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Opi1-mediated transcriptional modulation orchestrates genotoxic stress response in budding yeast

Giovanna Panessa, Eduardo Tassoni-Tsuchida, Marina Pires, Rodrigo Felix, Rafaella Jekabson, Nadja de Souza-Pinto, Fernanda da Cunha, Onn Brandman, and José Renato Cussiol

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GENETICS-2022-305709
The transcriptional repressor Opi1 modulates the DNA Damage Response by downregulation of inositol pyrophosphates in *Saccharomyces cerevisiae*

Dear Dr. Cussiol:

Two experts in the field have reviewed your manuscript, and I have read it as well. All agreed that this is an important area of study and that, while the connection between inositol and DNA damage response is not new, you have a system in hand with which to really reveal novel mechanistic information. While your manuscript is not currently acceptable for publication in GENETICS, we would welcome a substantially revised manuscript. Both reviewers have comments and concerns to be addressed in a revised manuscript. You can read their reviews at the end of this email.

The most important areas on which to focus are the following: 1) a more quantitative assessment of some of the growth phenotypes, as well as an assessment of cell death versus slow growth phenotypes in the presence of DNA damaging agents, 2) more robust assessment of different DNA damaging agents that incur different types of DNA damage and therefore may have different effects in the opi1 deletion background, 3) assessment of Rad53 phosphorylation as a marker for activation of the DNA damage response and 4) transcriptional analysis of other Opi1-regulated promoters. This is just a suggestion, but you may want to consider re-organizing the manuscript to highlight the Kcs1 result . Importantly, GENETICS strives to be of interest to a more general audience. Therefore it is important to provide more experimental and background information. For instance, describe the type of DNA damage incurred by MMS and bleomycin, define how the different localization of Opi1-GFP were scored (i.e. what does "transition" look like?), as well as experimental details, as indicated by both reviewers. For instance, YPG is not defined or described in the text. In addition to the minor points mentioned by the reviewers, please clarify the phenotype ofOpi1-GFP +INO+MMS localization phenotype - it is ambiguous as written. And getting a sense of the cell cycle progression of the opi1 deletion in the absence of MMS would be an important control.

We look forward to receiving your revised manuscript. Please let the editorial office know approximately how long you expect to need for revisions.

Upon resubmission, please include:
1. A clean version of your manuscript;
2. A marked version of your manuscript in which you highlight significant revisions carried out in response to the major points raised by the editor/reviewers (track changes is acceptable if preferred);
3. A detailed response to the editor's/reviewers' feedback and to the concerns listed above. Please reference line numbers in this response to aid the editor and reviewers.

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Sincerely,

Jennifer Surtees
Associate Editor
GENETICS

Approved by:
Jeff Sekelsky
Senior Editor
GENETICS

Reviewer #1 (Comments for the Authors (Required)):

This is a review of the manuscript entitled "The transcriptional repressor Opi1 modulates the DNA Damage Response by

downregulation of inositol pyrophosphates in Saccharomyces cerevisiae." The authors show that Op1-GFP migrates into the nucleus upon oxidative stress. The authors assert that the genotoxic sensitivity of opi1 mutants is conferred by upregulation of inositol pyrophosphates. In support of this hypothesis, the authors show that opi1 ino1 ino2 mutants are resistant to methyl methanesulfonate (MMS), implying that expression of genes controlled by Ino1 Ino2 transcriptional activator confer MMS sensitivity in opi1 mutants.. Deleting genes controlled by Opi1 indicate that ksc1 opi1 mutants are no longer MMS sensitive, while overexpression of KSC1 confers MMS sensitivity. Since Ksc1 functions as an inositol hexakisphosphate and inositol heptakisphosphate kinase, the overall picture is that inositol phosphates regulate the DNA damage response. The authors propose that cells must downregulate inositol pyrophosphate synthesis during replication stress to trigger an effective DNA Damage Response. The assertion that inositol pyrophosphates modulate DNA damage signaling has been previously reported (Biochem J (2009) 423 (1): 109-118 and other references). While the current study is novel concerning the role of OPI1, the authors only provide limited measurements of the DNA damage response after MMS exposure, such as H2A phosphorylation and cell cycle delay, and only yeast growth after bleomycin exposure. Overall, this reviewer feels that the authors need to include additional data to support their assertions but would be willing reconsider the manuscript after revision. These revisions are outlined below.

Major Comments:

1) The major concern with this manuscript is the limited endpoints used to determine the DNA damage response. MMS also alkylates proteins, is mutagenic and indirectly causes double-strand breaks. If the authors assert OPI1 controls the DNA damage response due to replication stress, they should also expose yeast to hydroxyurea, which chiefly causes replication stress.

2) Rad53 phosphorylation is a more robust indicator of the DNA damage response, compared to H2A phosphorylation. The authors use an antibody against anti-gamma H2A but it's unclear exactly how much H2A is phosphorylated. Western blots could detect both Rad53 and Rad53 phosphorylation and may give a better assessment of the persistence of the DNA damage response. Figure 2B seems to indicate that while H2A phosphorylation is initially more robust in wild-type cells, it seems to persist longer in opi1 cells at six hours. It is noted that authors indicate that the DNA damage response persists for less time in opi1 cells, compared to wild type. Figure 2C also doesn't appear quite as obvious to this reviewer; it seems the comparison should be made to unphosphorylated H2A. In addition, could recovery from MMS exposure also depend on how well the DNA damage response is deactivated?

3) It is unclear why the authors didn't follow up their studies on MMS with bleomycin. It should be noted that in a manuscript authored by Omnebe and Saiardi (2009), phleomycin exposure had different effects compared to hydrogen peroxide. Thus, it is possible that OPI1 has different functions in the DNA damage response depending on the genotoxic agent. The authors should compare the opi1 MMS sensitivity with the sensitivity to other DNA damaging or genotoxic agents. For example, is the opi1 mutant UV sensitive?

4) The authors assert that the cells are opi1 are MMS sensitive and at initial glance of Figure 2, this is indeed the case. However, as described by the authors, the plates were photographed after two-three days. Are the opi1 cells killed after MMS exposure, or could the plates just indicate that their growth is delayed? In other words, exactly lethal is MMS in the opi1 mutant and how do the cells die?

Minor Comments:

1. The authors should elaborate more on how the strains were constructed. Were they made by the Rothstein method of one-step gene replacement? Considering that there are multiple methods, a better description is required. In addition, the authors should list the primers on how the knockouts were identified by PCR in a supplementary table.

2. Hydrogen peroxide is a genotoxic agent, and one would expect peroxide exposure to also trigger a DNA damage response. If so, how would the authors interpret that the opi1 peroxide-associated sensitivity is similar to wild type?

3. There are some figure legends that need better explanation. Indeed, Figure 6 has a title but not a legend. Several figures give the reader a conclusion without describing exactly what is in the figure (see Figures 4 and 5). This also holds true for supplemental figures.

4. Was there an independent nuclear stain to indicate that Op1-GFP was transported into the nucleus?

Reviewer #2 (Comments for the Authors (Required)):

given in the attached file

The authors studied the role of the transcriptional repressor Opi1 (through inositol metabolism) in DNA Damage Response in *S. cerevisiae*. In order to investigate that, cells lacking Opi1 were exposed to genotoxic stress. By following the rescue and recapitulation of MMS sensitivity through genetic modifications, they investigated the involvement of DNA damage response under these conditions. The manuscript is well organized but the results presented do not provide strong support for the conclusions reached (some listed below).

Although the approach itself is not novel, the study provides set of data, potentially valuable to the community, but the interpretation of results is overestimated.

The following comments might help the authors to support their conclusions and improve their paper:

1. Introduction misses the reason(s) for the choice of yeast cells and the importance of the response of these cells to MMS. What is the relevance of MMS?

2. Materials and Methods: In most parts there is no citation at all, these are not new techniques and require proper citation of relevant reference(s).
Page 8, line 122: What is meant by drugS? What are they? Later it is understood that they are MMS and bleomycin but they should be mentioned here as well. How much is given and for how long?
Is only expression of INO1 measured by RT-qPCR? Why? Why not expression of other relevant genes measured?
Are the experiments done once? Any repetitions?

3. It is not explained why MMS and bleomycin are selected to study the response of yeast cells. What is the importance of them? And bleomycin: only shown in Figure 1C, not clear why it is used and then why the experiments done with MMS is not repeated with bleomycin as well.

4. The selection dosage/concentrations of MMS and/or inositol is not explained. How the amounts decided?

5. In general the suggestions/proposals are not based on strong evidence but rather on qualitative observations. Most of the results are shown in serial dilutions spotted on media. However, these observations, being qualitative and relative, are not enough. For example, by looking at MMS sensitivity how can it be said that "downregulation of inositol levels through Opi1 are important to trigger… "? Inositol levels were not measured. Such approach is seen in each subsection of the Results and Discussion parts. Another example: by looking at the translocation of OPi1-GFP to nucleus, how can it be known that localization is important to manage with genotoxic stress (page 15, line 307)?

I believe the study should be supported by quantitative data, such as measurement of intracellular and extracellular levels of inositol; measurement of PP-IPs, measurement of gene expression of more genes, preferably whole transcriptome or at least for the genes in inositol metabolism; and statistical analysis would provide the significance of the findings.

Manuscript GENETICS-2022-305709

RESPONSE TO REVIEWERS:

We would like to thank all the reviewers for your valuable feedback and comments on our manuscript. We have carefully considered each of the points raised by the reviewers and have made substantial revisions to address their concerns and improve the quality of the manuscript. In particular, we would like to highlight the significant changes that have been made to the text and figures as a result of new experiments, including transcriptome RNA-seq analysis, as suggested by one of the reviewers, which have not only expanded the number of figures and supplemental material but have also led us to propose new hypotheses. As a result, the conclusions drawn in the revised version differ in certain aspects from those of the original manuscript. In this letter, we aim to provide detailed responses to the main questions raised by the reviewers and to justify the changes that have been implemented in light of these new findings. Furthermore, we would like to inform you that we have decided to change the title of the manuscript from "The transcriptional repressor Opi1 modulates the DNA Damage Response by downregulation of inositol pyrophosphates in *Saccharomyces cerevisiae*" to "Opi1-mediated transcriptional modulation orchestrates genotoxic stress response in budding yeast." We believe that this revised title better reflects the comprehensive nature of our study and highlights the central role of Opi1 in coordinating the cellular response to genotoxic stress. We have attached two new versions of the manuscript: one is a marked version (Panessa et al resubmission_marked version) that highlights the changes made to the previous version in green, and the other is a clean version (Panessa et al resubmission_clean version). We look forward to address any further questions or concerns you may have regarding the revised manuscript.

SPECIFIC RESPONSE TO REVIEWER #1:

"This is a review of the manuscript entitled "The transcriptional repressor Opi1 modulates the DNA Damage Response by downregulation of inositol pyrophosphates in Saccharomyces cerevisiae." The authors show that Op1-GFP migrates into the nucleus upon oxidative stress. The authors assert that the genotoxic sensitivity of opi1 mutants is conferred by upregulation of inositol pyrophosphates. In support of this hypothesis, the authors show that opi1 ino1 ino2 mutants are resistant to methyl methanesulfonate (MMS), implying that expression of genes controlled by Ino1 Ino2 transcriptional activator confer MMS sensitivity in opi1 mutants.. Deleting genes controlled by Opi1 indicate that ksc1 opi1 mutants are no longer MMS sensitive, while

overexpression of KSC1 confers MMS sensitivity. Since Ksc1 functions as an inositol hexakisphosphate and inositol heptakisphosphate kinase, the overall picture is that inositol phosphates regulate the DNA damage response. The authors propose that cells must downregulate inositol pyrophosphate synthesis during replication stress to trigger an effective DNA Damage Response. The assertion that inositol pyrophosphates modulate DNA damage signaling has been previously reported (Biochem J (2009) 423 (1): 109–118 and other references). While the current study is novel concerning the role of OPI1, the authors only provide limited measurements of the DNA damage response after MMS exposure, such as H2A phosphorylation and cell cycle delay, and only yeast growth after bleomycin exposure. Overall, this reviewer feels that the authors need to include additional data to support their main conclusion. Suggested revisions are outlined below."

We acknowledge the reviewer for their valuable comments. Below, we provide our responses to the relevant points raised by the reviewer.

MAJOR POINT 1: *"The major concern with this manuscript is the limited endpoints used to determine the DNA damage response. MMS also alkylates proteins, is mutagenic and indirectly causes double-strand breaks. If the authors assert OPI1 controls the DNA damage response due to replication stress, they should also expose yeast to hydroxyurea, which chiefly causes replication stress".*

We appreciate the reviewer's comment regarding the endpoints used to determine the DNA damage response and the suggestion to expose yeast to hydroxyurea (HU) as an additional replication stress inducer. We agree that MMS can induce various types of DNA damage beyond replication stress. To address this concern, we conducted sensitivity assays with other genotoxins, including HU and camptothecin (CPT), which induce replication stress (New Figure 1C). Our new findings demonstrate that *opi1Δ* cells do not exhibit sensitivity to HU and CPT. It is important to note that the mechanism by which MMS induces replication stress differs from that of HU and CPT. Previous studies have shown that certain yeast mutants, such as *slx4Δ* and *pph3Δ*, display sensitivity to MMS but not to HU and CPT (Jablonowski *et al.* 2015). Additionally, we exposed *opi1Δ* cells to zeocin and 4NQO, which are known genotoxic agents with distinct mechanisms of action (New Figure 1C). Our

results demonstrate that *opi1Δ* cells exhibit heightened sensitivity to genotoxic agents such as MMS, zeocin, and 4NQO, indicating a broader role for Opi1 in the cellular response to genotoxic stress. Therefore, we decided to change the term “replication stress” to the more generic term “genotoxic stress”. Notably, we have shown that deletion of *INO2-INO4* rescues the sensitivity of *opi1Δ* cells to MMS and 4NQO, suggesting a potential shared mechanism through which these genotoxins affect cells in the context of Opi1-mediated genotoxin resistance (New Figure 5A). Interestingly, we found that deletion of *INO4* alone also rescues the zeocin/bleomycin sensitivity of *opi1Δ* cells, whereas deletion of *INO2* does not have the same effect (New Figure 5A). This observation supports the notion that Ino2 and Ino4 possess independent roles in the modulation of gene expression in different cellular contexts, as previously showed (Chumnanpuen *et al.* 2013). We acknowledge that further investigation is needed to unravel the specific molecular pathways and downstream effectors through which Opi1 contributes to the cellular response to these genotoxic stresses. We have incorporated additional experiments that shed light on the increased MMS sensitivity observed in *opi1Δ* cells (New Figure 6). Specifically, we have included evidence suggesting a link between Opi1 and mitochondrial DNA (mtDNA) instability, as MMS treatment has been previously shown to induce mtDNA damage. Furthermore, we have provided data demonstrating that the effect of MMS treatment in cells lacking Opi1 is not attributed to a defect in coping with increased proteotoxic stress (New Figure 1D), thereby excluding the possibility of heightened protein alkylation as the underlying cause.

MAJOR POINT 2: *" Rad53 phosphorylation is a more robust indicator of the DNA damage response, compared to H2A phosphorylation. The authors use an antibody against anti-gamma H2A but it's unclear exactly how much H2A is phosphorylated. Western blots could detect both Rad53 and Rad53 phosphorylation and may give a better assessment of the persistence of the DNA damage response. Figure 2B seems to indicate that while H2A phosphorylation is initially more robust in wild-type cells, it seems to persist longer in opi1 cells at six hours. It is noted that authors indicate that the DNA damage response persists for less time in opi1 cells, compared to wild type. Figure 2C also doesn't appear quite as obvious to this reviewer; it seems the comparison should be made to unphosphorylated H2A. In addition, could recovery*

from MMS exposure also depend on how well the DNA damage response is deactivated?

We appreciate the reviewer's comment regarding the use of Rad53 phosphorylation as an indicator of the DNA damage response (DDR) and the need to assess the persistence of the DDR. To address this concern, we have incorporated western blot analysis to evaluate Rad53 activation through Rad53 mobility shift, providing a more comprehensive assessment of the DDR dynamics (New Figure 2 and New Supplementary Figure 3). First, we have added a new result showing that there is no significant difference in Rad53 activation between asynchronous populations of wild-type and *opi1Δ* cells exposed to MMS, zeocin, and 4NQO (New Supplementary Figure 3). While *opi1Δ* cells show heightened sensitivity to these genotoxins, we did not observe changes in Rad53 activation, indicating that Opi1 is not directly involved in regulating the Rad53 axis of the DDR.

Regarding Figure 2B (now, New Figure 2F), the reviewer's observation suggests that gamma-H2A levels appears to persist longer in *opi1⊿* cells at six hours. We have repeated this experiment, but since gamma-H2A levels are already low in *opi1⊿* we believe that kinetics of gama-H2A disappearance does not change between strains. To better assess this point, we also monitor kinetics of Rad53 deactivation, and as we can see in new Figure 2G there is no change in downregulation of Rad53 between strains, thus we are convinced that this new result indicates that Opi1 does not affect the downregulation of the DNA damage signaling. In Figure 2C (New Figure 2F), we understand the reviewer's suggestion to compare the results to unphosphorylated H2A for better clarity. We have now included an additional panel in the figure to show the levels of total unphosphorylated histone H2A. Although obtaining a robust signal with this antibody proved to be difficult, requiring longer exposure times to achieve satisfactory detection levels, we had showed that total histone H2A levels doesn’t seem to change between strains. However, it is worth noting that we are detecting total histone H2A rather that histone H2A organized in nucleosomes bound to DNA. We were unable to perform chromatin immunoprecipitations analysis that would answer this specific point due to the

poor quality of the antibody. Therefore, we cannot exclude the possibility that Opi1 regulates histone recruitment to nucleosomes.

MAJOR POINT 3: *"It is unclear why the authors didn't follow up their studies on MMS with bleomycin. It should be noted that in a manuscript authored by Omnebe and Saiardi (2009), phleomycin exposure had different effects compared to hydrogen peroxide. Thus, it is possible that OPI1 has different functions in the DNA damage response depending on the genotoxic agent. The authors should compare the opi1 MMS sensitivity with the sensitivity to other DNA damaging or genotoxic agents. For example, is the opi1 mutant UV sensitive?*

We appreciate the reviewer's comment regarding the need to investigate the response of *opi1Δ* cells to other DNA damaging or genotoxic agents, including bleomycin and UV radiation. We fully acknowledge the importance of such comparisons in order to comprehend the specific functions of Opi1 in the DNA damage response (DDR) under different genotoxic stress conditions. We have already addressed some of these concerns in Major Points 1 and 2, and we will now expand upon our explanations. In response to the reviewer's suggestion, we conducted additional experiments to evaluate the sensitivity of *opi1Δ* cells to other genotoxins, including zeocin (which belongs to the phleomycin family of radiomimetic drugs) and 4NQO (a known UV mimetic). Our findings support the involvement of Opi1 in the cellular response to other genotoxic agents, as we observed increased sensitivity of *opi1Δ* cells to both zeocin and 4NQO (New Figure 1C). Moreover, we have considered the reviewer's comment by providing new results that demonstrate Rad53 activation in the presence of other genotoxins besides MMS (New Supplementary Figure 3), as discussed in the response to Major Point 2. However, since most of our experiments were done in MMS, including new RNA-seq analysis, we decided to focus our investigation in the response of Opi1 to genotoxic stress induced by MMS. Regarding the study by (Onnebo and Saiardi 2009), it is important to note the distinction between their investigation, which utilized *kcs1Δ* cells, and our study, which primarily focuses on *opi1Δ* cells. Therefore, while their work demonstrates that a decrease in inositol pyrophosphates leads to phleomycin sensitivity, our findings suggest that overexpression of Kcs1, and subsequently

an increase in inositol pyrophosphate levels, phenocopies the MMS sensitivity observed in *opi1Δ* cells (New Figure 5D). We propose that elevated production of inositol pyrophosphates may explain certain phenotypes, particularly the increased MMS sensitivity and delayed G1 to S-phase observed in *opi1Δ* cells. Furthermore, our results confirm some of the findings from the aforementioned study, as we demonstrate increased sensitivity of *kcs1Δ* cells to bleomycin and zeocin (New Figure 5E). Notably, in this revised version of the manuscript, we present evidence showing that deletion of *KCS1* rescues the MMS sensitivity of *opi1Δ* cells, but simultaneously enhances sensitivity to other genotoxins (New Figure 5E). These observations suggest a potential cooperative role between Opi1 and Kcs1 in coping with bulky DNA damage that is different from their opposite role during MMS treatment. However, the specific molecular mechanisms underlying this cooperation remain to be elucidated.

MAJOR POINT 4: "*The authors assert that the cells are opi1 are MMS sensitive and at initial glance of Figure 2, this is indeed the case. However, as described by the authors, the plates were photographed after two-three days. Are the opi1 cells killed after MMS exposure, or could the plates just indicate that their growth is delayed? In other words, exactly lethal is MMS in the opi1 mutant and how do the cells die?"*

This is an important point raised by the reviewer as serial dilution assays may not provide a definitive distinction between viability and delayed growth. To address this concern, we employed two alternative methods, flow cytometry and colony forming unit assays (CFU), to assess cell viability. Interestingly, both methods yielded comparable results, indicating that there is no significant difference in cell viability between the wild-type and *opi1Δ* cells. Furthermore, we have included a growth curve analysis that demonstrates that *opi1Δ* cells have a prolonged arrest of the cell cycle since cells start to divide (New Figure 1E). These findings align with other observation that Opi1 depletion leads to a delayed G1 to S-phase transition (New Figures 2A, D, and E), suggesting a crucial role for Opi1 in regulating cell cycle progression. Additionally, our RNA-seq analysis revealed the upregulation of several genes involved in mating signaling transduction pathway in *opi1Δ cells* during genotoxic stress (New Figure 4D and E). Notably, we identified proteins from the MAPK pathway such

as Far1 and Fus3 that are upregulated in *opi1Δ cells* under MMS-treatment. These proteins act in concert to inhibit the function of the Cln-Cdc28 complex during G1 phase, thereby impeding cell progression from G1 to START. These findings are thoroughly discussed in the revised version of the manuscript.

MINOR POINT 1: "*The authors should elaborate more on how the strains were constructed. Were they made by the Rothstein method of one-step gene replacement? Considering that there are multiple methods, a better description is required. In addition, the authors should list the primers on how the knockouts were identified by PCR in a supplementary table".*

All yeast strains were generated using the one-step PCR method. PCR genotyping using specific primers was conducted to confirm the successful generation of knockout strains. We have now included this information in the Material and methods (Yeast strains and plasmids section), along with a new table that provides a comprehensive list of all the primers utilized in this study (New Table S3).

MINOR POINT 2: "*Hydrogen peroxide is a genotoxic agent, and one would expect peroxide exposure to also trigger a DNA damage response. If so, how would the authors interpret that the opi1 peroxide-associated sensitivity is similar to wild type?"*

This question raises intriguing considerations. While hydrogen peroxide (H_2O_2) can indeed cause DNA damage, it is noteworthy that the most prevalent types of lesions induced by H_2O_2 are oxidations in adenine and guanine bases, resulting in the formation of non-bulky DNA adducts like 8-hydroxyadenine and 8-hydroxyguanine. These lesions are primarily repaired by the Base Excision Repair (BER) machinery. Consequently, unless there is a defect in the BER pathway, oxidative damage typically does not activate the DNA damage signaling pathway or induce homologous recombination repair as MMS does. This phenomenon is commonly referred to as "silent repair" (Leroy *et al.* 2001). Of greater significance is the fact that MMS treatment induces different types of lesions, particularly N7-methylguanine and N3-methyladenine, which can inhibit DNA synthesis and result in DNA breaks that activate DNA damage

signaling. The DNA repair pathways responsible for removing these DNA lesions also differ, with MMS-induced lesions requiring at least three pathways: Base Excision Repair, Homologous Recombination, and Replication Bypass. Although the possibility of a dose response exists, we tested millimolar amounts of H_2O_2 that proved insufficient to induce sensitivity in *opi1Δ* cells (New Figure 1D). In fact, an article from 1989 using filamentous fungi *Neurospora crassa* showed that very few of the MMS-sensitive were hypersensitive to H_2O_2. On the other hand, there was a great overlap in sensitive of these MMS-sensitive mutants with bleomycin and ionizing radiation (Käfer and Luk 1989). Additionally, another intriguing observation is that our findings propose a potential increase in mtDNA instability in *opi1Δ* cells following MMS treatment (New Figure 6). In support of this, we demonstrate in this revised manuscript that treatment with antimycin-A also sensitizes cells lacking Opi1. Hence, the question arises as to why antimycin-A sensitizes *opi1Δ* cells, but not H_2O_2. One possibility is that endogenous and localized production of reactive oxygen species (ROS) may lead to greater mtDNA instability compared to H_2O_2, which can be rapidly scavenged by various antioxidant enzymes such as catalases, peroxiredoxins, and glutathione peroxidases. In future investigations, it would be interesting to explore whether deletion of peroxidases, such as the cytosolic peroxiredoxin Tsa1 or the mitochondrial peroxiredoxin Prx1 in an *opi1Δ* strain enhances the sensitivity to H_2O_2.

MINOR POINT 3: "*There are some figure legends that need better explanation. Indeed, Figure 6 has a title but not a legend. Several figures give the reader a conclusion without describing exactly what is in the figure (see Figures 4 and 5). This also holds true for supplemental figures."*

We acknowledge that some figure legends lacked sufficient information to clearly convey the content and significance of the figures. In response to this feedback, we have revised the figure legends accordingly and made changes in order to provide more comprehensive explanations. It is also important to note that we have restructured some Figures and also increased the number of Supplementary Figures. We believe that in this new version of the manuscript

each legend accurately describes the content and highlights the key findings of the respective figure.

MINOR POINT 4: *"Was there an independent nuclear stain to indicate that Op1-GFP was transported into the nucleus?"*

In our study, we employed live-cell imaging to visualize the localization of Opi1-GFP. Unfortunately, utilizing a traditional nuclear stain such as DAPI was not feasible in this experimental setup. However, we acknowledge that alternative methods, such as fusing a yeast nuclear protein with RFP for nuclear labeling, could have been considered for nuclear visualization. Nonetheless, it is crucial to highlight that the dual localization of Opi1 has been extensively documented in previous studies. Numerous publications have reported that Opi1 is predominantly localized in the nucleus under conditions of inositol abundance, and it translocates to the endoplasmic reticulum (ER) upon depletion of inositol levels (Gaspar *et al.* 2017; Hofbauer *et al.* 2018). These studies, alongside a comprehensive body of literature, strongly support the established phenomenon of Opi1's dual localization. While we acknowledge that an independent nuclear stain would have further reinforced our findings regarding Opi1's migration to the nucleus under genotoxic stress, we firmly believe that our results are robust and well-supported by the existing literature on Opi1's dual localization.

SPECIFIC RESPONSE TO REVIEWER #2:

"The authors studied the role of the transcriptional repressor Opi1 (through inositol metabolism) in DNA Damage Response in S. cerevisiae. In order to investigate that, cells lacking Opi1 were exposed to genotoxic stress. By following the rescue and recapitulation of MMS sensitivity through genetic modifications, they investigated the involvement of DNA damage response under these conditions. The manuscript is well organized but the results presented do not provide strong support for the conclusions reached (some listed below). Although the approach itself is not novel, the study provides set of data, potentially valuable to the community, but the interpretation of results is overestimated. The following comments might help the authors to support their conclusions and improve their paper:"

We thank the reviewer for the comments and we have addressed the relevant and interesting points raised.

MAJOR POINT 1: *"Introduction misses the reason(s) for the choice of yeast cells and the importance of the response of these cells to MMS. What is the relevance of MMS?"*

We appreciate the valuable comment raised by the reviewer concerning the relevance of utilizing methyl methanesulfonate (MMS) as a tool to investigate the DNA damage response in yeast cells. MMS is widely recognized as a genotoxic agent capable of inducing DNA damage through the alkylation of DNA bases. Notably, studying the response to MMS offers insights into the impact it has on both nuclear and mitochondrial DNA. In yeast research, MMS has been extensively employed due to its ability to emulate endogenous DNA damage and robustly activate the DDR kinases Mec1 and Rad53 serving as a valuable resource for investigating the DNA damage response in yeast cells. By unraveling the intricacies of the cellular response to MMS, we can acquire fundamental knowledge about cellular processes that have implications not only for yeast but also for higher eukaryotes, including humans, as the basic principles governing the DNA damage response are conserved across species. We have incorporated in the introduction section further elucidation on the mechanism of MMS action and its significance in investigating the DDR in yeast (lines 29-38).

MAJOR POINT 2: *Materials and Methods: In most parts there is no citation at all, these are not new techniques and require proper citation of relevant reference(s). Page 8, line 122: What is meant by drugS? What are they? Later it is understood that they are MMS and bleomycin but they should be mentioned here as well. How much is given and for how long? Is only expression of INO1 measured by RT-qPCR? Why? Why not expression of other relevant genes measured? Are the experiments done once? Any repetitions?*

Based on the valuable observations raised by both reviewers, we have taken their feedback into consideration and thoroughly revised the Material and Methods section to ensure a comprehensive description of the experimental

conditions. In response to the specific issue raised about the lack of references in the Material and Methods section, we have included references to provide appropriate citations for the techniques employed. Regarding the specific point raised on page 8, line 122, we have made the following revision: *"Fourfold serial dilutions were spotted on yeast plates and grown for 1–3 days at 30°C in the presence or absence of specific chemical agents, including genotoxins and other drugs. The specific concentrations of these agents used in the assay are provided in the corresponding figure and/or figure legends, serving as a clear reference for the experimental setup."* (lines 145-153). We acknowledge the reviewer's concern about the clarity of the term "drugs", but we believe that the inclusion of Figures and corresponding figure legends in the revised version of the manuscript adequately addresses the concerns raised, clearly presenting all the chemical agents used and their respective concentrations. These modifications have significantly improved the clarity and comprehensiveness of the experimental descriptions in the manuscript.

Regarding the gene expression analysis, initially, we performed RT-qPCR to measure the expression of *INO1* as it is a key gene involved in the pathway under investigation and also serves as a reporter for other genes that contain a UAS_{INO} upstream of the promoter region. We acknowledge the reviewer's concern about the limited scope of gene expression analysis. To address this, we conducted a comprehensive RNA-seq experiment, which we consider a major improvement in this manuscript. The RNA-seq analysis allowed us to analyze the transcriptome of yeast cells under MMS treatment conditions in *opi1Δ* cells, providing valuable insights into the control of gene expression by Opi1 during genotoxic stress. Considering the constraints in terms of time and financial resources, we performed the RNA-seq experiment comparing wild-type and *opi1Δ* cells in SC medium supplemented with inositol in unchallenged and MMS-treated conditions. It is important to note that the experimental conditions used in the RNA-seq analysis were the same as those employed in our RT-qPCR analysis of *INO1* expression. We have provided an explanation in the "Results" section of our paper to clarify the rationale behind selecting this specific experimental condition. The following excerpt is included in the manuscript (lines 450-454):

"We selected this specific experimental condition based on two primary reasons: Firstly, our experiments have demonstrated the heightened sensitivity of Opi1-deficient cells to MMS when grown in medium supplemented with inositol (Figure 3C). Secondly, our own observation reveals that MMS-induced upregulation of INO1 expression in opi1Δ cells is independent of inositol supplementation (Figure 3D)."

Furthermore, to address the question of experiment repetition, we want to clarify that all experiments were performed with appropriate biological replicates to ensure the reliability of our findings. However, we realize that this was not explicitly mentioned in the manuscript, and we apologize for the omission. We revised the manuscript to clearly state that the experiments were repeated independently and included the number of replicated performed for each experiment and also the type of statistical analysis performed for each experiment. We appreciate the reviewer's valuable feedback, and we will make the necessary revisions to improve the clarity and rigor of our Materials and Methods section.

MAJOR POINT 3: *It is not explained why MMS and bleomycin are selected to study the response of yeast cells. What is the importance of them? And bleomycin: only shown in Figure 1C, not clear why it is used and then why the experiments done with MMS is not repeated with bleomycin as well.*

Regarding the selection of MMS and bleomycin as DNA damage agents, we understand the importance of providing a clear rationale for their use. Both MMS and bleomycin are well-established genotoxic agents capable of activating the DNA damage response (DDR) through different mechanisms. As explained in the response to Major Point 1, MMS induces DNA base methylation, leading to the formation of DNA adducts, while bleomycin causes DNA strand breaks through oxidative damage. Our choice of these agents was motivated by their ability to induce specific types of DNA damage that are relevant to the biological processes and pathways under investigation. In response to the reviewer's comment, we have expanded the information on the

genotoxins used in our study and included new experiments in the revised manuscript to further support our findings. Specifically, we have demonstrated that yeast cells lacking Opi1 exhibit sensitivity not only to MMS but also to zeocin (a member of the same family of radiomimetic drugs as bleomycin) and 4NQO (a UV mimetic). The results of these experiments are presented in new Figures 1D, 5A, and 5E. Furthermore, we have included a characterization of Rad53 activation kinetics in response to MMS, zeocin, and 4NQO treatments in the new Supplementary Figure 3. These additional experiments provide valuable insights into the role of Opi1 in mediating resistance to different genotoxins that induce distinct types of DNA damage. While we acknowledge that including additional experiments with bleomycin in our original set of experiments would have further enhanced the comprehensiveness of our study, we were limited by the availability of resources. However, we believe that the inclusion of zeocin and 4NQO, along with the comprehensive analysis of MMS-induced genotoxic stress and the RNA-seq data, provides a robust framework for understanding the importance of Opi1 in mediating genotoxin resistance.

MAJOR POINT 4:*The selection dosage/concentrations of MMS and/or inositol is not explained. How the amounts decided?*

The concentrations of inositol and MMS used in our study were carefully selected based on established literature and previous experimental findings. In the case of inositol supplementation, as described by (Hirsch and Henry 1986) it was experimentally established that a concentration of 75μM of inositol has been shown to support robust growth of inositol auxotrophic strains (e.g., *ino1Δ*) and effectively repress *INO1* expression. We have added this information to the manuscript (lines 424-426). Regarding MMS, we employed concentrations that are commonly used in the field and supported by relevant studies. Serial dilution assays, which are frequently used to monitor MMS sensitivity, typically utilize concentrations up to 0.02%. Moreover, a concentration of 0.033% MMS (approximately 3 mM) has been experimentally established as a standard for inducing replication stress and studying cell cycle progression in several research studies (Tercero and Diffley 2001; Tercero *et al.* 2003; M *et al.* 2007;

Cussiol *et al.* 2015). To further justify our choices, we have included references to these studies that support the selected concentrations of MMS in the revised manuscript (lines 380-381). Additionally, we acknowledge the work by (Jelinsky and Samson 1999), who employed a 0.1% MMS treatment for 1 hour to investigate global changes in gene expression (lines 425-426). We utilized this concentration to evaluate gene expression by RT-qPCR, RNA-seq and the cellular localization of Opi1-GFP. By providing the specific references and explanations for the concentrations chosen, we aim to offer a comprehensive understanding of our experimental design and demonstrate the rationale behind our decisions.

MAJOR POINT 5: *In general the suggestions/proposals are not based on strong evidence but rather on qualitative observations. Most of the results are shown in serial dilutions spotted on media. However, these observations, being qualitative and relative, are not enough. For example, by looking at MMS sensitivity how can it be said that "downregulation of inositol levels through Opi1 are important to trigger... "? Inositol levels were not measured. Such approach is seen in each subsection of the Results and Discussion parts. Another example: by looking at the translocation of OPi1-GFP to nucleus, how can it be known that localization is important to manage with genotoxic stress (page 15, line 307)? I believe the study should be supported by quantitative data, such as measurement of intracellular and extracellular levels of inositol; measurement of PP-IPs, measurement of gene expression of more genes, preferably whole transcriptome or at least for the genes in inositol metabolism; and statistical analysis would provide the significance of the findings.*

We appreciate the comment from the reviewer regarding the importance of quantitative measurements of intracellular inositol and PP-IPs to support our conclusions. We acknowledge that such measurements would provide valuable insights into the cellular dynamics of these metabolites. However, due to the complexity of the assay and the lack of necessary expertise, resources and collaborators with the required equipment, we were unable to perform these experiments at this stage. We apologize for this limitation and recognize the potential value of these measurements in future studies. However, in response to the reviewer's suggestion, we conducted whole transcriptome analysis using RNA-seq to explore differential gene expression between wild-type and *opi1Δ*

cells under both unchallenged and MMS-treated conditions. This analysis allowed us to identify significant changes in gene expression associated with various biological processes in response to genotoxic stress induced by MMS (New Figure 4 and New Supplementary Figure 5). These RNA-seq results have influenced several key aspects of our manuscript. For instance, regarding our initial assumption of upregulated genes in the inositol phosphate pathway, we observed a lack of significant changes in the expression of *KCS1*, *ARG82*, or other upstream genes in the pathway, which contradicted our previous hypothesis based on prior literature (Wimalarathna *et al.* 2011). However, we believe that our genetic analysis is valuable, although indirect, since it clearly demonstrates that deletion of *KCS1* rescues the MMS sensitivity of *opi1Δ* cells, and overexpression of *KCS1* phenocopies the MMS sensitivity observed in *opi1Δ* cells. Therefore, it is expected that by deleting *KCS1* you also deplete PP-IP, or at least considerably reduces its levels, while overexpression increases PP-IPs levels. These genetic findings are important to corroborate one of our hypotheses that Opi1 counteracts the effects of inositol pyrophosphates during genotoxic stress. We have made appropriate modifications to the manuscript, eliminating the previous idea that the absence of Opi1 leads to upregulation of the inositol pyrophosphate pathway, as least at gene expression levels, as we were unable to see upregulation of this pathway. However, it is known that deletion of Opi1 increases the levels of PI, which is a precursor for inositol polyphosphate molecules. Additionally, we have included new controls to demonstrate the functionality of our Kcs1 overexpression under the control of the *GAL1* promoter (New Supplementary Figure 8B and C).

Regarding the Opi1-GFP localization and the assumption made, we apologize for any confusion caused by the wording in our previous manuscript. We understand that it may have implied that the translocation of Opi1-GFP to the nucleus leading to gene repression was stated as a definitive fact. We want to clarify that it was not presented as a conclusive finding but rather as a hypothesis generated based on our initial observations. We have made slight changes to the text to better clarify the speculative nature of our hypothesis. However, with the RNA-seq data obtained, we were able to demonstrate that Opi1 plays a crucial role in modulating the expression of several genes in

response to MMS treatment. These findings support our hypothesis that Opi1 is involved in the regulation of gene expression during genotoxic stress. We appreciate the valuable input from the reviewer, and although we were unable to perform the suggested intracellular metabolite measurements, we believe that the RNA-seq analysis and the additional experimental controls provide substantial evidence to support our revised conclusions.

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June 21, 2023
RE: GENETICS-2023-306233

Dear Dr. Cussiol:

I am pleased to accept your manuscript entitled "**Opi1-mediated transcriptional modulation orchestrates genotoxic stress response in budding yeast**" for publication in GENETICS, pending minor revision.

Please submit your revision along with a response to the reviewers' concerns and suggestions, which can be viewed at the bottom of this email. Most important are being a little more careful with the interpretation of the MMS data with respect to DNA damage versus general damage/cells stress, and clarifying the data in Figure 1E. I expect this can be done within 30 days.

Upon resubmission, please include:
1. A clean version of your manuscript;
2. A marked version of your manuscript in which you highlight significant revisions carried out in response to the major points raised by the editor/reviewers (track changes is acceptable if preferred);
3. A detailed response to the editor's/reviewers' comments and to the concerns listed above. Please reference line numbers in this response to aid the editors.

Additionally, please ensure that your revision is formatted for GENETICS:. https://academic.oup.com/genetics/pages/general-instructions.

Follow this link to submit the revised manuscript: Link Not Available

Thank you for submitting your excellent research to Genetics!

Sincerely,

Jennifer Surtees
Associate Editor
GENETICS

Approved by:
Jeff Sekelsky
Senior Editor
GENETICS

Reviewer comments:

Reviewer #1 (Comments for the Authors (Required)):

This is the second review of the manuscript entitled "The Opi1-mediated transcriptional modulation orchestrates the genotoxic stress response in budding yeast." The authors assert that the genotoxic sensitivity of opi1 mutants is conferred by upregulation of inositol biosynthesis. In support of this notion, they perform transcriptomic analysis comparing MMS-associated transcripts in opi1 mutants and in wild type and show that Opi1 functions to repress phospholipid biosynthesis and mating signaling. Overall, the authors have done well in performing additional experiments to support the role of Opi1 signaling in conferring resistance to diverse genotoxins. The opi1 phenotypes described for diverse genotoxic agents are interesting and should be published. While additional experiments are not required, this reviewer feels that there are several "general assertions" posed by these authors should be revised or toned down and additional edits are necessary in the manuscript before publication. Thus, while the data appear acceptable for publication the interpretation requires some modification.
Major Points:
1. The authors have shown that Opi1 functions in the MMS-associated changes in gene expression. The transcriptomic analyses were performed in 0.1% MMS, which is a high concentration of MMS, also known to cause diverse types of cellular damage. As noted by the authors, MMS-associated Rad53 phosphorylation was not affected in asynchronous opi1 cells (lines 374-377). This implies that sensors and transducers of DNA damage signaling are still functioning. MMS also directly causes lipid damage: see

Ovejero, S., Soulet, C., & Moriel-Carretero, M. (2021). The alkylating agent methyl methanesulfonate triggers lipid alterations at the inner nuclear membrane that are independent from its DNA-damaging ability. International Journal of Molecular Sciences, 22(14), 7461.Thus, while this reviewer would accept the interpretation that Opi1 is a critical sensor of MMS-associated stress in budding yeast, the interpretation that Opi1 is a critical sensor for genotoxic stress caused by many types of DNA damaging agents is less clear. One suggestion is to entitle the article: "The Opi1-mediated transcriptional modulation orchestrates the MMS-associated stress response in budding yeast."

2. There are clearly differences between op1 phenotypes regarding 4NQO and MMS. This reviewer asked whether opi1 mutants were also sensitive to UV. The authors refer to 4-NQO as a UV-mimetic agent and did not report whether opi1 mutants were sensitive to UV. While many DNA repair genes function in repairing both UV and 4-NQO-associated DNA damage, 4-NQO causes a significant amount of oxidative stress, see Ramotar, Dindial, et al. "A yeast homologue of the human phosphotyrosyl phosphatase activator PTPA is implicated in protection against oxidative DNA damage induced by the model carcinogen 4-nitroquinoline 1-oxide." Journal of Biological Chemistry 273.34 (1998): 21489-21496. A manuscript by Zewail et al., suggest that opi1 mutants are not UV sensitive, see Zewail, A., Xie, M. W., Xing, Y., Lin, L., Zhang, P. F., Zou, W., ... & Huang, J. (2003). Novel functions of the phosphatidylinositol metabolic pathway discovered by a chemical genomics screen with wortmannin. Proceedings of the National Academy of Sciences, 100(6), 3345-3350.

3. On page 21, the authors assert there was no difference in kinetics of Rad53 deactivation. Considering the authors did not report ratios of phosphorylated Rad53 to unphosphorylated Rad53 and the time points are rather spaced apart, this quantitative assertion ("kinetics") seem too strong. However, from the Figure 2F, it does appear that the overall time course of deactivation is similar.

Other points:
1. Figure 1E growth curve is a bit confusing. At time zero, the WT and opi1 mutants treated with MMS have higher absorbance than the WT and opi1 mutant that was not treated. This seems to imply that the authors chose different cell densities for their experiments. Did the authors subtract the initial absorbance for the untreated cells but not for the treated cells? Does MMS contribute to the A600 reading?

2. The authors give the molarity for MMS but not for the other agents. The molarity for the other agents should be mentioned in the Materials and Methods.

3. The authors note that genotoxic stress induces filamentous growth in budding yeast and that these proteins associated with filamentous growth are downregulated in an Opi1-dependent manner. While certain genotoxins and DNA replication stress inducers do induce filamentous growth in haploid yeast, the phenomena are strain specific. Indeed, Jiang and Kang, 2003 note that they did not observe filamentous growth induced in S288c-derived strain. Considering that the authors use BY4741-derived strains that are essentially derived from S288c-related strains, this section needs some revision.

4. Camptothecin exposure does result in DNA breakage and not just replication stress.

5. The article is very long and some narrative in the Results section could be truncated or placed in the Discussion.

Reviewer #2 (Comments for the Authors (Required)):

The manuscript has been revised accordingly. In view of the considerable efforts of the authors in response to comments given before, I believe that the manuscript has improved a lot. And current manuscript is acceptable for publication in Genetics.

Manuscript GENETICS-2023-306233

RESPONSE TO REVIEWERS:

We would like to thank all the reviewers for your valuable feedback and comments on our manuscript. It is gratifying to know that the manuscript was considered acceptable for publication in Genetics. Based on the considerations of reviewer 1 we have attached two new versions of the manuscript: one is a marked version (Panessa et al resubmission_track version) that highlights the changes made to the previous version (with track changes visible), and the other is a clean version (Panessa et al resubmission_clean version).

SPECIFIC RESPONSE TO REVIEWER #1:

"This is the second review of the manuscript entitled "The Opi1-mediated transcriptional modulation orchestrates the genotoxic stress response in budding yeast." The authors assert that the genotoxic sensitivity of opi1 mutants is conferred by upregulation of inositol biosynthesis. In support of this notion, they perform transcriptomic analysis comparing MMS-associated transcripts in opi1 mutants and in wild type and show that Opi1 functions to repress phospholipid biosynthesis and mating signaling. Overall, the authors have done well in performing additional experiments to support the role of Opi1 signaling in conferring resistance to diverse genotoxins. The opi1 phenotypes described for diverse genotoxic agents are interesting and should be published. While additional experiments are not required, this reviewer feels that there are several "general assertions" posed by these authors should be revised or toned down and additional edits are necessary in the manuscript before publication. Thus, while the data appear acceptable for publication the interpretation requires some modification."

We appreciate your dedicated review of our manuscript entitled "The Opi1-mediated transcriptional modulation orchestrates the genotoxic stress response in budding yeast". We are pleased to learn that you have found our additional experiments supporting the role of Opi1 signaling in MMS-induced stress response to be interesting and suitable for publication. We have carefully considered your comments and suggestions, and we have made the necessary revisions to address the concerns raised regarding the general assertions and interpretation of our findings.

MAJOR POINT 1: *The authors have shown that Opi1 functions in the MMS-associated changes in gene expression. The transcriptomic analyses were performed in 0.1%*

MMS, which is a high concentration of MMS, also known to cause diverse types of cellular damage. As noted by the authors, MMS-associated Rad53 phosphorylation was not affected in asynchronous opi1 cells (lines 374-377). This implies that sensors and transducers of DNA damage signaling are still functioning. MMS also directly causes lipid damage: see Ovejero, S., Soulet, C., & Moriel-Carretero, M. (2021). The alkylating agent methyl methanesulfonate triggers lipid alterations at the inner nuclear membrane that are independent from its DNA-damaging ability. International Journal of Molecular Sciences, 22(14), 7461.Thus, while this reviewer would accept the interpretation that Opi1 is a critical sensor of MMS-associated stress in budding yeast, the interpretation that Opi1 is a critical sensor for genotoxic stress caused by many types of DNA damaging agents is less clear. One suggestion is to entitle the article: "The Opi1-mediated transcriptional modulation orchestrates the MMS-associated stress response in budding yeast".

We appreciate the reviewer for bringing this important reference to our attention. We acknowledge that the MMS-induced stress may not be directly associated with DNA damage. However, it is worth noting that our study also demonstrated the sensitivity of Opi1-deficient cells to zeocin, which, according to the reference provided, does not induce lipid stress. Additionally, based on the experiments conducted by Ovejero et al., it is difficult to exclude the possibility that alterations in the inner nuclear membrane impact genome stability. The authors of this manuscript highlights in the discussion section numerous instances where the inner nuclear membrane is known to play a crucial role in maintaining genome stability. Therefore, given the significance of this finding, we have included a paragraph in the discussion section to propose that Opi1 may play a role in mitigating lipid stress at the nuclear membrane.

Lines 747-754

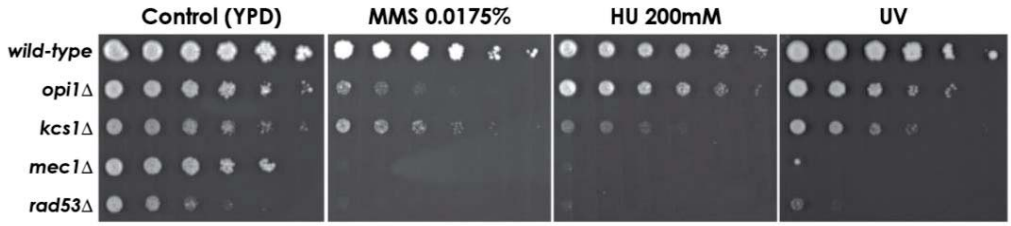
"Significantly, previous research has demonstrated that exposure to MMS leads to lipid stress specifically at the inner nuclear membrane of RPE-1 cells. This stress response is mitigated through a combination of nuclear membrane deformation and the emission of nuclear lipid droplets (Ovejero *et al.* 2021)*. Given that alterations in the nuclear membrane can impact critical nuclear processes including DNA replication, transcription, and repair* (Mekhail and Moazed 2010) *, it is of particular interest to explore the potential involvement of Opi1 in the preservation of nuclear membrane integrity."*

In response to the reviewer's suggestion, we have made the requested changes throughout the manuscript by replacing the term "genotoxic stress" with "MMS-induced stress" (please refer to the track changes). We appreciate the reviewer's input in this regard. However, we would like to respectfully express our different opinion regarding the proposed title change. While we acknowledge that our study primarily focused on MMS-induced stress and presented results from RNA-seq and fluorescence microscopy experiments conducted specifically in response to MMS, it is important to note that our findings extend beyond MMS. We demonstrated that Opi1-deficient cells also exhibit sensitivity to other genotoxic agents such as 4NQO and zeocin/bleomycin, which have the potential to directly or indirectly damage DNA molecules. Furthermore, we have shown that chemicals inducing environmental stress responses do not impact cell fitness in the absence of Opi1. Therefore, we believe that the term "genotoxic stress" in the title more accurately reflects the collective experimental evidence and the broader scope of our study.

MAJOR POINT 2: *"There are clearly differences between op1 phenotypes regarding 4NQO and MMS. This reviewer asked whether opi1 mutants were also sensitive to UV. The authors refer to 4-NQO as a UV-mimetic agent and did not report whether opi1 mutants were sensitive to UV. While many DNA repair genes function in repairing both UV and 4-NQO-associated DNA damage, 4-NQO causes a significant amount of oxidative stress, see Ramotar, Dindial, et al. "A yeast homologue of the human phosphotyrosyl phosphatase activator PTPA is implicated in protection against oxidative DNA damage induced by the model carcinogen 4-nitroquinoline 1-oxide." Journal of Biological Chemistry 273.34 (1998): 21489-21496. A manuscript by Zewail et al., suggest that opi1 mutants are not UV sensitive, see Zewail, A., Xie, M. W., Xing, Y., Lin, L., Zhang, P. F., Zou, W., ... & Huang, J. (2003). Novel functions of the phosphatidylinositol metabolic pathway discovered by a chemical genomics screen with wortmannin. Proceedings of the National Academy of Sciences, 100(6), 3345-3350.*

We acknowledge that zeocin can induce oxidative stress and can also affect mitochondrial DNA in addition to its genotoxic effects. Regarding the absence of experiments using UV irradiation, we apologize for not including those in our study. Unfortunately, we currently do not have a precise method for measuring the dosage of UV radiation. However, to address this limitation, we conducted a short-term sensitivity

assay using UV light from the laminar flow hood for a duration of 30 seconds. As a positive control, we included yeast mutants lacking Mec1 and Rad53 to ensure that the cells were indeed exposed to UV radiation. Interestingly, as depicted in the figure below, we observed a very modest sensitivity of Opi1-deficient cells to UV irradiation. However, due to the experimental constraints and the lack of quantitative UV dosage measurement, we made the decision to not include this result in the manuscript. We appreciate the reviewer's understanding in this matter. Based on the considerations mentioned, we have carefully reviewed the manuscript and excluded the term "UV-mimetic agent" from the text when referring to 4NQO.



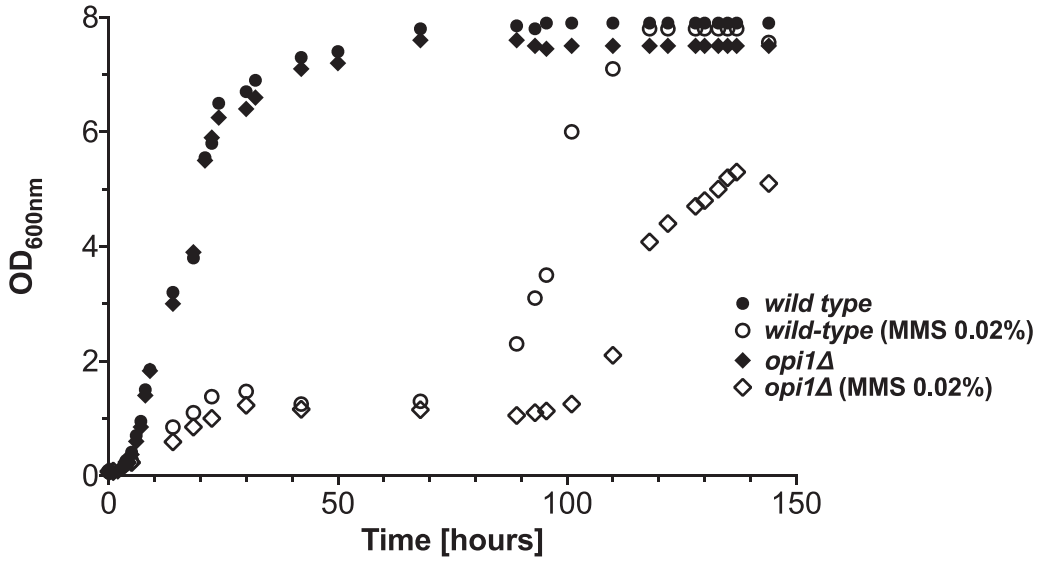
MAJOR POINT 3: *On page 21, the authors assert there was no difference in kinetics of Rad53 deactivation. Considering the authors did not report ratios of phosphorylated Rad53 to unphosphorylated Rad53 and the time points are rather spaced apart, this quantitative assertion ("kinetics") seem too strong. However, from the Figure 2F, it does appear that the overall time course of deactivation is similar.*

We appreciate the reviewer's feedback, and we agree with their suggestion. Therefore, we have revised the manuscript and removed the term "kinetics" when referring to the time course of disappearance of phosphorylated forms of histone H2A and Rad53.

MINOR POINT 1: *Figure 1E growth curve is a bit confusing. At time zero, the WT and opi1 mutants treated with MMS have higher absorbance than the WT and opi1 mutant that was not treated. This seems to imply that the authors chose different cell densities for their experiments. Did the authors subtract the initial absorbance for the untreated cells but not for the treated cells? Does MMS contribute to the A600 reading?*

We would like to emphasize that the solid line in the figure represents the non-linear regression curve fit generated by using GraphPad Prism version 9.0.0. It is worth noting that the program automatically selected this curve as the best fit, although it

may not accurately reflect the actual OD values at the initial time points. The geometrical symbols, on the other hand, represent the actual ODs. To ensure consistency, all strains were normalized to an OD_{600nm} of approximately 0.1 at time zero. Furthermore, it is important to clarify that MMS treatment does not influence the absorbance at 600nm. For ease of comparison, we have included a modified version of the figure without the non-linear regression curve fit, as shown in the Figure below. Please note that the open boxes representing MMS treatment may appear hidden at the initial time points due to overlap with the black boxes. Additionally, we collected fewer time points for the MMS-treated cells due to their slower growth kinetics. However, we have chosen to retain the figure with the non-linear regression curve fit as it provides a better visual representation of the prolonged cell proliferation arrest observed in *opi1Δ* cells following MMS treatment compared to the figure presented here.



MINOR POINT 2: "*The authors give the molarity for MMS but not for the other agents. The molarity for the other agents should be mentioned in the Materials and Methods.*

We understand that it is common practice in the scientific literature to indicate the concentrations of genotoxins in different manners depending on the specific compound. In the case of zeocin and bleomycin, as they are antibiotics, it is customary to express their concentrations in milligrams per milliliter (mg/ml) both in solution and on plates. We followed this convention to maintain consistency with the existing literature and facilitate comparison with previous studies. For 4NQO, we observed that

many articles exploring its genotoxic effects on yeast cells depict the concentration in micrograms per milliliter (µg/ml). Therefore, we adopted this convention to align with established practices in the field. On the other hand, compounds such as hydroxyurea (HU) and camptothecin (CPT), are commonly depicted in molar concentration (M) due to their specific mode of action. We apologize for any confusion caused by the variations in concentration units, but we aimed to align our methodology with the prevailing standards in the field. However, we understand that this variation in concentration units may have caused confusion, and we apologize for any inconvenience. To address this concern, we have decided to remove the information about the MMS concentration in molar units from the material and methods section since it is not the common representation in the DNA damage response field. We appreciate your understanding of this matter. We also want to emphasize that the material and methods section shows:

Lines 142 to 144:

"For the detailed concentrations and durations of specific chemical agents, including genotoxins and other drugs, please refer to the respective figure legends associated with the experimental data."

MINOR POINT 3: "*The authors note that genotoxic stress induces filamentous growth in budding yeast and that these proteins associated with filamentous growth are downregulated in an Opi1-dependent manner. While certain genotoxins and DNA replication stress inducers do induce filamentous growth in haploid yeast, the phenomena are strain specific. Indeed, Jiang and Kang, 2003 note that they did not observe filamentous growth induced in S288c-derived strain. Considering that the authors use BY4741-derived strains that are essentially derived from S288c-related strains, this section needs some revision.*

We would like to address the point raised by the reviewer and provide clarification. While our RNA-seq data demonstrate differential expression of genes involved in filamentous growth in yeast cells lacking Opi1 under MMS-induced stress, it is important to note that we did not claim direct effects on filamentous growth in our strains. We would like to highlight a specific paragraph from the discussion section that elaborates on this distinction:

Lines 703 to 706

"It is important to note that all of our results were obtained using BY4741, which is known to be a filamentation-deficient yeast strain. Thus, any phenotypes related to filamentous growth, such as biofilm formation and invasive growth on agar, were not observable in our study."

Here, we aim to emphasize that our study does not specifically investigate the impact of Opi1 on filamentous growth. Rather, we acknowledge the presence of genes related to filamentous growth among the differentially expressed genes identified in our RNA-seq analysis. We apologize if our previous wording led to any confusion and appreciate the opportunity to provide this clarification.

MINOR POINT 4: *"Camptothecin exposure does result in DNA breakage and not just replication stress"*

Camptothecin is known to induce replication stress by trapping topoisomerase I cleavage complexes, which subsequently results in the formation of replication-dependent DNA lesions, including double-strand breaks. Therefore, it is important to acknowledge that replication stress does not preclude the occurrence of DNA breaks.

MINOR POINT 5: *The article is very long and some narrative in the Results section could be truncated or placed in the Discussion"*

We agree that the article is long and we understand the importance of optimizing the article's length to enhance readability. In response to this suggestion, we have carefully reviewed the Results and Discussion sections and made necessary revisions to reduce any excessive or repetitive content.

SPECIFIC RESPONSE TO REVIEWER #2:

"The manuscript has been revised accordingly. In view of the considerable efforts of the authors in response to comments given before, I believe that the manuscript has improved a lot. And current manuscript is acceptable for publication in Genetics.:"

We would like to express our sincere appreciation to the reviewer for their positive assessment of the revised manuscript. We are grateful for the reviewer's valuable feedback and guidance throughout the review process, which has undoubtedly strengthened the quality and clarity of our work.

References

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