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DNA polymerase zeta contributes to heterochromatin replication to prevent genome instability

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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. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision 4th Mar 2020

Thank you for submitting your manuscript on Pol zeta heterochromatin replication roles to The EMBO Journal. I have now received the enclosed four reports from experts in replication timing/dynamics (refs 1 & 4) and specialized DNA polymerases (refs 2 & 3). As you will see, all referees acknowledge the potential interest of your study and appreciate the large amount of data presented, but they also raise a number of substantive concerns with the conclusiveness of some of the key data in the present form. In particular, the referees share major reservations regarding the utilized cell lines/systems and the conclusions that can be drawn from Tag-immortalized MEFs and HeLa cells, especially with respect to replication timing. Another shared major concern relates to the mutagenesis experiments and the clonal selections underlying them. In this light, I feel that the study is at the current stage not yet sufficiently compelling to warrant EMBO Journal publication.

Since all referees still acknowledge the potential of this work if properly revised, I would nevertheless like to give you an opportunity to address their criticisms by way of a major revision of this manuscript. I realize that decisively addressing the key issues will likely require significant further time and effort and may possibly also lead to alterations of some of the conclusions, making it difficult to predict the eventual outcome of re-review, but I do feel that the study could well become a much more compelling candidate for an EMBO Journal article if substantiated along the lines suggested by the referees. I would therefore be open to possible extensions of the resubmission deadline, as well as to discussing any questions related to the referee reports and proposals for addressing them at any stage during the revision period. I would further be available for discussing possibilities of publishing a revised version in one of our sister journals, in case a full revision for The EMBO Journal should not be possible or in fact result in weakening of some of the current conclusions.

I should add that it is our policy to allow only a single round of (major) revision, making it important to comprehensively answer to all points raised at this stage. Further information on preparing and uploading revised manuscript files can be found below and in our Guide to Authors.

Thank you again for the opportunity to consider this work for The EMBO Journal. I look forward to hearing from you in due time.

Referee #1:

This study reports that the translesion DNA polymerase zeta is localized to heterochromatin, and is essential for unperturbed and timely heterochromatin replication. This observation confirms and extends several recent reports proposing a role for translesion polymerases during unperturbed chromosome duplication. The observations reported in the paper suggest that REV3L, a catalytic subunit of Polζ, is recruited to prevent replication stress in heterochromatin is intriguing. The effects of interference with REV3L function in MEFs reportedly include: 1/disrupted replication timing leading to a higher frequency of mutagenesis, and 2/ Repression of development-regulated gene expression.

The manuscript provides important support to an interesting hypothesis. Some of the observations are clearly presented and have a potential to impact our understanding of the role of translesion polymerases. In particular, evidence for interaction of pol zeta with heterochromatin and DNA damage markers is strong (with the exception of a single PLA experiment, see below) and the paper provides convincing evidence for a selective role of pol zeta in protecting heterochromatin from replication stress. However, as currently written, several issues should be addressed, including the interpretation of key results such as the CGH and mutagenesis data. Importantly, the paper will benefit from a clarification of how the combined observations provide strong mechanistic insights for Polζ mediated embryonic lethality and mutagenesis. Experimental and interpretation issues that require addressing, as well as some minor typos, are listed below.

Major points:

1. Interpretation replication timing data: The paper reports an interesting observation, showing that the relative copy number of late-replicating regions is lower in Polζ deleted cells. This result is interpreted as a change in replication timing, which describes the order in which replication occurs during S-phase. The same observation, however, could also indicate that in the depleted cells, regions that normally replicate late (often heterochromatin) are not replicated (or undergo very slow and sparse DNA synthesis) in the absence of Polζ. To test for a potential change in replication timing, regions that undergo timing transitions (early to late as well as late to early) should be identified and the transitions should be quantified. The alternative hypothesis of no or slow replication in some late-replicating regions is consistent with the induction of selective replication stress in heterochromatin, and with the changing proportions of early- and late-phase cells reported in the paper.
The conclusion that replication delays (("changed replication timing") are particularly evident in temporal transition regions (TTRs) should also be tested for statistical significance. The representative replication timing tracks shown in Figure 2B are not all supportive of this hypothesis (for example, chromosome 2 contains a region undergoing replication delay (adjacent to the 78 Mb mark) that is not limited to the TTR.
2. Quality of the mutagenesis data: Stronger support is required for the hypothesis that mutagenesis is more frequent in TTRs. The data shown in Figure 3C report a higher statistical significance for increased mutational frequency in late-replicating regions than in both TTRs and regions of delayed replication, consistent with previous reports in the literature. Mutagenesis data in early replicating regions are very variable (as is evident by the size of the error bars).

3. For the experiments used in figures 4-7: which passage was used:
4. Fig. 2B: What are the criteria for 'disrupted' regions?
5. Fig. 3 C: Analyses of the p60 rev- cells shows similarity to the WT, is there an explanation for this? How reproducible is this observation?
6. Fig. 4D: The association with heterochromatin is interesting, however, the study will benefit from the inclusion of a control ChIP using an active mark ChIP (H3K27ac, H3K4me3).
7. The PLA staining in Fig. 5 exhibits a high background and it would be advisable to use a second method to measure physical interactions of Rev3L.
8. Fig. 6: In addition to the exogenous, truncated Rev3L constructs, would it be possible to determine the subnuclear localization of endogenous Rev3L?
9. It would be good to clarify the rationale underlying the use of p60 in this study.

Minor points:
1. Fig. 1A: Details of the gating strategy are missing. Statistical analysis is required to test the conclusions comparing Rev3L positive and negative cells.
2. Fig.1 B: The number of cells counted per condition should be reported. (also for other figures, including 7G).
3. Fig. 2A: Arrows should be defined in the legend. Why are they not shown for chr1 and 9?
4. Fig. 4A: The blue color is overwhelmingly strong and it is hard to observe specific signals.
5. Fig. 4 C : Error bars are missing
6. Parentheses should be closed on page 3, line 3.
7. A clarification is needed for the sentence in line 5 of the Figure 2 legend,

Referee #2:

This is an interesting study, which suggests that polz specifically functions to assist heterochromatin replication. A substantial amount of work is presented, covering various approaches and technologies, which is commendable. However, several issues need to be addressed by the authors, as detailed below.

1. It is my understanding that the authors used in this study MEFs immortalized with SV40-Large T-antigen, described in ref 23. These cells tolerate the Rev3L deletion, replicate much faster than primary MEFs, and unlike the primary MEFs do not undergo massive senescence or apoptosis. What is the number of spontaneous DSB in the Large Tag-immortalized MEF compared to the primary MEFs (both in Rev3L+/+ and Rev3L-/-)? These differences should be pointed out and discussed.
2. The authors claim that polz assist heterochromatin replication. Do they mean essentially all heterochromatin regions in cell, regardless of location/function? Or is it a specific involvement in the pericentromeric heterochromatin? Is polz involved in euchromatin replication as well? After all the latter also contains hard-to-replicate sequences. While this does not require additional experiments, the authors should clarify the way they see the broader function of polz during chromosomal replication, clearly pointing out which parts are supported by their (or other's) data.
3. The Tag-immortalized Rev3L-/- MEFs are presumably unstable, and accumulate mutations. It would be important to verify that the observed effects are indeed caused by the lack of polz by doing complementation experiments, for at least some of the main effects.
4. In analyzing S phase progression, the authors state that late S phase patterns are characterized by dots localized mainly at the nuclear periphery (Fig. 1B, C). This is not seen in the MEF IF image (Fig. 1B), and should be performed using confocal microscopy.
5. To which extent is the magnitude of the log ratio early fraction vs late fraction - a quantitative

variable? In Fig 2A a change in sign is seen only for Chr9.
6. What is the doubling time of the REV3L+/+ and Rev3L-/- MEFs?
7. Is there a difference in the number of DSB at p6 vs. p60 in each Rev3L+/+ and Rev3L -/- MEFs?
8. Can some of the effects observed in Rev3L-/- MEFs be caused by the DSB rather than the lack of active Rev3L?
9. In determining mutations using deep sequencing - how did the authors filter-out artifacts generated during processing (e.g., amplification)? Sequencing the libraries twice is not sufficient for artifact mutations present in the libraries.
10. The authors present the count of 'Mutational events'. They should present Mutational frequency. Otherwise it is difficult to evaluate the significance of the results, e.g., how do they compare to the frequency of mutations in other studies.
11. In Fig. 3C, most mutations were detected in early replicating regions, while according to the literature (which the authors cite), there are more mutations in late replicating regions. Having frequencies here (see item 10 above), might help clarify the issue.
12. A negative control is needed for the IP of Flag-Rev3L, which is a big protein, to which many proteins may stick. Perhaps CENP, which associates with minor satellites may be a good control.
13. The co-localization results in Fig. 6 are problematic. Merging the images of green and red fluorescence signals should create yellow signals if the proteins co-localize. This is not seen Fig. 6, B, C and E. The green and red stains indeed seem to be in the same general region - but in fact it appears that they do NOT co-localize, otherwise yellow dots would be visible.
14. In the PLA data in Fig. 7, controls for the specificity of each of the gH2AX-HP1b and 53BP1-HP1a interactions should be presented.
15. The authors state that repair of DSB in Rev3L-/- cells is slower than in Rev3L+/+, but do not present data showing it. This data should be presented, or the statement removed from the manuscript.

Referee #3:

The translesion synthesis (TLS)-associated DNA polymerase Rev3 has previously been shown to be involved in the replication of fragile sites. In this manuscript the authors further investigate the role of Rev3 in 'normal' replication. They describe that specifically replication of heterochromatic pericentric regions is delayed in the absence of Rev3. Rev3 appears to be recruited to these regions by binding to the HP1 proteins. Also, breaks, deletions and insertions in these regions are increased, imprinting is altered as well as the expression of (frequently development-related, genes encoded in these regions. Finally, the authors unveil a defect in homology-dependent double-strand breaks repair (DSBR)in Rev3-deficient cells.

These data are interesting and relevant, and most experiments have been well done and interpreted. However, not all conclusions appear warranted and some relevant literature has not been cited.

Major issues:
1. For many (including cell-cycle) experiments the authors have used a single Rev3 MEF line. Given the phenotypic variability of MEF lines this restricted approach might be risky. It would have been nice to use more than one line, or to complement the line with wt Rev3. Admittedly, the authors also use Rev3 siRNA-treated Hela but this is an entirely different cell type and the analysis of these cells was restricted to a cell cycle experiment.
2. The mutagenesis experiment has not been performed in an appropriate fashion. Thus, polyclonal pools of cells were sequenced at p=5 and p=60 generations. In this fashion, 'new' mutations will not

be identified as they most likely will remain subclonal. The experiment should have been performed by sequencing a (monoclonal) subclone at p-5, expanding this subclone to p=60, subclone again and then sequence one or more of these subclones. Also, it is not clear from which strain background the Rev3 (and wt) MEFs are derived so it is not certain whether it is appropriate to compare these sequences to the reference genome that may be of an entirely different strain. Furthermore, it is not logical nor expected why the number of mutations in temporal transition regions (TTR) regions is reduced in Rev3 MEFs at p=60 as compared with p=5 as in both cases it concerns polyclonal populations of the same genotype and thus the mutations at p=5 should also be present at p=60. The authors state that this can be explained by selective loss of heavily mutated Rev3 cells resulting in (oligo)clonality but this is an unsatisfactory explanation and would also suggest that loss of Rev3 leads to more mutations than in wt cells, which would be at variance with the proposed hypomutability of Rev3 cells. In this light, why do Rev3 cells appear to have more nucleotide substitutions (at p=5) than wt cells (Fig. S2)?
3. It was not clear to me (or I have missed it) whether in Rev3 cells the relative fraction of deregulated genes was higher in the RT zones than outside of these RT zones. This is expected if the deregulation of replication timing in these zones affects gene expression.
4. DSBR is perturbed in Rev3 cells. This is an interesting though not completely unexpected finding (DSBR is also perturbed in cells with defects in the Rev3 partners Rev7 and Rev1, why were these data not cited?). Importantly, rather than a defect in TLS, the defect in DSBR might explain the claimed propensity of rearrangements at pericentric chromatin in Rev3 cells. This should be discussed.

Minor issues:
1. The role of Rev3 in DSBR should be included in the abstract.
2. What fraction of the TTR requires Rev3 for efficient replication?
3. The Sale lab has shown imprinting defects in Rev1 cells. It would be appropriate to discuss the epigenetic alterations in Rev3 cells that are described here in the light of findings.
4. Fig. 1A bar graphs: Are the differences between the wt and Rev3 statistically significant? What is the reason that the cell cycle distribution is the same for both wt and Rev3 MEFs at T0h? I would expect that there would be a difference due to slower S phase progression in the Rev3 MEFs.
5. Fig. 1B. Pie charts: I assume that the colors corresponding to the SIII and SIV fraction are incorrect and should be swapped.
6. UV-C light is a much better agent than X-rays to analyze replication-associated DSB formation in a cytogenetic fashion.

Referee #4:

Starting from the observation that during embryonic development Rev3L is an essential gene, Yamin and colleagues have created Rev3L knock-out immortalised MEFs to analyse the function of this TLS polymerase during unperturbed cell cycle.
The authors analyse progression through S-phase in the KO cells, finding a delayed progression through the last part of S-phase. In agreement with this finding, they show a delayed replication of some of the replication timing transition zones and an accumulation of mutations in these regions. REV3L localises at heterochromatin through interaction with HP1s, through a canonical peptide located between residues 761-1029. Absence of REV3L correlates with increased endogenous DNA damage and a delayed repair of irradiation-induced double strand breaks.

I appreciate the amount of work that this paper contains and that manipulation of an essential and very large protein like REV3L is very difficult. I think that the results are interesting but over-

interpreted. There is not enough evidence to say that the role of Rev3L is SPECIFIC for heterochromatin, but certainly the data demonstrate that there is a role at heterochromatin during repair. The link to replication is less convincing, as the damage does not appear to be specific to S-phase.
The data on replication timing and gene expression are very weak (see below the detailed comments). However, I find that the part of the paper relative to the localisation of REV3L at heterochromatin through interaction with HP1b well done and convincing.

Overall, I think that the paper needs to be substantially revised, but it could be reconsidered once the comments are addressed.

MAJOR COMMENTS:

In general, I don't think the first part relative to replication timing, gene expression and accumulation of mutations is strong enough. The use of large T immortalised MEFs limits what claims can be made due to genomic instability and continuous clonal selection (see specifics below). One way around could be to use Large T immortalised MEFs carrying the conditional Rev3L allele, infect them with empty vector or Cre-encoding virus and compare them after just few days. This gives a much more reliable base-line.

Figure 1 A: T0 indicates that there are no differences in the cell cycle distribution between wt and KO cells. After 2 hours there is a green arrow pointing at BrdU- G2 cells. What is it meant to show? This experiment is designed to follow the progression of BrdU+ cells through cell cycle. At 4 hours there are less BrdU+ cells back in G1, indicating a possible G2/M delay. However, re-entry into S seems comparable in the two genotypes. This is all very logical in cells that are Large-T immortalised and therefore have no G1/S checkpoint. Hard to deduce a mid/late S-phase delay from this data. The HeLa siRNA experiment supports the idea that the problem is in G2/M, delaying re-entry into G1. Again, HeLa cells have no G1/S checkpoint.

Figure 1 B and C: show a change in the distribution of the spatial patterns of replication. It has already been shown that spatial organisation does not necessarily reflect replication timing (uncoupling in the Rif1 null cells). Therefore, this figure does not show a change in the replication timing program (as stated in the first sentence of the paragraph "Loss of Rev3L disrupts...").

Figure 2: please specify what "p5 and p7 or p60" mean. How were the passages counted? From derivation or from immortalisation? This is crucial for the whole paper. Large T immortalisation leads to clonal selection. Moreover, large T immortalised cells are genomically unstable. Subtle changes are therefore very hard to judge. For example, evaluating mutation burden is really unreliable with this type of immortalisation.
For the replication timing, how many biological and technical replicas were analysed? The shifts indicated in the figure could within clonal variability....
Why the shifts to later replication in TTR would be more significant than the switches to earlier or later replication also shown in the same figures? What is the threshold that has been applied to call a shift=change? What is the percentages of TTR that change?
What happens to the size of replication timing domains?

Figure 3: I don't think this type of analysis can be meaningful in Large T immortalised cells. Clonal selection is clearly evident.

Figure 4: changes in gene expression suffer from the same problem of analysing clonal and

genomically unstable populations. One way around this problem could be to employ the conditional immortalised MEFs and analyse mutation burden, gene expression and epigenetic profiling pre vs. post Cre infection. In this way there would be a base-line that is specific to each cell line.

For the genes in Table 1, there is no statistical analysis provided regarding the significance of this 7.6% of deregulated genes falling into changed TTR?

For example, in Figure 7, it is presented the analysis of co-localisation of DNA damage markers with HP1b. However, what would be the results by doing the same analysis with H3K4me3, for example? REV3L seems to significantly co-localise with H3K4me3 in Figure 5E. What is the general kinetics of 53BP1 foci removal from the Rev3L-/- cells compared to the wild type? In Figure 7G, what is defined "pericentromere proximal" is, in reality, far apart. The resolution on chromosomal spreads is far too low to claim anything about proximity.

• The last paragraph links the increased DNA damage burden to S-phase, but there is really no evidence for this. An S-phase specific analysis of DNA damage accumulation would be needed for this claim.

• I think the authors should limit the statement to a role during repair in heterochromatin, not necessarily specific.

I have some MINOR COMMENTS on the figures in the second part.

Figure 6 B: the figure should include non-transfected cells in the same field, to ensure that in Suv39h DN we are looking at transfected and delocalised REV3L and not just fluorescence background. Same for Figure 6 B. In addition, it could also be useful to have non-pre-extracted cells shown in Figure 6C.

Figure 6 F: GST-REV3L 700-900 in the left panel (WT and V802D). The figure legend states that is FH-tagged too. Is this a mistake? Molecular weight ladder should be indicated in the right panel.

Figure 7A and C: the difference of the PLA foci between NT in wt and Rev3L-/- in panel C is much higher than in panel A. Why? It should also be made clear in the paragraph relative to this figure that this comparison can be made because there are no major differences in the cell cycle distribution of the two genotypes.

Point-by-point response

We thank all four of the reviewers for their support of the importance of our reported findings and for the extensive constructive comments. Below is the point-by-point response, keyed to the revised manuscript.

Referee #1:

This study reports that the translesion DNA polymerase zeta is localized to heterochromatin, and is essential for unperturbed and timely heterochromatin replication. This observation confirms and extends several recent reports proposing a role for translesion polymerases during unperturbed chromosome duplication. The observations reported in the paper suggest that REV3L, a catalytic subunit of Polζ, is recruited to prevent replication stress in heterochromatin is intriguing. The effects of interference with REV3L function in MEFs reportedly include: 1/disrupted replication timing leading to a higher frequency of mutagenesis, and 2/ Repression of development-regulated gene expression. The manuscript provides important support to an interesting hypothesis. Some of the observations are clearly presented and have a potential to impact our understanding of the role of translesion polymerases. In particular, evidence for interaction of pol zeta with heterochromatin and DNA damage markers is strong (with the exception of a single PLA experiment, see below) and the paper provides convincing evidence for a selective role of pol zeta in protecting heterochromatin from replication stress.
However, as currently written, several issues should be addressed, including the interpretation of key results such as the CGH and mutagenesis data. Importantly, the paper will benefit from a clarification of how the combined observations provide strong mechanistic insights for Polζ mediated embryonic lethality and mutagenesis. Experimental and interpretation issues that required addressing, as well as some minor typos, are listed below.

Major points:

1. Interpretation replication timing data: The paper reports an interesting observation, showing that the relative copy number of late-replicating regions is lower in Polζ deleted cells. This result is interpreted as a change in replication timing, which describes the order in which replication occurs during S-phase. The same observation, however, could also indicate that in the depleted cells, regions that normally replicate late (often heterochromatin) are not replicated (or undergo very slow and sparse DNA synthesis) in the absence of Polζ.

The replication timing profile is based on the quantification of BrdU incorporation in cell fractions sorted in early and late S phase (i.e: measure of the log2 ratio of early *vs* late fractions). If a genomic region is not replicated or sparsely replicated, BrdU incorporation will be very weak, leading to no or low DNA immunoprecipitation so that the signal will be near background log2 ratio will be near zero. We do not observe this. Instead, the major effect of *Rev3l* loss (>80% of disturbed regions) is boundary shifts, corresponding to a delay in replication regions that lie between early and late replicating domains. These segments of DNA are still replicated, but with delayed timing.

To test for a potential change in replication timing, regions that undergo timing transitions (early to late as well as late to early) should be identified and the transitions should be quantified. The conclusion that replication delays ("changed replication timing") are particularly evident in temporal transition regions (TTRs) should also be tested for statistical significance.

This is an important point. All regions that have undergone timing transitions have been now identified and quantified. To perform a more rigorous analysis, profiles of replication timing have now been analyzed using the START-R program recently developed by the Cadoret lab (co-authors of this work). This software (Hadjadj et al., *NAR Genom Bioinform* 2020) is cited in our revised manuscript. To detect a significant difference between *Rev3l*$^{+/+}$ and *Rev3l*$^{-/-}$ mouse cells, we applied the classical mean method with a threshold of p value= 0.01. We found that in *Rev3l*-deficient mouse cells, about 5.7% of the whole genome was affected (examples in Fig. 2A). 19.2% (in bp) of these regions were advanced in timing and 80.8% of regions were delayed (EV2B). We also found that 18.7% of TTR were disrupted in *Rev3l*$^{-/-}$ mouse cells. This information has been included on pages 5 and 6, of the revised manuscript, with statistical results shown in the Figure panels.

The representative replication timing tracks shown in Figure 2B are not all supportive of this hypothesis (for example, chromosome 2 contains a region undergoing replication delay (adjacent to the 78 Mb mark) that is not limited to the TTR.

The previous Figure 3B is being referred to here, we believe. It is true that this curve suggests a difference between *Rev3l*$^{+/+}$ and *Rev3l*$^{-/-}$ cells in that genomic region. However, employing the START-R analysis with a threshold p value = 0.01 did not reveal a statistically significant difference for many genomic regions. Fig 2A and EV2C show the results and examples of our rigorous analysis.

2. Quality of the mutagenesis data: Stronger support is required for the hypothesis that mutagenesis is more frequent in TTRs. The data shown in Figure 3C report a higher statistical significance for increased mutational frequency in late-replicating regions than in both TTRs and regions of delayed replication, consistent with previous reports in the literature. Mutagenesis data in early replicating regions are very variable (as is evident by the size of the error bars).

As recommended by Reviewer #1, and by the other referees, we completely re-designed the experiments to investigate the mutational landscape in *Rev3l*$^{-/-}$ cells in collaboration with experts in bio-informatics (S. Nikolaev and A.Yurchenko, Gustave Roussy, France). In our previous experiment, we sequenced only 3 chromosomal regions corresponding to approx. 25 Mb (former Fig 3). In the new experiments, whole-genome sequencing was performed from 4 subclones of *Rev3l*$^{+/+}$ and *Rev3l*$^{-/-}$ cells after 90 doublings (about passage 30) to get a complete overview of the mutational landscape (Fig 6A). We found that nucleotide substitution rate in the disturbed TTR was not significantly different between *Rev3l*$^{-/-}$ and *Rev3l*$^{+/+}$ cells at the genome-wide level (in contrast to what we observed when we analyzed only 25 Mb). However, there was a significant increase of genomic deletions (ranging from 55 to 10 000 bp) in absence of REV3L function (Fig 6B,C), and these deletions preferentially occur in late replicating regions as well as in specific genomic regions that are disturbed in *Rev3l*$^{-/-}$ cells (Fig 6D). These results are consistent with the work reported by Volkova *et al* who observed an increased numbers of indels and deletions in *C.elegans* REV-3 mutant after genotoxic stresses (Volkova et al., *Nature Communications* 2020). Therefore, the previous Fig 3 has been removed and replaced by the new Fig 6 which focuses only on the structural variations detected in *Rev3l*$^{-/-}$ genomes.

3. For the experiments used in figures 4-7: which passage was used:

All experiments described in the revised version (except WGS) were performed with MEFs at passages between 10 and 20 (no more than 2 months in culture).

4. Fig. 2B: What are the criteria for 'disrupted' regions?

The word “disrupted” was used in the previous version to specify the genomic regions that showed a statistically significant change in replication timing. To be more clear and avoid confusion with gene disruption, we used the word “disturbed” in the revised version (Fig 2) and defined it on page 6 of the manuscript.

5. Fig. 3 C: Analyses of the p60 rev- cells shows similarity to the WT, is there an explanation for this? How reproducible is this observation?

As noted above (see point 2), we re-designed the experiments and used only one passage corresponding to about 90 doublings for both cell lines (*Rev3l*$^{+/+}$ and *Rev3l*$^{-/-}$ MEFs).

6. Fig. 4D: The association with heterochromatin is interesting, however, the study will benefit from the inclusion of a control ChIP using an active mark ChIP (H3K27ac, H3K4me3).

This is a valid point. We obtained data by ChIP for H3K27ac and H3K9ac and this is now included in the revised Fig 3D. A reduction in these active histone marks in *Rev3l*$^{-/-}$ cells was found for *Hox2*, *Hox8* and *WT1*.

7. The PLA staining in Fig. 5 exhibits a high background and it would be advisable to use a second method to measure physical interactions of Rev3L.

This is true. As shown in previous Fig 5 (now Fig 4C and EV4E), the H3K9ac antibody in particular has a high background. Nevertheless, there was no increase of PLA between H3K9ac and Flag-REV3L, while we observed a significant increase above background for PLA with other histone marks. The interaction of REV3L with these histone marks may be indirect (e.g. via HP1 and other proteins), or direct. Our result points to the possibility of future studies of the nature of such physical interactions with modified chromatin.

8. Fig. 6: In addition to the exogenous, truncated Rev3L constructs, would it be possible to determine the subnuclear localization of endogenous Rev3L?

Like others, we have not been able to detect endogenous REV3L with available antibodies by immunofluorescence staining. The authors have previously raised many antibodies for such test without success. For future work, we are working again on mouse monoclonal antibodies with the BIOTEM Company. Clones for two promising hybridomas are being amplified.

9. It would be good to clarify the rationale underlying the use of p60 in this study.

We cultured cells for a period to check the stability of our observations. Using START-R program, we compared the replication timing obtained at different passages for each cell line. For *Rev3l*$^{+/+}$ MEFs, there was a 1.9% difference between p5 and p60. For *Rev3l*$^{-/-}$ cells, we observed only 0.2% difference between p7 and p60, suggesting that the replication timing remains stable during cell culture passages in both cell lines, and can be considered as two independent replicates (shown now in Figure EV2A). As noted for comment 3, other experiments in this revised version have been performed with MEF passages 10-20.

Minor points:

1. Fig. 1A: Details of the gating strategy are missing. Statistical analysis is required to test the conclusions comparing Rev3L positive and negative cells.

In the revised Figure 1A, the gating strategy is now shown. Statistical analysis and testing is now shown in the bar graphs below the FACS plots.

2. Fig.1 B: The number of cells counted per condition should be reported. (also for other figures, including 7G).

Thank you, the number of cells counted is now noted in the respective figure legends.

3. Fig. 2A: Arrows should be defined in the legend. Why are they not shown for chr1 and 9?

Thank you, in the new Figure 2 and EV2 the arrows have been replaced and/or supplemented by color coding.

4. Fig. 4A: The blue color is overwhelmingly strong and it is hard to observe specific signals.

Yes – the range has now been toned down in the revised figure (now Figure 3A and EV3C)

5. Fig. 4 C : Error bars are missing

The bars are missing because the histograms represent signal intensity calculated by ImageJ for samples 1X on the western blot shown in above panel. This is now described in the Figure 3 legend. This ratio has been verified for samples 2X and in replicated experiments.

6. Parentheses should be closed on page 3, line 3.

corrected

7. A clarification is needed for the sentence in line 5 of the Figure 2 legend

corrected

Referee #2:

This is an interesting study, which suggests that polz specifically functions to assist heterochromatin replication. A substantial amount of work is presented, covering various approaches and technologies, which is commendable. However, several issues need to be addressed by the authors, as detailed below.

1. It is my understanding that the authors used in this study MEFs immortalized with SV40-Large T-antigen, described in ref 23. These cells tolerate the Rev3L deletion, replicate much faster than primary MEFs, and unlike the primary MEFs do not undergo massive senescence or apoptosis. What is the number of spontaneous DSB in the Large Tag-immortalized MEF compared to the primary MEFs (both in Rev3L+/+ and Rev3L-/-)? These differences should be pointed out and discussed.

Yes, as noted, the primary MEFs die within a few cell divisions from checkpoint-mediated senescence and apoptosis. At the first metaphase after cre-mediated disruption, 50% of primary mitotic spreads had visible chromosome breaks, compared with about 10% for wild type primary MEFs (Lange et al 2012). With T-antigen immortalization, ongoing breaks remain high, but the pathways leading to cell death are suppressed. Assays for micronuclei (arising from unrepaired chromosome breaks in the previous mitosis show about 22% of immortalized cells have micronuclei vs about 3% for Rev3l-proficient MEFs (Tomida et al 2015, Martin et al 2021). In the

revised version we discuss that the immortalized MEFs (like primary MEFs) are undergoing severe replicative break stress on page 13 and 14.

2. The authors claim that polz assist heterochromatin replication. Do they mean essentially all heterochromatin regions in cell, regardless of location/function? Or is it a specific involvement in the pericentromeric heterochromatin? Is polz involved in euchromatin replication as well? After all the latter also contains hard-to-replicate sequences. While this does not require additional experiments, the authors should clarify the way they see the broader function of polz during chromosomal replication, clearly pointing out which parts are supported by their (or other's) data.

This is an important suggestion, a summary is now included in the discussion on page 13 and Fig 7I. Overall, the replication timing data suggests that heterochromatic regions rely especially on Polζ, but it is likely that Polζ is involved with other difficult to replicate sequences such as common fragile site as shown by Bhat et al. NAR 2013. To reinforce our hypothesis that Polζ/REV3L contributes to the replication of heterochromatic regions, we examined the velocity of replication forks in pericentromeric heterochromatin (PHC) using a DNA combing/FISH approach. We found that in *Rev3l*$^{-/-}$ cells, DNA replication dynamics is strongly reduced in PHC while it remains nearly unaffected for the genome globally as compared to WT cells. This data has now been added as new Fig 5A. Consistent with that, we analyzed the stability of PHC in metaphase spreads by FISH in *Rev3l*$^{-/-}$ cells. We observed numerous abnormalities, including breaks, loss, duplication and rearrangements. This has been added as a new Fig. 5F.

3. The Tag-immortalized Rev3L-/- MEFs are presumably unstable, and accumulate mutations. It would be important to verify that the observed effects are indeed caused by the lack of polz by doing complementation experiments, for at least some of the main effects.

For the revised version we have now analyzed complemented MEFs as well as several human cell lines. Accumulation of spontaneous DSB in heterochromatin after depletion of REV3L has been detected in several cell lines including MEFs, RPE and HeLa (shown now in Fig 5D and E). These breaks are significantly reduced in REV3L-complemented cell lines. This is consistent with the idea that Polζ/REV3L facilitates the replication of heterochromatic regions, and is presented in the new Fig 7F-H.

4. In analyzing S phase progression, the authors state that late S phase patterns are characterized by dots localized mainly at the nuclear periphery (Fig. 1B, C). This is not seen in the MEF IF image (Fig. 1B), and should be performed using confocal microscopy.

As suggested, images have now been performed using confocal Leica SPE and a more through description is now given on manuscript page 5. Only the larger/dots foci tend be localized at the nuclear periphery.

5. To which extent is the magnitude of the log ratio early fraction vs late fraction - a quantitative variable? In Fig 2A a change in sign is seen only for Chr9.

This is the value of the log2 ratio of early *vs* late fractions. A detailed description is now described in (Hadjadj et al., *NAR Genom Bioinform* 2020).

6. What is the doubling time of the REV3L+/+ and Rev3L-/- MEFs?

The doubling time for *Rev3l*$^{+/+}$ and *Rev3l*$^{-/-}$ MEFs was measured as 19.1 and 17.2 hr, respectively (Wittschieben et al Cancer Res 2006) (see also graph in Fig. 5E of Lange et al Nucleic Acids Res 2012 for establishment of the Tag immortalized lines).

7. Is there a difference in the number of DSB at p6 vs. p60 in each Rev3L+/+ and Rev3L -/- MEFs?

The number of DSBs between p6 and p60 in each group was not significantly different. However, as mentioned above, we removed the Fig 3 that compared mutational events between the two passages (i.e. p6 and p60.) Therefore, we did not include this data in the revised manuscript.

8. Can some of the effects observed in Rev3L-/- MEFs be caused by the DSB rather than the lack of active Rev3L?

Additional DSB caused by lack of REV3L will contribute to increased deletion/rearrangement mutagenesis as we discuss. The replication timing shifts in *Rev3l-/-* cells may be caused partly by unrepaired breaks in heterochromatic regions; the two phenomena are closely connected.

9. In determining mutations using deep sequencing - how did the authors filter-out artifacts generated during processing (e.g., amplification)? Sequencing the libraries twice is not sufficient for artifact mutations present in the libraries.

As recommended by all Reviewers, we re-examined the mutational landscape in unchallenged *Rev3l-/-* cells as compared with *Rev3l+/+*cells after 90 doublings. In our previous version, we sequenced only 3 chromosomal regions corresponding to approx. 25 Mb. In response to the reviewers' critiques, we re-designed the experiments, expended 4 single clones and performed whole genome sequencing to get a complete overview of the mutational landscape. While nucleotide substitution rate was not significantly different genome-wide (in contrast to what we found when we analyzed only 25 Mb), we found a significant increase of genomic deletions (ranging from 55 to 10 000 bp) in the absence of REV3. These deletions are preferentially detected in late replicating regions as well as in specific genomic regions that are disturbed in Rev3l-/- cells. These data and analyses are now added as a new Fig 6. The analysis of WGS has been performed by bioinformatics experts (S. Nikolaev and A. Yurchenko) and the procedure is detailed as followed in Material and Methods: *Single cells from Rev3L*$^{-/-}$ *and Rev3L*$^{+/+}$ *fibroblasts were isolated, amplified and subjected to whole genome sequencing. The genomes were sequenced according to the manufacturer protocols (BGI Tech solutions, Hong Kong, Co., Ltd) with a mean coverage of 30x using 150 bp paired-end reads with BGISEQ-500 sequencer. Reads were then aligned to the mm9 mouse genome using BWA mem software (Li & Durbin, 2009) and sorted with samtools (Li et al, 2009). Then we removed duplicates from the BAM file according to the GATK Best Practices pipeline (Van der Auwera et al, 2013). To identify structural variants (SV) from genomic data we used GRIDSS2 (Cameron et al, 2017) and Manta (Chen et al, 2016) software and compared replicates 1 and 2 between each other for Rev3l*$^{+/+}$ *and Rev3l*$^{-/-}$ *samples. Resulting structural variants from two methods were intersected using BEDTools for each sample (Quinlan, 2014) with overlap at least 90% and used for further analysis.*

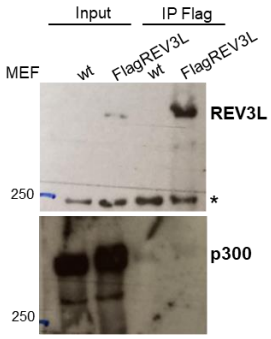
10. The authors present the count of 'Mutational events'. They should present Mutational frequency. Otherwise it is difficult to evaluate the significance of the results, e.g., how do they compare to the frequency of mutations in other studies.

11. In Fig. 3C, most mutations were detected in early replicating regions, while according to the literature (which the authors cite), there are more mutations in late replicating regions. Having frequencies here (see item 10 above), might help clarify the issue.

For comments 10 and 11, as indicated in comment 9, the strategy of the mutation experiments has now been altered.

12. A negative control is needed for the IP of Flag-Rev3L, which is a big protein, to which many proteins may stick. Perhaps CENP, which associates with minor satellites may be a good control.

A negative control (p300) has been performed as shown below. We confirmed that this protein does not stick to Flag-REV3L. However, because the figures in the revised version are very busy due to the introduction of the new set of experiments asked by the four reviewers, we decided to avoid to include this negative control.



13. The co-localization results in Fig. 6 are problematic. Merging the images of green and red fluorescence signals should create yellow signals if the proteins co-localize. This is not seen Fig. 6, B, C and E. The green and red stains indeed seem to be in the same general region - but in fact it appears that they do NOT co-localize, otherwise yellow dots would be visible.

To confirm the co-localization between GFP-REV3L and HP1α (or H3K9me3), immunofluorescence was processed as before and images were acquired using confocal Leica SPE. We now provide the fluorescence intensity profile analyses showing the overlap of the signals. We hope that the co-localization is now more obvious in the reformatted Fig 4E.

14. In the PLA data in Fig. 7, controls for the specificity of each of the gH2AX-HP1b and 53BP1-HP1a interactions should be presented.

As the previous Fig 7 has been removed as suggested by Referee #3, we now present in the revised version controls for the specificity of each of the γH2AX-HP1b and 53BP1-HP1a interactions in Fig 5B and C.

15. The authors state that repair of DSB in Rev3L-/- cells is slower than in Rev3L+/+, but do not present data showing it. This data should be presented, or the statement removed from the manuscript.

To provide data on this issue, we have performed new experiments now shown in Fig 7 and EV6. In contrast to *Rev3l+/+* cells, we show that UV irradiation exacerbates replication-associated DSBs in *Rev3l-/-* cells, leading to a strong accumulation of 53BP1 foci in S phase as well as RPA and RAD51 foci in cells. Of note, *Rev3l-/-* cells fail to resolve DSBs as shown by the persistence of S824-phosphorylated KAP1 as well as 53BP1 and RAD51 foci 24 h post UV.

Referee #3:

The translesion synthesis (TLS)-associated DNA polymerase Rev3 has previously been shown to be involved in the replication of fragile sites. In this manuscript the authors further investigate the role of Rev3 in 'normal' replication. They describe that specifically replication of heterochromatic pericentric regions is delayed in the absence of Rev3. Rev3 appears to be recruited to these regions by binding to the HP1 proteins. Also, breaks, deletions and insertions in these regions are increased, imprinting is altered as well as the expression of (frequently development-related, genes encoded in these regions. Finally, the authors unveil a defect in homology-dependent double-strand breaks repair (DSBR)in Rev3-deficient cells.

These data are interesting and relevant, and most experiments have been well done and interpreted. However, not all conclusions appear warranted and some relevant literature has not been cited.

Major issues:

1. For many (including cell-cycle) experiments the authors have used a single Rev3 MEF line. Given the phenotypic variability of MEF lines this restricted approach might be risky. It would have been nice to use more than one line, or to complement the line with wt Rev3. Admittedly, the authors also use Rev3 siRNA-treated HeLa but this is an entirely different cell type and the analysis of these cells was restricted to a cell cycle experiment.

In the revised version we have included experiments on further cell lines, including complemented REV3L MEFs, and we think they help validate the conclusions. We analyzed replication timing profiles in two independent cell lines after depletion of REV3L (MEFs and HeLa cells). We observed that in both cell lines, loss of REV3L disturbs replication timing in specific genomic regions. The new data for replication timing in HeLa cells are shown in figure EV2 C, D, and E. All statistics have now been included in the revised manuscript thanks to a new software dedicated to replication timing analysis which has been created by one of the authors (Hadjadj et al., *NAR Genom Bioinform* 2020). We also find that accumulation of spontaneous DSBs in heterochromatin after depletion of REV3L can be detected in several cell lines including MEFs, RPE and HeLa (Fig 5B-F). These breaks are significantly reduced in REV3L-complemented cell lines. This is consistent with the idea that Pol zeta/REV3L facilitates the replication of heterochromatic region, and is presented in a new Figure 7F-H.

2. The mutagenesis experiment has not been performed in an appropriate fashion. Thus, polyclonal pools of cells were sequenced at p=5 and p=60 generations. In this fashion, 'new' mutations will not be identified as they most likely will remain subclonal. The experiment should have been performed by sequencing a (monoclonal) subclone at p-5, expanding this subclone to p=60, subclone again and then sequence one or more of these subclones. Also, it is not clear from which strain background the Rev3 (and wt) MEFs are derived so it is not certain whether it is appropriate to compare these sequences to the reference genome that may be of an entirely

different strain. Furthermore, it is not logical nor expected why the number of mutations in temporal transition regions (TTR) regions is reduced in Rev3 MEFs at p=60 as compared with p=5 as in both cases it concerns polyclonal populations of the same genotype and thus the mutations at p=5 should also be present at p=60. The authors state that this can be explained by selective loss of heavily mutated Rev3 cells resulting in (oligo)clonality but this is an unsatisfactory explanation and would also suggest that loss of Rev3 leads to more mutations than in wt cells, which would be at variance with the proposed hypomutability of Rev3 cells. In this light, why do Rev3 cells appear to have more nucleotide substitutions (at p=5) than wt cells (Fig. S2)?

As recommended, we re-examined the mutational landscape in unchallenged Rev3l-/- cells as compared with Rev3l+/+ cells. In our previous version, we sequenced only 3 chromosomal regions corresponding to approx. 25 Mb. In response to the reviewers' critiques, we re-designed the experiments, expended 4 single clones and performed whole genome sequencing to get a complete overview of the mutational landscape. While nucleotide substitution rate was not significantly different genome-wide (in contrast to what we found when we analyzed only 25 Mb), we found a significant increased number of genomic deletions (ranging from 55 to 10 000 bp) in the absence of REV3. These deletions are preferentially detected in late replicating regions as well as in specific genomic regions that are disturbed in Rev3l-/- cells. These data and analysis are now added as a new Figure 6.

3. It was not clear to me (or I have missed it) whether in Rev3 cells the relative fraction of deregulated genes was higher in the RT zones than outside of these RT zones. This is expected if the deregulation of replication timing in these zones affects gene expression.

As written in the manuscript on page 7, we integrated the data on gene expression and genome-wide profiling of replication timing. We found 24 out of 317 genes (corresponding to 7.6% of total deregulated genes or 11.7% of downregulated genes) located in these specific genomic domains (Table 1). We speculate that deregulation of genes outside of these TTR regions might be due to indirect effect (trans-repression/activation). Indeed, transcription factors, such as GATA6, PAX3 or FOXG1 downregulated in *Rev3l-/-* are localized in TTR regions (Table 1), which can in turn impact expression of genes in the whole genome.

4. DSBR is perturbed in Rev3 cells. This is an interesting though not completely unexpected finding (DSBR is also perturbed in cells with defects in the Rev3 partners Rev7 and Rev1, why were these data not cited?).

This is true. In the revised version (page 12), we cite the study from the Canman Lab that showed that REV1, REV3 or REV7-depleted human cells displayed increased chromosomal aberrations, residual DSBs and sites of HR repair following exposure to ionizing radiation (Sharma et al., NAR 2012).

Importantly, rather than a defect in TLS, the defect in DSBR might explain the claimed propensity of rearrangements at pericentric chromatin in Rev3 cells. This should be discussed.

Yes, this connection between DSBR and pericentromeric heterochromatic regions is now discussed in a more orderly fashion in the revised Discussion (page 13 of the manuscript)

Minor issues:

1. The role of Rev3 in DSBR should be included in the abstract.

Thank you, this is now included

2. What fraction of the TTR requires Rev3 for efficient replication?

To perform a more rigorous analysis, profiles of replication timing have now been analyzed using the START-R program recently developed by the Cadoret lab (co-authors of this work). This software (Hadjadj et al., *NAR Genom Bioinform* 2020) is cited in our revised manuscript. We found that 18.7% of TTR were disturbed in *Rev3l*$^{-/-}$ mouse cells. This information has been included on page 6 of the revised manuscript.

3. The Sale lab has shown imprinting defects in Rev1 cells. It would be appropriate to discuss the epigenetic alterations in Rev3 cells that are described here in the light of findings.

That's true. This is now included in the Discussion, page 15

4. Fig. 1A bar graphs: Are the differences between the wt and Rev3 statistically significant?

We have now included the statistics for these experiments as shown on histograms in Fig 1A (lower panel). The difference between the *Rev3l*+/+ and *Rev3l*-/- cells are statistically significant. This is described in the manuscript on page 4 as followed: "*Rev3l*$^{-/-}$ *cells exhibited a significant higher percentage in late S/G2 phase at 4, 6 and 8 h as compared to control cells".*

What is the reason that the cell cycle distribution is the same for both wt and Rev3 MEFs at T0h? I would expect that there would be a difference due to slower S phase progression in the Rev3 MEFs.

Given that the S phase is slightly longer in the *Rev3l*$^{-/-}$ MEFs, it is difficult to observe a difference just after the 15 min BrdU pulse, at T0h. However, the difference appears during the progression of BrdU-positive cells in the S phase. It is what we observed at 4, 6 and 8 h post BrdU incorporation.

5. Fig. 1B. Pie charts: I assume that the colors corresponding to the SIII and SIV fraction are incorrect and should be swapped.

Yes, thank you. This is now corrected

6. UV-C light is a much better agent than X-rays to analyze replication-associated DSB formation in a cytogenetic fashion.

As suggested, we now show that UV irradiation exacerbates replication-associated DSBs in *Rev3l*$^{-/-}$ cells, leading to a strong accumulation of 53BP1 foci in S phase as well as RPA and RAD51 foci in cells. Of note, *Rev3l*$^{-/-}$ cells fail to resolve DSB as shown by the persistence of S824-phosphorylated KAP1 as well as 53BP1 and RAD51 foci 24 h post UV. The new data are in Fig 7 and EV6.

Referee #4:

Starting from the observation that during embryonic development Rev3L is an essential gene, Yamin and colleagues have created Rev3L knock-out immortalised MEFs to analyse the function of this TLS polymerase during unperturbed cell cycle.

The authors analyse progression through S-phase in the KO cells, finding a delayed progression through the last part of S-phase. In agreement with this finding, they show a delayed replication of some of the replication timing transition zones and an accumulation of mutations in these regions. REV3L localises at heterochromatin through interaction with HP1s, through a canonical peptide located between residues 761-1029. Absence of REV3L correlates with increased endogenous DNA damage and a delayed repair of γ irradiation-induced double strand breaks.

I appreciate the amount of work that this paper contains and that manipulation of an essential and very large protein like REV3L is very difficult. I think that the results are interesting but over-interpreted. There is not enough evidence to say that the role of Rev3L is SPECIFIC for heterochromatin, but certainly the data demonstrate that there is a role at heterochromatin during repair. The link to replication is less convincing, as the damage does not appear to be specific to S-phase. The data on replication timing and gene expression are very weak (see below the detailed comments). However, I find that the part of the paper relative to the localisation of REV3L at heterochromatin through interaction with HP1b well done and convincing. Overall, I think that the paper needs to be substantially revised, but it could be reconsidered once the comments are addressed.

MAJOR COMMENTS:

In general, I don't think the first part relative to replication timing, gene expression and accumulation of mutations is strong enough. The use of large T immortalised MEFs limits what claims can be made due to genomic instability and continuous clonal selection (see specifics below). One way around could be to use Large T immortalised MEFs carrying the conditional Rev3L allele, infect them with empty vector or Cre-encoding virus and compare them after just few days. This gives a much more reliable base-line.

Additional data is now presented in the revised manuscript: (i) we find that accumulation of spontaneous DSBs in heterochromatin after depletion of REV3L can be detected in several cell lines including MEFs, RPE and HeLa (Fig 5 B-E), (ii) these breaks are significantly reduced in REV3L-complemented cell lines (Fig 7 F-H). This is consistent with the idea that Pol zeta/REV3L facilitates the replication of heterochromatic regions.

Figure 1 A: T0 indicates that there are no differences in the cell cycle distribution between wt and KO cells. After 2 hours there is a green arrow pointing at BrdU- G2 cells. What is it meant to show?

This experiment is designed to follow the progression of BrdU+ cells through cell cycle. At 4 hours there are less BrdU+ cells back in G1, indicating a possible G2/M delay. However, re-entry into S seems comparable in the two genotypes. This is all very logical in cells that are Large-T immortalised and therefore have no G1/S checkpoint. Hard to deduce a mid/late S-phase delay from this data. The HeLa siRNA experiment supports the idea that the problem is in G2/M, delaying re-entry into G1. Again, HeLa cells have no G1/S checkpoint.

As mentioned above for Referee #3, it is difficult to observe a difference just after the 15 min BrdU pulse, at T0h for this kind of experiment. The difference appears during the progression of BrdU-positive cells in the S phase. We observed that *Rev3l*$^{-/-}$ cells exhibit a significant higher percentage in late S/G2 phase at 4, 6 and 8 h post BrdU incorporation as compared to control cells. The green arrow that points at BrdU-negative cells indicate that G2/M phase is also longer

in *Rev3l-/-* cells. The description and interpretation of these results are now discussed properly on page 4 of the manuscript.

Figure 1 B and C: show a change in the distribution of the spatial patterns of replication. It has already been shown that spatial organisation does not necessarily reflect replication timing (uncoupling in the Rif1 null cells). Therefore, this figure does not show a change in the replication timing program (as stated in the first sentence of the paragraph "Loss of Rev3L disrupts...").

The concluding sequence of the paragraph is now worded “These results suggest that lack of REV3L can affect the temporal control of DNA replication.”

Figure 2: please specify what "p5 and p7 or p60" mean. How were the passages counted? From derivation or from immortalisation? This is crucial for the whole paper. Large T immortalisation leads to clonal selection. Moreover, large T immortalised cells are genomically unstable. Subtle changes are therefore very hard to judge. For example, evaluating mutation burden is really unreliable with this type of immortalisation.

Passages were counted from immortalization. As recommended by all Reviewers, we re-examined the mutational landscape in unchallenged Rev3l-/- cells as compared with Rev3l+/+ cells. In our previous version, we sequenced only 3 chromosomal regions corresponding to approx. 25 Mb. We re-designed the experiments, expanding 4 single clones and performing whole genome sequencing to get a complete overview of the mutational landscape after 90 doublings. While nucleotide substitution rate was not significantly different genome-wide (in contrast to what we found when we analyzed only 25 Mb), we found a significant increase of genomic deletions (ranging from 55 to 10 000 bp) in the absence of REV3. These deletions are preferentially detected in late replicating regions as well as in specific genomic regions that are disturbed in Rev3l-/- cells. Note that none deletion was shared between samples, suggesting that Rev3l-/- population cells did not undergo clonal selection. These data and analysis are now added as a new Figure 6.

For the replication timing, how many biological and technical replicas were analysed? The shifts indicated in the figure could within clonal variability....

To perform a more rigorous analysis, profiles of replication timing have now been analyzed using the START-R program recently developed by the Cadoret lab (co-authors of this work). This software (Hadjadj et al., *NAR Genom Bioinform* 2020) is cited in our revised manuscript. All statistics have now been included in the revised manuscript. Using START-R program, we compared the replication timing obtained at different passages for each cell line. For *Rev3l*$^{+/+}$ MEFs, there was a 1.9% difference between p5 and p60. For *Rev3l*$^{-/-}$ cells, we observed only 0.2% difference between p7 and p60, suggesting that the replication timing remains stable during cell culture passages in both cell lines, and can be considered as two independent replicates. Moreover, we have now analyzed replication timing profiles in another cell line after depletion of REV3L (i.e. HeLa cells). We observed that in both cell lines, loss of REV3L disrupts replication timing in specific genomic regions. The new data for replication timing in HeLa cell is in EV2 C, D, and E.

Why the shifts to later replication in TTR would be more significant than the switches to earlier or later replication also shown in the same figures? What is the threshold that has been applied to call a shift=change? What is the percentages of TTR that change?

This is an important point. As mentioned above, profiles of replication timing have now been analyzed using the START-R program and all regions that have undergone timing transitions have been now identified and quantified. To detect a significant difference between *Rev3l*$^{+/+}$ and *Rev3l*$^{-/-}$ mouse cells, we applied the classical mean method with a threshold of p value= 0.01. We found that in *Rev3l*-deficient mouse cells, about 5.7% of the whole genome was affected (examples in Fig. 2A). 19.2% (in bp) of these regions were advanced in timing and 80.8% of regions were delayed (EV2B). We also found that 18.7% of TTR were disrupted in *Rev3l*$^{-/-}$ mouse cells. This information has been included on pages 5 and 6, of the revised manuscript, with statistical results shown in the Figure panels. Same analysis has been applied for HeLa cells depleted for REV3L (see EV2 C-E).

What happens to the size of replication timing domains?

As mentioned above, we found that in *Rev3l*-deficient mouse cells, 19.2% (in bp) of disturbed regions were advanced in timing and 80.8% of these regions were delayed (EV2B).

Figure 3: I don't think this type of analysis can be meaningful in Large T immortalised cells. Clonal selection is clearly evident.

As mentioned above, and recommended by all Reviewers, we re-examined the mutational landscape in unchallenged Rev3l-/- cells as compared with Rev3l+/+ cells. In our previous version, we sequenced only 3 chromosomal regions corresponding to approx. 25 Mb. We re-designed the experiments, expanding 4 single clones and performing whole genome sequencing to get a complete overview of the mutational landscape after 90 doublings. While nucleotide substitution rate was not significantly different genome-wide (in contrast to what we found when we analyzed only 25 Mb), we found a significant increase of genomic deletions (ranging from 55 to 10 000 bp) in the absence of REV3. These deletions are preferentially detected in late replicating regions as well as in specific genomic regions that are disturbed in Rev3l-/- cells. Note that none deletion was shared between samples, suggesting that Rev3l-/- population cells did not undergo clonal selection. These data and analysis are now added as a new Figure 6.

Figure 4: changes in gene expression suffer from the same problem of analysing clonal and genomically unstable populations. One way around this problem could be to employ the conditional immortalised MEFs and analyse mutation burden, gene expression and epigenetic profiling pre vs. post Cre infection. In this way there would be a base-line that is specific to each cell line.

We agree, this way may limit problem cause to T-Ag expression in MEFs. However, several effects due to the absence of REV3L in our study (including replication timing, epigenetic, mutational landscape) required numerous rounds of replication. Therefore, clonal selection might occur during cell culture after Cre-infection. To confirm results found in MEFs cells, several experiments have been repeated in the revised version using human cells depleted in REV3L (including cell cycle, replication timing and DSB monitoring).

For the genes in Table 1, there is no statistical analysis provided regarding the significance of this 7.6% of deregulated genes falling into changed TTR?

By integrating the data on gene expression and genome-wide profiling of replication timing, we found 24 out of 317 genes (corresponding to 7.6% of total deregulated genes) located in TTR domains. We were not able to introduce statistics for this observation. However, 100% of these genes showed a downregulation in *Rev3l-/-* MEFs, suggesting that boundary shifts observed in Rev3l-/- MEFs lead to repressive expression, as expected for regions that replicate in late S phase. This has been rewritten on page 7 in the manuscript and in discussion section.

For example, in Figure 7, it is presented the analysis of co-localisation of DNA damage markers with HP1b. However, what would be the results by doing the same analysis with H3K4me3, for example? REV3L seems to significantly co-localise with H3K4me3 in Figure 5E.

Bivalent domains simultaneously contain the opposing histone modification H3K4me3 and H3K27me3 and have been found in the promoters of a broad range of genes involved in development (Sachs et al, 2013). The analysis of transcriptome profile in *Rev3l-/-* MEFs showed that numerous of these genes are downregulated (i.e. HoxB clusters). This might suggest that REV3L can interact with this type of bivalent chromatin. Therefore, it is not surprising that REV3L co-localizes with H3K4me3 and H3K27me3 as shown in Fig 4C in the revised version. This hypothesis is now discussed on page 16, and experiments are ongoing to determine the link between REV3L and bivalent chromatin. We also tested the co-localization between REV3L and an active histone mark such as H3K9ac and we did not find any co-localization using PLA approach (now shown in Fig 4C).

What is the general kinetics of 53BP1 foci removal from the Rev3L-/- cells compared to the wild type?

Unchallenged *Rev3l*$^{-/-}$ MEFs exhibit constitutive DSBs. Therefore, it is impossible to monitor the general kinetics of 53BP1 foci removal. This is also observed after depletion of REV3L in human cells such as HeLa and RPE (now showed in Fig 5D-E). To try to answer to the question of 53BP1 kinetics, we challenged MEFs with UV radiation. Thus, new experiments along this general line are now shown in Fig 7 and EV6. UV irradiation exacerbates replication-associated DSBs in *Rev3l*$^{-/-}$ cells, leading to a strong accumulation of 53BP1 foci in S phase as well as RPA and RAD51 foci in cells. However, we were not able to monitor a decrease of 53BP1 foci in these experimental conditions. Indeed, *Rev3l*$^{-/-}$ cells fail to resolve DSBs as shown by the persistence of S824-phosphorylated KAP1 as well as 53BP1 and RAD51 foci 24 h post UV.

In Figure 7G, what is defined "pericentromere proximal" is, in reality, far apart. The resolution on chromosomal spreads is far too low to claim anything about proximity.

This is true. To confirm that breaks in *Rev3l*$^{-/-}$ MEFs partially localize in pericentromeric heterochromatin (PHC), we performed DNA fluorescence *in situ* hybridization (DNA-FISH) on metaphase chromosome spreads using major satellite DNA as probe. As now shown in Fig 5F, a higher level of DNA breaks in PHC in *Rev3l-/-* MEFs was detected as compared to *Rev3l+/+* cells. Moreover, various abnormalities were observed at PHC, including breaks/gaps, loss, duplication and rearrangements (Fig 5F, upper panel). Consistent with that, we examined the velocity of replication forks in pericentromeric heterochromatin (PHC) using a DNA combing/FISH approach. We found that in REV3L-depleted cells, DNA replication dynamics is strongly reduced in PHC while it remains nearly unaffected for the genome globally as compared to WT cells. This data has now been added as new Fig 5A.

The last paragraph links the increased DNA damage burden to S-phase, but there is really no evidence for this. An S-phase specific analysis of DNA damage accumulation would be needed for this claim.

We have now monitored 53BP1 foci formation during S phase as well as HR markers such as pRPA2 S4-S8 and RAD51 foci after UV irradiation. This new set of experiments is shown in Fig 7. Moreover, we have modulated our statement in the rewritten discussion section on page 13-14.

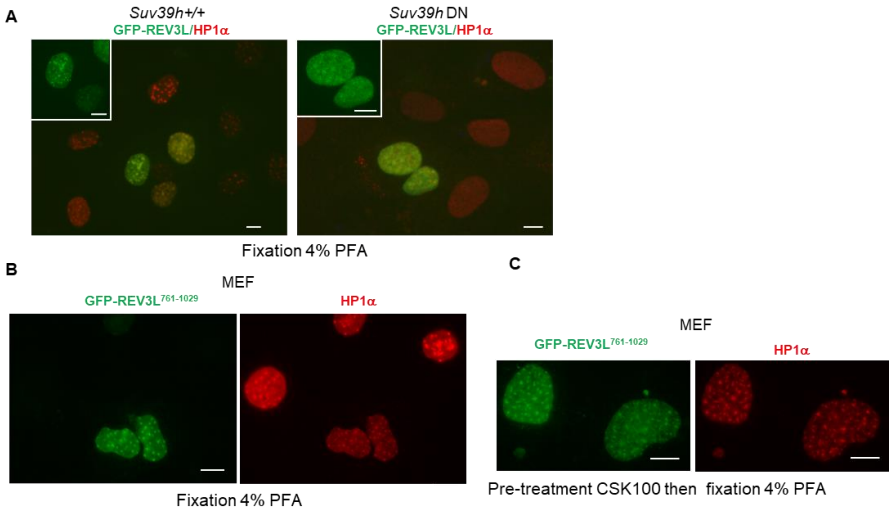
I think the authors should limit the statement to a role during repair in heterochromatin, not necessarily specific.

This is true and we no longer note that it is specific to only heterochromatin.

I have some MINOR COMMENTS on the figures in the second part.

Figure 6 B: the figure should include non-transfected cells in the same field, to ensure that in Suv39h DN we are looking at transfected and delocalised REV3L and not just fluorescence background. Same for Figure 6 B. In addition, it could also be useful to have non-pre-extracted cells shown in Figure 6C.

All these controls have been performed as shown below. However, because the figures in the revised version are very busy due to the introduction of the new set of experiments asked by the four reviewers, we decided to avoid to include these controls.



A: *Suv39h+/+ and Suv39h* DN transfected with vector expressing GFP-REV3L$^{761-1029}$ and fixed with 4%PFA
B: MEFs transfected with vector expressing GFP-REV3L$^{761-1029}$ and fixed with 4%PFA
C: MEFs transfected with vector expressing GFP-REV3L$^{761-1029}$ and permeabilized with CSK100 for 5 min before PFA fixation. Scale bar: 10 µm

Figure 6 F: GST-REV3L 700-900 in the left panel (WT and V802D). The figure legend states that is FH-tagged too. Is this a mistake? Molecular weight ladder should be indicated in the right panel.

Thank you. This has been corrected.

Figure 7A and C: the difference of the PLA foci between NT in wt and Rev3L-/- in panel C is much higher than in panel A. Why? It should also be made clear in the paragraph relative to this figure that this comparison can be made because there are no major differences in the cell cycle distribution of the two genotypes.

As mentioned above, the previous Fig 7 has been removed and replaced by the new set of experiments using UV radiation, as suggested the Referee #3. Moreover, we have modulated our statement in the rewritten discussion section on page 13 and 14.

Thank you for your patience during our re-evaluation of your revised manuscript, which unfortunately was delayed due to initial referee unavailability, and also by my recent absence from the office. We have now received comments from all four original reviewers, copied below for your information. All referees find the study overall substantially improved, although -perhaps unsurprisingly in light of the extensive revisions- they still list several remaining issues, partly arising from the new data. Nevertheless, given the considerable revision efforts acknowledged by all four reviewers, we would at this point be happy to pursue this work further for EMBO Journal publication, following a final revision round allowing you to answer the open points raised particularly by referees 3 and 4. In particular, it will be important to:

- strengthen the HP1-Rev3 colocalization data [ref 4 point (e)]
- comment on/clarify the differential Rev3 l.o.f. effects in human vs. mouse cells [ref 3 point 1]
- Respond to/discuss the issues raised in points 2 and 3 of referee 3, and either altering/tempering the respective conclusions or supporting them with more decisive evidence.
- finally, I would encourage you to follow referee 4's suggestions [points 1 and 2] for changing the order of presentation and interpretation of the hypotheses, data and conclusions, which may indeed lead to a more logical and compelling line of reasoning.

As always, please make sure to carefully answer to all (including minor) concerns also in the form of a detailed point-by-point response letter.

When preparing your final version, please also pay attention to the following editorial points (as well as our author guidelines for revised manuscripts), as this should greatly facilitate the editorial process at the time of resubmission:

Referee #1:

The revision, new analyses and additional data had addressed many of my concern. The new data concerning deletion rates are particularly timely and interesting. The revised paper is considerably improved. A minor point - the authors might want to consider removing the last paragraph of the Discussion section, which discusses mutation rates and appears somewhat out of place in the current version of the paper.

Referee #2:

The authors have satisfactory responded to the review comments, and I recommend that they manuscript be published in the EMBO J.

Referee #3:

Many comments of the reviewers have been properly addressed. However, the modified manuscript raises a number of new questions that I would like to see addressed:

Major issues
1. P. 6. Rev3 depletion in HeLa cells affects late replication whereas in mouse cells Rev3 deficiency affects replication of TTR that lie between early and late replication. This is surprising as it suggests important mechanistic differences between Rev3 function in mouse and human cells, or even between different cell types. Is the replication of pericentric heterochromatin affected in HeLa cells, similar to MEFs? Does HP1 bind to late replicating chromatin (in HeLa cells)??
2. P. 11. "Intriguingly, mild replication stress induced by a low dose of aphidicolin (APH) led to a similar genome-wide increase of breaks in both Rev3l-/- and Rev3l+/+ MEFs, suggesting that APH-induced DNA breaks are partially prevented by PolZ/REV3L. Consistent with this, we did not observe an increase of PHC breaks in Rev3l cells treated with aphidicolin (Fig 5F and G)." I do not agree with this conclusion since, in case Rev3 prevents APH-induced breaks they should specifically be increased in Rev3- cells. And why does APD not specifically induce even more breaks in pericentric heterochromatin (where Rev3 is recruited by HP1) in Rev3 KO cells than in global chromatin?
3. P. 12. UV is used to induce dsDNA breaks. But UV initially induces stalling of replication. The latter is much higher in Rev3 KO cells and therefore many more dsDNA breaks are induced in Rev3 KO cells than in wt cells. How can it be concluded that the protracted presence of these breaks reflects the direct involvement of Rev3 in dsDNA breaks repair rather than the delay in repair caused by saturation of the repair machinery due to the large number of breaks in Rev3 KO cells? Of note, the increased phosphorylation of RPA2 can very well be explained by the increased number of ssDNA at stalled forks in the absence of Rev3, rather that the presence of more (resected) dsDNA breaks. In brief, these experiments do not provide evidence for a role of Rev3 in dsDNA breaks repair, a statement that reappears at many places throughout the manuscript.

Minor (but not trivial!) issues:
1. P.4 describes that the late S phase is protracted in Rev3 cells. Surprisingly, according to the rebuttal, these cells proliferate faster that the wt controls (17.2 vs 19.1h). Can the authors explain this paradoxical result?
2. P. 7. WT1 expression was strongly downregulated in one Rev3 MEF line. Is this true also for other MEF lines? And for HeLa cells?
3. 3. P. 7. Figs. 3A and EV3C. It is not clear to me which lanes represent independent MEF lines and which lanes represent technical replicates. To me, most bands appear different when lanes for +/+ or -/- are compared. Also, the bands in the blue panel are difficult to see.
4. Similarly, P. 7. Figs. 3B and EV3D display the expression of different genes. These Figs show independent Rev3 MEF lines (as far as I understand). Why were not the same genes tested in these cell lines to compare the phenotypes?
5. P. 12. Do the deleted regions in Rev3 KO cells contain obvious hard-to-replicate sequences?

These deletions are found in late-replicating chromatin. This appears not to be this same chromatin as the pericentric chromatin (that replicates between early and late chromatin). Does HP1 (that recruits Rev3) bind to late chromatin? If not, what can the relevance of HP1 binding be?
6. P. 12. What does 'Relative deletion rate (Mb)' indicate? Why is there no increase in deletions at TTR (Fig. 6D)? And what is the significance in the difference between the # of breaks in early and late S? Are the # of deletions normalized to the size of the early vs late replicating regions?
7. P. 15. "Future studies may determine the mechanisms by which REV3L is involved in the repair of heterochromatin-associated DSBs by interaction with SCAI." There is no evidence provided that Rev3 interacts with SCA1.
8. The notion that in the absence of Rev3 increased levels of H3K27me3 levels and, to a lesser extent, H3K9me3 and H3K4me3 are linked to dsDNA repair is very speculative and not at all based on a mechanistic explanation.

Referee #4:

Yamin et al., have submitted a revised version of the paper that is very much improved. It is impressive, considering also the difficult year. My concerns have been satisfied. I have nevertheless some suggestions, mostly on reworking the text to give more emphasis to the more important data. I would like to see comment (e) addressed. It would be important to show that the co-localisation of HP1 and Rev3 is specific to S-phase.

Major points:
a. As a general comment, I agree with the hypothesis, explained in the discussion, proposing that the changes of replication timing could be a consequence of the problems in fork progression. Because of this, I am not sure I would start the paper with the replication timing data. It emphasises a consequence, and sets the wrong expectations. The data on the role of pol in the replication of heterochromatin are so well done and solid that should really be the main focus and the data on the replication timing should be a short observation in the middle/the end of the paper.
The same goes for the changes in gene expression. Again, I agree with the hypothesis that they are a secondary effect of the epigenetic changes, possibly as a consequence of problems in repair (this is one of many possibilities). I would therefore de-emphasise them.

b. Remaining on the topic of the changes of gene expression profile, I think that the authors' interpretation of the origin of the silencing is an argument in support of the conclusion that Rev3l deficiency induces DNA damage beyond heterochromatin. The authors propose that the silencing of genes normally expressed is induced by the permanence of heterochromatin marks deposited during repair in regions that have undergone DNA damage in absence of Rev3l. The consequential conclusion is that there is a lot of damage in genes that are normally expressed, and, therefore, within euchromatin. This is in agreement with the fact that the large majority of the genes whose expression is deregulated by Rev3l deletion do not fall within disturbed regions and supports the idea that the disturbed replication timing is a secondary consequence of the problems of replication in TTRs. I think the authors should revise the text in this direction. It would be much easier to link all the data.

c. Page 7 it should be discussed the fact that vast the majority of the deregulated genes do not fall within disturbed regions.

d. What percentage of the disturbed regions falls within TTR? This question is linked to the

changes in the gene expression profile.

e. Figure 4: the PLA data are still not very convincing. REV7 has a lot of background on its own. Moreover, the PLA with HP1 does not seem to correspond to chromocenters, although the quality of the pictures makes it hard to judge. Could the authors evaluate if and how much of the signal correlates with chromocenters? In addition, a PLA with EdU would be very informative, because it would make sense if REV3 co-localises with HP1 specifically in S-phase, and preferably, in presence of mid and late patterns. EdU staining should be compatible with PLA.

Minor points:
Figure 1A: -in the figure legend is still missing what the green and red arrows are pointing to. I know it is in the text, but it should be in the legend too.
-The name of the gates (P6, 8 and 11) should be removed.
Figure 1B: the "D" in EdU should not be capital.
Figure EV1: -the names of the gates R1, R2 and R3 should be removed.
-In the top half of the figure what do R2, R1 and R3 denote in all the FACS panels. (It might be better to remove these along with P6, P8 and P9 in Figure 1 and label the gates as G1/S, Mid-S and S/G2.)
-The bottom right panel shows PCR and not qPCR results (the legend says RT-qPCR).
Page 4: -end of the introduction. there is a mistake, "instability:" should read "stability".
-In the first paragraph of the results: "Moreover, analysis of BrdU-negative
cells showed that G2 phase was longer in Rev3l-/- cells as compared to Rev3l+/+". Technically, to say something like this the authors would need to track G2 in single cells. On a population-base, it would be more correct to say that cells tend to accumulate in G2?
Page 5: last sentence of the first paragraph. As I pointed out already, because there is not necessarily correspondence between spatial and temporal control of replication, the sentence should read: "These results suggest that lack of REV3L can COULD affect the temporal control of DNA replication."
Figure 2: in the legend it wrongly refers to Fig. EV1 instead of EV2.
Page 6: "For each parameter, we observed a molecular signature intermediate between that of early and late domains, strengthening our hypothesis that these disturbed regions correspond to TTR. In Rev3l-/- cells, 18.7% of TTR were disturbed.". I would say that every signature it is very close to the mid domains.
Figure EV2: what are the squinted, short grey dotted lines in the plots?
Figure 3: A The scale below the figure is unreadable. In addition, it is hard to see the changes in gene expression with the yellow versus blue colour .
E why are there no error bars, if the experiments were repeated twice, as the legend says? In addition, all the ChIP need unchanged controls, like genes that do not change RT or expression, genes that change RT but not expression, genes that become earlier replicating.
In all panel the asterisks need to be added to the figure.
Figure EV3: The scale under Expanded View Figure 3C is unreadable. In addition, it is hard to see the changes in gene expression with the yellow versus blue colour scheme (the blue is still too saturated, although in the response to reviewers the authors say this has been improved
Page 6: The text says that the paternally expressed gene Dcn is shown in blue in Expanded View Figure 3C whereas it is in fact shown in pink (and is referred to as maternally expressed in the legend to Expanded View Figure 3D).
Figure 4: E it would be better if the merged channels were shown to demonstrate the overlap in signals more clearly.
H & I refer to FH-REV3L constructs. These should be mentioned in the Plasmids section of the Materials and Methods as their construction is not described. Note also that these constructs are

referred to as FH-REV3L in the text, F-H-REV3L in Fig. 4 and Fig. EV5, but as Flag-HA-REV3L in the legend to Fig. EV5, so this should be rectified.
Figure 5: The legend should be altered to make clear the sequential nature of the IdU and CldU staining. E.g., 'Representative fibers of newly synthesised DNA labelled with IdU (red) for 30 minutes then CldU (green) 30 minutes...'
Figure EV5: C The text needs to be improved as the two Vs have merged to make them look like a W.

The empty vector and the construct referred to as REV3L should be described in the Materials and Methods 'Plasmid' section

Minor typos:

Page 7: at the bottom 'H3K9m3' should be replaced by 'H3K9me3'
Page 22: paragraph 2 'interested proteins' should be replaced by 'interesting proteins' and 'processes' by 'processed'.

Point-by-point response #2

Referee #1:

The revision, new analyses and additional data had addressed many of my concern. The new data concerning deletion rates are particularly timely and interesting. The revised paper is considerably improved.

A minor point - the authors might want to consider removing the last paragraph of the Discussion section, which discusses mutation rates and appears somewhat out of place in the current version of the paper.

Thanks for this positive comment. We think that the content in the last paragraph of the discussion is important since our work demonstrates that pol zeta contributes to duplicate heterochromatin regions that replicate in mid-late S phase. Several studies reported that these heterochromatin-associated late-replicating regions accumulate much more point mutations during species and cancer evolution than early-replicating regions. Therefore, it is reasonable to think that the error-prone activity of pol zeta might contribute to this elevated mutation rate. We agree that this remains speculative. However, two other studies suggested similar involvement of pol zeta in late-replicating regions using different approaches (Lang & Murray, 2011; Seplyarskiy et al, 2015). In response to the comment, we moved this more speculative paragraph to follow the replication timing paragraph in the Discussion. This allows us to end the Discussion section with points arising from our experiments on transcriptome analysis.

Referee #2:

The authors have satisfactory responded to the review comments, and I recommend that they manuscript be published in the EMBO J.

We thank the reviewer for the supportive comment regarding the paper and its suitability for publication.

Referee #3:

Many comments of the reviewers have been properly addressed.

Thanks for this positive comment.

However, the modified manuscript raises a number of new questions that I would like to see addressed:
Major issues

1. P. 6. Rev3 depletion in HeLa cells affects late replication whereas in mouse cells Rev3 deficiency affects replication of TTR that lie between early and late replication. This is surprising as it suggests important mechanistic differences between Rev3 function in mouse and human cells, or even between different cell types.

This does not necessarily reflect a mechanistic difference of REV3L function between mouse and human cells, but rather this can be inherent to the cell type. Indeed, the differences

observed in change of replication timing can be due to the difference of the (epi)genomic landscape between embryonic *vs* tumor cells (MEF and HeLa). The Gilbert Lab has shown that replication timing changes during cell differentiation and disease. Overall, about 50% of replication domains replicate at similar times in all cell types (constitutive domains), while the other 50% (developmental domains) switch replication timing at some point during development and/or in disease (reviewed in Vouzas AE and Gilbert DM. Cold Spring Harb Perspect Biol. 2021 Feb PMID: 33558366). This might explain the different genomic domains (e.g. TTR *vs* late) that change after the loss of REV3L in MEF as compared to HeLa cells. Nevertheless, we have observed that in both cell lines, replication of these regions was mainly delayed (Fig EV2 B-D), suggesting that the molecular mechanisms involved in these changes are similar.

Is the replication of pericentric heterochromatin affected in HeLa cells, similar to MEFs?

In the manuscript we have examined the stability of mouse pericentromeric heterochromatin using FISH approach on metaphase in mouse *Rev3l-/-* cells (now Fig 6F-G). However, our DNA combing-FISH experiments have been carried out on HeLa cells and showed clearly that REV3L depletion significantly reduces replication fork speed in human pericentromeric regions (now Fig 6A). Therefore, in both cell lines replication of pericentromeric heterochromatin is affected.

Does HP1 bind to late replicating chromatin (in HeLa cells)??

Heterochromatin protein 1 (HP1), a reader of the repressive histone mark, H3K9me3, is an essential component of the constitutive heterochromatin which replicates late in mammalian cells, including human cells. Therefore, HP1 binds late-replicating domains.

2. P. 11. "Intriguingly, mild replication stress induced by a low dose of aphidicolin (APH) led to a similar genome-wide increase of breaks in both Rev3l-/- and Rev3l+/+ MEFs, suggesting that APH-induced DNA breaks are partially prevented by PolZ/REV3L. Consistent with this, we did not observe an increase of PHC breaks in Rev3l cells treated with aphidicolin (Fig 5F and G)." I do not agree with this conclusion since, in case Rev3 prevents APH-induced breaks they should specifically be increased in Rev3- cells. And why does APD not specifically induce even more breaks in pericentric heterochromatin (where Rev3 is recruited by HP1) in Rev3 KO cells than in global chromatin?

We agree that this paragraph might be confusing. Therefore, we have re-phrased it in the revised version #2 of the manuscript.

3. P. 12. UV is used to induce dsDNA breaks. But UV initially induces stalling of replication. The latter is much higher in Rev3 KO cells and therefore many more dsDNA breaks are induced in Rev3 KO cells than in wt cells.

How can it be concluded that the protracted presence of these breaks reflects the direct involvement of Rev3 in dsDNA breaks repair rather than the delay in repair caused by saturation of the repair machinery due to the large number of breaks in Rev3 KO cells?

As explained in the manuscript, the high level of DSBs in *Rev3l-/-* cells visualized by 53BP1 marker might be due to a combination of increased replication associated-DSBs and inefficient repair of those breaks. The hypothesis that loss of REV3L causes inefficient DSB repair is based on the following results (now Fig 8): (i) a pronounced persistence of S824-

phosphorylated KAP1 after low doses of UV irradiation (e.g. 4 j/m2), (ii) an activation of phosphorylated (S4-S8) RPA2 (known to be a good surrogate of DSBs resection) in UV-irradiated *Rev3l-/-* cells, indicating that repair of DSBs by homologous recombination is initiated, (iii) persistence of RAD51 foci at late time points after UV-irradiation, suggesting that HR-associated DNA synthesis is inefficient, and (iv) Interaction of REV3L with SCAI (now Fig 5J-K), a mediator of 53BP1-dependent repair of heterochromatin-associated DSBs (Hansen et al, 2016). Moreover, other studies in the literature reported that Polζ/REV3L might be required for efficient HR-associated DNA synthesis to complete DSB repair (Sharma et al, 2012). There is also evidence that Polζ-mediated DNA synthesis occurs during DSB repair in some settings in different organisms (Kane et al, 2012).
Saying that, we cannot rule out that the high level of these breaks can also be due to the delay in repair caused by saturation of the repair machinery in *Rev3l* KO cells. Therefore, we have mitigated our statement by including this possibility in the Discussion section.

Of note, the increased phosphorylation of RPA2 can very well be explained by the increased number of ssDNA at stalled forks in the absence of Rev3, rather that the presence of more (resected) dsDNA breaks.

To investigate the effect of Polζ/REV3L in regulating repair of heterochromatin-associated DSBs, we examined the levels of S4/S8 RPA2 phosphorylation (now Fig 8A). We agree that S33 RPA2 phosphorylation by ATR is induced by ssDNA at stalled forks. However, RPA2 phosphorylation at S4/S8 indicates ATM/DNA-dependent protein kinase (DNA-PK) activation and is typically used as a DSB marker for ssDNA arising from resection at breaks (Wang H et al. Cancer Res. 2001; Liu S et al. Nucleic Acids Res. 2012).

In brief, these experiments do not provide evidence for a role of Rev3 in dsDNA break repair, a statement that reappears at many places throughout the manuscript.

We have broadened the interpretative statement in the Discussion section.

Minor (but not trivial!) issues:

1. P.4 describes that the late S phase is protracted in Rev3 cells. Surprisingly, according to the rebuttal, these cells proliferate faster that the wt controls (17.2 vs 19.1h). Can the authors explain this paradoxical result?

Apologies for this confusing response in the rebuttal letter. It is true that *Rev3l*$^{-/-}$*;p53*$^{-/-}$ MEFs grow faster than *Rev3L*$^{+/+}$*;p53*$^{-/-}$cells (17.2 vs 19.1h; Wittschieben et al Cancer Res 2006). However, *Rev3l*$^{-/-}$ MEFs immortalized with a T-antigen expression vector (same used in this study) grow slower than T-Ag immortalized *Rev3l*$^{\Delta/+}$ MEFs (see graph in Fig. 5E of Lange et al Nucleic Acids Res 2012). This difference might be due to the fact that for the first set of cell lines, p53 is lost while in the latter set of cell lines, T-Ag can sequester p53 protein, resulting to a partial loss of function of p53 which can be variable, depending on the expression level of T-Ag.

2. P. 7. WT1 expression was strongly downregulated in one Rev3 MEF line. Is this true also for other MEF lines? And for HeLa cells?

Unfortunately, we did not assess the gene expression profiles in other cell lines.

3. 3. P. 7. Figs. 3A and EV3C. It is not clear to me which lanes represent independent MEF lines and which lanes represent technical replicates.

Apologies for this. We have now modified the figure legend to distinguish biological and technical samples.

To me, most bands appear different when lanes for +/+ or -/- are compared. Also, the bands in the blue panel are difficult to see.

We have again modified the intensity of panel colors. For more details about reproducibility of replicates, microarray data have been deposited at EBI Array express database with the Accession Number E-MTAB-8338.

4. Similarly, P. 7. Figs. 3B and EV3D display the expression of different genes. These Figs show independent Rev3 MEF lines (as far as I understand). Why were not the same genes tested in these cell lines to compare the phenotypes?

In the manuscript, we wrote "*We therefore performed microarray-based transcriptome profiling from Rev3l*$^{+/+}$ *and Rev3l*$^{-/-}$ *MEFs (Fig 3A and EV3)*". Therefore, Fig 3B and EV3D display the microarray data from one pair of MEFs (same as used for cell cycle, replication timing, histone marks expression…). To validate these results, RTqPCR has been performed on specific genes from the same couple of cell lines (and not from independent Rev3l-/- MEF lines), but from independent cell cultures. To be more understandable, we have reorganized the set of figures. Now, Figure 3 contains all results concerning gene expression profiling (including data from Fig 3A and EV3**)** and Figure 4 contains results related to epigenomic features (including histone marks expression levels, ChIP-qPCR and DNA methylation of major satellites).

5. P. 12. Do the deleted regions in Rev3 KO cells contain obvious hard-to-replicate sequences?

Our analysis by WGS identified 61 deletions in *Rev3l-/-* samples and only 5 in *Rev3l+/+* samples (95%CI=7.6-13.4, P-value < 2.2e-16, Poisson test), with a mean deletion size of 2092 bp (ranged from 55 bp to 9115 bp). Taking into the account the low number of identified deleted regions and the relatively small size of deletions, it was impossible to reveal any common features related to "hard-to-replicate" sequences using bioinformatics tools.

These deletions are found in late-replicating chromatin. This appears not to be this same chromatin as the pericentric chromatin (that replicates between early and late chromatin). Does HP1 (that recruits Rev3) bind to late chromatin? If not, what can the relevance of HP1 binding be?

As mentioned above, HP1 is a component of heterochromatin and HP1 binds to H3K9me3, a repressive histone mark found in constitutive heterochromatin. So HP1 is enriched in heterochromatin-associated late replicating regions.

6. P. 12. What does 'Relative deletion rate (Mb)' indicate? Why is there no increase in deletions at TTR (Fig. 6D)? And what is the significance in the difference between the # of breaks in early and late S? Are the # of deletions normalized to the size of the early vs late replicating regions?

The "relative deletion rate" is the term we used for number of deletions in Rev3 -/- clones per megabase. A more correct term is "frequency" not rate, so this has been changed (now Fig 7D). Thus, this measure is normalized to the difference in the region size. The number of deletions in late-replicating regions relative to early-replicating regions is 2.24 times higher

and statistically significant (P=0.01454, Poisson test with the correction for different length of intervals through the ratio). We have modified the figure 7D legend as follows:
"Distribution of the deletions detected in Rev3l$^{-/-}$ subclones related to the replication timing in deletions per megabase. Difference between late and early regions is statistically significant (p=0.01454, Poisson test with the correction for different length of intervals through the ratio)."

7. P. 15. "Future studies may determine the mechanisms by which REV3L is involved in the repair of heterochromatin-associated DSBs by interaction with SCAI." There is no evidence provided that Rev3 interacts with SCA1.

The Result section and figure 5J and 5K, describe the interaction between REV3L and SCAI.

8. The notion that in the absence of Rev3 increased levels of H3K27me3 levels and, to a lesser extent, H3K9me3 and H3K4me3 are linked to dsDNA repair is very speculative and not at all based on a mechanistic explanation.

We agree, our hypothesis is speculative and lacks mechanistic explanation, but it remains one possibility among many others. Investigation is in progress to clarify the molecular mechanisms that can explain how loss of REV3L leads to changes in epigenetic landscape and transcriptional control of genes.

Referee #4:

Yamin et al., have submitted a revised version of the paper that is very much improved. It is impressive, considering also the difficult year. My concerns have been satisfied.

We thank the reviewer for carefully reading the manuscript again and we are delighted that he/she find it much improved and that concerns have been satisfied. The reviewer has some remaining suggestions that we have addressed as fully as possible below and in the revised manuscript #2. We hope that these data, text modifications and figure alterations will address these remaining concerns in a satisfactory manner.

I have nevertheless some suggestions, mostly on reworking the text to give more emphasis to the more important data. I would like to see comment (e) addressed. It would be important to show that the co-localisation of HP1 and Rev3 is specific to S-phase.

Major points:

a. As a general comment, I agree with the hypothesis, explained in the discussion, proposing that the changes of replication timing could be a consequence of the problems in fork progression. Because of this, I am not sure I would start the paper with the replication timing data. It emphasises a consequence, and sets the wrong expectations. The data on the role of pol ζ in the replication of heterochromatin are so well done and solid that should really be the main focus and the data on the replication timing should be a short observation in the middle/the end of the paper.
The same goes for the changes in gene expression. Again, I agree with the hypothesis that they are a secondary effect of the epigenetic changes, possibly as a consequence of problems in repair (this is one of many possibilities). I would therefore de-emphasise them.

This is a reasonable point regarding possible re-organization of the study. It is true that once one understands that REV3L associates with heterochromatin components and that polζ is involved with DNA repair events in those regions, our other observations then follow as consequences. In this spirit, we have organized the Abstract to describe the causes and consequences in this respect, consistent with the viewpoint of the referee. A reader of the paper will start with the Abstract and this should set the expectations clearly. For presentation of the Results, we decided that it is preferable to use the actual order of our experimental course and reasoning, which took place over many years. This guides the reader through the logic of the experiments, and we have used linking phrases between sections such as ""We reasoned that …", "this result prompted us to investigate", and so forth. We think that future readers, including trainees will appreciate this description of how the investigation actually took place over many years. The Discussion section is organized in a more cause-and-effect manner, along the lines suggested by the referee.

b. Remaining on the topic of the changes of gene expression profile, I think that the authors' interpretation of the origin of the silencing is an argument in support of the conclusion that Rev3l deficiency induces DNA damage beyond heterochromatin. The authors propose that the silencing of genes normally expressed is induced by the permanence of heterochromatin marks deposited during repair in regions that have undergone DNA damage in absence of Rev3l. The consequential conclusion is that there is a lot of damage in genes that are normally expressed, and, therefore, within euchromatin. This is in agreement with the fact that the large majority of the genes whose expression is deregulated by Rev3l deletion do not fall within disturbed regions and supports the idea that the disturbed replication timing is a secondary consequence of the problems of replication in TTRs. I think the authors should revise the text in this direction. It would be much easier to link all the data.

We are agreed with this interpretation. Nevertheless, we do not have any biological evidence to claim that many DSBs occur in euchromatin in absence of REV3L. However, we have reorganized our discussion in this way.

c. Page 7 it should be discussed the fact that vast the majority of the deregulated genes do not fall within disturbed regions.

This has been now mentioned in the Results and Discussion sections.

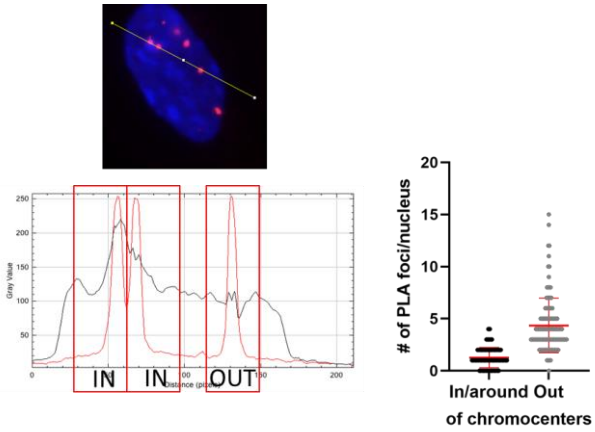
d. What percentage of the disturbed regions falls within TTR? This question is linked to the changes in the gene expression profile.

As mentioned in the manuscript, 19.2% (in bp) of disturbed regions were advanced in timing and 80.8% of regions were delayed in *Rev3l-/-* MEFs (EV2B). 67% of disturbed domains fall in TTR (corresponding to 83% of delayed regions). We added this information in the revised manuscript.

e. Figure 4: the PLA data are still not very convincing. REV7 has a lot of background on its own. Moreover, the PLA with HP1α does not seem to correspond to chromocenters, although the quality of the pictures makes it hard to judge. Could the authors evaluate if and how much of the signal correlates with chromocenters? In addition, a PLA with EdU would be very informative, because it would make sense if REV3 co-localises with HP1α specifically in S-phase, and preferably, in presence of mid and late patterns. EdU staining should be compatible with PLA.

The hypothesis that the interaction between REV3L and HP1 takes place in S phase, especially during mid-late S phase is timely and very interesting. We plan to decipher the dynamics of REV3L-HP1 interaction during the cell cycle. However, this set of experience will take time because technically it is not trivial. We have to set up the conditions to detect PLA foci with EdU, and the way to perform this experiment. Indeed, click chemistry for detecting EdU may affect PLA foci detection (and vice versa, starting doing PLA might alter EdU detection). For these reasons, the study cannot be reported in the current manuscript.

However, the referee also suggested that we evaluate if and how much of the PLA signal REV3L-HP1 correlates with chromocenters. This has been investigated by going back to images used for PLA quantification (Fig 5B and EV3D). We did not expect that the interaction REV3L-HP1 necessarily takes place IN, but rather at the periphery of (around) the chromocenters. As described by the Almouzni Lab, replication of pericentromeric regions occur mainly at the surface of the chromocenters where PCNA is located (Quivy et al., EMBO J 2004). Of note for REV3L-HP1 PLA experiment, we did not use chromocenter markers (e.g. H3K9me3 staining). Therefore, we used DAPI density to recognize these structures (see Figure below). We determined the localization of REV3L-HP1 PLA signals in 115 cells (from 2 independent experiments). We observed that 41/115 cells (36%) have ≥ 2 REV3L-HP1 PLA foci in/around chromocenters, suggesting that REV3L interacts with HP1 in pericentromeric regions in one third of the cells which are probably in S phase. The mean of REV3L-HP1 PLA foci in and out of chromocenters/nucleus was 1.25 and 4.4, respectively (see Figure below), consistent with the idea that REV3L interacts with HP1 in pericentromeric heterochromatin, but also in other heterochromatic regions localized throughout the genome. This quantification has been integrated in the revised version of the manuscript (Figure EV4G).



Minor points:

Figure 1A: -in the figure legend is still missing what the green and red arrows are pointing to. I know it is in the text, but it should be in the legend too.

corrected

-The name of the gates (P6, 8 and 11) should be removed.

done

Figure 1B: the "D" in EdU should not be capital.

corrected

Figure EV1: -the names of the gates R1, R2 and R3 should be removed.

-In the top half of the figure what do R2, R1 and R3 denote in all the FACS panels. (It might be better to remove these along with P6, P8 and P9 in Figure 1 and label the gates as G1/S, Mid-S and S/G2.)

done

-The bottom right panel shows PCR and not qPCR results (the legend says RT-qPCR).

corrected

Page 4: -end of the introduction. there is a mistake, "instability:" should read "stability".

corrected

-In the first paragraph of the results: "Moreover, analysis of BrdU-negative cells showed that G2 phase was longer in Rev3l-/- cells as compared to Rev3l+/+". Technically, to say something like this the authors would need to track G2 in single cells. On a population-base, it would be more correct to say that cells tend to accumulate in G2?

corrected

Page 5: last sentence of the first paragraph. As I pointed out already, because there is not necessarily correspondence between spatial and temporal control of replication, the sentence should read: "These results suggest that lack of REV3L can COULD affect the temporal control of DNA replication."

corrected

Figure 2: in the legend it wrongly refers to Fig. EV1 instead of EV2.

corrected

Page 6: "For each parameter, we observed a molecular signature intermediate between that of early and late domains, strengthening our hypothesis that these disturbed regions correspond to TTR. In Rev3l-/- cells, 18.7% of TTR were disturbed.". I would say that every signature it is very close to the mid domains.

You are right, mid domains and TTR replicate in the same time windows, and therefore have the same molecular signatures. Because we always compared early, late and TTR domains in the figures, we want to keep TTR in the text. Otherwise, the text can become confusing.

Figure EV2: what are the squinted, short grey dotted lines in the plots?

Concerning the grey dots in the plots in Fig EV2, they represent the value of the log2 ratio (early vs late) for a given probe. The lines are the smoothing profile calculated on a moving window of these values. we use the START-R software to make the smoothing with a window of 800kb (Hadjadj et al, 2020).

Figure 3: A The scale below the figure is unreadable. In addition, it is hard to see the changes in gene expression with the yellow versus blue colour.

We have increased the scale bar and modified the intensity of panel colors. For more details about reproducibility of replicates, microarray data have been deposited at EBI Array express database with the Accession Number E-MTAB-8338.

E why are there no error bars, if the experiments were repeated twice, as the legend says? In addition, all the ChIP need unchanged controls, like genes that do not change RT or expression, genes that change RT but not expression, genes that become earlier replicating. In all panel the asterisks need to be added to the figure.

Fig 7E has been added during the first revision of this article among many other experiments asked by the four reviewers. Because of time constraint, experiments have been repeated twice instead of three time. We found that the results of the two independent experiments had the same tendency, but the enrichment of histone marks (H3K9ac and H3K27ac) was quite different between experiments, leading to important error bars (see Figure below). We then decided to show representative graphs from one experiment.

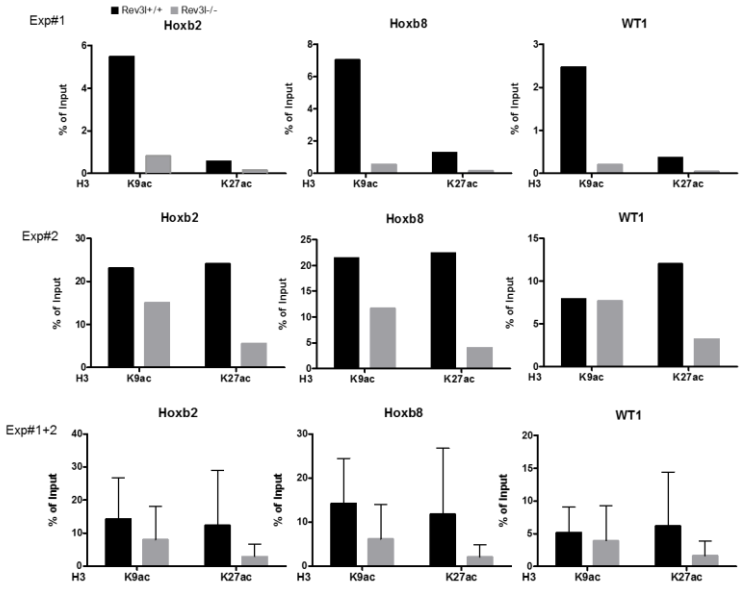


Figure EV3: The scale under Expanded View Figure 3C is unreadable. In addition, it is hard to see the changes in gene expression with the yellow versus blue colour scheme (the blue is still too saturated, although in the response to reviewers the authors say this has been improved

Same as Fig 3

Page 6: The text says that the paternally expressed gene Dcn is shown in blue in Expanded View Figure 3C whereas it is in fact shown in pink (and is referred to as maternally expressed in the legend to Expanded View Figure 3D).

Apologies about this mistake, DCN is a maternally expressed gene. We have modified the revised manuscript.

Figure 4: E it would be better if the merged channels were shown to demonstrate the overlap in signals more clearly.

We think that the overlap in signal is clearly demonstrated in the Fig 4E with the graph providing fluorescence intensity of each signal.

H & I refer to FH-REV3L constructs. These should be mentioned in the Plasmids section of the Materials and Methods as their construction is not described. Note also that these constructs are referred to as FH-REV3L in the text, F-H-REV3L in Fig. 4 and Fig. EV5, but as Flag-HA-REV3L in the legend to Fig. EV5, so this should be rectified.

Apologies for this mislabeling. Plasmid name (F-H-REV3L) has been homogenized in the text, Figures and Figure Legends. Description of plasmid constructs has also been added in the Materials and Methods section.

Figure 5: The legend should be altered to make clear the sequential nature of the IdU and CldU staining. E.g., 'Representative fibers of newly synthesised DNA labelled with IdU (red) for 30 minutes then CldU (green) 30 minutes...'

corrected

Figure EV5: C The text needs to be improved as the two Vs have merged to make them look like a W.

corrected

The empty vector and the construct referred to as REV3L should be described in the Materials and Methods 'Plasmid' section

corrected

Minor typos:

Page 7: at the bottom 'H3K9m3' should be replaced by 'H3K9me3'

corrected

Page 22: paragraph 2 'interested proteins' should be replaced by 'interesting proteins' and

'processes' by 'processed'.

corrected

2nd Revision - Editorial Decision 30th Jul 2021

Thank you for submitting your re-revised manuscript. I have now carefully checked your responses and final modifications, and I am pleased to say that I see no further conceptual reservations towards publication at this point.

However, there are still some open editorial issues that need to be addressed:

The authors performed the requested editorial changes.

3rd Revision - Editorial Decision 28th Aug 2021

Thank you for submitting your final revised manuscript for our consideration. I am pleased to inform you that we have now accepted it for publication in The EMBO 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: KANNOUCHE
Journal Submitted to: EMBO J
Manuscript Number: EMBOJ-2020-104543

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 χ2 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?	Sample size was determined empirically, no statistical test was used to predetermine the sample size.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	N/A
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 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.	N/A
For animal studies, include a statement about randomization even if no randomization was used.	N/A
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.	Samples of plated cells were randomly chosen for transfection, drug treatment, molecular biology and microscopy analysis. The experiments were performed by two colleagues in blinded fashion when possible.
4.b. For animal studies, include a statement about blinding even if no blinding was done	N/A
5. For every figure, are statistical tests justified as appropriate?	The statistical tests performed are stated in all figure legends.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	When possible, data were tested for normality using the Graphpad Prism software

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Is there an estimate of variation within each group of data?	Error bar was used to show variation.
Is the variance similar between the groups that are being statistically compared?	N/A

C- Reagents

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).	The source of all the antibodies used in this study is reported in the materials and methods section
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Immortalized mouse embryonic fibroblast (MEF) Rev3l-/- and Rev3l+/+ were generated in the Wood Lab and described in (Lange et al., NAR 2012). Rev3l-/- MEFs complemented with POZ empty vector (clone 4-5 POZN Cl2) or POZ-hREV3L (clone 4-5 POZRev3l2 Cl H11) were generated in the Wood Lab and described in (Lange et al, Plos Genetics, 2016). Suv39h double-null MEFs were generated and provided by T. Jenuwein (Peters et al., Cell 2001). NIH3T3 cells (CRL-1658), HEK293 (CRL-¬1573), Hela (CCL-2) and hTERT RPE-1 (CRL-4000) cells were obtained from ATCC´s collection. All cell lines are routinely checked for mycoplasma contamination using the MycoAlert detection kit (Lonza).

* 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.	N/A
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E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N/A
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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	• Microarray data: EMBL-EBI E-MTAB-8338 (https://ebi.ac.uk/arrayexoress/experiments/E-MTAB-8338) • Replication timing data: GSE 178927 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE178927)
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22. 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.	N/A