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DASH/Dam1 complex mutants stabilize ploidy in histone-humanized yeast by weakening kinetochore-microtubule attachments

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. 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.)

Thank you for formally submitting your study on ploidy stabilization by weakening of kinetochore attachments to The EMBO Journal. I sent it to three referees with expertise in kinetochores, synthetic biology and yeast chromatin/genetics, but have so far only received the reports of two of them. Since both of them are in fair agreement, and in the interest of time, I am forwarding you these reports now together with an invitation to start revising the study based on these overall supportive comments. I should stress that this remains currently still a preliminary decision, and that any specific concerns raised by the outstanding third report (should it come within the next two weeks) may still need to be incorporated before finalization and resubmission of the work.

Referee #1:

- General summary and opinion about the principal significance of the study, its questions, and findings

In the manuscript entitled "DASH/Dam1 complex mutants stabilize ploidy by weakening kinetochore-microtubule attachments" Haase et al. followed up on their work "humanizing" histones in yeast (Haase et al., 2019; Truong and Boeke, 2017). The authors previously found, using some genetic tricks in yeast, that replacing all core histones in yeast with their human counterparts led to severe growth defects. However, rare yeast colonies with humanized histones were able to grow. This manuscript investigated the adaptations these yeast strains developed to better tolerate human histones. The authors observed that chromosomal aneuploidy was a common occurrence in response to histone humanization, however, they concluded that these aneuploidies are in fact not adaptive, but a consequence of histone humanization leading to chromosomal missegregation. Interestingly, they found that the identity of aneuploid chromosomes was non-random, and concluded that this observation is likely due to a set of chromosomes having an evolutionary different centromeric origin. They also identified some adaptive missense mutations, and then focused on identifying how a select set of these mutations, specifically in the outer kinetochore DASH/Dam1 complex, led to a better tolerance of human histones. The authors proposed a mechanism for how their DASH/Dam1c mutants can tolerate humane histones - through decreased ability of the complex to oligomerize. This decrease in oligomerization leads to weaker kinetochore-microtubule attachments, which in the absence of robust error correction mechanisms, gives the chromosomes a chance to bi-orient more efficiently. These findings are important for understanding the mechanisms underlying chromosome segregation, the role of DASH/Dam1c oligomerization in this process, and what processes rely on species-specific histones. This work also further develops a platform (yeast with humanized histones) that can be used to study human histones. This work is a very nice body of work overall, and should be considered for publication after the authors address the following concerns:

- Specific major concerns essential to be addressed

1. In lines 234-236 and 505-509, the authors concluded that the subset of chromosomes (Paralog A chromosomes except chromosome 15) that has a higher frequency of aneuploidy has this feature because their centromeres are more sensitive to human histones. This claim does not align with their meta-analysis that showed the same bias in other studies that didn't involve histone humanization. If the centromeres of these chromosomes were sensitive to human histones specifically, you would expect to find this bias in histone-humanized yeasts but not in other evolution studies. Furthermore, previous studies (Torres et al 2007) have examined disomic yeast strains and their tolerance to contain a single extra chromosome. In almost all cases, the "Paralog A" chromosome was better tolerated than the Paralog B chromosome, suggested gene specific differences between the tolerance of these chromosomes - further reducing confidence in the authors claims. We recommend taking the conclusion that the centromeres of Paralog A chromosomes are especially sensitive to human histones out of the manuscript since it is not supported by the data and doesn't affect the other main conclusions, discussing the other possible explanations for the observed bias and using a less assertive tune. Alternatively, better support for these claims should be provided.

2. In figure 5D, lines 386-387, and line 540, we think that these data suggest that SAC activity is not required for the rare events of histone humanization in yeast, but it doesn't show that SAC is inactive in these mutants. We recommend rephrasing this conclusion and using less strong language in line 540.

- Minor concerns that should be addressed

1. In line 217, there is a grammatical mistake, "we therefore explicitly tested..."

2. In line 246, there is a grammatical mistake, "DNA and create more transcriptionally..."

3. In lines 275-278, The way the sentence is phrased is confusing and hard to comprehend. We recommend trying to rephrase.

4. In line 286, there is a grammatical mistake, "These data are consistent with centromere..."

5. In figure 3A, make it clear on the figure that the gray graph is the WT.

6. In figure 3E, we recommend having a key next to the figure explaining that the brown color is cells with clustered kinetochores, and purple is cells with de-clustered kinetochores.

7. In figure 4C, the annotation "W/O" and "W/" on the X-axis is confusing.

8. In lines 362-363, there is a grammatical mistake, "note: these experiments were done in a genetic background..."

9. In figures 5A and 5C, in 5A the wild type is annotated as "WT" while in 5C as "wt". We recommend being consistent with this.

10. In figure 6A, we recommend labeling the residues in all panels similar to how they are labeled in the panel to the left since that makes it easier to follow.

11. In figure 7B, we recommend replacing this figure with Figures S12 A and B since, in our opinion, it is more convincing of the point the authors are trying to make. This is just a suggestion, not a requirement.

12. In figure 7D, the schematic suggests that there is "free" DASH/Dam1c in the solution and oligomerizing around the microtubules, which according to the methods section is not the case (DASH/Dam1c is only loaded into the beads). We recommend this diagram be changed to better reflect the assay conducted to avoid confusion for the reader.

13. In figure 7E, we recommend commenting on why at low concentration of DASH complex (2.5nM), it seems like the rupture force of mutant Dad1 is higher than WT. Also use brighter colors for the survival curves since it is hard to see the 2.5nM curves.

14. In lines 479-480, we recommend rephrasing this sentence as we find it cumbersome to read.

15. In figure 8A and lines 485-490, the authors should consider whether keeping the sporulation efficiency data strengthens their claim. The expectation is that Dam1c mutations will specifically affect chromosome segregation, which is almost certainly the case based on the subsequent data in figure 8B-C. Sporulation efficiency, however, does not always reflect segregation defects. For example, spo11 mutants sporulate just fine but chromosome segregation is terrible. Minimally, the authors should mention how dam1 mutants could affect sporulation efficiency.

16. In lines 544-548, we think that you can suggest explicitly that due to the observed low occupancy of the centromeres in the histone humanized yeast, maybe Aurora B recruitment is reduced, leading to a significant reduction in error correction, causing an increased aneuploidy which is rescued in the DASH/Dam1c mutants due to decreased microtubule binding. Better "connecting the dots" for the reader would be helpful in the discussion.

17. In lines 563-564, the reference (Sarangapani et al., 2014) is not probably the best sole source to cite for this information. We recommend also citing other articles like (Petronczki et al., 2006).

18. In supplemental Figure 3 (the figure labeled "CRISPR-Cas9 mediated mutation of outer kinetochore genes and suppressor validation"), the figure is labeled "Figure 3." Instead of "Figure 3S."

Reference list;
Petronczki, M., Matos, J., Mori, S., Gregan, J., Bogdanova, A., Schwickart, M., Mechtler, K., Shirahige, K., Zachariae, W., & Nasmyth, K. (2006). Monopolar Attachment of Sister Kinetochores at Meiosis I Requires Casein Kinase 1. Cell, 126(6), 1049-1064. https://doi.org/10.1016/j.cell.2006.07.029
Torres, E. M., Sokolsky, T., Tucker, C. M., Chan, L. Y., Boselli, M., Dunham, M. J., & Amon, A. (2007). Effects of aneuploidy on cellular physiology and cell division in haploid yeast. Science (New York, N.Y.), 317(5840), 916-924. https://doi.org/10.1126/SCIENCE.1142210

Referee #3:

This manuscript is the follow-up to a study published in Cell (2017) in which strains of S. cerevisiae were created where all core histones (H2A, H2B, H3 and H4) were replaced by the equivalent human histones. This is an infrequent event, and strains initially grow very slowly. However, after extended periods of cultivation, the strains acquire secondary mutations and aneuploidies (duplication of chromosomes) that provide increased growth rates.
Here, the authors specifically ask whether the aneuploidies or the point mutations are responsible for improved growth. They report that the aneuploidies are restricted to a group of yeast chromosomes that share evolutionary history, though the molecular basis and significance of this is unclear. Furthermore, they study mutations in components of the outer kinetochore, specifically in Dad1 and Dam1, components of the DASH/Dam ring that connects the kinetochore to the microtubule, that arise in histone-humanized yeast, suggesting a link between kinetochore function, survival with human histones, and the aneuploidies. They find that the mutations alone (without the aneuploidies) are sufficient to increase the initial rate of "humanization" of yeast. On a molecular level, the mutations map to a protein-protein interface required for oligomerization of the DASH/ Dam1 ring. The authors show by electron microscopy and protein biochemistry that the mutations cause reduced oligomerization to form the DASH/ Dam1 ring and that they cause weakened microtubule attachment (as measured by a rupture force assay). The mutations alone do not reduce fitness of yeast cells during mitotic growth, but surprisingly, are detrimental in meiosis, the reason for this remaining unclear.
Altogether, the conclusion is that the human histones cannot properly assemble the centromeric nucleosome in yeast, which causes strong centromere and kinetochore defects. This can be compensated by weakening (!) the kinetochore - microtubule interface. The observed aneuploidies are a consequence of the centromere defect caused by the human histones, but they by themselves to not contribute to improved viability. The authors place their findings in the context of evolutionary "leaps" (rather than small steps) that might take advantage of such mechanisms.

The advance of this work is that it shows that reduced (rather than enhanced) interactions at the kinetochore - microtubule interface is beneficial for the compensation of a centromeric defect. This is, at least to me, unexpected. The conclusions are based on solid and sophisticated biophysical work demonstrating molecular defects in the DASH/ Dam components. On the whole, this is in my opinion a well-rounded and sound piece of work that really does not require much more experimentation.

Major comments:
- In Fig. 8C, it is shown that the DAD1-E50D mutation severely impairs sporulation efficiency in heterozygous diploids, and that spores with the DAD1-E50D mutation have a higher likelihood of survival. What about homozygous mutant diploids, do they also have decreased sporulation efficiency? From the above, one might expect that all spores survive, which would argue that an imbalance in microtubule attachment is what causes poor survival of spores.
- In Fig. 3C, PCRs are shown do quantitate the levels of transcription through the histone-humanized centromere. Do you have a more quantitative measurement, i.e. quantitative real-time PCR?

Minor comments:
- Title of Figure S10: DASH/ Dam1c mutants _partially_ rescue Aurora B kinase ipl1-2 mutant
- The authors cite a paper (Clarke et al, 2022) that reports similar conclusions regarding mutations in the oligomerization interface of DASH/ Dam components and that thus might somewhat reduce the novelty of the findings. I could only find the paper in BioRxiv, not in "Cell Biology", as indicated in the reference list.
- The part of the ms that describes the characterization of the aneuploidies is rather lengthy. The results are puzzling and a bit distracting to me, and there is no good explanation for why the evolutionary history of a chromosome might matter for aneuploidization. I think this part of the ms could be shortened to improve readability of the ms.

Other considerations/ thoughts:

- Given that the humanized yeasts have a centromeric defect, is this due to the inability of the human histones to adequately form a nucleosome with Cse4, the yeast CENP-A homolog? What about H2A.Z, is it incorporated into the humanized yeast chromatin?
- I am surprised that the authors isolated only mutations in the outer kinetochore (DASH/Dam, Ndc80, Spc105) and did not isolate mutations in components of the inner kinetochore that would increase humanization frequency. Is there any rationalization for this?

Some language/ typos I found:
- P 12, line 299: "We therefore sought _to_ understand ..."
- p 17 line 413: amino acid pairing (not paring)
- p 18 line 440: Figure (not Frigure)
- p 22 line 535: no "period" between the words "detachment and "is".
- p 22 line 546: should this read "... consistent with Aurora B being inactive _in_ histone-humanized yeasts ..."?
- p 23 line 565: may be (two words)

Referee #1:

- General summary and opinion about the principal significance of the study, its questions, and findings

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We thank the referee for their thoughtful reading of our manuscript. The concerns raised greatly helped strengthen our manuscript and clarify some of the logic of results and discussion sections. We have gone through each point raised by the referee and have addressed each point with either changes to the text or inclusion of additional analyses. Our comments and any graphs are provided directly below each concern in text that is colored blue.

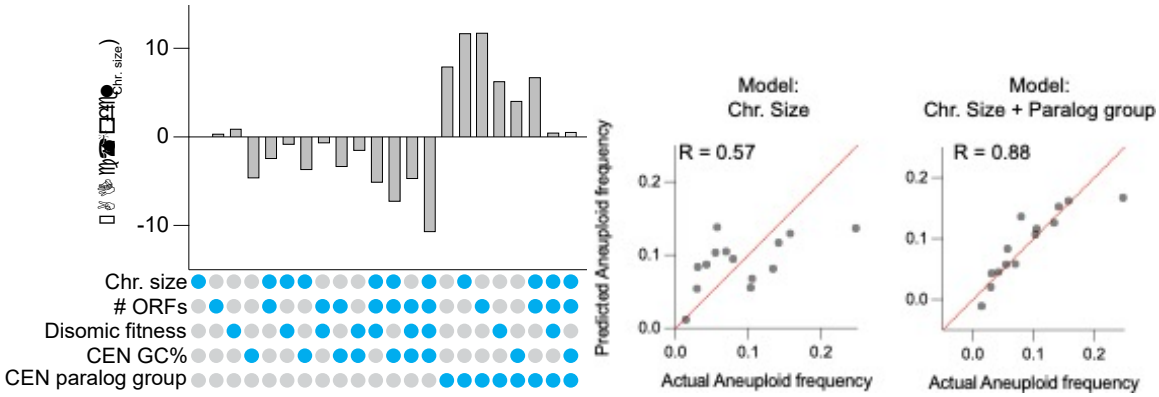
- Specific major concerns essential to be addressed

1. In lines 234-236 and 505-509, the authors concluded that the subset of chromosomes (Paralog A chromosomes except chromosome 15) that has a higher frequency of aneuploidy has this feature because their centromeres are more sensitive to human histones. This claim does not align with their meta-analysis that showed the same bias in other studies that didn't involve histone humanization. If the centromeres of these chromosomes were sensitive to human histones specifically, you would expect to find this bias in histone-humanized yeasts but not in other evolution studies. Furthermore, previous studies (Torres et al 2007) have examined disomic yeast strains and their tolerance to contain a single extra chromosome. In almost all cases, the "Paralog A" chromosome was better tolerated than the Paralog B chromosome, suggested gene specific differences between the tolerance of these chromosomes - further reducing confidence in the authors claims. We recommend taking the conclusion that the centromeres of Paralog A chromosomes are especially sensitive to human histones out of the manuscript since it is not supported by the data and doesn't affect the other main conclusions,

discussing the other possible explanations for the observed bias and using a less assertive tune. Alternatively, better support for these claims should be provided.

We have taken a look at our text and we recognize we may have written some results and conclusions in confusing manner. The major observation is that aneuploid frequency seems to be dependent on the evolutionary origins of each chromosome across 1,688 unrelated yeast strains (If you look at paralog pairs one is always more aneuploid than the other, excluding chromosomes 13 & 15). We asked how unlikely this is to be with a probability estimation. We randomly assign the aneuploid frequencies to chromosome pairs and test how extreme the difference in aneuploidy is between members of paralog pairs. This is the analysis we did from lines 181 – 191. Note we are comparing aneuploidy frequencies from both our study and seven earlier studies in this analysis. This analysis shows that the observed difference in aneuploid frequency between paralog pairs is greater than chance alone predicts across unrelated strains, the point being this is a general affect that is not specific to histone humanization. This is not an obvious conclusion to reach and there is no good reason to suspect such a basis between paralogous chromosome pairs a priori – so we find it to be surprising and noteworthy.

To show that the paralog groupings best explain aneuploid frequency across *all* studies examined here, including ours, we compared the relative performance of various linear regression models of aneuploid frequency (a more positive AIC is better). We considered a few factors, as suggested by the reviewer, including chromosome size, number of ORFs, fitness of strains with specific disomes (Beach et al. PMID: 28388408), and paralog grouping as defined in the manuscript (we excluded the pair Chr.15-Chr.13, as we 1) observed no aneuploidy and 2) Beach et al do not provide measurement for the Chr15 disome). The best performing models are those that include paralog group information, in fact a model with only this factor performs considerably well (note all models are compared to a model based solely on chromosome size). We show correlation plots of the actual aneuploid frequencies observed vs. predicted from each model, and this shows that adding paralog group information improves performance from the model based solely on chromosome size.



The general conclusion is that aneuploid frequency is influenced by the evolutionary origins of each centromere (the stark contrast between paralog pairs). This is particularly compelling in light of the evidence that the genome of *S. cerevisiae* likely arose from an *interspecies* hybridization event (Marcet-Houben and Gabaldón 2015). It thus had two sets of eight centromeres which evolved independently for some time (millions of years) before being rejoined in the hybridization event. We do not yet know which centromere came from what parent, but this could be a focus of future research. To our knowledge no prior work has made this intriguing observation – that the evolutionary origins of centromeres may still impart functional consequences today.

From the above we still feel strongly that taking into account the evolutionary origins of centromeres offer unexpected explanatory power as to why certain chromosomes are more frequently aneuploid in yeast. We soften our claims to make clear this is a hypothesis and not a settled topic, proposing that the evolutionary origins of centromeres may be a factor that influences aneuploidy like well documented factors such as chromosome size and gene-specific tolerances.

The referee noted that:

"Furthermore, previous studies (Torres et al 2007) have examined disomic yeast strains and their tolerance to contain a single extra chromosome. In almost all cases, the "Paralog A" chromosome was better tolerated than the Paralog B chromosome, suggested gene specific differences between the tolerance of these chromosomes - further reducing confidence in the authors claims."

We addressed this in the above linear regression modeling by looking the relative fitness (to euploid) values of disomic strains as a factor to the observed aneuploidy across 1,767 strains. The modeling showed that the fitness of disomic strains performs equally as well to chromosome size, but is substantially improved upon including the centromere paralog factor.

See lines: 190-197

We also have softened our tone to place emphasis on other potential explanations for the bias.

See lines: 201-203 and lines: 507-513

Lastly, the referee requested:

"We recommend taking the conclusion that the centromeres of Paralog A chromosomes are especially sensitive to human histones out of the manuscript since it is not supported by the data and doesn't affect the other main conclusions"

Form the added analysis and rewriting of the results we realized this conclusion is not supported by the data. We have removed this text. Instead, we propose that the data supports the idea aneuploid frequency in *S. cerevisiae* shows a characteristic bias between paralog pairs, regardless of strains examined, which is further informed by chromosome size.

Please also note our changes to Figure 2 and Supplemental Figure 5.

2. In figure 5D, lines 386-387, and line 540, we think that these data suggest that SAC activity is not required for the rare events of histone humanization in yeast, but it doesn't show that SAC is inactive in these mutants. We recommend rephrasing this conclusion and using less strong language in line 540.

We agree with the referee that our data do not directly support the idea that histone humanized yeast have an inactivated SAC. We have dialed back our claims and have modified the text as a result:

Please see lines 384-385 and Line 548

- Minor concerns that should be addressed

We thank the referee for these detailed corrections. We have fixed all minor concerns and provide additional comments where appropriate.

1. In line 217, there is a grammatical mistake, "we therefore explicitly tested..."

fixed

2. In line 246, there is a grammatical mistake, "DNA and create more transcriptionally..."

fixed

3. In lines 275-278, The way the sentence is phrased is confusing and hard to comprehend. We recommend trying to rephrase.

This paragraph has been rephrased to improve clarity. See lines: 271-273

4. In line 286, there is a grammatical mistake, "These data are consistent with centromere..."

fixed

5. In figure 3A, make it clear on the figure that the gray graph is the WT.

This has been corrected by adding colored labels within the figure panel.

6. In figure 3E, we recommend having a key next to the figure explaining that the brown color is cells with clustered kinetochores, and purple is cells with de-clustered kinetochores.

We added labels next to the far-right bar to indicate which are clustered/de-clustered.

7. In figure 4C, the annotation "W/O" and "W/" on the X-axis is confusing.

We have updated this labeling scheme to more clearly indicate that we are comparing lineages with and without DASH/Dam1c mutations.

8. In lines 362-363, there is a grammatical mistake, "note: these experiments were done in a genetic background..."

fixed

9. In figures 5A and 5C, in 5A the wild type is annotated as "WT" while in 5C as "wt". We recommend being consistent with this.

We have fixed this, now each is labeled as “WT”.

10. In figure 6A, we recommend labeling the residues in all panels similar to how they are labeled in the panel to the left since that makes it easier to follow.

Labeled residues have now been added for each panel as suggested.

11. In figure 7B, we recommend replacing this figure with Figures S12 A and B since, in our opinion, it is more convincing of the point the authors are trying to make. This is just a suggestion, not a requirement.

We agree with the referee that Figures S12A/B illustrate our point best. As such we reorganized Figure 7 and the text has been updated to accommodate the relabeled figure panels. Figure legends have also been updated.

12. In figure 7D, the schematic suggests that there is "free" DASH/Dam1c in the solution and oligomerizing around the microtubules, which according to the methods section is not the case (DASH/Dam1c is only loaded into the beads). We recommend this diagram be changed to better reflect the assay conducted to avoid confusion for the reader.

There is indeed free DASH/Dam1c in the solution, so the diagram is correct. Our original methods do report this:

“Lastly, ~ 2-2.5 mg/mL of purified bovine brain tubulin was added to the reaction mixture as well as 5 µM FLAG-tagged DASH complex.”

We reworded this sentence to the following:

“Lastly, ~ 2-2.5 mg/mL of purified bovine brain tubulin and 5 nM FLAG-tagged DASH complex were added to the reaction mixture.”

We note our original methods stated “5 µM of FLAG-tagged DASH complex”, we apologize for this error in the methods add have corrected it to the right concentration of 5 nM.

See lines 827 – 828.

13. In figure 7E, we recommend commenting on why at low concentration of DASH complex (2.5nM), it seems like the rupture force of mutant Dad1 is higher than WT. Also use brighter colors for the survival curves since it is hard to see the 2.5nM curves.

Although the mean rupture force at 2.5 nM is slightly higher for the Dam1 complex containing mutant Dad1 than for wild-type Dam1 complex the statistical significance is borderline (3.9 pN v. 4.9 pN, p-value = 0.0579). If we apply multiple tests corrections to this analysis the p-value = 0.1416 for this comparison (performed 2-way ANOVA, with an FDR correction). We note, when we compare WT-2.5nM to Dad1-E50D-10nM the difference in the mean rupture (3.9pN vs. 5.7 pM, respectively) is also insignificant (p = 0.2454), further highlighting that Dad1-E50D DASH does not form stronger attachments at higher concentrations of complex (for reference, the difference in mean rupture for of WT-2.5nM (3.9pN) vs WT-10nM (6.9 pN) is highly significant, $p < 0.0001$).

Whether or not the rupture force of the mutant is marginally higher at 2.5 nM than WT does not affect our major conclusion that Dad1-E50D's strength does not scale with increased density of complex on the beads.

Lastly, we have updated the colors to be brighter, the graphs were also slightly enlarged.

14. In lines 479-480, we recommend rephrasing this sentence as we find it cumbersome to read.

We have altered the sentence to the following, see lines 478 - 480:

"We have shown that the subtle *DAD1*^{E50D} mutation improves the fidelity of mitotic chromosome segregation in certain conditions. Why then is the glutamic acid at residue 50 of Dad1 nearly invariant across species (Figure 6E), if *DAD1*^{E50D} appears more fit in mitosis?"

15. In figure 8A and lines 485-490, the authors should consider whether keeping the sporulation efficiency data strengthens their claim. The expectation is that Dam1c mutations will specifically affect chromosome segregation, which is almost certainly the case based on the subsequent data in figure 8B-C. Sporulation efficiency, however, does not always reflect segregation defects. For example, spo11 mutants sporulate just fine but chromosome segregation is terrible. Minimally, the authors should mention how dam1 mutants could affect sporulation efficiency.

We have performed one additionally experiment (suggested to us by reviewer #3) where we look at sporulation and viability in the homozygotic Dad1-E50D mutant. In this case. Sporulation efficiency is only reduced to 50% and spore viability is nearly that of WT diploids. This is likely an effect of chromosome missegregation as suggested by the poor viability of the spores in heterozygous diploids. Further reduction in spore viability and sporulation efficiency may be exacerbated in heterozygous diploids due to an imbalance in microtubule attachments from an incompatible interaction of WT/Dad1-E50D complex. The Dad1-E50D mutant likely does not affect later events such as prospore membrane closure or spore wall assembly (Tetrads from the homozygous mutant show viability of WT). However, the increased abundance of asci with 2 or 3 spores and immature asci suggests that the Dad1-E50D mutant is detrimental to earlier events in sporulation – likely in meiotic division.

Please see changes to the section from lines 478-499.

And changes made to the discussion.

Lines 565-567

16. In lines 544-548, we think that you can suggest explicitly that due to the observed low occupancy of the centromeres in the histone humanized yeast, maybe Aurora B recruitment is reduced, leading to a significant reduction in error correction, causing an increased aneuploidy which is rescued in the DASH/Dam1c mutants due to decreased microtubule binding. Better "connecting the dots" for the reader would be helpful in the discussion.

We agree with the referee's comment that we should make this connection more apparent. As such we have modified the text to the following:

See lines 538-5543

"Of particular interest would be to assess whether kinetochores from histone-humanized yeasts can sense tension or whether Aurora B is inactive in histone-humanized yeasts. Our data suggests that the reduced occupancy of centromeric nucleosomes in histone humanized yeasts may lead to a reduction to the recruitment of Aurora B, thereby driving a significant reduction to error correction, and that the DASH/Dam1c mutants suppress the loss of Aurora B activity through decreased microtubule binding and increased kinetochore-microtubule turnover."

17. In lines 563-564, the reference (Sarangapani et al., 2014) is not probably the best sole source to cite for this information. We recommend also citing other articles like (Petronczki et al., 2006).

This point is well taken and we have expanded the list of sources for this particular claim; including the suggested citation alongside two additional citations (Corbett et al 2011 and Monje-Casas et al 2007).

See lines 570-571.

18. In supplemental Figure 3 (the figure labeled "CRISPR-Cas9 mediated mutation of outer kinetochore genes and suppressor validation"), the figure is labeled "Figure 3." Instead of "Figure 3S."

Fixed; it has been renamed to Figure EV2 (for embo formatting).

Reference list;

Petronczki, M., Matos, J., Mori, S., Gregan, J., Bogdanova, A., Schwickart, M., Mechtler, K., Shirahige, K., Zachariae, W., & Nasmyth, K. (2006). Monopolar Attachment of Sister Kinetochores at Meiosis I Requires Casein Kinase 1. Cell, 126(6), 1049-1064.https://doi.org/10.1016/j.cell.2006.07.029
Torres, E. M., Sokolsky, T., Tucker, C. M., Chan, L. Y., Boselli, M., Dunham, M. J., & Amon, A. (2007). Effects of aneuploidy on cellular physiology and cell division in haploid yeast. Science (New York, N.Y.), 317(5840), 916-924. https://doi.org/10.1126/SCIENCE.1142210

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This manuscript is the follow-up to a study published in Cell (2017) in which strains of S. cerevisiae were created where all core histones (H2A, H2B, H3 and H4) were replaced by the equivalent human histones. This is an infrequent event, and strains initially grow very slowly. However, after extended periods of cultivation, the strains acquire secondary mutations and aneuploidies (duplication of chromosomes) that provide increased growth rates.
Here, the authors specifically ask whether the aneuploidies or the point mutations are responsible for improved growth. They report that the aneuploidies are restricted to a group of yeast chromosomes that share evolutionary history, though the molecular basis and significance of this is unclear. Furthermore, they study mutations in components of the outer kinetochore, specifically in Dad1 and Dam1, components of the DASH/Dam ring that connects the kinetochore to the microtubule, that arise in histone-humanized yeast, suggesting a link between kinetochore function, survival with human histones, and the aneuploidies. They find that the mutations alone (without the aneuploidies) are sufficient to increase the initial rate of "humanization" of yeast. On a molecular level, the mutations map to a protein-protein interface required for oligomerization of the DASH/ Dam1 ring. The authors

show by electron microscopy and protein biochemistry that the mutations cause reduced oligomerization to form the DASH/ Dam1 ring and that they cause weakened microtubule attachment (as measured by a rupture force assay). The mutations alone do not reduce fitness of yeast cells during mitotic growth, but surprisingly, are detrimental in meiosis, the reason for this remaining unclear. Altogether, the conclusion is that the human histones cannot properly assemble the centromeric nucleosome in yeast, which causes strong centromere and kinetochore defects. This can be compensated by weakening (!) the kinetochore - microtubule interface. The observed aneuploidies are a consequence of the centromere defect caused by the human histones, but they by themselves to not contribute to improved viability. The authors place their findings in the context of evolutionary "leaps" (rather than small steps) that might take advantage of such mechanisms.

The advance of this work is that it shows that reduced (rather than enhanced) interactions at the kinetochore - microtubule interface is beneficial for the compensation of a centromeric defect. This is, at least to me, unexpected. The conclusions are based on solid and sophisticated biophysical work demonstrating molecular defects in the DASH/ Dam components. On the whole, this is in my opinion a well-rounded and sound piece of work that really does not require much more experimentation.

We thank the referee for their considerate comments and suggestions to our manuscript. We also appreciate the enthusiasm for the work. Below we provide responses to each comment raised by the referee.

Major comments:
- In Fig. 8C, it is shown that the DAD1-E50D mutation severely impairs sporulation efficiency in heterozygous diploids, and that spores with the DAD1-E50D mutation have a higher likelihood of survival. What about homozygous mutant diploids, do they also have decreased sporulation efficiency? From the above, one might expect that all spores survive, which would argue that an imbalance in microtubule attachment is what causes poor survival of spores.

We have now repeated the experiments in the homozygous DAD1-E50D background as the referee requested. We observed that the homozygous Dad1-E50D mutant sporulates significantly better than the heterozygous mutant, however it sporulates worse than the wild type diploid (see updated Figure 8A). We then dissected tetrads from homozygous mutants and observed that spore viability is substantially improved over that of the heterozygous diploid (WT/dad1-E50D). These data were added to figure 8 (panels A and B) and accompanying text was added where relevant.

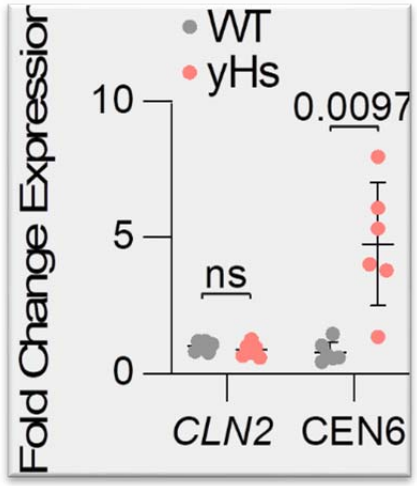
We thank the referee for suggesting this experiment as it helped make our conclusions on the meiosis data clearer. Overall, the data support the imbalance in microtubule attachment interpretation that the referee proposed. However, the homozygous mutant still sporulates worse than WT, so other mechanisms may be at play during meiosis (please see the above reviewer's question #15).

Please see changes to the section from lines 478-499 and changes made to the discussion - Lines 565-567

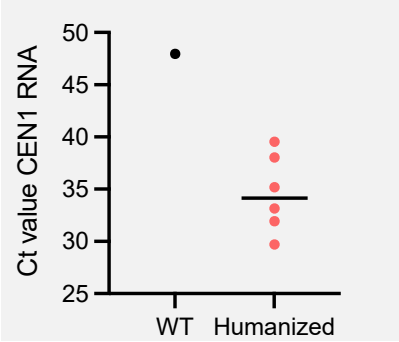
- In Fig. 3C, PCRs are shown do quantitate the levels of transcription through the histone-humanized centromere. Do you have a more quantitative measurement, i.e. quantitative real-time PCR?

We have performed RT-qPCR on *CEN6* RNA from six biological replicates of each strain (yeast histones or human histones) and these data are now provided in Appendix Figure S3B and pasted below. Additionally, methods were added describing these experimental procedures (See lines 758 - 763). As you can see from this experiment *CEN6* RNA is up regulated in histone humanized cell ~5 fold over that from WT cells. As a control we used *CLN2*, which showed no difference in expression. Expression data was normalized to the expression of *UBC6*.

See added text on lines 261-263.



We additionally performed qRT-PCR for *CEN1* RNA, but failed to detect any RNA in 5 out 6 WT samples (and in the 6th sample, the high Ct sample suggests the RNA is at the extreme limit of detection) as seen below (these data were not added to the manuscript). Again, this supports the notion that CEN RNAs are upregulated in histone humanized yeasts.



Additionally, we note that the data provided in Figure 3B shows quantification of transcription through all 16 CENs by RNA sequencing (data is the average log10 transcript per million from three biological replicates).

Minor comments:
- Title of Figure S10: DASH/ Dam1c mutants _partially_ rescue Aurora B kinase ipl1-2 mutant

Fixed.

- The authors cite a paper (Clarke et al, 2022) that reports similar conclusions regarding mutations in the oligomerization interface of DASH/ Dam components and that thus might somewhat reduce the novelty of the findings. I could only find the paper in BioRxiv, not in "Cell Biology", as indicated in the reference list.

We apologize for this error, indeed the paper we cite is on BioRxiv not “Cell Biology”. This has been corrected. Of course, we understand this paper is likely to be in press at EMBO J.

- The part of the ms that describes the characterization of the aneuploidies is rather lengthy. The results are puzzling and a bit distracting to me, and there is no good explanation for why the evolutionary history of a chromosome might matter for aneuploidization. I think this part of the ms could be shortened to improve readability of the ms.

This section came out of the question as to why certain chromosomes present aneuploidy in histone humanized strains. We agree with the referee, there is not yet a good explanation as to why the evolutionary origins might matter. However, it is a very clear trend we see across diverse strains. We think this observation is an important one to make and to our knowledge no such observation has been reached before. Alongside comments from reviewer #1 we have rewritten this section extensively to provide clarity in our conclusions. We hope the readability has improved from these edits. Specifically, we shorted this section from ~723 words to 596 words in the revised manuscript.

See changes made from lines 160-209 and 511-522.

Other considerations/ thoughts:

- Given that the humanized yeasts have a centromeric defect, is this due to the inability of the human histones to adequately form a nucleosome with Cse4, the yeast CENP-A homolog? What about H2A.Z, is it incorporated into the humanized yeast chromatin?

This is a great question and one we are very interested in. This question deserves its own investigation and is beyond the scope of this work and thus we do not focus on it in this manuscript. From our unpublished data we know that human CENP-A does not rescue any of these defects, in fact it only serves to make the yeast sicker (Ólafsson et al. Unpublished observations). We suspect that the centromere defect could be due to two mechanisms, 1) as the referee mentions, the inability of human nucleosomes to adequately form a nucleosome with Cse4 (perhaps due to species specific protein interactions) and 2) competition of the centromeric nucleosome position between canonical human nucleosomes and human/Cse4 nucleosomes.

As for yeast H2A.Z we do not know whether it is incorporated into humanized yeast chromatin. However, from unpublished data we know yeast H2A.Z is not needed for histone humanization as we have made histone humanized strains which have yeast H2A.Z replaced by the human ortholog (Haase et al. Unpublished observations).

This is ongoing work in the lab, as such we leave speculation to this point out of the manuscript/discussion as we will address it in future work.

- I am surprised that the authors isolated only mutations in the outer kinetochore (DASH/Dam, Ndc80,

Spc105) and did not isolate mutations in components of the inner kinetochore that would increase humanization frequency. Is there any rationalization for this?

This is the “surprise” that fixated our attention to study these mutants in the first place. Indeed, it is somewhat peculiar that we have never isolated an inner kinetochore mutant. We note the mechanism we describe here is just one such path to adaption with human histones (see Figure S2), however for this path it is indeed seemingly restricted to mutations of outer kinetochore genes (or KMT attachment more generally). One possible rationalization is simply that our screen for suppressor mutations is not yet saturated. This is likely the case, as we have only isolated mutants of the same gene twice (URA2 and GEX2) both of which seem to be inconsequential mutations (either synonymous or noncoding variants). We also note, these genes are rather large (~6kb and ~1.8kb respectively).

Additionally, any mutation of the inner kinetochore would be expected to improve humanization likely through improved centromere binding. We think, given the nature of the short period of growth we evolved our strains for (less than 50 generations), this is unlikely to occur through a single nonsynonymous mutation. First, a mutation of the human histone themselves could arise (to improve interaction with Cse4). However, the human histone genes are present in 2-4 copies per cell (they are on an episomal pRS vector), so any de novo mutation would likely not be fully penetrant. Additionally, we have shown part of the incompatibility of human nucleosomes is due to at least 2 residues of H3 and 5 residues of H2A (Truong and Boeke 2017), thus it is probably unlikely any single missense mutation in the human histones would rescue interaction with Cse4.

As for the inner kinetochore protein themselves…? The physical connection between DNA and kinetochore in budding yeasts (Saccharomycotaceaea, specifically) relies on a single specialized nucleosome. If that nucleosome is profoundly disturbed, as we think it is in histone humanized yeast, we find it hard to imagine that any single missense mutation of an inner kinetochore protein (CCAN) could rescue this defect directly. Perhaps mutations in proteins that directly interact with Cse4, such as Mif2, may have benefit. We think this to be unlikely as long as the issue of histone incompatibility remains. Perhaps, we might expect to eventually uncover gain of function (perhaps enhanced binding?) mutations in CBF1 or CBF3 complex, which directly bind to DNA elements CDEI and CDEIII respectively (although recent structural models suggested that CBF3 is not present at “mature” kinetochores – Yan et al. Nature 2019)). Further, our data suggests the major issue is not dysfunction of the Cse4-CCAN interaction (DASH/Dam1c mutants are perfectly euploid!), but the failure of the centromere to recruit key regulators of chromosome segregation (Aurora B). The outer kinetochore mutants likely directly rescue the issue, which we proposed is dysfunction in regulated kinetochore microtubule turnover (DASH mutants promote increased turnover).

We think these reasons may explain the high frequency of outer kinetochore mutants isolated and lack of any inner kinetochore mutants (so far!).

Please see the newly added text to the discussion that addressing these points. Lines 593 - 611

Some language/ typos I found:
- P 12, line 299: "We therefore sought _to_ understand ..."

Fixed

- p 17 line 413: amino acid pairing (not paring)

Fixed

- p 18 line 440: Figure (not Frigure)

Fixed

- p 22 line 535: no "period" between the words "detachment and "is".

Fixed

- p 22 line 546: should this read "... consistent with Aurora B being inactive _in_ histone-humanized yeasts ..."?

This comment is no longer relevant, as the text has changed based on reviewer's 1 suggestions (comment #16).

- p 23 line 565: may be (two words)

fixed

1st Revision - Editorial Decision

14th Dec 2022

Thank you for submitting your revised manuscript for our consideration. It has now been assessed once more by one of the original referees (see comments below), who, I am happy to say, was mostly satisfied with the revision and is now supportive of publication. As soon as a few remaining editorial points listed below have been addressed, we should therefore be ready to proceed with formal acceptance and production of the manuscript:

Referee #1:

In this revised manuscript, the authors addressed the points that this referee previously raised. Therefore, I am satisfied with revisions.

Some general comments:
• The authors tempered their discussion about the paralogous centromeres, and chose to leave the data in the MS which I think is fine.
• The authors did not address our comment about Fig 7E (which is now 7G), in which we asked them to comment on why the Dad1 mutant seems to have higher rupture force than the WT at low concentrations. Even though the authors didn't directly comment on this, the data is there for the readers, so I think this is okay as well.

EMBO Press Author Checklist

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- 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.
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  - are there adjustments for multiple comparisons?
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  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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Select "Not Applicable" only when the requested information is not relevant for your study.

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