EMBO reports

Centriolar subdistal appendages promote double-strand break repair through homologous recombination

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Review #1

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

Summary

Rodríguez-Real, Huertas and colleagues here explore the roles of centrosomes in DNA damage responses, focussing on DNA repair activities. They show that centrosome depletion by PLK4 inhibition leads to reduced levels of homologous recombination and increased nonhomologous end-joining, along with altered level of nuclear focus formation by DNA repair proteins. Knockdown of genes that encode components of centriolar subdistal appendages (SDAs) cause reduced levels of RPA foci, with CRISPR-generated CEP170 heterozygotes also showing defects in focus formation. Knockdown of CEP170 impairs homologous recombination, although NHEJ activities are unaffected. Some increase in sensitivity to DNA damaging agents is seen in CEP170- or centriole-deficient cells, albeit with a modest effect size. CEP170 status is shown to affect mutational signatures and patient prognosis in different cancer samples.

While the experiments are generally well-presented and controlled, the effects seen are not large, so that the the conclusions that the authors draw are not entirely substantiated by the data presented, even without the suggestion of a mechanism. There are several additional experiments and clarifications that I consider necessary to provide appropriate support for the phenomenon.

Major points

1. The lack of cell cycle arrest or phenotype in the U2OS cells after a week's treatment with centrinone is somewhat surprising, given their p53 status. The initial description of centrinone showed a distinct impact on U2OS proliferation, albeit after 2 weeks' treatment (although the present paper shows robust impact on centriole numbers after only 1 week in centrinone). It would be useful to know the percentage of mitotic cells, or if there is any increased cell death observed at this stage of treatment.
2. In the I-SceI assays, were transduction efficiencies or apoptosis within the experiment impacted by centrinone treatment? If not, it would be useful to state that this was examined and that there were no confounding effects; having only normalised data does not allow the reader to exclude these potential confounding factors.

3. The authors present binary data for a given type of nuclear focus (positive or negative for RPA/ BRCA1/ RAD51), while the supporting images show altered numbers/ intensities. For example, the BRCA1 signals shown in Fig. 3D are less readily distinguished than they are in Fig. 1D. These data should be reconsidered: it is possible that these observations reflect different kinetics of focus formation, rather than a change in IRIF formation capacity. Numbers and a timecourse should be provided, with details of how these are quantitated provided in the Methods.
4. Are the BRCA1 and RAD51 results seen with centrinone treatment of U2OS cells recapitulated in the Saos-2 and RPE1 lines?
5. Some additional analysis is needed of the extent to which cells are sensitised to DNA damaging treatments by CEP170 deficiency or centrinone treatment. It should be confirmed that these experiments were performed in biological triplicate, rather than a technical triplicate (within a single experiment); if this is not the case, these experiments should be done in triplicate.
Analysing p53-deficient hTERT-RPE1 clones, Kumar et al. (NAR Cancer 2020 PMID: 33385162) showed <10% survival with 100 ng/ml NCS. Hustedt et al. (Genes Dev 2019 PMID: 31467087) showed just over 50% survival with 10 nM CPT treatment, although their data for IR were comparable to the current study. Given the wide variation that these assays seem to incur, the extent to which a ≈20% difference in clonogenic survival is biologically significant may be limited. A rescue of the CEP170 siRNA, and/ or washout in the centrinone experiment would make these data more convincing.
The knockdown of CEP170 in Figure 4 should be correctly labelled (not as CEP170+/-); given that the authors have generated CEP170 heterozygotes in Figure 2, this is potentially confusing.
6. Direct data for the (centrosomal) phosphorylation of CEP170 are limited; it has not been demonstrated that the S637A mutants are fully functional in terms of the centrosome functions of CEP170, so that the conclusion regarding a requirement for centrosomal CEP170 phosphorylation is not sufficiently supported by the available data. The CEP170-dependent changes in RPA focus positive cell percentages shown in Figure 3 are not very marked. The relevant sections should be revised, or the authors should include additional experiments showing directly a phosphorylation of CEP170.
7. It is difficult to interpret the mutational spectrum data and their significance. These should be compared with data for mutations in NDEL1 mutant cells, and/or other SDA components.
8. The Kaplan-Meier curves data are intriguing, but their interpretation is highly speculative, given that there are no data on treatment groups included in this study. It is unclear whether other genes that affect SDAs might also impact survival (in the same, or different cancers), so the presentation of those patient groups where CEP170 status impacted survival seems selective, given the ubiquity of HR and centrosomes.

These data would be better included as Supplemental information.
9. The independence of p53 status/ responsiveness of the system is a crucial aspect of this study. Sir et al. (JCB 2013 PMID: 24297747) showed no DNA repair defect in centrosome-deficient chicken DT40 cells. This paper is very relevant to the current study and should be discussed. Similarly, the work by Lambrus et al (JCB 2015 PMID: 26150389) should also be considered.

Minor points

10. References for the RPE1 TP53/ SAS6 mutant cell lines should be provided (or controls for their generation presented).
11. Fig S1K should correct its x-axis to reflect the time intervals correctly.
12. Fig 2D should show blow-ups of the centrosomes.
13. To avoid any potential confusion, it would be helpful to indicate in the Figure proper which cells are used for the various analyses.
14. The 'basal side' of the centriole is not a standard term- this should be clarified. This may be confusing, given the role of centrioles in the basal body.
15. The consideration of Seckel syndrome seems somewhat speculative at this stage in the exploration of this phenomenon.

Referees cross commenting
I think the comments from Reviewers #2 and #3 are reasonable and justified; there is good convergence between the comments that we all made and I have no issues to raise in this cross-commentary.

2. Significance:

Significance (Required)

Strengths: Much previous work linking centrosomes and DNA damage responses has addressed cell cycle and checkpoint roles of the centrosome, so that a direct role in (nuclear) DNA repair is intriguing.
Limitations:The present study shows a relatively moderate impact of centrosome defects on DNA repair, without a clear mechanism. There are some technical details that should be addressed. The relatively limited sensitization to DNA damaging treatments caused by centrosome deficiency questions the biological significance of the phenomenon.

Advance: The current study presents some new findings that potentially show DNA repair defects resulting from the loss of centrioles (or SDA proteins). This has not been demonstrated to date.

Audience: The idea of subdistal appendage components contributing to homologous recombinational repair of DNA damage is of potential interest to several fields, ranging from basic centrosome biology through translational to clinical cancer research.

Reviewer's expertise: basic/ cell biology.

3. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 1 and 3 months

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Review #2

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

In this manuscript by Rodriguez-Real et al, the authors address the contribution of the centrosome to cellular process unrelated to organizing the microtubule cytoskeleton,

namely DNA repair. As many proteins contributing to the DNA damage response physically associate with centrosomes, this appears a relevant question that has been neglected so far and led to a number of studies that appeared controversial. To do so, the authors exploit a variety of tissue culture models that are well established in the fields of centrosomes and DNA repair, including U2OS and RPE1 cells, exposed to perturbations promoting DNA damage (such as ionizing radiation or pharmacologic perturbation of DNA stability) in conjunction with siRNA mediated depletion of candidate centrosomal proteins., followed by the visualization of repair events either using fluorescent reporters, or visualizing endogenous repair foci by immunofluorescence. On this basis, the authors propose that a discrete centrosomal sub-structure, namely sub-distal appendages and the CEP170 protein therein concur to promote a particular nuclear DNA repair process, namely homologous recombination.

The manuscript suffers of two main limitation:

1. the authors provide no mechanistic understanding of how CEP170, a protein that resides at centriolar subdistal appendages and shows no nuclear translocation upon DNA damage, concurs to regulate processes in the nucleus. The fact that all reported phenomena appear to be independent of microtubules suggests that neither the LINC complex nor the precise position of the centrosome in the vicinity of nuclear pore complexes contribute to the reported phenomena.
2. some of the experimental perturbations performed in the manuscript might elicit the reported phenotypes due to spurious effects on cellular processes that have not been considered with sufficient caution.

Given that uncovering the mechanism underlying the contribution of CEP170 to homologous recombination might prove very demanding, my comments will focus primarily on the second point.

Major comments:

The centriolar depletion using centrinone is known to impinge on cell proliferation in p53 WT cells. Thus, I am not convinced that the data shown in Figure S1B and S1C will sufficiently document that the observed unbalance between HDR and NHEJ are not simply reflecting a different cell cycling speed/behavior. Moreover, it would be important to address whether centrinone or depletion of CEP170 (an essential gene, according to the authors!) will trigger DNA damage by themselves. In fact, even a small extent of chronic genotoxic stress caused by the perturbations used in the manuscript might explain the reported differential proficiency of HDR.

Minor comments:

It is a pity that CEP170 is not amenable to functional dissection using a complete knockout. The fact that in PMID: 27818179 a complete knockout of CEP128 has been achieved, suggests however that subdistal appendage mediated DNA repair is not the essential process in itself. As the authors employ other cell lines stemming from the same laboratory, they could consider acquiring CEP128 KO to complement their own experiments.

The proposal that CEP170 phosphorylation of by ATM/ATR upon DNA damage might require SDA localization of the protein is plausible, yet not circumstantiated by any experimental evidence. If the authors could monitor the phosphorylation of the endogenous CEP170 protein in WT vs CEP128 KO cells (phosphor-specific antibody, MS-based proteomics or simply "phos-tag" gels), this could provide a first spark towards a mechanistic understanding of the reported phenomenon.

The entire Figure 4 is based on quantifications of clonogenic potential.

1. it would be helpful if the data were accompanied by images displaying representative crystal violet stained dishes.
2. clonogenic potential potential is discussed as a mere readout of cell survival, yet a combination between survival and proliferation concur to the reported differential clonogenic potential

Odf2 contribution to both DAs and SDAs: while Odf2 has been initially proposed to be necessary for the assembly of both types of appendages, its contribution to distal appendages has been disputed by Tanos et al using siRNA (PMID: 23348840), also confirmed by our group using CRISPR (unpublished). Thus, the role of Odf2 in SDA assembly appears more crucial than for DA assembly.

CEP164 contribution to ATM/ATR activation: this has been disputed in this paper by the Morrison lab (PMID: 26966185). Thus, a cautionary note should be mentioned when referring to this concept.

2. Significance:

Significance (Required)

Taken together, this manuscript addresses the contribution of the centrosome to DNA repair. This is in itself a very interesting topic with the potential to attract the interest of both cell/molecular biologists as well as cancer researchers. The major advance strength is represented by pinpointing a specific centriolar substructure, namely

subdistal appendages, in the control of HDR. CEP170 had been previously shown to be target of phosphorylation by ATM/R and the present study highlights that the abovementioned phosphorylation is not a mere passenger event during DNA repair, but that potentially reflects a decisive event informing the repair pathway of choice. However, several experiments have alternative explanations/interpretations and no understanding of the underlying mechanism is provided.

The expertise of this reviewer is the study of cell cycle regulation and on the centrosome structure/function.

3. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

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Review #3

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

Summary

In this manuscript, Rodríguez -Real and colleagues investigate how the centrosome may influence the repair of DNA double-stranded breaks (DSBs), building on the initial finding that relative HR frequencies (as measured using a standard split-GFP gene conversion reporter assay) are reduced in centrinone treated centrosome-depleted cells relative to mock treated controls cells. Such defects are found correlate to concordant reductions in immunofluorescence proxies for resection (RPA recruitment into foci) and upstream and downstream events in the HR cascade (BRCA1 and RAD51 recruitment, respectively), and a correlating increase in NHEJ repair of I-SceI induced repair in EJ5-like reporter assay. Taking a candidate approach to identifying which centrosome proteins link the centrosome to DSB repair regulation, the authors reveal cells depleted for subdistal appendage proteins show equivalent deviations in DSB repair reporter assays and show concordant defects in RPA recruitment, leading to the proposal that subdistal appendage proteins regulate DNA resection and thus optimal HR. Experiments are then used to show CEP170 (a subdistal appendage protein) may be phosphorylated by DDR kinases and some rescue experiments are used to support hypothesis that this phosphorylation may be involved in centrosome-DSB repair cross-talk signalling. Figure 3 experiments then show centrosome-depleted and heterozygous losses of CEP170 result in moderate sensitivities across a number of DSB-inducing treatments. Lastly meta-analyses of cancer datasets correlate low CEP170 expression to differences in cancer mutations signatures (Fig 4) and altered patient outcomes across a number of cancers (Fig 5), and propose that CEP170 - via a DSB repair repair function - may be causal in these alterations. Ultimately, the authors propose that the centrosome acts as a signalling node or 'centrosomal processing unit' (CPU) via distal appendage proteins to coordinate the signalling of DNA damage and its repair, and speculate this may link to the clinical phenotypic overlap between centrosome-related ciliopathies and DDR signalling disorders (e.g. ATR-Seckel).

Major comments

1. Concerning Figs 1-3, it is argued that the presented skews in pathway choice are not an indirect consequence of cell-cycle effects that accompany centrosome depletion (i.e. following centrinone treatments) or depleted centrosome factors. Indeed, S1B shows centrione depleted cell show reduced S-phase indices (where HR is most active) are concordant with increased G2(/M) cell indices, significant effects that may contribute (at least in part) to some of the reported. In the case of the reporter assays it will be difficult/impossible to normalise data vs cell cycle skew, however in the case of RAD51 IRIF frequencies and RPA recruitment, this can be done easily by

monitoring the relative frequencies of these events specifically S-phase (BrDU/EdU positive) cells. This should be done if the case for indirect cell-cycle effects is to be dismissed.
2. Related to point (1): RPA/RAD51/BRCA1 measurements made quantitatively (i.e. by QIBC or equivalent) given % IRIF positive cells can be misleading given it is completely subjective to user defined thesholds.
3. Fig 3 - The fact that CEP170 KD decreases BRCA1 IRIF but does not increase RIF1 IRIF, is not indicative of a lack of NHEJ stimulation, nor does it infer the existence of a/some distinct mechanism stimulating NHEJ, or an 'undiscovered factor', as is stated. This is important as RIF1 IRIF are not an accepted, nor accurate surrogate marker of NHEJ pathway activity, only an indicator of RIF1 recruitment downstream of 53BP1, whose role in resection control is clear, yet whose contribution to NHEJ is highly context-specific.
4. Is CEP170 Ser-637 an evolutionarily conserved ATM/ATR site? - Conservation, at least in mammals/vertebrates would be expected if a regulatory event in DSB pathway choice. This should be commented on with supplementary alignment included to demonstrate whether this is likely to be a universally conserved mechanism of repair regulation.
5. Fig 3F-G: Important to show appendage localisation of wild-type and mutant CEP170 S637A/D proteins to inform whether these are functional, expressed at equivalent levels and support equal centrosome localisation intensities. Immunoblot data in support of CEP170 siRNA depletion and CEP170 transgene complementation efficiencies is missing, and needs to be included to reassure a reader the results are specific to defects in the phosphorylation (not stability/expression level/other).
6. Do the CEP170 P'n nmutations affect its physiological centrosome functions? If separation of function is not experimentally defined, it should be at least discussed.

Comments on interpretation and accuracy of stated conclusions:

1. P12. - The manuscript is lacks the necessary evidence to support the section title: "CEP170 Ser647 phosphorylation is critical for HR double strand break repair", and as such I find this and related textual conclusions in the manuscript body to be inaccurate and misleading. To make this claim would require generating a cell-line knockin of the S647A mutation, preferably at the endogenous CEP170 locus (or a robust complementation system), and its utilisation to establish that standard measures of HR e.g. RAD51 recruitment, PARPi sensitivity, and/or SCE frequencies are all affected as expected in cells bearing this mutation.
2. Abstract reads: "we identify a centriolar structure, the subdistal appendages, and a specific factor, CEP170, as the critical centrosome component involved in the regulation of recombination and resection... " - I disagree with this statement given that the study has not excluded other centrosome components/features of the

centrosome in regulation of resection. Can the authors perform experiments to exclude a role for other centrosome components and substantiate the conclusion that this is a specific function of the subdistal appendages as is stated?
3. Based on the marginal sensitivity phenotypes shown in Fig 4 for heterozygous cell-lines, it seems unlikely that CEP170 is a central player in the DSB response.
4. The CPU model for DDR-centric role of the centrosome is premature based on the provided data, likewise the fact that a centrosome-regulated resection could explain the clinical overlap between seckel and and this model should be toned down. We probably don't need another acronym for the DDR.

Minor comments

- Abstract, lasts sentence needs correction: "suggesting this protein can act as a driver mutation but also..." - a protein cannot act as a driver mutation.
- Information regarding biological replicates, sample sizes, error bars should be made more clear throughout to better represent reproducibility; e.g. n=3 {plus minus} Dt. Dev, biological replicates consisting >500 cells/nuclei per condition

2. Significance:

Significance (Required)

General assessment

In exploring for functional links between DSB repair and the centrosome, the results encompass a series of corelating results that collectively hint at a potential role for the centrosome in repair regulation. The indirect and perhaps boring explanation for the presented DSB repair imbalances is these are an indirect consequence of the inevitable cell cycle defects that accompany centrosome depletion. In S1 the authors make some effort towards dispelling this less interesting (indirect) explanation for the presented results, yet not really far enough to dismiss it as the unifying explanation. A major consequence of centrosome-loss is prolonged time spent in G2/M dues to sub-optimal spindle nucleation and assembly kinetics, and an extended transit through mitosis, defects that occur independently of the p53-dependent checkpoint to centrosome loss (in fact the defects have long been speculated precede and perhaps propagate p53 activation). Indeed, supplementary data indicates that in centrosome-depleted cells a reduction in S-phase index (when HR activity is highest) correlates to greater proportion of cells with DNA with G2(/M) content. While I agree that these cell-cycle skews are unlikely to be great enough fully account for the reductions in HR reporter and IF proxies, more targeted approaches to control for indirect cell cycle effects (one suggestion below) could strengthen the case for a direct role in repair regulation. The

manuscript also falls short of a identifying a discrete mechanism that explains centrosome-repair crosstalk, and on this basis I feel some of the conclusions are too preliminary and speculative and thus the authors would benefit from being more nuanced in their conclusions. One clear example is the authors's oversimplistic attribution of DSB regulation to distal appendage components of the centrosome/cilia, yet doing so having only tested the appendage proteins on the basis of literature based exercise of protein segregation of DDR and centrosome proteins (S2A). I also find it premature to propose "CPU" models of DDR regulation, the results (while interesting) haven't gone far enough to rigorously challenge this hypothesis, and define its mechanistic basis. I also question the importance and relevance of the analyses in Figs 4-5: in the absence of scientific evidence to establish causation for low CEP170 expression in tumour mutation signature burden or patient prognosis, the presented remain correlates that might equally result from a number of phenomena unrelated to DSB repair. As such, I feel the manuscript does encompass results worthy of report that would be of interest to cell cycle and DNA repair biologists, it would be greatly improved by being more rigorous, objective and nuanced in its interpretation.

3. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 1 and 3 months

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Revision Plan

Manuscript number: #RC-2022-01727
Corresponding author(s): Pablo Huertas

[The "revision plan" should delineate the revisions that authors intend to carry out in response to the points raised by the referees. It also provides the authors with the opportunity to explain their view of the paper and of the referee reports.

The document is important for the editors of affiliate journals when they make a first decision on the transferred manuscript. It will also be useful to readers of the reprint and help them to obtain a balanced view of the paper.

If you wish to submit a full revision, please use our "Full Revision" template. ***It is important to use the appropriate template to clearly inform the editors of your intentions.****]*

1. General Statements [optional]

Significance and target audience:

The main message of this paper is that the subdistal appendages of the centrioles are directly involved in the finetuning of the balance between DNA double strand break repair pathways. This has implications in the cellular homeostasis and the capacity of cells to survive to DNA damaging agents. Furthermore, we present evidence that this might be at the root of the development, and more importantly, the treatment response, of cancer. Thus, we think this represent a compelling message that will have a broad and diverse audience. Indeed, despite their specific criticisms to our paper and the weaknesses they have uncovered (and that we plan to address, see below), all three reviewers, despite their clearly different expertise, have stated their agreement with our assessment. Indeed, reviewer 1 states that "*Much previous work linking centrosomes and DNA damage responses has addressed cell cycle and checkpoint roles of the centrosome, so that a direct role in (nuclear) DNA repair is intriguing*", "*The current study presents some new findings that potentially show DNA repair defects resulting from the loss of centrioles (or SDA proteins). This has not been demonstrated to date*", and finally that "*The idea of subdistal appendage components contributing to homologous recombinational repair of DNA damage is of potential interest to several fields, ranging from basic centrosome biology through translational to clinical cancer research*." Reviewer 2 points out that "*As many proteins contributing to the DNA damage response physically associate with centrosomes, this appears a relevant question that has been neglected so far and led to a number of studies that*

appeared controversial.“ but also said that “*This is in itself a very interesting topic with the potential to attract the interest of both cell/molecular biologists as well as cancer researchers. The major advance strength is represented by pinpointing a specific centriolar substructure, namely subdistal appendages, in the control of HDR. CEP170 had been previously shown to be target of phosphorylation by ATM/R and the present study highlights that the abovementioned phosphorylation is not a mere passenger event during DNA repair, but that potentially reflects a decisive event informing the repair pathway of choice*”. Finally, for reviewer 3 feels that “*the manuscript does encompass results worthy of report that would be of interest to cell cycle and DNA repair biologists*”.

2. Description of the planned revisions

Insert here a point-by-point reply that explains what revisions, additional experimentations and analyses are <u>planned</u> to address the points raised by the referees.

Careful examination of the reviewers comments led us to propose the following plan of action, explained here point by point as a response to the reviewers comments: (Reviewers comments in blue)

<u>Reviewer #1 (Evidence, reproducibility and clarity (Required)):</u>

Summary

Rodríguez-Real, Huertas and colleagues here explore the roles of centrosomes in DNA damage responses, focussing on DNA repair activities. They show that centrosome depletion by PLK4 inhibition leads to reduced levels of homologous recombination and increased nonhomologous end-joining, along with altered level of nuclear focus formation by DNA repair proteins. Knockdown of genes that encode components of centriolar subdistal appendages (SDAs) cause reduced levels of RPA foci, with CRISPR-generated CEP170 heterozygotes also showing defects in focus formation. Knockdown of CEP170 impairs homologous recombination, although NHEJ activities are unaffected. Some increase in sensitivity to DNA damaging agents is seen in CEP170- or centriole-deficient cells, albeit with a modest effect size. CEP170 status is shown to affect mutational signatures and patient prognosis in different cancer samples.

While the experiments are generally well-presented and controlled, the effects seen are not

large, so that the the conclusions that the authors draw are not entirely substantiated by the data presented, even without the suggestion of a mechanism. There are several additional experiments and clarifications that I consider necessary to provide appropriate support for the phenomenon.

Major points

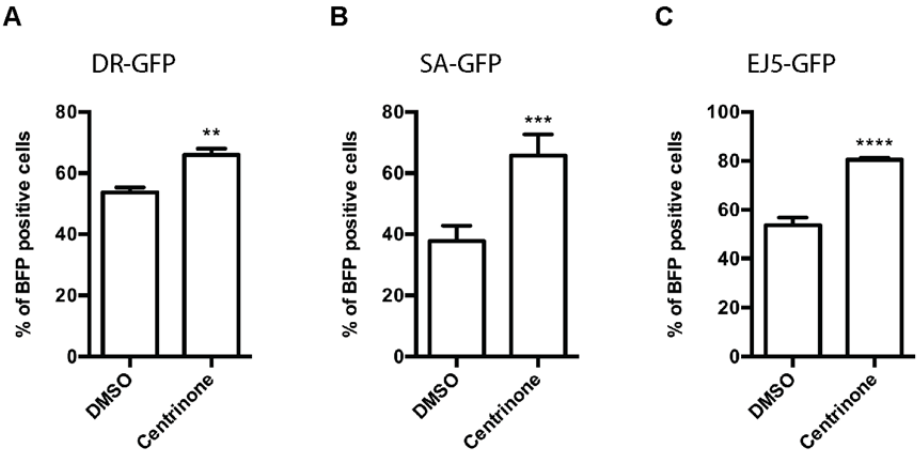
1. The lack of cell cycle arrest or phenotype in the U2OS cells after a week's treatment with centrinone is somewhat surprising, given their p53 status. The initial description of centrinone showed a distinct impact on U2OS proliferation, albeit after 2 weeks' treatment (although the present paper shows robust impact on centriole numbers after only 1 week in centrinone). It would be useful to know the percentage of mitotic cells, or if there is any increased cell death observed at this stage of treatment.

As mentioned by the reviewer, there is a distinct difference in our experimental setup and the initial description of centrinone. We plan to appease this reviewer's in two fronts. On the one hand, by including a more detailed discussion on these mentioned differences. Secondly, by scoring the number of mitotic cells and assessing the cell death using microscopy-based approaches. Albeit we need to perform the experiments in that regard, it is worth pointing out that we have not observed, so we do not expect, major changes in any of those aspects. Additionally, even if we observe those changes, they do not impact greatly in our findings as a great part of our experiments focus on the direct observation of interphase cells that are alive, therefore disregarding both mitotic cells and dead cells.

2. In the I-SceI assays, were transduction efficiencies or apoptosis within the experiment impacted by centrinone treatment? If not, it would be useful to state that this was examined and that there were no confounding effects; having only normalised data does not allow the reader to exclude these potential confounding factors.

We apologize if this was not clear in the initial manuscript, but this is not a real issue. We use an I-SceI vector that contains a BFP for these experiments, and we count only BFP-positive cells. So, we control and normalize all our experiments taking into consideration transduction efficiencies. Indeed, as shown in the figure, centrinone treatment increases transduction

efficiency in all cases, so even if we would not normalize as we do, in any case we would have been underestimating the effect.



3. The authors present binary data for a given type of nuclear focus (positive or negative for RPA/ BRCA1/ RAD51), while the supporting images show altered numbers/ intensities. For example, the BRCA1 signals shown in Fig. 3D are less readily distinguished than they are in Fig. 1D. These data should be reconsidered: it is possible that these observations reflect different kinetics of focus formation, rather than a change in IRIF formation capacity. Numbers and a timecourse should be provided, with details of how these are quantitated provided in the Methods.

In this point there is a mix of different issues with differential answers. First, it is true that we usually score the percentage of foci-positive cells and we have not stated in the methods how this is scored. Indeed, we count as positive cells with over 10 foci. Additionally, we are now re-analyzing our data considering number of foci per cell, intensity of the nuclear signal and intensity of individual foci using a automatic computerized approach. Then, the comparison in intensities between figures 1D and 3D the reviewer suggest is simply not appropriate from our point of view. These experiments were performed completely independently, therefore small changes in the primary or secondary antibodies affinities due to batch, the room temperature, small changes in the buffer, or even the age of the microscope lamp can account for those changes. Indeed, the images were taken with different microscopes. Finally, regarding kinetics, it is formally true that there might be changes, but it is well stablished that the times we take our images are relevant for our observations. Performing kinetics of all the foci we analyzed in all the conditions will be unmanageable with very little added value, as least in our opinion.

4. Are the BRCA1 and RAD51 results seen with centrinone treatment of U2OS cells recapitulated in the Saos-2 and RPE1 lines?

This is an interesting question, and we will perform the experiment as suggested.

5. Some additional analysis is needed of the extent to which cells are sensitised to DNA damaging treatments by CEP170 deficiency or centrinone treatment. It should be confirmed that these experiments were performed in biological triplicate, rather than a technical triplicate (within a single experiment); if this is not the case, these experiments should be done in triplicate.

Again, apologies if this was not clear in our original submission. The experiments were performed in biological triplicates, in each one of them in technical triplicates. I.e. We repeated the experiments three completely independently times, and each time we performed a technical triplicate. So, the average we show is the mean of the three averages.

Analysing p53-deficient hTERT-RPE1 clones, Kumar et al. (NAR Cancer 2020 PMID: 33385162) showed <10% survival with 100 ng/ml NCS. Hustedt et al. (Genes Dev 2019 PMID: 31467087) showed just over 50% survival with 10 nM CPT treatment, although their data for IR were comparable to the current study. Given the wide variation that these assays seem to incur, the extent to which a ≈20% difference in clonogenic survival is biologically significant may be limited. A rescue of the CEP170 siRNA, and/ or washout in the centrinone experiment would make these data more convincing.

It is complicated to compare different experiments in different labs. So, in order to address this point, and as suggested, we are performing a rescue experiment using heterozygous +/- cells complemented with either an ectopic version of CEP170 or an empty vector.

The knockdown of CEP170 in Figure 4 should be correctly labelled (not as CEP170+/-); given that the authors have generated CEP170 heterozygotes in Figure 2, this is potentially confusing.

Apologies if this is, somehow misleading. Indeed, these are CEP170+/- RPE cells, so there is no mistake in the labelling of the figure. We realize that is probably the main text what might be confusing. We refer as them as knockdown as there is a reduction, but not loss, of CEP170. We will instead change the main text to say heterozygous instead of knockdown to avoid confusion.

6. Direct data for the (centrosomal) phosphorylation of CEP170 are limited; it has not been demonstrated that the S637A mutants are fully functional in terms of the centrosome functions of CEP170, so that the conclusion regarding a requirement for centrosomal CEP170 phosphorylation is not sufficiently supported by the available data. The CEP170-dependent changes in RPA focus positive cell percentages shown in Figure 3 are not very marked. The relevant sections should be revised, or the authors should include additional experiments showing directly a phosphorylation of CEP170.

As we mentioned in the response to reviewer 2, we are going to address this point and add further support. Also, we would like to point out that what the reviewer claim to be “not very marked” changes are similar or even greater to what us and other has shown previously for BRCA1 depletion.

7. It is difficult to interpret the mutational spectrum data and their significance. These should be compared with data for mutations in NDEL1 mutant cells, and/or other SDA components.

This is a very interesting suggestion. We have already repeated this in silico experiments and seen that cancer samples with low levels CEP170 share the same exposure to mutational signatures than samples with low levels of Centriolin or NIN but not NDEL1. These data will be added and discussed in a revised version.

8. The Kaplan-Meier curves data are intriguing, but their interpretation is highly speculative, given that there are no data on treatment groups included in this study. It is unclear whether other genes that affect SDAs might also impact survival (in the same, or different cancers), so the presentation of those patient groups where CEP170 status impacted survival seems selective, given the ubiquity of HR and centrosomes. These data would be better included as Supplemental information.

Again, as suggested, we are analyzing other SDA components to strengthen this point. As if this should be included as a main figure or a supplementary, we disagree with the reviewer. In that regard, neither of the two other referees seem to agree with him/her. In any case, we are open to discuss this with the reviewers and the editor and keep it as it is or send it to the supplementary information once we have reached a consensus.

9. The independence of p53 status/ responsiveness of the system is a crucial aspect of this

study. Sir et al. (JCB 2013 PMID: 24297747) showed no DNA repair defect in centrosome-deficient chicken DT40 cells. This paper is very relevant to the current study and should be discussed. Similarly, the work by Lambrus et al (JCB 2015 PMID: 26150389) should also be considered.

We are happy to include these two papers in our discussion.

Minor points

10. References for the RPE1 TP53/ SAS6 mutant cell lines should be provided (or controls for their generation presented).

We will include it

11. Fig S1K should correct its x-axis to reflect the time intervals correctly.

We will change it accordingly

12. Fig 2D should show blow-ups of the centrosomes.

We will add them.

13. To avoid any potential confusion, it would be helpful to indicate in the Figure proper which cells are used for the various analyses.

We will add it as it is already in most of the cases.

14. The 'basal side' of the centriole is not a standard term- this should be clarified. This may be confusing, given the role of centrioles in the basal body.

We will change the nomenclature to “proximal side of centrioles” to avoid confusions.

15. The consideration of Seckel syndrome seems somewhat speculative at this stage in the exploration of this phenomenon.

That is why is in the discussion and we state is a possibility. Therefore, we would prefer to keep it as it is, but again we are open to discuss this point with the editor and the rest of the reviewers.

Revision Plan

Referees cross commenting

I think the comments from Reviewers #2 and #3 are reasonable and justified; there is good convergence between the comments that we all made and I have no issues to raise in this cross-commentary.

Reviewer #1 (Significance (Required)):

Strengths: Much previous work linking centrosomes and DNA damage responses has addressed cell cycle and checkpoint roles of the centrosome, so that a direct role in (nuclear) DNA repair is intriguing.

Limitations:The present study shows a relatively moderate impact of centrosome defects on DNA repair, without a clear mechanism. There are some technical details that should be addressed. The relatively limited sensitization to DNA damaging treatments caused by centrosome deficiency questions the biological significance of the phenomenon.
As described, we will strive to address those technical details. Additionally, the sensitivities we observe are not really minor, as again they are similar to what is observed upon depletion of even core resection factors as CtIP. The fact that NHEJ can take care of more than 80% of the DSBs in cells is what makes resection deficiency cause this survival reductions. However, the consequences in terms of mutagenesis and the long-term accumulation of unrepaired breaks is what makes HR so relevant, especially in a cancer setup.

Advance: The current study presents some new findings that potentially show DNA repair defects resulting from the loss of centrioles (or SDA proteins). This has not been demonstrated to date.

Audience: The idea of subdistal appendage components contributing to homologous recombinational repair of DNA damage is of potential interest to several fields, ranging from basic centrosome biology through translational to clinical cancer research.

Revision Plan

Reviewer's expertise: basic/ cell biology.

Reviewer #2 (Evidence, reproducibility and clarity (Required)):

In this manuscript by Rodriguez-Real et al, the authors address the contribution of the centrosome to cellular process unrelated to organizing the microtubule cytoskeleton, namely DNA repair. As many proteins contributing to the DNA damage response physically associate with centrosomes, this appears a relevant question that has been neglected so far and led to a number of studies that appeared controversial. To do so, the authors exploit a variety of tissue culture models that are well established in the fields of centrosomes and DNA repair, including U2OS and RPE1 cells, exposed to perturbations promoting DNA damage (such as ionizing radiation or pharmacologic perturbation of DNA stability) in conjunction with siRNA mediated depletion of candidate centrosomal proteins., followed by the visualization of repair events either using fluorescent reporters, or visualizing endogenous repair foci by immunofluorescence. On this basis, the authors propose that a discrete centrosomal sub-structure, namely sub-distal appendages and the CEP170 protein therein concur to promote a particular nuclear DNA repair process, namely homologous recombination.

The manuscript suffers of two main limitation:

1. the authors provide no mechanistic understanding of how CEP170, a protein that resides at centriolar subdistal appendages and shows no nuclear translocation upon DNA damage, concurs to regulate processes in the nucleus. The fact that all reported phenomena appear to be independent of microtubules suggests that neither the LINC complex nor the precise position of the centrosome in the vicinity of nuclear pore complexes contribute to the reported phenomena.

The referee is absolutely right, and we do not hide it in the manuscript. We even mentioned it in the discussion. We lack this molecular connection, but as he/she points out finding it will be far from straight forward. In any case, we think the advance we observe is enough to grant publication.

2. some of the experimental perturbations performed in the manuscript might elicit the reported phenotypes due to spurious effects on cellular processes that have not been considered with sufficient caution.

We do not completely agree with the reviewer, but as mentioned below we will tackle experimentally his/her concerns.

Given that uncovering the mechanism underlying the contribution of CEP170 to homologous recombination might prove very demanding, my comments will focus primarily on the second point.

Major comments:

The centriolar depletion using centrinone is known to impinge on cell proliferation in p53 WT cells. Thus, I am not convinced that the data shown in Figure S1B and S1C will sufficiently document that the observed unbalance between HDR and NHEJ are not simply reflecting a different cell cycling speed/behavior. Moreover, it would be important to address whether centrinone or depletion of CEP170 (an essential gene, according to the authors!) will trigger DNA damage by themselves. In fact, even a small extent of chronic genotoxic stress caused by the perturbations used in the manuscript might explain the reported differential proficiency of HDR.

We understand the reviewer concerns, and this is the reason we used p53 -/- cells in some of our critical experiments. Thus, we can show our observations do not reflect this effect. Also, we validate everything with CEP170 depletion, that does not affect p53, strengthening our observations. Additionally, we are always very careful controlling cell cycle changes, as stated in the manuscript. A different, and very valid, issue is the idea that centrinone or CEP170 depletion might cause directly DNA damage. We do not think it is the case, as it can be already observed in supplementary figure 1K that no increase of DSBs follows centrinone treatment in non-irradiated cells, and this has been previously documented (Lambrus et al 2016). Also, we always check for DNA damage presence in un-irradiated samples, albeit those data were not included in the original submission. In any case, we are going to add further indication of this aspect by carefully scoring and representing gamma-H2AX foci in noniradiated cells.

Revision Plan

Minor comments:

It is a pity that CEP170 is not amenable to functional dissection using a complete knockout. The fact that in PMID: 27818179 a complete knockout of CEP128 has been achieved, suggests however that subdistal appendage mediated DNA repair is not the essential process in itself. As the authors employ other cell lines stemming from the same laboratory, they could consider acquiring CEP128 KO to complement their own experiments.

This is a very good suggestion. We have reached and asked for the CEP128 KO and we will repeat critical experiments with it.

The proposal that CEP170 phosphorylation of by ATM/ATR upon DNA damage might require SDA localization of the protein is plausible, yet not circumstantiated by any experimental evidence. If the authors could monitor the phosphorylation of the endogenous CEP170 protein in WT vs CEP128 KO cells (phosphor-specific antibody, MS-based proteomics or simply "phos-tag" gels), this could provide a first spark towards a mechanistic understanding of the reported phenomenon.

Again, a very fair point. We will perform IP-western using CEP170 antibody for IP and phospho-specific antibodies and/or phos-tag in cells irradiated or not. Additionally, albeit we agree with the reviewer that endogenous CEP170 will be better, we will take the same approach using our GFP-tagged versions. This will allow us to test if the phosphorylation is gone in the S327A mutant.

The entire Figure 4 is based on quantifications of clonogenic potential.
1. it would be helpful if the data were accompanied by images displaying representative crystal violet stained dishes.

We have the images and will add them.

2. clonogenic potential potential is discussed as a mere readout of cell survival, yet a

combination between survival and proliferation concur to the reported differential clonogenic potential.

We will change the text accordingly.

Odf2 contribution to both DAs and SDAs: while Odf2 has been initially proposed to be necessary for the assembly of both types of appendages, its contribution to distal appendages has been disputed by Tanos et al using siRNA (PMID: 23348840), also confirmed by our group using CRISPR (unpublished). Thus, the role of Odf2 in SDA assembly appears more crucial than for DA assembly.

We will change the text accordingly.

CEP164 contribution to ATM/ATR activation: this has been disputed in this paper by the Morrison lab (PMID: 26966185). Thus, a cautionary note should be mentioned when referring to this concept.

We will change the text accordingly.

Reviewer #2 (Significance (Required)):

Taken together, this manuscript addresses the contribution of the centrosome to DNA repair. This is in itself a very interesting topic with the potential to attract the interest of both cell/molecular biologists as well as cancer researchers. The major advance strength is represented by pinpointing a specific centriolar substructure, namely subdistal appendages, in the control of HDR. CEP170 had been previously shown to be target of phosphorylation by ATM/R and the present study highlights that the abovementioned phosphorylation is not a mere passenger event during DNA repair, but that potentially reflects a decisive event informing the repair pathway of choice. However, several experiments have alternative explanations/interpretations and no understanding of the underlying mechanism is provided.

We will add the indicated experiments to strengthen the manuscript

The expertise of this reviewer is the study of cell cycle regulation and on the centrosome structure/function.

Revision Plan

Reviewer #3 (Evidence, reproducibility and clarity (Required)):

Summary

In this manuscript, Rodríguez -Real and colleagues investigate how the centrosome may influence the repair of DNA double-stranded breaks (DSBs), building on the initial finding that relative HR frequencies (as measured using a standard split-GFP gene conversion reporter assay) are reduced in centrinone treated centrosome-depleted cells relative to mock treated controls cells. Such defects are found correlate to concordant reductions in immunofluorescence proxies for resection (RPA recruitment into foci) and upstream and downstream events in the HR cascade (BRCA1 and RAD51 recruitment, respectively), and a correlating increase in NHEJ repair of I-SceI induced repair in EJ5-like reporter assay. Taking a candidate approach to identifying which centrosome proteins link the centrosome to DSB repair regulation, the authors reveal cells depleted for subdistal appendage proteins show equivalent deviations in DSB repair reporter assays and show concordant defects in RPA recruitment, leading to the proposal that subdistal appendage proteins regulate DNA resection and thus optimal HR. Experiments are then used to show CEP170 (a subdistal appendage protein) may be phosphorylated by DDR kinases and some rescue experiments are used to support hypothesis that this phosphorylation may be involved in centrosome-DSB repair cross-talk signalling. Figure 3 experiments then show centrosome-depleted and heterozygous losses of CEP170 result in moderate sensitivities across a number of DSB-inducing treatments. Lastly meta-analyses of cancer datasets correlate low CEP170 expression to differences in cancer mutations signatures (Fig 4) and altered patient outcomes across a number of cancers (Fig 5), and propose that CEP170 - via a DSB repair repair function - may be causal in these alterations. Ultimately, the authors propose that the centrosome acts as a signalling node or 'centrosomal processing unit' (CPU) via distal appendage proteins to coordinate the signalling of DNA damage and its repair, and speculate this may link to the clinical phenotypic overlap between centrosome-related ciliopathies and DDR signalling disorders (e.g. ATR-Seckel).

Major comments

1. Concerning Figs 1-3, it is argued that the presented skews in pathway choice are not an

indirect consequence of cell-cycle effects that accompany centrosome depletion (i.e. following centrinone treatments) or depleted centrosome factors. Indeed, S1B shows centrione depleted cell show reduced S-phase indices (where HR is most active) are concordant with increased G2(/M) cell indices, significant effects that may contribute (at least in part) to some of the reported. In the case of the reporter assays it will be difficult/impossible to normalise data vs cell cycle skew, however in the case of RAD51 IRIF frequencies and RPA recruitment, this can be done easily by monitoring the relative frequencies of these events specifically S-phase (BrDU/EdU positive) cells. This should be done if the case for indirect cell-cycle effects is to be dismissed.

This is an important point, so we will repeat the IRIF experiments using S-phase and G2 markers as suggested.

2. Related to point (1): RPA/RAD51/BRCA1 measurements made quantitatively (i.e. by QIBC or equivalent) given % IRIF positive cells can be misleading given it is completely subjective to user defined thesholds.

As mentioned above, we are repeating the experiments using automated methods to quantify the number and intensity of the foci.

3. Fig 3 - The fact that CEP170 KD decreases BRCA1 IRIF but does not increase RIF1 IRIF, is not indicative of a lack of NHEJ stimulation, nor does it infer the existence of a/some distinct mechanism stimulating NHEJ, or an 'undiscovered factor', as is stated. This is important as RIF1 IRIF are not an accepted, nor accurate surrogate marker of NHEJ pathway activity, only an indicator of RIF1 recruitment downstream of 53BP1, whose role in resection control is clear, yet whose contribution to NHEJ is highly context-specific.

The reviewer is right. We will rephrase the text accordingly.

4. Is CEP170 Ser-637 an evolutionarily conserved ATM/ATR site? - Conservation, at least in mammals/vertebrates would be expected if a regulatory event in DSB pathway choice. This should be commented on with supplementary alignment included to demonstrate whether this is likely to be a universally conserved mechanism of repair regulation.

An interesting point. Indeed, the site, and the surrounding region, is conserved in all vertebrates (see attached image, phosphorylated ATM/ATR site inside the red box). In non-vertebrate

animals, plants or microorganisms there is no clear homologue of CEP170 that we can analyze. Even using the protein as a query in homology search programs do not render positive results.

5. Fig 3F-G: Important to show appendage localisation of wild-type and mutant CEP170 S637A/D proteins to inform whether these are functional, expressed at equivalent levels and support equal centrosome localisation intensities.

Again, an interesting point. We will show this localization.

Immunoblot data in support of CEP170 siRNA depletion and CEP170 transgene complementation efficiencies is missing, and needs to be included to reassure a reader the results are specific to defects in the phosphorylation (not stability/expression level/other).

This is an oversight on our side writing the manuscript. We will provide the western blot as indicated, as this has controlled in all our experiments.

6. Do the CEP170 P'n nmutations affect its physiological centrosome functions? If separation of function is not experimentally defined, it should be at least discussed.

Subdistal appendages and CEP170 has been reported to be involved in MTs anchoring to the Centrosome. This is not an evident phenotype to score. The best option to test this might be the quantification of alpha tubulin signal surrounding the centrosome which has been described to be reduced when specific SDA proteins such as ODF2 or CEP128 are absent. So, we will test this phenotype in CEP170 heterozygous RPE1 cells complemented with the different versions of CEP170. Additionally, we will strive to show the localization of this variants is correct as mentioned. In any case, we will discuss that we could not exclude that these mutations might impact other, so far not well defined, centriolar functions of SDAs.

Comments on interpretation and accuracy of stated conclusions:

1. P12. - The manuscript is lacks the necessary evidence to support the section title: "CEP170 Ser647 phosphorylation is critical for HR double strand break repair", and as such I find this and related textual conclusions in the manuscript body to be inaccurate and misleading. To make

this claim would require generating a cell-line knockin of the S647A mutation, preferably at the endogenous CEP170 locus (or a robust complementation system), and its utilisation to establish that standard measures of HR e.g. RAD51 recruitment, PARPi sensitivity, and/or SCE frequencies are all affected as expected in cells bearing this mutation.

The reviewer is right. It is more accurate to suggest this Serine modulates resection. We will change the title and rephrase the text accordingly.

2. Abstract reads: "we identify a centriolar structure, the subdistal appendages, and a specific factor, CEP170, as the critical centrosome component involved in the regulation of recombination and resection... " - I disagree with this statement given that the study has not excluded other centrosome components/features of the centrosome in regulation of resection. Can the authors perform experiments to exclude a role for other centrosome components and substantiate the conclusion that this is a specific function of the subdistal appendages as is stated?

The evidence we have is that Cep170 depletion and Centrinone treatment show the same degree of impairment in resection, recombination and that centrinone treatment do not further reduces resection (Figure 3A for example). So, genetically, those data suggest that they are in the same pathway and, furthermore, there are no further centriolar proteins involved in modulating resection independently of CEP170. If this was not the case, centrinone treatment should show a stronger defect in resection and adding centrinone to CEP170 should reduce resection up to this hypothetical lower level. We could rephrase the text discussing this point in more detail and stating that the genetic data agree with this idea. Having said that, it is likely that other centriolar proteins are involved in this regulation, but through CEP170. Indeed we propose this is the case for Ninenin, Cep128 or centriolin, for example. Furthermore, we have now similar results with the combination of NIN depletion and centrinone treatment, reinforcing this idea of SDAs been the critical centriolar component in the regulation of resection.

3. Based on the marginal sensitivity phenotypes shown in Fig 4 for heterozygous cell-lines, it seems unlikely that CEP170 is a central player in the DSB response.

This is a point worth discussing further in the text. The moderate reduction we observed upon CEP170 reduction in the heterozygous cell lines is on a similar level of what is observed upon, for example, CtIP depletion. As mentioned before in our response to comments to reviewer 1, this is because NHEJ is particularly active in human cells, and takes care of the bulk of DSBs. So, following the reviewer line of thought, CtIP itself is not a central factor of DSB response,

what will be a controversial idea in the field. A major difference is what can be observed upon depletion of factors that act downstream of resection (RAD51, BRCA1, BRCA2, etc), in which resected DNA is committed to be repaired by HR so the lack of those proteins might cause the accumulation of toxic recombination intermediates. To put the things into context, the resection defect we observe upon depletion of CEP170 is similar, or even stronger, of what can be observed upon BRCA1 depletion. In any case, we can discuss this more in depth in the revised version of the paper.

4. The CPU model for DDR-centric role of the centrosome is premature based on the provided data, likewise the fact that a centrosome-regulated resection could explain the clinical overlap between seckel and and this model should be toned down. We probably don't need another acronym for the DDR.

We are open to reshape this part of the discussion, but only after a more in-depth discussion involving the editor and the rest of the referees to see if they agree with this reviewer. It is worth to point out that only this reviewer seems to have this opinion. So, although he/she is obviously entitled to it, we are also legitimized to disagree with him/her. We like the idea that the centrosome acts as a hub for computing signals, what is in agreement with other people suggestions. Also, considering that ATR signaling defects, caused by resection impairment, has been demonstrated to cause Seckel Syndrome, we do not feel it is too big of a leap suggest that this might explain, at least partially, why a centriolar protein such NIN, that we clearly show regulates resection, has been associated with this disease.

Minor comments

• Abstract, lasts sentence needs correction: "suggesting this protein can act as a driver mutation but also..." - a protein cannot act as a driver mutation.

We will change the text as suggested.

• Information regarding biological replicates, sample sizes, error bars should be made more clear throughout to better represent reproducibility; e.g. n=3 {plus minus} Dt. Dev, biological replicates consisting >500 cells/nuclei per condition

We will include more detailed information in this regard.

Revision Plan

Reviewer #3 (Significance (Required)):

General assessment

In exploring for functional links between DSB repair and the centrosome, the results encompass a series of corelating results that collectively hint at a potential role for the centrosome in repair regulation. The indirect and perhaps boring explanation for the presented DSB repair imbalances is these are an indirect consequence of the inevitable cell cycle defects that accompany centrosome depletion. In S1 the authors make some effort towards dispelling this less interesting (indirect) explanation for the presented results, yet not really far enough to dismiss it as the unifying explanation. A major consequence of centrosome-loss is prolonged time spent in G2/M dues to sub-optimal spindle nucleation and assembly kinetics, and an extended transit through mitosis, defects that occur independently of the p53-dependent checkpoint to centrosome loss (in fact the defects have long been speculated precede and perhaps propagate p53 activation). Indeed, supplementary data indicates that in centrosome-depleted cells a reduction in S-phase index (when HR activity is highest) correlates to greater proportion of cells with DNA with G2(/M) content. While I agree that these cell-cycle skews are unlikely to be great enough fully account for the reductions in HR reporter and IF proxies, more targeted approaches to control for indirect cell cycle effects (one suggestion below) could strengthen the case for a direct role in repair regulation. The manuscript also falls short of a identifying a discrete mechanism that explains centrosome-repair crosstalk, and on this basis I feel some of the conclusions are too preliminary and speculative and thus the authors would benefit from being more nuanced in their conclusions. One clear example is the authors's oversimplistic attribution of DSB regulation to distal appendage components of the centrosome/cilia, yet doing so having only tested the appendage proteins on the basis of literature based exercise of protein segregation of DDR and centrosome proteins (S2A). I also find it premature to propose "CPU" models of DDR regulation, the results (while interesting) haven't gone far enough to rigorously challenge this hypothesis, and define its mechanistic basis. I also question the importance and relevance of the analyses in Figs 4-5: in the absence of scientific evidence to establish causation for low CEP170 expression in tumour mutation signature burden or patient prognosis, the presented remain correlates that might equally result from a number of phenomena unrelated to DSB repair. As such, I feel the manuscript does encompass results worthy of report that would be of interest to cell cycle and DNA repair

biologists, it would be greatly improved by being more rigorous, objective and nuanced in its interpretation.

As mentioned, we will address the major concerns of this reviewer and also tone down some of our statements.

3. Description of the revisions that have already been incorporated in the transferred manuscript

Please insert a point-by-point reply describing the revisions that were already carried out and included in the transferred manuscript. If no revisions have been carried out yet, please leave this section empty.

We have not yet included any revision on the manuscript, at the expectative to know if a suitable revised version will fit in the journal.

4. Description of analyses that authors prefer not to carry out

Please include a point-by-point response explaining why some of the requested data or additional analyses might not be necessary or cannot be provided within the scope of a revision. This can be due to time or resource limitations or in case of disagreement about the necessity of such additional data given the scope of the study. Please leave empty if not applicable.

Reviewer 1 ask for a "Numbers and a timecourse should be provided, with details of how these are quantitated provided in the Methods" for our foci analysis. As stated above in our tentative response to his/her comments, we do not think this will have any added value and will be unmanageable due to the sheer number of timepoints to test.

Additional, there are a couple of points of contention about the inclusion of some data or some speculation on the mansucript either with reviewer 1 or 3. As discussed above, we think inall cases it is better to keep it as it is, but are open to a further discussion with the editor and to see if the other reviewers agree with us or not.

Dear Dr. Huertas,

Thank you for submitting your manuscript for consideration by EMBO reports. I apologize for my delayed response, but we have now carefully assessed it and discussed it with the other members of our editorial team, alongside the reports of the referees who evaluated it and your revision plan. In addition, I have also consulted an expert advisor, who is familiar both with the field and with our journal and its scope. I am sorry to say that our conclusion is that the manuscript is not well suited for publication in EMBO reports.

We appreciate that your study investigates how centrosomes and their components may influence repair of DNA double-strand breaks (DSBs). Your findings suggest that the centriolar subdistal appendage protein CEP170 is involved in the regulation of DSB repair pathway choice. You present evidence that the absence of centrioles, subdistal appendages or CEP170 lowers the levels of homologous recombination (HR). This protein has been identified as a phosphorylation target of the DNA damage response (DDR) kinases ATM/ATR, and your study suggests that this phosphorylation event is important to inform repair pathway choice. We agree with the referees and acknowledge that the topic is interesting and significant, and that the proposed concept of centrosomes functioning as a "CPU" to integrate and relay signals generated by the DDR is intriguing.

We also note, however, that the presented data do not rule out the possibility that the observed reduced efficiency of HR is the result of spurious cell cycle impairments due to long-term centrosome depletion or CEP170 knockdown. Because of that, the main conclusion of the study is not fully supported by the available results, and this is a concern that is shared by the referees and the advisor that we contacted (please find the relevant comments we received appended below). Therefore, we unfortunately cannot proceed with further consideration of your manuscript.

I am sorry to disappoint you on this occasion. In the interest of your manuscript and your time, I am providing you with a decision on your manuscript that will allow you to submit it elsewhere without further delay.

I would like to thank you once more for your interest in our journal.

Yours sincerely,

Ioannis Papaioannou, PhD
Editor
EMBO reports

Comments of our advisor:

"I agree with the prevailing feeling by all three reviewers that the topic is interesting and important, and that the presented concept of centrosomes in general, and SDAs in particular, is intriguing. At the same time, I also share their major concern that the effect of long-term centrosome depletion (7-day treatment by cetrinone) or CEP170 knockdown (also 'long-term' as the cells were exposed to siRNAs for several days) can cause spurious cell cycle impairments, which can indirectly translate to reduced HR efficiency. In my view, this major concern has not been dispelled by the current experiments and I actually think it cannot be done with the assays applied in this manuscript; as mentioned above, all treatments used to disrupt centrosome/SDA integrity is based on long-term exposure of cells to centrosome deficiency that can cause unpredictable impairments of cellular homeostasis. Because of that, the proposed concept of centrosomes functioning as a "centrosomal processing unit" to integrate and relay signals generated by the DNA damage response has not been proven. As much as I like the concept and find it intriguing, I think that compelling evidence for its existence would require acute disruption of SDAs directly in the HR-permissive cell cycle stages (S and G2 phase). This, in principle, can be done - for instance by CEP170 degron."

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Rev_Com_number: RC-2022-01727
New_manu_number: EMBOR-2022-56724V1
Corr_author: Huertas
Title: Centriolar subdistal appendages promote double strand break repair through homologous recombination

Dear Dr. Papaioannou,

Thanks a lot for your response. I understand your reluctance with the manuscript as it is. However, and with no intention of undermine the reviewers and advisor valid concerns, I am a bit puzzled with the fact that is the possibility of a spurious cell cycle effect what is the deterrent for consideration in your journal. As stated in the putative response to the reviewers, there is already quite a lot of data that suggest this is not the case, including the fact that we see the same in p53 + and - cells, but also that the FACs profile do not look so different and, even if the mitotic cells are not considered, the numbers simply do not add up. Also, and as explained in our letter, there are other, simple, experiments that can be done to address the issue in more depth, including quantification of mitotic cells or co-satining with cell cycle markers to analysise specifically cells in S and G2. So, while I can understand that your journal might not be interested in the manuscript by a number of reasons, this specific issue of the cell cycle does not seems, in my opinion, strong enough for not letting us to, at least, attempt to submit in the future a revised version addressing such concern.

So, I will like to ask you to reconsider your position and open the possibility of us attempting to address that reviewers comments. In any case, we will abide by your decision, and if you still consider the manuscript a weak candidate for your journal we will submit elsewhere.

Best regards

Pablo

Dear Pablo,

Thank you for the submission of your research manuscript to EMBO reports through Review Commons. It has been evaluated by three experts in the field, and we have also consulted an expert advisor who is familiar both with the field and with our journal and its scope.

The referees and the advisor raised the important concern that the altered levels of homologous recombination could be an indirect consequence of cell cycle impairment due to long-term centrosome depletion or CEP170 knockdown, which would invalidate the main conclusion of the study. In your reply to this criticism, you explained that you are willing to address the concern by attempting to rule out the possibility of a non-specific effect with additional experimentation that is described in your revision plan and in your letter to us. We contacted two of the original referees who evaluated your plan and found it likely sufficient for a conclusive answer. Furthermore, they provided a few additional related suggestions:

Referee #2
With respect to the choice of p53 KO as a means to blunt the cell cycle perturbations induced centrosome depletion:
"I don't deem this choice as particularly appropriate as p53 has a very pleiotropic impact on many processes beyond cell cycle regulation, most notably DNA repair. Given that centrosome depletion leads to cell cycle delay in a manner that depends on p53, 53BP1, USP28 and p21 and given that both p53 and 53BP1 have strong impact on DNA repair, I would deem the choice of USP28 and/or p21 KO as more appropriate for addressing the issue in a cleaner fashion."

Referee #3
1. With respect to point 2 of referee #1:
"I was encouraged to see that the authors did actually use a BFP gating strategy to normalise the HR/repair reporter frequencies, and mitigate non-specific changes due to sample-associated fluctuations in I-SceI transfection efficiency. This gives me more confidence in the assay, but I would want to see a flow cytometry primary data representation as evidence of this gating strategy/normalisation strategy included as a pre-requisite for publication (this is not an unreasonable request)."
2. With respect to point 1 of referee #3:
"Their proposal to address this by monitoring IRIF frequencies, if done specifically in S-phase cells, would satisfy my previous concern. I would stress that S-phase and G2 events are distinct and should be treated as such in this analysis. Pooling S/G2 events (potentially implied by the author response to this query) is likely to skew results owing to the fact that pathway choice reverts somewhat to a pro-NHEJ state in G2, and because by delaying MTOC licensing in late G2 centrinone-treatments will inevitably increase frequencies of G2/early-M phase events, relative to S phase events."
3. With respect to point 3 of referee #3 and the closely related concern of reviewer 1 (point 3):
"The authors aim to "repeating the experiments using automated methods to quantify the number and intensity of the foci." and "reanalyzing our data considering number of foci per cell, intensity of the nuclear signal and intensity of individual foci using a automatic computerized approach." - I think this would be a reasonable and necessary revision plan that if performed to a sufficient standard would address this previous more important concern of mine."

In addition, the referees have also identified and described in their original reports other limitations and raised concerns that should be addressed, and they have provided a number of suggestions for the improvement of the study and the manuscript. Please note that mechanistic insight will not be required for further consideration of your manuscript, although we agree with the referees that it would be desirable for strengthening the study.

Given these constructive comments, we would like to invite you to revise your manuscript along the lines you have proposed with the understanding that the referee concerns (as detailed above and in their reports) must be fully addressed and their suggestions taken on board. In particular, I would like to stress again that the issue of the cell cycle skew must be sufficiently and convincingly addressed. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript. If you have any questions or comments, we can also discuss the revisions in a video chat, if you like.

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

*****
IMPORTANT NOTE:
We perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the

handling will be DELAYED if the following APPLIES:

1) A data availability section providing access to data deposited in public databases is missing. If you have not deposited any data, please add a sentence to the data availability section that explains that (see below for more information).

2) Your manuscript contains statistics and error bars based on n=2. Please use scatter plots in these cases. No statistics should be calculated if n=2.

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

When submitting your revised manuscript, we will require:

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

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).
Please download our Figure Preparation Guidelines (figure preparation pdf) from our Author Guidelines pages https://www.embopress.org/page/journal/14693178/authorguide for more info on how to prepare your figures.

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper unless you opt out of this (please see below for further information).

4) a complete author checklist, which you can download from our author guidelines (). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines
()

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

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

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

7) Please note that a Data Availability section at the end of Materials and Methods is now mandatory. In case you have no data that require deposition in a public database, please state so instead of refereeing to the database.
See also < https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

8) We updated our journal's competing interests policy in January 2022 and request authors to consider both actual and perceived competing interests. Please review the new policy () and update your competing interests statement if necessary. Please name this section 'Disclosure and competing interests statement' and place it after the Acknowledgements section.

9) Figure legends and data quantification:
The following points must be specified in each figure legend:

- the name of the statistical test used to generate error bars and P values,
- the number (n) of independent experiments (please specify technical or biological replicates) underlying each data point,
- the nature of the bars and error bars (s.d., s.e.m.)
- If the data are obtained from n {less than or equal to} 2, use scatter plots showing the individual data points.

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

See also the guidelines for figure legend preparation:
https://www.embopress.org/page/journal/14693178/authorguide#figureformat

- Please also include scale bars in all microscopy images.

10) We now request the publication of original source data with the aim of making primary data more accessible and transparent to the reader. Our source data coordinator will contact you to discuss which figure panels we would need source data for and will also provide you with helpful tips on how to upload and organize the files.

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

12) Please also note our reference format:
.

13) We now use CRediT to specify the contributions of each author in the journal submission system. CRediT replaces the author contribution section, which should be removed from the manuscript. Please use the free text box to provide more detailed descriptions. See also guide to authors:
.

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

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

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

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

Best regards,

Ioannis

Ioannis Papaioannou, PhD
Editor
EMBO reports

Dear Dr. Papaioannou,

We would like you to thank you for the opportunity to submit our revised manuscript "**Centriolar subdistal appendages promote double strand break repair through homologous recombination"** for consideration in EMBO Reports.
We want to thank all three referees and the editor for their constructive criticisms to our work. We believe that the new set of data in response to reviewers comment have significantly improved the original manuscript and cemented our model. Find below a point-by-point response to the reviewer's initial comments (after the first round of revision in Review Commons platform) and additional point-by-point response to your comments and further notes of referee #2 and #3 received by email.

Reviewer #1 (Evidence, reproducibility and clarity (Required)):

Summary

Rodríguez-Real, Huertas and colleagues here explore the roles of centrosomes in DNA damage responses, focussing on DNA repair activities. They show that centrosome depletion by PLK4 inhibition leads to reduced levels of homologous recombination and increased nonhomologous end-joining, along with altered level of nuclear focus formation by DNA repair proteins. Knockdown of genes that encode components of centriolar subdistal appendages (SDAs) cause reduced levels of RPA foci, with CRISPR-generated CEP170 heterozygotes also showing defects in focus formation. Knockdown of CEP170 impairs homologous recombination, although NHEJ activities are unaffected. Some increase in sensitivity to DNA damaging agents is seen in CEP170- or centriole-deficient cells, albeit with a modest effect size. CEP170 status is shown to affect mutational signatures and patient prognosis in different cancer samples.

While the experiments are generally well-presented and controlled, the effects seen are not large, so that the the conclusions that the authors draw are not entirely substantiated by the data presented, even without the suggestion of a mechanism. There are several additional experiments and clarifications that I consider necessary to provide appropriate support for the phenomenon.

We would like to start by thanking the referee for their fair and constructive criticisms. We have aimed to address all the raised issues, as described below.

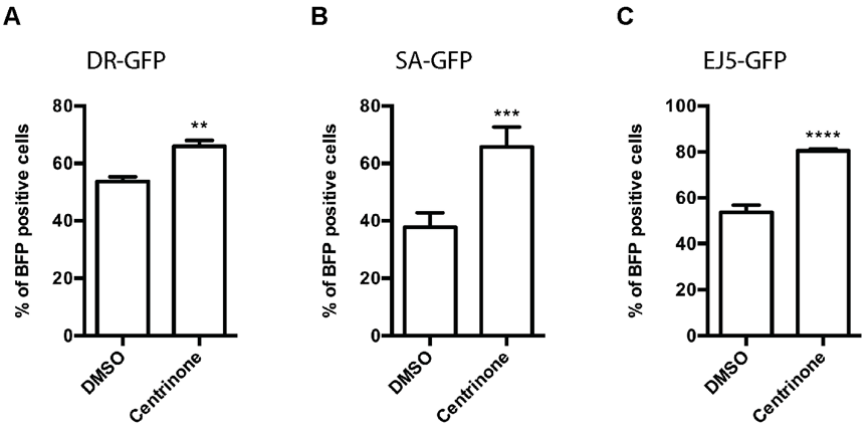
Major points

1. The lack of cell cycle arrest or phenotype in the U2OS cells after a week's treatment with centrinone is somewhat surprising, given their p53 status. The initial description of centrinone showed a distinct impact on U2OS proliferation, albeit after 2 weeks' treatment (although the present paper shows robust impact on centriole numbers after only 1 week in centrinone). It would be useful to know the percentage of mitotic cells, or if there is any increased cell death observed at this stage of treatment.
Albeit the original manuscript already presented data indicating that the phenotype was independent of cell cycle, in general, the reviewer is right in the sense that mitotic cells well not formally accounted for. So, we have included a new result´s section to cover this and related concerns of the reviewers as it was one of the main critics to our work. In this new section we monitor cell cycle parameters as % of mitotic cells

directly or through H3S10 presence (EV1O and P), % cell death (EV1Q) and a detailed analysis of DNA end resection throughout the cell cycle by Quantitative Image Based Cytometry (QIBC) (Figure 1F-H). Additionally, we have reproduced the resection defect upon camptothecin treatment, that only created DSBs in S phase, in cells treated with centrinone (EV1C). We have also extended our results to a battery of cells defective on p53 or p21 (EV1R-Z), in which no cell cycle arrest occurs. All these results have been grouped in the section: DNA double strand break repair imbalance upon centrosome lost is not a consequence of cell cycle perturbance line 242-268 and related Figures Fig. 1 I and J and EV1N-Z.

2. In the I-SceI assays, were transduction efficiencies or apoptosis within the experiment impacted by centrinone treatment? If not, it would be useful to state that this was examined and that there were no confounding effects; having only normalised data does not allow the reader to exclude these potential confounding factors.

We apologize if this was not clear in the initial manuscript, but this is not a real issue. We use an I-SceI vector that contains a BFP for these experiments, and we count only BFP-positive cells. So, we control and normalize all our experiments taking into consideration transduction efficiencies. Indeed, as shown in the figure, centrinone treatment increases transduction efficiency in all cases, so even if we would not normalize as we do, in any case we would have been underestimating the effect.



The above shown control charts have not been included in the figures of the manuscript for simplicity. However, they could be included in the appendix upon request by the editor or referees if required.

3. The authors present binary data for a given type of nuclear focus (positive or negative for RPA/ BRCA1/ RAD51), while the supporting images show altered numbers/ intensities. For example, the BRCA1 signals shown in Fig. 3D are less readily distinguished than they are in Fig. 1D. These data should be reconsidered: it is possible that these observations reflect different kinetics of focus formation, rather than a change in IRIF formation capacity. Numbers and a timecourse should be provided, with details of how these are quantitated provided in the Methods.

We have re-analyzed all our images considering number of foci per cell, intensity of the nuclear signal and intensity of individual foci using an automatic computerized approach for all our experiments (i. e. Fig. 1C-D, Fig.EV1C-F, L, M, R-Z, Fig. 2C, D, F, G, Fig. EV2E, F, H-J, Fig. 3A, D, E F, H, and Fig. EV3B, C, H, I) . In this new version of the manuscript for simplicity for each condition we show number of foci per cell. Similar results were found for other parameters (intensity of the nuclear signal and intensity of individual foci) and charts showing these data could be included upon request in the appendix, although we consider

then redundant with the data showed in the main and EV figures. Regarding the comparison in intensities between figures 1D and 3D, as initially express in our review plan, we believe it is simply not appropriate. These experiments were performed completely independently, therefore small changes in the primary or secondary antibodies affinities due to batch, the room temperature, small changes in the buffer, or even the age of the microscope lamp can account for those changes. Indeed, the images were taken with different microscopes. Finally, regarding kinetics, it is formally true that there might be changes, but it is well stablished that the times we take our images are relevant for our observations. Performing kinetics of all the foci we analyzed in all the conditions will be unmanageable with very little added value, as least in our opinion.

4. Are the BRCA1 and RAD51 results seen with centrinone treatment of U2OS cells recapitulated in the Saos-2 and RPE1 lines?

They are, as shown in Fig. EV1R-Z and mentioned in the main text lines 265-267.

5. Some additional analysis is needed of the extent to which cells are sensitised to DNA damaging treatments by CEP170 deficiency or centrinone treatment. It should be confirmed that these experiments were performed in biological triplicate, rather than a technical triplicate (within a single experiment); if this is not the case, these experiments should be done in triplicate.

This is an important point to clarify. The experiments were performed in biological triplicates, and in each one of them in technical triplicates. I.e. We repeated the experiments three completely independently times, and each time we performed a technical triplicate. So, the average we show is the mean of the three averages. This has been clarified in Figure text lines 794-795 and in the figure legend. We thank the reviewer for bringing this to our attention.

Analysing p53-deficient hTERT-RPE1 clones, Kumar et al. (NAR Cancer 2020 PMID: 33385162) showed <10% survival with 100 ng/ml NCS. Hustedt et al. (Genes Dev 2019 PMID: 31467087) showed just over 50% survival with 10 nM CPT treatment, although their data for IR were comparable to the current study. Given the wide variation that these assays seem to incur, the extent to which a ≈20% difference in clonogenic survival is biologically significant may be limited. A rescue of the CEP170 siRNA, and/ or washout in the centrinone experiment would make these data more convincing.

Again, an interesting experiment. Indeed, as suggested, we have performed a rescue experiment depleting CEP170 with an siRNA and ectopically expressing a siRNA resistant version of CEP170. Figure 4I and J and main text lines 453-456. The results support our model.

The knockdown of CEP170 in Figure 4 should be correctly labelled (not as CEP170+/-); given that the authors have generated CEP170 heterozygotes in Figure 2, this is potentially confusing.

They are indeed the heterozygous we described before and not knockdowns. We have changed the main text to say heterozygous instead of knockdown to avoid confusion. Main text line 443.

6. Direct data for the (centrosomal) phosphorylation of CEP170 are limited; it has not been demonstrated that the S637A mutants are fully functional in terms of the centrosome functions of CEP170, so that the conclusion regarding a requirement for centrosomal CEP170 phosphorylation is not sufficiently supported by the available data. The CEP170-dependent changes in RPA focus positive cell percentages shown in Figure 3 are not very marked. The relevant sections should be revised, or the authors should include additional experiments showing directly a phosphorylation of CEP170.

As we mentioned in our previous correspondence with the editor, we want to reiterate what the reviewer claim to be “not very marked” changes are similar or even greater to what us and other has shown previously for BRCA1 depletion. A reference is now included. To support that CEP170 mutant versions (S367A and S367D) are functional we have assessed their precise localization at the centriole with Ultrastructure Expansion Microscopy. Both mutant versions localized with the same pattern to the endogenous CEP170 and a ectopically expressed *wt* CEP170. These data are shown in Figure 3G and Figure EV3F and in main text lines 406-415. Unfortunately, we have been unable to prove CEP170 phosphorylation in S367 in response to DNA damage. Our experimental approach were either by GFP:CEP170 immunoprecipitation and blotting with a generic ATM/ATR phosphorylation site antibody (Phospho-(Ser/Thr) ATM/ATR Substrate Antibody; Cell Signaling; REF: #2851) or by using Phos-tag gel (Phos-tag (TM) Acrylamide AAL-107; FUJIFILM Wako Pure Chemical Corporation; REF: 304-93521).

7. It is difficult to interpret the mutational spectrum data and their significance. These should be compared with data for mutations in NDEL1 mutant cells, and/or other SDA components.

We have expanded our analysis on mutational signatures to several subcentriolar appendages proteins (i.e. CEP170, NDEL1, CEP128, Centriolin and NIN). Interestingly, NDEL1 seems to show a similar pattern to CEP170, NIN or Centriolin), but to a lesser extent (or at least with less statistical power). This might indicate that the whole SDA affects repair in an in vivo tumoral set up. Strikingly too, CEP128 behaves differently to the others. Therefore, our data suggest a more complex relationship between SDA and mutagenesis in cancer. These data are presented in Fig. EV5 and in the Apendix Figure S2 and in main text lines 489-494 and discussed in main text lines 689-698.

8. The Kaplan-Meier curves data are intriguing, but their interpretation is highly speculative, given that there are no data on treatment groups included in this study. It is unclear whether other genes that affect SDAs might also impact survival (in the same, or different cancers), so the presentation of those patient groups where CEP170 status impacted survival seems selective, given the ubiquity of HR and centrosomes. These data would be better included as Supplemental information.

As it was discussed in our first response (see below in italic) we think this data should stay in main Figure 6.

(Again, as suggested, we are analyzing other SDA components to strengthen this point. As if this should be included as a main figure or a supplementary, we disagree with the reviewer. In that regard, neither of the two other referees seem to agree with him/her. In any case, we are open to discuss this with the reviewers and the editor and keep it as it is or send it to the supplementary information once we have reached a consensus.)

9. The independence of p53 status/ responsiveness of the system is a crucial aspect of this study. Sir et al. (JCB 2013 PMID: 24297747) showed no DNA repair defect in centrosome-deficient chicken DT40 cells. This paper is very relevant to the current study and should be discussed. Similarly, the work by Lambrus et al (JCB 2015 PMID: 26150389) should also be considered.

We have included these two papers in our discussion. Main text lines 553-555 and in line 74. Thanks for bringing it to our attention.

Minor points

10. References for the RPE1 TP53/ SAS6 mutant cell lines should be provided (or controls for their generation presented).

It is now included in main text line 721-722.

11. Fig S1K should correct its x-axis to reflect the time intervals correctly.

It has been corrected as suggested.

12. Fig 2D should show blow-ups of the centrosomes.

Blow ups of the centrosomes have been included.

13. To avoid any potential confusion, it would be helpful to indicate in the Figure proper which cells are used for the various analyses.

We have added this information in all figures and/or figure legends as suggested.

14. The 'basal side' of the centriole is not a standard term- this should be clarified. This may be confusing, given the role of centrioles in the basal body.

We have changed the nomenclature to “proximal side of centrioles” to avoid confusions. Main text lines 304, 409, 571, 573, 575 and 1027.

15. The consideration of Seckel syndrome seems somewhat speculative at this stage in the exploration of this phenomenon.

As stated in our previous response this is why it is in the discussion and we state it is a possibility. Therefore, we would prefer to keep it as it is, but again we are open to discuss this point with the editor and the rest of the reviewers.

Referees cross commenting

I think the comments from Reviewers #2 and #3 are reasonable and justified; there is good convergence between the comments that we all made and I have no issues to raise in this cross-commentary.

Reviewer #1 (Significance (Required)):

Strengths: Much previous work linking centrosomes and DNA damage responses has addressed cell cycle and checkpoint roles of the centrosome, so that a direct role in (nuclear) DNA repair is intriguing.

Limitations:The present study shows a relatively moderate impact of centrosome defects on DNA repair, without a clear mechanism. There are some technical details that should be addressed. The relatively limited sensitization to DNA damaging treatments caused by centrosome deficiency questions the biological significance of the phenomenon.

As shown above, we hope we have answered the main concerns of reviewer #1 and address most technical details. Additionally, as stated in our previous response, the sensitivities we observe are not really minor, as again they are similar to what is observed upon depletion of even core resection factors as

CtIP. The fact that NHEJ can take care of more than 80% of the DSBs in cells is what makes resection deficiency cause this survival reductions. However, the consequences in terms of mutagenesis and the long-term accumulation of unrepaired breaks is what makes HR so relevant, especially in a cancer setup.

Advance: The current study presents some new findings that potentially show DNA repair defects resulting from the loss of centrioles (or SDA proteins). This has not been demonstrated to date.

Audience: The idea of subdistal appendage components contributing to homologous recombinational repair of DNA damage is of potential interest to several fields, ranging from basic centrosome biology through translational to clinical cancer research.

Reviewer's expertise: basic/ cell biology.

Reviewer #2 (Evidence, reproducibility and clarity (Required)):

In this manuscript by Rodriguez-Real et al, the authors address the contribution of the centrosome to cellular process unrelated to organizing the microtubule cytoskeleton, namely DNA repair. As many proteins contributing to the DNA damage response physically associate with centrosomes, this appears a relevant question that has been neglected so far and led to a number of studies that appeared controversial. To do so, the authors exploit a variety of tissue culture models that are well established in the fields of centrosomes and DNA repair, including U2OS and RPE1 cells, exposed to perturbations promoting DNA damage (such as ionizing radiation or pharmacologic perturbation of DNA stability) in conjunction with siRNA mediated depletion of candidate centrosomal proteins., followed by the visualization of repair events either using fluorescent reporters, or visualizing endogenous repair foci by immunofluorescence. On this basis, the authors propose that a discrete centrosomal sub-structure, namely sub-distal appendages and the CEP170 protein therein concur to promote a particular nuclear DNA repair process, namely homologous recombination.

The manuscript suffers of two main limitation:

1. the authors provide no mechanistic understanding of how CEP170, a protein that resides at centriolar subdistal appendages and shows no nuclear translocation upon DNA damage, concurs to regulate processes in the nucleus. The fact that all reported phenomena appear to be independent of microtubules suggests that neither the LINC complex nor the precise position of the centrosome in the vicinity of nuclear pore complexes contribute to the reported phenomena.

The referee is absolutely right, and we do not hide it in the manuscript. We even mentioned it in the discussion. We lack this molecular connection, but as he/she points out finding it will be far from straight forward. In any case, we think the advance we observe is enough to grant publication. Indeed, upon email discussion with the editor, he indicated that advancing on the mechanistic insight of the described connection was not required to grant publication on EMBO Rep.

2. some of the experimental perturbations performed in the manuscript might elicit the reported

phenotypes due to spurious effects on cellular processes that have not been considered with sufficient caution.

We have performed further experiments to rule out this possibility and as shown below we believe they tackle experimentally reviewer´s concerns. See below for specific responses.

Given that uncovering the mechanism underlying the contribution of CEP170 to homologous recombination might prove very demanding, my comments will focus primarily on the second point.

As discussed with the editor, and suggested by this reviewer, the first point lays outside of the scope of this manuscript and the journal. In any case, we want to extend our thanks to the referee for helping us to strengthen our paper.

Major comments:

The centriolar depletion using centrinone is known to impinge on cell proliferation in p53 WT cells. Thus, I am not convinced that the data shown in Figure S1B and S1C will sufficiently document that the observed unbalance between HDR and NHEJ are not simply reflecting a different cell cycling speed/behavior. Moreover, it would be important to address whether centrinone or depletion of CEP170 (an essential gene, according to the authors!) will trigger DNA damage by themselves. In fact, even a small extent of chronic genotoxic stress caused by the perturbations used in the manuscript might explain the reported differential proficiency of HDR.

We agree with the reviewer(s) that this is an important issue. So, and despite that our original manuscript already presented data against an effect mediated by the cell cycle, we have studied in more detail this issue. As it has been presented above in response to reviewer #1, we have included a whole new result´s section to cover this and related concerns of the reviewers as it was one of the main critics to our work. In this new section we monitor cell cycle parameters as % of mitotic cells (EV1O and P), % cell death (EV1Q) and a detailed analysis of DNA end resection throughout the cell cycle by Quantitative Image Based Cytometry (QIBC). Also, we have tested a battery of cells that are Kos for either p53 or p21. All these results have been grouped in the section: “DNA double strand break repair imbalance upon centrosome lost is not a consequence of cell cycle perturbance”, main text lines 248-278 and related Figures Fig. 1I and J and EV1N-Z.

The idea that centrinone and/or CEP170 depletion might lead to an increase of spontaneous DNA damage was an intriguing one. As suggested by the referee, this might affect the interpretation of some of our results. So, we have also included experiments to measure DNA damage after centrinone treatment or CEP170 depletion and results (i.e. no damaged observed) are shown in Fig. EV1J, EV2I and J, and in main text lines 219-22 and 335-337. Thanks for this interesting suggestion.

Minor comments:

It is a pity that CEP170 is not amenable to functional dissection using a complete knockout. The fact that in PMID: 27818179 a complete knockout of CEP128 has been achieved, suggests however that subdistal appendage mediated DNA repair is not the essential process in itself. As the authors employ other cell

lines stemming from the same laboratory, they could consider acquiring CEP128 KO to complement their own experiments.

We want to thank the reviewer for bringing this to our attention. These experiments have been included with results supporting our model in Fig. EV2E-F and in main text lines 329-332, and in Fig. EV3B-C and main text lines 388-389. The results support our main findings with CEP170.

The proposal that CEP170 phosphorylation of by ATM/ATR upon DNA damage might require SDA localization of the protein is plausible, yet not circumstantiated by any experimental evidence. If the authors could monitor the phosphorylation of the endogenous CEP170 protein in WT vs CEP128 KO cells (phosphor-specific antibody, MS-based proteomics or simply "phos-tag" gels), this could provide a first spark towards a mechanistic understanding of the reported phenomenon.

As stated in response to reviewer #1, unfortunately, we have been unable to prove CEP170 phosphorylation in S367 in response to DNA damage. Our experimental approach were either by GFP:CEP170 immunoprecipitation and blotting with a generic ATM/ATR phosphorylation site antibody (Phospho-(Ser/Thr) ATM/ATR Substrate Antibody; Cell Signaling; REF: #2851) or by using Phos.tag gel (Phos-tag (TM) Acrylamide AAL-107; FUJIFILM Wako Pure Chemical Corporation; REF: 304-93521). However, upon the discussion with the editor by email we think the data are strong enough to support our claim and no further mechanistic insight should be essential for publication in EMBO Reports.

The entire Figure 4 is based on quantifications of clonogenic potential.

1. it would be helpful if the data were accompanied by images displaying representative crystal violet stained dishes.

We show the images now in Fig, EV4. Thanks for the suggestion.

2. clonogenic potential potential is discussed as a mere readout of cell survival, yet a combination between survival and proliferation concur to the reported differential clonogenic potential.

We have changed the text accordingly. Main text line 442

Odf2 contribution to both DAs and SDAs: while Odf2 has been initially proposed to be necessary for the assembly of both types of appendages, its contribution to distal appendages has been disputed by Tanos et al using siRNA (PMID: 23348840), also confirmed by our group using CRISPR (unpublished). Thus, the role of Odf2 in SDA assembly appears more crucial than for DA assembly.

We have changed the text accordingly. Main text lines 81-83.

CEP164 contribution to ATM/ATR activation: this has been disputed in this paper by the Morrison lab (PMID: 26966185). Thus, a cautionary note should be mentioned when referring to this concept.

We have included the reference and a comment following this suggestion. Main text line 95-98.

Reviewer #2 (Significance (Required)):

Taken together, this manuscript addresses the contribution of the centrosome to DNA repair. This is in itself a very interesting topic with the potential to attract the interest of both cell/molecular biologists as well as cancer researchers. The major advance strength is represented by pinpointing a specific centriolar

substructure, namely subdistal appendages, in the control of HDR. CEP170 had been previously shown to be target of phosphorylation by ATM/R and the present study highlights that the abovementioned phosphorylation is not a mere passenger event during DNA repair, but that potentially reflects a decisive event informing the repair pathway of choice. However, several experiments have alternative explanations/interpretations and no understanding of the underlying mechanism is provided.
We hope to cover the major concerns of reviewer #2 with the additional data supported in this new version of the manuscript.

The expertise of this reviewer is the study of cell cycle regulation and on the centrosome structure/function.

Reviewer #3 (Evidence, reproducibility and clarity (Required)):

Summary
In this manuscript, Rodríguez -Real and colleagues investigate how the centrosome may influence the repair of DNA double-stranded breaks (DSBs), building on the initial finding that relative HR frequencies (as measured using a standard split-GFP gene conversion reporter assay) are reduced in centrinone treated centrosome-depleted cells relative to mock treated controls cells. Such defects are found correlate to concordant reductions in immunofluorescence proxies for resection (RPA recruitment into foci) and upstream and downstream events in the HR cascade (BRCA1 and RAD51 recruitment, respectively), and a correlating increase in NHEJ repair of I-SceI induced repair in EJ5-like reporter assay. Taking a candidate approach to identifying which centrosome proteins link the centrosome to DSB repair regulation, the authors reveal cells depleted for subdistal appendage proteins show equivalent deviations in DSB repair reporter assays and show concordant defects in RPA recruitment, leading to the proposal that subdistal appendage proteins regulate DNA resection and thus optimal HR. Experiments are then used to show CEP170 (a subdistal appendage protein) may be phosphorylated by DDR kinases and some rescue experiments are used to support hypothesis that this phosphorylation may be involved in centrosome-DSB repair cross-talk signalling. Figure 3 experiments then show centrosome-depleted and heterozygous losses of CEP170 result in moderate sensitivities across a number of DSB-inducing treatments. Lastly meta-analyses of cancer datasets correlate low CEP170 expression to differences in cancer mutations signatures (Fig 4) and altered patient outcomes across a number of cancers (Fig 5), and propose that CEP170 - via a DSB repair repair function - may be causal in these alterations. Ultimately, the authors propose that the centrosome acts as a signalling node or 'centrosomal processing unit' (CPU) via distal appendage proteins to coordinate the signalling of DNA damage and its repair, and speculate this may link to the clinical phenotypic overlap between centrosome-related ciliopathies and DDR signalling disorders (e.g. ATR-Seckel).

As for the others reviewers, we want to thank referee 3 for their interesting suggestion. Upon taking them onboard, we think we have strengthened our results.

Major comments

1. Concerning Figs 1-3, it is argued that the presented skews in pathway choice are not an indirect consequence of cell-cycle effects that accompany centrosome depletion (i.e. following centrinone treatments) or depleted centrosome factors. Indeed, S1B shows centrione depleted cell show reduced S-phase indices (where HR is most active) are concordant with increased G2(/M) cell indices, significant effects that may contribute (at least in part) to some of the reported. In the case of the reporter assays it will be difficult/impossible to normalise data vs cell cycle skew, however in the case of RAD51 IRIF frequencies and RPA recruitment, this can be done easily by monitoring the relative frequencies of these events specifically S-phase (BrDU/EdU positive) cells. This should be done if the case for indirect cell-cycle effects is to be dismissed.

As stated above we have included a new result´s section to cover this and related concerns of the reviewers as it was one of the main critics to our work. In this new section we monitor cell cycle parameters as % of mitotic cells (EV1O and P), % cell death (EV1Q) and a detailed analysis of DNA end resection throughout the cell cycle by Quantitative Image Based Cytometry (QIBC). Also, we have analyzed DSBs induced by camptothecin (that only creates DSBs during S phase) and tested

the effect on p53 or p21 KO cells. All these results have been grouped in the section: DNA double strand break repair imbalance upon centrosome lost is not a consequence of cell cycle perturbance line 248-278 and related Figures Fig. 1 I and J and EV1N-Z.

2. Related to point (1): RPA/RAD51/BRCA1 measurements made quantitatively (i.e. by QIBC or equivalent) given % IRIF positive cells can be misleading given it is completely subjective to user defined thesholds.

As mentioned above, we have re-analyzed all our images considering number of foci per cell, intensity of the nuclear signal and intensity of individual foci using an automatic computerized approach for all our experiments (i. e. Fig. 1C-D, Fig.EV1C-F, L, M, R-Z, Fig. 2C, D, F, G, Fig. EV2E, F, H-J, Fig. 3A, D, E F, H, and Fig. EV3B, C, H, I) . In this new version of the manuscript for simplicity for each condition we show number of foci per cell. Similar results were found for other parameters (intensity of the nuclear signal and intensity of individual foci) and charts showing these data could be included upon request in the appendix, although we consider then redundant with the data showed in the main and EV figures.

3. Fig 3 - The fact that CEP170 KD decreases BRCA1 IRIF but does not increase RIF1 IRIF, is not indicative of a lack of NHEJ stimulation, nor does it infer the existence of a/some distinct mechanism stimulating NHEJ, or an 'undiscovered factor', as is stated. This is important as RIF1 IRIF are not an accepted, nor accurate surrogate marker of NHEJ pathway activity, only an indicator of RIF1 recruitment downstream of 53BP1, whose role in resection control is clear, yet whose contribution to NHEJ is highly context-specific.

We have rephrased the text accordingly.

4. Is CEP170 Ser-637 an evolutionarily conserved ATM/ATR site? - Conservation, at least in mammals/vertebrates would be expected if a regulatory event in DSB pathway choice. This should be commented on with supplementary alignment included to demonstrate whether this is likely to be a universally conserved mechanism of repair regulation.

An interesting point. Indeed, the site, and the surrounding region, is conserved in all vertebrates (see attached image, phosphorylated ATM/ATR site inside the red box). In non-vertebrate animals, plants or microorganisms there is no clear homologue of CEP170 that we can analyze. Even using the protein as a query in homology search programs do not render positive results. This data is shown in Fig. EV3E and in main text line 402-403.

5. Fig 3F-G: Important to show appendage localisation of wild-type and mutant CEP170 S637A/D proteins to inform whether these are functional, expressed at equivalent levels and support equal centrosome localisation intensities.

WT and CEP170 mutant versions (S367A and S367D) were express and their precise localization was assessed at the centriole with Ultrastructure Expansion Microscopy. Both mutant versions localized with a extremely similar pattern to the endogenous CEP170 and a ectopically expressed *wt* CEP170. These data are shown in Figure 3G and Figure EV3F and in main text lines 406-415.

Immunoblot data in support of CEP170 siRNA depletion and CEP170 transgene complementation efficiencies is missing, and needs to be included to reassure a reader the results are specific to defects in the phosphorylation (not stability/expression level/other).

This data can be found now in Fig. EV3D.

6. Do the CEP170 P'n nmutations affect its physiological centrosome functions? If separation of function is not experimentally defined, it should be at least discussed.

We have discussed this point in main text lines 615-616.

Comments on interpretation and accuracy of stated conclusions:

1. P12. - The manuscript is lacks the necessary evidence to support the section title: "CEP170 Ser647 phosphorylation is critical for HR double strand break repair", and as such I find this and related textual conclusions in the manuscript body to be inaccurate and misleading. To make this claim would require generating a cell-line knockin of the S647A mutation, preferably at the endogenous CEP170 locus (or a robust complementation system), and its utilisation to establish that standard measures of HR e.g. RAD51 recruitment, PARPi sensitivity, and/or SCE frequencies are all affected as expected in cells bearing this mutation.

The reviewer is right. We now suggest this Serine modulates resection. Main text line 395.

2. Abstract reads: "we identify a centriolar structure, the subdistal appendages, and a specific factor, CEP170, as the critical centrosome component involved in the regulation of recombination and resection... " - I disagree with this statement given that the study has not excluded other centrosome components/features of the centrosome in regulation of resection. Can the authors perform experiments to

exclude a role for other centrosome components and substantiate the conclusion that this is a specific function of the subdistal appendages as is stated?

As stated in our first response: The evidence we have is that CEP170 depletion and Centrinone treatment show the same degree of impairment in resection, recombination and that centrinone treatment do not further reduces resection (Figure 3A for example). So, genetically, those data suggest that they are in the same pathway and, furthermore, there are no further centriolar proteins involved in modulating resection independently of CEP170. If this was not the case, centrinone treatment should show a stronger defect in resection and adding centrinone to CEP170 should reduce resection up to this hypothetical lower level.

We have rephrased the text discussing this point in more detail and stating that the genetic data agree with this idea. Main text lines 595-600.

3. Based on the marginal sensitivity phenotypes shown in Fig 4 for heterozygous cell-lines, it seems unlikely that CEP170 is a central player in the DSB response.

This is a point now discussed in the text, Main text lines 617-619.

4. The CPU model for DDR-centric role of the centrosome is premature based on the provided data, likewise the fact that a centrosome-regulated resection could explain the clinical overlap between seckel and and this model should be toned down. We probably don't need another acronym for the DDR.

We do not agree here with the reviewer. We like the idea that the centrosome acts as a hub for computing signals, what is in agreement with other people suggestions. Also, considering that ATR signaling defects, caused by resection impairment, has been demonstrated to cause Seckel Syndrome, we do not feel it is too big of a leap suggest that this might explain, at least partially, why a centriolar protein such NIN, that we clearly show regulates resection, has been associated with this disease.

Minor comments

• Abstract, lasts sentence needs correction: "suggesting this protein can act as a driver mutation but also..." - a protein cannot act as a driver mutation.

We have changed the text as suggested. Main text line 47

• Information regarding biological replicates, sample sizes, error bars should be made more clear throughout to better represent reproducibility; e.g. n=3 {plus minus} Dt. Dev, biological replicates consisting >500 cells/nuclei per condition

We have included more detailed information in text figures.

Reviewer #3 (Significance (Required)):

General assessment

In exploring for functional links between DSB repair and the centrosome, the results encompass a series of corelating results that collectively hint at a potential role for the centrosome in repair regulation. The indirect and perhaps boring explanation for the presented DSB repair imbalances is these are an indirect consequence of the inevitable cell cycle defects that accompany centrosome depletion. In S1 the authors make some effort towards dispelling this less interesting (indirect) explanation for the presented results,

yet not really far enough to dismiss it as the unifying explanation. A major consequence of centrosome-loss is prolonged time spent in G2/M dues to sub-optimal spindle nucleation and assembly kinetics, and an extended transit through mitosis, defects that occur independently of the p53-dependent checkpoint to centrosome loss (in fact the defects have long been speculated precede and perhaps propagate p53 activation). Indeed, supplementary data indicates that in centrosome-depleted cells a reduction in S-phase index (when HR activity is highest) correlates to greater proportion of cells with DNA with G2(/M) content. While I agree that these cell-cycle skews are unlikely to be great enough fully account for the reductions in HR reporter and IF proxies, more targeted approaches to control for indirect cell cycle effects (one suggestion below) could strengthen the case for a direct role in repair regulation. The manuscript also falls short of a identifying a discrete mechanism that explains centrosome-repair crosstalk, and on this basis I feel some of the conclusions are too preliminary and speculative and thus the authors would benefit from being more nuanced in their conclusions. One clear example is the authors's oversimplistic attribution of DSB regulation to distal appendage components of the centrosome/cilia, yet doing so having only tested the appendage proteins on the basis of literature based exercise of protein segregation of DDR and centrosome proteins (S2A). I also find it premature to propose "CPU" models of DDR regulation, the results (while interesting) haven't gone far enough to rigorously challenge this hypothesis, and define its mechanistic basis. I also question the importance and relevance of the analyses in Figs 4-5: in the absence of scientific evidence to establish causation for low CEP170 expression in tumour mutation signature burden or patient prognosis, the presented remain correlates that might equally result from a number of phenomena unrelated to DSB repair. As such, I feel the manuscript does encompass results worthy of report that would be of interest to cell cycle and DNA repair biologists, it would be greatly improved by being more rigorous, objective and nuanced in its interpretation.

We hope to have answered to the main concerns of reviewer #3 with this new version of the manuscript.

Additional notes from the editor and referee #2 and #3 received by email

Dear Pablo,

Thank you for the submission of your research manuscript to EMBO reports through Review Commons. It has been evaluated by three experts in the field, and we have also consulted an expert advisor who is familiar both with the field and with our journal and its scope.

The referees and the advisor raised the important concern that the altered levels of homologous recombination could be an indirect consequence of cell cycle impairment due to long-term centrosome depletion or CEP170 knockdown, which would invalidate the main conclusion of the study. In your reply to this criticism, you explained that you are willing to address the concern by attempting to rule out the possibility of a non-specific effect with additional experimentation that is described in your revision plan and in your letter to us. We contacted two of the original referees who evaluated your plan and found it likely sufficient for a conclusive answer. Furthermore, they provided a few additional related suggestions:

Referee #2

With respect to the choice of p53 KO as a means to blunt the cell cycle perturbations induced centrosome depletion:

"I don't deem this choice as particularly appropriate as p53 has a very pleiotropic impact on many processes beyond cell cycle regulation, most notably DNA repair. Given that centrosome depletion leads to cell cycle delay in a manner that depends on p53, 53BP1, USP28 and p21 and given that both p53 and 53BP1 have strong impact on DNA repair, I would deem the choice of USP28 and/or p21 KO as more appropriate for addressing the issue in a cleaner fashion."

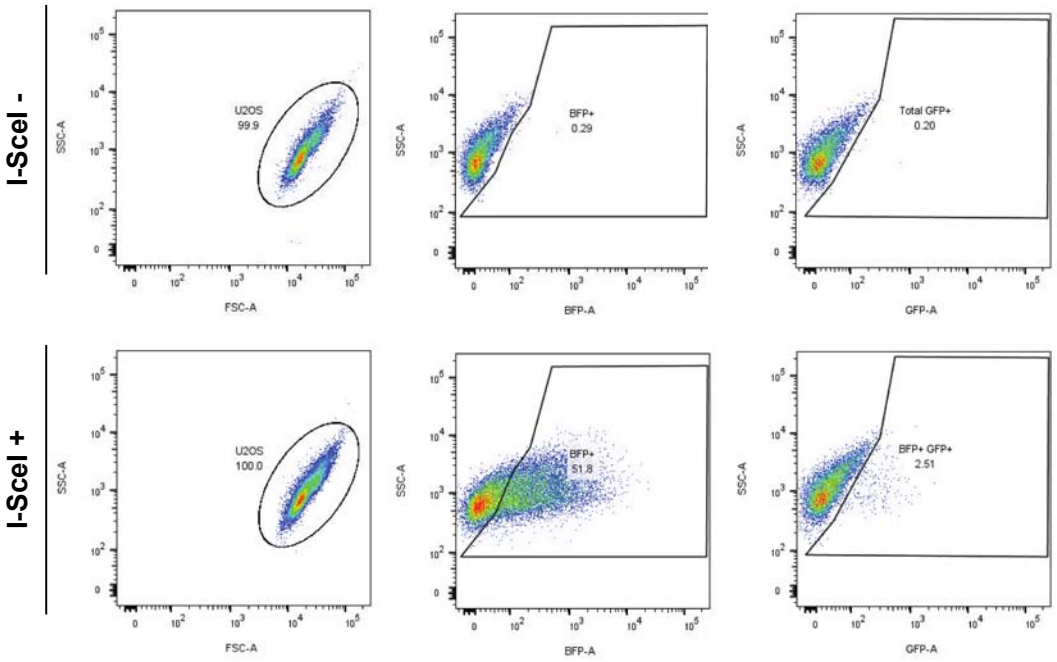
We have performed similar experiments with a p21 KO finding similar results to the ones observed for p53 KO. Results are shown in Fig. EV1X-Z. See our previous answer to the reviewers for details.

Referee #3

1. With respect to point 2 of referee #1:

"I was encouraged to see that the authors did actually use a BFP gating strategy to normalise the HR/repair reporter frequencies, and mitigate non-specific changes due to sample-associated fluctuations in I-SceI transfection efficiency. This gives me more confidence in the assay, but I would want to see a flow cytometry primary data representation as evidence of this gating strategy/normalisation strategy included as a pre-requisite for publication (this is not an unreasonable request)."

We show here an example of the gating we have done for the SceI experiments. If the referee finds it relevant, we are happy to include it as an appendix.



This is an example of the gating strategy for the analysis of I-SceI transduced cells. Cells were transduced with a I-SceI tagged to BFP expressing plasmid (I-SceI +) and only BFP positive cells were selected (middle plot) to then analyze the percentage of them that become GFP positive (Right chart).

2. With respect to point 1 of referee #3:

"Their proposal to address this by monitoring IRIF frequencies, if done specifically in S-phase cells, would satisfy my previous concern. I would stress that S-phase and G2 events are distinct and should be treated as such in this analysis. Pooling S/G2 events (potentially implied by the author response to this query) is likely to skew results owing to the fact that pathway choice reverts somewhat to a pro-NHEJ state in G2, and because by delaying MTOC licensing in late G2 centrinone-treatments will inevitably increase frequencies of G2/early-M phase events, relative to S phase events."

We believe we have covered this concern with the above presented QIBC experiments and by checking the effect on Camptothecin-induced DSBs.

3. With respect to point 3 of referee #3 and the closely related concern of reviewer 1 (point 3):
"The authors aim to "repeating the experiments using automated methods to quantify the number and intensity of the foci." and "reanalyzing our data considering number of foci per cell, intensity of the nuclear signal and intensity of individual foci using a automatic computerized approach." - I think this would be a reasonable and necessary revision plan that if performed to a sufficient standard would address this previous more important concern of mine."

This point has also been covered as presented above.

In addition, the referees have also identified and described in their original reports other limitations and raised concerns that should be addressed, and they have provided a number of suggestions for the improvement of the study and the manuscript. Please note that mechanistic insight will not be required for further consideration of your manuscript, although we agree with the referees that it would be desirable for strengthening the study.

We believe we have covered all the main critics of the initial manuscript with special focus on ruling out a possible impact of cell cycle perturbance in the reported phenotype.

Given these constructive comments, we would like to invite you to revise your manuscript along the lines you have proposed with the understanding that the referee concerns (as detailed above and in their reports) must be fully addressed and their suggestions taken on board. In particular, I would like to stress again that the issue of the cell cycle skew must be sufficiently and convincingly addressed. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript. If you have any questions or comments, we can also discuss the revisions in a video chat, if you like.

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

Dear Fiona,

Thanks a lot for your careful check of our manuscript. I am submitting the documents with the changes you suggested. Regarding that, here is the description of the things we have altered:

1. During a standard image analysis we detected potential aberrations in the figure set, and we would like to clarify these issues before sending your paper back to referees. We kindly invite you to check the composition of Figure EV4 yourself, and to send us the related source data. If you make changes to the figure set, please include a point-by-point describing what you have changed and why.

Please see the attached figure check report for clarification..

Image source data should be provided as one file per figure that contains the original, uncropped and unprocessed scans of all or key gels/microscopy images used in the figure. The file(s) should be labelled with the appropriate figure/panel number, and should display molecular weight markers; further annotation may be useful but is not essential. Source data files will be published online with the article as supplementary "Source Data."

Apologies for the misunderstanding. There was, indeed, some duplications on the images we used as controls. The reason is that, experimentally, we did all the experiments in parallel and some conditions shared controls. That means that, cells treated with Camptothecin and Etoposide, both dissolved in DMSO, share a control cell line exposed only to DMSO and, similarly, irradiated and NCS treated cells also share a control plate. In any case, we realized that this might be misleading. So, as not only we repeated the experiment three times, but each time we used three technical replicates we have altered the figure so in each case a different technical replicate is showed.

2. We only allow up to 5 keywords, they should be below the Abstract.

I have changed the position in the text and reduced to 5 keywords

3. The journal does not permit citation of "Data not shown". All data referred to in the paper should be displayed in the main or Expanded View figures. "Unpublished observations" may be referred to in exceptional cases. (pages 12 and 20).

We have eliminated one of the “Data not shown”. We have kept one as unpublished observations. We prefer to keep it that way as is a relevant point in the discussion and we think it does not make sense to show a completely negative result. This refer to the lack of recruitment of CEP170 to laser line, so the actual image is a cell with a lack of signal. But if required, we can provide an expanded view showing this lack of recruitment. Please, let me know

4. Please add tex callouts to Fig. 1F-H.

Added. Sorry for the mistake, there was a callout stating Figure F-H in which the number was missing.

5. Please add a Table of Contents including page numbers missing to your Appendix. The nomenclature should be Appendix Figure S1-S2.

Added

6. Source data files for Fig. 6 is missing - please check

As mentioned in the Source Data Checklist, we cannot provide it as we downloaded the images directly from GEPIA, similar to Figure 5.

7. The Reference list should be before the Figure Legends.

Changed

8. Add heading 'Expanded View Figure Legends'.

Added as requedted.

Dear Pablo,

Thank you for the submission of your revised manuscript to EMBO reports and for your patience during peer review. We have now received the full set of reports from the three referees that were asked to re-evaluate your study. Please see their detailed comments below.

As you will see, all three referees find the revised version significantly improved and the new data compelling, and they are now positive about the study and the manuscript. They have a few remaining concerns that we need you to address with a minor revision before we can proceed with acceptance of the manuscript. Please address all comments in a detailed point-by-point response, and make sure that all changes in the revised manuscript are highlighted (or "tracked") to be clearly visible.

From the editorial side, there are also a few things that we need from you before we can proceed to acceptance of the manuscript:

- Please correct "Material and Methods" to "Materials and Methods".

- Please update your Data availability statement to: "Our study includes no data deposited in public repositories. Original microscopy images can be found at the BioImage Archive". If there is a BioImage Archive ID or link associated with your microscopy images, please include it in the last sentence of this statement.

- Please correct the heading of your conflict-of-interest statement: it should be "Disclosure and competing interests statement".

- Please remove the remaining "unpublished observations" and show, instead, the negative result of CEP170 recruitment in a new Appendix Figure S#, according to your suggestion. Figure callouts of the Appendix Figures in the main text should be updated, if necessary.

- Please correct the nomenclature of your Appendix Figures in their legends (in the Appendix, under each figure) to Appendix Figure S#.

- Please note that EMBO press papers are accompanied online by:
A) a short (1-2 sentences) summary of the findings and their significance,
B) 2-4 short bullet points highlighting the key results, and
C) a synopsis image that is exactly 550 pixels wide and 300-600 pixels high (the height is variable). You can either show a model or key data in the synopsis image. Please note that the text needs to be readable at the final size.
Please upload this information along with your revised manuscript (the text for A and B should be provided in a separate Word file).

- Your Figure legends have been inspected by our data editors for completeness and accuracy. Please see the required changes in the attached Word file and address all comments in your revised manuscript (please make sure that changes are tracked and/or the comments in the Word file answered).

Please also note that as part of the EMBO publications' Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

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

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

We look forward to seeing a final version of your manuscript as soon as possible.

Best regards,

Ioannis

Ioannis Papaioannou, PhD
Editor
EMBO reports

Referee #1:

I consider that this paper now presents a convincing case for a role for centrioles in modulating homologous recombinational repair of DNA damage, with a key activity residing in the proteins of the subdistal appendages, particularly CEP170. These findings will be of interest in several areas of basic biology- the centrosome, the cell cycle, DNA damage responses- as well as pointing towards some clinically interesting possibilities. I am enthusiastic about this study.

In the revision, the authors have addressed the major concerns of all three reviewers. They have performed controls for the cell cycle impacts of the various treatments which may have provided alternative explanations for the impact of centrosome/ SDA loss on DNA repair activities. The use of numerical data for foci makes these important datasets more convincing. Additional technical controls for the I-SceI experiments have been provided. The manuscript text has undergone significant revision and rewording, which now makes more balanced claims around the interpretation of the data.

1. The absence of a mechanism makes it very important that the CEP170 (SDA) dependency of the phenomenon be completely convincing, as one imagines that this paper will be the basis of a good deal of future work. Thus, despite the very clear correlation of RPA focus formation and CEP170 levels shown in Fig 2E, the CEP170 siRNA rescue experiment is crucial- there is no rescue for the CEP170 heterozygotes, or for the CEP128 nulls, or for the other SDA knockdown experiments. There are some details of this experiment that should be tidied up:
a. The control blot for the critical rescue experiment in Fig 2F, which is shown in Fig. EV2G is not clear (a similar issue applies to EV3F). Which antibodies were used for each panel? If the upper panel is with anti-GFP, why is a signal in the GFP only lanes seen at the size of GFP-CEP170. Also, why is only a single band seen in those cells expressing GFP-CEP170 in the middle panel? One would have hoped to get an idea of the transgene expression levels relative to the endogenous.
b. CEP170 rescue experiments are not shown for RAD51 or BRCA1 foci in Fig. 3. If available, these should be included.
c. The U2OS survival assay shown in Figure 4i measures 'colony area', rather than % survival, which is more typically done (e.g. in the rest of the Figure and in previous work from the authors, such as in the cited reference by Cruz-Garcia et al.). These data should be presented in a format that maps to previous work and to the rest of the Figure.

2. I remain unconvinced that the Kaplan-Meier data merit inclusion in the main body of the paper. (I note that Referee #3 also notes that these data are open to other interpretations).
a. I continue to think that these data should be moved to a supplemental figure, as they potentially dilute the main story. My reasoning is that, at present, these are highly selective data and, while potentially interesting, there is not enough depth here to present a convincing clinical aspect to this study.
b. I reiterate my previous comment that there is no analysis of the treatment that the patients received, so that the observation that improved survival of patients with lower levels of CEP170 is 'likely because of a heightened sensitivity to treatment' is not appropriate. This sentence should be rephrased to emphasize that this is a speculation that is consistent with the authors' model.
c. As an aside, did the authors find similar sensitivity profiles to high/ low levels of other SDA components? (This analysis was mentioned in the rebuttal letter and could be mentioned, if the outcome is consistent with the models advanced in the paper)

Minor points
3. There are some areas where the clarity of the phrasing could be improved (e.g., line 35 'albeit it does not control '; line 179 'infection efficiency for all conditions were monitored '; line 219 'We wonder if centrosome lost itself could induce '; line 390 'plays a critical role on the centriole '; line 402 'is an evolutionary conserved site '; line 413 'overexpression of the protein rendered the accumulation in additional centrosomal locations' etc.)- the MS. would be improved by a thorough check for such issues.

4. The cells examined should be specified in the legends to Figs. 2 and 3.

Referee #2:

The revision of the manuscript has strengthened the link between centrosomes, particularly subdistal appendages, and homologous recombination. The authors did thorough work in addressing my criticism relating to this point. It remains mysterious how this interaction works mechanistically, and it is a pity that the authors had no hint to offer. This should, however, not preclude publication at this stage.
The new sentence "Notably, extended centrinone treatment has been reported to prolong mitosis through a 53BP1-USP28-

mediated activation of p53 and p21, which eventually blocks the cell cycle in G1 (Meitinger et al, 2016; Wong et al, 2015; Fong et al, 2016; Lambrus et al, 2016), line 252, is wrong.
Prolonged mitosis activates 53BP1-USP28 and not the opposite. To this end, the authors could also consider citing the latest preprint by Meitinger and colleagues, which sheds important new light on this issue (https://doi.org/10.1101/2022.11.14.515741).

Referee #3:

The authors have clearly gone a long way to tackle the prior issues regarding the potentially confounding issue of centrinone-dependent cell-cycle skews indirectly altering DSBR pathway choice balance (a unifying critique of all referees). In particular Figs 1F-H further support the author's proposition of a centrosome-repair interplay and the camptothecin data lends further credence to this notion. I also find the interpretations to be more nuanced and concede that some of the more daring propositions can fine for a discussion section. s such, this paper raises the interesting possibility of a centrosome-DDR crosstalk as is (in my opinion) now ready for publication in my opinion

Final comments:
In the new section (lines 265-267) they state: "We obtained similar results to the ones observed in U2OS andRPE-1 cells with all cell lines (Figure EV1R-Z), supporting the idea that the effect of centriolar loss on HR is independent of an accumulation in G1 or mitosis" - this is not very helpful since they do not state/specify the key effects that are replicated in these new cells lines. The needs updating as a reader should not have to do detective work to understand the nature of experiments being referred to.

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Rev_Com_number: RC-2022-01727
New_manu_number: EMBOR-2022-56724V3
Corr_author: Huertas
Title: Centriolar subdistal appendages promote double strand break repair through homologous recombination

Dear Dr. Papaioannou,

We would like you to thank you for the opportunity to submit our revised manuscript "**Centriolar subdistal appendages promote double strand break repair through homologous recombination"** for consideration in EMBO Reports.

We want to thank all three referees and the editor for their constructive criticisms to our work. Also, the thorough revision by the data editor. Find below a point-by-point response to the editorial concerns and reviewer's comments.

Editorial comments:

- Please correct "Material and Methods" to "Materials and Methods".

Done

- Please update your Data availability statement to: "Our study includes no data deposited in public repositories. Original microscopy images can be found at the BioImage Archive". If there is a BioImage Archive ID or link associated with your microscopy images, please include it in the last sentence of this statement.

Changed as suggested.

- Please correct the heading of your conflict-of-interest statement: it should be "Disclosure and competing interests statement".

Corrected

- Please remove the remaining "unpublished observations" and show, instead, the negative result of CEP170 recruitment in a new Appendix Figure S#, according to your suggestion. Figure callouts of the Appendix Figures in the main text should be updated, if necessary.

Added as EV3 panel A. EV callouts have been updated accordingly.

- Please correct the nomenclature of your Appendix Figures in their legends (in the Appendix, under each figure) to Appendix Figure S#.

Changed as requested.

- Please note that EMBO press papers are accompanied online by:
A) a short (1-2 sentences) summary of the findings and their significance
B) 2-4 short bullet points highlighting the key results, and
C) a synopsis image that is exactly 550 pixels wide and 300-600 pixels high (the height is variable). You can either show a model or key data in the synopsis image. Please note that the text needs to be readable at the final size.
Please upload this information along with your revised manuscript (the text for A and B should be provided in a separate Word file).

Done. The text is uploaded as a separate file named additional text.

- Your Figure legends have been inspected by our data editors for completeness and accuracy. Please see the required changes in the attached Word file and address all comments in your revised manuscript (please make sure that changes are tracked and/or the comments in the Word file answered).

All the issues raised have been clarified and changed in the text. Changes throughout the text have been highlighted.

Regarding the additional text required:
A) a short (1-2 sentences) summary of the findings and their significance,

CEP170 promotes double strand break repair by homologous recombination from the subdistal appendages. Therefore, CEP170 presence modulates the sensitivity to DNA damaging agents.

B) 2-4 short bullet points highlighting the key results, and

- Centrioles are required for fully proficient double strand break repair by homologous recombination.
- Subdistal appendage proteins promote DNA end resection from the centriole, mostly through the presence of CEP170 at this structure.
- CEP170 phosphorylation at Ser 637 is required for efficient homologous recombination DNA repair.
- Centrioles and CEP170 are required for cell survival upon exposure to agents that cause DNA double strand breaks.

Referee #1:

I consider that this paper now presents a convincing case for a role for centrioles in modulating homologous recombinational repair of DNA damage, with a key activity residing in the proteins of the subdistal appendages, particularly CEP170. These findings will be of interest in several areas of basic biology- the centrosome, the cell cycle, DNA damage responses- as well as pointing towards some clinically interesting possibilities. I am enthusiastic about this study.

In the revision, the authors have addressed the major concerns of all three reviewers. They have performed controls for the cell cycle impacts of the various treatments which may have provided alternative explanations for the impact of centrosome/ SDA loss on DNA repair activities. The use of numerical data for foci makes these important datasets more convincing. Additional technical controls for the I-SceI experiments have been provided. The manuscript text has undergone significant revision and rewording, which now makes more balanced claims around the interpretation of the data.

We would like to thank the reviewer for his/her enthusiasm and for supporting our manuscript.

1. The absence of a mechanism makes it very important that the CEP170 (SDA) dependency of the phenomenon be completely convincing, as one imagines that this paper will be the basis of a good deal of future work. Thus, despite the very clear correlation of RPA focus formation and CEP170 levels shown in Fig 2E, the CEP170 siRNA rescue experiment is crucial- there is no rescue for the CEP170 heterozygotes, or for the CEP128 nulls, or for the other SDA knockdown experiments. There are some details of this experiment that should be tidied up:

We understand the reviewer's points. However, it is worth noticing that we have observed similar results were obtained both with heterozygotes and siRNA depletion. In this later case, key experiments have been done with expression of an ectopic version of the wildtype protein to rescue the phenotype. Chiefly both RPA and survival have been complemented. So, upon discussion with the editor, we have agreed not to perform additional experiments. This also apply to the CEP128 KO, that incidentally was a gift from Dr. Pedersen and was checked by them. This information was lacking in our manuscript. For that, we apologize and also thanks the reviewer for helping us correcting this oversight.

a. The control blot for the critical rescue experiment in Fig 2F, which is shown in Fig. EV2G is not clear (a similar issue applies to EV3F). Which antibodies were used for each panel? If the upper panel is with anti-GFP, why is a signal in the GFP only lanes seen at the size of GFP-CEP170. Also, why is only a single band seen in those cells expressing GFP-CEP170 in the middle panel? One would have hoped to get an idea of the transgene expression levels relative to the endogenous.

Thanks for bringing us this o our attention. Indeed, in the GFP only we see a faint unspecific band at the same size that the GFP-CEP17. Also, using the GFP antibody, and due to the high molecular weight of the protein, we are not able to resolve the endogenous and GFP-labelled version of CEP170 (see a thicker band when GFP-170 is expressed). This calrification has been noted in the legend of the figure.

b. CEP170 rescue experiments are not shown for RAD51 or BRCA1 foci in Fig. 3. If available, these should be included.

As discussed with the editor, we are not including further experiments.

c. The U2OS survival assay shown in Figure 4i measures 'colony area', rather than % survival, which is more typically done (e.g. in the rest of the Figure and in previous work from the authors, such as in the cited reference by Cruz-Garcia et al.). These data should be presented in a format that maps to previous

work and to the rest of the Figure.

We understand the referee point. Both methods can be used to measure survival, but there are nuances on what they mean. In this case, we have recently moved to measure area coverage as a way to automate the process and avoid any possible subjective bias. This is way this new data uses this method. In any case, as both measure survival and we are note comparing results between panels A-H with panle I, but comparing in this panel I between expression of different versions of the protein, we think we can claim our interpretation. In any case, the difference in the methods is clearly stated in the text to avoid misinterpretations.

2. I remain unconvinced that the Kaplan-Meier data merit inclusion in the main body of the paper. (I note that Referee #3 also notes that these data are open to other interpretations).
a. I continue to think that these data should be moved to a supplemental figure, as they potentially dilute the main story. My reasoning is that, at present, these are highly selective data and, while potentially interesting, there is not enough depth here to present a convincing clinical aspect to this study.

We have moved this data to a supplementary figure as suggested.

b. I reiterate my previous comment that there is no analysis of the treatment that the patients received, so that the observation that improved survival of patients with lower levels of CEP170 is 'likely because of a heightened sensitivity to treatment' is not appropriate. This sentence should be rephrased to emphasize that this is a speculation that is consistent with the authors' model.

The referee is right. We have rephrased the text as suggested.

c. As an aside, did the authors find similar sensitivity profiles to high/ low levels of other SDA components? (This analysis was mentioned in the rebuttal letter and could be mentioned, if the outcome is consistent with the models advanced in the paper)

We have not tested this idea. It is something that we aim to keep studying. In the rebuttal we mentioned that we have analyzed the mutational signature of all the SDA components.

Minor points
3. There are some areas where the clarity of the phrasing could be improved (e.g., line 35 'albeit it does not control '; line 179 'infection efficiency for all conditions were monitored '; line 219 'We wonder if centrosome lost itself could induce '; line 390 'plays a critical role on the centriole '; line 402 'is an evolutionary conserved site '; line 413 'overexpression of the protein rendered the accumulation in additional centrosomal locations' etc.)- the MS. would be improved by a thorough check for such issues.

We have checked the text. Thanks for pointing out these issues.

4. The cells examined should be specified in the legends to Figs. 2 and 3.

This information has been included in the legends, Thanks for pointing out this oversight.

Referee #2:

The revision of the manuscript has strengthened the link between centrosomes, particularly subdistal appendages, and homologous recombination. The authors did thorough work in addressing my criticism relating to this point. It remains mysterious how this interaction works mechanistically, and it is a pity that the authors had no hint to offer. This should, however, not preclude publication at this stage.

We completely agree with the reviewer sentiment, and specially thanks that even if the mechanism is still undiscovery he/she is supporting publication at this stage.

The new sentence "Notably, extended centrinone treatment has been reported to prolong mitosis through a 53BP1-USP28-mediated activation of p53 and p21, which eventually blocks the cell cycle in G1 (Meitinger et al, 2016; Wong et al, 2015; Fong et al, 2016; Lambrus et al, 2016), line 252, is wrong. Prolonged mitosis activates 53BP1-USP28 and not the opposite. To this end, the authors could also consider citing the latest preprint by Meitinger and colleagues, which sheds important new light on this issue (https://doi.org/10.1101/2022.11.14.515741).

Thanks for pointing out this issue. We have corrected the sentence and included the reference as suggested.

Referee #3:

The authors have clearly gone a long way to tackle the prior issues regarding the potentially confounding issue of centrinone-dependent cell-cycle skews indirectly altering DSBR pathway choice balance (a unifying critique of all referees). In particular Figs 1F-H further support the author's proposition of a centrosome-repair interplay and the camptothecin data lends further credence to this notion. I also find the interpretations to be more nuanced and concede that some of the more daring propositions can fine for a discussion section. s such, this paper raises the interesting possibility of a centrosome-DDR crosstalk as is (in my opinion) now ready for publication in my opinion

We want to thank this referee for his/her positive outlook on our revised manuscript.

Final comments:
In the new section (lines 265-267) they state: "We obtained similar results to the ones observed in U2OS andRPE-1 cells with all cell lines (Figure EV1R-Z), supporting the idea that the effect of centriolar loss on HR is independent of an accumulation in G1 or mitosis" - this is not very helpful since they do not state/specify the key effects that are replicated in these new cells lines. The needs updating as a reader should not have to do detective work to understand the nature of experiments being referred to.

We have clarified this point as suggested.

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- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
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Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
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