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Error-free DNA damage tolerance pathway is facilitated by the Irc5 translocase through cohesin

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(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 20th December 2017

Thank you for submitting your manuscript on Irc5 and cohesin in error-free DNA damage tolerance in yeast to our journal. It has now been assessed by three expert referees, whose reports are copied below for your information. As you will see, all referees find your results potentially interesting, but especially referees 1 and 3 raise a number of serious concerns, including the correlative nature of the roles of Irc5 and cohesin, and the decisiveness of the data implicating Irc5 in template switching. Given these issues with the conclusiveness of the presented genetic data and the absence of stronger data to connect Irc5 and cohesin functions, I am afraid we have to consider the study still somewhat too preliminary to warrant publication in The EMBO Journal.

Nevertheless, we notice that the study may in time become a more compelling candidate for an EMBO Journal article if improved and extended along the lines suggested in the referees' reports. Therefore, should you be able to decisively address all key concerns raised by the reviewers, we shall be happy to consider a revised version of this work further for The EMBO Journal. Since this may require substantial additional efforts and it is also hard to predict the results and insightfulness of these required further experiments, I am currently not able to make strong predictions on the outcome of an eventual re-review, and I would therefore understand if you should choose to rather publish this work rapidly and without major modifications elsewhere. Should you decide to revise the manuscript for The EMBO Journal, we would be open to discuss an extension of our regular three-months revision period, during which publication of any competing work elsewhere would not affect our final assessment of your own study. Please note that our policy to allow only a single major revision round would make it important to carefully and comprehensively answer to all the points raised during this round. Additional information on how to format and submit a revised manuscript can be found below.

Thank you again for the opportunity to consider this work for The EMBO Journal, and please do not hesitate to contact me in case you should have any questions regarding this decision, or would like to discuss specific revision plans ahead of a resubmission.

REFEREE REPORTS.

Referee #1:

Litwin et al., present data to characterize Irc5 in the error-free DNA damage tolerance pathway via regulating cohesin through regulating (contributing to) Scc2/Scc4 function. They showed previously that there is decreased interaction between Scc1 and Scc2 in irc5Δ-1 mutants.

When assessing the scc2-4 allele, all of the functional assays such as PFGE and ChIP for Scc1 and Pol2 and forks are performed with the single mutant. The genetic data with the irc5Δ-1 scc2-4, which shows additive sensitivity to DNA damaging agents, is interpreted to argue that Irc5 compensates for Scc2/Scc4-mediated cohesin loading in the scc2-4 background. It is true that an additive impact would not be detected with either PFGE or ChIP because the scc2-4 allele is so severe with these readouts, but the following experiments need to be performed to support the paper.
1. The 2D gels need to be performed in sgs1Δ scc2-4 (double) and sgs1Δ scc2-4 irc5Δ-1 (triple) mutant cells. % of cells with Rad52 - YFP and Rfa1- YFP in scc2-4 and scc2-4 irc5Δ-1.
2. These should also be performed with the irc5DAEA mutant too to support their claim that it is the enzymatic activity.
3. To understand the role of cohesin regulation for DTT and TS, and integrate Fig. 5 with the rest of the paper the following experiments should be performed a.) sensitivity on genotoxins and b.)2D gel analysis in wild type and sgs1Δ mutant background, and c.) Rad52/Rfa1 foci in the following mutant combinations:
rad18Δ scc2-4 (double) and sgs1Δ rad18Δ scc2-4 (triple) mutant cells.
rev3Δ scc2-4 (double) and sgs1Δ rev3Δ scc2-4 (triple) mutant cells.
Minor points:
The title should be modified cohesion is not measured anywhere in this paper.
There are a number of grammatical changes throughout - please proofread carefully.

These are doable experiments that are important to support their claim and understand the interplay (epistatic or additive) between Scc2/Scc4 and Irc5 in cohesin regulation during replication stress and fork associated DNA damage. If the following experiments are incorporated I would be very supportive of publication.

Referee #2:

The manuscript "Error-free DNA damage tolerance pathway is facilitated by the cohesion function of Irc5 translocase" by Litwin et al investigates the role of the Snf2 family translocase Irc5 for DNA damage tolerance through the Rad18/Rad5/Sgs1 pathway. They find that Irc5 promotes Translesion Synthesis, and prevents that regions of single-stranded DNA accumulates during replication. They also show that Irc5 enables replication progression by aiding in recruitment of Cohesin complexes to the vicinity of blocked replication forks, in an Scc2/Scc4-dependent manner. All in all this suggests an important function for Cohesin in completion of DNA duplication in the presence of replication stress, and links the Rad18/Rad5- DDT response to SNF2 dependent recruitment of Cohesin to regions where replication is problematic or disrupted. This study provides a number of interesting findings and broadens the knowledge in the Cohesin field. The authors are connecting information that has been available since some time into a new way of looking at both Cohesin recruitment and the pre-requisites of chromatin interaction by Scc2/Scc4, the Cohesin loader. I do not have any major issues with the paper, rather I find it interesting, and mostly the experiments are both carefully performed and well described, as well as support the conclusions.

However I have a few concerns listed below that I suggest are dealt with:

Throughout the paper please check that the number of experiments performed is indicated.

Also throughout the paper check the text for consistency in using definitive article (the) and of, which is lacking for example at page 11, second line from the bottom.

Describe the irc5-Δ1 allele, the reader should not need to go back to the original study to understand how the deletion is done / why the -Δ1.

The figure legend to Figure 1 does not match the labeling in the Figure; D, E and F are in the legend E, F and G.

The level of recovery after exposure to MMS in Fig 1C does not really correlate with the level of Rad53 Phosphorylation seen in Fig 1B. Is this because of the lower MMS concentration? and is there a reason for the different MMS concentrations used?

The quantification of the ChEC analysis could be described in greater details (Fig 1E).

Out of curiosity; why are the numbers of Rad52 and Rfa1 foci detected by live cell imaging? And more importantly how many cells are analyzed (fig 1C and 1F)? Or does n=5 mean 5 cells? This is potentially an important concern since the differences between WT and irc5Δ cells are (despite being significant) not that large.

Fig 2A. How many times was this experiment performed with similar/identical result? A strong delay in the cell cycle arrest is somewhat difficult to appreciate in these very small histograms.

Fig 2C and F, the quantification of the Chr 12 and 3 bands could be described better. Are the bands correlated to the total amount of DNA/lane or just to the same Chr band in the G1 lane? How do the authors assure equal loading? The same concern is also valid for Fig 5A and B.

Referee #3:

In this study Litwin et al. explore the involvement of the Irc5 translocase in the tolerance to DNA damage. The authors report that Irc5-deficient cells accumulate Rad52/Rfa1 foci and checkpoint activation signals during the recovery from DNA damage induced in S-phase by MMS treatment. These cells also exhibit delayed S-phase progression and chromosome replication completion following MMS treatment, as judged by FACS and PFGE. Altogether indicating that Irc5 plays a relevant function in the completion of alkykated treated DNA.

Next, genetic interactions with DNA damage tolerance mutants are analysed, revealing synthetic sensitivity to MMS or irc5 alleles in combination with rad51/rad52 and rev3 deletion mutants. By 2D gel analysis, a reduction in X-shaped template switch intermediates accumulation in sgs1 cells is observed upon impairment of Irc5 function. Previous observations linking Irc5 function to cohesin association to chromatin prompt the authors to analyse cohesin association to chromatin at regions close to replication origins. They observe decreased cohesin levels close to origins, accompanied by increased Pol epsilon (Pol2) levels in irc5 cells, which they interpret as reduced cohesin loading in the context of fork stalling.

Lastly, authors observe chromosome replication defects following MMS treatment in scc2-4 cohesin loader mutants and describe synthetic sensitivity of scc2-4 and irc5 alleles in response to different agents inducing DNA damage. Based on these results the authors propose that Irc5 is a novel factor acting to support replication within the Rad18/Rad5/Sgs1 DNA damage tolerance pathway. They also conclude that this error free pathway is linked to Irc5-dependent enrichment of cohesin at perturbed replication forks.

The reported work is original and provides insight into a role of Irc5 in supporting replication of damaged chromosomes. However, there are important concerns on the interpretation of genetic data linking Irc5 and template switch factor's and cohesin loader's functions, as well as on the experimental design on the cohesin ChIP experiments. In its current form the manuscript fails to convincingly support the central conclusions and would need to be significantly improved to justify publication in EMBO Journal.

Major concerns:

- Authors ascribe Irc5 to the Rad18/Rad5/Sgs1 DDT pathway based on genetic analyses. These are performed at a variety of MMS concentrations in which double mutants show sensitivities equivalent to rad18, rad5 and sgs1 single deletants. However, since irc5Δ-1 cells do not exhibit MMS sensitivity at these concentrations an epistatic behaviour cannot be concluded. The analysis should be performed at concentrations in which irc5 cells do show sensitivity to be able to establish meaningful genetic interactions.

In addition, while a contribution of Irc5 to sister chromatid junctions accumulating in sgs1 cells is observed, would it not be expected to find epistasis with rad51/rad52 mutations if Irc5 was involved in a template switch DDT branch?

- ChIP experiments were performed in the presence of hydroxyurea and MMS. HU is also inducing fork stalling, which complicates the interpretation of the results. The experiments should be repeated in cells treated with MMS only and more time points should be analysed to provide accurate information on the timing of origin firing and replisome stalling (e.g. at 15', 30', 45', 60').

- The inferred link between Irc5 function in MMS and cohesin association to forks is correlational. Stronger evidence is required to conclude that the defects in MMS of irc5 cells are a consequence of reduced cohesin association to forks. On the same lines, the synthetic sensitivity conferred by irc5Δ-1 and scc2-4 combination argues that these genes share a function in MMS survival. However, since cohesin association to forks seems fully dependent on Scc4, if Irc5 contribution reflected a function in cohesin loading an epistatic effect should be expected. Authors should check if epistasis exists between cohesin-defective and irc5 alleles in terms of MMS sensitivity.

Minor concerns:

- Authors need to explain why replication progression does not seem delayed in the FACS profiles corresponding to the 2D gel experiments in MMS.

- A cohesin-bound region not actively replicated should be included in experiments analysing Scc1 chromatin binding in irc5 and scc2-4 cells, to ascertain if the effect observed is specific to cohesin associated to stalled forks.

- Basic information is not provided about the quantification and normalization of replication intermediates in 2D experiments. What are X-molecules levels relative to?

- Authors state that Irc5 disruption does not have an effect on PCNA poly-ubiquitylation. However, differences in U1 and U2 bands kinetics are observed in WT vs irc5 cells in Figure 3C. How does this relate to Irc5 function in MMS?

- There are some apparent mistakes in the spelling of references and in the description of panels in figure legends.

1st Revision - authors' response 11th June 2018

Referee #1:

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When assessing the scc2-4 allele, all of the functional assays such as PFGE and ChIP for Scc1 and Pol2 and forks are performed with the single mutant. The genetic data with the irc5Δ-1 scc2-4, which shows additive sensitivity to DNA damaging agents, is interpreted to argue that Irc5

compensates for Scc2/Scc4-mediated cohesin loading in the scc2-4 background. It is true that an additive impact would not be detected with either PFGE or ChIP because the scc2-4 allele is so severe with these readouts, but the following experiments need to be performed to support the paper.

1. The 2D gels need to be performed in sgs1Δ scc2-4 (double) and sgs1Δ scc2-4 irc5Δ-1 (triple) mutant cells. % of cells with Rad52 - YFP and Rfa1- YFP in scc2-4 and scc2-4 irc5Δ-1.

As suggested by the Referee we analyzed the levels of MMS-induced Rfa1-YFP and Rad52-YFP foci in *scc2-4* and *scc2-4 irc5Δ-1* mutants. Interestingly, we show that while disruption of *IRC5* in *scc2-4* background leads to increased sensitivity to MMS it has no effect on DNA repair foci levels (Figure 7 and Appendix Figure 4). In addition, we added the analysis of genetic interactions between *irc5Δ-1* and *scc1-73*, defective for cohesin subunit, showing that both alleles are epistatic in all tests (Figure 7 and Appendix Figure 4). Having known that the Scc2/Scc4 complex performs cohesin-independent functions in the cell, we propose that Scc2/Scc4 and Irc5 exhibit overlapping (cohesin loading) and independent roles in response to MMS-induced DNA damage. Similarly, we found that deletion of *RSC2*, encoding non-essential subunit of the RSC complex that also contributes to cohesin loading, in the *scc2-4* background results in increased sensitivity to MMS compared to single mutants (our unpublished data). We added a new paragraph to the Discussion section (pp. 20-21) where we discussed genetic interactions between cohesin mutants and *irc5*Δ*-1*.

Following Referees suggestions, we constructed *sgs1*Δ *scc2-4* and *sgs1*Δ *scc2-4 irc5*Δ*-1* strains but we found out that they display slow growth phenotype. Importantly, these mutants accumulate in G2/M phase preventing efficient G1 synchronization that is required for proper analysis of recombination intermediates. This is the reason why we have not performed 2D gel experiments for these strains. We also note that other cohesin mutants, such as *scc1-73*, have been shown to impair sister chromatid junction formation in response to DNA damage during replication. The epistasis we reveal between *irc5*Δ*-1* and *scc2-4*, *scc1-73* in regard to Rfa1-YFP and Rad52-YFP DNA damage foci accumulation, suggests that these factors act jointly with regard to gap-filling.

2. These should also be performed with the irc5DAEA mutant too to support their claim that it is the enzymatic activity.

According to reviewer's suggestion we assessed levels of Rfa1-YFP and Rad52-YFP foci in the *irc5*^{DAEA} mutant (Figure 3). Like in the case of *irc5*Δ*-1* mutant, ATPase deficient cells accumulated more DNA repair foci during MMS treatment and recovery period suggesting that translocase activity of Irc5 required for completion of DNA replication in the presence of MMS-induced damage (see pp 9-10). We were willing to analyze replication intermediates in *irc5*^{DAEA} *sgs1*Δ double mutant. However, due to technical problems were not able to finish these experiments and meet already extended deadline for resubmission. We hope that the *irc5*^{DAEA} mutant phenotypes, including levels of Rfa1-YFP and Rad52-YFP foci and replication kinetics of alkylated DNA (Figure 3), that are very similar to phenotypes of Irc5 deletion mutant, are sufficient to conclude that ATPase activity of Irc5 is required to facilitate replication completion and DNA repair during exposure to MMS.

3. To understand the role of cohesin regulation for DTT and TS, and integrate Fig. 5 with the rest of the paper the following experiments should be performed a.) sensitivity on genotoxins and b.)2D gel analysis in wild type and sgs1Δ mutant background, and c.) Rad52/Rfa1 foci in the following mutant combinations: rad18Δ scc2-4 (double) and sgs1Δ rad18Δ scc2-4 (triple) mutant cells. rev3Δ scc2-4 (double) and sgs1Δ rev3Δ scc2-4 (triple) mutant cells. The 2D gels need to be performed in sgs1Δ scc2-4 (double) and sgs1Δ scc2-4 irc5Δ-1 (triple) mutant cells. % of cells with Rad52 - YFP and Rfa1- YFP in scc2-4 and scc2-4 irc5Δ-1.

A suggested by the Referee we investigated genetic interactions between cohesin loader, cohesin and DDT mutants. Our new data, presented in Figure 8, show that disruption of *RAD18* in *scc2-4* or *scc1-73* background results in slight or no increase of MMS sensitivity as well as no increase of Rfa1-YFP and Rad52-YFP foci. On the other hand, lack of *REV3* in *scc2-4* or *scc1-73* mutants

resulted in additive accumulation of DNA repair foci and strong increase of MMS sensitivity. These data suggest that cohesin loader/cohesin work with Rad18, independently from TLS polymerases, to avoid DNA damage accumulation (see also pp. 19-20 in the Discussion section).

We would like to emphasize that since it has been previously shown that *rad18*Δ cells are strongly deficient in the formation of X-molecules and deficiencies in translesion synthesis polymerases do not affect X-molecule formation (Branzei *et al.*, 2008; Vanoli *et al.*, 2010), we think that 2D gel analysis of recombination intermediates in *rad18* and *rev3* backgrounds would not be informative.

Minor points:

The title should be modified cohesion is not measured anywhere in this paper

We changed the title as follows: "Error-free DNA damage tolerance pathway is facilitated by the Irc5 translocase through cohesin".

There are a number of grammatical changes throughout - please proofread carefully.

We've proofread the manuscript carefully. We hope that the changes we've made will satisfy the Referee.

Referee #2:

The manuscript "Error-free DNA damage tolerance pathway is facilitated by the cohesion function of Irc5 translocase" by Litwin et al investigates the role of the Snf2 family translocase Irc5 for DNA damage tolerance through the Rad18/Rad5/Sgs1 pathway. They find that Irc5 promotes Translesion Synthesis, and prevents that regions of single-stranded DNA accumulates during replication. They also show that Irc5 enables replication progression by aiding in recruitment of Cohesin complexes to the vicinity of blocked replication forks, in an Scc2/Scc4-dependent manner. All in all this suggests an important function for Cohesin in completion of DNA duplication in the presence of replication stress, and links the Rad18/Rad5- DDT response to SNF2 dependent recruitment of Cohesin to regions where replication is problematic or disrupted. This study provides a number of interesting findings and broadens the knowledge in the Cohesin field. The authors are connecting information that has been available since some time into a new way of looking at both Cohesin recruitment and the pre-requisites of chromatin interaction by Scc2/Scc4, the Cohesin loader. I do not have any major issues with the paper, rather I find it interesting, and mostly the experiments are both carefully performed and well described, as well as support the conclusions.

However I have a few concerns listed below that I suggest are dealt with:

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We have carefully proofread the manuscript and correct the errors. We hope that the corrections we've made will satisfy the Referee.

Describe the irc5-Δ1 allele, the reader should not need to go back to the original study to understand how the deletion is done / why the -Δ1.

As suggested by the Referee we added a paragraph to the Results section describing the *irc5*Δ*-1* allele (pp. 5-6): "We have previously shown that the complete deletion of *IRC5* open reading frame results in reduced expression of essential *RSC8* gene located 194 bp downstream from *IRC5*. As a result of this, the *irc5*Δ strain displayed phenotypes of slow growth and increased sensitivity to DNA damaging agents that were not complemented by the wild type *IRC5*. To overcome this problem, we deleted the 3' end of *IRC5* gene, containing both SNF2_N and Helic C domains, generating the *irc5*-Δ*1* allele. The *irc5*-Δ*1* mutant grew normally and exhibited wild type levels of *RSC8* transcript. Interestingly, *irc5*-Δ*1* cells showed increased sensitivity to MMS, so we decided to

investigate which aspect(s) of replication stress response is defective in this mutant (Litwin *et al.*, 2017)".

The figure legend to Figure 1 does not match the labeling in the Figure; D, E and F are in the legend E, F and G.

This was corrected.

The level of recovery after exposure to MMS in Fig 1C does not really correlate with the level of Rad53 Phosphorylation seen in Fig 1B. Is this because of the lower MMS concentration? and is there a reason for the different MMS concentrations used?

Indeed, the fact that the level of Rad53 phosphorylation seen in Fig. 1B does not correlate with the level of recovery measured by percentage of cells with Rad52-YFP or Rfa1-YFP foci, presented in Figures 1C and F, is due to lower concentration of MMS (0.01%) used for microscopic analysis versus 0.03% MMS used in all other experiments. We had to use lower concentration of MMS for foci detection as in our hands virtually all wild type cells treated with 0.03% MMS exhibited Rad52-YFP or Rfa1-YFP focus formation.

The quantification of the ChEC analysis could be described in greater details (Fig 1E).

The following paragraph was added to Materials and Methods section (p. 24): "Equal concentrations of total DNA were loaded and resolved on 0.8% agarose gel and stained with ethidium bromide. The DNA intensity in each line was quantified with Bio-Rad ChemiDoc MP System and plotted on the histograms with Image Lab software (Bio-Rad). Each experiment was repeated three times with similar results".

Out of curiosity; why are the numbers of Rad52 and Rfa1 foci detected by live cell imaging? And more importantly how many cells are analyzed (fig 1C and 1F)? Or does n=5 mean 5 cells? This is potentially an important concern since the differences between WT and irc5Δ cells are (despite being significant) not that large.

Live cell imaging is a standard approach used commonly in the field. This method is relatively easy, fast, cheap and allows to observe DNA damage repair centers *in vivo*. Moreover, fixing the cells with cross-linkers can sometimes produce artefacts.

Rad52-YFP and Rfa1-YFP foci levels were analysed in 5 independent experiments. At least 300 cells were counted in each experiment.

Fig 2A. How many times was this experiment performed with similar/identical result? A strong delay in the cell cycle arrest is somewhat difficult to appreciate in these very small histograms.

FACS analysis was performed at least 3 times with similar results. A representative result is shown. To make the difference in S-phase progression between wild type and *irc5*Δ*-1* more visible, we magnified the histograms in Figure 2.

Fig 2C and F, the quantification of the Chr 12 and 3 bands could be described better. Are the bands correlated to the total amount of DNA/lane or just to the same Chr band in the G1 lane? How do the authors assure equal loading? The same concern is also valid for Fig 5A and B.

We addressed it by modifying Materials and Methods section (p. 25) as follows: "To assure equal amount of DNA in the agarose plugs, at each time point yeast cultures samples were taken, the number of cells were determined with hemacytometer and adjusted to $3x10^7$ cells per sample (…). After electrophoresis, the gel was stained with ethidium bromide. Signal detection and quantification was performed using Bio-Rad ChemiDoc MP System and Image Lab software. For quantification, the chromosome III and XII intensity after MMS treatment and during recovery was correlated to intensities obtained for the same chromosomes in G1."

Referee #3:

In this study Litwin et al. explore the involvement of the Irc5 translocase in the tolerance to DNA damage. The authors report that Irc5-deficient cells accumulate Rad52/Rfa1 foci and checkpoint activation signals during the recovery from DNA damage induced in S-phase by MMS treatment. These cells also exhibit delayed S-phase progression and chromosome replication completion following MMS treatment, as judged by FACS and PFGE. Altogether indicating that Irc5 plays a relevant function in the completion of alkykated treated DNA.

Next, genetic interactions with DNA damage tolerance mutants are analysed, revealing synthetic sensitivity to MMS or irc5 alleles in combination with rad51/rad52 and rev3 deletion mutants. By 2D gel analysis, a reduction in X-shaped template switch intermediates accumulation in sgs1 cells is observed upon impairment of Irc5 function. Previous observations linking Irc5 function to cohesin association to chromatin prompt the authors to analyse cohesin association to chromatin at regions close to replication origins. They observe decreased cohesin levels close to origins, accompanied by increased Pol epsilon (Pol2) levels in irc5 cells, which they interpret as reduced cohesin loading in the context of fork stalling.

Lastly, authors observe chromosome replication defects following MMS treatment in scc2-4 cohesin loader mutants and describe synthetic sensitivity of scc2-4 and irc5 alleles in response to different agents inducing DNA damage. Based on these results the authors propose that Irc5 is a novel factor acting to support replication within the Rad18/Rad5/Sgs1 DNA damage tolerance pathway. They also conclude that this error free pathway is linked to Irc5-dependent enrichment of cohesin at perturbed replication forks.

The reported work is original and provides insight into a role of Irc5 in supporting replication of damaged chromosomes. However, there are important concerns on the interpretation of genetic data linking Irc5 and template switch factor's and cohesin loader's functions, as well as on the experimental design on the cohesin ChIP experiments. In its current form the manuscript fails to convincingly support the central conclusions and would need to be significantly improved to justify publication in EMBO Journal.

Major concerns:

- Authors ascribe Irc5 to the Rad18/Rad5/Sgs1 DDT pathway based on genetic analyses. These are performed at a variety of MMS concentrations in which double mutants show sensitivities equivalent to rad18, rad5 and sgs1 single deletants. However, since irc5Δ-1 cells do not exhibit MMS sensitivity at these concentrations an epistatic behaviour cannot be concluded. The analysis should be performed at concentrations in which irc5 cells do show sensitivity to be able to establish meaningful genetic interactions.

To examine better genetic interactions between *irc5*Δ-*1* and mutants important for DDT (*rad18*Δ, *rad5*Δ, *sgs1*Δ and *rev3*Δ), we performed survival assays after acute MMS treatment using single and double mutants. These experiments confirmed that *irc5*Δ-*1* is epistatic with *sgs1*Δ and additive with *rev3*Δ suggesting that Irc5 works with Sgs1 in a pathway parallel to translesion synthesis, most probably in template switch. Indeed, double *irc5*Δ-*1 rad18*Δ and *irc5*Δ-*1 rad5*Δ double mutants are not more sensitive to MMS compared to single mutants (Figure 4 and Appendix Figure 1). However, also in the case of survival test, MMS concentrations that cause viability loss of *irc5*Δ-*1* cells are lethal for *rad18*Δ and *rad5*Δ mutants preventing us to conclude that *irc5*Δ-*1* is epistatic to *rad18*Δ and *rad5*Δ. We changed the text accordingly. Considering our new results showing that both *irc5*Δ-*1 rad18*Δ and *rad18*Δ, or *irc5*Δ-*1 sgs1*Δ and *sgs1*Δ, exhibit similar levels of Rfa1-YFP and Rad52-YFP foci, whereas *irc5*Δ-*1 rev3*Δ exhibits increased incidence of Rfa1-YFP and Rad52-YFP foci compared to single mutants (Appendix Figure 2), we feel that it is justified to ascribe Irc5 to the error-free branch of DDT.

In addition, while a contribution of Irc5 to sister chromatid junctions accumulating in sgs1 cells is observed, would it not be expected to find epistasis with rad51/rad52 mutations if Irc5 was involved in a template switch DDT branch?

In addition to already shown spot assays, we performed survival curves for *irc5*Δ-*1*, *rad51*Δ, *rad52*Δ and *irc5*Δ-*1 rad51*Δ and *irc5*Δ-*1 rad5*2Δ mutants in the presence of MMS. In agreement

with our previous results, disruption of *IRC5* in *rad51*Δ or *rad52*Δ mutants causes increased sensitivity to MMS when compared to single mutants (Figure 4 and Appendix Figure 1). As was shown before in several studies (e.g., Liberi *et al.*, 2005; Motegi *et al*., 2006; Branzei *et al.*, 2008; Vanoli *et al.*, 2010; Daee *et al.*, 2012; Fumasoni *et al.*, 2015) deletion of *rad18*Δ or *rad5*Δ in the *rad51*Δ background leads to additive sensitivity to DNA damaging agents even though Rad52 and Rad51 are required for SCJ formation during template switch. Importantly, Rad52/Rad51-mediated HR can be performed independently of Rad18/Rad5 ubiquitin ligases (Branzei *et al.*, 2008; Karras et al, 2011). These data show that Rad18/Rad5 and Rad52/Rad51 have both overlapping and independent functions. We propose that Irc5 works with Rad52/Rad51 in TS but has no role in canonical HR and that this is the cause of additive sensitization of *irc5*Δ*-1 rad51*Δ and *irc5*Δ*-1 rad52*Δ mutants to MMS.

- ChIP experiments were performed in the presence of hydroxyurea and MMS. HU is also inducing fork stalling, which complicates the interpretation of the results. The experiments should be repeated in cells treated with MMS only and more time points should be analysed to provide accurate information on the timing of origin firing and replisome stalling (e.g. at 15', 30', 45', 60').

According to the reviewer's suggestion we performed ChIP experiments in the presence of MMS only, analysing Scc1 and Pol2 association with chromatin at additional time points (Figure 5 and 6). In agreement with the previous experiments performed in the presence of both MMS and HU, we detected decreased accumulation of Scc1 at early replication origins in cells lacking Irc5. In the case of ChIP analysis for Pol2, we noticed that Pol2 binding to early replication origins is almost the same in *irc5*Δ*-1* and wild type cells, whereas we have previously shown that cells lacking Irc5 accumulate more Pol2 in the presence of MMS and HU, indicating replication fork stalling. We believe that in the absence of HU individual cells progress through S-phase less synchronously reducing Pol2 ChIP sensitivity and by extension the differences between wild type and *irc5*Δ*-1*.

- The inferred link between Irc5 function in MMS and cohesin association to forks is correlational. Stronger evidence is required to conclude that the defects in MMS of irc5 cells are a consequence of reduced cohesin association to forks. On the same lines, the synthetic sensitivity conferred by irc5Δ-1 and scc2-4 combination argues that these genes share a function in MMS survival. However, since cohesin association to forks seems fully dependent on Scc4, if Irc5 contribution reflected a function in cohesin loading an epistatic effect should be expected. Authors should check if epistasis exists between cohesin-defective and irc5 alleles in terms of MMS sensitivity.

We followed the reviewer's suggestion and analyzed genetic interactions between *irc5*Δ*-1* and *scc1-73* which is a temperature sensitive allele of *SCC1*. Interestingly, we found that in contrast to the *irc5*Δ*-1 scc2-4* mutant, *irc5*Δ*-1 scc1-73* strain is viable at 30˚C and is no more sensitive than single mutants when treated with MMS (Figure 7 and Appendix Figure 4). Moreover, disruption of *IRC5* does not exacerbate the chromosome replication defect of *scc1-73* mutant and does not lead to additional accumulation of DNA damage repair foci (Figure 7). Importantly, also *irc5*Δ*-1 scc2-4* mutant did not display increased incidence of DNA repair foci compared to single mutants (Figure 7). Taken into account above results, we propose that Irc5 works in the cohesin pathway, not only during an unperturbed cell cycle (Litwin *et al*., 2017), but also following MMS-induced replication stress. Having known that the Scc2/Scc4 complex performs cohesin-independent functions in the cell, we propose that Scc2/Scc4 and Irc5 exhibit overlapping (cohesin loading) and independent roles in response to MMS-induced DNA damage. Similarly, we found that deletion of *RSC2*, encoding a non-essential subunit of the RSC complex that also contributes to cohesin loading, in the *scc2-4* background results in increased sensitivity to MMS compared to single mutants (our unpublished data). We added a new paragraph to the Discussion section (pp. 20-21) where we discussed genetic interactions between cohesin mutants and *irc5*Δ*-1*.

Minor concerns:

- Authors need to explain why replication progression does not seem delayed in the FACS profiles corresponding to the 2D gel experiments in MMS.

We added two vertical lines, which mark maximal fluorescence intensity of G1 and G2 cells, to FACS graphs in Figure 4D to visualize better that, *irc5*Δ*-1* cells progress slower through S-phase under replication stress compared to wild type, similarly to data shown in Figure 2A. Please, bear in mind that different time points were used in both experiments (90, 150, 210 and 240 min in Figure 4D and 20, 40, 60, 80, 100, 120, 140 min in Figure 2A).

- A cohesin-bound region not actively replicated should be included in experiments analysing Scc1 chromatin binding in irc5 and scc2-4 cells, to ascertain if the effect observed is specific to cohesin associated to stalled forks.

We analysed the Scc1 levels at *POA1* locus which is located on chromosome II about 30,000 bp from the nearest replication origin and enriched in cohesin in logarithmically growing wild type cells. We show that 1h after release from G1 block to fresh medium containing MMS the replisome did not reach *POA1* resulting in no accumulation of cohesin (see Figure 5A and C). These results show that the cohesin defects observed for *irc5*Δ*-1* and *scc2-4* cells is specific to cohesin associated with stalled replication forks.

- Basic information is not provided about the quantification and normalization of replication intermediates in 2D experiments. What are X-molecules levels relative to?

We have added information on the quantification method, specifying that the levels of X-molecules are relative to the monomer spot signal (see Materials and Methods section, p. 26). We are also citing a paper, Fumasoni *et al*, 2015, in which examples of quantification are shown in the Supplemental Information.

- Authors state that Irc5 disruption does not have an effect on PCNA poly-ubiquitylation. However, differences in U1 and U2 bands kinetics are observed in WT vs irc5 cells in Figure 3C. How does this relate to Irc5 function in MMS?

We have shown here that *irc5*Δ*-1* cells accumulate RPA-coated ssDNA regions. This likely leads to increased levels of chromatin bound Rad18 that boosts PCNA ubiquitylation (Davies *et al.*, 2008; Huttner and Ulrich, 2008). The inability of *irc5*Δ*-1* cells to efficiently use the sister chromatid to bypass replication blocking DNA lesion may result in prolonged ubiquitylation and/or delayed ubiquitin disassembly. We modified description and interpretation of Figure 4C on page 11 as follows: “We found that disruption of *IRC5* leads to slightly higher and prolonged PCNA ubiquitination levels compared to wild type, probably as a result of increased DNA damage accumulation observed in *irc5*-Δ*1* (see Figure 1). Thus, in contrast to INO80 and RSC, Irc5 does not promote DDT at the level of PCNA posttranslational modifications”

- There are some apparent mistakes in the spelling of references and in the description of panels in figure legends.

We have proofread the manuscript carefully. We hope that the changes we made will satisfy the Referee.

Reference list:

Branzei D, Vanoli F, Foiani M (2008) SUMOylation regulates Rad18-mediated template switch. *Nature* 456: 915-920

Daee DL, Ferrari E, Longerich S, Zheng XF, Xue X, Branzei D, Sung P, Myung K (2012) Rad5-dependent DNA repair functions of the Saccharomyces cerevisiae FANCM protein homolog Mph1. *J Biol Chem* 287: 26563-26575

Davies AA, Huttner D, Daigaku Y, Chen S, Ulrich HD (2008) Activation of ubiquitin-dependent DNA damage bypass is mediated by replication protein a. *Mol Cell* 29: 625-636

Fumasoni M, Zwicky K, Vanoli F, Lopes M, Branzei D (2015) Error-free DNA damage tolerance and sister chromatid proximity during DNA replication rely on the Polα/Primase/Ctf4 Complex. *Mol Cell* 57: 812-823

Huttner D, Ulrich HD (2008) Cooperation of replication protein A with the ubiquitin ligase Rad18 in DNA damage bypass. *Cell Cycle* 7: 3629-33

Liberi G, Maffioletti G, Lucca C, Chiolo I, Baryshnikova A, Cotta-Ramusino C, Lopes M, Pellicioli A, Haber JE, Foiani M (2005) Rad51-dependent DNA structures accumulate at damaged replication forks in sgs1 mutants defective in the yeast ortholog of BLM RecQ helicase. *Genes Dev* 19: 339-350

Litwin I, Bakowski T, Maciaszczyk-Dziubinska E, Wysocki R (2017) The LSH/HELLS homolog Irc5 contributes to cohesin association with chromatin in yeast. *Nucleic Acids Res 45*: 6404-6416

Motegi A, Kuntz K, Majeed A, Smith S, Myung K (2006) Regulation of gross chromosomal rearrangements by ubiquitin and SUMO ligases in *Saccharomyces cerevisiae*. *Mol Cell Biol* 26: 1424-1433

Vanoli F, Fumasoni M, Szakal B, Maloisel L, Branzei D (2010) Replication and recombination factors contributing to recombination-dependent bypass of DNA lesions by template switch. *PLoS Genet* 11;6(11):e1001205

2nd Editorial Decision 6th July 2018

Thank you for submitting your revised manuscript for our consideration. We have now received the re-reviews from all three original referees, and I am pleased to inform you that they all consider the manuscript significantly improved and the majority of originally raised issues addressed. However, referee 3 retains a few major concerns regarding interpretation and experimental data. Given the mentioned conclusiveness concern, I therefore feel it would be important to still attempt the originally requested 2D gel experiments utilizing the setup proposed by referee 3. Furthermore, referee 1 raises a caveat with the quantifications of Figures 2B and 3C that seem to be identical, so please check whether these panels may have been by mistake duplicated, and also provide us with the tables (values) behind the respective graphs.

REFEREE REPORTS

Referee #1:

In general the manuscript is improved and most of recommendations were addressed. It still requires proofreading as there are multiple grammatical errors.

Importantly, Figures 2C and 3D are IDENTICAL, but the gels (2B and 3C) are slightly different, I would request the raw data that was used to support these graphs.

Referee #2:

In this revised version of the manuscript "Error-free DNA damage tolerance pathway is facilitated by the Irc5 translocase through cohesin" by Ireneusz Litwin et al, I find that the issues I raised in the primary revision have been dealt with to my satisfaction.

Referee #3:

In general, the revised version of the manuscript addresses the concerns raised during revision. I therefore find this work suitable for publication in EMBO Journal.

It should be noted that epistasis between icr5 and TS mutants is not demonstrated. In addition, the interpretation of correlations between Rad52/Rfa1 foci accumulation and the function of the TS pathway is not straightforward (e.g. Rad52/Rfa1 foci are augmented in both rad18 and sgs1 mutants that either lack or accumulate TS intermediates, and icr5 mutation does not alter the abundance in combination with either). Thus, authors should be conservative when ascribing Irc5 to this pathway (e.g. "...we establish that the Snf2 family translocase Irc5 is a novel factor in the Rad18/Rad5/Sgs1 pathway of DDT..." in the abstract).

It would be advisable to carry out the 2D gel experiments proposed by referee #1, time permitting. Cells could be released from a nocodazole block to overcome G2/M accumulation problems, as this experimental setup has been used in very similar experiments (Branzei et al., Cell 2006).

2nd Revision - authors' response 20th July 2018

Referee #1:

In general the manuscript is improved and most of recommendations were addressed. It still requires proofreading as there are multiple grammatical errors.

The manuscript was proof-read by a native English speaker.

Importantly, Figures 2C and 3D are IDENTICAL, but the gels (2B and 3C) are slightly different, I would request the raw data that was used to support these graphs.

As noted by Referee 1, we indeed included identical PFGE quantifications (Figures 2C and 3D). We are very sorry for this mistake. Please find enclosed source data containing original raw data of DNA intensity quantifications for both gels and the incorrect graph in Figure 3D was replaced with the original one.

Referee #2:

In this revised version of the manuscript "Error-free DNA damage tolerance pathway is facilitated by the Irc5 translocase through cohesin" by Ireneusz Litwin et al, I find that the issues I raised in the primary revision have been dealt with to my satisfaction.

Referee #3:

In general, the revised version of the manuscript addresses the concerns raised during revision. I therefore find this work suitable for publication in EMBO Journal.

It should be noted that epistasis between icr5 and TS mutants is not demonstrated. In addition, the interpretation of correlations between Rad52/Rfa1 foci accumulation and the function of the TS pathway is not straightforward (e.g. Rad52/Rfa1 foci are augmented in both rad18 and sgs1 mutants that either lack or accumulate TS intermediates, and icr5 mutation does not alter the abundance in combination with either). Thus, authors should be conservative when ascribing Irc5 to this pathway (e.g. "...we establish that the Snf2 family translocase Irc5 is a novel factor in the Rad18/Rad5/Sgs1 pathway of DDT..." in the abstract).

We agree with Referee 3 that it is not fully justified to genetically ascribe Irc5 to Rad18/Rad5/Sgs1 pathway. Irc5 has a mild phenotype on MMS and we could not conclude epistatic relationship with Rad18 and Rad5. We also agree that correlations between Rad52/Rfa1 foci accumulation and a function in the TS pathway is not straightforward. However, we showed strong additive effect of *IRC5* disruption in *rev3*Δ background and an epistatic interaction between *icr5*Δ*-1* and *sgs1*Δ.

Moreover, Irc5 promotes formation of sister chromatid junctions that are the essence of template switch as shown by the 2D gel experiment. Taking these results into account, we feel that it is rightful to conclude that Irc5 is linked to Rad18/Rad5/Sgs1 pathway of DDT and promotes template switching (TS). To clarify this issue, we propose to change following sentences in the manuscript as follows:

Before:
"Here, we establish that the Snf2 family translocase Irc5 is a novel factor in the Rad18/Rad5/Sgs1 pathway of DDT, promoting TS and averting single-stranded DNA persistence during replication." (Abstract).
After:
“Here, we establish that the Snf2 family translocase Irc5 is a novel factor that promotes TS and averts single-stranded DNA persistence during replication.”

Before:
"Genetic analysis suggests that Irc5 is involved in the Rad18-Rad5-dependent error-free DDT pathway, but acts in parallel with canonical HR and TLS." (p. 5, Introduction section).
After:
“Genetic analysis suggests that Irc5 is linked to Rad18-Rad5-Sgs1-dependent error-free DDT pathway, but acts in parallel with canonical HR and TLS”

Before:
"Remarkably, disruption of *IRC5* in the *sgs1*Δ background resulted in decreased levels of X-molecules (Fig 4D and Appendix Fig S3). Thus, Irc5 contributes to the formation of MMS-induced SCJs. Consistent with the notion that Irc5 works with Sgs1 in the same pathway mediated by Rad18/Rad5 (Branzei et al, 2008, Karras et al, 2010), the *irc5*-Δ*1 sgs1*Δ mutant was no more sensitive to MMS than *sgs1*Δ (Fig 4E and Appendix Fig S1F)." (p. 12, Results section).
After:
"Remarkably, disruption of *IRC5* in the *sgs1*Δ background resulted in decreased levels of X-molecules (Fig 4D and Appendix Fig S3). Thus, Irc5 contributes to the formation of MMS-induced SCJs. Consistently, the *irc5*-Δ*1 sgs1*Δ mutant was no more sensitive to MMS than *sgs1*Δ (Fig 4E and Appendix Fig S1F)."

It would be advisable to carry out the 2D gel experiments proposed by referee #1, time permitting. Cells could be released from a nocodazole block to overcome G2/M accumulation problems, as this experimental setup has been used in very similar experiments (Branzei et al., Cell 2006).

If we understand correctly, Referee 3 suggests performing 2D gel experiments with *scc2-4sgs1*Δ and *scc2-4 sgs1*Δ *irc5*Δ*-1* mutants to determine the relationship between Irc5 and Scc2 (additive or epistatic) in the DDT by investigating the X-molecules levels. We think that 2D gel experiments would not give a clear answer to this question. First, 2D gel assay is more qualitative then quantitative method that is not suitable for conclusive epistasis tests regarding additive/epistatic effect, unless suppression is observed, which is not the case here. Secondly, it has been already shown by Tittel-Elmer *et al*. (2012, Mol Cell) as well as Fumasoni *et al*. (2015, Mol Cell) that dysfunction of cohesin results in a strong reduction of X-molecule signal to a background level as it is the case for *RAD18* deletion. Performing similar experiment with the *scc2-4* allele will likely result in comparable or even stronger decrease of X-molecule signal. Moreover, this would most likely prevent detection of possible modest changes caused by an additional *IRC5* disruption. Third, *scc2-4 sgs1*Δ *irc5*Δ*-1* cells, that accumulate in G2/M phase probably due to genomic instability, would not synchronously begin a new cell cycle, even with nocodazole pretreatment and release. This lack of synchronicity between *scc2-4 sgs1*Δ and *scc2-4 sgs1*Δ *irc5*Δ*-1* cultures would also add to inconclusiveness of this test.

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Corresponding Author Name: Ireneusz Litwin
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2017-98732

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

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.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

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?	A minimum of 2 biological repeats were performed for each experiment. Unpaired T-test was used to determine statistical significance. See Materials and Methods section for details.
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?	No samples were excluded from the data analysis.
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
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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
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5. For every figure, are statistical tests justified as appropriate?	Yes, see Figure Legends and Materials and Methods
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Unpaired T-test
Is there an estimate of variation within each group of data?	Yes, calculated standard deviations shown by error bars
Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

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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 antibodies used are commercially available. All suppliers are provided in Materials and Methods
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	All yeast strains and their sources are listed in Appendix Table 1

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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
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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
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F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for '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
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