EMBO reports

Manuscript EMBO-2016-42352

Loss of CHD1 causes DNA repair defects and enhances prostate cancer therapeutic responsiveness

Vijayalakshmi Kari, Wael Mansour, Sanjay Raul, Simon Baumgart, Andreas Mund, Marian Grade, Hüseyin Sirma, Ronald Simon, Hans Will, Matthias Dobbelstein, Ekkehard Dikomey, and Steven Johnsen

Corresponding author: Steven Johnsen, University Medical Center Goettingen

Review timeline:

Submission date:	09 March 2016
Editorial Decision:	23 March 2016
Revision received:	17 June 2016
Editorial Decision:	14 July 2016
Revision received:	29 July 2016
Editorial Decision:	04 August 2016
Revision received:	07 August 2016
Accepted:	11 August 2016

Editor: Esther Schnapp / Achim Breiling

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 23 March 2016

Thank you for the transfer of your manuscript to our journal. We have now received the comments from the referees, as well as referee cross-comments, which are pasted below.

As you will see, all referees acknowledge that the findings are interesting and novel and of potential therapeutic relevance. However, they also suggest several experiments to strengthen the study. While we think that all suggestions are useful, they do not all have to be addressed. As referee 3 points out in her/his cross-comments below, while the data on CtIP recruitment should be improved, this does not necessarily have to be done by laser-microirradiation experiments for CtIP. Points 9 and 16 by referee 2 and confirming the main results in non-transformed cells (referee 3) do also not have to be addressed. Instead, point 23 by referee 2 is an interesting suggestion, I think, and if you feel this is doable, this experiment should be performed. Also, the recruitment of CHD1 to DSB and the resection phenotype need to be better characterized, a second shRNA targeting CHD1 should be used, all missing controls, information, quantifications and statistical analyses must be added, and the discussion should be improved. As referee 1 points out, the splicing of gel bands must be clearly indicated and source data (see below) of uncropped gels should be provided.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the

manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of 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.

Regarding data quantification, please specify the number "n" for how many experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. This information is currently incomplete and must be provided in the figure legends. Please also include scale bars in all microscopy images.

We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure or per figure panel.

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

REFEREE REPORTS

Referee #1:

In the manuscript 'Loss of CHD1 confers DNA repair defects and alters prostate cancer therapeutic responsiveness' Kari et al. investigate the role of CHD1 in the DNA damage response. From their experiments the authors conclude that CHD1 plays a role in the repair of DNA double strand breaks (DSBs) by homologous recombination (HR). In their model, CHD1 helps to open up chromatin around sites of DNA damage, promoting the early DNA end resection step of HR through facilitating the recruitment of CtIP. This model is consistent with the strong hypersensitivity to olaparib that the authors observe for CHD1-depleted cells when using olaparib as a stand-alone agent or in combination with ionizing radiation. Strikingly, CHD1 is mutated or deleted in a high percentage of prostate cancers. Thus, the results of this study could in the future contribute to the stratification of prostate cancer patients based on the cancer's CHD1 status.

The olaparib data in this study are strong and convincing. Moreover, they are in line with the role that the authors are proposing for CHD1 in DSB repair, which is novel and interesting to a broad readership. However, the experimental evidence to support the authors' conclusions regarding the mechanistic role of CHD1 in HR are far from sufficient. Therefore, I cannot support this manuscript for publication in EMBO Reports at this stage.

The authors would have to thoroughly address the following major points to strengthen their hypothesis:

1. Recruitment of CHD1 to sites of DNA damage: the pan-nuclear staining of CHD1 (see especially Fig. S1A) makes it impossible to judge whether CHD1 is actually recruited to DNA damage foci. This also holds true for the PLAs in Figure 1D. Due to CHD1's global presence along chromatin, CHD1 is expected to show a positive signal at gammaH2AX-positive sites, even if it is not specifically recruited to sites of DNA damage. Moreover, the representative image used to show recruitment of CHD1 to I-SceI-induced DSBs marked by GFP-lacR (Fig. 1B), is not convincing: it appears that doxycycline-mediated induction of I-SceI expression results in generation of the high background levels of DNA damage as the nucleus is full of gammaH2AX staining. I-SceI should specifically induce a DSB at the recognition site. These issues could and should potentially be resolved by performing laser micro-irradiation studies for CHD1. The latter assay would be more sensitive and might allow the authors to unequivocally detect CHD1 accumulation at sites of DNA damage even over a high pan-nuclear CHD1 background.

2. Delayed DNA damage repair in CHD1-depleted cells: the effects on gammaH2AX appear rather minor in the IF analyses (Fig. 2A-C), but fairly strong by Western blot (Fig. 2C). It is usually the other way around due to the higher sensitivity of the IF over immunoblot assays. Can the authors explain this discrepancy? Also, it seems that the authors base their conclusions on one mix of siRNA oligos targeting CHD1. It is essential to deconvolute this mix and demonstrate that independent siRNAs lead to the same effects in various assays that are key to this study e.g. HR assay, DNA repair defects etc.

3. General recruitment of CHD1 to chromatin in response to DNA damage: the chromatin fractionation studies in Figures 1E, S1D and S1E support the idea of CHD1 accumulation on chromatin following DSB induction. However, in Figure 2C, the levels of CHD1 seem to increase after treatment with NCS, especially at the 24 h time point. Thus the question is whether CHD1 accumulation on chromatin following DNA damage treatment is due to its specific recruitment or merely a consequence of increased expression of CHD1 after DNA damage?

4. HR/NHEJ assays: the dynamic range is very low for the assays (plus the comprehensive labeling of the y axes is missing in Figures 3A-B). Did the authors normalize for I-SceI transfection efficiencies? In addition, when describing the kinetics of RAD51 focus formation and disappearance (Figure 3D), the authors state that the reduced number of RAD51 foci in CHD1-depleted cells at the 3h time point strongly suggests the role for CHD1 in the early stage of HR, namely DNA-end resection. To confirm that this is indeed the case, the authors should perform assays that would directly assess the extent of DNA-end resection in CHD1-depleted cells. The most convincing would be either high-throughput IF- or FACS-based assays to quantify RPA or BrDU signal in gammaH2AX-positive/cyclin A-positive cells following DNA damage induction. The data on RPA reduction in Figure 4D is not sufficient both due to the nature of the LacR assay and due to the problems with these particular data mentioned below (see point 5). With regard to Figure 3D, it is also striking that 24h after damage, RAD51 foci persist in CHD1-depleted cells. This could mean that CHD1 plays a role at the later stage of HR. Such observations deserve more attention.

5. CtIP recruitment: the PLA in Figure 4A is not conclusive due to the pan-nuclear and chromatin-bound nature of CHD1 and also due to the known difficulties in visualizing CtIP foci after IR. To resolve this ambiguity, the authors would have to perform laser-microirradiation studies and check for CtIP recruitment specifically in S/G2-phase cells, as CtIP is not recruited to DSBs in G1 phase. In this regard, it is surprising that the authors detect CtIP-positive foci in 100% of the control cells in Figure 4C. The same goes to RPA foci in Figure 4D. One would expect the percentage to correlate with the percentage of cells in S/G2 phase. These are important issues that the authors need to address.

6. CHD1 complementation: The assay in Figure 4E is confusing. What are the pan-nuclear signals? This is difficult to explain, as gammaH2AX would be expected to show foci and not pan-nuclear staining, in all conditions tested.

7. The role for MRE11 in CHD1 recruitment: the results in Figure 5A are surprising and the blot is not convincing overall. Why do the CHD1 and CtIP signals go down at 4 and 6 hours in the mirin-treated cells while the signals are really high at 2h? To test if there are any MRE11-dependent effects, this experiment should also be performed with siRNAs targeting MRE11. Moreover, it looks like the membranes for gammaH2AX are spliced together for the three 4-time-point conditions. This should be clearly indicated.

Referee #2:

This study by Kari et al seeks to address the role of CHD1 in DNA double strand breaks in prostate cancer cells. The paper provide direct evidence that CHD1 is recruited to DSBs and is necessary for end resection, its depletion in combination with DSBs subsequently leads to reduced CtIP and RPA recruitment to DSBs and reduced RAD51 foci and sustained yH2AX levels. Thus CHD1 is important for HR but not NHEJ, and prostate cancer cells depleted of CHD1 show increased sensitivity to PARP inhibition. Thus, the authors show that they have identified a synthetic lethal relationship between CHD1-deficiency and PARP inhibition. In light of the usage of PARP inhibitors in treatment of castration defective prostate cancers these data are extremely important as

it could indicate that the CHD1 mutated cancers respond to PARP inhibitors.

It has previously been shown by Boulton et al 2009, Science, that the CHD1L (also called ALC1) that shows substantial sequence similarity to CHD1 is targeted to sites of DNA damage through interaction with poly(ADP-ribose) and functions to regulate chromatin during DNA repair. It is able to catalyze nucleosome sliding in an ATP-dependent manner and its helicase activity is strongly stimulated upon poly(ADP-ribose)-binding. Thus the findings the authors provide that CHD1 has a similar role as CHD1L in HR is not totally surprising. Yet the authors do not discuss their findings in relation to this reference or investigate the potential overlapping roles of CHD1L and CHD1 in PARP dependent recruitment of factors for chromatin relaxation in HR.

The majority of the experiments in the paper are done using shCDH1 but the authors seem to have used only one hairpin targeting CDH1, the authors should perform the same experiments with at least one more shRNA targeting CHD1 to exclude that the results are not due to unspecific off-target effects by the hairpins. For the siRNA, four different siRNA sequences are shown in the supplementary table but only one seems to have been used in the figures. The authors should specify which one of the four CDH1 siRNAs that has been used the figures, or if a mix of the siRNAs have been used, as well as show the effects on the individual siRNAs on CDH1 protein and mRNA level. The authors should repeat some experiments using 2 different siRNAs targeting CDH1 to avoid that results are due to unspecific off-target effects by the siRNAs. In many cases there is also a lack of information regarding how many times experiments have been repeated, sometimes representative confocal images of one nuclei per treatment is shown without any quantifications, and it is therefore unclear if some of the data is reproducible.

The authors should discuss the potential overlapping roles of CHD1L and CHD1 in PARP dependent recruitment of factors for chromatin relaxation in HR.
The recruitment of CHD1 to strand breaks should be characterized in more detail, e.g. look at kinetics for CHD1 recruitment to breaks as well as colocalization with yH2AX upon micro-irradiation. The impairment of the DNA damage response induced by CHD1-depletion upon should be investigated in more detail (e.g. look checkpoint activation and cell cycle progression).

Specific comments
1) Figure 1 B, C. The authors claim that CHD1 localizes to DNA double strand breaks and colocalizes with yH2AX, quantification is shown in C. However, the number of cells used per condition for the quantification in C) is missing.

2) Figure 1 D: It would be helpful for the readers if the authors could quantify the co-localization if yH2AX/CHD1 in Edu positive and negative cells as it is hard to draw conclusions from a set of representative images.

3) Figure 1 E, S2A-C, S3D-I, S4D-J and S5B: For the siRNA, four different siRNA sequences are shown in the supplementary table but only one seems to have been used in the figures. The authors should specify which one of the four CDH1 siRNAs that has been used the figures, or if a mix of the siRNAs have been used, as well as show the effects on the individual siRNAs on CDH1 protein and mRNA level. The authors should repeat the experiments using 2 different siRNAs targeting CDH1 to avoid that the results are due to unspecific off-target effects by the siRNAs.

4) Figure 1 E: It seems that the label for the treatment has disappeared, I assume the number indicate release after NCS treatment as in figure S1D, E?

5) Figure S1 A: Reference to this figure is missing in the main text. The authors claim that CHD1 and yH2AX interact using the PLA assay. It would be helpful for the readers if the authors could quantify the interaction of yH2AX/CHD1 in NCS treated and non-treated cells as it is hard to draw conclusions regarding interaction upon DSB-induction from a representative image of one nuclei per treatment.

6) Figure S1 C: Similar to Figure S1 A, please quantify the interaction detected in the PLA assays and provide information on how many cells that were quantified per condition.

7) Figure 2A: The text in the results sections states that the irradiation was 2 Gy but the figure

legend states 3 Gy, which dose was used?

8) Figure 2C: The authors should complement the NCS treatment to look at prolonged yH2AX signaling on western blot with irradiation, as done in the rest of the panels in the figure which investigate sensitivity to ionizing radiation upon CDH1 depletion.

9) In Figure 2, the authors show prolonged yH2AX activation upon CHD1 depletion in combination with irradiation, the author should complement this data with investigating checkpoint signaling and cell cycle progression.

10) Figure 2D: P-values are missing for the colony formation assays.

11) Figure 4A: It would be helpful for the readers if the authors could quantify the interaction between CtIP and CDH1 in the PLA assay with and without induction of DSBs with NCS to be able to compare the effects on DSBs on the interaction.

12) Figure 4B and S3A and B: The authors claim that CtIP does not increase over time in CHD1 depleted cells, however it is absent at early (2h) timepoint but increase over time in Fig. 4B (PC3 cells). The authors should acknowledge the difference in CtIP recruitment to chromatin between cell lines (VcAP S3A, U-2 OS S3B and PC3 Fig4B).

13) Figure 4C and D showed be moved to be under panel A and B and panel E should be where panel C and D currently are.

14) It is unclear if the quantification in Figure 4 is from the chromatin fractionations in S3H?

15) Figure 4E: The authors claim a decreased PLA signal in panel E upon CDH1 depletion and this can be rescued by WT but not mutant CDH1, as this is difficult to interpret from the images the authors should include quantifications of the PLA signals in the figure. Also, this panel is without NCS treatment/induction of DSBs and the authors should investigate if CDH1 depletion also affect DSB-induced interaction between yH2AX and CHD1.

16) Figure 5A: The authors show that inhibition of PARP does not affect CHD1 recruitment to chromatin upon induction of DSBs. To further characterize the signaling pathways and show that the CHD1 is involved in HR pathway and not NHEJ the authors should compare CHD1 recruitment upon ATM, ATR and DNA-PK inhibition in combination with NCS treatment.

17) Figure 5B: The authors claim that the yH2AX/CHD1 PLA signal is not affected by CtIP depletion, the authors should include quantifications of the PLA signals in the figure as a complement to the representative images.

18) Figure S2B,C: P-values are missing for the colony formation assays.

19) Figure 5C: Please also clearly mark which bars are siCDH1.

20) Figure 5D showed be moved out as a separate figure as it at the moment is not referred to in the result section and is described after figure 6.

21) Figure 6A, 6C 6D ands S5A: P-values are missing for the colony formation assays. The authors should also acknowledge the difference in response between the two cell lines.

22) Reference to figure 6F is missing.

23) Although the survival data in figure 2D is not impressive, the survival data when combining shCDH1 with Olaparib in figure 6C and D are striking. As PARP is known to As the authors connect the decrease in chromatin opening upon CHD1 depletion with the decreased HR activity and cell survival upon siCHD1 it would be most interesting if the authors could investigate how combination of siCHD1 and Olaparib affects chromatin opening at DSB sites employing the FAIRE assay.

Referee #3:

This manuscript from Kari et al deals with a new role of the chromatin remodeller CHD1 in homology-directed recombination (HDR). The authors find that CHD1 is recruited to DNA double strand break sites, and that CHD1 depletion decreases the recruitment of the key DNA end resection protein CtIP to chromatin. Hence, CHD1 depleted cells display impaired end resection during DNA double strand break repair and HDR is reduced. CHD1 is frequently deleted in prostate cancer, correlating with poor prognosis, and the authors now find that PARP1 inhibition eradicates CHD1-depleted cells. PARP1 inhibition is efficient in eliminating HDR deficient cancers, thus, the authors identify a potential therapeutic possibility for targeting CHD1-deleted tumours through PARP1i.

Major comments:
This is clearly a work of interest to several fields due to the identification of a new role of CHD1 in HDR that links chromatin remodeling, DNA repair and cancer. The manuscript is well written, and experiments are generally well executed and controlled.

Minor comments:
*The resection phenotype as detected by the RPA staining is not very apparent (4D), yet, it is a key point in this study. The authors should address this aspect in more detail by a more complete ssDNA analysis (RPA staining and non-denatured BrdU staining after high-dose IR, such as 10 Gy 2h).
*To generalise the findings, normal (non-transformed cell lines) should be assayed for CHD1 impact on DNA end resection.
*A recent publication identified SRCAP as an important chromatin remodeller in CtIP driven resection, this should be discussed and referenced, PMID: 25176633 .
*The impact of CHD1 status on yH2AX levels is modest and yH2AX is an indirect damage marker. The authors should detect actual DNA damage after CHD1 depletion by a more direct approach (Neutral comet or PFGE).

Cross-comments from Referee 3:

In general, the reviewers comments are of course highly relevant and useful, their inclusion will clearly lead to a much improved manuscript. However, I do find that a few of the points/suggestions are too demanding relative to the scope of the manuscript. In particular:

*Rev #1, Point 5: The laser-microirradiation experiments for CtIP are very demanding and far from straightforward (the temporal CtIP recruitment pattern is complex, laser irradiation is not equivalent to IR/NCS treatment, CtIP expression levels affect its behaviour). However, I do agree that the manuscript will be strengthened by improved analysis of CtIP recruitment. Perhaps the authors can improve the quality of data with their current approach, including analysis of CtIP foci formation upon IR/NCS in different cell cycle phases (based on cell cycle markers or synchronization of cells).

*Rev#2, Point 9: The scope of the manuscript is clearly on DNA repair aspects, I really find the checkpoint aspect of minor importance.

*Rev #2, Point 16: Although the potential kinase mediated regulation of CHD1 recruitment is of interest and relevance, I also do not find this of major importance. This line of research would require a much more dedicated analysis with kinase targeting siRNA and perhaps CHD1 mutants to really improve the manus significantly.

*Rev #2, Point 23: In the context of the manuscript, this is not an important point.

1st Revision - authors' response 17 June 2016

We kindly thank you and the reviewers for your enthusiasm for our manuscript and your helpful comments and suggestions. In the revised manuscript we have sought to address each of the reviewer's concerns to the best of our ability. Below you will find a point-to-point response to each critique.

I hope that you will find this revised version of the manuscript suitable for publication. If you have any questions or require any additional information, please feel free to contact me again.

We thank the reviewer for pointing out the oversight of having left of the labeling. We have now included the labeling in the y-axis. Indeed, all HR and NHEJ assays were normalized to the transfection efficiency of I-SceI vector.

In addition, when describing the kinetics of RAD51 focus formation and disappearance (Figure 3D), the authors state that the reduced number of RAD51 foci in CHD1-depleted cells at the 3h time point strongly suggests the role for CHD1 in the early stage of HR, namely DNA-end resection. To confirm that this is indeed the case, the authors should perform assays that would directly assess the extent of DNA-end resection in CHD1-depleted cells. The most convincing would be either high-throughput IF- or FACS-based assays to quantify RPA or BrDU signal in gammaH2AX-positive/cyclin A-positive cells following DNA damage induction. The data on RPA reduction in Figure 4D is not sufficient both due to the nature of the LacR assay and due to the problems with these particular data mentioned below (see point 5). With regard to Figure 3D, it is also striking that 24h after damage, RAD51 foci persist in CHD1-depleted cells. This could mean that CHD1 plays a role at the later stage of HR. Such observations deserve more attention.

In order to address the reviewer's concern regarding the CHD1 role in DNA-end resection process we performed the native BrdU assay in control and siCHD1 transfected cells 2 h after NCS treatment. Our results show that depletion of CHD1 in PC3 cells results in decreased DNA-end resection as further indicated by decreased BrdU staining after NCS treatment (Fig. 5E and EV5J). To further assess the role of CHD1 in RPA1 recruitment we performed IF studies for RPA1 and CenpF (an established marker for S/G2) with PC3 cells depleted with siCHD1 (four individual siRNAs). As now shown in Fig. EV4J-K, CHD1 depletion reduced the RPA1 foci formation more specifically in S/G2-phase of cells, further confirming a role for CHD1 in HR-mediated DSB repair. Depletion of CtIP was used as a positive control.

5. CtIP recruitment: the PLA in Figure 4A is not conclusive due to the pan-nuclear and chromatin-bound nature of CHD1 and also due to the known difficulties in visualizing CtIP foci after IR. To resolve this ambiguity, the authors would have to perform laser-microirradiation studies and check for CtIP recruitment specifically in S/G2-phase cells, as CtIP is not recruited to DSBs in G1 phase.

Cross-comment from Referee 3:

**Rev #1, Point 5: The laser-microirradiation experiments for CtIP are very demanding and far from straightforward (the temporal CtIP recruitment pattern is complex, laser irradiation is not equivalent to IR/NCS treatment, CtIP expression levels affect its behaviour). However, I do agree that the manuscript will be strengthened by improved analysis of CtIP recruitment. Perhaps the authors can improve the quality of data with their current approach, including analysis of CtIP foci formation upon IR/NCS in different cell cycle phases (based on cell cycle markers or synchronization of cells).*

In order to address the phase specific recruitment of CtIP and its regulation by CHD1, we performed immunofluorescence analyses for CtIP and CenpF 2 h after irradiation in cells transfected with CHD1 depletion using siRNA (four individual siRNAs) in PC3 cells. The results clearly indicate that CHD1 depletion decreased CtIP focus formation specifically in S/G2-phase cells. This is shown in Fig. EV4H-I.

In this regard, it is surprising that the authors detect CtIP-positive foci in 100% of the control cells in Figure 4C. The same goes to RPA foci in Figure 4D. One would expect the percentage to correlate with the percentage of cells in S/G2 phase. These are important issues that the authors need to address.

In Fig.4C and 4D, the "100%" refers to the normalization to cells which show the CtIP or RPA1 foci in the control condition. To avoid confusion, in the revision we have represented the cells positive for CtIP or RPA1 foci according to the actual percentage of cells in the total cell population.

6. CHD1 complementation: The assay in Figure 4E is confusing. What are the pan-nuclear signals? This is difficult to explain, as gammaH2AX would be expected to show foci and not pan-nuclear staining, in all conditions tested.

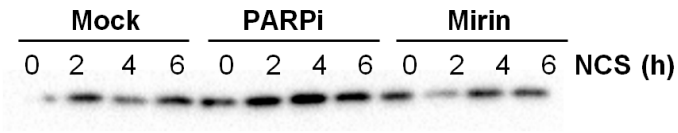
Indeed, as the reviewer rightly notes, we indeed observe a pan-nuclear PLA signal in all the cells. The data represented in Fig. 4E are PLA analyses examining the interaction of γH2AX and CtIP. While we do not fully understand this phenomenon, we note that it only occurs in the absence of CHD1 and can be fully rescued to a punctate staining pattern with the re-expression of full-length mChd1, but not an ATPase-defective mChd1 mutant. Thus, while there appears to be some unexplained effects of the effects of CHD1 depletion on the PLA staining pattern of γH2AX/CtIP, we feel that the quantitative data provide support to our hypothesis that CHD1 function in HR-mediated DSB repair relies on its ATPase activity.

7. The role for MRE11 in CHD1 recruitment: the results in Figure 5A are surprising and the blot is not convincing overall. Why do the CHD1 and CtIP signals go down at 4 and 6 hours in the mirin-treated cells while the signals are really high at 2h? To test if there are any MRE11-dependent effects, this experiment should also be performed with siRNAs targeting MRE11. Moreover, it looks like the membranes for gammaH2AX are spliced together for the three 4-time-point conditions. This should be clearly indicated.

To further confirm the role of MRE11 in CHD1 recruitment, we depleted MRE11 in PC3 cells with siRNA and performed chromatin fractionation studies at different time points following NCS treatment. Our data clearly indicate that MRE11 depletion decreased the recruitment of CHD1 and CtIP, suggesting a role for CHD1 downstream of MRE11 (Fig. 5A).

Moreover, it looks like the membranes for gammaH2AX are spliced together for the three 4-time-point conditions. This should be clearly indicated.

Below is the original blot for the γH2AX where the loading order is different compared to the other blots. For this reason the blot was cut in order to place the blots above one another.



Referee #2:

1) Figure 1 B, C. The authors claim that CHD1 localizes to DNA double strand breaks and colocalizes with yH2AX, quantification is shown in C. However, the number of cells used per condition for the quantification in C) is missing.

We have now included the number of cells used per condition for the quantification in the figure legend.

2) Figure 1 D: It would be helpful for the readers if the authors could quantify the co-localization if yH2AX/CHD1 in Edu positive and negative cells as it is hard to draw conclusions from a set of representative images.

We have now included the quantification of the co-localization of γH2AX/CHD1 in EdU positive and negative cells from control and NCS treated cells (Fig.1E).

3) Figure 1 E, S2A-C, S3D-I, S4D-J and S5B: For the siRNA, four different siRNA sequences are shown in the supplementary table but only one seems to have been used in the figures. The authors should specify which one of the four CDH1 siRNAs that has been used the figures, or if a mix of the siRNAs have been used, as well as show the effects on the individual siRNAs on CDH1 protein and

mRNA level. The authors should repeat the experiments using 2 different siRNAs targeting CDH1 to avoid that the results are due to unspecific off-target effects by the siRNAs.

Please see the response for the comment # 2 from reviewer 1. Briefly, the experiments with PC3 cells for HR, chromatin fractionation and IF analysis for CtIP and RPA1 experiments were performed with four individual siRNAs.

4) Figure 1 E: It seems that the label for the treatment has disappeared, I assume the number indicate release after NCS treatment as in figure S1D, E?

We thank the reviewer for pointing out this oversight. We have now included the label in the Fig.1F (which is NCS).

5) Figure S1 A: Reference to this figure is missing in the main text.

We have included the reference for Fig.S1A in the text.

The authors claim that CHD1 and yH2AX interact using the PLA assay. It would be helpful for the readers if the authors could quantify the interaction of yH2AX/CHD1 in NCS treated and non-treated cells as it is hard to draw conclusions regarding interaction upon DSB-induction from a representative image of one nuclei per treatment.

We agree with the reviewer's comment that it would be important to quantitate the co-localization of γH2AX and CHD1 in control and NCS treated cells. However, the data represented in the Fig.S1A is not from a PLA assay. It is rather from normal immunofluorescence microscopy to obtain insight into potential co-localization between γH2AX and CHD1 upon NCS treatment. We observe that CHD1 is partially co-localized with γH2AX. Given the limitations of this approach (see also the response to comment #1 from reviewer #1) we have focused more on the use of PLA for co-localization studies.

6) Figure S1 C: Similar to Figure S1 A, please quantify the interaction detected in the PLA assays and provide information on how many cells that were quantified per condition.

The data represented in Fig. S1C is from the PLA assay to check the co-localization of γH2AX/CHD1 upon DNA DSB induction. Further to check the specificity of this interaction we depleted CHD1 using siRNA, the depletion of CHD1 completely abolished the PLA signal. The data is similar to the data represented in Fig. 1D where we included the EdU to further analyze the cell cycle dependent CHD1 localization to DNA DSB. The quantification of γH2AX/CHD1 PLA signal is represented in Fig. 1E.

7) Figure 2A: The text in the results sections states that the irradiation was 2 Gy but the figure legend states 3 Gy, which dose was used?

We thank the reviewer for pointing this out and have now corrected the text and mention the exact dose (3 Gy) used for the experiment.

8) Figure 2C: The authors should complement the NCS treatment to look at prolonged yH2AX signaling on western blot with irradiation, as done in the rest of the panels in the figure which investigate sensitivity to ionizing radiation upon CDH1 depletion.

Please see also comment # 3 from Reviewer 1. As the reviewer has suggested, we have repeated the experiment with irradiation and analyzed for γH2AX and CHD1 levels (Fig. 2C is now replaced with new data).

9) In Figure 2, the authors show prolonged yH2AX activation upon CHD1 depletion in combination with irradiation, the author should complement this data with investigating checkpoint signaling and cell cycle progression.

Cross-comment from Referee 3:

**Rev#2, Point 9: The scope of the manuscript is clearly on DNA repair aspects, I really find the checkpoint aspect of minor importance.*

Although the authors agree that this would, indeed, be a very interesting additional aspect to study, a potential role of CHD1 in controlling check point signaling and cell cycle regulation lies outside the major focus of the current work in which we have mainly focused our studies on the role of CHD1 in DNA DSB repair and its therapeutic relevance in CHD1 deleted tumors. As it is also cross commented by reviewer 3 and by the editor, due to time limitation we have not addressed this point in this manuscript.

10) Figure 2D: P-values are missing for the colony formation assays.

We have now included p-valves for the colony formation assays

11) Figure 4A: It would be helpful for the readers if the authors could quantify the interaction between CtIP and CDH1 in the PLA assay with and without induction of DSBs with NCS to be able to compare the effects on DSBs on the interaction.

We have now included the quantification of PLA signal for CHD1/CtIP from PLA assay in Fig. 4A and represented the data in a graph in Fig. S4A.

12) Figure 4B and S3A and B: The authors claim that CtIP does not increase over time in CHD1 depleted cells, however it is absent at early (2h) timepoint but increase over time in Fig. 4B (PC3 cells). The authors should acknowledge the difference in CtIP recruitment to chromatin between cell lines (VcAP S3A, U-2 OS S3B and PC3 Fig4B).

As the reviewer has suggested, a statement has been added to the description of the figures stating that the kinetics of recruitment varied slightly between the cell lines. We have not specifically added a statement regarding the 2 h time point since this apparent absence is not seen in the other cell systems and is probably not representative of general mechanisms.

13) Figure 4C and D showed be moved to be under panel A and B and panel E should be where panel C and D currently are.

As reviewer suggested we have now rearranged the panels in Fig.4.

14) It is unclear if the quantification in Figure 4 is from the chromatin fractionations in S3H?

The graph which represented Figure 4F, is the quantification form the Western blot data from the rescue experiment which was shown in S3H (now Fig. EV4O).

15) Figure 4E: The authors claim a decreased PLA signal in panel E upon CDH1 depletion and this can be rescued by WT but not mutant CDH1, as this is difficult to interpret from the images the authors should include quantifications of the PLA signals in the figure.

We have quantified the PLA single from Fig. 4E and represented these in a graph (Fig. EV4M) as a fraction of cells containing punctate staining from all the cells as well as the percentage of cells which contain HA signal (i.e, those transfected with the respective expression vectors).

Also, this panel is without NCS treatment/induction of DSBs and the authors should investigate if CDH1 depletion also affect DSB-induced interaction between yH2AX and CHD1.

The specificity of PLA signal and the interaction between γH2AX/CHD1 was confirmed by CHD1 depletion followed PLA assay for γH2AX/CHD1, which is was shown in Fig. EV1C. The depletion of CHD1 completely abolished the PLA signal which shows the specificity of interaction between γH2AX/CHD1.

16) Figure 5A: The authors show that inhibition of PARP does not affect CHD1 recruitment to chromatin upon induction of DSBs. To further characterize the signaling pathways and show that the

CHD1 is involved in HR pathway and not NHEJ the authors should compare CHD1 recruitment upon ATM, ATR and DNA-PK inhibition in combination with NCS treatment.

Cross-comment from Referee 3:

**Rev #2, Point 16: Although the potential kinase mediated regulation of CHD1 recruitment is of interest and relevance, I also do not find this of major importance. This line of research would require a much more dedicated analysis with kinase targeting siRNA and perhaps CHD1 mutants to really improve the manus significantly.*

The authors agree with both reviewers that the additional study of the role of DNA-repair related PI3 kinase family members in CHD1 recruitment would, indeed, be very interesting. However, at the suggestion of the editor, due to time limitations and the scope of current study, we have not sought to address this question here.

17) Figure 5B: The authors claim that the yH2AX/CHD1 PLA signal is not affected by CtIP depletion, the authors should include quantifications of the PLA signals in the figure as a complement to the representative images.

Quantification of PLA signals from Fig. 5B is now represented in a graph in Fig. 5C.

18) Figure S2B,C: P-values are missing for the colony formation assays.

We have now included p-values for figures EV2B and C.

19) Figure 5C: Please also clearly mark which bars are siCDH1.

We have now mentioned the bars as siCHD1 in Fig. 5C (now 5D)

20) Figure 5D showed be moved out as a separate figure as it at the moment is not referred to in the result section and is described after figure 6.

Indeed, we agree with the reviewer's comment and have moved the model to Figure 6 (6G).

21) Figure 6A, 6C 6D and S5A: P-values are missing for the colony formation assays. The authors should also acknowledge the difference in response between the two cell lines.

As suggested by the reviewer, we have now included the p-values for the data represented in the figures.

22) Reference to figure 6F is missing.

We thank the reviewer for pointing out this oversight and have now appropriately referenced Fig. 6F in the text.

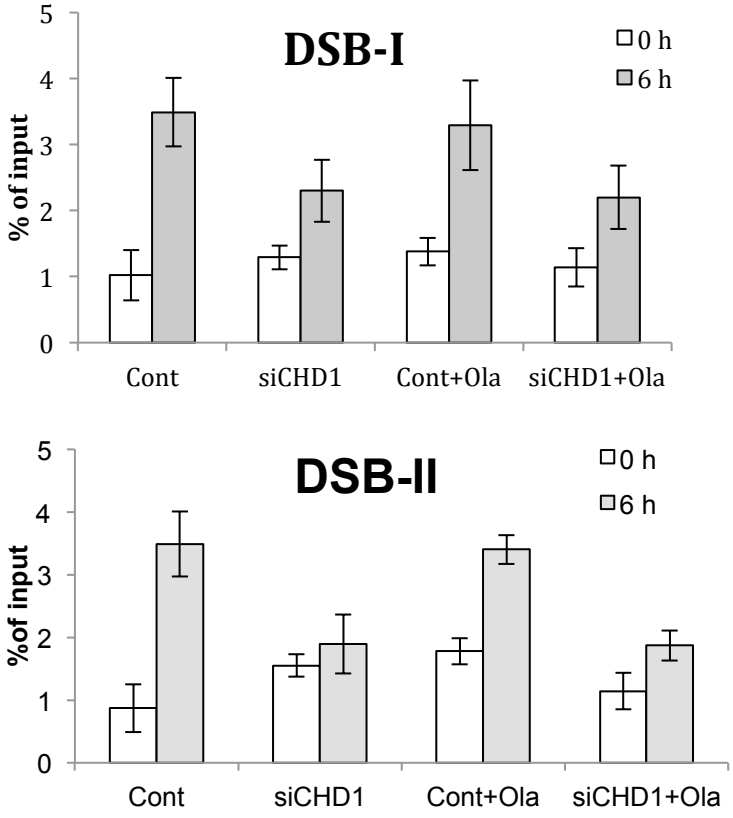
23) Although the survival data in figure 2D is not impressive, the survival data when combining shCDH1 with Olaparib in figure 6C and D are striking. As PARP is known to As the authors connect the decrease in chromatin opening upon CHD1 depletion with the decreased HR activity and cell survival upon siCHD1 it would be most interesting if the authors could investigate how combination of siCHD1 and Olaparib affects chromatin opening at DSB sites employing the FAIRE assay.

Cross-comment from Referee 3:

**Rev #2, Point 23: In the context of the manuscript, this is not an important point.*

The authors agree that studying the effects of combining CHD1 depletion and PARP inhibition in the opening of chromatin at the DNA damage site may potentially be of interest. In order to test this we have performed the FAIRE experiment using AsiSI-U2OS cells in control and CHD1 depleted cells with or without treatment with the PARP inhibitor Olaparib. We tested for the chromatin

openness at the DSB sites I and II where it was shown to be affected by CHD1 depletion. Interestingly, the PARP inhibition did not alter the open chromatin status compared to CHD1 depletion (see below). However, since it is too preliminary to make definitive conclusions about the role of PARP in the chromatin opening in the context of CHD1 depletion, we have not included these data in the manuscript.



Referee #3:

Minor comments:

*The resection phenotype as detected by the RPA staining is not very apparent (4D), yet, it is a key point in this study. The authors should address this aspect in more detail by a more complete ssDNA analysis (RPA staining and non-denatured BrdU staining after high-dose IR, such as 10 Gy 2h).

Please see also comment # 4 from reviewer 1. Briefly, we have performed native BrdU assay in PC3 cells in mock and siCHD1 depleted cells after 2 h of NCS treatment (Fig. 5E, S5J).

*To generalise the findings, normal (non-transformed cell lines) should be assayed for CHD1 impact on DNA end resection.

While we agree that it may be interesting to study the impact of CHD1 on non-transformed cells, the models available for normal prostate epithelial cells are very limited. Thus, as cross commented by the editor, we could not address this in a timely manner in the current study.

*A recent publication identified SRCAP as an important chromatin remodeller in CtIP driven resection, this should be discussed and referenced, PMID: 25176633.

Indeed, the authors agree that this is an interesting paper which shows the importance of chromatin remodelers during the end resection process. We have included the reference in our discussion.

*The impact of CHD1 status on yH2AX levels is modest and yH2AX is an indirect damage marker. The authors should detect actual DNA damage after CHD1 depletion by a more direct approach (Neutral comet or PFGE).

To address this we have performed neutral comet assay in control and CHD1-depleted cells upon NCS treatment for 30 min and 6h. The data are shown in Fig. EV2A-B and indicate that loss of CHD1, indeed, leads to defects in DNA repair.

Cross-comments from Referee 3:

We kindly thank the referee for their helpful insights and suggestions.

2nd Editorial Decision 14 July 2016

Thank you for the submission of your revised manuscript to our journal. We have now received comments from all three referees, as well as cross-comments, that are pasted below.

As you will see, while referee 1 is more critical, both referees 2 and 3 support the publication of your revised study, despite referee 3's concerns. However, the remaining concerns need to be clearly addressed in the manuscript text, i.e. overstatements regarding Chd1 recruitment to DSB must be avoided, blot quality must be improved, and all information on materials and methods must be included.

Please specify the number "n" for how many experiments were performed, the bars and error bars and the tests used to calculate p-values for Figure 1C, 2B, 3A, 3D, 4F, 6A-D, EV3, EV4, EV5. If the information is the same for all figure panels, a single sentence at the end of the legend is sufficient. Please also add scale bars to all microscopy images.

In figure 2C the second row of bands has a very different background. Are all bands derived from the same gel/blot? Please send us the source data with the full gels to show where the bands come from. Please also leave some white space around all spliced bands in order not to give the impression that the figure panel is one piece of gel.

Please change figure 3 into portrait format, as we cannot layout figures in landscape format.

In figure EV1 the top right image of the bottom panel seems to be empty. Some background staining of the cell should be visible. Please explain what happened and send us a new figure with the primary data.

In figure EV2 the bottom middle panel has a nmber of horizontal lines on it. Can you please explain what this is?

I would also like to suggest some changes to the manuscript title and abstract:

Loss of CHD1 causes DNA repair defects and enhances prostate cancer therapeutic responsiveness

The CHD1 gene, encoding the Chromo-domain Helicase DNA-binding protein-1, is one of the most frequently deleted genes in prostate cancer. Here we examine the role of CHD1 in DNA double strand break (DSB) repair in prostate cancer cells. We show that CHD1 is required for the recruitment of CtIP to chromatin and subsequent end resection during DNA DSB repair. Our data support a role for CHD1 in opening the chromatin around the DSB to facilitate the recruitment of homologous recombination proteins. Consequently, depletion of CHD1 specifically affects homologous recombination (HR)-mediated DNA repair but not non-homologous end joining. Together, we provide evidence for a previously unknown role of CHD1 in DNA DSB repair via HR, and show that CHD1 depletion sensitizes cells to olaparib and PARP inhbitors, which has potential therapeutic relevance. Our findings suggest that CHD1 deletion, like BRCA1/2 mutation in ovarian cancer, may serve as a marker for prostate cancer patient stratification and the utilization of targeted therapies such as PARP inhibitors, which specifically target tumors with HR defects.

Please let me know whether you agree with these changes.

I am looking forward to receiving a final version of your manuscript as soon as possible. Let me know please if you have any questions.

REFEREE REPORTS

Referee #1:

The revised manuscript by Kari et al. is definitely improved over the original submission. However, there remain issues with this study, including lack of mechanistic insight, that prevent me from recommending this work for publication in EMBO Reports.

1. Lack of convincing demonstration of CHD1 DSB recruitment still remains a key issue:
- The argument in the rebuttal letter that CHD1 distribution across genome is not random and that it associates with a subset of gene promoters and enhancers does not mean that CHD1 localises to the sites of DNA damage.
- The very high background staining of γH2AX upon doxycycline-mediated induction of I-SceI expression (Fig. 1B) can't be due to the I-SceI cutting at "endogenous" sites as the authors suggest in the rebuttal letter: there are no endogenous I-SceI sites in either human or murine genomes.
- The PLA assays are not at all informative due to the fact that CHD1 is evenly chromatin bound before and after DSB induction and therefore some of it will co-localize with γH2AX. Moreover, as CHD1 accumulates on chromatin upon damage (these data are very strong), the PLA signal is expected to increase. Overall, this reviewer is not convinced by the conclusions that the authors draw from the PLA data.

2. In Fig. 2C, persistence of γH2AX signal upon CHD1 depletion was previously demonstrated by the immunoblot following treatment of cells with NCS. In the revised manuscript, this has been replaced with the immunoblot following IR, which is the same treatment as used in IF data in Fig. 2A. Unfortunately, the new blot is of very poor quality and, interestingly, over time the levels of CHD1 in siCont go up after NCS (old Fig.2C) and down after IR (new Fig. 2C).

3. In the previous version of Figs. 4C and 4D, the proportion of cells with either CtIP or RPA foci, respectively, was presented as 100% in control cells. As the authors explain in the rebuttal letter, those were normalized values and that in the revised manuscript, the actual percentages are now plotted. The concern however is that in the former version the difference between siCont and siCHD1 was about 5-fold for CtIP foci (from 100 to 20%) and now it is approximately 2.5 fold (from 17 to 7%). Isn't it expected that upon normalization the relative differences should remain the same? It is also somewhat surprising that the fraction of CtIP and RPA focus forming cells is only 20-25% because usually, the proportion of S/G2 cells in U2OS culture is about 2-2.5 fold higher. However, the reviewer accepts that the latter might be due to the nature of the particular assay.

4. As suggested by the reviewers, the authors performed experiments to address the role of CHD1 in DNA end-resection. However, the data in the main Fig. 5E do not allow fair judgement of the effect CHD1 has on resection as it's an image of a single cell. The quantitative data in Fig. EV4 is far more informative. The reviewer is still mystified though how the authors manage to detect clear multiple foci of endogenous CtIP following the 2Gy dose, as others in the field are not able to do this.

As a general comment, the authors don't seem to include information on the antibodies used in the study.

Referee #2:

The authors have properly addressed all concerns

Referee #3:

The Authors have adequately addressed my points and I support its publication in EMBO Reports.

Referee Cross-comments:

Referee 1:

As I was the harshest of the reviewers, I
have softened my review. I would be happy for this work to be published
if the authors can address the following points:

• Make at least some statement about the ISCe1 data being surprising as
there are reportedly no endogenous sites.

• Point 3; they explain how their data were quantified and normalised
compared to the previous data.

• Point 4: they include the quantitative data in the main figure.

Referee 2:

I remain saying this is an important study and the overall conclusion well supported. I disagree that recruitment of CHD1 to DSB is important, the role in HR more important and of clinical relevance.

Referee 3:

Reviewer 1 is clearly far more critical of the study than I am; indeed the points are relevant, but I don't find that they should preclude publication of the manuscript.

2nd Revision - authors' response 29 July 2016

We kindly thank you, the other editors and the reviewers for your support and suggestions to further improve the manuscript. As suggested, in the second version of the revised manuscript we have now avoided overstating the recruitment of CHD1 to DSB sites. We have also included the changes in the title and the abstract as suggested (with one exception related to the redundancy of Olaparib an PARP inhibitors).

Below is a list of the suggested changes and how these have been addressed in the new version of the manuscript.

Editorial comments

1.Please specify the number "n" for how many experiments were performed, the bars and error bars and the tests used to calculate p-values for Figure 1C, 2B, 3A, 3D, 4F, 6A-D, EV3, EV4, EV5. If the information is the same for all figure panels, a single sentence at the end of the legend is sufficient. Please also add scale bars to all microscopy images.
We have now included the "n" number, p-vlaues and the method to calculate the significance in the figure legends (at the end). The scale bars for the microscopy images are added to the images.

2. In figure 2C the second row of bands has a very different background. Are all bands derived from the same gel/blot? Please send us the source data with the full gels to show where the bands come from. Please also leave some white space around all spliced bands in order not to give the impression that the figure panel is one piece of gel.
In Fig. 2C, indeed the bands are derived from the same gel. We have how re-done these blots and have also provided the source data as to where the bands come from. As suggested by the editor we

have now changed the figure with proper background and space around the spliced bands (as well as boxes around the bands of all blots to clearly delineate the edges of the cut regions).

3. Please change figure 3 into portrait format, as we cannot layout figures in landscape format.
We have now changed the Figure 3 into portrait format. We will also upload a PPT file which may potentially be of use for formatting the final version of the figures for publication.

4. In figure EV1 the top right image of the bottom panel seems to be empty. Some background staining of the cell should be visible. Please explain what happened and send us a new figure with the primary data.
In figure EV1C, the top right image of the bottom panel which shows the mock and CHD1 depleted cells without NCS treatment indeed appeared blank. We have now changed to contrast (in all the panels) to make sure that background is visible. We have also included the raw data.

5. In figure EV2 the bottom middle panel has a number of horizontal lines on it. Can you please explain what this is?
The horizontal lines in the bottom panel are not present in original figure. The lines appear to have arisen during the conversion of figure to TIF.

Reviewer 1

1. Lack of convincing demonstration of CHD1 DSB recruitment still remains a key issue:
- The argument in the rebuttal letter that CHD1 distribution across genome is not random and that it associates with a subset of gene promoters and enhancers does not mean that CHD1 localises to the sites of DNA damage.
In the original concerns the reviewer implied that CHD1 is localized indiscriminately across the genome and that therefore a colocalization would not necessarily indicate sites of double-strand breaks. The point of our rebuttal was to state that CHD1 localization is highly specific across the genome. Thus, localization with sites of DNA damage (as shown by the PLAs) and the increased recruitment of CHD1 (i.e., via chromatin fractionation) are not simply due to non-specific localization of CHD1. If CHD1 were localized non-specifically across the genome (e.g., like core histone proteins), then a co-localization by PLA would not have any real meaning. On the other hand, since CHD1 is highly specifically localized to transcriptional start sites (mostly) and some enhancers (although we don't see much of this in our ChIP-seq data), the observed co-localization is most likely due to increased recruitment to DSB sites. Apart from this, in the case that CHD1 were already present at DSB sites, it would not diminish its mechanistic importance and necessity for DSB repair.

- The very high background staining of gH2AX upon doxycycline-mediated induction of I-SceI expression (Fig. 1B) can't be due to the I-SceI cutting at "endogenous" sites as the authors suggest in the rebuttal letter: there are no endogenous I-SceI sites in either human or murine genomes.
We agree with the reviewers comment that there are no I-SceI endogenous sites in the human genome. Surprisingly we see that upon doxycycline treatment we observe that increased γH2AX signal, however, we have seen clearly observed that only in doxy treated cells CHD1 is co-localized with the lac array. We have now mentioned the background induction of γH2AX in doxy treated cells in the new version of the manuscript.

- The PLA assays are not at all informative due to the fact that CHD1 is evenly chromatin bound before and after DSB induction and therefore some of it will co-localize with gH2AX. Moreover, as CHD1 accumulates on chromatin upon damage (these data are very strong), the PLA signal is expected to increase. Overall, this reviewer is not convinced by the conclusions that the authors draw from the PLA data.
Again, this is a point that the authors disagree with (see above). An even chromatin binding of CHD1 across the genome is not correct. We know (1) that CHD1 is localized to specific sites in the genome in untreated cells and (2) we know that there is increased recruitment of CHD1 following DNA damage (e.g., through chromatin fractionation studies).

2. In Fig. 2C, persistence of gH2AX signal upon CHD1 depletion was previously demonstrated by the immunoblot following treatment of cells with NCS. In the revised manuscript, this has been replaced with the immunoblot following IR, which is the same treatment as used in IF data in Fig.

2A. Unfortunately, the new blot is of very poor quality and, interestingly, over time the levels of CHD1 in siCont go up after NCS (old Fig.2C) and down after IR (new Fig. 2C).
We agree with the reviewer's comment that the blot is not of a very good quality. This blot has been redone and included (together with source data) in the new version of the manuscript.

3. In the previous version of Figs. 4C and 4D, the proportion of cells with either CtIP or RPA foci, respectively, was presented as 100% in control cells. As the authors explain in the rebuttal letter, those were normalized values and that in the revised manuscript, the actual percentages are now plotted. The concern however is that in the former version the difference between siCont and siCHD1 was about 5-fold for CtIP foci (from 100 to 20%) and now it is approximately 2.5 fold (from 17 to 7%). Isn't it expected that upon normalization the relative differences should remain the same? It is also somewhat surprising that the fraction of CtIP and RPA focus forming cells is only 20-25% because usually, the proportion of S/G2 cells in U2OS culture is about 2-2.5 fold higher. However, the reviewer accepts that the latter might be due to the nature of the particular assay.
For the revision the counts were performed again, thus the actual nuclei numbers and differences in absolute values can vary slightly from experiment to experiment. However, the significance and general effects clearly remain the same. Regarding the faction of CtIP and RPA foci with relation to S/G2 cells, the authors agree with the reviewer that due to the nature of the particular assay a direct relationship between S/G2 and cells with CtIP and RPA foci cannot be made in this case.

4. As suggested by the reviewers, the authors performed experiments to address the role of CHD1 in DNA end-resection. However, the data in the main Fig. 5E do not allow fair judgement of the effect CHD1 has on resection as it's an image of a single cell. The quantitative data in Fig. EV4 is far more informative. The reviewer is still mystified though how the authors manage to detect clear multiple foci of endogenous CtIP following the 2Gy dose, as others in the field are not able to do this.
With regard to the end resection data, we have now included the quantitative data in the figure as well. Regarding the CtIP staining we agree that, it is difficult to obtain clear CtIP foci. In our hands the antibody seems to be a major determinant of the quality and reliability of the staining.

As a general comment, the authors don't seem to include information on the antibodies used in the study.
Indeed, the supplemental information file was inadvertently forgotten in the last upload.

Additional comments from Referee 1:
As I was the harshest of the reviewers, I have softened my review. I would be happy for this work to be published if the authors can address the following points:
• Make at least some statement about the ISCe1 data being surprising as
there are reportedly no endogenous sites.
As suggested by the reviewer and described above we have now included the statement in the manuscript about the back ground staining of γH2AX in doxy treated cells.

• Point 3; they explain how their data were quantified and normalized compared to the previous data.
We have now explained in the test how data were quantified and normalized in the text.

• Point 4: they include the quantitative data in the main figure.

As suggested by the reviewers we have now included the quantification for Fig. 5E in the main figure as Fig. 5F
We hope that these additional changes address all concerns and qualify the manuscript for publication in EMBO reports. We again thank you for your excellent suggestions and support and look forward to working together with you again in the future.

3rd Editorial Decision 04 August 2016

Thank you for the submission of your revised manuscript to our editorial offices, which is now suitable for publication in EMBO reports. However, before we can proceed with the formal acceptance of your manuscript, I would like to ask you for some further minor revisions.

Could you please go through all the panels (including those indicated by my colleague in her

decision letter) and add the relevant statistical testing and the information regarding this to the figure legends where these are still missing (the number "n" for how many experiments were performed, the bars and error bars and the test used to calculate p-values, and the value of the p-values). In the present version of the manuscript this is incomplete. For example in Figure 2B there are p-values indicated (asterisks), but in the legend these p-values, their actual value and the test used are not mentioned. It is also not clear what significant difference to which value was tested in 2B. Please fix this for all the other relevant panels.

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

3rd Revision - authors' response 07 August 2016

I sincerely apologize for not addressing the necessary points in the previous version.

We have now took care to address all the points that have been mentioned in your email.

The number of replicates are mentioned in the figure legend for Fig.4C/D, EV3B and EV4A/I.

For Fig.6D, the p-values are calculated and mentioned in the figure legend and the Fig.6D is modified according to that.

In Fig.4F, the quantification is from the particular blots shown in Fig.EV4O and we mentioned that in the figure legend.

Please find the attached the revised version of text and the new figure for Fig.6 (TIFF format) with this email.

Please let us know if we need to provide any further details.

Thank you for your patience.

4th Editorial Decision 11 August 2016

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Steven A. Johnsen and Vijayalakshmi Kari
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2016-42352V3

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

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
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	For the experiments we used biological triplcates to ensure the observed data is true. All experiments were also repeated at least three times.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes, we have calculated the statistical significance for the data where it is required with the approprite test.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes.
Is there an estimate of variation within each group of data?	No
Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	All the antibodies used in this study were listed in the expanded information along with the catalog numbers.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

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

D- Animal Models

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

E- Human Subjects

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

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	NA
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NO