

CIP2A mediates mitotic recruitment of SLX4/MUS81/XPF to resolve replication stress-induced DNA lesions

Corresponding Author: Professor Marcel van Vugt

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Haan et al investigate the role of the CIP2A-TOPBP1 complex during mitosis, particularly in processing incompletely replicated DNA. The authors show that CIP2A-TOPBP1 forms large filamentous structures at sites of incomplete DNA replication during mitosis, recruiting the SMX tri-nuclease complex (SLX4, MUS81, ERCC1/XPF). While CIP2A is not required for mitotic DNA synthesis itself, its C-terminal domain is essential for filament formation and SMX complex recruitment. Importantly, BRCA1^{-/-} and BRCA2^{-/-} cells exhibit increased mitotic lesions involving CIP2A and SLX4, and the C-terminal domain of CIP2A, as well as SLX4, are critical for genome stability and survival of BRCA2^{-/-} cells.

Overall, the work is of high quality and the findings are novel and relevant, particularly for understanding genome maintenance mechanisms in BRCA-deficient contexts. The link between CIP2A filament formation and SMX recruitment during mitosis provides an interesting advance.

The manuscript is generally well written and experiments well designed, but I would suggest that the authors consider addressing the minor issues below:

1. Abstract: "We show that CIP2A-TOPBP1 forms large filamentous structures at sites of incomplete DNA replication during mitosis, which recruit the SMX tri-nuclease complex members SLX4, MUS81 and ERCC1/XPF." I don't think that the authors show that specifically filaments of CIP2A-TOPBP1 recruit SLX4, etc. The formulation could be slightly revised to avoid misinterpretation by the reader.
2. Line 173: "CIP2A interacts with the SMX complex upon perturbed replication." I don't think the authors show that there is an interaction between CIP2A and SMX complex. XPF was slightly enriched in IP-MS, but the others not. Having said this, the authors may have a different interpretation of what the term "interaction" means. Is the presence at mitotic structures + dependency for recruitment the evidence for interaction?
3. Figure 3H, etc. I am not sure what to make of the EdU labelling of the CIP2A structures. There is clearly a correlation, but the spatial relationship in super-resolution is confusing. Do the authors have any proposal for how such structures might look like? Perhaps a speculative cartoon could be drawn?
4. Figure 5/S5A: Expression levels of the mutants is not very consistent. Phenotype description of all mutants, even of S904A, which shows lower expression in one of the experiments, should be made with the expression levels in mind. The authors write "All CIP2A mutants showed stable expression, 283 except for CIP2AGlob (Suppl. Fig. 5A)." Stable is not a meaningful way of describing the expression. Identical expression levels to the control full length is required for all conclusions made. Otherwise conclusions have to be adjusted.
5. Figure 5B, low signal in the MUS81 WB makes it hard to judge how efficient the depletion is, which is important for some of the conclusions made.

6. 335-337: "Clearly, formation of higher order CIP2A-TOPBP1 complexes that can recruit the SMX complex is required to resolve the mitotic DNA lesions in BRCA1/2 mutant cells.". I don't think that this is so "clearly" shown. The mutants used do not unambiguously demonstrate that the only property of CIP2A changed by the mutations is the formation of higher order structures.

Reviewer #2

(Remarks to the Author)

In this manuscript, de Haan et al have focused on the CIP2A-TOPBP1 complex and its role in coordinating mechanisms to cope with mitotic cells with incompletely replicated DNA, which can compromise proper chromosome segregation. The authors demonstrate that both aphidicolin and irradiation result in mitotic DNA lesions and CIP2A foci. Loss of CIP2A also resulted in loss of TOPBP1 foci, as expected. ATM and ATR inhibition had differential effects on aphidicolin- versus IR-induced CIP2A foci, indicating distinct upstream signaling. The authors used STED to produce super-resolution images of CIP2A structures formed by either aphidicolin or IR induced damage and traced these structures through mitosis. They observed 3 distinct classes of CIP2A structure (unstructured, loop-containing and filamentous) and noted changes to the prevalence of each class through mitosis. The authors found CIP2A structures formed by aphidicolin (incomplete replication) specifically recruited the SMX complex, consisting of SLX4, XPF-ERCC1 and MUS81-EME1, at or near regions of perturbed DNA replication. CIP2A was not required for MiDAS in this system, nor did depletion of MUS81. Using deletion constructs, the authors demonstrated that the C-terminal domain of CIP2A is required for CIP2A-TOPBP1 structure formation and for SMX recruitment. They also demonstrated the importance of CIP2A-TOPBP1-SMX structures in BRCA-deficient cell lines. They propose that CIP2A-TOPBP1 structures recruit SMX to under-replicated regions to generate breaks that allow chromosome segregation and can be repaired by POLQ microhomology-mediated end-joining. Overall the data are convincing and make a strong argument for CIP2A-TOPBP1 being important for roles beyond holding DSB ends together.

Major comments:

1. Was SLX4 or MUS81 found in the mass spectrometry analysis? If not, why might that be? To what extent do the foci for XPF overlap with SLX4 and/or MUS81? Is there a role for XPF outside of SMX in this context? Are there XPF foci that do not colocalize with CIP2A?
2. The dependence on CIP2A-TOPBP1 for recruitment of SMX is clear, as is dependence of MUS81 recruitment on SLX4. I would like to see dependence of XPF on SLX4. Also of interest is whether recruitment of either endonuclease (XPF-ERCC1 or MUS81-EME1) is dependent on the other or if perhaps SMX subcomplexes can be recruited and are perhaps functional in this context. Is the % localization of SLX4, XPF and MUS81 comparable? Looking at Figure 3 D, E F, there is a higher % of structures with SLX4 than XPF and MUS81. And the quantification of that colocalization – similar amount of each? – is not shown. Is it possible to be more quantitative to assess the potential for subcomplex recruitment?
3. Similarly, is there any differential recruitment of XPF vs MUS81 in any of the CIP2A truncation mutations?
4. While colocalization of CIP2A and SLX4 was observed in BRCA1 depleted cells, the percent co-localization was significantly reduced compared to BRCA+ cells (30% vs. 60%). Does this mean that BRCA1 is involved in recruitment of SLX4 or stabilization of those interactions? The same was not observed for BRCA2- cells.

Reviewer #3

(Remarks to the Author)

Noteworthy results | Originality and significance.

This is an exciting and timely study that provides us with new molecular insights into mitotic DNA repair. It builds on previous studies that identified the CIP2A-TOPBP1 complex as a molecular tether that connects broken DNA ends that get transmitted from interphase into mitosis (PMID: 30898438, 35842428, 35842428). The results are also largely complementary with those of Martin et al. (BioRxiv preprint at <https://doi.org/10.1101/2024.11.12.621593>).

The authors report two main findings, as summarized below.

1) Through the use of super-resolution microscopy (STED), they provide the first high-resolution details into the architecture of the large, filamentous structures marked by CIP2A-TOPBP1 in mitosis (PMID: 30898438, 35842428, 35842428). They observe three general types of CIP2A-TOPBP1 structures – unstructured, loop-containing, and filamentous.

1.a) Through structure-function studies of CIP2A, they show that the unstructured C-terminal region is required for the formation of these structures in mitosis and the genome stability functions of CIP2A in RPE1-hTERT p53^{-/-} and DLD1 cells (WT and BRCA2^{-/-}).

1.b) Interestingly, these structures form in response to IR and aphidicolin, pointing towards a general feature of the DNA damage response in mitosis. However, the protein composition of CIP2A-TOPBP1 complexes differs between IR and aphidicolin. Although not fleshed out in this manuscript, the observation suggests that the CIP2A-TOPBP1 structures can regulate downstream repair through protein-protein interactions tailored to the nature of the DNA lesion. This will be an important area for future research.

2) Three proteins of the SMX tri-nuclease (i.e. SLX4, MUS81 and XPF) colocalize with the mitotic CIP2A-TOPBP1 structures seen by STED. The authors also show that CIP2A (and in particular the unstructured C-terminal region) is required for the ability of SLX4, MUS81, and XPF to form foci on mitotic chromatin. Although the requirement of TOPBP1 for mitotic SLX4 foci formation has been known for some time (PMID: 26283799), this study shows that CIP2A is involved in the requirement/stabilization of SMX proteins at replication-stress induced DNA lesions in mitosis.

In general, this is a solid body of work that includes appropriate cell models and experimental controls. The manuscript is well-written, and the data is presented coherently. The main findings will have important implications for researchers interested in mitotic DNA damage, genome stability, and cancer biology. After addressing the points below, I believe this manuscript will be a strong candidate for publication in Nature Communications.

Validity | Data & methodology | Suggested improvements.

The experimental approaches are valid, and the data and their presentation are generally of good quality. My specific concerns are detailed below.

- Figure 1. What is the frequency of the CIP2A-TOPBP1 structures seen in mitosis? In other words, what % of the mitotic cells contain these structures? Is there any correlation with multipolar spindles?
- Figure 1F-I. This reviewer found it difficult to decipher what data was inputted and outputted in these panels and would have appreciated a more detailed discussion in the manuscript. For example, what is the definition of a CIP2A-TOPBP1 structure in terms of pixels? Additionally, panels H and I may be better represented as stacked bar charts, showing the mean of each replicate.
- Figure 2. One of the major results in this study is that CIP2A-TOPBP1 recruits SLX4, MUS81 and XPF to replication-induced DNA lesions in mitosis (where lesions are inferred by the presence of CIP2A-TOPBP1). The complete loss of foci containing SLX4, MUS81, and XPF is striking. Since SLX4, MUS81 and XPF form foci in interphase cells treated with genotoxic agents, the authors should verify that these foci form similarly in WT and CIP2A^{-/-} cells. It would also be helpful to have a western blot showing the total protein levels of SLX4, MUS81, and XPF in WT and CIP2A^{-/-} cells.
- Supplementary Figure 2. Experiments similar to those performed in Supplementary Figure 2F-J should be performed with siCTRL/siMUS81 and siCTRL/siXPF to confirm antibody specificity. Additionally, since XPF has SLX4-independent roles in the cell, it would be helpful for the authors to quantify the number of XPF foci per mitotic cell in siCTRL/siSLX4 cells (as they did for MUS81).
- Figure 4. The main message in this figure is that CIP2A is not required for MiDAS, with a sub-text that SLX4 and MUS81 have cell line-dependent roles in APH-induced MiDAS. While I agree that EdU is the most direct marker of MiDAS, it would be helpful if the authors could use a second molecular marker to probe MiDAS (e.g. twin FANCD2 foci).
- Figure 4B. The authors report a very high number of EdU foci in some of the APH-treated cells. At what point does this become pan-nuclear staining? It would be helpful if they could comment on the extensive distribution of EdU foci per cell (e.g. ranges from < 10 to > 100). Can they provide representative images that span the distribution?
- Line 282 (related to Figure 5 and Supp Figure 5). The authors claim that all CIP2A mutants showed stable expression, except for CIP2A dGlob. This should be rephrased since the data in Supp Figure 5A shows that: i) CIP2A dCC and Glob are expressed several fold less than the other mutants; and ii) CIP2A dGlob is not detected (what evidence do they have that this protein is expressed?). Along these lines, the data with the CIP2A dGlob mutant should be removed unless they can provide evidence that the protein is expressed.
- Figure 6. The main message is that the mitotic recruitment of SMX to CIP2A foci is required for the survival of BRCA1^{-/-} and BRCA2^{-/-} cells (lines 324-326). However, the authors don't provide data to support their conclusion for BRCA1^{-/-} cells. Instead, the authors imply that what's shown for BRCA2^{-/-} cells in Figures 6E-J will hold true for BRCA1^{-/-} cells because BRCA1^{-/-} cells contain elevated numbers of mitotic CIP2A foci. This should be corrected either editorially or experimentally by expanding the assays conducted with BRCA1^{-/-} cells.
- Line 323 (related to Figure 6C-D). The authors state that a large fraction of mitotic CIP2A foci recruit SLX4 in BRCA1^{-/-} and BRCA2^{-/-} cells. While I agree that the data support this conclusion for BRCA2^{-/-} cells (>65% of CIP2A foci contain SLX4), this is not the case for the BRCA1^{-/-} cells: only 30% of CIP2A foci contain SLX4 and there are fewer CIP2A/SLX4 positive foci in BRCA1^{-/-} cells than in BRCA1 WT cells (likely due to the significant increase in CIP2A foci in BRCA1^{-/-} cells). What do the numbers look like if the authors measure the number of CIP2A/SLX4 colocalizations in cells that contain at least 1 CIP2A/SLX4 colocalization?
- This reviewer would appreciate it if the authors were consistent with how they present quantified colocalizations in the main figures. For example, Figure 1B shows the number of CIP2A/gH2AX foci per cell, while Figure 2C shows CIP2A/XPF foci as a % (of what?). While I appreciate that the supp figures contain the reciprocal data, addressing this comment may improve the general readability of the manuscript.
- The authors should provide more methodological details on how they detected and quantified foci and colocalized foci from confocal images (e.g. thresholds and definition of colocalization, whether foci had to overlap with DAPI, etc). Is this the same pipeline that was used for V5 and TOPBP1 quantification?

- In the figure legends, it would be helpful to indicate which microscopy images were collected by confocal microscopy (as they do for STED). This would remove any potential ambiguity about methodology.

Appropriate use of statistics and treatment of uncertainties (if applicable).

- Sample size. The number of mitotic cells analyzed is quite low given that the authors performed a mitotic enrichment by releasing from RO3306 (G2 arrest). To facilitate meaningful comparisons across the phases of mitosis, it is important to capture a large enough sample size and to ensure that each phase contains equal (or nearly equal) numbers of cells. The authors should consider this caveat in their discussion of the prevalence of the different structures during each stage of mitosis. If they wish to make quantitative comparisons, it may be helpful to perform a power analysis and determine the smallest sample size required for this type of comparison.
- Replicates. Figure 2C is the results of one experiment (line 712). This should be repeated twice for a total of three biological replicates.

Conclusions. Once the authors have conducted the experiments and editorial changes listed above, I believe that they will be in a strong position to make robust, valid, and reliable conclusions about the data presented.

Suggested improvements: Minor editorial corrections are listed below.

- Title: The authors should change 'SLX1/XPF/MUS81' to 'SLX4/MUS81/XPF' in recognition of the fact that they did not test SLX1 in this study.
- Abstract (line 31): I believe the conventional nomenclature is to put the catalytic subunit before the regulatory subunit, eg XPF-ERCC1.
- Line 97: inconsistent use of capital letters in the full spelling of STED.
- Line 201: suggest replacing 'foci was' with 'were'.
- Line 334: 'rescue' should be replaced with 'rescued'.
- Line 362 – since they did not perform STED of cells co-stained with EdU and SMX, the authors should clarify the underlying assumptions when they state that the SMX complex is recruited to sites of under-replicated DNA.
- Line 703 (title for Figure 2). For general readability, the authors should not write statements like 'SLX4-ERCC1-XPF-MUS81 complex', which could be interpreted to mean that this is a stable complex whose composition is different than that of the SMX tri-nuclease (made up of SLX1-SLX4, MUS81-EME1, and XPF-ERCC1). The manuscript would benefit from editing throughout to fix these minor issues.

References. Appropriate.

Clarity and context. Abstract is appropriate.

Reviewer #4

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #5

(Remarks to the Author)

In this paper the authors have investigated the function of the CIP2A-TOPBP1 complex to resolve incomplete replication during mitosis. They uncovered that the CIP2A-TOPBP1 complex is required for accumulation of the SMX complex in mitotic foci.

The authors made use of STED super-resolution microscopy to show the formation of different structures of the CIP2A-TOPBP1 complexes changing over the course of mitosis. Given the size of the structures this seems a good choice as STED provides a high resolution, with the ability to apply multicolor imaging without the need of registration of the images. The use of a combination of confocal microscopy and STED microscopy only to look at high resolution at the structures of interest is also an interesting approach.

Overall, the study is setup well and the paper is clearly written. The main conclusion that formation of CIP2A-TOPBP1 foci is required for SMX recruitment is well supported by their data. The focus of the paper on using super-resolution imaging to describe the structures is not essential for these conclusions, but does add to the existing literature showing the complexity in the organization of DNA repair complexes.

However, there are several issues the authors would need to address.

Major issues:

- The authors solely did their experiments in p53 deficient cell lines. While this approach seems understandable given their interest in studying the mechanisms of under-replication in cancer cells, and clearly marked throughout the text, it would be

useful if the authors could explain more carefully in the paper the potential consequences of the loss of p53 on their observations and whether their observations can also be relevant in conditions with functional p53. Do CIP2A-TOPBP1 foci also form in p53 wildtype cells under given IR and APH conditions?

- The classification of CIP2A-TOPBP1 foci acquired by STED microscopy is done manually. It is unclear to me what criteria the authors used to classify the different structures (is this based on their size?). While manual classification is not inherently problematic, it would be good to mention the objective criteria the authors have used to select the structures in the methods section.

Furthermore, the authors could make use of additional shape and structure metrics to describe the structures, such as their elongation, circularity and solidity. Additionally, I would encourage the authors to share the STED images of all classified structures in a supplementary figure or for example via Zenodo or the BioImageArchive.

- The different structures the authors observe by super-resolution are interesting, but I do think the relative localization of other proteins such as the SMX proteins, gH2AX and EdU are also compelling. The significance of these observations are not mentioned in the discussion. The authors also mention the possibility that filamentous structures reflect ultrafine bridges. Would it not be possible to show this directly by immunostaining combined with STED microscopy?

Minor issues

- Figure 1C: Could you please clarify what are the data points plotted within the barplots? In the legend is written that the bar plots in the mean and SEM of 3 biological replicates, however more than 3 dots are shown per condition.

- Figure 1D-E: The difference in scaling in the crops of the different structures is quite confusing, I would reconsider displaying them all at the same scale, so it is clear they differ in size.

- Figure 2A: It is mentioned in the legend that proteins marked red are enriched after either IR or APH, however XPF and MDC1 seems not enriched after IR treatment. Furthermore, apart from the marked proteins there are more proteins that are enriched at either IR or APH treatment. Please clarify.

- In Figure 2D-G, the labelling and legend of this figure is confusing as the first row of the panels is marked as TP53^{-/-} and the second CIP2A^{-/-} whereas both cell lines are TP53 deficient.

- "These three classes of structures occurred both in response to APH or IR treatment, which all showed a high degree of CIP2A and TOPBP1 co-localization within these structures, indicating that these proteins are less than 30 nm apart from each other (Fig. 1D, E, Suppl. Fig. 1G-I)."

Given the homogenous localization at apparent high concentration for both proteins in these structures I do think the claim that these proteins localize only 30 nm apart, suggesting a direct interaction, is problematic. By examining at the images of CIP2A and TOPBP1 differences in the spatial distribution of intensities throughout the structures can actually be observed.

- "The observation that the abundance of CIP2A-TOPBP1 structures gradually decreases during the course of mitotic progression already suggested that CIP2A-TOPBP1 is not only involved in tethering DNA ends, but also functions in processing of DNA lesions."

This is an interesting statement, but I think this needs to be rephrased.

If CIP2A-TOPBP1 is only present to tether the DNA ends and other proteins are resolving the lesions one would also observe a reduction in the number of foci as the lesions are being resolved. The data in the paper suggests that CIP2A-TOPBP1 can be used as a scaffold for other proteins to process DNA lesions, so their presence might be a prerequisite for repair, but the reduction in number of CIP2A foci is not evidence for their function in processing DNA lesions.

- Personally I do not see much difference between the raw and Wiener deconvoluted STED images shared in the paper. Could the authors explain the need to make use of deconvolution. In the supplementary methods they mention a wide variety of settings the authors used to deconvolve the images, some explanation on the choice of those different parameters would be useful, given the images are shown in the main panels of the figures. Are all images processed with the same settings or on what basis different settings have been used?

- I do think full western blot images should be provided as supplementary images to the paper.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have addressed my comments appropriately.

(Remarks on code availability)

N/A

Reviewer #2

(Remarks to the Author)

I appreciate the thoughtful responses to all the comments. The new results that have been added in response to my comments, as well as the comments of the other reviewers, have provided additional mechanistic details and have improved

and strengthened this manuscript. Overall, the data remain convincing and present a strong argument for a novel role of CIP21-TOPBP1 in responding to replication-stress induced lesions by facilitating the recruitment of SMX to under-replicated DNA during mitosis.

(Remarks on code availability)

I followed the link but have no way of evaluating the code.

Reviewer #3

(Remarks to the Author)

The authors have done an excellent job at addressing my concerns or attempting to address my concerns. I am satisfied with their rebuttal and approve the manuscript for publication in Nature Communications.

(Remarks on code availability)

Reviewer #4

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

(Remarks on code availability)

Reviewer #5

(Remarks to the Author)

The authors have carefully addressed the raised issues. The only remaining issue is that the URL (<https://doi.org/10.34894/O3KAFO>) to the Dataverse repository containing the raw imaging data is not working (yet) in my hands.

(Remarks on code availability)

The code contains scripts to process the super-resolution images. I managed to run the code, however it required manually installing the required dependencies. For reproducibility it would be useful to add some installation instructions in the README or provide a pip or conda requirements file to ease installation of the required python libraries. The Jupyter notebook itself is very clear, easy to use and the provided test data made it easy to validate the software.

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Point-by-point rebuttal for NCOMMS-25-25109-T

Reviewer #1 (Remarks to the Author):

Comment: *‘Haan et al investigate the role of the CIP2A-TOPBP1 complex during mitosis, particularly in processing incompletely replicated DNA. The authors show that CIP2A-TOPBP1 forms large filamentous structures at sites of incomplete DNA replication during mitosis, recruiting the SMX tri-nuclease complex (SLX4, MUS81, ERCC1/XPF). While CIP2A is not required for mitotic DNA synthesis itself, its C-terminal domain is essential for filament formation and SMX complex recruitment. Importantly, BRCA1-/- and BRCA2-/- cells exhibit increased mitotic lesions involving CIP2A and SLX4, and the C-terminal domain of CIP2A, as well as SLX4, are critical for genome stability and survival of BRCA2-/- cells.’ Overall, the work is of high quality and the findings are novel and relevant, particularly for understanding genome maintenance mechanisms in BRCA-deficient contexts. The link between CIP2A filament formation and SMX recruitment during mitosis provides an interesting advance. The manuscript is generally well written and experiments well designed, but I would suggest that the authors consider addressing the minor issues bellow:’*

Reply: We thank the reviewer for the positive remarks.

Comment 1: *‘Abstract: “We show that CIP2A-TOPBP1 forms large filamentous structures at sites of incomplete DNA replication during mitosis, which recruit the SMX tri-nuclease complex members SLX4, MUS81 and ERCC1/XPF.” I don’t think that the authors show that specifically filaments of CIP2A-TOPBP1 recruit SLX4, etc. The formulation could be slightly revised to avoid mis-interpretation by the reader.’*

Reply: We agree with the reviewer. We show that CIP2A is required for localization of SMX components SLX4, MUS81, XPF, ERCC1 to mitotic DNA lesions, but do not specifically show that the filament formation is specifically required for this recruitment. We have altered the text in the abstract on line 35/36.

Comment 2: *‘Line 173: “CIP2A interacts with the SMX complex upon perturbed replication.” I don’t think the authors show that there is an interaction between CIP2A and SMX complex. XPF was slightly enriched in IP-MS, but the others not. Having said this, the authors may have a different interpretation of what the term “interaction” means. Is the presence at mitotic structures + dependency for recruitment the evidence for interaction?’*

Reply: We agree with the reviewer that our data indicate a dependency, rather than proof for a direct interaction between CIP2A and SMX complex members. We have altered the text to clarify this throughout the manuscript, including on lines 105, 240-252, 298, 414, 437.

Comment 3: *'Figure 3H, etc. I am not sure what to make of the EdU labelling of the CIP2A structures. There is clearly a correlation, but the spatial relationship in super-resolution is confusing. Do the authors have any proposal for how such structures might look like? Perhaps a speculative cartoon could be drawn?'*

Reply: To clarify the spatial relationship, we now have included an extended super-resolution analysis of EdU staining, including co-staining with γ H2AX and CIP2A. We observed that in unstructured CIP2A complexes, when CIP2A co-localizes with γ H2AX, EdU is localized at the perimeter of this γ H2AX/CIP2A staining. As soon as loop-containing structure emerge, EdU is in close proximity to CIP2A, with EdU signal being adjacent to the most intense CIP2A staining. In later stages of mitosis, γ H2AX does not appear to co-localize with CIP2A, but is rather localized to the core of the loop-containing CIP2A structures, while it was excluded from filamentous CIP2A structures. This could indicate that local chromatin remodeling is ongoing, possible with histone eviction, leading to local removal of γ H2AX. These analyses have been included in the New Suppl. Figure 6, and discussed on lines 279-289.

Comment 4: *'Figure 5/S5A: Expression levels of the mutants is not very consistent. Phenotype description of all mutants, even of S904A, which shows lower expression in one of the experiments, should be made with the expression levels in mind. The authors write "All CIP2A mutants showed stable expression, 283 except for CIP2AGlob (Suppl. Fig. 5A)." Stable is not a meaningful way of describing the expression. Identical expression levels to the control full length is required for all conclusions made. Otherwise conclusions have to be adjusted.'*

Reply: We agree with the reviewer. As suggested, we have altered the text (lines 334-348) and have removed 'stable' as a description for protein expression. We also removed the data on CIP2A-Glob, as expression of this variant was hardly detected and these data are not informative for analysis of domain function of CIP2A. Finally, we have included a statement that the expression levels of the Glob and Δ CC mutants were significantly lower, and that this could explain lack of rescue. We have added this statement on lines 350-353.

Comment 5: *'Figure 5B, low signal in the MUS81 WB makes it hard to judge how efficient the depletion is, which is important for some of the conclusions made.'*

Reply: We have repeated the WB analysis of MUS81 and have included new Western blots in Suppl. Fig. 4C. Also, we have quantified MUS81 foci over multiple experiments and have included the quantifications in Suppl. Fig. 4D.

Comment 6: *'335-337: "Clearly, formation of higher order CIP2A-TOPBP1 complexes that can recruit the SMX complex is required to resolve the mitotic DNA lesions in BRCA1/2 mutant cells." I don't think that this is so "clearly" shown. The mutants used do not*

unambiguously demonstrate that the only property of CIP2A changed by the mutations is the formation of higher order structures.'

Reply: We agree with the reviewer and have adjusted our wording on line 413-415.

Reviewer #2 (Remarks to the Author):

Comment: *'In this manuscript, de Haan et al have focused on the CIP2A-TOPBP1 complex and its role in coordinating mechanisms to cope with mitotic cells with incompletely replicated DNA, which can compromise proper chromosome segregation. The authors demonstrate that both aphidicolin and irradiation result in mitotic DNA lesions and CIP2A foci. Loss of CIP2A also resulted in loss of TOPBP1 foci, as expected. ATM and ATR inhibition had differential effects on aphidicolin- versus IR-induced CIP2A foci, indicating distinct upstream signaling. The authors used STED to produce super-resolution images of CIP2A structures formed by either aphidicolin or IR induced damage and traced these structures through mitosis. They observed 3 distinct classes of CIP2A structure (unstructured, loop-containing and filamentous) and noted changes to the prevalence of each class through mitosis. The authors found CIP2A structures formed by aphidicolin (incomplete replication) specifically recruited the SMX complex, consisting of SLX4, XPF-ERCC1 and MUS81-EME1, at or near regions of perturbed DNA replication. CIP2A was not required for MiDAS in this system, nor did depletion of MUS81. Using deletion constructs, the authors demonstrated that the C-terminal domain of CIP2A is required for CIP2A-TOPBP1 structure formation and for SMX recruitment. They also demonstrated the importance of CIP2A-TOPBP1-SMX structures in BRCA-deficient cell lines. They propose that CIP2A-TOPBP1 structures recruit SMX to under-replicated regions to generate breaks that allow chromosome segregation and can be repaired by POLQ microhomology-mediated end-joining. Overall the data are convincing and make a strong argument for CIP2A-TOPBP1 being important for roles beyond holding DSB ends together.'*

Reply: We thank the reviewer for the positive remarks

Major comments:

Comment 1: *'Was SLX4 or MUS81 found in the mass spectrometry analysis? If not, why might that be? To what extent do the foci for XPF overlap with SLX4 and/or MUS81? Is there a role for XPF outside of SMX in this context? Are there XPF foci that do not colocalize with CIP2A?'*

Reply: We have updated the CIP2A IP-MS graphs in Fig. 2A, B and Suppl. Fig. 3B, C, and have now indicated SLX4 and other SMX members. SLX4 was identified as significantly enriched in the MS analysis cells of untreated CIP2A cells (Suppl. Fig. 3C), other SMX complex members were identified, but were not significantly enriched. We also think that the indirect interaction of CIP2A with SMX (through TOPBP1) explains why SLX4 interactors within the SMX complex were not all identified. Consistently, the number of identified TOPBP1 peptides was much higher when compared to identified SLX4 peptides. Lastly, we highlighted the top 25 enriched proteins for the described CIP2A IP-MS comparisons in Suppl. Table 1.

Concerning the overlap between XPF and SLX4/MUS81 foci: we have performed the proposed experiment and observed that the far majority of SLX4 foci and MUS81 foci co-localizing with XPF (now included in Suppl. Fig. 3I, J).

Concerning the amount of XPF foci that do not co-localize with CIP2A; we have quantified this and rarely observed XPF foci that do not co-localize with CIP2A. These data are now included in new Fig. Suppl. Fig 3E.

Comment 2: *'The dependence on CIP2A-TOPBP1 for recruitment of SMX is clear, as is dependence of MUS81 recruitment on SLX4. I would like to see dependence of XPF on SLX4. Also of interest is whether recruitment of either endonuclease (XPF-ERCC1 or MUS81-EME1) is dependent on the other or if perhaps SMX subcomplexes can be recruited and are perhaps functional in this context. Is the % localization of SLX4, XPF and MUS81 comparable? Looking at Figure 3 D, E F, there is a higher % of structures with SLX4 than XPF and MUS81. And the quantification of that colocalization – similar amount of each? – is not shown. Is it possible to be more quantitative to assess the potential for subcomplex recruitment?'*

Reply: This is an important point by the reviewer, and relates to the presence of sub-complexes, possible pointing at CIP2A-independent roles of XPF or MUS81. We quantified the degree of co-localization of SLX4, ERCC1 and MUS81 with CIP2A structures in STED images, and observed only a minor difference between localization of SLX4 (92%) and the ERCC1 or MUS81 nucleases (82%). These data are now included in Suppl. Fig. 5A. We also tested subcomplex recruitment by quantifying the recruitment of XPF after depleting SLX4 or MUS81 using siRNA. We now show that XPF recruitment is dependent on SLX4, but not on MUS81. Likewise, we show data that MUS81 recruitment is not dependent on XPF. We also quantified the degree of co-localization between XPF and SLX4, and XPF and MUS81. Here we see that all XPF foci co-localize with both MUS81 and SLX4. These data are now included in Suppl. Fig. 3I and J. Together, these data indicate that SLX4 is recruited to CIP2A where it functions as being the scaffold that subsequently recruits the other complex members. These data are now included in Suppl. Fig. 4G-L.

Comment 3: *‘Similarly, is there any differential recruitment of XPF vs MUS81 in any of the CIP2A truncation mutations?’*

Reply: We have now analyzed mitotic XPF foci formation in cells expression CIP2A mutants. New data is included in Fig. 5I and representative figures are included in Suppl. Fig. 9D. We observed that both XPF and MUS81 nucleases are not properly recruited in cells expressing the deltaC CIP2A mutant, whereas both XPF and MUS81 are normally recruited in cells expressing the S904A mutant. In conclusion, we do not see differential recruitment of XPF and MUS81 in the analyzed CIP2A mutants.

Comment 4: *‘While colocalization of CIP2A and SLX4 was observed in BRCA1 depleted cells, the percent co-localization was significantly reduced compared to BRCA+ cells ((30% vs. 60%). Does this mean that BRCA1 is involved in recruitment of SLX4 or stabilization of those interactions? The same was not observed for BRCA2- cells.’*

Reply: The reviewer is correct that SLX4-CIP2A co-localization is reduced in *BRCA1*-mutant cells, when compared to aphidicolin-treated *BRCA1*-WT cells. We have tested whether *BRCA1* is required for SLX4 recruitment to CIP2A structures. We see that in *BRCA1*-mutant cells, aphidicolin is still able to induce mitotic CIP2A foci that are positive for SLX4, indicating that *BRCA1* is not required for SLX4 recruitment (Supp. Fig. 10A, B). We now also included analysis of the co-localization of SLX4 to CIP2A foci and see that all SLX4 foci are colocalizing with CIP2A (Suppl. Fig 10B).

We argue that *BRCA1*-mutant cells acquire a variety of DNA lesions that are all recognized by CIP2A, but do not all require SLX4 for processing. To further test this, we used an orthogonal approach to analyze *BRCA1* inactivation, using AID-BARD1 cell lines. Upon auxin-mediated BARD1 inactivation, *BRCA1* is destabilized. In these cells, we also observed an increase in mitotic CIP2A foci, again with a relatively low percentage of CIP2A foci being SLX4-positive (now included in Suppl. Figure 10D, F).

Reviewer #3 (Remarks to the Author):

Comment: *‘Noteworthy results | Originality and significance.’*

This is an exciting and timely study that provides us with new molecular insights into mitotic DNA repair. It builds on previous studies that identified the CIP2A-TOPBP1 complex as a molecular tether that connects broken DNA ends that get transmitted from interphase into

mitosis (PMID: 30898438, 35842428, 35842428). The results are also largely complementary with those of Martin et al. (BioRxiv preprint at <https://doi.org/10.1101/2024.11.12.621593>).’

The authors report two main findings, as summarized below.

1) Through the use of super-resolution microscopy (STED), they provide the first high-resolution details into the architecture of the large, filamentous structures marked by CIP2A-TOPBP1 in mitosis (PMID: 30898438, 35842428, 35842428). They observe three general types of CIP2A-TOPBP1 structures – unstructured, loop-containing, and filamentous.

1.a) Through structure-function studies of CIP2A, they show that the unstructured C-terminal region is required for the formation of these structures in mitosis and the genome stability functions of CIP2A in RPE1-hTERT p53^{-/-} and DLD1 cells (WT and BRCA2^{-/-}).

1.b) Interestingly, these structures form in response to IR and aphidicolin, pointing towards a general feature of the DNA damage response in mitosis. However, the protein composition of CIP2A-TOPBP1 complexes differs between IR and aphidicolin. Although not fleshed out in this manuscript, the observation suggests that the CIP2A-TOPBP1 structures can regulate downstream repair through protein-protein interactions tailored to the nature of the DNA lesion. This will be an important area for future research.

2) Three proteins of the SMX tri-nuclease (i.e. SLX4, MUS81 and XPF) colocalize with the mitotic CIP2A-TOPBP1 structures seen by STED. The authors also show that CIP2A (and in particular the unstructured C-terminal region) is required for the ability of SLX4, MUS81, and XPF to form foci on mitotic chromatin. Although the requirement of TOPBP1 for mitotic SLX4 foci formation has been known for some time (PMID: 26283799), this study shows that CIP2A is involved in the requirement/stabilization of SMX proteins at replication-stress induced DNA lesions in mitosis.

In general, this is a solid body of work that includes appropriate cell models and experimental controls. The manuscript is well-written, and the data is presented coherently. The main findings will have important implications for researchers interested in mitotic DNA damage, genome stability, and cancer biology. After addressing the points below, I believe this manuscript will be a strong candidate for publication in Nature Communications.’

Reply: We thank the reviewer for the accurate summary and positive remarks.

Comment: ‘Validity | Data & methodology | Suggested improvements.

The experimental approaches are valid, and the data and their presentation are generally of good quality. My specific concerns are detailed below.’

Comment: ‘Figure 1. What is the frequency of the CIP2A-TOPBP1 structures seen in

mitosis? In other words, what % of the mitotic cells contain these structures? Is there any correlation with multipolar spindles?’

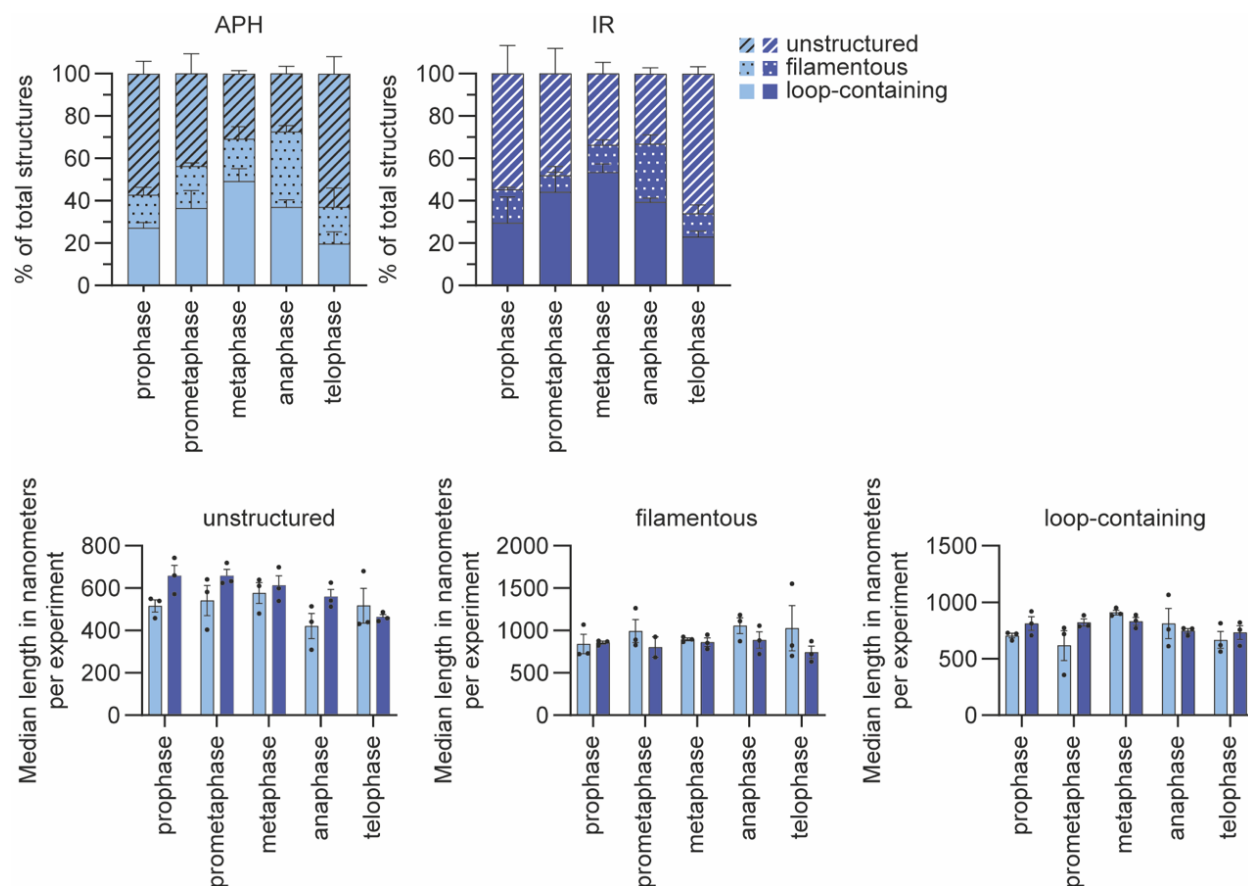
Reply: We have now quantified the percentage of mitotic cells with/without CIP2A-TOPBP1 structures. In unperturbed cells, we find 3 out of 34 (91.2%) cells have at least one CIP2A-TOPBP1 structure (Suppl. Fig. 1D). In aphidicolin-treated cells, we observed that initially 100% of mitotic cells have at least one CIP2A-TOPBP1 structure (Suppl. Fig. 1D), and this percentage lowers to 88.5% and 95.7% in the final stages of mitosis. These data have now been included in Suppl. Fig. 2B.

In our IF microscopy analysis, we did not include staining of spindle components, and therefore were not able to systematically record the presence of multi-polar spindles in our analyses. For STED analysis, no cells with multipolar spindles were included for analysis.

Comment: *‘Figure 1F-I. This reviewer found it difficult to decipher what data was inputted and outputted in these panels and would have appreciated a more detailed discussion in the manuscript. For example, what is the definition of a CIP2A-TOPBP1 structure in terms of pixels? Additionally, panels H and I may be better represented as stacked bar charts, showing the mean of each replicate.’*

Reply: As the reviewer suggested, we replotted the data as stacked bar graphs (included below). In discussing these plots with co-authors and colleagues, the consensus was that these plots did not make the data easier to understand. We have therefore kept the original data plots.

Concerning the question what the definition of a CIP2A-TOPBP1 structure is in terms of pixels; we did not use a threshold to define structure boundaries. We used manual classification to determine the structure type. We have now clarified this procedure in the supplemental methods. Additionally, we determined the size of the CIP2A-TOPBP1 structures by measuring the longest and shortest axis of CIP2A, as now explained in more detail in Suppl. Fig 2C.



Rebuttal Figure 1. Data from Figure 1 plotted as stacked bar graphs.

Comment: ‘Figure 2. One of the major results in this study is that CIP2A-TOPBP1 recruits SLX4, MUS81 and XPF to replication-induced DNA lesions in mitosis (where lesions are inferred by the presence of CIP2A-TOPBP1). The complete loss of foci containing SLX4, MUS81, and XPF is striking. Since SLX4, MUS81 and XPF form foci in interphase cells treated with genotoxic agents, the authors should verify that these foci form similarly in WT and CIP2A^{-/-} cells. It would also be helpful to have a western blot showing the total protein levels of SLX4, MUS81, and XPF in WT and CIP2A^{-/-} cells.’

Reply: These are important points raised by the reviewer. We found the SLX4, MUS81 and XPF protein levels to be similar between WT vs CIP2A^{-/-} RPE1 cells, as analyzed by western blot (now included in Suppl. Fig. 3G). Hence, loss of SLX4/MUS81/XPF foci in the CIP2A^{-/-} cells was not caused by changes in levels of these proteins.

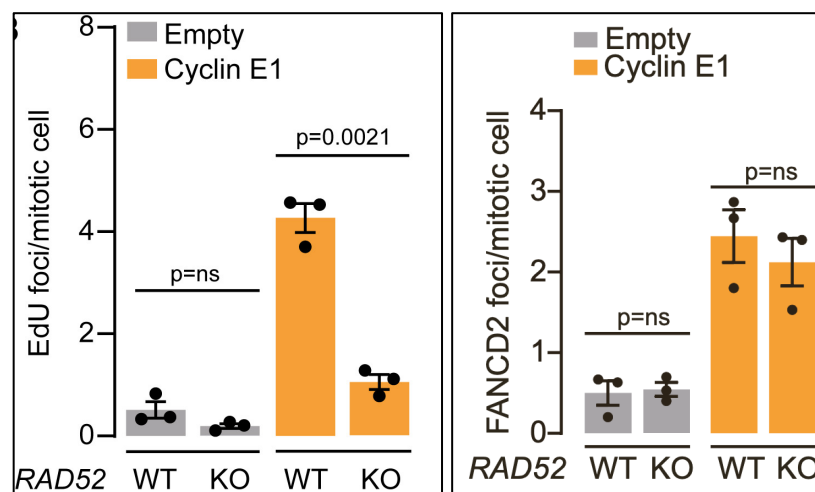
For MUS81 and XPF foci analysis in interphase cells; we typically do not see distinct foci of these proteins. We were able to analyze SLX4 foci formation in interphase cells, and observed that SLX4 foci form normally in interphase CIP2A^{-/-} RPE1 cells, pointing at differential regulation of SLX4 foci formation between interphase and mitotic cells, with a mitosis-specific requirement for CIP2A. These results are included in Suppl. Fig. 3H of the revised manuscript.

Comment: ‘Supplementary Figure 2. Experiments similar to those performed in Supplementary Figure 2F-J should be performed with siCTRL/siMUS81 and siCTRL/siXPF to confirm antibody specificity. Additionally, since XPF has SLX4-independent roles in the cell, it would be helpful for the authors to quantify the number of XPF foci per mitotic cell in siCTRL/siSLX4 cells (as they did for MUS81).’

Reply: We validated the specificity of MUS81 and XPF antibodies by depleting these proteins by siRNA and simultaneously assessing protein levels by western blot and foci formation. These data are now included in Suppl. Fig. 4C-F. We also quantified XPF foci in SLX4-depleted cells and observed a clear reduction in XPF foci upon SLX4 depletion, indicating that SLX4 is recruiting both MUS81 and XPF to replication stress lesions. These data are included in Suppl. Fig. 4I/J.

Comment: ‘Figure 4. The main message in this figure is that CIP2A is not required for MiDAS, with a sub-text that SLX4 and MUS81 have cell line-dependent roles in APH-induced MiDAS. While I agree that EdU is the most direct marker of MiDAS, it would be helpful if the authors could use a second molecular marker to probe MiDAS (e.g. twin FANCD2 foci).’

Reply: We agree the reviewer that EdU incorporation is the most direct read-out of MiDAS, as it reflects DNA synthesis. To the best of our knowledge, no robust alternative measure of MiDAS has been described. Concerning twin FANCD2 foci as a measure of MiDAS; FANCD2 foci merely reflect the presence of underreplicated DNA, rather than ongoing MiDAS. In fact, we previously showed that RPE1 *RAD52*^{-/-} cells, which cannot perform MiDAS, still show mitotic FANCD2 foci [Audrey *et al*, Cell reports, 2024; PMID:38625790], indicated below. In addition, FANCD2 foci persist in late-stage mitotic cells at the edge of ultra-fine bridges [Chan *et al*, Nature Cell Biology, 2009; PMID: 19465922], again arguing that FANCD2 twin foci do not per se read out mitotic DNA synthesis, but rather mark sites of unresolved DNA lesions.

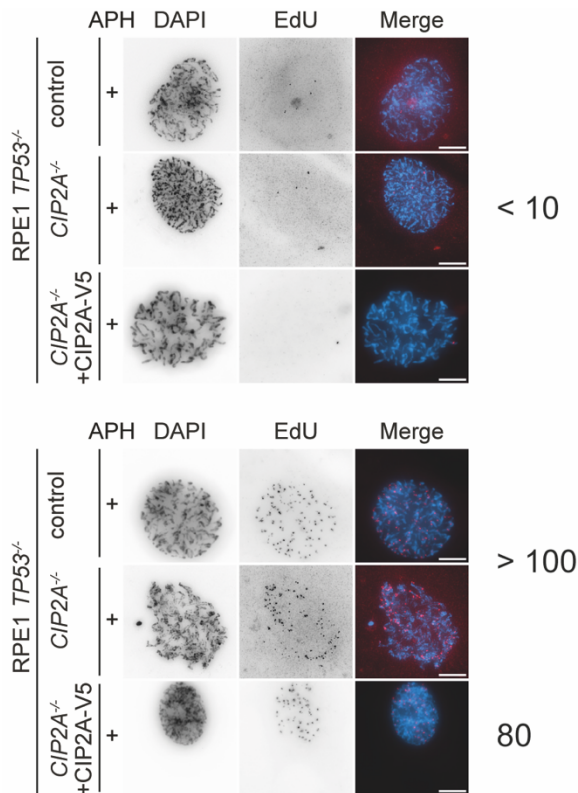


Rebuttal Figure 2. *RAD52 inactivation prevents MiDAS induced by Cyclin E1 overexpression, but does not affect mitotic FANCD2 foci formation (PMID: 38625790 DOI: 10.1016/j.celrep.2024.114116).*

As an alternative approach, we tested if RAD52 foci were still present in *CIP2A^{-/-}* cells. We observed the CIP2A inactivation does not prevent RAD52 foci formation in mitosis, further strengthening our conclusion that MiDAS does not require CIP2A. This data is now included in Suppl. Fig. 7B.

Comment: ‘Figure 4B. The authors report a very high number of EdU foci in some of the APH-treated cells. At what point does this become pan-nuclear staining? It would be helpful if they could comment on the extensive distribution of EdU foci per cell (e.g. ranges from < 10 to > 100). Can they provide representative images that span the distribution?’

Reply: We have included below pictures of cells with <10 EdU foci, as well as cells with high amounts of foci (80 to >100), still showing multiple individual foci rather than pan-nuclear staining.



Rebuttal Figure 3. *Examples of cells with low and high numbers of EdU foci.*

Comment: ‘Line 282 (related to Figure 5 and Supp Figure 5). The authors claim that all CIP2A mutants showed stable expression, except for CIP2A dGlob. This should be rephrased since

the data in Supp Figure 5A shows that: i) CIP2A dCC and Glob are expressed several fold less than the other mutants; and ii) CIP2A dGlob is not detected (what evidence do they have that this protein is expressed?). Along these lines, the data with the CIP2A dGlob mutant should be removed unless they can provide evidence that the protein is expressed.'

Reply: We agree with the reviewer and have removed all results describing cells with the Δ Glob mutant, as this mutant is indeed non-informative. For the other mutants, we have included discussion on the lower expression levels on lines 334-338.

Comment: *'Figure 6. The main message is that the mitotic recruitment of SMX to CIP2A foci is required for the survival of BRCA1^{-/-} and BRCA2^{-/-} cells (lines 324-326). However, the authors don't provide data to support their conclusion for BRCA1^{-/-} cells. Instead, the authors imply that what's shown for BRCA2^{-/-} cells in Fig. 6E-J will hold true for BRCA1^{-/-} cells because BRCA1^{-/-} cells contain elevated numbers of mitotic CIP2A foci. This should be corrected either editorially or experimentally by expanding the assays conducted with BRCA1^{-/-} cells.'*

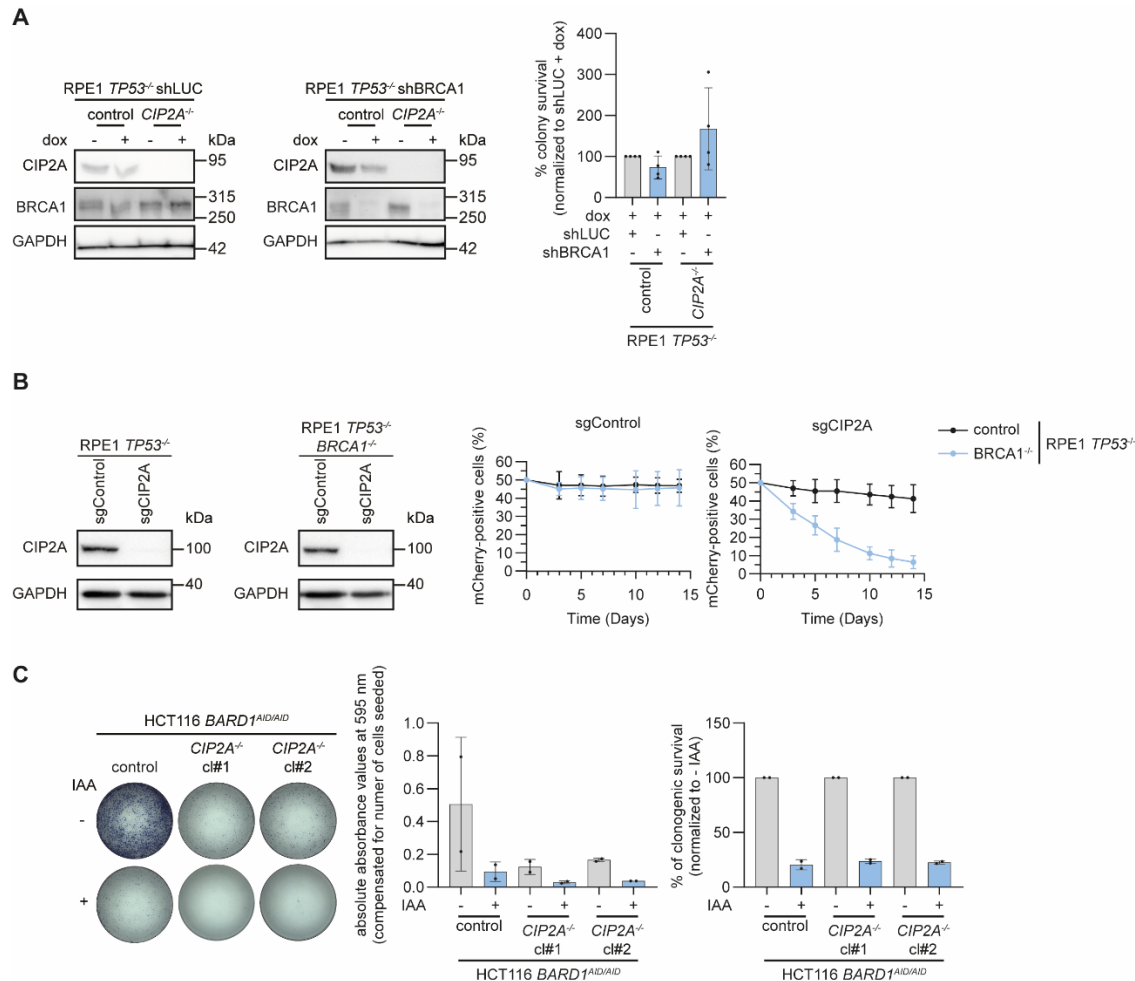
Reply: We have extended our analysis of homologous recombination deficient models.

Firstly, we attempted to take a similar approach as we used for BRCA2. Using doxycycline-inducible shBRCA1 in RPE1-TP53^{-/-} CIP2A^{-/-} cells, we observed reasonable BRCA1 depletion (Rebuttal Figure 4, panel A), but this was not sufficient to induce synthetic lethality (Rebuttal Figure 4, panel A), and precluded add-back experiments with CIP2A mutants. This was not due to a lack of synthetic lethality in RPE1 TP53^{-/-} cells, as CAS9-mediated inactivation of CIP2A in BRCA1^{-/-} cells clearly reduced viability in these cells (Rebuttal Figure 4, panel B). Unfortunately, the efficient SL in these BRCA1^{-/-} sgCIP2A cells, prevented us from establishing cell lines with CIP2A mutants.

We next attempted to use an alternative approach using HCT116 BARD1-AID cells, in which the BRCA1 co-factor BARD1 is endogenously tagged with an auxin-inducible degron. Upon auxin addition, BARD1 is degraded, leading to a non-functional instable BRCA1. We have included analysis of mitotic CIP2A/SLX4 foci in these cells, and observed increased numbers of mitotic CIP2A/SLX4 foci upon BARD1 inactivation (Suppl. Fig. 10D, F), mimicking observations in BRCA1^{-/-} cells.

Unfortunately, CIP2A depletion impacted the long-term viability of these cells, even in absence of auxin. Transduction of these HCT116 BARD1-AID CIP2A^{-/-} with CIP2A mutants resulted in poorly growing cells, which again precluded effective analysis of CIP2A mutants. Of note, BARD1 inactivation affected clonogenic survival in these cells, which was further decreased when CIP2A was inactivated, pointing at SL effects between BARD1 and CIP2A (Rebuttal Figure 5, panel C).

In conclusion, despite our efforts, technical setbacks prevented us to conclusively show that the C-terminus of CIP2A is required for survival of BRCA1^{-/-} cells. We have therefore adjusted the conclusions in our manuscript concerning SL effects to only BRCA2 mutant cells.



Rebuttal Figure 4.

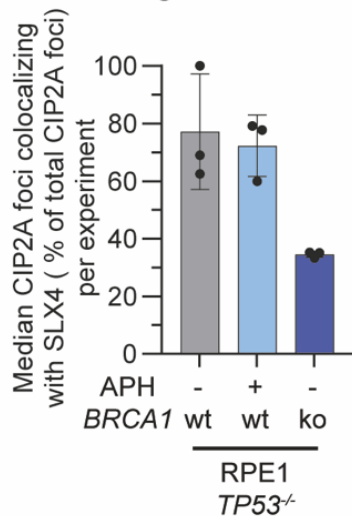
Concerning effects on the formation of micronuclei formation and cytoplasmatic bridges, we were able to use RPE1 *TP53*^{-/-} *BRCA1*^{-/-} cells in combination with CIP2A and SLX4 siRNAs. We now show that depletion of either CIP2A or SLX4 results in micronuclei and interphase bridges RPE1 *TP53*^{-/-} *BRCA1*^{-/-} cells. We also observed no significant difference between siCIP2A and siSLX4 effects, pointing at similar consequences of CIP2A and SLX4 inactivation. These data are now included in Figure 6, and extend our previous data in *BRCA2* mutant cells to *BRCA1* mutant cells.

Comment: 'Line 323 (related to Figure 6C-D). The authors state that a large fraction of mitotic CIP2A foci recruit SLX4 in *BRCA1*^{-/-} and *BRCA2*^{-/-} cells. While I agree that the data support this conclusion for *BRCA2*^{-/-} cells (>65% of CIP2A foci contain SLX4), this is not the case for the *BRCA1*^{-/-} cells: only 30% of CIP2A foci contain SLX4 and there are fewer CIP2A/SLX4 positive foci in *BRCA1*^{-/-} cells than in *BRCA1* WT cells (likely due to the significant increase in CIP2A foci in *BRCA1*^{-/-} cells). What do the numbers look like if the authors measure the

number of CIP2A/SLX4 colocalizations in cells that contain at least 1 CIP2A/SLX4 colocalization?’

Reply: This indeed is an interesting finding, and suggests that *BRCA1* vs *BRCA2* mutant cells accumulate different types of DNA lesions. As the reviewer suggested, we have re-analyzed our data, only looking at cells that contain at least 1 CIP2A/SLX4 focus. These data are shown below for the reviewers’ appreciation (Rebuttal Figure 5), and are very similar to the data in Figure 6C.

data when cells without any CIP2A⁺/SLX4⁺ colocalizing are excluded



Rebuttal Figure 5. Analysis of cells with at least 1 CIP2A/SLX4 focus.

Importantly, we have now included a second model in which *BRCA1* is functionally impaired by degradation of *BARD1* (HCT116-*aid-BARD* cells). In these cells we also find that only a minority of the CIP2A foci induced by *BARD1* inactivation are positive for SLX4, resembling the *BRCA1*^{-/-} data. These data are now included in Suppl. Figure 10D,F.

Comment: ‘This reviewer would appreciate it if the authors were consistent with how they present quantified colocalizations in the main figures. For example, Figure 1B shows the number of CIP2A/gH2AX foci per cell, while Figure 2C shows CIP2A/XPF foci as a % (of what?). While I appreciate that the supp figures contain the reciprocal data, addressing this comment may improve the general readability of the manuscript.’

Reply: This is a good point of the reviewer, and we now changed the labeling of the graphs.

Comment: ‘The authors should provide more methodological details on how they detected and quantified foci and colocalized foci from confocal images (e.g. thresholds and definition of colocalization, whether foci had to overlap with DAPI, etc). Is this the same pipeline that was used for V5 and TOPBP1 quantification?’

Reply: We have added information on the methodological details of foci and co-localization quantification in the Supplemental Document. All quantification of foci numbers and co-localization of foci was done by manual counting in ImageJ (FIJI). To determine overlap, foci channels were compared and scored. V5 and TOPBP1 foci intensity quantification was done with the ImageJ plugin 'Foci Analyzer' as described in the methods section.

Comment: *'In the figure legends, it would be helpful to indicate which microscopy images were collected by confocal microscopy (as they do for STED). This would remove any potential ambiguity about methodology.'*

Reply: We have now indicated in the figure legends which images were taken with confocal, wide-field or STED microscopy.

Comment: *'Appropriate use of statistics and treatment of uncertainties (if applicable). Sample size. The number of mitotic cells analyzed is quite low given that the authors performed a mitotic enrichment by releasing from RO3306 (G2 arrest). To facilitate meaningful comparisons across the phases of mitosis, it is important to capture a large enough sample size and to ensure that each phase contains equal (or nearly equal) numbers of cells. The authors should consider this caveat in their discussion of the prevalence of the different structures during each stage of mitosis. If they wish to make quantitative comparisons, it may be helpful to perform a power analysis and determine the smallest sample size required'*

Reply: We agree with the reviewer that sufficient numbers of cells in each mitotic phase need to be included to perform meaningful analyses. Due to the labor-intensive nature of STED microscopy, we have focused on imaging large quantities of individual CIP2A structures, rather than focusing on imaging many individual cells. For this reason, we provide descriptive metrics in Figure 1 on the many observed CIP2A structures, but refrained from making statistical statements in the description of CIP2A structures in Figure 1F-I. Conversely, for all wide-field and confocal analysis, we have analyzed foci from many individual cells, allowing statistical analysis.

Comment: *'Replicates. Figure 2C is the results of one experiment (line 712). This should be repeated twice for a total of three biological replicates.'*

Reply: This is a good point of the reviewer, and we now repeated this experiment twice more to get to a total of three biological replicates and added statistics. We have updated figure 2C with the new biological replicates and show that there is a significant difference in

colocalization between untreated and APH-treated cells, while there is no significant difference after IR.

Comment: *‘Conclusions. Once the authors have conducted the experiments and editorial changes listed above, I believe that they will be in a strong position to make robust, valid, and reliable conclusions about the data presented.’*

Reply: Thank you.

Comment: *‘Suggested improvements: Minor editorial corrections are listed below.
• Title: The authors should change ‘SLX1/XPF/MUS81’ to ‘SLX4/MUS81/XPF’ in recognition of the fact that they did not test SLX1 in this study.’*

Reply: Thank you for these suggestions, we have corrected the title accordingly.

Comment: *‘Abstract (line 31): I believe the conventional nomenclature is to put the catalytic subunit before the regulatory subunit, eg XPF-ERCC1.’*

Reply: We have now corrected this.

Comment: *‘Line 97: inconsistent use of capital letters in the full spelling of STED.’*

Reply: We have changed the full spelling of STED at multiple instances into Stimulated Emission Depletion microscopy, which capitalizes S, E and D.

Comment: *‘Line 201: suggest replacing ‘foci was’ with ‘were’.*

Reply: We have corrected this.

Comment: *‘Line 334: ‘rescue’ should be replaced with ‘rescued’.*

Reply: This has been corrected.

Comment: *‘Line 362 – since they did not perform STED of cells co-stained with EdU and SMX, the authors should clarify the underlying assumptions when they state that the SMX complex is recruited to sites of under-replicated DNA.’*

Reply: Although the reviewer is correct that we did not perform STED of EdU and SMX components, this statement was inferred from the STED data that CIP2A structures are induced by perturbed replication, are formed in the close vicinity of EdU foci, and STED data that SMX members are localized at these CIP2A structures.

Comment: *'Line 703 (title for Figure 2). For general readability, the authors should not write statements like 'SLX4-ERCC1-XPF-MUS81 complex', which could be interpreted to mean that this is a stable complex whose composition is different than that of the SMX tri-nuclease (made up of SLX1-SLX4, MUS81-EME1, and XPF-ERCC1). The manuscript would benefit from editing throughout to fix these minor issues.'*

Reply: we agree with the reviewer and have changed this into 'SMX complex'.

Comment: *'References. Appropriate.'*

Reply: Thank you.

Comment: *'Clarity and context. Abstract is appropriate.'*

Reply: Thank you.

Reviewer #4 (Remarks to the Author):

Comment: *'I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.'*

Reply: Thank you for co-reviewing the manuscript with the other reviewer.

Reviewer #5 (Remarks to the Author):

Comment: *'In this paper the authors have investigated the function of the CIP2A-TOPBP1 complex to resolve incomplete replication during mitosis. They uncovered that the CIP2A-TOPBP1 complex is required for accumulation of the SMX complex in mitotic foci. The authors made use of STED super-resolution microscopy to show the formation of different structures of the CIP2A-TOPBP1 complexes changing over the course of mitosis. Given the size of the structures this seems a good choice as STED provides a high resolution, with the ability to apply multicolor imaging without the need of registration of the images.'*

The use of a combination of confocal microscopy and STED microscopy only to look at high resolution at the structures of interest is also an interesting approach. Overall, the study is setup well and the paper is clearly written. The main conclusion that formation of CIP2A-TOPBP1 foci is required for SMX recruitment is well supported by their data. The focus of the paper on using super-resolution imaging to describe the structures is not essential for these conclusions, but does add to the existing literature showing the complexity in the organization of DNA repair complexes.'

Reply: We thank the reviewer for the constructive and positive remarks.

Comment *'However, there are several issues the authors would need to address. Major issues:*

'The authors solely did their experiments in p53 deficient cell lines. While this approach seems understandable given their interest in studying the mechanisms of under-replication in cancer cells, and clearly marked throughout the text, it would be useful if the authors could explain more carefully in the paper the potential consequences of the loss of p53 on their observations and whether their observations can also be relevant in conditions with functional p53. Do CIP2A-TOPBP1 foci also form in p53 wildtype cells under given IR and APH conditions?'

Reply: We predominantly used RPE1 *TP53*^{-/-} cells, because of the relevance of this genetic background for studying genomic instability (e.g. *BRCA1/2* mutations almost invariably occur in *TP53* mutant backgrounds). We have now also included analysis of CIP2A-TOPBP1 recruitment in *TP53* wt cells under IR and APH conditions and now show in Suppl. Fig. 1E/F that CIP2A-TOPBP1 is also recruited to mitotic foci in RPE1 *TP53* wt cells (line 127-132). Baseline levels in untreated *TP53* wt cells were slightly lower when compared to *TP53*^{-/-} RPE1 cells, possibly pointing at higher levels of replication-induced DNA lesions, or an impaired cell cycle checkpoint in *TP53*^{-/-} RPE1 cells.

Comment: *'The classification of CIP2A-TOPBP1 foci acquired by STED microscopy is done manually. It is unclear to me what criteria the authors used to classify the different structures (is this based on their size?). While manual classification is not inherently problematic, it would be good to mention the objective criteria the authors have used to select the structures in the methods section.'*

Reply: We have manually classified the various CIP2A structures. Loop-containing structures were defined by having a continuous ring-like structure, with absence of labeling in the inner core. Filamentous structures were based on their line-like appearance. Remaining structures were classified as unstructured, and no obvious other categories were observed.

For Figure 5J-L, we also included 'loop-like' and 'filament-like' for cells expressing delta-CIP2A. In these experiments, signal that appeared in a ring-like shape, but was not continuous was labeled as 'loop-like'. Similarly, CIP2A staining that resembled line-like appearances, but was not continuous was labeled as 'filament-like'. Information on scoring CIP2A structures has been included in the Suppl. Methods section on page 6.

Comment: *'Furthermore, the authors could make use of additional shape and structure metrics to describe the structures, such as their elongation, circularity and solidity. Additionally, I would encourage the authors to share the STED images of all classified structures in a supplementary figure or for example via Zenodo or the BioImageArchive.'*

Reply: Concerning additional shape metrics, we have now included an analysis of the elongation of loop-containing CIP2A structures (Suppl. Fig 2D), which shows that while the average loop structure does not change dramatically through mitotic progression, there is a large variation in elongation of individual structures.

We feel that additional descriptive metrics without mechanistic insight what drives these metrics does not significantly strengthen the paper, and should be studied in follow-up studies. However, to facilitate analysis by others, and as suggested by the reviewer, we have deposited all raw STED images on DataverseNL, and have provided links in the supplemental document and in the data availability section. In addition, all size measurements of individual CIP2A structures are included in the source data to facilitate follow-up analysis.

Comment: *'The different structures the authors observe by super-resolution are interesting, but I do think the relative localization of other proteins such as the SMX proteins, γ H2AX and EdU are also compelling. The significance of these observations are not mentioned in the discussion. The authors also mention the possibility that filamentous structures reflect ultrafine bridges. Would it not be possible to show this directly by immunostaining combined with STED microscopy?'*

Reply: To address whether the filamentous CIP2A structures overlap with ultrafine bridges, we analyzed co-localization with PICH. This data is now included in Suppl. Fig. 2E-G and described in lines 186-195 and lines 496-500. While we observed that the majority of CIP2A structures in anaphase were positive for PICH, only a subset of PICH-positive UFBs showed CIP2A co-localization. Of note, filamentous CIP2A structures occasionally overlapped with parts of PICH-decorated UFBs, whereas the majority of filamentous CIP2A structures involved CIP2A localization at or in between PICH foci. Combined, these data show that although CIP2A frequently co-localizes with PICH, CIP2A filaments do not per se reflect UFB structures.

Minor issues:

Comment: *‘Figure 1C: Could you please clarify what are the data points plotted within the barplots? In the legend is written that the bar plots in the mean and SEM of 3 biological replicates, however more than 3 dots are shown per condition.’*

Reply: Thank you for this observation. We adjusted the legend text with the correct number of experiments.

Comment: *‘Figure 1D-E: The difference in scaling in the crops of the different structures is quite confusing, I would reconsider displaying them all at the same scale, so it is clear they differ in size.’*

Reply: Indeed, some of the scaling is different, which is needed to illustrate the entire shape of the structures. For the overview confocal microscopy images, the scale was kept relatively equal, to also indicate the differences in size.

Comment: *‘Figure 2A: It is mentioned in the legend that proteins marked red are enriched after either IR or APH, however XPF and MDC1 seems not enriched after IR treatment. Furthermore, apart from the marked proteins there are more proteins that are enriched at either IR or APH treatment. Please clarify.’*

Reply: the reviewer is correct that the proteins in red (XPF, MDC1) are not enriched in the IR setting. We have now adjusted the color labeling accordingly. Concerning other proteins that were enriched either in IR or APH, we have now updated the list of enriched proteins in the Supplementary Table 1.

Comment *‘In Figure 2D-G, the labelling and legend of this figure is confusing as the first row of the panels is marked as TP53-/- and the second CIP2A-/- whereas both cell lines are TP53 deficient.’*

Reply: We apologize for the labeling error, which we have adjusted, also in other panels throughout the manuscript.

Comment: *“These three classes of structures occurred both in response to APH or IR treatment, which all showed a high degree of CIP2A and TOPBP1 co-localization within these structures, indicating that these proteins are less than 30 nm apart from each other (Fig. 1D, E, Suppl. Fig. 1G-I).”*

Given the homogenous localization at apparent high concentration for both proteins in these structures I do think the claim that these proteins localize only 30 nm apart, suggesting a direct interaction, is problematic. By examining at the images of CIP2A and TOPBP1 differences in the spatial distribution of intensities throughout the structures can actually be observed.'

Reply: We agree that the co-localization of CIP2A and TOBP1 cannot be used as an argument for direct interaction. We have acknowledged in the text that differences in spatial distribution exist (line 152/153), and do not claim that co-localization suggests a direct interaction (although a direct interaction has previously been described for CIP2A and TOPBP1 in PMID:35121901, with the interaction site mapped in PMID:35121901 and PMID:34145035).

Comment: *'The observation that the abundance of CIP2A-TOPBP1 structures gradually decreases during the course of mitotic progression already suggested that CIP2A-TOPBP1 is not only involved in tethering DNA ends, but also functions in processing of DNA lesions.'* This is an interesting statement, but I think this needs to be rephrased.

If CIP2A-TOPBP1 is only present to tether the DNA ends and other proteins are resolving the lesions one would also observe a reduction in the number of foci as the lesions are being resolved. The data in the paper suggests that CIP2A-TOPBP1 can be used as a scaffold for other proteins to process DNA lesions, so their presence might be a prerequisite for repair, but the reduction in number of CIP2A foci is not evidence for their function in processing DNA lesions.

Reply: We agree with the reviewer, and did not want to imply that a reduction in CIP2A during mitotic progression is evidence of DNA damage processing role of CIP2A, but merely a suggestion for DNA damage during mitosis per se, and a possible facilitating role beyond a tethering role. We have rephrased this sentence (lines 491-493).

Comment: *'Personally I do not see much difference between the raw and Wiener deconvoluted STED images shared in the paper. Could the authors explain the need to make use of deconvolution. In the supplementary methods they mention a wide variety of settings the authors used to deconvolve the images, some explanation on the choice of those different parameters would be useful, given the images are shown in the main panels of the figures. Are all images processed with the same settings or on what basis different settings have been used?'*

Reply: Due to the nature of STED microscopy, Wiener deconvolution is a common method of image processing. We applied this deconvolution in a rather conservative manner, such that the image clarity is improved but the underlying structural features remain the same. For clarity, we now removed the raw figures from the supplement. To still enable the reader to verify that the deconvolution is applied in an appropriate manner, we now uploaded the

corresponding raw figures to DataverseNL, with a link to this data in the supplementary methods and the data availability section.

The deconvolution settings were chosen based on signal intensities, signal-to-noise ratio, and resolution. We made the python script used for the Wiener deconvolution available on Zenodo, with a link in the supplementary methods and code availability section.

Comment: *'I do think full western blot images should be provided as supplementary images to the paper.'*

Reply: We have provided all uncropped western blots in a resource file.

Point-by-point rebuttal for NCOMMS-25-25109-T

Reviewer #1 (Remarks to the Author):

'The authors have addressed my comments appropriately.

Reviewer #1 (Remarks on code availability):

N/A'

Reply: We thank the reviewer for the positive response.

Reviewer #2 (Remarks to the Author):

'I appreciate the thoughtful responses to all the comments. The new results that have been added in response to my comments, as well as the comments of the other reviewers, have provided additional mechanistic details and have improved and strengthened this manuscript. Overall, the data remain convincing and present a strong argument for a novel role of CIP21-TOPBP1 in responding to replication-stress induced lesions by facilitating the recruitment of SMX to under-replicated DNA during mitosis.

Reviewer #2 (Remarks on code availability):

I followed the link but have no way of evaluating the code.'

Reply: We thank the reviewer for the positive response.

Reviewer #3 (Remarks to the Author):

The authors have done an excellent job at addressing my concerns or attempting to address my concerns. I am satisfied with their rebuttal and approve the manuscript for publication in

Nature Communications.

Reply: We thank the reviewer for the positive response.

Reviewer #4 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reply: We thank the reviewer for the positive response.

Reviewer #5 (Remarks to the Author):

'The authors have carefully addressed the raised issues. The only remaining issue is that the URL (<https://doi.org/10.34894/O3KAFO>) to the Dataverse repository containing the raw imaging data is not working (yet) in my hands.'

Reply: We waited with opening the dataverse repository until the manuscript was finally accepted, and we could enter all data in the final manuscript. The repository has now been made accessible.

Reviewer #5 (Remarks on code availability):

'The code contains scripts to process the super-resolution images. I managed to run the code, however it required manually installing the required dependencies. For reproducibility it would be useful to add some installation instructions in the README or provide a pip or conda requirements file to ease installation of the required python libraries. The Jupyter notebook itself is very clear, easy to use and the provided test data made it easy to validate the software.'

Reply: Thank you for testing the code.