EMBO reports

An AlphaFold2 map of the 53BP1 pathway identifies a direct SHLD3-RIF1 interaction for shieldin activity

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Dear Dan,

Thank you for the submission of your manuscript to EMBO reports. We have now received the full set of referee reports that is pasted below.

As you will see, all referees acknowledge that the findings are interesting. They only have relatively minor concerns that should be addressed. Please let me know in case you have any questions or comments regarding the revisions.

I would thus like to invite you to revise your manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of major revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (11th May 2023). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions.

You can either publish the study as a short report or as a full article. For short reports, the revised manuscript should not exceed 27,000 characters (including spaces but excluding materials & methods and references) and 5 main plus 5 expanded view figures. The results and discussion sections must further be combined, which will help to shorten the manuscript text by eliminating some redundancy that is inevitable when discussing the same experiments twice. For a normal article there are no length limitations, but it should have more than 5 main figures and the results and discussion sections must be separate. In both cases, the entire materials and methods must be included in the main manuscript file.

IMPORTANT NOTE: we perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:
1) A data availability section providing access to data deposited in public databases is missing. If you have not deposited any data, please add a sentence to the data availability section that explains that.
2) Your manuscript contains statistics and error bars based on n=2. Please use scatter blots in these cases. No statistics should be calculated if n=2.

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure). See https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf for more info on how to prepare your figures.

3) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: <https://www.embopress.org/page/journal/14693178/authorguide#expandedview>

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

4) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

5) a complete author checklist, which you can download from our author guidelines <https://www.embopress.org/page/journal/14693178/authorguide>. Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

6) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (<https://orcid.org/>). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines
<https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines>

7) Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database (see https://www.embopress.org/page/journal/14693178/authorguide#datadeposition). Please remember to provide a reviewer password if the datasets are not yet public. The accession numbers and database should be listed in a formal "Data Availability" section placed after Materials & Method (see also https://www.embopress.org/page/journal/14693178/authorguide#datadeposition). Please note that the Data Availability Section is restricted to new primary data that are part of this study. * Note - All links should resolve to a page where the data can be accessed. *
If your study has not produced novel datasets, please mention this fact in the Data Availability Section.

8) At EMBO Press we ask authors to provide source data for the main manuscript figures. Our source data coordinator will contact you to discuss which figure panels we would need source data for and will also provide you with helpful tips on how to upload and organize the files.

9) Our journal also encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at https://www.embopress.org/page/journal/14693178/authorguide#referencesformat

10) Regarding data quantification (see Figure Legends:
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The following points must be specified in each figure legend:

- the name of the statistical test used to generate error bars and P values,

- the number (n) of independent experiments (please specify technical or biological replicates) underlying each data point,

- the nature of the bars and error bars (s.d., s.e.m.),

- If the data are obtained from n Program fragment delivered error ``Can't locate object method "less" via package "than" (perhaps you forgot to load "than"?) at //ejpvfs23/sites23b/embor_www/letters/embor_decision_revise_and_review.txt line 56.' 2, use scatter blots showing the individual data points.

Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.

- Please also include scale bars in all microscopy images.

11) The journal requires a statement specifying whether or not authors have competing interests (defined as all potential or actual interests that could be perceived to influence the presentation or interpretation of an article). In case of competing interests, this must be specified in your disclosure statement. Further information: https://www.embopress.org/competing-interests

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File (RPF) to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

I look forward to seeing a revised form of your manuscript when it is ready. Please use this link to submit your revision:
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Best wishes,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #1:

Sifri et al. used AlphaFold-Multimer to comprehensively define the protein interactions within the 53BP1-Rif1-Shieldin-CST (Ctc1, Stn1, Ten1)-Polalpha/Primase complex, which is a prominent player in DSB repair. Alphafold-multimer faithfully predicted known interfaces (including one dependent on phosphorylation!) as well as previously unknown protein interactions, such as between Ctc1 and Shld2. In addition, they predict a new interface between Rif1 and Shld3 and do all the proper controls in vivo to show that Alphafold-multimer indeed has detected an interaction that was missed by the field. Although the findings do not present a new functional insight, this study provides valuable information for the DDR field that justifies publication in EMBO Reports. Below are a number of points that the authors will want to address before publication.

1. Regarding the AF2 methodology
The authors cut up some of the reading frames. A more rigorous practice is to include short overlaps in the fragments for AF2. Avoiding cuts at structured sites is a good start, but it is always possible that there is a disordered region that folds upon binding (a la Shld1-Ctc1).
Where the authors use AF2 to predict known complexes (CST, SHLD3-REV7) to support the utility of their approach, they should clarify whether AF2 was used in the template free setting (or with max template date prior to the release of the structures in the PDB).
The authors do not mention AMBER relaxation which should be done for any of their predicted structures where side chains are analyzed.

2. The authors mention that Stn1C position is not predicted (Fig 1)
There are several different positions Stn1C can occupy. It appears that their comparison is to PDB 6w6w, where Stn1C is far from Ten1 and Ctc1 OBG. But there are other predicted positions. Does Stn1C match any of the other locations (e.g., CST monomer "head" conformation or the recent PIC conformation (PDB 7u5c)? If Stn1C is predicted with low positional confidence, it shouldn't be shown in Fig. 1.

3. Figure 1 general
The labeling leaves a lot to be desired, making the figure very hard to understand. Where is what? Please label which domain are shown in the structures and the N- and C- termini of each peptide. It is also unclear which region of each subunit is modeled. This could be indicated more clearly in this figure.

4. Figure 3
This reviewer is bewildered by the lines connecting side chains. Is it a salt bridge line between S131 and E70? Also the Q and S in the upper right corner of A have some strange lines. Please label more clearly/unequivocally.

5. Rev7
Several fragments of 53BP1 and RIF1 are predicted to interact with Rev7 via a HORMA-seatbelt interaction. It would be interesting to retry these predictions in the context of Rev7 bound to Shld3 to exclude the possibility of seatbelt-type binding.

6. Interactions with CST
CST has numerous interactions that are not considered in the discussion/analysis of the Shld2-Ctc1 contacts. This reviewer could not work out where in Ctc1 this interaction takes place and whether this interaction could affect the binding of Ctc1 to Stn1, ssDNA, and Pola/primase (in two configurations, RC and PIC) and/or CST dimerization. Please look at this closely and describe.

7. Shld3
Is the Shld3 DNA binding mutant H242A/K243A still able to bind to Rif1? Is this a potential switch for binding? Similarly, does H242A/K243A disrupt Rif1 bindin/localization of Shld3?

8. Model of the complex (Fig. 5)
Please fix the model to more properly reflect where Pola/primase is in the complex. As drawn, it hangs out with Stn1/Ten1 which is not accurate. Particularly, the figure should reflect the binding of Pola/primase to Ctc1 (not just Stn1/Ten1) and the potential clashes between Shld2 v Pola/primase binding to Ctc1 (see point 6).

9. Supplemental Figure 1

Please take out the lines representing rank 1, 2, and 3. They are likely meaningless and a reader not familiar with AF2 might think these are real interactions. Panel A is also presented in Figure 1 and should be removed.

10. Text fix
Where the authors refer to Polalpha they should write Polalpha/primase. Polalpha is not active on its own.

Referee #2:

In this manuscript, Sifri et al. use AlphaFold to map protein-protein interactions within the 53BP1-RIF1-Shieldin complex, a key regulator of DNA double-strand break repair. This allows them to predict key residues mediating interaction between domains in RIF1 and SHLD3, which they then verify using immunoprecipitations and cell-based assays.

This is a nice piece of work that highlights how useful AlphaFold can be to map protein-protein interactions within a complex/pathway, and will thus be of general interest rather than just being confined to those interested in the molecular details of the 53BP1 pathway. That said, I have a few points that should be addressed prior to publication:

1. ASTE1 was not predicted to interact with any of the proteins tested. Could the authors confirm whether ASTE1 recruitment is Shieldin-dependent in human cells? If there are question marks over the results in the Zhao et al. paper, this is important for the field to know.

2. Given that the interaction between 53BP1 and RIF1 is phospho-dependent, could the authors show what effect phospho-mimetic substitution of known S/TQ phosphorylation sites in 53BP1 with aspartic and glutamic acid has in AlphaFold predictions of 53BP1-RIF1 binding?

3. As additional negative controls, can the authors show whether AlphaFold predicts interactions between SHLD3 and the HEAT repeats from proteins other than RIF1, and whether it predicts interactions between RIF1 and eIF4E domains from other proteins? I.e. are these controls we should all be doing when testing AlphaFold predictions?

4. Can AlphaFold predict why the charge reversal mutations for one set of mutations works (to some extent) but the other doesn't? I.e., run predictions with RIF1-D28R and SHLD3-R166D, and with RIF1-R36D and SHLD3-D216R.

5. There is a slight discrepancy between the CSR assay results and binding assays. Could this be because human SHLD3 is being expressed in mouse cells? Would it be informative to show AlphaFold predictions of human SHLD3 binding to mouse RIF1?

6. The title of the paper mentions that direct RIF1-SHLD3 interaction is "critical for DNA repair activity", however the manuscript does not contain any DNA repair assays. Consider changing title.

7. Please annotate the residues in Fig. 1B, panel ii

8. Page 4, 8th line from the bottom: "that binds" should be "that bind". Can the authors please include line numbers in their manuscript as this makes it easier for reviewers.

Referee #3:

This manuscript describes a new method AlphaFold2-Multimer (AF2) to predict an novel binding between the HEAT-repeat domain of RIF1 and the eIF4E-like domain of SHLD3. Both in vitro pulldown analysis and cellular assays demonstrates that RIF1-SHLD3 binding is essential for shieldin recruitment to sites of DNA damage and shieldin-dependent CSR.

This manuscript is interesting and carefully written. Most experiments are well designed. Most data are clear and well-controlled. However, there are some major points that need to addressed.

1、Dose the mutants of RIF1 or SHLD3 reduce the formation of SHLD3, SHLD2, SHLD1 foci upon DNA damage? Why do authors have to show it in complicated mCherry-LacR-FokI system, why not the foci staining widely used in this paper? It should be added the foci results in this paper.

2、Why do authors used different RIF1 mutants (D28A, D28R, D28N) at residues D28 in the figure 4?

3、In figure 5, authors investigated whether mutations abolishing SHLD3-RIF1 binding abrogates shieldin function through CSR. The authors should check whether NHEJ is altered or not. Furthermore, CSR and NHEJ also should be tested in the CH12F3

RIF1-/- cells transduced with lentivirus expressing wild-type RIF1 and mutations.

4, Page 13 line 14 - "bonds with SHLD3 residues W132, R166 + N201, and D216, respectively (Fig 3A)." Please check the "+".

Response to reviewers

Overview

We thank the reviewers for their constructive and valuable comments which we address in the revised manuscript. In addition to our responses to the reviewer comments below, the revised manuscript contains additional data that further support our conclusions, as well as converting the manuscript to the Research Article format to conform to the editorial guidelines of EMBO Reports. Since this required reformatting the figures, we have included a Table for Reviewers summarizing the changes for the benefit of the reviewers. Substantive changes to the manuscript are highlighted for the convenience of the editor and reviewers. We summarize below several key improvements in the manuscript for the benefit of the editor and reviewers:

A) Reviewers 1 and 2 proposed several excellent suggestions to improve our AlphaFold2 (AF2) methodology that we have integrated. First, to avoid missing interactions taking place in the junctions between the fragments of long proteins, we performed a second round of pairwise predictions using fragments spanning these junctions (Fig EV1A). Second, we previously detected multiple 53BP1 and RIF1 fragments predicted to interact with the REV7 seatbelt region which we interpreted as false positives. Repeating the AF2 analysis using these fragments in the presence of SHLD3—the bona fide REV7 interacting partner—completely displaced the 53BP1 and RIF1 fragments and supporting our initial interpretation that these are false positive interaction (Appendix Fig S1). Finally, to increase our confidence in the specificity of the predicted RIF1 HEAT-SHLD3 eIF4E-like interaction, we show that the same RIF1 region is not predicted to interact with five structural paralogues of the SHLD3 eIF4E-like domain (Fig EV2D, Table EV2).

B) Reviewer 1 provided some key insights into the exploration and discussion of the predicted CST-shieldin interfaces that we have integrated into the manuscript. Specifically, we included discussion of two known conformations of the STN1 C-terminus (‘Arm’ and ‘Head’) compared to the AF2-predicted structure (Appendix Fig S2A). We also analyzed the compatibility of the predicted SHLD1- and SHLD2-CTC1 binding sites with Pol α-Primase and determined that they are not mutually exclusive (Appendix Fig S2B-E).

C) Both reviewers 1 and 2 commented on the poor annotation of the protein structures we present in this manuscript, which we have taken to heart. Accordingly, we have labeled all displayed residues, the N- and C-termini, the nature of the electrostatic bonds, as well as the domains and residue boundaries in the figure panels depicting protein structures (Fig 1B, 3A).

D) Reviewer 3 suggested that we show additional validation that the RIF1-SHLD3 interface discovered in this study is biologically relevant. To address this suggestion, we have included new data showing that mutations disrupting this interface results in defective localization of SHLD2 to ionizing radiation (IR)-induced foci (Fig 3E-F) as well as defective complementation of PARP inhibitor (PARPi) toxicity in BRCA1-mutated cells (Fig 6C-D).
E) To conform with the editorial guidelines of EMBO Reports, we have renamed the supplementary figures into Expanded View figures (e.g. Figure S1 to Figure EV1). We have also included an Appendix for additional data that are tangential to the primary findings of the study.

Please find below our response to the comments and suggestions from the reviewers. The comments and suggestions from reviewers are italicized, while our responses to them are organized into bullet points.

Point by point response

Referee #1:

Sifri et al. used AlphaFold-Multimer to comprehensively define the protein interactions within the 53BP1-Rif1-Shieldin-CST (Ctc1, Stn1, Ten1)-Polalpha/Primase complex, which is a prominent player in DSB repair. Alphafold-multimer faithfully predicted known interfaces (including one dependent on phosphorylation!) as well as previously unknown protein interactions, such as between Ctc1 and Shld2. In addition, they predict a new interface between Rif1 and Shld3 and do all the proper controls in vivo to show that Alphafold-multimer indeed has detected an interaction that was missed by the field. Although the findings do not present a new functional insight, this study provides valuable information for the DDR field that justifies publication in EMBO Reports. Below are a number of points that the authors will want to address before publication.

- We are grateful for the reviewer's positive assessment of the manuscript and the many insightful suggestions found below.

1. Regarding the AF2 methodology
The authors cut up some of the reading frames. A more rigorous practice is to include short overlaps in the fragments for AF2. Avoiding cuts at structured sites is a good start,

but it is always possible that there is a disordered region that folds upon binding (a la Shld1-Ctc1).

- The reviewer's assessment that there is the potential for the disordered junctions between the input fragments we used in our AlphaFold2 analysis to coincidentally contain binding sites is correct. In our first analysis, we ascribed a low probability to this occurrence, but in light of this suggestion we performed a second round of pairwise analysis where we predicted potential interactions with fragments spanning +/- 200 residues from the original junction locations. This analysis predicted two new interactions which we show in Figures 1B and EV1A.

Where the authors use AF2 to predict known complexes (CST, SHLD3-REV7) to support the utility of their approach, they should clarify whether AF2 was used in the template free setting (or with max template date prior to the release of the structures in the PDB).

- We have added new text in both the Results and Methods section clarifying that the analysis was performed in the template-free setting.

The authors do not mention AMBER relaxation which should be done for any of their predicted structures where side chains are analyzed.

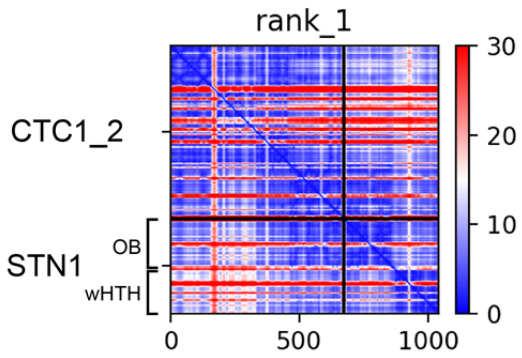
- We thank the reviewer for the suggestion and performed AMBER relaxation for every structure we display that show side chains. Accordingly, we include a description of how we performed AMBER relaxation in the Methods section.

2. The authors mention that Stn1C position is not predicted (Fig 1)
There are several different positions Stn1C can occupy. It appears that their comparison is to PDB 6w6w, where Stn1C is far from Ten1 and Ctc1 OBG. But there are other predicted positions. Does Stn1C match any of the other locations (e.g., CST monomer "head" conformation or the recent PIC conformation (PDB 7u5c)? If Stn1C is predicted with low positional confidence, it shouldn't be shown in Fig. 1.

- This suggestion prompted us to repeat our analysis of the position of the STN1 C-terminal wHTH domain in light of two conformations of CST. In our initial analysis, we compared the AF2 model to the 'Arm' conformation seen in the decameric CST structure (PDB: 6W6W; Lim et al., 2020). CST adopts an

alternative ‘Head’ conformation (shown in high resolution with Pol α-Primase bound in PDB: 8D0K; He et al., 2022). Neither of these two models are consistent with the AF2 prediction, though the ‘Head’ conformation is more similar (Appendix Fig S2A). The CST-Pol α-Primase structure in the recruitment state (PDB: 7U5C; Cai et al., 2022) has a poorly resolved density for STN1C that is consistent with the ‘Head’ conformation.

- The reviewer brings up an excellent point on not displaying STN1C if the confidence is low, however the positional confidence of STN1C in the CTC1_2-STN1 AF2 model is high (see below for the corresponding PAE plot). Accordingly, we believe it is most informative to include it even if it may be inconsistent with experimental data. Instead, we have changed the model used in the comparison in Figure 1B to PDB: 8D0K, since the ‘Head’ conformation is more similar to the AF2 prediction than the previously used ‘Arm’ conformation.



3. Figure 1 general
The labeling leaves a lot to be desired, making the figure very hard to understand. Where is what? Please label which domain are shown in the structures and the N- and C- termini of each peptide. It is also unclear which region of each subunit is modeled. This could be indicated more clearly in this figure.

- We have improved the labeling of the structures displayed in the manuscript. Specifically, the domains and residue boundaries depicted are labeled, with the locations of N- and C-termini indicated as suggested by the reviewer.

4. Figure 3
This reviewer is bewildered by the lines connecting side chains. Is it a salt bridge line between S131 and E70? Also the Q and S in the upper right corner of A have some strange lines. Please label more clearly/unequivocally.

- We agree that the initial depiction can be unclear, therefore we altered our depiction of the predicted electrostatic bonds to improve clarity. First, we use black dotted lines for hydrogen bonds and red dotted lines for salt bridges in Figures 1B and 3A. Second, we reduced the number of electrostatic interactions shown between RIF1 and SHLD3 in Figure 3A to only those further analyzed in this study to reduce the clutter in this panel and improve its readability.

5. Rev7
Several fragments of 53BP1 and RIF1 are predicted to interact with Rev7 via a HORMA-seatbelt interaction. It would be interesting to retry these predictions in the context of Rev7 bound to Shld3 to exclude the possibility of seatbelt-type binding.

- We pursued this excellent suggestion by repeating the same predictions in the presence of SHLD3(1-100) which encompasses the SHLD3-REV7 binding interface (Appendix Fig S1). Interestingly, in all cases the SHLD3(1-100) fragment displaces the 53BP1 and RIF1 fragments that, in the absence of SHLD3, are predicted to bind within the REV7 seatbelt. This analysis supports our initial supposition that the predicted 53BP1/RIF1-REV7 seatbelt interactions are likely spurious.

6. Interactions with CST
CST has numerous interactions that are not considered in the discussion/analysis of the Shld2-Ctc1 contacts. This reviewer could not work out where in Ctc1 this interaction takes place and whether this interaction could affect the binding of Ctc1 to Stn1, ssDNA, and Pola/primase (in two configurations, RC and PIC) and/or CST dimerization. Please look at this closely and describe.

- We have included additional discussion on the predicted SHLD1- and SHLD2-CTC1 binding interface positions in the Results section. SHLD1 binds CTC1 on the face consisting of OB-ABC and the hinge three-helix bundle (Appendix Fig S2B), an interface which is not occupied by Pol α-Primase or other CST subunits.

- SHLD2 binds CTC1 on the face consisting of OB-DFG in the same binding site as ssDNA (Appendix Fig S2C). Interestingly, in the context of the PIC conformation (PDB: 8D0K) SHLD2 binds CTC1 directly adjacent to the POLA1 exonuclease and catalytic domain in a non-mutually exclusive manner (Appendix Fig S2D). In the recruitment conformation (PDB: 7U5C), Pol α-Primase binds an entirely separate face of CTC1 that is compatible with SHLD2 (Appendix Fig S2E).

- CST dimerizes with the ssDNA-binding site of two monomers oriented towards each other (Lim et al., 2020). As SHLD2 binds in the ssDNA-binding site of CTC1, it is sterically incompatible with CST dimerization.

7. Shld3
Is the Shld3 DNA binding mutant H242A/K243A still able to bind to Rif1? Is this a potential switch for binding? Similarly, does H242A/K243A disrupt Rif1 bindin/localization of Shld3?

- This is an interesting suggestion as it may speak to a link between the putative ssDNA-binding activity of SHLD3 with its RIF1-mediated recruitment to sites of DNA breaks. We tested the SHLD3 DNA-binding H242A/K243A mutant using the LacO-LacR assay to test whether this mutant can interact with RIF1 (Fig EV3F-H). The SHLD3^{C} H242A/K243A is efficiently recruited to the LacO array by LacR-RIF1^{N}, suggesting that H242/K243 do not participate in RIF1 binding or SHLD3 localization.

8. Model of the complex (Fig. 5)
Please fix the model to more properly reflect where Pola/primase is in the complex. As drawn, it hangs out with Stn1/Ten1 which is not accurate. Particularly, the figure should reflect the binding of Pola/primase to Ctc1 (not just Stn1/Ten1) and the potential clashes between Shld2 v Pola/primase binding to Ctc1 (see point 6).

- To better depict the schematic representation of CST-Pol α-Primase, we altered Fig 5E (now Fig 6E) to show Pol α-Primase in contact with CTC1, STN1, and TEN1. Since we did not detect potential clashes with the predicted SHLD1 and SHLD2 binding sites, we depicted Pol α-Primase as distal to SHLD1 and SHLD2 for the sake of clarity.

9. Supplemental Figure 1
Please take out the lines representing rank 1, 2, and 3. They are likely meaningless and a reader not familiar with AF2 might think these are real interactions. Panel A is also presented in Figure 1 and should be removed.

- In accordance with the reviewer's suggestion, we have removed lines in Fig EV1A (previously S1B) corresponding to 1-3 predicted interactions except for the 53BP1 oligomerization domain that is supported by experimental evidence. We have also removed the scatterplot of pDockQ and interface PAE scores from Fig EV1 (previously S1A) since it is shown in Fig 1A.

10. Text fix
Where the authors refer to Polalpha they should write Polalpha/primase. Polalpha is not active on its own.

- We thank the reviewer for this clarification and accordingly changed all instances of CST-Pol α to CST-Pol α-Primase.

Referee #2:

In this manuscript, Sifri et al. use AlphaFold to map protein-protein interactions within the 53BP1-RIF1-Shieldin complex, a key regulator of DNA double-strand break repair. This allows them to predict key residues mediating interaction between domains in RIF1 and SHLD3, which they then verify using immunoprecipitations and cell-based assays.

This is a nice piece of work that highlights how useful AlphaFold can be to map protein-protein interactions within a complex/pathway, and will thus be of general interest rather than just being confined to those interested in the molecular details of the 53BP1 pathway. That said, I have a few points that should be addressed prior to publication:

- We thank the reviewer for their positive assessment of our study, especially since as the reviewer suggests, proposing a generalizable framework for interrogating a pathway for novel protein-protein interactions using AlphaFold2 is one of our goals with this manuscript.

1. ASTE1 was not predicted to interact with any of the proteins tested. Could the authors confirm whether ASTE1 recruitment is Shieldin-dependent in human cells? If there are question marks over the results in the Zhao et al. paper, this is important for the field to know.

- We understand the reviewer's concern with regards to the lack of predicted interaction between ASTE1 and the other members of the 53BP1-RIF1-shieldin-CST pathway. However, we are careful to not interpret the lack of predicted interaction as evidence of a true lack of interaction, especially since we focused on a set of stringent criteria for predicted interactions to minimize false positives. For example, in the study calibrating the pDockQ score utilized in our manuscript, Bryant et al. describes a true positive rate of 66% at a false positive rate of 5% on a validation set of heterodimeric crystal structures (Bryant et al., 2022). Although extremely powerful, the current AlphaFold2 is far from being able to predict every protein binding event.

- Instead of trying to explore undetected interactions, we instead focused our manuscript on the high-confidence RIF1-SHLD3 interaction which we validated through a series of in vitro experiments and cellular assays. Although we agree that verifying the ASTE1 involvement in this pathway is an important task, we believe that it is beyond the scope of our study.

2. Given that the interaction between 53BP1 and RIF1 is phospho-dependent, could the authors show what effect phospho-mimetic substitution of known S/TQ phosphorylation sites in 53BP1 with aspartic and glutamic acid has in AlphaFold predictions of 53BP1-RIF1 binding?

- We agree that if AlphaFold2 can accurately predict the structural effect of phosphomimetic mutations, the suggested analysis would be an excellent approach to characterize potential phosphorylation-dependent interactions. However, due to the importance of natural amino acid residue covariation encoded within the sequence alignments used as inputs to the AlphaFold2 neural network, artificially substituting residues to simulate point mutations is ineffective. A recent study by Pak et al. determined a very poor correlation between the predicted ΔpLDDT and the measured ΔΔG values of individual mutations (Pak et al., 2023). In our own experience, introducing point mutations that are known to completely disrupt an interaction has little effect on the predicted protein-protein interface. For these reasons, we believe that using aspartic or glutamic acid to model protein phosphorylation will be uninformative.

3. As additional negative controls, can the authors show whether AlphaFold predicts interactions between SHLD3 and the HEAT repeats from proteins other than RIF1, and whether it predicts interactions between RIF1 and eIF4E domains from other proteins? I.e. are these controls we should all be doing when testing AlphaFold predictions?

- This is a very interesting idea to search for potential biases in AlphaFold2 between the RIF1 HEAT-repeats and eIF4E-like domains in general. To test this approach, we used AF2-Multimer to predict RIF1 residues 1-615 with five different paralogues of the SHLD3 eIF4E-like domain: three known human paralogues (eIF4E, eIF4E-2, and eIF4E-3) and two structural paralogues of uncharacterized proteins (A0A1Y2WIF9 from Hypoxylon sp. And A0A6A4RT61 from Scophthalmus maximus) identified from the AlphaFold2 database by the FoldSeek algorithm (Kempen et al., 2023).

  Out of the 25 models predicted, only 1 meets the pDockQ and interface PAE criteria in our study. Since we used a cutoff of four out of five models containing a

predicted interaction, none of these pairs would have been categorized as a hit in our study (Fig EV2D).

- Using the same approach for HEAT repeat paralogues is complicated, since it is a configuration of alpha-helical repeats that occur in many conformations and sizes as opposed to a well-defined fold.
- We believe that using structural orthologues to detect false positives may be useful in initial validation of AF2-predicted interactions. A previous study of an all vs. all protein-protein interaction prediction in yeast using a similar neural network (RoseTTAFold) identified a subset of proteins that formed hubs of spurious predicted interactions (Humphreys et al., 2021). Indeed, REV7 in our analysis behaves like this, with five predicted interactions through the same seatbelt interface, only one of which is supported by experimental data. Developing a framework using structural or sequence paralogues to detect erroneous interaction hubs will be an intriguing topic for future study.

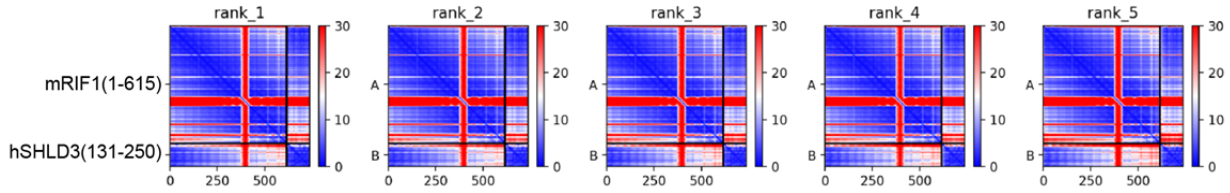
4. Can AlphaFold predict why the charge reversal mutations for one set of mutations works (to some extent) but the other doesn't? I.e., run predictions with RIF1-D28R and SHLD3-R166D, and with RIF1-R36D and SHLD3-D216R.

- Although this would have been a tremendously useful feature, please see our response to point 2 on the limitation of AlphaFold2 with artificially introduced mutations.

5. There is a slight discrepancy between the CSR assay results and binding assays. Could this be because human SHLD3 is being expressed in mouse cells? Would it be informative to show AlphaFold predictions of human SHLD3 binding to mouse RIF1?

- We thank the reviewer for their suggestion and performed the AF2 prediction of human SHLD3 with mouse RIF1. As shown by the PAE plot below, AF2 still predicts a high-confidence interaction between the mouse and human proteins. RIF1 is a generally a highly conserved protein, and mouse RIF1 share 71.4% sequence identity with human RIF1 leading us to our initial belief that it is compatible with human SHLD3 which is supported by the ability of human SHLD3 to complement CH12F3 *Shld3*$^{-/-}$ mouse B cell lymphomas in class switch recombination.

  Our interpretation of the discrepancy in the CSR assay is that the partial DNA damage recruitment and RIF1 interaction observed in the SHLD3 N201A and D216A variants that is absent in the W132A and R166A variants (Fig 3C-D) are sufficient to recapitulate SHLD3-mediated CSR, at least in the context of SHLD3 overexpression.



6. The title of the paper mentions that direct RIF1-SHLD3 interaction is "critical for DNA repair activity", however the manuscript does not contain any DNA repair assays. Consider changing title.

- The reviewer's point is well received. We have therefore changed "DNA repair activity" to "shieldin function", especially in light of the new data we include showing that the SHLD3-RIF1 interface is essential for SHLD3's role in mediating PARPi sensitivity in BRCA1-mutated cells (Fig 6C-D).

7. Please annotate the residues in Fig. 1B, panel ii

- We have annotated the residues shown in Fig 1B panel ii and also included an analysis of their sequence conservation in Fig EV1H-I.

8. Page 4, 8th line from the bottom: "that binds" should be "that bind". Can the authors please include line numbers in their manuscript as this makes it easier for reviewers.

- We apologize for the inconvenience and have added line numbers in the manuscript and have integrated the suggested change.

Referee #3:

This manuscript describes a new method AlphaFold2-Multimer (AF2) to predict an novel binding between the HEAT-repeat domain of RIF1 and the eIF4E-like domain of SHLD3. Both in vitro pulldown analysis and cellular assays demonstrates that RIF1-SHLD3 binding is essential for shieldin recruitment to sites of DNA damage and shieldin-dependent CSR.

This manuscript is interesting and carefully written. Most experiments are well designed. Most data are clear and well-controlled. However, there are some major points that need to addressed.

- We thank the reviewer for their positive appraisal of the manuscript and address their major points below.

1、Dose the mutants of RIF1 or SHLD3 reduce the formation of SHLD3, SHLD2, SHLD1 foci upon DNA damage? Why do authors have to show it in complicated mCherry-LacR-FokI system, why not the foci staining widely used in this paper? It should be added the foci results in this paper.

- Our initial choice to use the LacR-FokI system is due to our previous observation that shieldin IR-induced foci are weaker in intensity relative to 53BP1 and RIF1 foci. Using the LacR-FokI system allowed for quantification of small differences in DNA damage recruitment due to the strong and focused signal, such as in the case of the N201A and D216A mutants (Fig 3D). However, shieldin does form IR-induced foci, which we show in Fig 3E-F. Consistent with the LacR-FokI experiments, disrupting the predicted RIF1-SHLD3 interface results in loss of SHLD3 and SHLD2 IR-induced foci (Fig 3E-F).

2、Why do authors used different RIF1 mutants (D28A, D28R, D28N) at residues D28 in the figure 4?

- We regret the confusion and hope that the following explanation will clarify the mutations for the reviewer. The D28A mutant was designed as an alanine substitution which is a standard mutation for testing the function of specific residues.
- The D28R mutant was designed as a charge reversal experiment: If the predicted salt bridge between RIF1^{D28} and SHLD3^{R166} residues is correct, then switching the charges by reversing the involved residues (Through RIF1 D28R and SHLD3 R166D mutations) may still be compatible with RIF1-SHLD3 binding. The charge reversal rescue was not observed for RIF1^{D28R} and SHLD3^{R166D}, likely due to additional roles for SHLD3^{R166} within the interface, while the charge reversal pair of RIF1^{R36D} and SHLD3^{D216R} was able to rescue RIF1-SHLD3 interaction (Fig 3D).
- The RIF1 D28N mutation was introduced endogenously in the U2OS 2-6-3 cell line using base editors targeted through a nuclease-dead Cas9. Due to the nature of the base editor used, we were limited in which base substitutions we can induce, thus excluding the same D28A mutation used in previous experiments. Based on this limitation, we chose to induce the D28N mutation.

3、In figure 5, authors investigated whether mutations abolishing SHLD3-RIF1 binding abrogates shieldin function through CSR. The authors should check whether NHEJ is altered or not. Furthermore, CSR and NHEJ also should be tested in the CH12F3 RIF1-/- cells transduced with lentivirus expressing wild-type RIF1 and mutations.

- We fully agree with the reviewer's opinion that additional experiments interrogating the effect of disrupting RIF1-SHLD3 interaction on shieldin function should be performed. However, although 53BP1-associated processes positively affect NHEJ, 53BP1 and its associated proteins are not core NHEJ factors as evidenced by its modest role in IR sensitivity and V(D)J recombination compared to LIG4 (Grawunder et al., 1998; Riballo et al., 2004).

  Instead of assaying NHEJ activity—which is partially reflected in the CSR efficiency that we already tested—we decided to evaluate the ability of SHLD3 variants to complement PARPi sensitivity, which is a characteristic function of the 53BP1-RIF1-shieldin-CST pathway. We complemented RPE *BRCA1-KO SHLD3-KO* cells with SHLD3 variants and measured their relative growth in the presence of the PARPi, olaparib, and found that the W132A and R166A mutants are unable to restore PARPi sensitivity (Fig 6C-D). These results are fully consistent with our pulldown, DNA recruitment, and CSR experiments, and further strengthens our conclusion that RIF1-SHLD3 binding through the AF2-predicted interface is essential for shieldin function.

- Although we concur with the reviewer that repeating the functional experiments from the point of view of RIF1 complementation could bolster our conclusions, these experiments are nontrivial. RIF1 is a massive 2472 residue protein, making complementation by viral transduction extremely challenging. Since we showed that disrupting the RIF1 residues involved in the RIF1-SHLD3 interface disrupts their interaction and the DSB localization of shieldin, and the corresponding mutations in SHLD3 disrupts both CSR and PARPi resistance, we are confident in our conclusion that this interface is essential for shieldin function.

4, Page 13 line 14 - "bonds with SHLD3 residues W132, R166 + N201, and D216, respectively (Fig 3A)." Please check the "+".

- We apologize for the confusion and have changed the text to "both R166 and N201" to clarify that both these residues are predicted to form electrostatic bonds with $RIF1^{D28}$.

Table for Reviewers 1. List of changes to figures and tables

Original	Revised
Fig 1B	Labeled termini, residues, domains. Added panel vi (new SHLD2-SHLD2 interaction). Changed H-bond and salt bridge colors.
Fig 3A	Removed non-conserved bonds. Changed H-bond and salt bridge colors.

-	Introduced Fig 3E-F.
Fig 3E-F	Moved to Fig 4A-B.
Fig 3G-H	Moved to Fig 4D-E
Fig 4	Moved to Fig 5.
Fig 5A	Moved to EV5C.
Fig 5B-C	Moved to Fig 6A-B.
Fig 5D	Moved to Fig 6E.
-	Introduced Fig 6C-D.
Fig EV1A	Removed.
Fig EV1B	Moved to Fig EV1A, added junction fragments, removed lines corresponding to 1-3 predicted interactions.
Fig EV1C-L	Moved to Fig EV1B-K. Added more conservation analysis to Fig EV1H-I.
-	Introduced Fig EV1L.
Fig EV2D-I	Moved to Fig EV2E-J.
-	Introduced Fig EV2D.
Fig EV3F	Moved to Fig EV3I.
Fig EV3G	Moved to Fig 4C.
-	Introduced Fig EV3F-H and EV3J.
Fig EV5C-D	Moved to Fig EV5D-E.
-	Introduced Fig EV5C.
-	Introduced Fig EV5F.
-	Introduced Appendix Figures 1 and 2, Tables EV1 and EV2.

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I have looked at the revisions and the authors have addressed each of my concerns.

Referee #2:

The authors have done a great job addressing all reviewers' comments and I would like to congratulate them for the innovative and elegant work described in this manuscript.

Referee #3:

The author solved my concerns and the revision meet the requirements of the review.

The authors have addressed all minor editorial requests.

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Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Yes	Materials and Methods
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.	Not Applicable	
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.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Yes	Data Availability
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Yes	Data availability
If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	