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JOURNAL

Metabolism-Induced Oxidative Stress and DNA Damage Selectively Trigger Genome Instability in Polyploid Fungal Cells

Gregory J. Thomson, Claire Hernon, Nicanor Austriaco, Rebecca S. Shapiro, Peter Belenky and Richard J. Bennett.

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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. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 1st March 2019

Thank you for submitting your manuscript for our editorial consideration. It has now been reviewed by three expert referees, whose comments are copied below for your information. As you will see, the referees consider your study and its results interesting in principle, but they also bring up two substantive criticisms that in our view currently preclude publication, at least in a broad general journal such as The EMBO Journal: as pointed out by especially by referee 1, the studies with ROS sensors and quenchers remain ambiguous and at present fail to explain differences between diploid and tetraploid cells; and referee 3 notes that the increased cell death of tetraploid cells in CCL conditions is well likely to confound interpretation, with hypermetabolism during CCL potentially only reflecting a bulk population of dying cells. Given that especially the former concern appears to go to the essence of the study and its message, I am afraid we cannot consider this study a sufficiently strong candidate for an EMBO Journal article, at least not at the present stage.

REFEREE REPORTS

Referee #1:

This represents a potentially interesting study connecting ROS to loss of chromosome stability in tetraploid cells of Candida albicans. The paper is extremely well written and if true, the findings would represent a major advance in our understanding of eukaryotic ploidy cycles. My main concern is that the evidence that ROS is key is not yet water-tight based on the data provided. Enhanced ROS by itself cannot explain the difference between diploids and tetraploids.

Major concerns:
1) For the most part, diploids and haploids look the same in terms of ROS. They induce the same pattern of gene expression on PRE-SPO media, they both induce the CAP1 marker of ROS and they both generate ROS as seen by CellROX Green. Although by flow cytometry, there may appear to be more CellROX green in the tetraploids, these cells have more DNA for CellROX Green interactions and more mitochondria/cell for ROS. If markers of ROS are the same in tetraploids and diploids, then how can ROS explain the chromosome instability and inviability of tetraploids?
2) There is an apparent disconnect between the different effects of various chemical anti-oxidants that weakens the proposed connection between ROS and chromosome instability of tetraploids. For example, DTT and ascorbate rescued viability to same extent but only DTT but not ascorbate rescued CAP1 localization and genome instability. Additionally, Zn does not affect ROS nor viability, but it protects against DSBs. Such disconnects makes the reader feel there is not a strong connection between ROS, viability and chromosome loss.
3) Dose responses are lacking for any of the chemical anti-oxidants or generators of ROS. How did the authors choose these doses - how was maximal efficacy determined, toxicity etc? Some sort of dose response should at least be presented in supplemental material. Dose responses would improve the rigor of these studies.
4) Does overexpression of SOD3 rescue markers of ROS and genome stability?
5) Fig. 7E - do these ROS generating agents have the same effect on diploids?
6) Mitochondrial superoxide is not a substrate for SOD3; any superoxide made in the mitochondrial is dealt with by SOD1 in the IMS and SOD2 in the matrix. However, H_2O_2 could leave the mitochondria for cytosolic ROS. How do the authors explain an effect of cytosolic SOD3 if it cannot act on superoxide derived from mitochondria.

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1) All the experiments were conducted with PRE-SPO media. How physiological is this media with regard to the natural conditions of C. albicans in the host that would induce chromosome loss in tetraploids?
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3) Is the oxygen consumption of Fig. 3 cyanide inhibitable? That is the only way to insure you are measuring mitochondrial respiration and not oxygen consumption by numerous other oxygen consuming processes in the cell.
4) Fig. 4A - should show images of 4N and 2N cells stained with CellROX green side by side rather than putting 2N cells in the sup material. Are these cells really any different other than one having more DNA for the probe to interact with?
5) Fig. 5, same concern. The Gam-GFP signal in cell images should be shown side by side for tetraploids vs diploids as opposed to having one in supplemental material. Is there a difference?
6) Why is Zn considered an anti-oxidant? Zn can also cause oxidative stress at higher doses. Again, there is concern about the lack of appropriate dose response tests.
7) There is no information on the type of statistical analysis used for any of the graphs. Also no mention of how many experimental trials for any of the studies.

Referee #2:

The reviewed manuscript "Metabolism-Induced Stress and DNA Damage Selectively Trigger Genome Instability in Polyploid Cells" by Thomson end colleagues from the Bennett group focuses on understanding the conditions triggering so called "concerted chromosome loss" (CCL), a state of high chromosomal instability that may be observed in tetraploid Candida albicans cells and that eventually leads to re-diploidization of the original tetraploids. CLL is considered to be a primitive meiosis-like process that allows genome reshuffling. The authors show that on high-glucose media, C.albicans become highly metabolically active, which leads to accumulation of ROS and extensive DNA damage. This, in turn, triggers extensive chromosome loss that leads to accumulation of near-

diploid cells. This is a highly interesting manuscript that addresses some of the key questions of the interplay between ploidy, genome stability and metabolism. The manuscript will be interesting for the EMBO Journal readership and I have only minor suggestions that should be addressed before the acceptance.

1.
Figure 1E: The figure has to be complemented by showing the ploidy levels of 2N strains growing on Pre-SPO as well as 2N and 4N strains on YPD. 4N strains are inherently unstable and it has to be demonstrated that the instability on Pre-SPO is significantly larger than the instability on YPD.

2.
Figure 2B: In the GSEA, it should be clarified which pathways are upregulated and which are downregulated.

3.
Figure 3D: In the text it states: ". .. tetraploid cells showed a 1.5- fold increase in OCR and 2.1- increase in ECAR when . . . . . . , and diploid cells showed similar fold differences between the two media." This sentence can be interpreted that diploid cells showed also a 1.5-fold and 2.1 increase as tetraploids. This is, however, not the case, as in the figure the diploids behave identically on both media. Rewording would be useful.

4.
Figure 4G: Does the overexpression of antioxidant genes also affects the percentage of 2-DOG(+) colonies in 4N?

5.
It would be interesting to explain what can be the cause of the differences between different antioxidant treatments.

6.
Figure 5A - the staining does not look like nuclear. It either needs better picture or a better explanation.

7.
I find the chapter on "Polyploidy and elevated ROS levels in mammalian cells" too speculative and not reflecting the complexity of the issue. For example, tetraploid hepatocytes decrease mitochondrial oxidative phosphorylation. Many cancers actually suffer from mitochondrial dysfunction. Finally, the authors completely avoid one important aspect of polyploidy and this is the increased size that alone, without changing ploidy, affects metabolism. I would therefore strongly suggest to edit this chapter to better reflect the various aspects of this research.

Referee #3:

The manuscript of Thomson et al. describes a study of the process by which tetraploid C. albicans cells undergo concerted chromosome loss under a specific set of environmental/media conditions. They have found that the tetraploid cells show evidence of increased metabolic activity on the pre-SPO medium relative to 2n cells. The medium contains 5X as much glucose as standard media but 2n cells show little evidence of increased metabolic activity. This increased metabolic activity includes both fermentative and respiratory arms of carbon metabolism based on Seahorse data. The authors argue that the increased oxidative metabolism leads to increased ROS which in turn causes genotoxic stress which ultimately causes DNA damage induced reduction in ploidy. Genetic and chemical modulators of ROS have the expected effects on ploidy supporting their model.

The main complication/major concern has to do with the fact that most of the tetraploid cells die under the conditions of CCL. Since many of the phenotypes are measured in bulk cells (metabolism/gene expression ect), these phenotypes and characteristics may simply be the signatures of dying cells and the cells that survive and reduce ploidy may be the ones that are not hypermetabolic. The effects of the antioxidants are mostly assayed using viability and only one

(DTT) of those tested had an effect on chromosome loss. It seems that hypermetabolism represents a significant selective pressure on tetraploid cells which may yield cells with reduced ploidy from a completely different mechanism simply by providing conditions that kill cells that do not successfully reduce their ploidy. It is not clear to me that the authors can firmly eliminate this alternatively hypothesis.

A minor concern is the speculative nature of the authors assertion for the generalizability of these findings to primordial meiosis. At least some measurements on other tetraploid cells with a seahorse should be done before embarking on this sort of argument in print.

Details
1. The GAM-GFP construct seems to be a novel application in C. albicans. Some positive controls with WT cells and known inducers of DSB should be included.

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We would like to thank the three reviewers for their detailed comments on the manuscript. Reviewer 2 was very positive about the paper overall and suggested only minor corrections. In contrast, Reviewers 1 and 3 raised several concerns, including a need for better understanding of how ROS levels vary between diploid and tetraploid *C. albicans* cells, as well as the efficiency of different ROS quenchers in protecting against both cell death and genome instability. We have therefore undertaken a large number of additional experiments to address these concerns as outlined below and believe that the new data supports a model involving a link between elevated metabolism, ROS production and ploidy reduction in *C. albicans*.

Referee #1:
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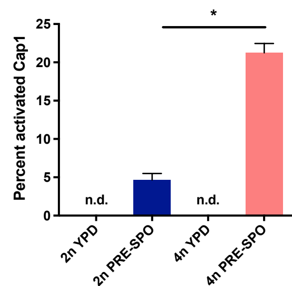
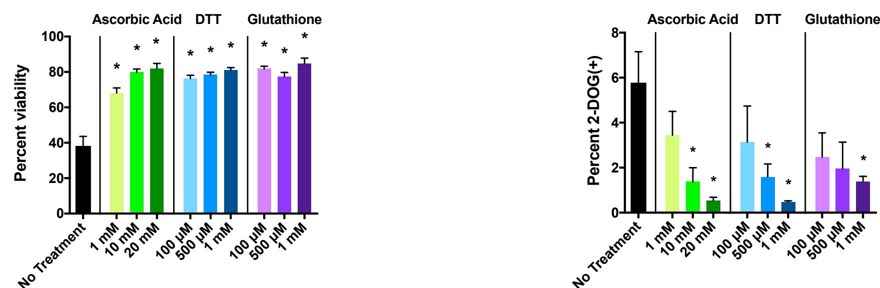
Major concerns:
1) For the most part, diploids and haploids look the same in terms of ROS. They induce the same pattern of gene expression on PRE-SPO media, they both induce the CAP1 marker of ROS and they both generate ROS as seen by CellROX Green. Although by flow cytometry, there may appear to be more CellROX green in the tetraploids, these cells have more DNA for CellROX Green interactions and more mitochondria/cell for ROS. If markers of ROS are the same in tetraploids and diploids, then how can ROS explain the chromosome instability and inviability of tetraploids?

We now include new data demonstrating that while both diploid and tetraploid cells show increased ROS on PRE-SPO medium, significantly more tetraploid cells activate oxidative stress responses based on the Cap1 reporter assay. This data establishes that 4-fold as many tetraploid cells display Cap1 activation compared to diploid cells and supports the model that tetraploid cells experience more ROS on PRE-SPO medium than diploid cells. This is reported in Figure 4D and shown below.

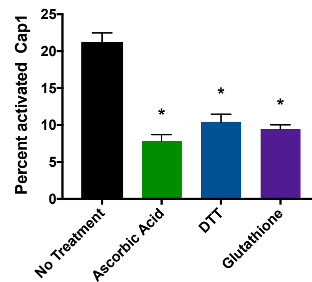


2) There is an apparent disconnect between the different effects of various chemical anti-oxidants that weakens the proposed connection between ROS and chromosome instability of tetraploids. For example, DTT and ascorbate rescued viability to same extent but only DTT but not ascorbate rescued CAP1 localization and genome instability. Additionally, Zn does not affect ROS nor viability, but it protects against DSBs. Such disconnects makes the reader feel there is not a strong connection between ROS, viability and chromosome loss.

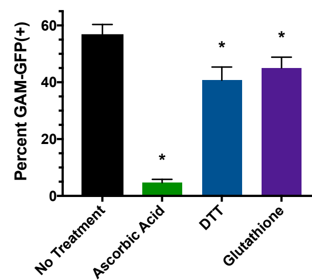
We agree with the reviewer that the initial data showed variable responses to an array of antioxidants. We looked at this in detail and focused on performing careful titrations of a subset of antioxidants. As shown below, the new data establishes that <u>3 different antioxidants significantly protect *C. albicans* tetraploid cells against both cell death and chromosome instability</u>. Thus, ascorbic acid, DTT and glutathione all increase viability on PRE-SPO medium and also reduce chromosome loss, and do so in a dose-dependent manner. This is shown in new Figure 5A and 5B.



We also examined whether the three anti-oxidants evaluated could protect tetraploid cells from oxidative stress during growth on PRE-SPO medium. As shown below, all three reduced activation of Cap1 by more than 50% and this data is in a new Figure 5C.

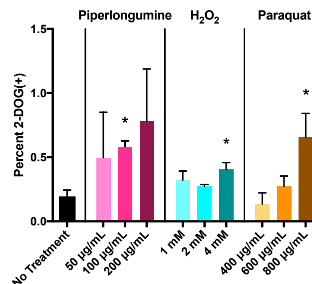


Moreover, we examined whether the three antioxidants could protect tetraploid cells from the DNA damage encountered during growth on PRE-SPO medium using the Gam-GFP reporter assay. All three reduced DNA damage on PRE-SPO medium, with ascorbic acid abrogating nearly all Gam-GFP signal, and this data is in a new Figure 6C.



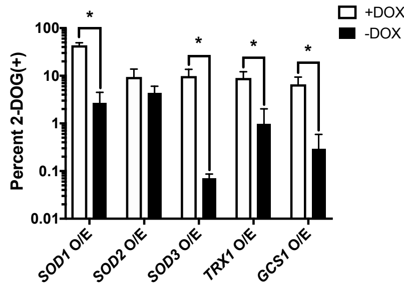
3) Dose responses are lacking for any of the chemical anti-oxidants or generators of ROS. How did the authors choose these doses - how was maximal efficacy determined, toxicity etc.? Some sort of dose response should at least be presented in supplemental material. Dose responses would improve the rigor of these studies.

We agree and therefore performed a dose response for the three anti-oxidants chosen for detailed analysis (see above and Figure 5). We also performed a dose response for the chosen generators of ROS. The data shown below demonstrates that all three inducers of ROS increased chromosome instability. The induced genome instability in the presence of generators of ROS was also dose-dependent, albeit modestly so.



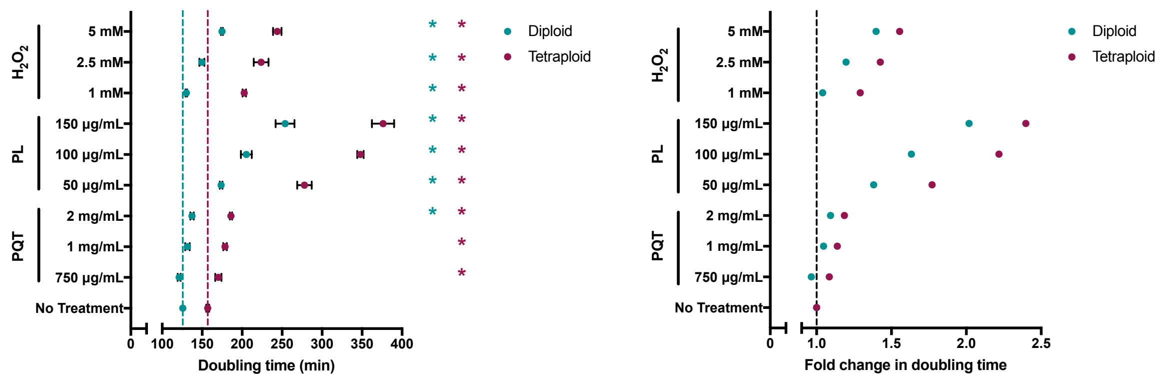
4) Does overexpression of SOD3 rescue markers of ROS and genome stability?

To address this question, we re-examined the ability of different antioxidant genes to protect against genome instability. Genome stability was examined after seven days of growth on PRE-SPO medium using 2-DOG selection. Interestingly, overexpression of *SOD1, SOD3, GCS1,* and *TRX1* all significantly protected tetraploid cells against genome instability and this is Figure 5D in the revised manuscript.



5) Fig. 7E - do these ROS generating agents have the same effect on diploids?

We examined doubling times of diploid/tetraploid cells grown in the presence of the oxidative stress-inducing agents paraquat (PQT), piperlongumine (PL), and hydrogen peroxide(H_2O_2). We find that tetraploid cells are more sensitive to each of the oxidative stress-inducing agents. As shown below, each agent increased the doubling times of both diploid and tetraploid cells compared to untreated medium. However, tetraploid cells showed a greater fold increase in doubling times with oxidative stress compared to diploid cells when normalized to growth rates without treatment. This is consistent with tetraploid cells being more sensitive to ROS generating agents than diploid cells.



6) Mitochondrial superoxide is not a substrate for SOD3; any superoxide made in the mitochondrial is dealt with by SOD1 in the IMS and SOD2 in the matrix. However, H2O2 could leave the mitochondria for cytosolic ROS. How do the authors explain an effect of cytosolic SOD3 if it cannot act on superoxide derived from mitochondria.

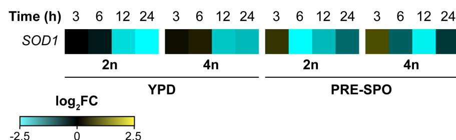
As discussed above, we now show that both *SOD1* and *SOD3* overexpression significantly rescue PRE-SPO-induced genome instability in tetraploid cells. While Sod2 is a mitochondrial superoxide dismutase, Sod1 and Sod3 are both cytosolic. We interpret this result as indicating that cytosolic ROS is capable of being scavenged by Sod1/Sod3. It is not clear why Sod2 is also not reducing the levels of ROS from the mitochondria. While these are interesting ideas to examine more closely, a more in depth analysis on the functions of *SOD1-3* is outside the scope of the current work, particularly given that *GCS1* and *TRX1* also protect tetraploid cells from PRE-SPO phenotypes.

Minor concerns:
1) All the experiments were conducted with PRE-SPO media. How physiological is this media with regard to the natural conditions of C. albicans in the host that would induce chromosome loss in tetraploids?

It is unclear whether PRE-SPO conditions are found in nature. However, our study highlights how both endogenous and exogenous cues can influence genome stability in *C. albicans*. It is feasible that other environmental conditions could lead to an increase in metabolic activity and produce sufficient ROS to increase genome instability. In addition, *C. albicans* encounters both endogenous and exogenous sources of oxidative stress during infection. Macrophages and neutrophils respond to *C. albicans* through ROS production and oxidative bursts that curtail fungal invasion (Warris and Ballou 2018; PMID 29522807). In fact, the axis between *C. albicans* and host immune cells during infection has been referred to as a “superoxide superstorm” (Rossi et al. 2017; PMID 29194441). Future experiments will therefore look to examine whether conditions in the mammalian host can induce chromosome instability in *C. albicans* polyploid cells and if these involve elevated ROS levels.

2) The authors are missing literature on SOD3. This is not considered to be an oxidative stress associated gene as indicated on page 9. This gene is induced under Cu starvation conditions when the Cu containing SOD1 is repressed. SOD3 is not involved in protecting against mitochondrial oxidative stress. Instead it functions in cytosolic glucose signaling as a substitute for Cu-SOD1 when cells are starved for Cu. The authors need to consider this information and cite the relevant papers. In this regard, what happens to SOD1 expression in cells switched to PRE-SPO?

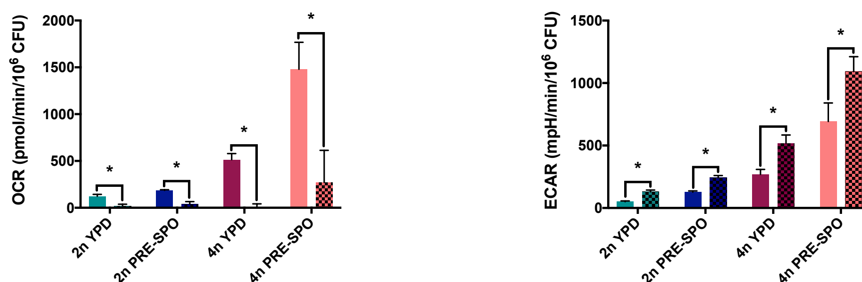
Examination of *SOD1* expression illustrates that *SOD1* has higher levels of expression on PRE-SPO medium relative to YPD medium at 3 h, but that the gene is repressed under all conditions at later time points (shown below).



We note that *SOD3* has been documented to protect against oxidative stress (Lamarre et al., 2001; PMID 11562375) and we have added this citation. The Lamarre et al. study showed that *SOD3* is not only induced under copper starvation conditions, but is also induced when cells enter stationary phase (Lamarre et al., 2001) and the authors state that "Sod3p …. is involved in the protection of *C. albicans* against reactive oxygen species during the stationary phase". Indeed, their data shows that heterologous expression of *CaSOD3* in *S. cerevisiae* protected those cells against treatment with menadione or paraquat (Lamarre et al., Fig. 4). As we are using an ectopic expression approach we also would expect that *SOD3* would increase protection against oxidative stress in *C. albicans* cells. As mentioned above, we have now included experiments examining genome stability in a *SOD1* O/E strain cultured on PRE-SPO medium and found that it also protects cells against genome instability.

3) Is the oxygen consumption of Fig. 3 cyanide inhibitable? That is the only way to insure you are measuring mitochondrial respiration and not oxygen consumption by numerous other oxygen consuming processes in the cell.

To address this point, we conducted Seahorse assays to examine whether or not the oxygen consumption we see is due to mitochondrial respiration. We examined oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) in untreated cells under each growth condition, then introduced a cocktail of rotenone, an inhibitor of Complex I of the electron transport chain (ETC), antimycin A, an inhibitor of Complex III of the ETC, and salicylhydroxamic acid (SHAM), an alternative oxidase inhibitor. Addition of antimycin A and rotenone is conventionally used to measure non-mitochondrial oxygen consumption and, because *C. albicans* also possesses an alternative oxidase which can contribute to oxygen consumption despite ETC inhibition (Aoki and Ito-Kuwa, 1984; PMID 6379382), we also added this to completely inhibit mitochondrial oxygen consumption. Immediately after treatment, OCR and ECAR were re-measured. The OCRs we observed in diploids and tetraploids on YPD and PRE-SPO media were greatly inhibited by addition of antimycin A/rotenone/SHAM, indicating that the oxygen consumption was due to mitochondrial respiration. While addition of these compounds inhibited oxygen consumption, they did not inhibit ECAR indicating that the treatment did not stop all cellular activity. In the graphs shown below, the second column in each pair with a checkered pattern indicates OCR/ECAR after antimycin A/rotenone/SHAM treatment. These data are now also included as Supplemental Figures EV2A and EV2B.



4) Fig. 4A - should show images of 4N and 2N cells stained with CellROX green side by side rather than putting 2N cells in the sup material. Are these cells really any different other than one having more DNA for the probe to interact with?

We have now amended the figure to include diploid and tetraploid cells side by side. The intensity of CellROX green signal is not dependent on DNA content in cells as the stoichiometry of oxidized dye to total DNA in cells is very unlikely to reach a point where the DNA is saturated with oxidized dye (information received directly from company). We therefore believe that the original CellROX green signal, in addition with the newly collected data illustrating that a higher percentage of tetraploid cells activate Cap1 on PRE-SPO medium compared to diploid cells, illustrates that tetraploid cells experience elevated levels of ROS and oxidative stress during growth on PRE-SPO medium.

5) Fig. 5, same concern. The Gam-GFP signal in cell images should be shown side by side for tetraploids vs diploids as opposed to having one in supplemental material. Is there a difference?

We have amended the figure to include diploid and tetraploid cells side by side.

6) Why is Zn considered an anti-oxidant? Zn can also cause oxidative stress at higher doses. Again, there is concern about the lack of appropriate dose response tests.

We agree with this point and therefore dropped this metal from our experiments. We instead focused on the three established anti-oxidants described above.

7) There is no information on the type of statistical analysis used for any of the graphs. Also no mention of how many experimental trials for any of the studies.

We apologize for this omission and have included information regarding the type of statistical analysis used in each graph, as well as the number of experimental trials, in the figure legends. Some of this information is also provided in the methods section.

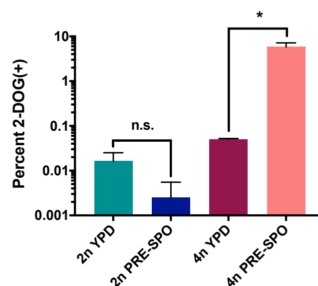
Referee #2:

The reviewed manuscript "Metabolism-Induced Stress and DNA Damage Selectively Trigger Genome Instability in Polyploid Cells" by Thomson end colleagues from the Bennett group focuses on understanding the conditions triggering so called "concerted chromosome loss" (CCL), a state of high chromosomal instability that may be observed in tetraploid Candida albicans cells and that eventually leads to re-diploidization of the original tetraploids. CLL is considered to be a primitive meiosis-like process that allows genome reshuffling. The authors show that on high-glucose media, C. albicans become highly metabolically active, which leads to accumulation of ROS and extensive DNA damage. This, in turn, triggers extensive chromosome loss that leads to accumulation of near-diploid cells. This is a highly interesting manuscript that addresses some of the key questions of the interplay between ploidy, genome stability and metabolism. The manuscript will be interesting for the EMBO Journal readership and I have only minor suggestions that should be addressed before the acceptance.

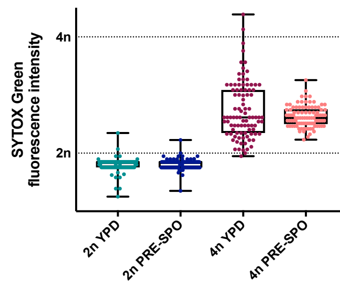
We would like to thank this reviewer for their positive comments on the paper.

1. Figure 1E: The figure has to be complemented by showing the ploidy levels of 2N strains growing on Pre-SPO as well as 2N and 4N strains on YPD. 4N strains are inherently unstable and it has to be demonstrated that the instability on Pre-SPO is significantly larger than the instability on YPD.

To address this point we conducted a 2-DOG assay using a *GAL1/gal1* diploid strain grown on YPD or PRE-SPO medium for seven days at 30°C or 37°C, respectively. We found no significant increase in the percentage of 2-DOG+ colonies between YPD and PRE-SPO media in diploid cells, whereas tetraploid cells produced more than 100 times as many 2-DOG+ progeny on PRE-SPO than on YPD. This is now included in the paper as Figure 1D.

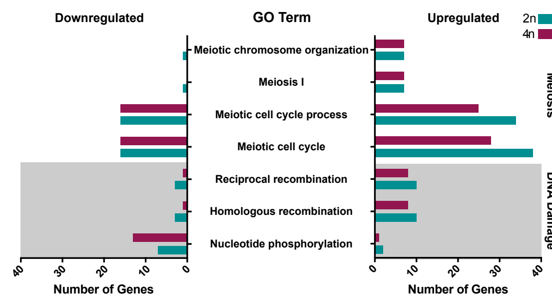


We performed an analysis of 2-DOG+ progeny from diploids and tetraploids grown on both YPD and PRE-SPO as shown below. As we expected, 2-DOG+ cells derived from tetraploids have undergone a reduction in ploidy whereas those from diploids showed minimal change. This parallels what was seen for ploidy change on PRE-SPO medium in Bennett et al. 2003. We therefore re-emphasize that 2-DOG+ selection is simply used as a proxy for ploidy reduction in tetraploids where chromosomes can readily be lost. Furthermore, 2-DOG+ cells can be recovered from tetraploids grown on both PRE-SPO and YPD, but many more are recovered from PRE-SPO indicating that this medium enhances chromosomal instability.



2. Figure 2B: In the GSEA, it should be clarified which pathways are upregulated and which are downregulated.

We have added a portion into the GSEA graph which indicates which pathways are upregulated and which are downregulated in diploids and tetraploids, as shown below. This is now included in the paper as Figure 2C.



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This may have been hard to see in the original figure as the data points for diploid cells are close together, but diploid cells also show higher OCR/ECAR on PRE-SPO than on YPD media. We have amended the Seahorse data so that OCR and ECAR measurements are displayed as two separate bar graphs, which may make it easier to discern this difference. On PRE-SPO medium, diploid cells exhibit a 1.5-fold increase in OCR and a 1.9-fold increase in ECAR. These numbers were added to the text.

4. Figure 4G: Does the overexpression of antioxidant genes also affect the percentage of 2-DOG(+) colonies in 4N?

As noted in our response to Reviewer 1, we conducted a 2-DOG assay in *SOD1, SOD2, SOD3, GCS1,* and *TRX1* O/E strains to monitor their effect on genome stability on PRE-SPO medium. Overexpression of each of these (except *SOD2*) significantly protected against genome instability. This data is now included in the paper as Figure 5D.

5. It would be interesting to explain what can be the cause of the differences between different antioxidant treatments.

We more thoroughly investigated the roles of DTT, glutathione, and ascorbic acid and found all 3 protected against oxidative stress, cell death, and instability.

6. Figure 5A - the staining does not look like nuclear. It either needs better picture or a better explanation.

We have amended the figure to include a better picture of a cell with a clear nuclear signal.

7. I find the chapter on "Polyploidy and elevated ROS levels in mammalian cells" too speculative and not reflecting the complexity of the issue. For example, tetraploid hepatocytes decrease mitochondrial oxidative phosphorylation. Many cancers actually suffer from mitochondrial dysfunction. Finally, the authors completely avoid one important aspect of polyploidy and this is the increased size that alone, without changing ploidy, affects metabolism. I would therefore strongly suggest to edit this chapter to better reflect the various aspects of this research.

We have completely edited the Discussion to better indicate the complexities of metabolism and mammalian genome instability.

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The manuscript of Thomson et al. describes a study of the process by which tetraploid C. albicans cells undergo concerted chromosome loss under a specific set of environmental/media conditions. They have found that the tetraploid cells show evidence of increased metabolic activity on the pre-SPO medium relative to 2n cells. The medium contains 5X as much glucose as standard media but 2n cells show little evidence of increased metabolic activity. This increased metabolic activity includes both fermentative and respiratory arms of carbon metabolism based on Seahorse data. The authors argue that the increased oxidative metabolism leads to increased ROS which in turn causes genotoxic stress which ultimately causes DNA damage induced reduction in ploidy. Genetic and chemical modulators of ROS have the expected effects on ploidy supporting their model.

The main complication/major concern has to do with the fact that most of the tetraploid cells die under the conditions of CCL. Since many of the phenotypes are measured in bulk cells (metabolism/gene expression ect), these phenotypes and characteristics may simply be the signatures of dying cells and the cells that survive and reduce ploidy may be the ones that are not hypermetabolic. The effects of the antioxidants are mostly assayed using viability and only one (DTT) of those tested had an effect on chromosome loss. It seems that hypermetabolism represents a significant selective pressure on tetraploid cells which may yield cells with reduced ploidy from a completely different mechanism simply by providing conditions that kill cells that do not successfully reduce their ploidy. It is not clear to me that the authors can firmly eliminate this alternatively hypothesis.

We agree that responses of *C. albicans* cells to PRE-SPO medium are complex and that we cannot completely rule out that different subpopulations are behaving differently. However, we contend that hypermetabolism is key in driving inviability/chromosome instability due to production of high ROS levels. In support of this, our new data indicates that 3 antioxidants clearly result in (1) increased survival of tetraploid cells on PRE-SPO medium, (2) reduced tetraploid genome instability, and (3) reduced ROS levels (see above). We believe that the new data is convincing in showing that multiple anti-oxidants (as well as overexpression of antioxidant genes) show similar effects, and that these support a model in which increased metabolism/ROS is linked to instability and cell death.

We also argue that the increased metabolism of *C. albicans* cells on PRE-SPO medium is unlikely to be due to massive cell death. We note that the increased metabolic activity of tetraploid cells relative to diploid cells is similar on both YPD (where cells remain viable) and PRE-SPO (where cell death is observed in tetraploid cells). On the former, metabolism is 4-fold higher in tetraploids than diploids, and in the latter, it is 8-fold higher (Seahorse data in Figure 3D). The similar fold differences on the two media suggest metabolic differences are due to increased ploidy, rather than hypermetabolism being due to extensive cell death on PRE-SPO.

A minor concern is the speculative nature of the authors assertion for the generalizability of these findings to primordial meiosis. At least some measurements on other tetraploid cells with a seahorse should be done before embarking on this sort of argument in print.

We have revised the Discussion to emphasize the model is speculative and we will look for more evidence of the proposed mechanism in other cell types moving forward. We think the additional experiments will be substantial and are therefore beyond the scope of the current work.

Details

1. The GAM-GFP construct seems to be a novel application in C. albicans. Some positive controls with WT cells and known inducers of DSB should be included.

We demonstrate that treatment of *C. albicans* cells with MMS or HU induces a strong GAM-GFP signal and this data is provided in Figure EV4.

2nd Editorial Decision 24th July 2019

Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by all three original referees, and I am pleased to communicate that they all find the revisions generally satisfactory and the paper now in principle suitable for The EMBO Journal. We shall therefore be happy to publish the study, after a few minor editorial modifications.

Referee 1 points out some remaining issues that will require textual modifications - please make those in the attached document, with "Track Changes" option activated.

REFEREE REPORTS

Referee #1:

The authors have done an excellent job in addressing the comments of the previous review with much more data. I only have one minor concern.

They did not address the previous concern 2 of reviewer 1 regarding the literature on SOD1 and SOD3. The one paper they added is outdated. The 2001 Lamarre paper did not show SOD3 induction by Cu starvation, only that SOD3 is induced by stationary phase. It was subsequently shown by Li et al PNAS 2015 that this stationary phase induction of SOD3 and repression of SOD1 are actually due to Cu starvation. And there are followup papers on SOD3 in glucose signaling as opposed to ROS protection that are also missing. The fact that SOD3 can protect against menedione and paraquat protection as Lamarre showed in 2001 is not evidence for a role in endogenous oxidative stress protection.

Referee #2:

I am satisfied with the revised version of the manuscript and would recommend it for a publication in EMBO Journal.

Referee #3:

All my concerns were addressed.

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THE EMBO JOURNAL

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

Corresponding Author Name: Richard Bennett
Manusript Number: EMBOJ-2019-101597

Reporting Checklist For Life Sciences Articles

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

A- Figures
1. Data
The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars only for independent experiments and sample sizes where the application of statistical tests is warranted (error bars should not be shown for technical replicates)
- when n is small (n < 5), the individual data points from each experiment should be plotted alongside an error bar.
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation (see link list at top right).

2. Captions
Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ^2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

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

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

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Question	Please fill out these boxes ↓
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	NA
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3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	No randomization was used.
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4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No steps were taken to minimize the effects of subjective bias.
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7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

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Question	Answer
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Question	Answer
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