mitomycin C (MMC), validating these clones.

5. FANCD2 KO cells (Fig. 3H-3I) should also be tested for their HR or mutagenic repair defects in the reporter system like what the authors have done for Ube2T and FANCL (Fig. 2)

Reply: As suggested by the reviewer, we depleted FANCD2 by RNAi and observed a significant reduction in HR (new figure S3e, f of the revised manuscript). This is consistent with reported data showing reduced HR in reporter assays upon FANCD2 loss (Yamamoto et al., Mol. Cell. Biol., 2005; Smogorzewska et al., Cell, 2007; Howard et al., Plos Genet., 2015, Eccles et al., DNA repair, 2018) and with FANCD2 being a strong hit in the HR screen published by Wienert et al. (Nat. Commun., 2020). Notably, the phenotypes that we observe after FANCD2 depletion are generally less severe

than those observed after FANCL/Ube2T loss. We discuss this in the revised manuscript (lines 506-513).

6. Fig. 4 results indicate that FANCD2 monoubiquitination by Ube2T and FANCL is the key for its recruitment to damage sites. The data would be stronger if the authors test a FANCD2 L561 mutant that cannot be monoubiquitinated.

Reply: We thank the reviewer for this suggestion. To address this point, we lentivirally introduced GFP-tagged FANCD2 WT or FANCD2 K561R into the U2OS FANCD2 KO cells and assessed their recruitment to DSBs induced by UV-A laser micro-irradiation. As shown in new figure S4c, d in the revised manuscript, GFP-FANCD2 WT was clearly recruited to the DNA damage stripes, but no recruitment of GFP-FANCD2 K561R could be observed. This further supports that FANCD2 mono-ubiquitination by the FA core complex is required for its accumulation at DSBs. This is now discussed in lines 335-340.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have included considerable new data and revisions, which thoroughly address all of this reviewer's comments. This work is improved and is appropriate for publication in Nature Communications.

Reviewer #2 (Remarks to the Author):

We thanks the author to have consider all the comments and believe now the paper quality is improved and suitable for publication.

Reviewer #3 (Remarks to the Author):

The authors have adequately addressed all the concerns.