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SCAI promotes error-free repair of DNA interstrand crosslinks via the Fanconi anemia pathway

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I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Kind regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee 1

The manuscript is generally well presented and results support the main conclusions. The authors have identified SCAI as a new regulator of ICL repair. SCAI act downstream FANCD2 and regulates by it interaction with pol zeta TLS/HR preventing mmeJ mediated by pol Q.

The presentation of SCAI as a potential new FA protein is interesting however the authors fall short in providing direct comparison of cell phenotypes. The molecular mechanisms presented are convincing however a few (minor) controls are missing. The identification of a patient SCAI with FA or FA-like or the use of mouse model (SCAI, ALDH2 double KO mice) would make the paper stronger.

Major concerns:
-For Figure 1F to K a cell KO for a FA genes should be used as a positive control especially since the model present SCAI as an actor of the FA pathway. This will allow to compare the phenotype of SCAI KO cells with FA KO cells.

-Could author discuss the non FA genes identified in the list of 11 the inhibit the sensitivity of SCAI KO cells? Could they be involved in FA? Are they involved in other mechanisms that could explain the reversal of the phenotype?

-Could authors explain/discuss why shFANCA and shFANCD2 are not sensitive in Fig 2E and S1J. Wy is SCAI KO cell more sensitive than FA cells? The shRNA efficiency appears to be sufficient to rescue the sensitivity of SCAI KO cells so it should be good enough to induce sensitivity.

-Fig4B Fig S2B :why does the lower band (*) detected by the antibodies is mentioned as antibodies cross reactivity since it disappear after depletion with c-ter antibody. Both N-ter and C-ter antibodies should be used for WB and not only for the depletion.

-IP of endogenous SCAI should be used to demonstrate the interactions in human cells

Minor concerns:
-Fig 1C Fig4B Fig S2B should have a supplementary win uncropped blot to control the specificity of the antibody.

-Author should replace Fig3C FANCD2 blot by another replicate to improve quality.

-Differences in S2D and S2E are not obvious and quantifications should be provided

-GFP alone is missing in the Fig 4D

Referee 2

The manuscript by Mailand, Duxin, and colleagues investigates the role of SCAI (Suppressor of Cancer Cell Invasion) in DNA interstrand crosslink (ICL) repair mediated by the Fanconi anemia (FA) pathway. Using a genome-wide CRISPR-Cas9 dropout screen, the authors have identified SCAI as a strong hit that, when knocked out, sensitizes cells to MMC, an ICL-inducing cytotoxic drug. By employing a Xenopus egg extract ICL repair assay, they further showed that SCAI works downstream of FANCD2 monoubiquitin to promote translesion DNA synthesis (TLS) and homologous recombination (HR). Specifically, SCAI was shown to directly interact with the Rev1-Polz (Rev3-Rev7) TLS complex, although the function of SCAI appears to be independent of its association with Pol . On the other hand, depletion of Polz or its pharmacological inhibition suppressed error-prone double-stranded DNA (dsDNA) repair caused by SCA1 deficiency, suggesting that one of the main roles of SCA1 in the FA pathway is to ensure the execution of faithful DNA ICL repair once incision occurs downstream of FANCD2 ubiquitination, presumably by promoting TLS, HR, or both, thereby preventing deletions and chromosome aberrations originated from Polq-mediated microhomology-mediated end-joining (MMEJ).

This work nicely combines both cellular analysis and in vitro ICL repair assays to elucidate the previously unappreciated role of SCA1 in regulating the FA pathway. Given that SCAI was identified as a 53BP1-binding protein implicated in double-strand break (DSB) repair besides its implication in transcriptional regulation, expanding its role in DNA ICL resolution is considered an important advance in the field. Data are of high quality, and experiments are well controlled both in cells and in Xenopus egg extracts. However, the major concern is that these individual results from in vitro and in vivo experiments do not lead to a coherent and convincing conclusion that can explain how SCAI influences the outcome of DNA ICL repair, especially without any clear mechanistic insight. The most important discovery in cells would be the identification of the Rev3-SCAI interaction, which implies the function of SCAI in TLS; however, Xenopus studies revealed that SCAI works independently of the Rev1-Polz complex, while exhibiting a profound defect in HR. Thus, it is not clear what this novel interaction would mean in DNA ICL repair; in other words, no connection between the SCAI-Rev1-Polz complex and its functional relevance in TLS or HR is described. As a result, the key question of how SCAI channels DNA ICL repair to error-free TLS/HR, while suppressing MMEJ, is not addressed. It is rather confusing to understand whether SCAI directly controls TLS or HR, and whether any of its interaction with the Rev1-Polz complex functionally matters. The authors have indeed mentioned in the discussion that the function of SCAI might be related to regulation of resected ssDNA ends and RAD51 loading, and considering several literatures that support the notion that Rev1 and Pol promote HR repair, SCAI may contribute to those processes. However, again, there is no hypothesis or model on this matter, and their discovery of the Rev3-SCAI interaction does not contribute to the understanding of any potential mechanism behind SCAI in DNA ICL repair. In terms of the role of SCAI in DSB repair, elegant molecular mechanisms have been proposed, which connect its physical and functional interactions with 53BP1 and RIF1, in regards to its association with heterochromatin and regulation by phosphorylation (Hanson 2016; Isobe 2017). Similar levels of mechanistic insight should be present to define the novel role of SCAI in DNA ICL repair, and some of the major points described below need to be addressed to achieve suitability for publications.

1. The Xenopus ICL repair assay shows that SCAI depletion results in a mild TLS delay but complete loss of HR-mediated repair (Figure 3). However, how SCAI loss leads to those defects is not addressed, especially in the context of its interaction with the Rev1-Pol complex. In Figure 4I, it was concluded that the Rev1-Pol interaction is not related to the function of SCAI to suppress deletions. Without making any clear connection, these in vivo interaction data are downgrading in vitro results, almost distracting the key point of how SCAI antagonizes Polq-mediated MMEJ. Conversely, any potential Rev1-Polz dependent function is not addressed at all. It would considerably strengthen the manuscript if the authors focus on what the Rev1-Polz complex interaction of SCAI would mean in antagonizing Polq activity or directly stimulating HR processes (with some insight into the reason behind its only mild TLS delay despite its stable association with the TLS machinery).

2. The survival curve of Figure 2E claims that preventing upstream FA pathway activation alleviates the ICL hypersensitivity of SCAI-deficient cells. Alternatively, this could be interpreted that FANCA knockdown and SCAI knockout are not epistatic. As a

control, downstream FA genes (e.g. BRCA2, FANCJ, FANCP, or FANCV) should be tested in order to determine whether there is a differential survival outcome between genes that regulate FANCD2 activation and execute downstream TLS or HR.

3. In Figure 3H, it is not clearly described what the prolonged presence of the nascent strand product and the increase of extension product means let alone how to interpret those data. For those readers without sufficient background on the Xenopus cell extract assay, a little more detailed explanation should be accompanied in interpreting DNA gels.

4. In Figure 4, one important experiment should be to show the interaction between endogenous SCAI and endogenous Rev3 (in addition to the Rev1 and SCAI interaction in Figure 4C, which is supposed to be indirect). Moreover, in Figure 4D, a considerable amount of Rev7 was shown to be immunoprecipitated by GFP-SCAI even without its proposed direct interacting partner Rev3. What is the explanation for this?

5. The lack of a unified model to describe the role of SCAI in DNA ICL repair is evident in the proposed scheme in Figure 6, where the Rev1-Polz complex is not shown in any of the steps engaged in TLS, HR, or MMEJ. Additional experiments may help establish the role of SCAI that forms a complex with the Rev1-Polz complex, and how its interaction contributes to the pathway choice. Being able to position Rev1 and Polz into the proposed model could significantly strengthen the manuscript.

6. To understand whether the Rev1-Pol interaction is important in promoting TLS/HR, one strategy would be to identify and use a SCAI mutant that has lost the interaction with Rev3 and determine whether the mutant is loss of function in the Xenopus ICL repair assay (e.g. suppressing the generation of extension product). Ideally, rescuing SCAI depletion in egg extracts needs to be included to exclude the possibility of non-specific co-depletion of other proteins.

Referee 3

In this study, the authors seek to investigate the mechanisms that regulate ICL repair downstream of FA pathway activation. They describe a potential role for SCAI at the intersection of translesion synthesis (TLS) and homologous recombination (HR), which, when defective, leads to aberrant repair that creates deletions through microhomology-mediated end joining. Although these findings provide novel insight into the mechanism of ICL repair and identify a new consequence of its dysfunction, evidence specifically pointing to SCAI's role is limited. Great care is taken to separate SCAI's function from REV1/3. However, as shown in Figure 4A, IPs of SCAI are enriched for numerous factors involved in ICL repair. It is not clear whether SCAI merely co-depletes another important factor, or if reducing the level of multiple repair proteins has a cumulative effect on repair efficiency. Both are interesting possibilities, but need to be explored further.

Additional specific comments:
As the focus of the paper, some description of SCAI and its known activities/functions should be provided.

As a 53BP1-interacting protein, how does SCAI knockout affect 53BP1 levels? Mutational inactivation of SCAI would preserve cellular complexes that rely on SCAI and help to specifically implicate SCAI in the repair defects described.

SCAI knockout cells are vastly more sensitive to MMC than FANCA knockouts. Is this sensitivity specific to ICLs compared to other forms of DNA damage, as classically seen with knockout of various FA genes?

Why does loss of SCAI (and increased mmEJ) lead to increased recombination? What is the structure of these intermediates and how does it change?

The authors note that depletion of SCAI may co-deplete CtIP (depending on the antibody used). This seems to reinforce the importance of characterizing what proteins are removed from the extract system and confirming their contributions to the observed defect in some way, ideally using a rescue experiment.

Point-by-point response to the reviewers' comments

We greatly appreciate the reviewers' constructive comments and helpful suggestions. All the reviewers' comments are shown in their entirety below, with our responses highlighted in bold italics. Although we comment on all reviewer points, we would like to emphasize that our experimental revisions were focused on addressing the **specific major and minor concerns of Referee #1, specific points 2, 3 and 4 by Referee #2, and the 'additional specific comments' by Referee #3**, according to the Editor's instructions.

Before we respond to the reviewers' points in detail, we would like to comment on a newly published manuscript from the Elledge laboratory, which aligns with and supports our findings that SCAI and REV3 interact with each other and ensure efficient ICL repair (Adeyemi *et al*, 2021). In their study, Adeyemi et al. provide evidence that SCAI interacts with REV3 to protect stalled forks that arise during ICL repair. They propose that the SCAI-REV3 complex, which they named Protexin, acts at stalled forks independently of other Polζ components (REV1 and REV7) and protects forks from hyper-resection by EXO1, thereby promoting HR. Additionally, Protexin may also counteract resection via REV3 catalytic activity, presumably primed by RNA POL2. While we were pleased to see that an independent study came to the same overall conclusions, we would like to highlight how our work complements and extends the findings by Elledge and co-workers, which we also discuss in the revised version of our manuscript (page 12-15):

1. We provide a molecular explanation for how SCAI interacts with Polζ, which is completely missing in the study by Adeyemi et al. We identify a SCAI-binding motif within the PCD domain of REV3, which does not overlap with its REV7- and POLD2-interacting regions. Accordingly, SCAI is not expected to compete with other Polζ components for binding to REV3, and although the study by Adeyemi et al. points to a role of SCAI-REV3 during ICL repair that is independent of other Polζ components, our MS data identified all REV1-Polζ subunits in SCAI immunoprecipitates (this was, in fact, also seen by Adeyemi et al. in their proteomic analysis of SCAI-interacting proteins). Thus, contrary to Adeyemi et al. we do not envision that REV3 forms a complex with SCAI in the absence of its accessory subunits, which greatly stimulate REV3 catalytic activity *in vitro* (Nelson *et al*, 1996), and further work will be needed to address whether SCAI can form a functional polymerase complex with REV3 that excludes other Polζ components.

2. In their work Adeyemi et al. propose that Protexin's main function during ICL repair is to protect stalled forks from hyper-resection by EXO1, thereby promoting downstream repair by HR. They further showed that this protective function depends on FANCM translocase activity, which is presumed to generate reversed forks during ICL repair (Gari *et al*, 2008; Zellweger *et al*, 2015; Amunugama *et al*, 2018). This suggests that the relevant substrate protected by SCAI is a reversed fork, which generates a one-ended DSB. Importantly, while we have not measured resection in our assays, our work clearly shows that one additional outcome of the de-protection caused by the absence of SCAI is the inappropriate fusion of DSBs by MMEJ. Based on these studies, we posit that one critical function of SCAI in ICL repair is to protect the reversed fork from improper processing (i.e. fusion to another DSB, which would be highly detrimental for cells, and/or hyper-resection by EXO1). This is reinforced by our findings showing that cells become critically reliant on SCAI for faithful repair of ICLs once their processing by the FA pathway is initiated.

Overall, our work provides important insights into the role of SCAI in ICL repair that are not only aligned with but also extend the recent findings of Elledge and co-workers.

Referee #1:

The manuscript is generally well presented and results support the main conclusions. The authors have identified SCAI as a new regulator of ICL repair. SCAI act downstream FANCD2 and regulates by it interaction with pol zeta TLS/HR preventing mmeJ mediated by pol Q.

The presentation of SCAI as a potential new FA protein is interesting however the authors fall short in providing direct comparison of cell phenotypes. The molecular mechanisms presented are convincing however a few (minor) controls are missing. The identification of a patient SCAI with FA or FA-like or the use of mouse model (SCAI, ALDH2 double KO mice) would make the paper stronger.

We agree with the reviewer that identification of a patient with mutated SCAI manifesting with FA-like phenotypes or generation of a SCAI/ALDH2 double KO mice will ultimately be required to address whether SCAI is a novel FA gene. Despite some efforts and contacts with researchers with access to patients with FA-like symptoms, we have not succeeded in identifying an FA patient with SCAI mutations, so this will remain an open question for the field. We have followed the reviewer's advice and initiated the generation and characterization of SCAI/ADH5 double KO mice, however we hope the reviewer will appreciate that this is a long-term undertaking that goes beyond the scope of the current manuscript.

Major concerns:
-For Figure 1F to K a cell KO for a FA genes should be used as a positive control especially since the model present SCAI as an actor of the FA pathway. This will allow to compare the phenotype of SCAI KO cells with FA KO cells.

To address this point, we generated U2OS cells with targeted knockout of FANCA, an integral component of the FA core complex (new Figure 2H). Consistent with our previous data obtained with siRNAs and CRISPR-Cas9 screens, we found that FANCA KO cells are indeed hypersensitive to MMC, but considerably less so than SCAI KO cells in a U2OS background (new Figure 2H-J). Moreover, we confirmed that FANCD2 monoubiquitylation is ablated in FANCA KO cells as expected (new Figure 2K). Since it is well established that FANCA-deficient cells show increased levels of chromosome breakage and radial formation upon MMC treatment, we have not performed chromosome spread analyses with our FANCA KO cells, as we believe inclusion of such data would add little overall value to our conclusion that SCAI is essential for replication-coupled ICL repair.

-Could author discuss the non FA genes identified in the list of 11 the inhibit the sensitivity of SCAI KO cells? Could they be involved in FA? Are they involved in other mechanisms that could explain the reversal of the phenotype?

GO term analysis identified FA genes as the main group of proteins that alleviate the hypersensitivity of SCAI KO cells to MMC (Figure 2C). In addition to this, a few genes involved in chromatin remodeling and cell survival/proliferation also scored as hits in this screen (Figure 2C). We have now added a sentence in the text (page 5-6) to highlight these genes, as follows:

"Genes encoding factors involved in chromatin remodeling via histone acetylation and regulators of cell proliferation and survival via p53 also conferred resistance to MMC in SCAI KO cells but were far less represented than FA genes (Figure 2C; Table EV2)."

It is, however, not possible to assert whether these genes are specific to SCAI loss or whether they also confer resistance to a near-lethal (LD80) dose of MMC in the parental U2OS cell line, which was not included in the screen. Because chromatin remodelers such as SMARCAD1 are known to participate in DSB repair by modulating DNA resection, it is possible that some of these hits are indeed specific to SCAI loss, but this will require further experimentation to be validated, and in our opinion this goes beyond the scope of the current study.

-Could authors explain/discuss why shFANCA and shFANCD2 are not sensitive in Fig 2E and S1J. Wy is SCAI KO cell more sensitive than FA cells? The shRNA efficiency appears to be sufficient to rescue the sensitivity of SCAI KO cells so it should be good enough to induce sensitivity.

We agree with the reviewer that the lack of apparent MMC hypersensitivity of FANCA- or FANCD2-depleted cells was surprising and needed further clarification. To eliminate the possibility that these observations were a consequence of residual FA pathway activity due to incomplete knockdown efficiency of the siRNAs, we generated FANCA KO and SCAI/FANCA double KO cell lines (new Figure 2H). This confirmed that SCAI KO leads to stronger MMC hypersensitivity than FANCA KO in a U2OS background and that FANCA deletion alleviates the hypersensitivity of SCAI KO cells to MMC (new Figure 2J), in accordance with our CRISPR-Cas9 screen and siRNA data. We confirmed that FA pathway activation is indeed abolished in FANCA KO cells, as evidenced by the disappearance of FANCD2 monoubiquitylation upon MMC treatment (new Figure 2K). Moreover, although less sensitive than SCAI KO cells, FANCA KO cells are still hypersensitive to MMC, as is evident when these cells are treated with higher doses of the drug (new Figure 2I). Together, these new results reinforce our conclusion that SCAI becomes essential for repair of ICLs once their resolution is initiated by the FA pathway.

We envision that the greater sensitivity of SCAI KO cells to MMC compared to FANCA KO cells could reflect the multiple critical functions of SCAI in ICL repair (i.e. protection of DSB intermediates from toxic repair by MMEJ, and potentially also stimulation of TLS) and/or additional lesions induced by MMC such as DNA mono-adducts and intra-strand crosslinks, which would depend on TLS but not the FA pathway for bypass.

-Fig4B Fig S2B :why does the lower band (*) detected by the antibodies is mentioned as antibodies cross reactivity since it disappear after depletion with c-ter antibody. Both N-ter and C-ter antibodies should be used for WB and not only for the depletion.

Because the indicated band is immunodepleted by the C-terminal but not the N-terminal antibody and both antibodies efficiently immunodeplete SCAI from extracts as determined by whole proteome analysis (new Figure 4C and Table EV4), we conclude that this band corresponds to a non-specific protein immunodepleted and recognized by Western blotting by the C-terminal antibody. Although both N- and C-terminal antibodies immunodeplete SCAI efficiently, the C-terminal antibody is better for Western blotting, and we therefore used this antibody throughout the study for immunoblotting. We have generated over 100 peptide antibodies in the laboratory, and it is not uncommon that some antibodies work well for immunoprecipitation or immunodepletion but are quite poor at recognizing the protein in Western blotting under denaturing conditions. To clarify this issue, we have now added panels comparing depletions and Western blotting with both the N- and C-terminal antibodies as requested by the reviewer (new Figure EV2B,C).

-IP of endogenous SCAI should be used to demonstrate the interactions in human cells

We note that REV3 is an extremely low abundant protein in human cells; for instance, proteomic analysis of absolute protein copy numbers has shown that in HeLa cells REV3 is present at only ~130 copies/cell and is among the 100 least abundant quantified proteins in this cell line, with both SCAI and REV7 being present at approx. 100-fold higher levels (Bekker-Jensen et al, 2017). Moreover, commercially available antibodies to detect human REV3 generally show poor performance (Kawamura et al, 2001; Martin & Wood, 2019), likely explaining why we have been unable to detect interaction between the endogenous SCAI and REV3 proteins in human cells. However, we now show that endogenous SCAI co-IPs with GFP-REV3 and endogenous REV1 and REV7 (new Figure EV3F). Moreover, we generated antibodies against Xenopus REV3 and now show by Western blotting that SCAI co-purifies with REV3 IPs in egg extracts (new Figure 4B).

Given our robust data in Xenopus egg extracts, human cells and in vitro binding assays showing that SCAI physically interacts with Polζ, we have provided extensive evidence for conserved interactions between these proteins in vertebrates. These findings were independently confirmed by the recent work from the Elledge laboratory (Adeyemi et al, 2021). However, as mentioned above, our work significantly extends the findings of Adeyemi et al., as we further show that SCAI interacts with Polζ by binding to a conserved peptide motif within the REV3 PCD domain (Figure 4E-G; Figure EV3I), providing a molecular understanding of how this complex is formed.

Minor concerns:
-Fig 1C Fig4B Fig S2B should have a supplementary win uncropped blot to control the specificity of the antibody.

As mentioned above, we have now added uncropped panels comparing depletions and Western blotting with both the N- and C-terminal antibodies against Xenopus SCAI as requested by the reviewer (new Figure EV2B,C).

However, because of limited space (only 5 Extended View figures are allowed), we cannot add uncropped versions of all blots shown in the manuscript. Figure 1C corresponds to human SCAI and because this antibody is commercially available and previously described (Hansen et al, 2016), we did not add the uncropped blot to the manuscript. Instead, the primary data is presented below to the reviewer (Figure R1). If deemed necessary, we will upload all our primary data to a publicly available server (i.e. Mendeley).

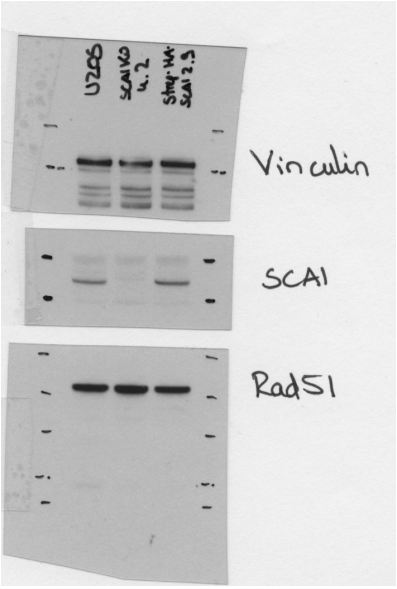

Figure R1: Uncropped immunoblots of Figure 1C.

-Author should replace Fig3C FANCD2 blot by another replicate to improve quality.

We have replaced this blot with another replicate of better quality (new Figure 3E).

-Differences in S2D and S2E are not obvious and quantifications should be provided

The quantification of SapI regeneration denoting repair by HR in the gels shown in Figure EV2F and EV2G is provided in Figure 3H, and a sentence in the legend for these panels (Figure EV2F,G in the revised manuscript) indicates this as follows: "the quantification of this experiment is shown in Figure 3H".

-GFP alone is missing in the Fig 4D

We have added the missing control (new Figure EV3E).

Referee #2:

The manuscript by Mailand, Duxin, and colleagues investigates the role of SCAI (Suppressor of Cancer Cell Invasion) in DNA interstrand crosslink (ICL) repair mediated by the Fanconi anemia (FA) pathway. Using a genome-wide CRISPR-Cas9 dropout screen, the authors have identified SCAI as a strong hit that, when knocked out, sensitizes cells to MMC, an ICL-inducing cytotoxic drug. By employing a Xenopus egg extract ICL repair assay, they further showed that SCAI works downstream of FANCD2 monoubiquitin to promote translesion DNA synthesis (TLS) and homologous recombination (HR). Specifically, SCAI was shown to directly interact with the Rev1-Polz (Rev3-Rev7) TLS complex, although the function of SCAI appears to be independent of its association with Polz. On the other hand, depletion of Polz or its pharmacological inhibition suppressed error-prone double-stranded DNA (dsDNA) repair caused by SCA1 deficiency, suggesting that one of the main roles of SCA1 in the FA pathway is to ensure the execution of faithful DNA ICL repair once incision occurs downstream of FANCD2 ubiquitination, presumably by promoting TLS, HR, or both, thereby preventing deletions and chromosome aberrations originated from Polq-mediated microhomology-mediated end-joining (MMEJ).

This work nicely combines both cellular analysis and in vitro ICL repair assays to elucidate the previously unappreciated role of SCA1 in regulating the FA pathway. Given that SCAI was identified as a 53BP1-binding protein implicated in double-strand break (DSB) repair besides its implication in transcriptional regulation, expanding its role in DNA ICL resolution is considered an important advance in the field. Data are of high quality, and experiments are well controlled both in cells and in Xenopus egg extracts. However, the major concern is that these individual results from in vitro and in vivo experiments do not lead to a coherent and convincing conclusion that can explain how SCAI influences the outcome of DNA ICL repair, especially without any clear mechanistic insight. The most important discovery in cells would be the identification of the Rev3-SCAI interaction, which implies the function of SCAI in TLS; however, Xenopus studies revealed that SCAI works independently of the Rev1-Polz complex, while exhibiting a profound defect in HR. Thus, it is not clear what this novel interaction would mean in DNA ICL repair; in other words, no connection between the SCAI-Rev1-Polz complex and its functional relevance in TLS or HR is described. As a result, the key question of how SCAI channels DNA ICL repair to error-free TLS/HR, while suppressing MMEJ, is not addressed. It is rather confusing to understand whether SCAI directly controls TLS or HR, and whether any of its interaction with the Rev1-Polz complex functionally matters. The authors have indeed mentioned in the discussion that the function of SCAI might be related to regulation of resected ssDNA ends and RAD51 loading, and considering several literatures that support the notion that Rev1 and Polz promote HR repair, SCAI may contribute to those processes. However, again, there is no hypothesis or model on this matter, and their discovery of the Rev3-SCAI interaction does not contribute to the understanding of any potential mechanism behind SCAI in DNA ICL repair. In terms of the role of SCAI in DSB repair, elegant molecular mechanisms have been proposed, which connect its physical and functional interactions with 53BP1 and RIF1, in regards to its association with heterochromatin and regulation by phosphorylation (Hanson 2016; Isobe 2017). Similar levels of mechanistic insight should be present to define the novel role of SCAI in DNA ICL repair, and some of the major points described below need to be addressed to achieve suitability for publications.

1. The Xenopus ICL repair assay shows that SCAI depletion results in a mild TLS delay but complete loss of HR-mediated repair (Figure 3). However, how SCAI loss leads to those defects is not addressed, especially in the context of its interaction with the Rev1-Polz complex. In Figure 4I, it was concluded that the Rev1-Polz interaction is not related to the function of SCAI to suppress deletions. Without making any clear connection, these in vivo interaction data are downgrading in vitro results, almost distracting the key point of how

SCAI antagonizes Polq-mediated MMEJ. Conversely, any potential Rev1-Polz dependent function is not addressed at all. It would considerably strengthen the manuscript if the authors focus on what the Rev1-Polz complex interaction of SCAI would mean in antagonizing Polq activity or directly stimulating HR processes (with some insight into the reason behind its only mild TLS delay despite its stable association with the TLS machinery).

The reviewer raises the valid criticism that we have not been able to address the functional significance of the SCAI-Polζ interaction in ICL repair. Unfortunately, SCAI immunodepletion co-depletes the REV1-Polζ complex in egg extracts (Figure 4H and new Figure 4C), precluding us from assessing Polζ function with or without SCAI. Despite our efforts, we have not been able to produce functional recombinant Polζ, which would allow us to address this question. Generation of full-length vertebrate Polζ remains a major challenge in the field, and to our knowledge no group has so far been able to achieve this.

Importantly, while SCAI may influence Polζ function during ICL repair, our work identifies an additional function of SCAI in preventing illegitimate repair via MMEJ, which is independent of REV1-Polζ, since REV1-Polζ depletion does not recapitulate these effects (Figure 4I). Thus, although SCAI is clearly a direct interactor of REV1-Polζ and may possibly regulate REV1-Polζ activity and/or localization during ICL repair, we show that SCAI has an additional important function in protecting DSBs from MMEJ activity, which is not shared by REV1-Polζ. We concur with the reviewer that describing the SCAI-Polζ interaction could be seen as distracting somewhat from the key point of how SCAI antagonizes Polθ-mediated MMEJ. However, considering the critical role of REV1-Polζ in ICL repair (Kim et al, 2012; Budzowska et al, 2015a; Bezalel-Buch et al, 2020; Hara et al, 2010) and the co-depletion of the complex following SCAI immunodepletion, we believe it is important to report on this interaction, which may further contribute to the strong sensitivity of SCAI knockout cells to MMC and/or explain why Polθ knockdown only partially reverts this sensitivity.

2. The survival curve of Figure 2E claims that preventing upstream FA pathway activation alleviates the ICL hypersensitivity of SCAI-deficient cells. Alternatively, this could be interpreted that FANCA knockdown and SCAI knockout are not epistatic. As a control, downstream FA genes (e.g. BRCA2, FANCJ, FANCP, or FANCV) should be tested in order to determine whether there is a differential survival outcome between genes that regulate FANCD2 activation and execute downstream TLS or HR.

The downstream genes mentioned by the reviewer were targeted by our sgRNA library, yet their loss did not significantly alleviate sensitivity of SCAI knockout cells to MMC (Figure 2) but did increase MMC sensitivity of parental cells (Figure 1) in our genome-scale CRISPR screens. These unbiased screening approaches strongly suggest that the hypersensitivity of SCAI KO cells can be partially reverted by FA pathway activators but not effectors.

3. In Figure 3H, it is not clearly described what the prolonged presence of the nascent strand product and the increase of extension product means let alone how to interpret those data. For those readers without sufficient background on the Xenopus cell extract assay, a little more detailed explanation should be accompanied in interpreting DNA gels.

We have provided additional explanations in the text (page 7-8) to facilitate readers' interpretation of this data. In particular, we now emphasize that the -1 and 0 positions represent nascent strand synthesis across the adduct, and we refer the nascent strand intermediates observed in the gel back to the model in Figure 3A (page 8).

4. In Figure 4, one important experiment should be to show the interaction between endogenous SCAI and endogenous Rev3 (in addition to the Rev1 and SCAI interaction in Figure 4C, which is supposed to be indirect). Moreover, in Figure 4D, a considerable amount of Rev7 was shown to be immunoprecipitated by GFP-SCAI even without its proposed direct interacting partner Rev3. What is the explanation for this?

During the revision of the manuscript, we managed to generate good antibodies against Xenopus REV3. Using these new antibodies, we now show that SCAI co-immunoprecipitates with REV3 in Xenopus egg extracts (new Figure 4B).

The residual interaction between SCAI and REV7 observed in REV3-depleted cells could be due to incomplete knockdown, and/or additional interactions of SCAI with the REV1-POLζ complex that are independent of REV3. Although our data clearly identifies a direct interaction between SCAI and the REV3 PCD domain, we cannot rule out that additional interactions of SCAI with REV1-POLζ components may occur, and we now acknowledge this in the legend for Figure 4D.

5. The lack of a unified model to describe the role of SCAI in DNA ICL repair is evident in the proposed scheme in Figure 6, where the Rev1-Polz complex is not shown in any of the steps engaged in TLS, HR, or MMEJ. Additional experiments may help establish the role of SCAI that forms a complex with the Rev1-Polz complex, and how its interaction contributes to the pathway choice. Being able to position Rev1 and Polz into the proposed model could significantly strengthen the manuscript.

As mentioned above, although SCAI interacts with REV1-Polζ, this interaction is not needed to prevent erroneous repair via MMEJ since no deletions are observed in the absence of REV1-Polζ (Figure 4I). At this point we do not think that we can provide additional experiments regarding the role of REV1-Polζ in ICL repair that go beyond a previous study in Xenopus egg extracts demonstrating its roles in bypassing cisplatin adducts and in homologous recombination (Budzowska et al, 2015b). However, we have further defined our model by pinpointing two potential intermediates during replication-coupled ICL repair that are likely dependent on SCAI function for error-free resolution (new Figure 6). We also discuss this in more detail in the text, taking into account the recent study from the Elledge laboratory, which suggests that the relevant substrate protected by SCAI is a reversed fork (Adeyemi et al, 2021) (page 12-15).

6. To understand whether the Rev1-Polz interaction is important in promoting TLS/HR, one strategy would be to identify and use a SCAI mutant that has lost the interaction with Rev3 and determine whether the mutant is loss of function in the Xenopus ICL repair assay (e.g. suppressing the generation of extension product). Ideally, rescuing SCAI depletion in egg extracts needs to be included to exclude the possibility of non-specific co-depletion of other proteins.

We agree with the reviewer that identifying a SCAI mutant that selectively abrogates its interaction with REV3 would be extremely valuable for separation of function

studies. Despite extensive structure-function analysis of SCAI, we have not been able to pinpoint functional domains and the REV3-binding site in the protein, as most deletions and truncations within SCAI appear to strongly impair its overall functionality (our unpublished observations). One possible explanation for this is that as predicted by the AlphaFold2 protein structure database (https://alphafold.ebi.ac.uk/entry/Q8N9R8) SCAI might exist in a compact and fully folded conformation, which could be prone to disruption by mutations or truncations. Accordingly, at this point we are unfortunately unable to generate and study a mutant SCAI protein that would selectively abolish its interaction with REV3.

To exclude the possibility that the observed effects of SCAI immunodepletion are caused by the co-depletion of other critical factors we performed a whole proteome analysis of SCAI-depleted extracts. As can be seen in new Figure 4C, SCAI and REV3 are the only two proteins that are significantly depleted from these extracts by both antibodies (new Figure 4C; new Table EV4). REV1 and REV7 were co-depleted to a lesser extent, due to their higher abundance in extracts than REV3.

Referee #3:

In this study, the authors seek to investigate the mechanisms that regulate ICL repair downstream of FA pathway activation. They describe a potential role for SCAI at the intersection of translesion synthesis (TLS) and homologous recombination (HR), which, when defective, leads to aberrant repair that creates deletions through microhomology-mediated end joining. Although these findings provide novel insight into the mechanism of ICL repair and identify a new consequence of its dysfunction, evidence specifically pointing to SCAI's role is limited. Great care is taken to separate SCAI's function from REV1/3. However, as shown in Figure 4A, IPs of SCAI are enriched for numerous factors involved in ICL repair. It is not clear whether SCAI merely co-depletes another important factor, or if reducing the level of multiple repair proteins has a cumulative effect on repair efficiency. Both are interesting possibilities, but need to be explored further.

Additional specific comments:
As the focus of the paper, some description of SCAI and its known activities/functions should be provided.

We agree and have now added additional descriptions of SCAI and its previously reported functions in genome maintenance and other processes to the results section (page 4). We have also extended our discussion to contextualize our results in relation to these previous reports by adding a new section on SCAI and repair pathway choice during ICL repair (page 14).

As a 53BP1-interacting protein, how does SCAI knockout affect 53BP1 levels? Mutational inactivation of SCAI would preserve cellular complexes that rely on SCAI and help to specifically implicate SCAI in the repair defects described.

As explained in our response to Referee #2 above, we have not been able to generate SCAI mutants that specifically disrupt its interaction with 53BP1 or REV3, precluding us from studying separation of function mutants. However, we investigated whether the role of SCAI in ICL repair is promoted by 53BP1, which was previously shown to

recruit SCAI to DSBs (Hansen et al, 2016; Isobe et al, 2017). We found that 53BP1 levels were neither affected by SCAI KO in human cells nor SCAI immunodepletion in egg extracts (new Figure EV5A,B). Furthermore, unlike immunodepletion of SCAI, 53BP1 immunodepletion from egg extracts did not induce detectable defects in replication-coupled ICL repair (new Figure EV5C). This suggests that the role of SCAI in ensuring error-free ICL repair is not shared by 53BP1.

SCAI knockout cells are vastly more sensitive to MMC than FANCA knockouts. Is this sensitivity specific to ICLs compared to other forms of DNA damage, as classically seen with knockout of various FA genes?

Yes, this is indeed what we observe - in contrast to their strong sensitivity to ICL-inducing agents, SCAI knockout cells are only mildly sensitive to UV (Figure EV4I) and IR (Hansen et al, 2016), similar to what has been observed for many known FA genes. By generating FANCA KO cells, we also provide further evidence that loss of SCAI gives rise to stronger MMC sensitivity than FANCA KO in a U2OS background (new Figure 2H,J).

Why does loss of SCAI (and increased mmEJ) lead to increased recombination? What is the structure of these intermediates and how does it change?

We are unsure what the reviewer is referring to here. SCAI depletion in egg extracts leads to an absence of recombination (Figure 3H) but elevated amounts of DNA structures migrating in the well (Figure 3F), which we find are strongly dependent on MMEJ (Figure EV5H,I) but have also been suggested to arise from homologous recombination intermediates (Long et al, 2011; Semlow et al, 2016). The precise nature of these well products has not been elucidated by the field, but based on our results we consider it likely that they could be generated via illegitimate fusions occurring between different plasmids.

The authors note that depletion of SCAI may co-deplete CtIP (depending on the antibody used). This seems to reinforce the importance of characterizing what proteins are removed from the extract system and confirming their contributions to the observed defect in some way, ideally using a rescue experiment.

Despite several attempts, our rescue experiments involving add-back of recombinant SCAI protein to SCAI-immunodepleted extracts have been unsuccessful. To characterize in an unbiased way which proteins may be co-depleted by the SCAI antibodies, we have performed whole proteome analysis by label-free MS of extracts following mock, SCAI-N and SCAI-C immunodepletion. Importantly, while both the SCAI N- and C-terminal antibodies co-deplete a few factors in addition to SCAI (which is common for peptide antibodies), combined analysis shows that only SCAI and REV3 are efficiently depleted from extracts by both antibodies (new Figure 4C; new Table EV4). REV1 and REV7 were co-depleted to a lesser extent, due to their higher abundance in extracts than REV3. Importantly, although SCAI IPs also contained many FA pathway activators (Figure 4A), depletion of SCAI did not functionally co-deplete the FA core complex as evidenced by intact ID2 monoubiquitylation during replication-coupled ICL repair (Figure 3C; Figure EV2D; Table EV4) Together, these results suggest that the effects observed are very likely specific to SCAI loss.

Adeyemi RO, Willis NA, Elia AEH, Clairmont C, Li S, Wu X, D'Andrea AD, Scully R & Elledge SJ (2021) The Protexin complex counters resection on stalled forks to promote homologous recombination and crosslink repair. *Mol. Cell*

Amunugama R, Willcox S, Wu RA, Abdullah UB, El-Sagheer AH, Brown T, McHugh PJ, Griffith JD & Walter JC (2018) Replication Fork Reversal during DNA Interstrand Crosslink Repair Requires CMG Unloading. *Cell Rep* **23:** 3419–3428

Bekker-Jensen DB, Kelstrup CD, Batth TS, Larsen SC, Haldrup C, Bramsen JB, Sørensen KD, Høyer S, Ørntoft TF, Andersen CL, Nielsen ML & Olsen JV (2017) An Optimized Shotgun Strategy for the Rapid Generation of Comprehensive Human Proteomes. *Cell Syst* **4:** 587–599.e4

Bezalel-Buch R, Cheun YK, Roy U, Schärer OD & Burgers PM (2020) Bypass of DNA interstrand crosslinks by a Rev1-DNA polymerase ζ complex. *Nucleic Acids Res.* **48:** 8461–8473

Budzowska M, Graham TG, Sobeck A, Waga S & Walter JC (2015a) Regulation of the Rev1-pol complex during bypass of a DNA interstrand cross-link. *The EMBO Journal*

Budzowska M, Graham TGW, Sobeck A, Waga S & Walter JC (2015b) Regulation of the Rev1-pol zeta complex during bypass of a DNA interstrand cross-link. *The EMBO Journal* **34:** 1971–1985

Gari K, Décaillet C, Delannoy M, Wu L & Constantinou A (2008) Remodeling of DNA replication structures by the branch point translocase FANCM. *Proc Natl Acad Sci USA* **105:** 16107–16112

Hansen RK, Mund A, Poulsen SL, Sandoval M, Klement K, Tsouroula K, Tollenaere MAX, Räschle M, Soria R, Offermanns S, Worzfeld T, Grosse R, Brandt DT, Rozell B, Mann M, Cole F, Soutoglou E, Goodarzi AA, Daniel JA, Mailand N, et al (2016) SCAI promotes DNA double-strand break repair in distinct chromosomal contexts. *Nat. Cell Biol.* **18:** 1357–1366

Hara K, Hashimoto H, Murakumo Y, Kobayashi S, Kogame T, Unzai S, Akashi S, Takeda S, Shimizu T & Sato M (2010) Crystal structure of human REV7 in complex with a human REV3 fragment and structural implication of the interaction between DNA polymerase zeta and REV1. *J. Biol. Chem.* **285:** 12299–12307

Isobe S-Y, Nagao K, Nozaki N, Kimura H & Obuse C (2017) Inhibition of RIF1 by SCAI Allows BRCA1-Mediated Repair. *Cell Rep* **20:** 297–307

Kawamura K, O-Wang J, Bahar R, Koshikawa N, Shishikura T, Nakagawara A, Sakiyama S, Kajiwara K, Kimura M & Tagawa M (2001) The error-prone DNA polymerase zeta catalytic subunit (Rev3) gene is ubiquitously expressed in normal and malignant human tissues. *Int J Oncol* **18:** 97–103

Kim H, Yang K, Dejsuphong D & D'Andrea AD (2012) Regulation of Rev1 by the Fanconi anemia core complex. *Nature Structural & Molecular Biology* **19:** 164–170

Long DT, Raschle M, Joukov V & Walter JC (2011) Mechanism of RAD51-Dependent DNA Interstrand Cross-Link Repair. *Science* **333:** 84–87

Martin SK & Wood RD (2019) DNA polymerase zeta in DNA replication and repair. *Nucleic Acids Res.* **47:** 8348–8361

Nelson JR, Lawrence CW & Hinkle DC (1996) Thymine-thymine dimer bypass by yeast DNA polymerase zeta. *Science* **272:** 1646–1649

Semlow DR, Zhang J, Budzowska M, Drohat AC & Walter JC (2016) Replication-Dependent Unhooking of DNA Interstrand Cross-Links by the NEIL3 Glycosylase. *Cell* **167:** 498–511.e14

Zellweger R, Dalcher D, Mutreja K, Berti M, Schmid JA, Herrador R, Vindigni A & Lopes M (2015) Rad51-mediated replication fork reversal is a global response to genotoxic treatments in human cells. *The Journal of Cell Biology* **208:** 563–579

Dear Julien,

Thank you for the submission of your revised manuscript. We have now received the enclosed reports from the referees who all support the publication of your work now. Referee 3 still has a minor suggestions that I would like you to incorporate before we can proceed with the official acceptance of your manuscript.

A few other editorial requests also need to be addressed:

- Please add up to 5 keywords to the manuscript file.

- Please add the heading "Data Availability Section" to this section.

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- Keerthana Viswalingam is missing from the authors contributions. Also is MSL Michael Lisby, and the 'S' should be added on the first page also.

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Referee #1:

Authors have improved significantly the manuscript and argued convincingly when they could not provide additional experiments.

The recent. publication of Elledge laboratory should not impact negatively the publication of this paper. Both research are complementary and make the presented work stronger.

Referee #2:

The authors addressed most of the key concerns raised in the previous review, and the manuscript is now much improved. It is great to see an independent work from the Elledge group that supports the new role of the SCA1-REV3 complex in DNA ICL repair, and this work nicely complements their findings.

Referee #3:

Overall, the authors were quite responsive and have made good effort to address each of the reviewer comments. Although this study does not answer all questions about the role of SCAI at this time, it provides a compelling story that is a useful foundation for future study. As noted, another story from the Elledge lab was recently published on SCAI. I agree with the authors that the findings presented here complement the published story and are worth publication on their own merits due to broad interest from the field.

I would ask that the authors add some discussion about the well products, which are formed in SCAI-depleted reactions. The consequences of repair failure are interesting and could be speculated further. In the authors' general response point 2, they describe how fork reversal may create DSB ends that could be fused by mmEJ. These structures would be highly branched and may explain formation of well products by another mechanism than recombination.

The authors have addressed all minor editorial requests.

Julien Duxin
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Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

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C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	All antibodies used in this study are reported in Methods section with their catalog number or previously used citation.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	All parental cell lines were obtained from ATCC. All cell lines used in this study were regularly tested negative for mycoplasma infection. The cell lines were not authenticated.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD024280. Username: reviewer_pxd024280@ebi.ac.uk Password: TmexTG1y
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	All mass spectrometry proteomics data obtained in this study have been provided (Dataset EV1). In addition, as described above, all mass spectrometry data have been deposited to the ProteomeXchange Consortium.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA