nature portfolio

Peer Review File

Evidence for improved DNA repair in long-lived Bowhead Whale

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

Version 1:

Reviewer comments:

Referee #1

(Remarks to the Author)
Longer lived species are believed to have additional layers of protections against carcinogenesis compared to shorter lived species. These additional layers of protection could be related to the number of genetic hits it takes to initiate cancer growth and indeed previous reports have indicated mouse cells only require two hits to generate cancer while human cells require five hits. The authors here ask whether longer-lived bowhead whale cells require even more than five hits to become carcinogenic and surprisingly find that it only takes four hits for them to become carcinogenic. They then propose that cells from these long-lived animals may have increased DNA repair efficiencies compared to human and even shorter-lived mammals. They find no appreciable differences in NER or BER rates between bowhead whales and humans, but they do find that DSBs induced in a reporter assay are repaired more efficiently in bowhead whale cells, and, moreover, these cells have fewer micronuclei following treatment with ionizing irradiation compared to human cells. When DSBs are introduced in whale, human, mouse, or cow fibroblasts, via a reporter or via Cas9 cleavage at endogenous loci, the authors find that whale fibroblasts have better repair and less mutations compared to other species. They account for this heightened efficiency largely due to the CIRBP factor which is highly expressed in bowhead whale cells.

While the topic of this manuscript is of general interest, there is a lack of definitive data to support the authors' conclusions. Specifically, the link between faster and more faithful NHEJ and increased longevity in bowhead whale cells needs to be solidified for consideration at Nature. The manuscript in its current form lacks the depth expected for publication and is more suited for submission to a more specialized journal.

Major comments:

• Much of the measurement of DSB repair efficiency in this manuscript relies upon a reporter assay. Can the authors perform a complementary assay to show DSBs are repaired more efficiently in bowhead whale cells? A commonly used assay like yH2AX immunofluorescence would be suitable. Do bowhead cells have less endogenous yH2AX than human cells? Is yH2AX resolved more rapidly in bowhead cells following ionizing radiation treatment? Does CIRBP depletion or supplementation affect yH2AX levels?
• The authors should be more systematic in identifying which DNA repair pathways are more/less efficient or faithful in bowhead whale cells. They perform reporter assays to study NER, BER, and NHEJ in these fibroblasts and based on these experiments conclude that more efficient NHEJ is the notable feature of bowhead whale cells that may allow them to live longer. Is it possible that the more mutagenic MMEJ pathway is less active in whale cells and thus these cells undergo less mutation? In general, they do not examine other DNA repair pathways including HR, alternative end-joining (MMEJ), as well as mismatch repair (MMR). It would strengthen this manuscript if the authors queried the repair efficiencies of these additional pathways between bowhead whale and human fibroblasts.
• The authors perform an assay where they treat whale or human primary fibroblasts with the DNA alkylating agent ENU and measure the mutation rates in each cell line. They find that in response to this agent whale cells mutate less than human cells, and thus have more accurate DNA repair. The authors should mention what types of lesions ENU induces onto DNA and the different DNA repair pathways that repairs these lesions. They conclude that NER and BER rates are not different between human and whale cells, and yet it is commonly believed that ENU induces lesions dealt with by the BER, NER,

MMR repair mechanisms. Why is it that whale cells mutate less to ENU than human cells, even if their excision repair efficiencies are similar? Are repair polymerases less faithful in whales?
• Additionally, are fewer mutations specific to treatment with ENU or is it a universal feature of these cells to many different mutagens. This section of the manuscript would be strengthened if the authors repeated the same assay with mutagen other than ENU.
• In lines 214-215 of the text and Extended Data Fig 2g, the authors demonstrate that bowhead whale cells are more sensitive to ENU. If these cells have better DNA repair and fewer mutagenesis compared to human cells, where does the increased sensitivity arise from? The higher sensitivity to ENU in whale cells suggests that some agents do promote more apoptosis in whale compared to human, as opposed to the data shown in Figure 1.
• The authors perform one experiment where they demonstrate PARP activity is increased in whale cells treated with H2O2 compared to human cells. This forms a large part of their model (Figure 5D). It is unclear why PARP activity higher. Is it that basal PARP levels are greater in bowhead whales? Is PARP activity also much higher in whale cells in response to DSBs, or is this unique to H2O2? It would be helpful if the authors included a control showing that H2O2 treatment worked equally well in both cell lines (perhaps a TUNEL assay or Comet assay showing induction of breaks). What are the consequences of increased PARP activity in these cells? Are SSBs induced by H2O2 repaired faster in bowhead whale fibroblasts compared to human fibroblasts.
• The authors show via RNA-seq that SASP markers are expressed less in senescent bowhead whale cells compared to human cells. While this observation is interesting, how does it fit with the remainder of the paper which proposes that it is increased DNA repair efficiency and fidelity that accounts for increased longevity of bowhead whale cells?
• The bowhead whale fibroblasts have a distinct mutational profile in response to CRISPR-induced DSBS (that being single-base insertions). Can the authors comment on where this distinct mutation profile comes from? Since NHEJ could be responsible for large deletion formation, it is theoretically possible that the phenotype is related to decreased rather than increased NHEJ. These cells indeed have very low levels of Ku/DNA-PKcs (Figure 4a).
• In Figure 4f, the authors demonstrate that human cells overexpressing bowhead whale CIRBP have fewer micronuclei than wild type cells. Is this result specific to the bowhead whale protein? Does overexpressing human CIRBP also lead to the same result?
• In Figure 4g, the authors interestingly show that human cells exposed to hypothermia have increased NHEJ efficiency in their reporter assay. The authors imply that this could be due to an increase in human CIRBP expression. Evidence that CIRBP expression has increased in these cells is missing though. A western blot demonstrating this would suffice and is an important control for this experiment.
• They describe two known functions of CIRBP in DNA damage response – increased translation of proteins involved in stress and DNA damage, and PAR-binding – but do not provide evidence for either mechanism in whales nor a link with NHEJ. At minimum, they should show levels of known CIRBP targets in whale cells after DNA damage and whether whale CIRBP has increased PAR-binding. It is also unclear if CIRBP is found at DNA lesions.
• The model in Figure 5 overstates the link with cancer resistance and longevity. While it may turn out that bowhead whale fibroblasts are able to repair DNA breaks faster and more efficiently than human fibroblasts, this doesn't demonstrate a connection with aging or cancer.

Minor comments
• The Discussion is sprawling and should be slimmed to the points that are most relevant to the core findings of this manuscript.

Referee #2

(Remarks to the Author)
Summary of key results:
This paper makes a compelling case that the long lifespan of bowhead whales has entailed mitigating the risk of cancer (arising from their very large body size) by elevation of efficiency and accuracy of DNA repair. Elimination of transformed cells via apoptosis was the route taken by elephants, but whales instead evolved enhanced capability for efficient and accurate repair of DNA damage.

Originality and significance:
This is a highly original paper with the potential to lead to entirely novel strategies to reduce cancer incidence in humans. The manuscript is well written, and presents a huge compilation of data.

Data & methodology:
The data are quite comprehensive and mutually supportive. Experiments are well designed and capably executed. The description of methods is somewhat underwhelming and would be improved by addition of greater detail -- which could be easily accommodated within the Supplementary Methods section.

Appropriate use of statistics and treatment of uncertainties:
The authors frequently present data for which N=2 or N=3, and assess significance of inter-group differences with a "two-tailed t-test". The standard ("Student's") t test requires that each N be large enough to reasonably estimate the population variance – typically N of at least 6 (but preferably at least 10), and that the variances not differ between groups. With an N of 3 – 5, it is preferable to use the Fisher-Behrens heteroscedastic t test (e.g., type 3 within Excel), which makes allowance for

unknown or unequal variances. When N = 2, no version of a t test is meaningful, and it would be preferable to plot the replicates separately, as evidence of reproducibility. Given the arduous nature of the assays involved, that would be a perfectly acceptable compromise.

Conclusions -- robustness, validity, reliability:
Because the experimental data are so extensive, the conclusions are quite robust. The preceding comments on assessment of statistical significance are effectively obviated by close agreement within duplicate or triplicate assays.

Suggested improvements:
This manuscript is exceptionally thorough and convincing. I would not request any improvement except to address a number of minor issues (see statistics section above, and minor points listed below).

References:
The references cited are appropriate. Although one paper is cited from the de Magalhaes group (Keane et al., 2015), I would have expected the prior contributions from this team (4 papers, 2015 - 2021) to have been discussed more thoroughly.

Clarity and context:
The abstract, introduction, and conclusions are all on target and written with commendable clarity and focus.

ADDITIONAL MINOR POINTS TO BE ADDRESSED BY THE AUTHORS:

1. Figure 1c should not show “150% of cells” on the ordinate.

2. Figure 1f could be made clearer and more informative to readers, for example if more than 2 symbol pairs were labeled with gene names. For the 3 rightmost genes, irradiated/control is 4 – 6 in humans, but ≤1 in bowhead whales, most likely signifying that BW do not respond to ɣ-irradiation by increasing transcription of SASP genes. However, too much is left to the reader’s imagination. What gene does the third pair of dots represent? What can be inferred from the dot pairs that show 50- to 200-fold induction in humans but *suppression* in BW? What could/should be a powerful argument for SASP blunting in BW ends up being at best suggestive, and at worst confusing.

3. Figure 3a is presented as an assay of NHEJ. Without a clear statement as to *how long* after I-Sce1 cleavage the fibroblasts were imaged, the reader could be forgiven for thinking that these results reflect an unknown mixture of NHEJ and HR events.

4. In Figure 5 a – c, it is impossible to discern to which categories of events the asterisks pertain. Again, with N=3, 2-way ANOVA is not an adequate measure of significance. In this case, it would be sufficient simply to acknowledge this in the figure legend, e.g. by referring to the P values as “nominal indices of significance”.

5. Extended Data Figure 2b appears to reveal an intriguing difference in the kinetics of pyrimidine-dimer repair, between human and BW fibroblasts — and yet the data are summarized in the main text as showing that human and BW are “similar” in this regard. Even with an N of 2, I strongly suspect that an appropriate comparison (e.g., by some version of the Wilcoxon log-rank test) would establish that these curves differ quite significantly. This looks just a bit like allowing the desired (or most readily assimilated) conclusion dictate the interpretation of data.

Reviewer: Robert J. Shmookler Reis

Referee #3

(Remarks to the Author)
Firsanov et al. discovered that bowhead whales, the second largest-living mammal, have a highly efficient DNA repair machinery, and suggest that DNA repair, rather than tumor suppression, is a likely cellular mechanism underlying the extreme longevity of this cetacean. Using proteomics, transcriptomics and experimental manipulations in cell culture, the authors identify two genes, Redacted Minor point: what is “RS” in Figure 1c?

Referee #4

(Remarks to the Author)
In this manuscript, the authors used bowhead whale fibroblasts to investigate why bowhead whales have extraordinarily long lifespan. They show that whale cells do not bypass senescence by activating telomerase and do not display increased p53 activity. Furthermore, whale cells require fewer hits to undergo oncogenic transformation compared to human cells. These findings argue against some previous models that were proposed to explain why large mammals have long lifespans. Interestingly, the authors also find that whale cells have higher NHEJ repair activity and lower levels of large deletions. Using LCMS, RNA-seq and western, they found that CIRBP Redacted. Although the model of this study is quite interesting, the data onDNA repair are not yet convincing. It is still premature to conclude that enhanced DNA repair is responsible for the longevityof bowhead whale.

1. In Fig. 3a, the authors show that NHEJ activity is much higher in whale cells than in other mammalian cells. However, there is no evidence that this heightened NHEJ activity in whale cells is responsible for the low levels of mutations and arrangements in whale cells (the data only show a correlation between the two). NHEJ is known to be a major driver of deletions/insertions in the genome. High NHEJ activity does not necessarily mean lower mutations and chromosomal arrangements.

2. The authors correctly stated that HR and NHEJ are the two main pathways that repair DSBs, but the HR activity in whale cells was not tested in Fig. 3. Why? This is puzzling especially because RPA is known to bind ssDNA and facilitate HR. If RPA2 is high in whale cells, HR is more likely to be stimulated than NHEJ.

3. The differences in DNA break junctions in human and whale cells are not adequately explained. Does heightened NHEJ promote 1 bp insertions and reduce MMEJ, which generates larger deletions/insertions? This is a possibility that can be directly tested.

4. How NHEJ is stimulated by bwCIRBP is not adequately explained. A previous study (Chen et al. PNAS 2017) already showed that hCIRBP is recruited to DSBs in a PARP-1 dependent manner. The previous study also showed that hCIRBP knockdown reduces HR and NHEJ activity. This study shows that bwCIRBP is sufficient to stimulate HR and NHEJ, and they need to better explain this phenomenon. Whether bwCIRBP affects HR activity in whale cells should be tested.

Redacted

7. Although bowhead whale fibroblasts provide a good model to study how whale cells are different from other mammalian cells, the main findings of this study should be confirmed in a more physiologically relevant context. Are CIRBP Redacted Are the levels of largedeletions/insertions much lower in whale cells compared to other mammals? If large deletions/insertions (instead of 1 bpinsertions) are drivers of aging, do bowhead whales accumulate these types of arrangements more slowly than other mammals as they age? I understand that these experiments may be difficult to perform, but some in vivo validation wouldmake the story much more convincing.

Referee #5

(Remarks to the Author)
In this manuscript Firsanov et al investigate mechanisms of improved DNA repair in the longest lived mammalian species the bowhead whale. To do this they generate fibroblast cell lines from bowhead whale skin samples. They demonstrate that bowhead whale fibroblasts require disruption of fewer tumour suppressor genes than humans to undergo oncogenic transformation. They investigate differences in activity and abundance of DNA repair genes and proteins. They find that bowhead whales have more efficient and accurate double-strand break repair than humans through a series of DNA damage and repair assays. Comparing the abundance of different DNA repair proteins in humans and bowhead whales theyi dentify CIRBP Redacted

The work in this manuscript is highly original and significant. It is, to my knowledge, the first mechanistic investigation of DNA repair in a species with a longer lifespan than humans. The demonstration that bowhead whales have evolved superior mechanisms of double-strand break repair is both evolutionarily of great interest and, as the authors mention in their discussion, has implications for potential therapeutic strategies to enhance the efficiency of DNA repair in humans to reduce

the risk of cancer and potentially slow the rate of ageing (though the connection between DNA damage and ageing remains contentious).

I am not an expert in the methods used for interrogating the DNA repair mechanisms in these cell lines. Therefore I cannot make an expert judgement about their appropriateness and whether the conclusions the authors draw are valid, which will be crucial for assessing the manuscript. However from reading through their methods and results my impression is that the authors were rigorous in their investigations and used multiple different assays to investigate DNA repair in the bowhead whale cell lines, which may mitigate limitations from any single specific method. If experts more familiar with the cell line assays used judge that they have been conducted and interpreted appropriately then I believe that this manuscript is highly significant.

I have some minor comments and suggestions for improving the manuscript:

1. More information could be provided about the samples used. It would be helpful if more information was included about the number of cell lines used in each assay and whether there was significant variation between cell lines. As one example, in Figure 3f where the most frequent allele types are shown after CRISPR induced DSBs it is unclear how different bowhead whale individuals were used in this analysis. The more individual whales that were used the more convincing the results would be, rather than depending on results from a single cell line or multiple cell lines from a single individual. Where multiple individuals and cell lines are used, a description of whether there was significant variation in results between individuals would be of interest to get a sense of how universal the results are for bowhead whales versus how dependent they may be on a single individual cell line.
For example I could not find it clearly stated in the manuscript whether the same number of bowhead whale cell lines were used for each assay. Furthermore what percentage of bowhead whale tissue samples that were collected were successfully transformed into cell lines? Does the study include one cell line per whale or multiple independently derived cell lines from each individual? Do the authors have any information about the estimated age-range of the whales from which tissue samples were collected? It is possible that DNA repair capabilities may vary with age in some species, in which case this information may be relevant.
The methods mention that skin and lung samples were collected for primary fibroblast isolation but the Primary cell cultures section only mentions isolation of fibroblasts from skin. Was there an attempt to culture from lung but this was not successful?

2. I extended figure 7b the authors show a comparison of RNA expression for a set of DNA repair genes in human and bowhead whale cell lines, which is part of how they identified CIRBP Redacted For this analysis I have a few questions. First ly, did the authors consider adding
data from other species such as mouse, or cow, as included in their analysis of protein abundance? Given the power of comparative methods to identify specific differences in bowhead whales that may underlie their improved DNA repair much might be gained from inclusion of data from a few additional species beyond this pairwise comparison. Secondly, how was the list of genes involved in DNA repair selected? It seems that many genes involved in DNA repair are not included (see for example https://www.mdanderson.org/documents/Labs/Wood-Laboratory/human-dna-repair-genes.html for a list of genes thought to be involved in DNA repair). A wider analysis of DNA repair genes could help to show that the genes the authors selected as their candidates really do stand out compared to other genes and might identify novel genes putatively involved in differences in DNA repair that the authors missed. Redacted. Is there a reason the
authors chose not to investigate RBBP8 further based on these results?

3.Redacted Did the authors investigate whether there was evidence for accelerated evolution in these genes in bowhead whales or if any amino acid substitutions were in highly conserved sites? Does the location of any amino acid changes provide insights into their likely function? Any such evidence might help to strengthen the evidence that these proteins have evolved to improve DNA repair in the bowhead whale.

4. Analysis of somatic mutations in healthy tissues in humans and other species has thus far focused on single nucleotide variants, primarily because of the difficulty of distinguishing between sequencing artefacts and mapping errors from true large structural variants present at low frequency in polyclonal tissues. From these studies it has emerged that normals cells in humans and other species accumulate point mutations due to processes such as the spontaneous deamination of 5-methylcytosine, incorrect repair of damage caused by reactive oxygen species and other mechanisms that have not yet been determined but are likely to include translesion synthesis and base excision repair. While the improved double strand break repair in bowhead whales would account for the reduction in some types of somatic mutations it is unlikely to cause a reduction in these other mutation types that have been shown to increase with age in humans and other mammalian species. Do the authors think that there may be additional mechanisms to reduce these types of mutations in bowhead whales or that it is only DSBs that are likely to be relevant for cancer risk and ageing in bowhead whales? While it is possible that DBBs might be more relevant for ageing it seems unlikely that bowhead whales could reduce their risk of cancer sufficiently without also reducing the mutation rate from other mutational processes besides DSBs, particularly given the authors's findings that bowhead whales do not require many mutations in tumour suppressor genes to trigger oncogenesis. I think the discussion would benefit from the authors' thoughts on this.

5. While intriguing I find the authors theories about linking CIRBP and DSB repair to the benefits of cold as a therapeutic agent highly speculative. While it is possible that there may be some mechanistic link there are many other ways that cold temperature may mediate health benefits beyond improvements to double strand break repair. Without additional experiments demonstrating that the benefits observed from cold therapies are mediated by improved DSB repair I think that the amount of space in the discussion dedicated to this is probably unwarranted. It could perhaps be mentioned briefly in the discussion and expanded upon as a supplementary note.

Version 2:

Reviewer comments:

Referee #1

(Remarks to the Author)

The authors have done several experiments to address concerns raised in the previous revision. Unfortunately, the data remains far from convincing. The authors present evidence that NHEJ, HR, and MMR all proceed more quickly and more faithfully in bowhead whales compared to human or other mammalian cells, and this is due to the fact that bowhead whalece lls have much greater expression of the factor CIRBP. It is very difficult to believe that expression of one factor is enough to boost all these different forms of repair to the extent that bowhead whales age slower. Redacted. Thus, I believe they are overselling their results. Also problematic is making all these conclusions about the mechanism of slower aging in bowhead whales based on the comparison of one bowhead whale fibroblast cellline with just one human, mouse, and cow fibroblast cell line. The authors did not conclusively prove that mutation rates are universally lower in bowhead whales (they use one mutagen, ENU, in one whale fibroblast cell line), nor did they thoroughly convince this reviewer that CIRBP is a master drive r of many forms of DNA repair and that higher expression of this gene isresponsible for slower mutation rates in bowhead whales.

Major concerns:

1. The authors claim to demonstrate that the mutation rate is lower in bowhead whales due to more efficient mismatch repair (they see no difference in base excision repair or nucleotide excision repair between bowhead whale cells and human cells). The evidence for this relies on one assay in which they stress cells with the mutagen ENU and see less mutations in the bowhead whale cells compared to human cells (via HPRT or SMM-seq). In their revision, the authors claim no other mutagen was suitable in their HPRT assay other than IR but fail to include that data where they use IR in the revised manuscript. One cannot make a general conclusion that whale cells have a lower mutation rate and better MMR based on their differential response to one DNA damaging agent (and a single MMR reporter assay). If the HPRT assay is not suitable for other DNA damaging agents, the authors should have used a different assay such as SMM-seq.

2. The manuscript relies on reporter assays to make conclusions about efficiency of DSB repair between bowhead whale and human cells. The authors should have also included simple survival experiments demonstrating that bowhead whale fibroblasts can withstand DSBs (via DNA damaging agents like etoposide, bleomycin, IR, etc) better than human fibroblasts. If CIRBP is a master DSB factor that can contribute to both NHEJ and HR, they should have demonstrated that the human fibroblasts that overexpress CIRBP can resolve exogenous DSBs better. They could have done basic survival experiments showing overexpression of CIRBP leads to better resistance to the DSB-inducing agents listed above. They also could have shown that yH2AX/53BP1 foci induced by these agents are resolved faster in human cells overexpressing CIRBP. It is very unclear how CIRBP could at the same time affect HR, NHEJ and MMR.

3. Expanding on Concern #3 above, in Extended Data 4E it appears DSB repair occurs more slowly in bowhead whale cells compared to human cells. This is a Pulse Field Electrophoresis Gel experiment. One of the main arguments in the manuscript is that repair occurs faster/more efficiently in bowhead whale cells compared to human cells though. This experiment is thus confusing and contradicts one of their central points. This is an example of why making conclusions that DSB repair is more efficient in bowhead whale cells based on reporter assays in the Main Figures may not be fully accurate.

4. It does not seem like overexpressing hCIRBP in human fibroblasts worked, technically-speaking. The authors claim in the text that overexpression of hCIRBP was done just as it was done with bwCIRBP, and that only bwCIRBP led to better NHEJ or HR efficiency (Figures 4b and 4c). However, if one examines Figure 4F, it is very clear that they did not successfully overexpress hCIRBP. One cannot make conclusions that bwCIRBP, but not hCIRBP, makes DSB repair more efficient when the hCIRBP overexpression did not work.

5. The authors make conclusions that CIRBP directly acts in NHEJ based on a biochemical NHEJ assay using purified proteins (Figure 4J). However, there is no control protein gel showing that purification of CIRBP worked and had no contaminating repair factors co-purifed. It is highly standard when purifying a new protein to demonstrate the quality of the purification (by Coomassie stain, western blot, and even mass spec analysis of the final purified products) and it is extremely problematic that these controls are missing. The results of this biochemical figure cannot be interpreted at all without this necessary data presented.

Redacted

Less major concerns:

1. Other essential controls are missing in this manuscript. Examples:
a. A Western blot showing that PTEN was actually knocked-out by CRISPR/Cas9 editing (Figure 2)
b. qPCR or Western blots showing that hTERT, Ras, p53, Rb, and PP2A/PTEN were successfully altered in Figures 2A/B.
Redacted
2. The supplementary figures are not presented in linear order through the manuscript which makes reading the manuscript unnecessarily confusing.

Referee #2

(Remarks to the Author)

Referee #3

(Remarks to the Author)
I am fully satisfied by the way the authors have addressed my questions/comments and I look forward to reading this manuscript in press soon.

Referee #4

(Remarks to the Author)
The authors have significantly improved the manuscript by adding new experiments. The sequencing data of mouse xenografts of bowhead whale cells in Extended Data Figure 1 has greatly strengthened the in vivo relevance of this study. Moreover, the in vitro biochemistry data showing that hCIRBP stimulates the DNA end ligation by XRCC4-LIG4 provide new evidence to explain how CIRBP promotes DSB repair. However, I still have some remaining questions about how CIRBP and RPA2 may contribute to the low genomic instability in whale cells. Addressing these questions is important for making this study mechanistically compelling.

Although the authors emphasized that CIRBP enhances NHEJ, it is not clear whether effects of CIRBP in cells is dependent on NHEJ. In Fig. 3c-i, ED4c, ED8f, ED8g, and ED8i, the authors showed a range of phenotypes to suggest that CIRBP promotes DSB repair and reduces more severe types of genomic alternations. While these phenotypes are clear, it is still unclear whether the differences caused by CIRBP are made through heightened NHEJ. The conclusion that CIRBP acts through NHEJ is only based on correlations. Given that CIRBP also increased HR (Fig. 3b), the overall picture of how CIRBP suppresses genomic alterations remains unclear.

I am still not convinced how CIBRP can promote NHEJ but avoid large deletions. Does CIBRP affect the processing of DNA ends? The authors provided some evidence that CIRBP can hold chromatin fragments together, and they speculated that this could be done through a LLPS-mediated mechanism. This model still cannot explain why large deletions are suppressed. In addition, if DSBs are clustered through LLPS, one would expect an increase in chromosomal translocations through error-prone NHEJ. Did the authors analyze translocations in whale cells?

Redacted

Referee #5

(Remarks to the Author)
I thank the authors for their responses to my comments. I am satisfied that they have addressed my concerns. The additional work on ENU induced mutational load and MMR repair efficiency is particularly interesting. In particular the response to the in-depth comments on the mutational assays from Reviewer 1 appear to have substantially strengthened the manuscript and added new dimensions that will are of great interest. My recommendation would be to accept the manuscript for publication.

Minor comment: Detecting low-frequency somatic mutations can be technically challenging. It would be nice to see the the trinucleotide mutational spectra from each species for the SMM-seq analyses to increase confidence that the somatic mutations detected are not highly enriched for artefacts as ENU induced mutagenesis has expected spectra. This is investigated in Extended Figure 2f but trinucleotide spectra can provide a more sensitive investigation of differences in mutational patterns.

Version 3:

Reviewer comments:

Referee #1

(Remarks to the Author)
This paper makes the bold claim that CIRBP is a major factor in determining longevity in the bowhead whale by simultaneously promoting mismatch repair and double strand break repair both by homologous recombination and non-homologous end joining. Although this is an interesting hypothesis, the data do not sufficiently support this claim.

For example, it is entirely unclear how CIRBP can promote end-joining and homologous recombination simultanously. Based on their in vitro data, CIRBP promotes end-ligation and protects DNA ends from exonuclease-mediated degradation. However, exonuclease mediated degradation (ie. End-resection) is exactly what is needed for homologous recombination. The point is that end-joining and homologous recombination are competing pathways, so how does CIRBP do both, let alone promote mismatch repair? Overexpression of other DNA repair factor (eg. CTIP) does not mean that HR is enhanced. If CIRPB is a key factor in determining longevity by facilitating DNA repair, it is important to know how it facilitates DNA repair.

If CIRPB promotes repair, why do fibroblast senesce and die by apoptosis at similar frequency after irradiation (Figures 1d-f)? One would think that repair would prolong their survival and inhibit senescence/cell death.

Another issue is longevity: Is the effect of overexpression of CIRBP in flies actually real? The results in figure 5a are not striking, especially when you compare these results to the life extension that genuine genetic aging interventions provide (one example is the Indy gene, mutation of which reportedly doubles the average lifespan of the fly) . If CIRBP is a driver of why bowhead whales live so long, surely we would expect to see it have more of an effect when overexpressed in flies.

• Extended Data 14v. They overexpress CIRBP in drosophilia at low, medium, or high levels. At high levels, there is no improved viability from CIRBP overexpression. But, they claim high expression of this factor in whales is what helps them live longer. This is thus conflicting and confusing.

Other points:
The authors demonstrate that in their hands, CIRBP association with chromatin is dependent on RNA. This fits in line with previous literature. The authors show that CIRBP facilitates end joining in vitro, this time in the absence of RNA.

Confusing when they say Each data point in comparative assays represents an independent cell line derived from a different individual. The comparative assays in Figure 1 and 2 only have three points and they claim they are biological replicates, so where do the 6 bowhead whale cell lines factor into this?

For example in Figure 3c (yH2AX foci), why are they plotting the data as averages of an experiment? They should be showing the full data, not the average of the experiment.

Same can be said for most of the data in Figure 3. How does the data look across individuals in the bowhead whale species. Is it consistent across individual cell lines?

bwCIRBP doesn't greatly improve IR resistance in flies, but hCIRBP does? I thought bwCIRBP was more potent.

In supplementary Figure 12h and i, CIRBP binds DNA? And yet in Supp. Figure 12a-c, they show in cells it doesn't bind DNA without RNA present.

Referee #4

(Remarks to the Author)
The authors have performed additional experiments and strengthened the manuscript. In particularly, they showed that expression of bwCIRBP and hCIRBP in Drosophila extended lifespan after IR, strengthening the in vivo impact of CIRBP overexpression. They also carried out detailed biochemical characterizations of CIRBP in vitro, providing further support to its role in enhancing NHEJ. These new results indeed make the conclusions more convincing.

However, the overall model of this study still pears "too good to be true". CIRBP enhances DNA repair and suppresses mutagenesis in all reporters, markers, and assays, even though the pathways affecting these readouts are very different. It is surprising that a factor enhancing NHEJ can stimulate HR at the same, and even increases the expression of MMR genes. CIRBP has to be a "magical" protein to do all these jobs at the same time. I don't think that the authors have providing an convincing and mechanistic explanation for this.

Nonetheless, the authors have provided several striking phenotypes resulting from CIRBP overexpression, including the new Drosophila data. Perhaps these results can inspire followup studies to explain the mechanisms. The model proposed by this study may bring new ideas to the research of aging. I do not object the publication of this manuscript in Nature.

Referee #1 (Remarks to the Author)

The authors have done several experiments to address concerns raised in the previous revision. Unfortunately, the data remains far from convincing. The authors present evidence that NHEJ, HR, and MMR all proceed more quickly and more faithfully in bowhead whales compared to human or other mammalian cells, and this is due to the fact that bowhead whale cells have much greater expression of the factor CIRBP. It is very difficult to believe that expression of one factor is enough to boost all these different forms of repair to the extent that bowhead whales age slower. Furthermore, the authors briefly mention that RPA2 is also overexpressed in bowhead whale cells and that it may also drive better repair, but provide no experimental evidence. Thus, I believe they are overselling their results. Also problematic is making all these conclusions about the mechanism of slower aging in bowhead whales based on the comparison of one bowhead whale fibroblast cell line with just one human, mouse, and cow fibroblast cell line. The authors did not conclusively prove that mutation rates are universally lower in bowhead whales (they use one mutagen, ENU, in one whale fibroblast cell line), nor did they thoroughly convince this reviewer that CIRBP is a master driver of many forms of DNA repair and that higher expression of this gene is responsible for slower mutation rates in bowhead whales.

- "*just one cell line*": the reviewer may have overlooked that **majority of experiments were performed on at least 3 primary fibroblast cell lines from 3 different individuals.** The list of cell lines used in this work can be found on pages 9-10 of Supplementary Information. We worked with 6 different cell lines isolated from the bowhead whale, 4 - from humans, 3 – from cows, and 4 - from mice. Each dot in comparative DNA repair assays represents different individual.
- "*only one mutagen*" the reviewer may have overlooked that we used (1) ENU, (2) gamma-irradiation, two different types of site-specific DNA breaks (3) I-SceI and (4) CRISPR. All gave consistent results of more efficient repair and reduced mutations in the bowhead whale. **We can readily include additional mutagenic treatments** although we think we included a diverse set of mutagens and therefore additional experiments with different mutagens are unlikely to change the conclusions as their mechanisms of action will at least in part overlap with the mutagen treatments already included.
- We do not claim that CIRBP is the master driver of all forms of DNA repair; only double-strand break repair. Furthermore, we do not claim that this fully explains the longevity of the bowhead whale as the reviewer implies.

Major concerns:

1. The authors claim to demonstrate that the mutation rate is lower in bowhead whales due to more efficient mismatch repair (they see no difference in base excision repair or nucleotide excision repair between bowhead whale cells and human cells). The evidence for this relies on one assay in which they stress cells with the mutagen ENU and see less mutations in the bowhead whale cells compared to human cells (via HPRT or SMM-seq). In their revision, the authors claim no other mutagen was suitable in their HPRT assay other than IR but fail to include that data where they use IR in the revised manuscript. One cannot make a general conclusion that whale cells have a lower mutation rate and better MMR based on their differential response to one DNA damaging agent (and a single MMR reporter assay). If the HPRT assay is not suitable for other DNA damaging agents, the authors should have used a different assay such as SMM-seq.

- **The data on IR followed by HPRT assay is shown in Extended Data Figure 2j-k, of the revised manuscript.** The reviewer may have overlooked it.
- The reviewer may have overlooked that we show data for two mutagens: **ENU and IR with the HPRT assay, and performed SMMseq on ENU-treated cells.** These assays show concordant results**.**
- **We can readily perform SMM-seq on cells treated with additional mutagens.** However, analysis of MMR was performed on the request of this reviewer. MMR is unlikely to be CIRBP dependent, therefore further investigation of MMR may not fit well into this manuscript.

2. The manuscript relies on reporter assays to make conclusions about efficiency of DSB repair between bowhead whale and human cells. The authors should have also included simple survival experiments demonstrating that bowhead whale fibroblasts can withstand DSBs (via DNA damaging agents like etoposide, bleomycin, IR, etc) better than human fibroblasts. If CIRBP is a master DSB factor that can contribute to both NHEJ and HR, they should have demonstrated that the human

fibroblasts that overexpress CIRBP can resolve exogenous DSBs better. They could have done basic survival experiments showing overexpression of CIRBP leads to better resistance to the DSB-inducing agents listed above. They also could have shown that yH2AX/53BP1 foci induced by these agents are resolved faster in human cells overexpressing CIRBP. It is very unclear how CIRBP could at the same time affect HR, NHEJ and MMR.

We can readily perform the requested survival experiments. In fact, many of these experiments have already been performed and show indeed that cellular survival is improved when CIRBP is overexpressed in the face of DNA damage. Note, due to the limitation of the number of figures we can include (also including the Extended data), we could not fit everything in the revised manuscript due to the very large number of additional experiments requested by the 5 reviewers that we included in our revision.

3. Expanding on Concern #3 above, in Extended Data 4E it appears DSB repair occurs more slowly in bowhead whale cells compared to human cells. This is a Pulse Field Electrophoresis Gel experiment. One of the main arguments in the manuscript is that repair occurs faster/more efficiently in bowhead whale cells compared to human cells though. This experiment is thus confusing and contradicts one of their central points. This is an example of why making conclusions that DSB repair is more efficient in bowhead whale cells based on reporter assays in the Main Figures may not be fully accurate.

We discuss this point in our previous revision. More efficient DNA repair is best defined by the total number of breaks repaired when given sufficient time, rather than the speed of repair. Whale cells are generally slower in terms of division, and we also assume overall metabolism, than human cells and repair rate may therefore appear slower at early time points. However, in the end more breaks are repaired by whale cells while in human cells breaks remain unrepaired resulting in acentric fragments.

4. It does not seem like overexpressing hCIRBP in human fibroblasts worked, technically-speaking. The authors claim in the text that overexpression of hCIRBP was done just as it was done with bwCIRBP, and that only bwCIRBP led to better NHEJ or HR efficiency (Figures 4b and 4c). However, if one examines Figure 4F, it is very clear that they did not successfully overexpress hCIRBP. One cannot make conclusions that bwCIRBP, but not hCIRBP, makes DSB repair more efficient when the hCIRBP overexpression did not work.

The reviewer is missing an important point that reflects interesting biology. We show that human CIRBP cannot achieve the same level of expression as whale CIRBP. This is the case not only for endogenously expressed CIRBP protein but also the same pattern is observed for recombinant expression. We made many careful experiments using multiple approaches before arriving to this conclusion such as: we expressed human and whale CIRBP ORF transiently from a plasmid in human cells, from a lentiviral construct, and repeated each experiment multiple times. All experiments consistently showed that human ORF is expressed at much lower level than whale CIRBP ORF. We then proceeded to investigate this phenomenon further and show that (Extended Data Figure 9D) replacing individual amino acids within the human CIRBP sequence to the amino acids from the whale CIRBP sequence improves protein expression. To explain the reason for low expression of human CIRBP we demonstrate that whale protein has more optimal codon usage (Extended Data Figure 9E) and higher mRNA structural stability (currently not shown but can be readily included).

5. The authors make conclusions that CIRBP directly acts in NHEJ based on a biochemical NHEJ assay using purified proteins (Figure 4J). However, there is no control protein gel showing that purification of CIRBP worked and had no contaminating repair factors co-purified. It is highly standard when purifying a new protein to demonstrate the quality of the purification (by Coomassie stain, western blot, and even mass spec analysis of the final purified products) and it is extremely problematic that these controls are missing. The results of this biochemical figure cannot be interpreted at all without this necessary data presented.

We can readily provide this control.
The NHEJ experiment shown in Figure 4j was performed with commercially purified CIRBP from Millipore that includes certificate of analysis. We also independently ran a gel and mass spec on this reagent, and this showed it was pure.

6. The section on RPA2 is purely descriptive and doesn't fit well with the rest of the manuscript. The authors

Redacted

Less major concerns:

1. Other essential controls are missing in this manuscript. Examples:
a. A Western blot showing that PTEN was actually knocked-out by CRISPR/Cas9 editing (Figure 2)
We have done this control. **We can readily provide this Western blot.**

b. qPCR or Western blots showing that hTERT, Ras, p53, Rb, and PP2A/PTEN were successfully altered in Figures 2A/B.
The reviewer overlooked that most of these controls are already included:

- **We show in Figure 1A that cells became immortalized by hTERT.** We also performed qPCR and **can readily provide this result.**
- **Westerns blots for p53 and Rb are shown in Figures 2C, D Extended data Figure 1 A, B**
- **In Extended data Figure 1 C, D we provide the functional assay for p53 and Rb using reporter.**
- We did not do assay for PP2A because it was optional for whale cell transformation. **We can readily provide it, although it would not affect the conclusions.**

c. A Western blot showing that overexpression of bwCIRBP increases RPA2 expression (Extended Data 8C)

We can readily provide the Western blot. The reviewer may have overlooked that extended Data Figure 8C is showing **Ion intensity by LC-MS of RPA2** in human fibroblasts with and without lentiviral overexpression of bwCIRBP.

2. The supplementary figures are not presented in linear order through the manuscript which makes reading the manuscript unnecessarily confusing.

We can readily address this.

Referee #4 (Remarks to the Author)

The authors have significantly improved the manuscript by adding new experiments. The sequencing data of mouse xenografts of bowhead whale cells in Extended Data Figure 1 has greatly strengthened the in vivo relevance of this study. Moreover, the in vitro biochemistry data showing that hCIRBP stimulates the DNA end ligation by XRCC4-LIG4 provide new evidence to explain how CIRBP promotes DSB repair. However, I still have some remaining questions about how CIRBP **Redacted** Addressing these questions is important for making this study mechanistically compelling.

Although the authors emphasized that CIRBP enhances NHEJ, it is not clear whether effects of CIRBP in cells is dependent on NHEJ. In Fig. 3c-i, ED4c, ED8f, ED8g, and ED8i, the authors showed a range of phenotypes to suggest that CIRBP promotes DSB repair and reduces more severe types of genomic alternations. While these phenotypes are clear, it is still unclear whether the differences caused by CIRBP are made through heightened NHEJ. The conclusion that CIRBP acts through NHEJ is only based on correlations. Given that CIRBP also increased HR (Fig. 3b), the overall picture of how CIRBP suppresses genomic alterations remains unclear.

This question is easy to address. We will use siRNAs to NHEJ and HR factors to test whether CIRBP effects on the cells are dependent on NHEJ or HR. It is also possible that CIRBP is upstream of the separation of DSB repair to NHEJ and HR sub pathways of DSB repair, in which case we will adjust our conclusions.

I am still not convinced how CIBRP can promote NHEJ but avoid large deletions. Does CIBRP affect the processing of DNA ends? The authors provided some evidence that CIRBP can hold chromatin fragments together, and they speculated that this could be done through a LLPS-mediated mechanism. This model still cannot explain why large deletions are suppressed. In addition, if DSBs are clustered through LLPS, one would expect an increase in chromosomal translocations through error-prone NHEJ. Did the authors analyze translocations in whale cells?

We are currently performing **optical tweezers experiment using C-trap** to provide additional support to our hypothesis that CIRBP holds the ends together. Keeping "correct" ends together should protect the ends from degradation and prevent rather than promote translocations as we do not expect multiple broken ends (in close proximity) to be present in normal cells under physiological conditions. **We will perform analysis of translocation by our new version of SMMseq SV and by chromosome banding.**

Redacted

Referee #5 (Remarks to the Author)

I thank the authors for their responses to my comments. I am satisfied that they have addressed my concerns. **The additional work on ENU induced mutational load and MMR repair efficiency is particularly interesting. In particular the response to the in-depth comments on the mutational assays from Reviewer 1 appear to have substantially strengthened the manuscript and added new dimensions that will are of great interest. My recommendation would be to accept the manuscript for publication.**

Minor comment: Detecting low-frequency somatic mutations can be technically challenging. It would be nice to see the the trinucleotide mutational spectra from each species for the SMM-seq analyses to increase confidence that the somatic mutations detected are not highly enriched for artefacts as ENU induced mutagenesis has expected spectra. This is investigated in Extended Figure 2f but trinucleotide spectra can provide a more sensitive investigation of differences in mutational patterns.

We have this data available and can readily include it.

First, we would like to express our appreciation to the Nature editors for skillfully guiding us through a vigorous process of peer review. We realize that our results: *the first direct evidence that DNA repair – since long a major candidate longevity assurance system - is a major factor in the extremely long lifespan and cancer resistance of the bowhead whale*, would give rise to extreme scrutiny. We are happy that all but one of the **five** reviewers are now satisfied that our manuscript is ready for publication. The exception is Reviewer 1 and while we appreciate their criticisms at previous rounds of review, we are also puzzled as to why this reviewer remains aloof from what we consider a major advance in our understanding of the control and evolution of animal longevity. After all, we addressed every single request of this reviewer with a multitude of additional experiments. Still worryingly, she/he continues to look for inconsistencies where there is none, now questioning if our results "are real", and continuing to express doubt about the role of DNA repair in the whale's longevity. Doubt is a good feeling, but in science, the data speak so that science can progress. We are convinced our manuscript already addresses the few new concerns Reviewer 1 raises, as we show below.

Referee #1 (Remarks to the Author)

This paper makes the bold claim that CIRBP is a major factor in determining longevity in the bowhead whale by simultaneously promoting mismatch repair and double strand break repair both by homologous recombination and non-homologous end joining. Although this is an interesting hypothesis, the data do not sufficiently support this claim.

We do **not** claim that CIRBP is a major determinant of bowhead longevity, nor that bowhead mismatch repair (MMR) efficiency is explained by CIRBP. Our claim is narrower: **CIRBP—highly expressed in bowhead fibroblasts and tissues—is a significant modulator of double-strand break (DSB) repair, enhancing both NHEJ and HR in human cells**.

The set of experiments involving MMR was conducted during the first revision, solely at the request of this reviewer who wanted us to "also test the MMR". We found MMR to be more efficient in the whale and **explicitly stated in our response that enhanced MMR is not related to CIRBP and pointed to elevated expression of several MMR factors** in the whale. Also see line 552 of the manuscript where we discuss enhanced MMR, and page 33 of the Rebuttal 2 where we state that CIRBP is not involved in enhanced MMR.

The reviewer, strangely and repeatedly, ignores our responses misrepresents our conclusions.

For example, it is entirely unclear how CIRBP can promote end-joining and homologous recombination simultanously. Based on their in vitro data, CIRBP promotes end-ligation and protects DNA ends from exonuclease-mediated degradation. However, exonuclease mediated degradation (ie. End-resection) is exactly what is needed for homologous recombination. The point is that end-joining and homologous recombination are competing pathways, so how does CIRBP do both, let alone promote mismatch repair? Overexpression of other DNA repair factor (eg. CTIP) does not mean that HR is enhanced. If CIRPB is a key factor in determining longevity by facilitating DNA repair, it is important to know how it facilitates DNA repair.

Our data and prior work indicate that **CIRBP acts at an *early*, pathway-agnostic step** by stabilizing damaged chromatin and promoting DSB signaling rather than enforcing the NHEJ/HR decision. In such an upstream role, a single factor can **facilitate both outcomes**: transient protection of DNA ends and enhanced recruitment/activation of DDR factors improve repair competence generally. This idea is not new, as there are examples of such factors, for example SIRT6 acts upstream to promote both NHEJ and HR (Mao et al, *Science* 2011).
Consistent with the role of CIRBP in both NHEJ and HR, Chen *et al.*, PNAS 2018 independently showed that CIRBP accumulates at breaks in a PARP1-dependent manner, and **CIRBP**

depletion impairs both HR and NHEJ while reducing MRN/ATM chromatin association and ATM-substrate phosphorylation - hallmarks of an upstream facilitator rather than a pathway-specific effector.

Importantly (and again), we **do not claim** that CIRBP promotes mismatch repair; any MMR differences observed between species are not ascribed to CIRBP in our manuscript.

If CIRPB promotes repair, why do fibroblast senesce and die by apoptosis at similar frequency after irradiation (Figures 1d-f)? One would think that repair would prolong their survival and inhibit senescence/cell death.

This question is puzzling to us and suggests a lack of expertise in cellular senescence and cell cycle checkpoints.

1. Replicative senescence is determined by telomere shortening and cannot be overcome by enhanced DNA repair.
2. 10-20 Gy of gamma-irradiation used for induction of Stress-Induced Premature Senescence is too high of a dose to be overcome by DNA repair. At such saturating doses of irradiation normal fibroblasts induce cell cycle checkpoints by activating p53-p21/p16 axis. Our claim is that CIRBP promotes DSB repair, and certainly not that it abolishes tumor-suppressive checkpoints or globally suppresses senescence/apoptosis.

Another issue is longevity: Is the effect of overexpression of CIRBP in flies actually real? The results in figure 5a are not striking, especially when you compare these results to the life extension that genuine genetic aging interventions provide (one example is the Indy gene, mutation of which reportedly doubles the average lifespan of the fly). If CIRBP is a driver of why bowhead whales live so long, surely we would expect to see it have more of an effect when overexpressed in flies.

• Extended Data 14v. They overexpress CIRBP in Drosophila at low, medium, or high levels. At high levels, there is no improved viability from CIRBP overexpression. But, they claim high expression of this factor in whales is what helps them live longer. This is thus conflicting and confusing.

Questioning if "the effect is actually real" after being shown all the evidence, is hardly data-driven! Our aim for the fly experiments was to test whether the effects of CIRBP extend beyond our *in vitro* data and they do. We do not agree with the premise of the reviewer's concerns. For example, would the reviewer expect that CIRBP should increase life span in the fly by a lot because it comes from the longest-lived mammal? Should it therefore increase life span of the fly to the same extent as the best lifespan extension treatment known in the fly (the indy mutant?). Furthermore:

1. The observed effect of CIRBP expression on radiation resistance is very strong.
2. The effect on lifespan is statistically significant and consistent. We report hazard ratios as effect size as they can be compared across contexts, and across species. The effect size of CIRBP overexpression in the fly are not small when compared to other proposed or even highly accepted interventions in the field. For example, it is half of that seen with mTOR suppression seen across model organisms (using rapamycin, for a meta-analysis including flies, see Garratt et al. 2016).
3. We expected a relatively small effect on lifespan as DNA repair may not be the major limiting factor for Drosophila life span, which is short-lived and mostly a postmitotic organism.
4. In mammalian cells high expression levels of CIRBP are beneficial for DNA repair. However, Drosophila does not even have a CIRBP homolog. It is not surprising that for

Drosophila high CIRBP expression may be too much, due to e.g. “off-target” binding to Drosophila proteins.

Other points:
The authors demonstrate that in their hands, CIRBP association with chromatin is dependent on RNA. This fits in line with previous literature. The authors show that CIRBP facilitates end joining in vitro, this time in the absence of RNA.

These observations are complementary rather than conflicting. Our data indicate two separable steps:

1. **Recruitment to damaged chromatin is RNA-dependent.** In cells, RNase treatment reduces CIRBP’s chromatin association after DSBs, consistent with prior work that damage-induced RNAs help recruit RNA-binding proteins at breaks.
2. **Stimulation of NHEJ is RNA-independent once CIRBP is recruited.** In the purified system, CIRBP is premixed with DNA ends and NHEJ factors, thereby **bypassing the recruitment step**.

Confusing when they say Each data point in comparative assays represents an independent cell line derived from a different individual. The comparative assays in Figure 1 and 2 only have three points and they claim they are biological replicates, so where do the 6 bowhead whale cell lines factor into this?

In Figure 1 we correctly state that n=3 biological replicates (three different bowhead whales). The total number of independent bowhead whales sampled was 6, but not all 6 cell lines were used in each assay. **n is indicated for each figure panel.** We do not agree this is confusing as all the information is provided in detail.

For example, in Figure 3c (yH2AX foci), why are they plotting the data as averages of an experiment? They should be showing the full data, not the average of the experiment.
Same can be said for most of the data in Figure 3. How does the data look across individuals in the bowhead whale species. Is it consistent across individual cell lines?

This is a minor point. We have all the data and can show it. We chose this way of presenting the data because our inference is made at the level of biological replicates (independent primary lines/individuals), not individual cells. Plotting hundreds of single-cell points (for yH2AX foci) and testing on those values would inflate n and constitute pseudo replication, a basic but regrettably still common statistical error. For this reason, we summarized per line per experiment (averaging many cells per condition) and used those biological replicates for statistics. We also show the images of the foci and the difference is clear.

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CIRBP likely exhibits an optimal window: a certain level of expression up to a certain threshold is sufficient to promote resistance in flies. Drosophila does not have a CIRBP homolog. It is not surprising that for Drosophila high CIRBP expression may be too much.

In supplementary Figure 12h and i, CIRBP binds DNA? And yet in Supp. Figure 12a-c, they show in cells it doesn’t bind DNA without RNA present.

These are different *in vivo* and *in vitro* assays using different DNA substrates. There is no contradiction. **RNA is required for CIRBP recruitment to chromatin *in vivo*, but not *in vitro* in the purified system.**

Response to Referees and Editors

We thank the referees and editors for their careful evaluation of our manuscript and for their helpful suggestions. We have revised the manuscript, Supplementary Information, and Reporting Summary accordingly. Below we provide a point-by-point response to all comments.

Responses to Referees

Referee #1 (Remarks to the Author)

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