nature portfolio

Peer Review File

DSS1 Restrains BRCA2's Engagement with dsDNA for Homologous Recombination, Replication Fork Protection, and R-loop Homeostasis

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

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BRCA2 is a critical tumor suppressor gene whose alterations are involved in breast, ovarian, and other cancers. BRCA2 plays important roles in homologous recombination (HR), replication fork protection (RFP), and R-loop suppression. DSS1 binds to the helical domain and OB fold1 in the C-terminal region of BRCA2 and functions in HR together with BRCA2. In this study, the authors found ss/dsDNA binding abilities in the HD-OB1 subdomain of BRCA2 and that DSS1 negatively regulates HD-OB1's dsDNA binding to promote ssDNA targeting of the BRCA2-RAD51 complex. The cancer-derived DSS mutation R57Q and the C-terminus deletant abolish this DSS function and enhance the dsDNA binding of HD-OB1/BRCA2-DBD. These mutants impair BRCA2/RAD51 ssDNA loading and focus formation, decrease HR activity, destabilize stalled forks, cause R-loop accumulation, and make cells sensitive to DNA-damaging agents. Thus, they proposed the model for DSS in shutting down the DNA binding ability of HD-OB1 to promote BRCA2's function.
The experiments in this study were very well done, the data are high quality, and the manuscript is clearly written. These findings have outstanding importance, and their models of the regulatory mechanism of DSS and BRCA2 for HR, RFP and R-loop suppression are fascinating. Minor points should be fixed in the revised manuscript for publication in Nature Communications.

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General concerns:
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dsDNA binding would be essential to provide experimental evidence to support the claim of dsDNA regulation in majority of cell-based phenotypes. Comparison to CTRB mutants would be also essential to analyse the contribution of individual domains involved in this process (Kwon et al 2023, with this lab contributing to the paper).
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We are extremely pleased that the Reviewers recognize the outstanding importance of our findings regarding the role of DSS1 in restricting BRCA2's ability to bind to dsDNA, acknowledging that it makes a significant new contribution to the field and will be of great interest to many researchers. We have considered the comments carefully, and have, in the past three months, performed a large number of new experiments to address these comments. Currently, we have 7 Figures and 12 Supplemental Figures in our revised manuscript. All changes in the revised manuscript are highlighted in blue.

Our point-by-point response to the critiques follows.

Reviewer #1

Overall: The Reviewer noted that our study makes the surprising discovery of a new role for DSS1 in regulating BRCA2, providing an important new contribution to the field that will be of great interest to many researchers. Furthermore, the Reviewer found our study to be thorough and experimentally sound, with results that convincingly demonstrate DSS1's role in promoting homologous recombination, stabilizing replication forks, and suppressing R-loop accumulation.

We are grateful to the Reviewer for his/her enthusiasm and support. Below, we detail how we have addressed the Reviewer's thoughtful points in the revised manuscript.

1. Page 4. 1st paragraph: "…we found that miBRCA2-DSS18A exhibits a similar ability to target RAD51 on ssDNA for RAD51 presynaptic filament formation as miBRCA2-DSS1 and is much more efficient that miBRCA2 in the same regard." Just a simple point of clarification, it's probably good to note in the main text that RPA is not present in the magnetic bead pulldown assays.

Response: Thank you for the suggestion. The description "in the absence of RPA" is now included in the revised manuscript (page 4) for clarification.

2. *The study reports numerous different DNA binding assays but only reports an apparent Kd value for OB23. It would be very important for the DNA repair community if the authors could report Kd values for all of the proteins that are assayed.*

Response: Thank you for your suggestion. As requested, the apparent Kd values for all the proteins assayed in this study are summarized and shown in Supplementary Fig. 3e and 4c.

3. *Page 6, top: "…increasing the KCL concentration in the EMSA leads to expression of DNA binding…" Maybe the word expression should be changed to "enhanced".*

Response: Thank you for pointing this out and we have changed "expression of" to "enhanced" (page 6).

4. Given the totality of the data, do the authors predict that BRCA2 mutants in the HDOB1 domain that disrupt dsDNA binding should exhibit in vitro and in vivo behaviors similar to WT BRCA2 plus or minus DSS11-54 or DSS1R57Q? Is it known whether such mutants exist in patient databases? Or, could such

mutants be constructed? Such mutants could be important to test because given the data presented in the manuscript, there is seemingly no known function for BRCA2 dsDNA binding activity.

Response: Thank you for bringing up this point. We suspect that there are two potential scenarios: In a straightforward model (Model 1), the DNA binding activity of HDOB1 must be shielded for any active role of BRCA2 (*i.e.,* the open status is an inactive form for storage or other purposes) in cells. We believe that the BRCA2 SOF (separation of function) mutants that specifically disrupt only dsDNA binding of HDOB1 (termed BRCA2$^{HDOB1\text{-}dsDNA\text{-}SOF}$) should behave similarly to BRCA2^{WT}-DSS1 or BRCA2 PIR (PARP-inhibitor-resistant) mutants (Edwards et.al., *Nature*. 2008 Feb 28;451(7182):1111-5, PMID: 18264088; Sakai et. al., *Nature*. 2008 Feb 28;451(7182):1116-20, PMID: 18264087) both *in vitro* and in cells. On the other side (Model 2), it is also plausible that the transition between open (high affinity for DNA binding) and closed (low affinity for DNA binding) configurations is dynamic and crucial for BRCA2-DSS1 to effectively engage and disengage with dsDNA or ssDNA substrates during different phases of homologous recombination reactions, replication fork maintenance, and R-loop management. Under this Model 2, BRCA2$^{HDOB1\text{-}dsDNA\text{-}SOF}$ mutants would lack the high-affinity binding state necessary for engagement, potentially leading to BRCA2 deficiency and genome instability.

It is not yet known whether such mutants exist in patient databases, but it is clear that numerous patient pathogenic mutations occur in this HDOB1 region (see Figure R1a), which might affect either DNA binding, DSS1 interaction, or both. It is therefore of significant interest to systematically test these pathogenic mutants and maybe also variants of uncertain significance (VUS) in the same region to construct the required SOF mutants. We fully concur that such SOF mutants, especially BRCA2$^{HDOB1\text{-}dsDNA\text{-}SOF}$ mutants, are crucial for elucidating the physiological function of BRCA2's dsDNA binding activity. An intriguing potential role for this HDOB1 dsDNA binding and its regulation by DSS1 is to facilitate the initial engagement of the BRCA2-RAD51 complex on the dsDNA region, which enables dynamic sliding along dsDNA to the ds-ssDNA junction, as outlined in a recent paper by the group of Dr. Simon Boulton (Belan et.al., *Mol Cell*. 2023 Aug 17;83(16):2925-2940.e8. PMID: 37499663). This diffusion-facilitated delivery mechanism may trigger RAD51 nucleation on ssDNA emanating from the ds-ssDNA junction. In this context, our hypothesis is that the novel dsDNA binding capability inherent to HDOB1, as discovered in this study, could contribute significantly to the sliding dynamics of BRCA2. This process might play a vital role in not only BRCA2-DSS1 mediated homologous recombination, but also R-loop resolution and safeguarding DNA replication fork integrity as suggested in this study.

Interestingly, based on the DBD-DSS1 crystal structure reported by Yang et al. (*Science*, 2002 Sep 13;297(5588):1837-48. PMID: 12228710), we predicted and constructed several OB1 mutations (*e.g.*, mutant1: L2736A, V2739A, R2744A, L2745A; mutant2: W2725A, F2801A; mutant3: K2750A) to weaken its interaction with DSS1. While there was a solubility issue with mutant2 and mutant3, we were able to purify HDOB1^{mut1}-DSS1 and HDOB1^{mut1}-DSS1$^{1\text{-}54}$, and found that these two mutants exhibit stronger ds/ssDNA binding than their wild type counterparts respectively (Figure R1b & c and data not shown). This suggests that weakening the DBD-DSS1 complex through OB1 mutations (in addition to the DSS1 C-terminal helix mutations shown in our manuscript) can also release the DNA binding capacity of HDOB1. Consistently, Alvaro-Aranda, Lucia et al. recently reported that the BRCA2 R2645G variant in the HD domain weakens its binding to DSS1 but increases ssDNA binding (*Nucleic Acids Research*, 2023, Dec 24: gkad1222. PMID: 38142462). Thus, we believe a list of positive and aromatic residues (*e.g.,* R2625I,

K2729N, W2619) that are not involved in DSS1 binding but are defective in homologous recombination, as demonstrated by the DR-GFP reporter assays (*e.g.*, Hu et. al., *Clin Cancer Res*. 2022 Sep 1;28(17):3742-3751. PMID: 35736817), could be potential candidates for the SOF mutants in scenario of Model 2. Should Model 1 is correct, we may also need to consider variants that cause no deficiencies in homologous recombination (see above hypothesis) for identifying the BRCA2$^{HDOB1\text{-}dsDNA\text{-}SOF}$ mutants. Of note, DSS1 is negatively charged and has been reported to function by mimicking DNA (Yang et. al., *Science*, 2002 Sep 13;297(5588):1837-48. PMID: 12228710 Science, 2002; Zhao et. al., *Mol Cell*. 2015 Jul 16;59(2):176-87. PMID: 26145171). Therefore, it is conceivable that some residues involved in DSS1 binding might also play a role in DNA binding when in the open state. The optimal approach to clarify this relationship would be to determine the structure of the DBD-DSS1 complex in its open state bound to dsDNA, as further discussed in addressing the point (#1-3) of the Reviewer 3.

Figure R1

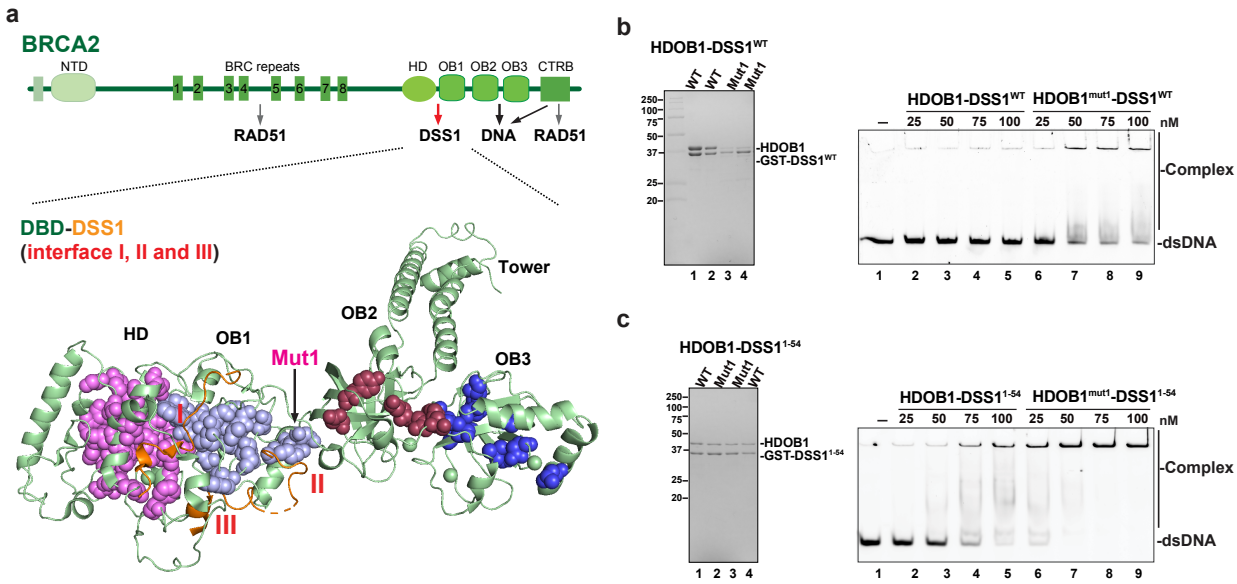

Figure R1. Pathogenic mutations enriched in HDOB1 and characterization of HDOB1^{mut1} in complex with DSS1 or DSS1$^{1\text{-}54}$. a. Schematic of BRCA2 and its domains (HD, OB1, OB23) and partners (DSS1, RAD51 and DNA) (top). Germline pathogenic variants (PVs), which have also been demonstrated to impair BRCA2's function in homologous recombination assays, in DBD subdomains of BRCA2 are highlighted in crystal structures of DBD-DSS1 (PDB: 1MIU; bottom). Pale green: DBD; Orange: DSS1; Violet: PVs in HD domain; Sky-blue: PVs in OB1 domain; Raspberry: PVs in OB2 domain; Tv-blue: PVs in OB3 domain. b. SDS-Page of purified HDOB1-DSS1^{WT} and HDOB1^{mut1}-DSS1^{WT} under 500 mM KCl condition (left, note the mut1 reduces its ability to form complex with DSS1^{WT} at high salt) and binding of dsDNA by HDOB1-DSS1^{WT} (lanes 2-5) and HDOB1^{mut1}-DSS1^{WT} (lanes 6-9). c. SDS-Page of purified HDOB1-DSS1$^{1\text{-}54}$ and HDOB1^{mut1}-DSS1$^{1\text{-}54}$ under 150 mM KCl condition (left, note the mut1 retains its ability to form complex with DSS1$^{1\text{-}54}$ at low salt) and binding of dsDNA by HDOB1-DSS1$^{1\text{-}54}$ (lanes 2-5) and HDOB1^{mut1}-DSS1$^{1\text{-}54}$ (lanes 6-9).

5. There are some minor typos and grammatical errors throughout that could be addressed with further proofreading.

Response: Thanks for pointing this out and we've now fixed typos and grammatical errors throughout the manuscript in blue, such as
page2 paragraph1 line3: "that" to "which".
page3 paragraph2 line18: "dsDNA" to "the dsDNA"; line19: "proper" to "the proper".

page4 paragraph3 line4: “DNA mobility shift” to “A DNA mobility shift assay”.
page5 paragraph3 line3: add “that” after “DSS1 mutants”.
page6 paragraph1 line2: “salt” to “the salt”; line6 “cancer associated” to “cancer-associated”; last line: “fullfil” to “fulfill”.
page6 paragraph2 line9: “template” to “a template”.
page8 paragraph1 line4: “comparation” to “comparison”.
page8 paragraph2 line14: “production” to “the production”; line18: “leads” to “lead”.
page11 paragraph2 line6: “regiments” to regimens”.

Reviewer #2

Overall: The Reviewer noted the outstanding importance of our findings and described our models of the regulatory mechanisms of DSS1 and BRCA2 in HR, RFP, and R-loop suppression as fascinating. Additionally, the Reviewer commended the high quality of our data, the thoroughness of our work, and the clarity of our manuscript.

We are grateful for the Reviewer's enthusiasm and recognition of our work. Below, we detail how we have addressed all the points raised.

1. Statistical significances should be presented in Figures 1c, 1d, 2d, 2e, 3d, 3f, and 5c, and Extended Data Fig.1f, 1h, 5b, 7g, and 5G.

Response: Thank you for this suggestion. The statistical significances have been included in the Figures 1c, 2d, 2e, 3d, 3f, 5c, 5d, and Supplementary Figs.1f, 1h, 1j, 6b, 7b, 9a, 11c of the revised manuscript. For Figure 1d, the statistical comparisons for miBRCA2 vs. miBRCA2-DSS1 and miBRCA2-DSS1^{8A} are detailed in the figure legend due to the inconvenience of labeling them directly in the figure.

2. In Extended Data Fig.7a, expression levels of DSS-WT and DSS-R57Q are markedly low compared with DSS-1-54. The authors should comment on this.

Response: Thank you for giving us the opportunity to explain it as we also noticed the differences for the expression level of endogenous DSS1 in different stable cell lines. It is intriguing and we have also previously reported (Zhao et. al., *Mol Cell*. 2015 Jul 16;59(2):176-87. PMID: 26145171) that ectopic overexpression of DSS1, particularly the wild type form, typically reduces the levels of endogenous DSS1. In this study, we repeated and confirmed that the exogenous expression of DSS1 (whether wild type, 1-54, or R57Q) significantly inhibits the expression of endogenous DSS1 to maintain a balanced total protein level, and have now included these results in another improved blot in Supplementary Fig. 10a (originally Extended Data Fig. 7a). Furthermore, we consistently observed higher levels of endogenous DSS1 in cells expressing DSS1$^{1-54}$ compared to those expressing DSS1^{WT}, similar to previous findings with DSS1^{8A} (Zhao et. al., *Mol Cell*. 2015 Jul 16;59(2):176-87. PMID: 26145171). This suggests that the lower the functionality of an ectopic DSS1 mutant, the more endogenous DSS1 remains. We suspect it could be due to the essentiality of wild type DSS1 for cell proliferation.

Notably, performing a Western blot for DSS1 presents unique challenges in the field due to its small size (70 amino acids) and high acidity (28 of the 70 amino acids are either D or E). Specific requirements include the use of a 1x PBS buffer (pH 7.0) instead of the standard Tris/glycine buffer with 20% methanol and the application of a 0.2 µm PVDF membrane fixed with 0.2% (v/v) glutaraldehyde (Li et. al., *Oncogene*. 2006 Feb 23;25(8):1186-94. PMID: 16205630).

3. Molecular weights should be presented in Western blots.

Response: Thanks for your suggestion and the molecular weights in all western blots have been included in the revised manuscript.

4. On page 2, Line 5, "unexpected" should be removed.

Response: Following the suggestion, we have removed it in the revised manuscript.

5. On page 2, Line 8, "new" should be removed.

Response: Following the suggestion, we have removed it in the revised manuscript.

Reviewer #3

Overall: We are grateful to the Reviewer for highlighting that our study addresses very important biological questions in the field and for pointing out the limitation of our study: 1) lack of generation of BRCA2 mutants affecting dsDNA binding for comparison to CTRB mutants, and 2) concern about the experimental/physical evidence for our claims regarding open/close conformation. We acknowledge this limitation, which is primarily due to our inability to resolve the structure of DBD-DSS1 in its open state bound to dsDNA, despite our extensive efforts over two years. However, we hope the Reviewer appreciates the novelty of our discovery concerning the DNA binding activity of HDOB1, its regulation by DSS1, and its unexpected roles in reducing homologous recombination, destabilizing replication forks, and causing R-loop accumulation. We also want to emphasize that this study represents the initial step in a long journey to elucidate many significant questions in this direction.

We are grateful to the Reviewer for his/her support. Below, we document how we have addressed each point raised.

General concerns:

1. Given previous reports implicating DSS1 in BRCA2 stabilisation, it is possible that many phenotypes could be attributed to this effect. Therefore, generation of BRCA2 mutants affecting dsDNA binding would be essential to provide experimental evidence to support the claim of dsDNA regulation in majority of cell-based phenotypes. Comparison to CTRB mutants would be also essential to analyse the contribution of individual domains involved in this process (Kwon et al 2023, with this lab contributing to the paper).

Response: Thank you for raising the question regarding BRCA2 stabilization. We had the same concern thus performed Western blot analyses to detect the total protein level of BRCA2 in cells expressing either wild type or mutants (R57Q and 1-54) of DSS1 (see the original Extended Data Fig. 7c and Supplementary Fig. 10c of the revised manuscript). Consistent with previous reports, knock-down DSS1 indeed causes destabilization of BRCA2. However, we did not observe a significant difference of BRCA2 levels in cells with DSS1 mutants (R57Q or 1-54) in comparison to cells with $DSS1^{WT}$. Additionally, we further investigated whether the nuclear localization of BRCA2 is affected by these mutations. As illustrated in Supplementary Fig. 10 b & c of the revised manuscript, BRCA2 nuclear localization and chromatin association in cells with either wild type or mutant (R57Q and 1-54) of DSS1 is similar. Therefore, we concluded that our DSS1 mutants (R57Q and 1-54) do not affect BRCA2 stabilization and nuclear localization. Importantly, we found that BRCA2/RAD51 foci are reduced in our DSS1 mutant (either $DSS1^{R57Q}$ and $DSS1^{1-54}$) cells, suggesting that the release of HDOB1's dsDNA binding activity by the DSS1 mutations prevents BRCA2/RAD51 assembly on ssDNA.

In terms of generation of dsDNA binding mutants in HDOB1/DBD to strengthen the claim of dsDNA regulation in cells, this is a very important point and also our goal since the first day we revealed the DNA binding activity of HDOB1. As mentioned later in addressing a related point (#3), we had spent over 2 years but been unable to solve the structure of DBD-DSS1 in the open status (induced by DSS1 mutations) with dsDNA bound, where we can not only resolve the open/close confirmation but also reveal the residues that are involving in dsDNA binding. We highly believe that studying those mutants (especially $BRCA2^{HDOB1\text{-}dsDNA\text{-}SOF}$ mutants) *in vitro* and in cells will be very valuable, including helping us to clarify which model (1 vs. 2) is correct as we discussed in point (#4) of the Reviewer 1. Of note, the increased DNA binding activity observed from our new HDOB1mut1 (Figure R1) and R2645G variant (Alvaro-Aranda, Lucia et al. *Nucleic Acids Research*, 2023, Dec 24: gkad1222. PMID: 38142462) has further supported our model and conclusion in this study that DNA binding ability of HDOB1 is shielded by its complex with DSS1. Thus, more investigations (biochemical characterization of more mutations in the HDOB1 and structure determinations of the DBD-DSS1 complex in its open state bound to dsDNA) are greatly needed to fully elucidate the dsDNA regulation of HDOB1 and its significance in cells. I hope the reviewer can agree with Reviewers 1 and 2 that our study represents the first and novel discovery regarding the HDOB1 DNA binding activity and its regulation by DSS1. Further research from our team and the broader scientific community will be essential to advance our understanding of this activity and regulation. Recognizing the importance of this goal, we are actively collaborating with the Dr. Yuan He group at Northwestern University to determine the structures and reveal the dsDNA binding residues, although we acknowledge that this is a challenging and long-term objective.

As for comparison of the dsDNA binding contributions of HDOB1/DBD and CTRB in BRCA2's functions, we noted the Reviewer has a related point (#2). Unfortunately, we were unable to conduct these comparisons due to the current unavailability of HDOB1 dsDNA binding mutants yet, as discussed in above section. Also please note the biochemical difference and biological significance about dsDNA binding by HDOB1 vs CTRB based on our current knowledge: HDOB1 possesses a robust ability to bind both ssDNA and dsDNA, displaying a slight preference for the former. Functionally, the release of HDOB1 dsDNA binding activity facilitates the loading of BRCA2-RAD51 onto dsDNA, not ssDNA, thereby inactivating BRCA2-DSS1 functions both *in vitro* and in cells. Conversely, CTRB binds dsDNA and ssDNA without preference, and this DNA binding activity is required for promoting RPA-RAD51 exchange and also

targeting BRCA2-RAD51 to ssDNA for RAD51-ssDNA presynaptic filament formation. In short, HDOB1-DNA binding gain (induced by DSS1 mutations) and CTRB-DNA binding loss (induced by 4A mutation) both lead to the same consequence: BRCA2 deficiency. Therefore, comparing the contributions of HDOB1 and CTRB to BRCA2 functions in various biological processes is complicated and of great interest but also presents significant challenges. A systematic study requiring a few years of work will be necessary to gain insight into their relationship.

While we have not been able to conduct the suggested experiments (generation of BRCA2 mutants and comparison to CTRB mutants), we purified CTRB and HDOB1-DSS1$^{1-36}$ side by side from bacterial cells and compared the dsDNA binding affinity of HDOB1-DSS1$^{1-36}$, CTRB and DBD-DSS1$^{1-36}$ in EMSA experiments (Figure R2a). Consistent with our previous publication (Kwon et. al., *Nat Commun*. 2023 Jan 26;14(1):432. PMID: 36702902), CTRB binds dsDNA with an affinity of about 300 nM. On the contrast, the dsDNA binding by HDOB1/DBD is much higher (<20 nM) at the same condition. Furthermore, we examined the relative contributions of HDOB1-DNA binding gain (induced by DSS1 mutations) versus CTRB-DNA binding loss (induced by 4A mutation) to cell survival following olaparib treatment. We requested the available DLD1 cell lines expressing 2xMBP-BRCA2-CTRB^{WT} (referred to as BRCA2^{WT}) or 2xMBP-BRCA2-CTRB^{4A} (referred to as BRCA2^{4A}) from Dr. Sung lab (Kwon et. al., *Nat Commun.* 2023 Jan 26;14(1):432. PMID: 36702902) for this purpose. Despite several attempts, we were unable to establish stable cell lines expressing wild type or its mutants of GFP-DSS1 yet. However, we managed to explore this comparison through transient overexpression of GFP-DSS1 (Wild type and R57Q mutant) combined with endogenous DSS1 knockdown by siDSS1. As depicted in Figure R2b, cells expressing the DSS1^{R57Q} mutant are more sensitive to olaparib than those with the BRCA2^{4A} mutation. Notably, cells harboring both DSS1^{R57Q} and BRCA2^{4A} mutations exhibit significantly increased sensitivity, indicating a synergistic effect of HDOB1-DNA binding gain and CTRB-DNA binding loss in supporting BRCA2's functions in cells.

Figure R2

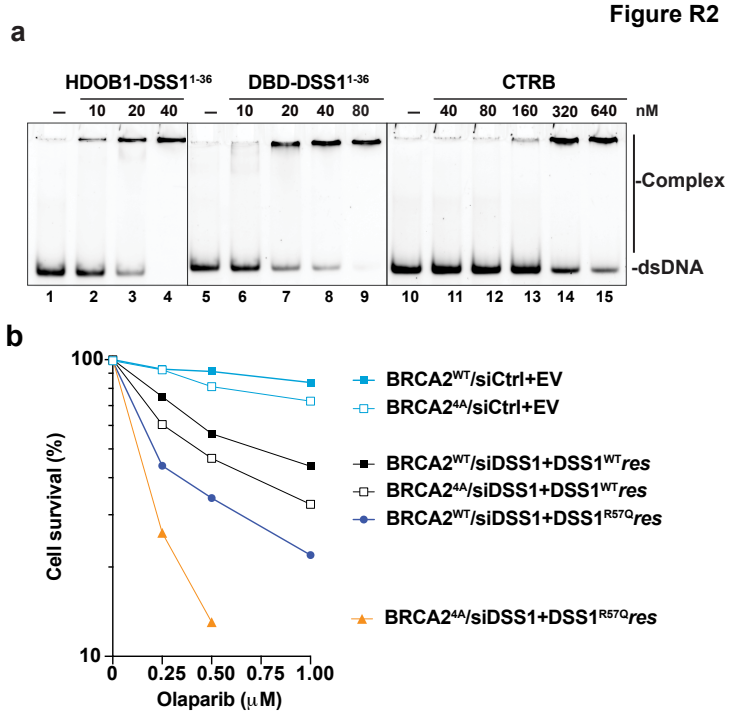

Figure R2: Comparison of dsDNA binding attributes of DBD/HDOB1 and CTRB *in vitro* and in cells. a. Binding of dsDNA (5nM) by HDOB1-DSS1$^{1-36}$ (lanes 2-4), DBD-DSS1$^{1-36}$ (lanes 6-9), and CTRB (lanes 11-15) at 90 mM KCl condition. b: Olaparib

survival curves of DLD1 cells with stable expression of BRCA2^{WT} or BRCA2^{4A} (CTRB DNA binding mutant 4A) and with transient transfection GFP-DSS1^{WT} or GFP-DSS1^{R57Q} after endogenous DSS1 knockdown by siDSS1 treatment.

In sum, we have made a sincere effort to address the point of the Reviewer and hope she/he would agree that the task for generation of BRCA2 SOF mutants affecting dsDNA binding and comparison to CTRB mutants to analyze their contributions represents a longer-term goal and is beyond the scope of this study.

2. The use of various BRCA2 fragments is confusing. In particular, as selection of individual domains of BRCA2 has significant effect on ss/dsDNA binding. Why HDOB1 is used in majority of the experiments? Probably the reason might be that DBD shows already very low dsDNA binding activity (seems 10-fold lower compared to ssDNA). The affinity of DBD fragment needs to be included in Fig 2a. Therefore, it is important to clarify the significance of dsDNA binding by DBD in relations to the full length or miniBRCA2. In particular, the CTRB domain also contains ss and dsDNA binding domain that alone can promote in vitro RAD51 loading and strand exchange activity, sensitivity to DNA damage, DSB repair and RAD51 foci formation (Kwon et al 2023, with this lab contributing to the paper).

Response: Thank you for raising this point. In response, we have added all the necessary labels in the related figures and have also summarized the BRCA2 fragments in Supplementary Figs. 3e and 4c of the revised manuscript to avoid any confusion. Please also note that, in addressing Reviewer 1's point (#2), we have also included the apparent Kd values of all DBD fragments, DBD-DSS1 complexes, and HDOB1-DSS1 complexes in Supplementary Figs. 3e and 4c.

In addition to the minimal dsDNA binding activity exhibited by DBD as the Reviewer noted, there are two primary reasons why we mainly use HDOB1: 1) The data from HDOB1 provides a clearer path to study the DNA binding activity and its regulation by DSS1, as DSS1 predominantly interacts with both HD and OB1 domains. The HDOB1-DSS1^{WT} complex completely lacks DNA binding activity, whereas mutants (*e.g.*, HDOB1-DSS1$^{1-54}$ and HDOB1-DSS1^{R57Q}) display robust ds/ssDNA binding. The conformation change of HDOB1-DSS1^{WT} upon salt titration is remarkably distinct. Conversely, DBD contains an additional ssDNA binding domain (OB2-OB3), and interactions between OB1 and OB2, along with potential oligomerization from the OB2 tower domain, make its behavior more complicated than just DSS1 regulation. 2) The yield of HDOB1 and HDOB1-DSS1 purified from bacterial cells is significantly higher, whereas DBD is mostly insoluble in either bacterial or insect cell expression system and the soluble fraction tends to oligomerize and even precipitate during purification. In contrast, the yield of DBD-DSS1 complexes, including DBD-DSS1$^{1-36}$, is 50-fold higher, approaching milligram levels. As shown in Figure R2a and Supplementary Fig. 8b, c, the DNA binding affinity of DBD-DSS1$^{1-36}$ is robust, and their behavior is more reliable than DBD: DBD-DSS1$^{1-36}$ possesses a similar dsDNA binding affinity as HDOB1 does, but a higher ssDNA binding affinity, presumably due to the involvement of OB2-3 in ssDNA binding.

Regarding clarifying the significance of dsDNA binding by DBD/HDOB1 vs CTRB, we noted that the Reviewer has raised a related point (#1) and we have discussed about this issue in detail in the above section. Although we were unable to systematically compare them due to the current unavailability of HDOB1 dsDNA binding mutants, we have performed an EMSA assay to evaluate the dsDNA binding affinities of DBD/HDOB1 and CTRB, and found that the dsDNA binding by HDOB1/DBD is much higher than that of CTRB (<20 nM vs >300 nM) (Figure R2a). Moreover, we have demonstrated that cells expressing

DSS1^{R57Q}, which releases dsDNA binding by HDOB1, are more sensitive compared to cells with the ds/ssDNA binding defect mutant CTRB-4A (Figure R2b), indicating their differences in contribution to cell survival. As mentioned earlier in addressing a related point (#1), once the HDOB1 dsDNA binding mutants (*i.e.*, BRCA2$^{HDOB1\text{-}dsDNA\text{-}SOF}$) are identified, we will investigate them with CTRB mutants systematically to clarify the significance of dsDNA binding by DBD/HDOB1 vs. CTRB in the context of miBRCA2 (*e.g.,* miBRCA2$^{HDOB1\text{-}dsDNA\text{-}SOF}$/DSS1$^{1\text{-}36}$ vs. miBRCA2$^{HDOB1\text{-}dsDNA\text{-}SOF+CTRB\text{-}4A}$/DSS1$^{1\text{-}36}$ vs. miBRCA2$^{CTRB\text{-}4A}$/DSS1$^{1\text{-}36}$ vs. miBRCA2/DSS1$^{1\text{-}36}$) for *in vitro* experiments and of full length BRCA2 *for cellular studies.*

3. The terms of "closed" and "open" conformation of DSS1/BRCA2 complex are used, however no data supporting these claims are provided. It is important to include experimental evidence validating role of these conformational states.

Response: Thank you for raising this point and we concur that our current dataset does not furnish direct evidence for these conformational states. Accordingly, we have amended the relevant sections of the text (e.g., "which we suspect results in an open configuration. However, we still lack direct experimental evidence, such as structural determination, to confirm this" in page 10 of the revised manuscript) to clarify that the open conformation is hypothesized, and currently lacks direct experimental support.

To define the open/close confirmation, we have endeavored for over two years to determine the structural details of the BRCA2-ds/ssDNA complex in association with DSS1 C-terminal helix mutants, employing both crystallization and cryo-EM techniques. These efforts, however, have been hampered by specific difficulties: in crystallization, we have only been able to produce needle-like crystals, necessitating further optimization of our conditions. Similarly, in cryo-EM analysis, the small particle size of the DBD-DSS1 complex has required us to develop a platform to increase the molecular weight to enhance structural resolution. Moreover, we planned to use FRET to dynamically monitor the open/closed states of the complex. Accurately labeling the appropriate sites for FRET remains a highly skilled task, underscoring the complexity of these experiments. Then, we spent considerable effort to perform limited digestion of various DBD/HDOB1-DSS1 complexes with chymotrypsin and trypsin. Despite repeated attempts, we failed to obtain conclusive results. Therefore, we anticipate that several additional years of dedicated research will be necessary to gather conclusive evidence regarding the opening and closing states and mechanisms of this BRCA2-DSS1 complex.

On a more positive note, our AlphaFold predictions have offered insightful clues into the potential existence of an open conformation for the DBD-DSS1 complex (see Supplementary Fig. 5). Furthermore, our EMSA experiments with HDOB1/DBD in complex with either wild type or mutant DSS1 under salt titration have consistently indicated variations in their conformations. These structural differences are substantiated by functional and cellular data, showing that BRCA2 in complex with DSS1 mutants demonstrates a diminished capacity to load BRCA2/RAD51 onto ssDNA *in vitro* and in cells, leading to deficiencies in homologous recombination, replication fork protection, and R-loop resolution. These findings align with our structural hypotheses and support the functional significance of the observed conformational states.

Detail comments:

1. The authors should provide detail description of the concentrations of proteins and their tagged forms, DNA (both concentration and type), and salt conditions for each experiment to ensure reproducibility and clarity.

Response: Thank you for this suggestion. We have now provided descriptions about proteins' tag and salt condition in each figure/figure legend, and also summarized them together in Supplementary Figs. 3e and 4c. The information about substrate concentration and types in our EMSA assays has been added in the legend of each figure.

2. Ext. Data Fig. 1b appears to be composed of two gels or two different exposures. Clarification or separate presentation may be necessary.

Response: We apologize for the confusion and have now included a line in the figure and added the text in the figure legend to indicate that they are from two gels.

3. Statistical analysis is required, particularly for Fig. Extended Data 1f, and statistical comparisons to control experiments (Fig.4 a, c) to strengthen the validity of the findings.

Response: Thank you for the suggestion. We also note that Reviewer 2 raised a similar point (#1). In response, we have now included the statistical analysis for all required figures, including Supplementary Fig.1f and Fig. 4a, c, in comparison to control experiments by Student's t test or/and two-way ANOVA. These statistical analyses, either labeled in the figure or described in the figure legend of the revised manuscript, fully support our original conclusions and further strengthen the validity of our findings.

4. The use of GST-fused fragments raises concern about dimerization effect on DNA binding, especially when various BRCA2 domain are employed.

Response: Thank you for pointing out this concern. We added a GST tag to enhance solubility and facilitate purification. After purifying the fragments (*e.g.*, HD and OB1), we assessed their DNA-binding capacity both with and without the tag. As shown in Figure R3, the binding affinity of either HD or OB1 remains very similar (less than 2-fold difference) under both conditions. Thus, we believe that the GST tag does not significantly affect the DNA binding of the DBD fragments in our study. Importantly, to maintain consistency, all our HDOB1/DBD-DSS1 complexes contain a GST tag on the DSS1 subunit. As our study does not aim to precisely quantify the binding affinity of these fragments, we hope the Reviewer will agree that our extensive EMSA efforts (Fig. 2 and Supplementary Figs. 3, 4, 6, and 7) provide robust evidence that HD, OB1, and HDOB1 can effectively bind DNA. More importantly, we further demonstrated that this DNA-binding activity of HDOB1 needs to be restricted to better target BRCA2-RAD51 to ssDNA *in vitro* and in cells, and the disruption of this restriction by DSS1 mutations leads to defective in homologous recombination, replication fork protection, and R-loop suppression.

Figure R3

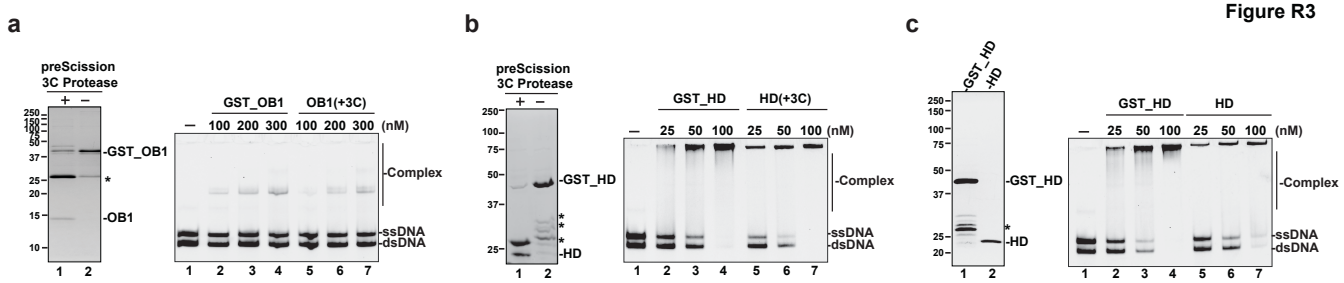

Figure R3. DNA binding ability of OB1 and HD with or without GST-tag. a. SDS-Page gel showing the untagged OB1 and GST-tagged OB1 (after and before adding preScission 3C protease to cleave the N-terminal GST-tag) (left panel) and ds/ssDNA binding by GST-OB1 (lanes 2-4) and OB1 (lanes 5-7) (right panel). b. SDS-Page gel showing the untagged HD and GST-tagged HD (after and before adding preScission 3C protease to cleave the N-terminal GST-tag) (left panel) and ds/ssDNA binding by GST-HD (lanes 2-4) and HD (lanes 5-7) (right panel). c. SDS-Page gel showing the GST-tagged HD and untagged HD which was treated with preScission 3C protease and further purified by size exclusion chromatography (left panel) and ds/ssDNA binding by GST-HD (lanes 2-4) and HD (lanes 5-7) (right panel). *No specific band or GST.

5. BRCA2 nuclear localization upon DSS1 depletion or expression of the DSS1-RQ mutant shows comparable results, but the reliability of BRCA2 "foci" needs verification.

Response: Thanks for the Reviewer to point out that "BRCA2 nuclear localization upon DSS1 depletion or expression of the DSS1-RQ mutant shows comparable results" and to suggest verifying the reliability of BRCA2 "foci". As shown in Supplementary Fig. 10e of the revised manuscript and Figure R4 below, BRCA2 foci were diminished when BRCA2 is knocked down by its specific siRNA in all cell lines tested. This suggests that the BRCA2 "foci" detected by the antibody in our assay is specific. This antibody was developed by Dr. Bing Xia and has been used in many previous publications (*e.g.*, Xia et. al., *Mol Cell.* 2006 Jun 23;22(6):719-729. PMID: 16793542; Zhang et. al., *Curr Biol.* 2009 Mar 24;19(6):524-9. PMID: 19268590).

Figure R4

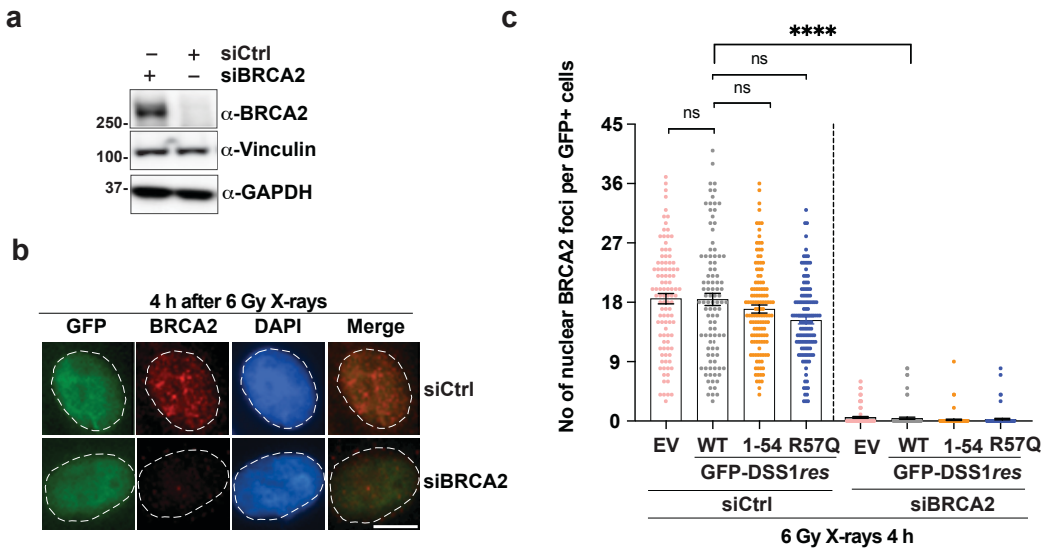

Figure R4. Specificity of BRCA2 foci by α-BRCA2 antibody. a, Western blot analysis to detect endogenous BRCA2 in HeLa-GFP-DSS1 cells after treatment of siBRCA2, Vinculin and GAPDH were used as the loading control. b. Representative

micrographs of BRCA2 foci (red) in HeLa cell nuclei at 4 hr after exposure to 6 Gy X-rays. Blue: DAPI. c. Quantification of BRCA2 foci number per cell at 4h in HeLa cells expressing wild type or mutants of DSS1 after exposure to 6 Gy X-rays. Scale bar:10 μm. The mean values ± SEM of at least three independent experiments is shown. ns, not significant and ****p < 0.0001.

6. a) In Fig 2.d and e, it seems from quantification that HDOB1 alone binds dsDNA better then ssDNA, which contradicts panel b.

Response: We apologize for the confusion caused by using different protein concentration ranges for the X axis: In the original manuscript, we plotted 0-100 nM for ssDNA and 0-200 nM for dsDNA (Figure R5). To avoid confusion, we now present the quantification with the same range of 0-100 nM (Figure R5). Based on all the EMSA data in this study, we found that HDOB1 binds ssDNA and dsDNA with the same affinity (6 nM) at 45 mM KCL condition, but binds ssDNA stronger than dsDNA at 90 mM salt (8±2 nM vs. 18±2 nM), as indicated in Supplementary Figs. 3e and 4c of the revised manuscript.

Figure R5

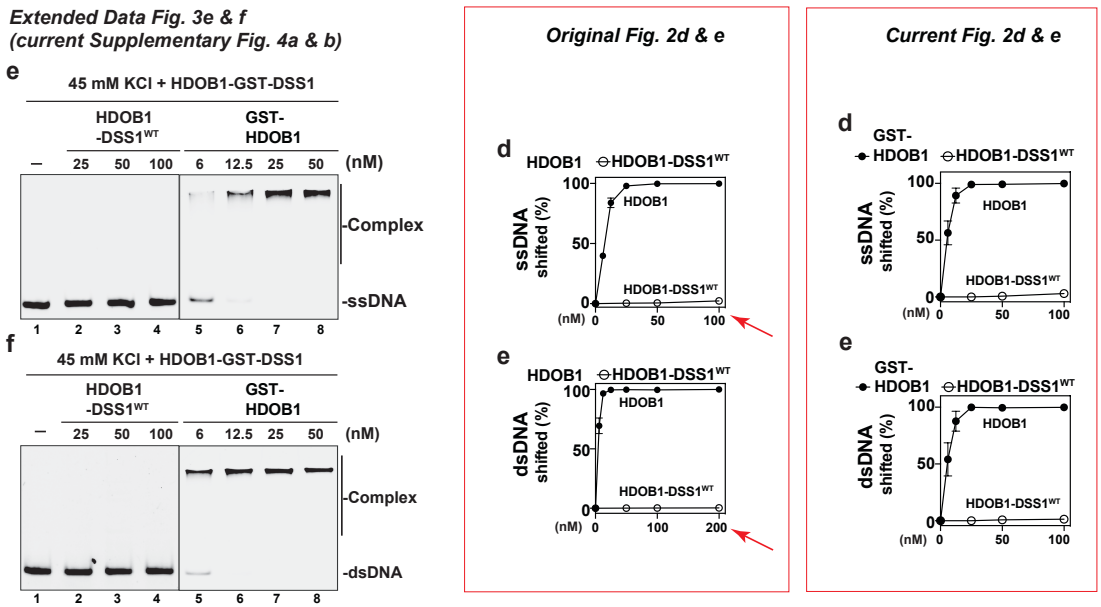

Figure R5. Comparison of ssDNA and dsDNA binding affinity of HDOB1. Left part: Binding of ssDNA by HDOB1-DSS1^{WT} (lanes 2-4) and HDOB1 (lanes 5-8) from original Extended Data Fig 3e & f (current Supplementary Fig. 4a & b). Right part: Comparison of the quantification shown in the original Fig. 2d & e and revised Fig. 2d & e).

b) In addition, the binding of HDOB1-alone to ssDNA presented in panel b, is the same or lower compered to one in complex with DSS1(1-36), which differs from Ext data Fig.3e.

Response: We appreciate the Reviewer's careful observation regarding a potential discrepancy in the ssDNA binding affinity of HDOB1 vs. HDOB1-DSS1$^{1-36}$ in Fig. 2b and Extended Data Fig. 3e (now Supplementary Fig. 4a). We apologize for any confusion arising from the different reaction conditions used: 90 mM KCl and a mixture of ssDNA and dsDNA in Fig. 2b, while DNA binding assays with only ssDNA substrate at 45 mM KCl were applied in Supplementary Fig. 4a.

As shown in Figure R6a & b, by comparing HDOB1 and HDOB1-DSS1$^{1-36}$ at the same protein concentration and salt condition (indicated by red arrows), we observed that the ssDNA binding affinity of

HDOB1 is slightly stronger than that of HDOB1-DSS1$^{1-36}$ under both 45 mM and 90 mM conditions, which is consistent with our conclusion in the study. However, at the 18 nM concentration point (indicated by green arrows), we agree with the Reviewer that HDOB1 shows slightly less ssDNA binding than HDOB1-DSS1$^{1-36}$. This observation might suggest a potential discrepancy; however, we believe that it is attributed to the competition from dsDNA in the reaction as HDOB1 has higher affinity for dsDNA than HDOB1-DSS1$^{1-36}$. Conversely, HDOB1-DSS1$^{1-36}$ exhibits a bit more specific affinity for ssDNA over dsDNA than HDOB1 does, resulting in faster ssDNA binding by HDOB1-DSS1$^{1-36}$ even in the competition reaction. Please note that, at 90 mM KCl condition, HDOB1 and HDOB1-DSS1$^{1-36}$ have relatively similar affinities for ssDNA (8±2 nM vs. 10±3 nM), while HDOB1 has approximately two-fold stronger dsDNA binding affinity compared to HDOB1-DSS1$^{1-36}$ (18±2 nM vs. 32 ±8 nM), as summarized in Supplementary Fig. 3e. Lastly, as shown in Figure R6c, we noticed that HDOB1 has stronger ssDNA binding than HDOB1-DSS1$^{1-36}$ when only ssDNA is used in the assay at 90 mM KCl condition.

Figure R6

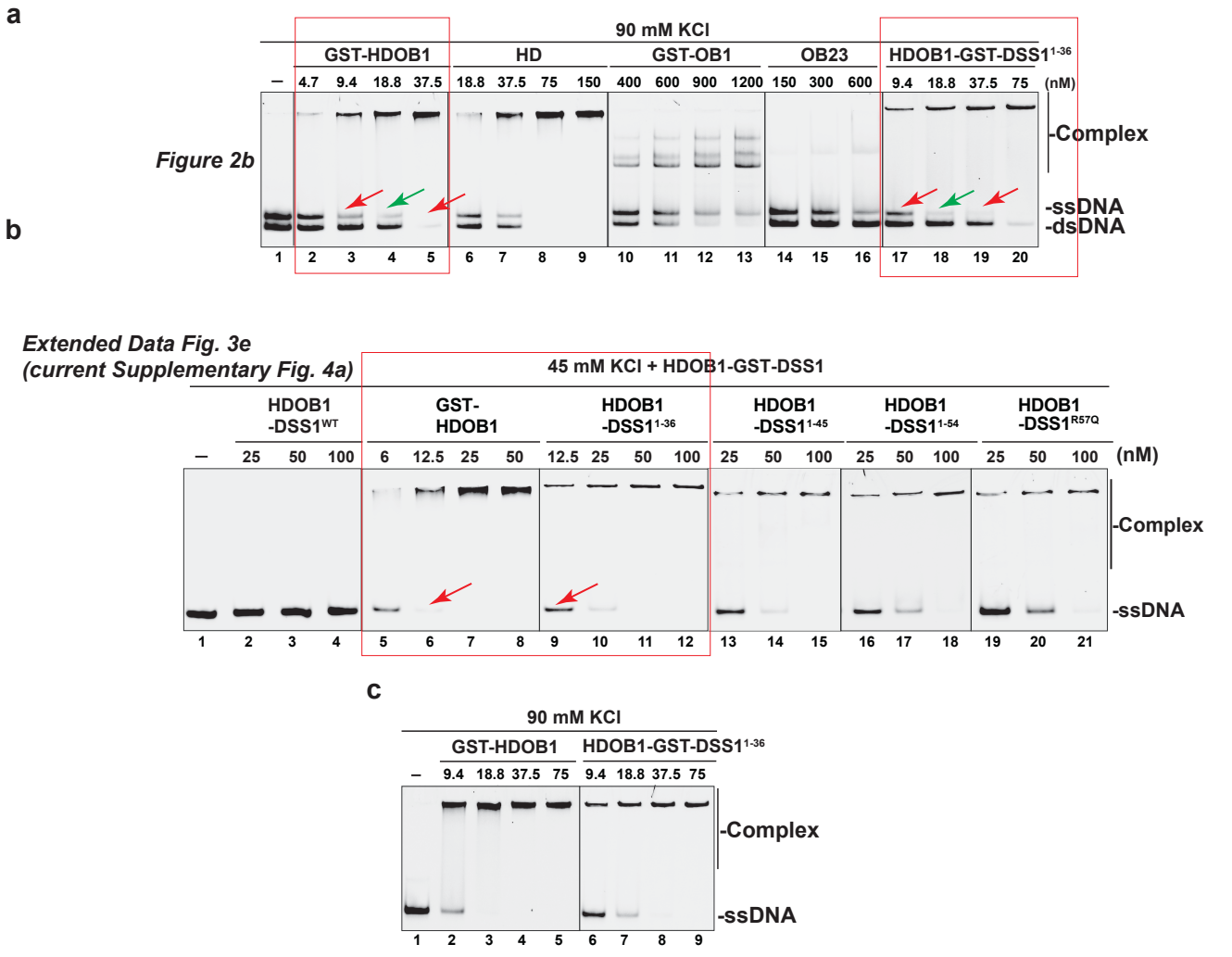

Figure R6. Comparison of ssDNA binding by HDOB1 and HDOB1-DSS1$^{1-36}$. a, Binding of ssDNA and dsDNA by HDOB1 (lanes 2-5) and HDOB1-DSS1$^{1-36}$ (lanes 17-20) from Figure 2b. b, Binding of ssDNA by HDOB1 (lanes 5-8) and HDOB1-DSS1$^{1-36}$ (lanes 9-12) from original Extended Data Fig. 3e (current Supplementary Fig. 4a). c, Binding of ssDNA by HDOB1 (lanes 2-5) and HDOB1-DSS1$^{1-36}$ (lanes 6-9) at 90 mM KCl condition.

c) *Furthermore, quantification of gels from Ext data Fig.3e frequently contains gels with only three concentration points, but graphs contain four concentration points.*

Response: We apologize for the confusion caused by including quantification data from more concentration points that may not correspond to the representative gels shown in the original Extended Data Fig. 3e & f. They were from repeated experiments at various concentration points. To avoid any confusion, we have omitted these data from the graphs in updated Fig. 2d & e (now they only contain three concentration points of each mutant complexes) of the revised manuscript.

7. Ext. Data Fig.5, why salt titration only for ssDNA is provided? How salt affect dsDNA binding of the constructs? Furthermore, 360 mM KCl is selected for further testing, but this is not physiological relevant salt concentration and under this condition the protein complexes do not enter gels anymore.

Response: We appreciate the feedback and have now incorporated dsDNA binding data under various salt concentrations. As illustrated in Supplementary Figs. 6 and 7, we observed that increased salt concentrations enhance the DNA binding (both dsDNA and ssDNA) of HDOB1-DSS1^{WT}, but inhibit HDOB1's ability to bind DNA. This indicates that salt can induce a conformational change in HDOB1-DSS1^{WT}, enhancing its DNA binding capacity, potentially through a transition from a closed to an open state. Importantly, the salt effect on dsDNA binding for the complexes involving DSS1 mutants (whether in combination with HDOB1 or DBD) differs from that observed with their wild-type counterparts (Supplementary Figs. 6 and 7). This suggests variable statuses and readiness for conformational changes among the different complexes.

Based on this premise, we selected a 360 mM KCl condition, which can induce significant alter DNA binding abilities of the DBD-DSS1 complexes, as a condition to better reveal potential differences between wild type (wt) and mutants as an indicator of their conformational change potential. Of note, we strongly believe that this confirmation conversion and dsDNA binding property also occurs under normal salt conditions, but it is just harder to detect it and show it. Consistent with this belief, we conducted the BRCA2-RAD51 targeting (ssDNA vs dsDNA) experiments at 45 mM KCl condition and observed that the mutants (miBRCA2-DSS1$^{1-54}$ and miBRCA2-DSS1^{R57Q}) exhibit a greater ability to target BRCA2-RAD51 to dsDNA compared to miBRCA2-DSS1^{WT}, while demonstrating similar capabilities in mediating RPA-RAD51 exchange.

8. Ext. Data Fig.6d and e, the quantification does not seem to align to gels in panel d, indicating a need for clarity and consistency.

Response: Thanks for pointing out and we apology for the mistake about concentration in original Extended Data Fig.6e and now we have revised them in the Supplementary Fig. 8e of the revised manuscript. Note: the concentrations listed in original Extended Data Fig.6d (current Supplementary Fig. 8d) are correct.

9. Ext. Data Fig.6f, no information about protein and DNA concentration, salt concentration is provided. Quantification and repetition are also required, since this assay represent more closely the physiological reaction in cells. miBRCA2 alone reaction is missing. How the authors explain different mobility of the product with RAD51 alone compared to other reactions? Does this rather correspond to two different gels

combined into one?

Response: Thanks for pointing this out. Following the suggestion, we have now repeated the experiments with miBRCA2 alone as the control over 3 times and included the quantification data and all information (protein/DNA/salt concentrations) in the Supplementary Fig. 9a and its figure legends in the revised manuscript. Note: The original figure showing different mobility of the D-loop product was combined from two different gels. We should have included a line between them. Thank you for catching this omission.

10. Ext. Data Fig.6g, a control reaction with GST-DSS1 alone should be included.

Response: Following the suggestion, we conducted pull-down experiments comparing GST-DSS1 with miBRCA2-DSS1 and observed that GST-DSS1 exhibits a similar or slightly stronger interaction with RPA than miBRCA2-DSS1, aligning with our previous findings. Additionally, we compared the DSS1 mutants (1-54 and R57Q) and wild-type within the context of GST-DSS1 alone and found that mutations in the C-helix of DSS1 do not affect its ability to interact with RPA. These results, along with the original data, have been included in Supplementary Fig.9b-d of the revised manuscript.

11. The overexpression of DSS1 seems to be quite toxic (increased BRCA2 staining, RAD51 foci etc without DNA damage). Comparison to endogenous levels of DSS1 and possible finetuning of the expression is thus required. Anti-DSS1 WB for all constructs need to be shown (Ext. Data Fig.7a and f).

Response: Thank you for your suggestion. In response, we have performed anti-DSS1 Western blots to compare the levels of ectopically overexpressed tagged DSS1 with those of endogenous DSS1. As shown in Supplementary Fig. 11a of the revised manuscript, the exogenous level of Flag-DSS1 is comparable to that of endogenous DSS1 (Note: the lower signals of Flag-DSS1 mutants (1-54 and R57Q) in related to Flag-DSS1^{WT} is very likely due to the effect of these DSS1 mutations on their recognition by α-DSS1 rabbit polyclonal antibody raised against 1-70 aa of full length human DSS1). Unfortunately, for the GFP-tagged version, we were unable to perform a similar comparison as the specific transfer buffer (1x PBS) required for endogenous DSS1 (see detailed special requirements in addressing Point (#2) of the Reviewer 2) does not accommodate the GFP-DSS1 protein, and vice versa. Additionally, BRCA1, BRCA2 and RAD51 foci can be resulted from endogenous breaks or stressed replication forks (in absence of the exogenous DNA damage), which has been observed and reported before (*e.g.,* Xia et. al., *Mol Cell*. 2006 Jun 23;22(6):719-729. PMID: 16793542). Thus, we suspect that the BRCA2 staining and RAD51 foci without DNA damage seen in cells expressing GFP-DSS1^{WT}, but not in cells with GFP-DSS1 mutants (1-54 and R57Q), highlights the unique capability of DBD-DSS1^{WT} to bind ssDNA over dsDNA specifically as proposed in our model (Fig. 7).

12. In Ext Data Fig6, panel g, there is no mention of using WB to detect the bound fraction in the pull-down.

Response: We appreciate the point made by the Reviewer. We now mention this information in the figure and its legend of Supplemental Fig. 9d (original Extended Data Fig. 6g).

13. Fig. 5B, FACS data for HDR frequency should be shown.

Response: Thanks for pointing this out and we have included FACS data for HDR frequency in Supplemental Fig. 11b.

14. *Fig. 5c and d as well as Ext. Data Fig. 7g, a comparison to DSS1 depleted cells should be shown.*

Response: We appreciate the suggestion and indeed hope to include cell survival data for DSS1-depleted cells in Fig. 5c and d, as well as in Extended Data Fig. 7g (now Supplementary Fig. 11c in the revised manuscript). However, the depletion of DSS1 by either siDSS1 or shDSS1, where the knock-down efficiency reaches over 90% in our experiments, significantly reduces plating efficiency (0.005-0.01), which prevents us from obtaining reliable and meaningful cell survival results. In contrast, cells treated with control siRNA/shRNA show a plating efficiency of 0.5-0.6. This super-low plating efficiency phenomenon has been previously reported for BRCA2 depletion (Feng et., al., *Proc Natl Acad Sci U S A*. 2011 Jan 11;108(2):686-91. PMID: 21148102) and was also observed with DSS1 depletion in our previous work (Zhao et. al., *Mol Cell*. 2015 Jul 16;59(2):176-87. PMID: 26145171).

15. In Fig. 6f and g, given the selectivity of S9.6 antibodies, a control reaction treated with RNAseH1 should be provided for validation.

Response: We appreciate the suggestion and have now included control reactions treated with RNaseH1 in the two assays using S9.6 antibodies. The results, which validated the specificity of the PLA signal (original Fig. 6f) and the R-loop blot (original Fig. 6g), are presented in Fig. 6f and Supplementary Fig. 12d-e of the revised manuscript.

SUMMARY

We would like to thank the Reviewers again for the time and effort spent in reading and critiquing our submission and for their insightful comments and experimental suggestions. As documented above and in the revised manuscript, we have expended considerable effort to address the points raised which, in our opinion, has resulted in a significantly improved revised manuscript. We sincerely hope that the Reviewers will find our revisions satisfactory and that the revised manuscript, which is to report our discovery of HDOB1's DNA binding activity and its regulation by DSS1 and of its important role for proper BRCA2's function in various processes, is suitable for publication.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have addressed all of my prior comments. This paper will make a nice addition to the literature.

Reviewer #2 (Remarks to the Author):

The revised manuscript significantly improved and the authors adequately answered to my previous concerns. I think that this manuscript is acceptable in Nature Communication.

Reviewer #3 (Remarks to the Author):

While the authors, in their revised manuscript titled "DSS1 Restrains dsDNA Engagement of BRCA2 to Promote Homologous Recombination, Stabilize Replication Forks, and Suppress R-loop Accumulation," have addressed many minor comments, they did not provide additional information necessary to understand the mechanism of dsDNA binding regulation by DSS1 or justify key claims to support publication in Nature Communications.

1. I understand the difficulty in generating dsDNA binding mutants; however, there are alternative approaches beyond X-ray crystallography that can be employed. These include footprinting techniques, cross-linking with mass spectrometry, NMR, mutagenesis (such as alanine scanning), and computational methods combined with site-directed mutagenesis.
2. The authors fail to provide clear evidence as to why dsDNA binding in the context of HDOB1 is more biologically relevant than in larger fragments like DBD and mini or full-length BRCA2. Clarification on this point is essential.
3. The authors did not provide any evidence to support the proposed closed or open conformations of the protein. To address this, they could attempt to utilise additional available techniques such as Circular Dichroism (CD), nanoDSF, cross-linking mass spectrometry (XL-MS), Small Angle X-ray Scattering (SAXS), and NMR.

Reviewer #4 (Remarks to the Author):

DSS1, a binding partner of BRCA2, was thought to primarily support BRCA2 mediator function in homologous recombination (i.e. ability to overcome RPA to support RAD51 binding on resected DNA) (PMID: 26145171). Here, the authors show that DSS1 also reduces the dsDNA biding and increases ssDNA binding of BRCA2 (a smaller version, termed miBRCA2 was used for initial experiments) without or with complex with RAD51. The interplay could then be mapped to a region in BRCA2 (termed HDOB1) and in DSS1 (specific deletion mutations and cancer associated mutations are presented). The authors then conclude that the switch from dsDNA towards ssDNA

is necessary for both HR and replication fork protection functions. These reported data are interesting and important, but care should be taken to avoid overinterpretation.

I was specifically asked to comment on the exchange between the authors and Reviewer #3. Reviewer #3 notes that it is important to distinguish the observed effects in dsDNA/ssDNA binding and BRCA2 stabilization (known from previous studies). I agree that it is a very important point: here, the authors observed that the DSS1 mutants affected BRCA2 foci formation, which may be indeed an indicator of BRCA2 destabilization; and this effect could be independent from the newly described function in balancing dsDNA/ssDNA binding. The reviewer #3 asked to disentangle the two phenomena.

Based on previous literature, it is known that RAD51 binding to ssDNA promotes homologous recombination, while RAD51 binding to dsDNA is inhibitory. The BRC repeats of BRCA2, for example, have a key function to target BRCA2-RAD51 to ssDNA (PMID: 19303847, followed by other studies, and generally well accepted in the field). Whether ssDNA or dsDNA biding of RAD51-BRCA2 is needed for replication fork protection is much less known and somewhat controversial (some reports, e.g. PMID: 36707518 and PMID: 36070766 indicate that dsDNA binding function might be more important). One of the key conclusions of the manuscript, i.e. that ssDNA binding of RAD51/BRCA2 is needed for both homologous recombination and protection, hence, could be wrong, if the reported DSS1 mutations also affect BRCA2 stabilization (which would e.g. take out its function in dsDNA binding via the N-terminal DNA binding domain PMID: 36707518). At the same time, I see that the experiments suggested by Reviewer #3 go well above a normal revision, plus a success is not guaranteed. A possible solution could be to tone down the conclusions. I suggest that the current conclusion that ssDNA binding of BRCA2 is needed for replication fork protection should only be a discussion point, noted alongside the experimental limitations.

I do not understand well comment #2 (the authors analyzed the effect of DSS1 also on miBRCA2 in Figure 1) and I am not very concerned about comment #3 (it is clear that these are models).

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Thank the Reviewer for his/her enthusiasm and support.

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Thank you to the Reviewer for noticing that we have made great efforts and performed many experiments to address the detailed/minor comments and general concerns during the last revision of over three months. We are grateful to the Reviewer for agreeing that our revision work has addressed the 15 detailed/minor comments, except for the remaining three general concerns below.

1. I understand the difficulty in generating dsDNA binding mutants; however, there are alternative approaches beyond X-ray crystallography that can be employed. These include footprinting techniques, cross-linking with mass spectrometry, NMR, mutagenesis (such as alanine scanning), and computational methods combined with site-directed mutagenesis.

Response: Thank the Reviewer for not being concerned any more about BRCA2 stabilization in DSS1 mutant cells, which was an important part of this general concern. As shown in Supplementary Fig. 10b & c of the revised manuscript, BRCA2 nuclear localization and chromatin association in cells with either wild type or mutant (R57Q and 1-54) DSS1 is similar.

We are grateful to the Reviewer for providing detailed suggestions about generating dsDNA binding

mutants and will conduct further investigations to reveal more insights in future manuscripts. During the last revision of over 3 months, we have cloned and tested three new mutants in HDOB1. Additionally, we have compared HDOB1, DBD, and CTRB of BRCA2 both *in vitro* and in cells. We agree with Reviewer #4 that the experiments suggested are beyond a normal revision and are also not guaranteed for success. As such, we have toned down the conclusion about dsDNA regulation in Title and Abstract, and provided discussions about the limitations on pages 9 and 10 of the revised manuscript, highlighted in blue.

2. The authors fail to provide clear evidence as to why dsDNA binding in the context of HDOB1 is more biologically relevant than in larger fragments like DBD and mini or full-length BRCA2. Clarification on this point is essential.

Response: We also do not understand this comment as Reviewer 4 does. As pointed out by Reviewer #4, we have analyzed the effect of DSS1 on both miBRCA2 and full-length BRCA2 *in vitro*, as shown in Figure 1 and Supplementary Figure 1. After our initial analysis with HDOB1 (see Figure 2 and Supplementary Figure 4), we have checked the effect of DSS1 and its mutants in the context of DBD and miniBRCA2 (see Figure 3 and Supplementary Figures 6-9) *in vitro*. Importantly, all the biological and cellular assays in our study were conducted in the context of full-length BRCA2.

3. The authors did not provide any evidence to support the proposed closed or open conformations of the protein. To address this, they could attempt to utilise additional available techniques such as Circular Dichroism (CD), nanoDSF, cross-linking mass spectrometry (XL-MS), Small Angle X-ray Scattering (SAXS), and NMR.

Response: We are grateful to the Reviewer for the suggestions on various methods to reveal more evidence for the proposed closed or open conformations. In spite of our extensive biochemical analysis providing strong support to propose these models, we fully agree that structural determination is required to confirm them. Such discussion about the limitation of our study has been included on page 10 of the revised manuscript, highlighted in blue. However, we also totally agree with Reviewer #4 that the experiments suggested are well above a normal revision, and that it is reasonable to propose these models for describing different states/conformations of the complex.

Reviewer #4 (Remarks to the Author):

DSS1, a binding partner of BRCA2, was thought to primarily support BRCA2 mediator function in homologous recombination (i.e. ability to overcome RPA to support RAD51 binding on resected DNA) (PMID: 26145171). Here, the authors show that DSS1 also reduces the dsDNA biding and increases ssDNA binding of BRCA2 (a smaller version, termed miBRCA2 was used for initial experiments) without or with complex with RAD51. The interplay could then be mapped to a region in BRCA2 (termed HDOB1) and in DSS1 (specific deletion mutations and cancer associated mutations are presented). The authors then conclude that the switch from dsDNA towards ssDNA is necessary for both HR and replication fork protection functions. These reported data are interesting and important, but care should be taken to avoid overinterpretation.

I was specifically asked to comment on the exchange between the authors and Reviewer #3. Reviewer #3 notes that it is important to distinguish the observed effects in dsDNA/ssDNA binding and BRCA2 stabilization (known from previous studies). I agree that it is a very important point: here, the authors observed that the DSS1 mutants affected BRCA2 foci formation, which may be indeed an indicator of BRCA2 destabilization; and this effect could be independent from the newly described function in balancing dsDNA/ssDNA binding. The reviewer #3 asked to disentangle the two phenomena.

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I do not understand well comment #2 (the authors analyzed the effect of DSS1 also on miBRCA2 in Figure 1) and I am not very concerned about comment #3 (it is clear that these are models).

We are very grateful to the Reviewer for his/her great support and suggestion. Following the suggestion, we have toned down the conclusion in Title and Abstract, and provided discussions about the limitations in page 9 and 10 of the revised manuscript, highlighted in blue. Thank you for providing this solution.