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A Sterol-PI(4)P exchanger modulates the Tel1/ATM axis of the DNA Damage Response

Sara Ovejero, Sylvain Kumanski, Caroline Soulet, Julie Azarli, Benjamin Pardo, Olivier Santt, Angelos Constantinou, Philippe Pasero, and María Moriel-Carretero
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Review COMMONS

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Review #1

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

Ovejero et al. report an increase in lipid droplet (LD) abundance after long (from 120' on) exposure of budding yeast cells to DNA damaging agents zeocin and camptothecin (CPT). Next, they analyze DNA damage signaling in yeast mutants that impair triacylglycerol (TAGs) or sterol (STEs) esterification. They observe a slight anticipation in Rad53/CHK2 phosphorylation (indicative of DDR signaling) in yeast stem mutants, as well as in yeast cells or human cells lines pre-treated with oleate upon zeocin treatment. Yeast stem mutants are sensitive to zeocin and captothecin, but only confer sensitivity to hydroxyurea upon combination with tagD mutations. Authors relate these phenotypes to a somewhat decreases DSB resection in yeh2D mutants (expected to have reduced steryl esters pools) and RPA-foci in steD yeast cells. Next, a reduction in single strand annealing recombination repair events upon zeocin treatment is reported using a genetic reporter in steD mutants and oleate-treated cells. From these data they conclude that inability to process sterols in response to DSBs leads to an exacerbated DDR and prevents DNA repair. Next, it is shown that Flag-tagged Tel1 distinctly interacts with mono-phosphate phosphoinositides, including PI(4)P. An interaction in vivo is also inferred through Proximity Ligation Assays (PLA) using anti-PI(4)P and anti-ATM antibodies in human cell lines, which was moderately downregulated upon treatment with MMS or zeocin. Over-expression of the Osh4/OSBP1 transporter, which consumes PI(4)P, increased the number of Tel1 (nuclear) foci upon zeocin treatment. Conversely Sac1 ablation, in which accumulation of PI(4)P is expected, abrogated nuclear Tel1 foci formation and reduced telomere length (a phenotype related to lack of Tel1 function). From these results authors conclude that Tel1 availability in the nucleus is influenced by PI(4)P availability. Lastly, treatment with an OSBP1 inhibitor led to a cell line and damaging agent -variable reduction of ATM phosphorylation and a mostly non-significant reduction of DNA resection, measured by native BrdU detection, in response to CPT treatment.
Overall, authors conclude that i) biding of Tel1/ATM to PI(4)P modulates its functional availability in the nucleus, and that ii) DNA damage elicits the esterification and storage of sterols toward LDs, which contributes to tritate Tel1/ATM away from the nucleus dampening the DDR and affecting long-range resection.

Minor comments:

- Figure S1D and E, experiments should be carried out to include time points in which LD accumulation and cell cycle arrest are observed upon zeocin treatment (i.e., up to 210' as in Figure 1A)

- How do authors explain increased single strand annealing recombination frequencies in steD and oleate-treated wild type cells (Figure 4A). Should it not be expected that increased STEs also impair recombination induced by endogenous damage?

- Data presented in figure 4B and 4C are not fully convincing. Performing time course experiments might help concluding if the differences observed represent a relevant defect in DSB processing.

- Is Figure 5B referring to Flag-tagged Tel1 or GFP-tagged Tel1 as stated in the figure legend?

- Treatment with the ATM inhibitor AZD0156 increased PI(4)P-ATM PLA signals. From these authors conclude that "association of ATM and PI(4)P inversely correlated with the need for ATM within the nucleus. Do they imply that treatment with ATM-inhibitors reduces the requirement for ATM function in the nucleus? The interpretation of this result should be further elaborated to sustain this conclusion.

- An increase of GFP-Tel1 foci upon OSH4 overexpression is described on Figure 7B. These are described as nuclear in the results, but no reference is made in the figure or legend as to how nucleus positions are addressed in these experiments. This should be clarified. Also, WT controls and quantifications should be included in the experiments shown on Figure 7C.

Major comments:

- While the conclusion that Tel1/ATM binds PI(4)P and this interaction modulates Tel1/ATM functional availability at the nucleus is convincing, the conclusion that DSBs elicit a change in the metabolism of this lipid to "control" Tel1/ATM function is not demonstrated.

- The notion that sterol processing occurs in response to DSBs is not sufficiently supported by the data presented, as the increase in LD numbers is observed much after activation of the DDR (Rad53 phosphorylation) in Zeozin-treated yeast cells. In addition, evidence is not provided on the mechanisms by which PI(4)P metabolism would be controlled, which would be expected to be DDR-independent as they are

placed upstream of this signaling pathway in the author's model. The damaging agents used have been suggested to alter the redox metabolism and even lipid peroxidation (Kitanovic 2009, Mizumoto 1993, Krol 2015, Todorova 2015, Ren 2019, Singh 2014). Hence it is possible that PI(4)P changes are not due to DSBs, but an indirect though relevant effect.

- In absence of direct evidence supporting an active regulation of PI(4)P dynamics in response to DNA breaks, this conclusion remains speculative and this should be noted in the manuscript.

- Authors conclude that LD is specific to DSB induction. This seems an overstatement as they just reported LD increases in response to two agents that also induce other kinds of DNA damage. To also strengthen the link between DSBs and PI(4)P modulation of Tel1 function, authors should analyze LD numbers, Rad53 phosphorylation and Tel1 nuclear re-localization in response to HO-induced DNA breaks (e.g., using the system employed in Figure 3C)

- In addition, on figure 5A, significant differences in GFP-Tel1 foci abundance between WT and steD or yeh2D cells are only observed after 210', way after the slight effect on Rad53 phosphorylation is observed. This is at odds with the conclusion that Tel1 association to STEs modulates DDR signaling.

Minor comments:

- Figure S1D and E, experiments should be carried out to include time points in which LD accumulation and cell cycle arrest are observed upon zeocin treatment (i.e., up to 210' as in Figure 1A)

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2. Significance:

Significance (Required)

While the conclusion of lipid metabolism responding to DSBs is not convincing, the observation that Tel1/ATM function is modulated by PI(4)P biding is significant and advances the understanding on the function and regulation of this key kinase in promoting genome integrity maintenance. This is an unanticipated result which is highly novel and has implications for the modulation of Tel1/ATM function through pharmacological manipulation of lipid metabolism. This finding would be of broad interest for scientists working on the response to DNA damage and the maintenance of genome integrity. This reviewer belongs to that group and has limited expertise to evaluate the lipid metabolism genetic manipulation in the manuscript.

3. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 1 and 3 months

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Reviewer Publons

Yes

Review #2

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

The authors show that cytoplasmic PI4P have a regulatory role on ATM response to DNA double strand breaks. The process involves a balance between exchange of PI4P between Golgi and ER in exchange of esterified sterols. The study is of interest, however provides indirect evidences to support their conclusions.

Major comments:

1). Since the major conclusion relates to PI4P association with ATM in basal conditions to keep ATM outside nucleus and known presence of PI4P, ATM in other organelles of a cell, further experiments such as cell fractionation experimental that show golgi specific interaction would support the main conclusion.

2). In continuation of 1st comment, since PI4P in substrate of PI4 phosphoinositol kinases, is there a competition between PI4kinases and ATM for PI4P binding should be addressed through immunoprecipitation studies.

3). The authors claim that the ATM retention is the main function of PI4P in Golgi. The authors should rule out the possibility that the phenotype observed on DNA damage response is not due to non availability of PI4P substrate for PI4P kinases, that have recently been shown to participate in genome integrity maintenance.

4). Does Oleate treatment influences Rad53 protein levels in addition to its phosphorylation that affect DNA damage response may be addressed.

5). Does Yeh2 deletion reduces LDS should be checked.

6). Figure 4D representation should show % of phospho reduction of initial activation and a bettier western blot image should be shown that show equal loading of samples.

7). In ammunoprecipitation experiments, kindly include isotypee IgG controls as well to rule out non-specificity.

Minor points:

1). Figure S1F do not show oleate treatment as presented in results section.

2). A better gel for S4B should be presented with ponceau of the same gel.

3). Nuclear PI4Ps has also been previously reported, an explanation to the specific interaction of ATM and PI4P in the Golgi should be addressed/discussed.

2. Significance:

Significance (Required)

The current work definitely adds a layer in our understanding to ATM regulation and cross-talk between different PIKK family of kinases. ATM localisation in extra nuclear regions of a cell has been described earlier with significant impact on cell physiology such as mitochondria etc., ATM retention at golgi and limiting nuclear ATM levels is significant advance at ATM activity regulation, while signifying non canonical function of PI4P.

3. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 3 and 6 months

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Reviewer Publons

Yes

Review #3

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

Summary:

In this manuscript, the authors propose that ATM/Tel1 signaling is regulated in a spatiotemporal manner during genotoxic stress both in yeast and mammalian cells. They show that Lipid droplets accumulate in response to genotoxic stress. As a consequence, there is a decrease of exchange of PI4P from the Golgi to ER, thus dampening ATM/Tel1 signaling by sequestering this kinase into the Golgi. The authors combined findings in yeast and mammals showing that this mechanism is conserved throughout eukaryotes. For this purpose, they use a vast number of techniques that support their proposed model.

Major comments:

The conclusions were made based on evidence combining yeast genetics, immunofluorescence, DNA end resection analysis and pharmacological interventions. The hypothesis that ATM is kept away from the nucleus by physically interacting with PI4P at the Golgi, thus allowing processive repair is bold and contributes for a better understanding of the choreography of the DDR kinases during DSB repair. However, many of the experiments in yeast and mammals show only mild phenotypes and there is no evidence that this mode of ATM dampening impact cell viability in mammals. Therefore, I have some comments and suggestions of experiments that I think could improve the quality of the manuscript. I believe that most of these new experiments does not require much time and resources.

- Does oleate treatment in RPE-1/Huh-7 cells induce loss of viability? An experiment showing loss of viability like MT-assay or decreased cell proliferation would reinforce the importance of the mechanism proposed.
- In yeast there is evidence that a ste delta strain show sensitivity to zeocin/CPT, but

there is no experiment showing the same effect on cells lacking Yeh2. Since both strains share similar phenotypes, it would be interesting to show that increased kinetics of Rad53 signaling leads to sensitivity to genotoxins.
- The conclusion that ste delta cells exposed to zeocin leads to unproductive events due to defects in DNA-end resection could be reinforced by a decrease in Rad52 foci. It has been previously shown by the group of Dr. Marcus Smolka, that inhibition of DNA-end resection decreases Rad52 foci (https://doi.org/10.1083/jcb.201607031). Since the authors were able to monitor Rad52-YFP (Figure S1A), it shouldn't consume time and resources.
- Since the authors propose that there is a DNA repair defect due to inhibition of long-range DNA-end resection, it would be important to monitor gamma-H2A(X) signal either in yeast or mammals.
- How do the authors exclude the possibility that yeast mutants or oleate treatment in yeast/mammalian cells change membrane permeability allowing an increase in genotoxin concentration?
- It would be interesting to investigate genetic interactions between ste delta (or yeh2delta) and yeast mutants with DNA-end resection problems (exo1delta; sae2delta). For instance, it has been shown that Sae2 antagonizes checkpoint signaling by competing with Rad9 to DSB sites (https://doi.org/10.1073/pnas.1816539115). Also, cells lacking Sae2 show an increase in Rad53 signaling due to increased Tel1 Signaling. Therefore, an epistatic effect between these two pathways would reinforce the hypothesis of the manuscript.
- The authors showed that Tel1-GFP does not accumulate in the nucleus in cells lacking Sac1 (Figure 7C). Tel1 is important to cope with increased DSBs in the absence of Mec1, thus avoiding genomic instability. Cells lacking both Mec1 and Tel1 show a sick phenotype with an exponential increase in gross chromosomal rearrangements and sensitivity to genotoxins. Therefore, does deletion of Mec1 (and Sml1) in sac1 delta phenocopies a mec1tel1 delta? Alternatively, does pharmacological inhibition of ATR in the presence of the OSBP1 inhibitor causes loss of viability or chromosomal aberrations?
- Finally, it seems strange to me that ATR/Mec1 signaling is not mentioned throughout the entire manuscript. Does PI4P pathway affect only ATM/Tel1? In Figure 2D, an antibody against phospho-CHK1 could be used to monitor ATR signaling. In line with that, I would like to see in the discussion how these new findings are in line with evidence from a 2019 paper showing that phophoinositides PIP2 and PIP3, but not PI4P are important for ATR signaling (DOI: 10.1038/s41467-017-01805-9). They showed that a nuclear pool of PIP2 increases upon DNA damage induction and rapidly accumulates at DNA lesions. This event is important for the recruitment of ATR. Since PI4P is substrate for PIP2 synthesis and there is a nuclear pool of PI4P and PIP2, I think it is important to discuss if the results presented here are in line with these previous findings.

Minor comments:

- Line 124: The correct is Figure S1E, lower panel and not Figure S1F
- Lines 127-128: Figure S2A does not show zeocin treatment

2. Significance:

Significance (Required)

Together, these new findings, if corroborated by others, might be important to open new lines of investigation in basic and translational research regarding human diseases as explored in the discussion section. I believe this paper will attract attention not only from the DDR field but also from other areas of research such as nutrient and lipid signaling both in yeast and mammals. I hope I was able to collaborate in this review, since my main expertise is in the area of DNA damage signaling using budding yeast as an organism model.

3. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 1 and 3 months

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Reviewer Publons

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Review #4

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

This is a very interesting study where Sara et al. demonstrated a link between lipid metabolism with DNA repair response (DDR). In this study, they have proposed ATM as a novel PI4P-effector. The sterol deposition into lipid droplets impacts the Golgi PI4P level due to lipid exchange machinery facilitated by OSBP1, therefore regulating the cytosolic retention of ATM due to PI4P binding. Although how lipid droplets in the cytosol sense the DNA damage and control the initiation of DDR by regulating ATM is still unclear, this study linked lipid biology/PI signaling to DNA damage repair and showed the evolutionary conservation of PI signaling and DNA repair machinery from yeast to humans. The experiments are well designed, nicely controlled, with a high quality of data presentation. With some improvements, this work could be a very interesting study attracting a broad readership.

In their model, ATM is PI4P-bound and sequestered inside the cytosol under basal conditions. Upon genotoxic stress, activation of OSBP1 removes PI4P and free PI4P-bound ATM for nuclear translocation of DNA repair. This could also be interpreted as genotoxic stress-induced PIP-kinase activity, where PI4P is processed into PIP2 or PIP3, somehow redirecting ATM into the nucleus to initiate its activation for DDR. Those aspects should be discussed and improved.

Upon stress, there is nuclear activation of p53-phosphoinositide (PI) signalosomes and PIP-kinases. Also, there is a significant PIP2 pool inside the nucleus with an involvement in DNA damage repair. Those papers and their relevance to the current study need to be discussed. If ATM is a novel PI4P-effector, there is also nuclear PI4P formation or nuclear PI4P accumulation upon stresses based on recent studies; how the ATM interacts with PIPn in the nucleus upon translocation? A know ATM substrate p53 is PIP2/PIP3 bound in the nucleus based on recent studies. Will ATM prefer to interact with other PIPn-bound proteins in the nucleus or PIPn regulate their interaction needs to be discussed.

Major points:

1. The PI4P-ATM complex is supported only by PLA and PIP strips. Need more robust biochemical characterization of the interaction: co-IP, lipid binding, and/or in vitro constitution.

2. The use of drug inhibitors only in the final figure is problematic. KD or KO experiments should be performed to confirm that ATM and the exchanger are the relevant targets.
3. Poor quality of some WBs (e.g Fig. S1F).
4. Lack of statistical analyses for some data (e.g. Fig. 1B-E)

Additional clarification points:

- Figure 1: No representative images were shown for quantifications in Figure 1C, D, E.

- Line 121: Should be Figure S1E, upper panel.

- Line 124: Should be Figure S1E, lower panel.

- Figure 2D-E, please show the quantification of the ratio of pCHK2/CHK2 with an N=3

- Figure S2B: needs quantification of NileRed staining to conclude induction in LD formation.

- Figure 3C, to show the selectivity of ATM-binding toward PI4P, PLA of ATM with other PIPn species should be assessed, such as PI3P, PI4,5P2, and PI3,4,5P3.

- Figure S6A, PI4P level could be assessed by IF staining using PI4P antibody besides using PI4P sensor.

2. Significance:

Significance (Required)

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(Decision Recommendation)

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Reviewer Publons

No

Revision Plan

Manuscript number: RC-2022-01580
Corresponding author(s): María MORIEL-CARRETERO

[The "revision plan" should delineate the revisions that authors intend to carry out in response to the points raised by the referees. It also provides the authors with the opportunity to explain their view of the paper and of the referee reports.

The document is important for the editors of affiliate journals when they make a first decision on the transferred manuscript. It will also be useful to readers of the reprint and help them to obtain a balanced view of the paper.

If you wish to submit a full revision, please use our "Full Revision" template. ***It is important to use the appropriate template to clearly inform the editors of your intentions.****]*

1. General Statements [optional]

This section is optional. Insert here any general statements you wish to make about the goal of the study or about the reviews.

We are very grateful about the thorough reading and deep understanding of the work that these 4 reviewers have provided. Although it is evident that they still request an improved profiling of some aspects, it is very encouraging that all four think the work is very interesting, original, insightful and adds a new layer of knowledge to the regulation of DNA damage sensing and repair. It is also very rewarding that the four reviewers estimate that this work will sew connections between different fields and interest a broad readership.

This is why we have designed here a very deep revision, tailored to satisfy all the raised concerns except one, and this just for technical reasons. Please note that, in spite of the multiple improvements suggested by the reviewers, the final amount of task is, while important, completely feasible. This is due to three reasons:

1. Several concerns are raised by most reviewers, making of each of them a single one.
2. Many of the concerns request **experiments that we have now already performed**, highlighted below in pink.
3. We are four authors already prepared to accomplish, each, her/his corresponding part of this revision.

Overall, if the editor has a look to the experiments that are yet to be done, shown in the text below as underlined, the view that emerges is that of a completely feasible (and yet much comprehensive) revision. Thank you for your positive consideration.

2. Description of the planned revisions

Insert here a point-by-point reply that explains what revisions, additional experimentations and analyses are planned to address the points raised by the referees.

Revision Plan

Our rebuttal plan is composed of three types of elements, all three included in this same section:
1. Some reviewers' concerns can be addressed by discussion, use of existing bibliography or logical argumentation.
2. When new experiments are needed but still need to be done, we have underlined the planned experiments that we will do.
3. When new experiments are requested by the reviewers but we have already done them, we will include them in pink font.
Please find below the original reviewers' comments and our answers to them preceded by the symbol ">":

Reviewer #1 (Evidence, reproducibility and clarity (Required)):
Ovejero et al. report an increase in lipid droplet (LD) abundance after long (from 120' on) exposure of budding yeast cells to DNA damaging agents zeocin and camptothecin (CPT). Next, they analyze DNA damage signaling in yeast mutants that impair triacylglycerol (TAGs) or sterol (STEs) esterification. They observe a slight anticipation in Rad53/CHK2 phosphorylation (indicative of DDR signaling) in yeast stem mutants, as well as in yeast cells or human cells lines pre-treated with oleate upon zeocin treatment. Yeast stem mutants are sensitive to zeocin and captothecin, but only confer sensitivity to hydroxyurea upon combination with tagD mutations. Authors relate these phenotypes to a somewhat decreases DSB resection in yeh2D mutants (expected to have reduced steryl esters pools) and RPA-foci in steD yeast cells. Next, a reduction in single strand annealing recombination repair events upon zeocin treatment is reported using a genetic reporter in steD mutants and oleate-treated cells. From these data they conclude that inability to process sterols in response to DSBs leads to an exacerbated DDR and prevents DNA repair. Next, it is shown that Flag-tagged Tel1 distinctly interacts with mono-phosphate phosphoinositides, including PI(4)P. An interaction in vivo is also inferred through Proximity Ligation Assays (PLA) using anti-PI(4)P and anti-ATM antibodies in human cell lines, which was moderately downregulated upon treatment with MMS or zeocin. Over-expression of the Osh4/OSBP1 transporter, which consumes PI(4)P, increased the number of Tel1 (nuclear) foci upon zeocin treatment. Conversely Sac1 ablation, in which accumulation of PI(4)P is expected, abrogated nuclear Tel1 foci formation and reduced telomere length (a phenotype related to lack of Tel1 function). From these results authors conclude that Tel1 availability in the nucleus is influenced by PI(4)P availability. Lastly, treatment with an OSBP1 inhibitor led to a cell line and damaging agent -variable reduction of ATM phosphorylation and a mostly non-significant reduction of DNA resection, measured by native BrdU detection, in response to CPT treatment.
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Major comments:
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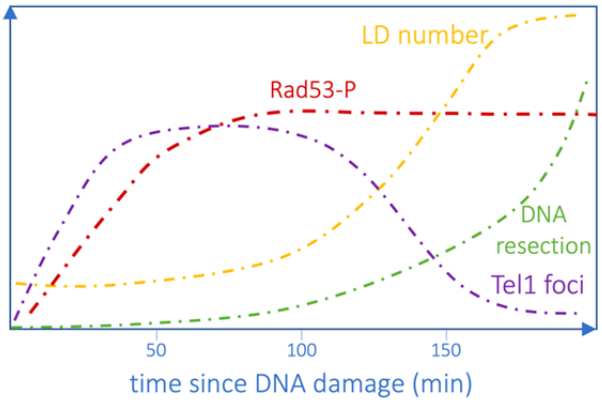
this lipid to "control" Tel1/ATM function is not demonstrated. The notion that sterol processing occurs in response to DSBs is not sufficiently supported by the data presented, as the increase in LD numbers is observed much after activation of the DDR (Rad53 phosphorylation) in Zeozin-treated yeast cells.

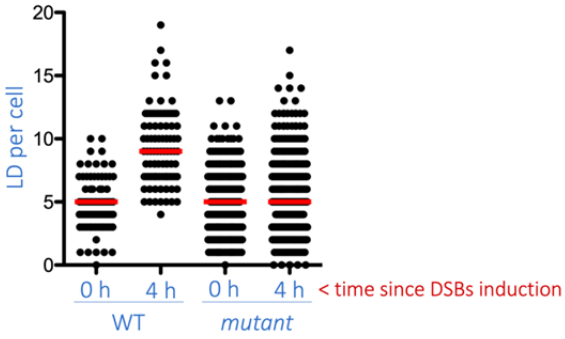
> We are afraid that we have not been clear enough in explaining the kinetics giving rise to our model. As indicated by the reviewer, our work shows, through kinetic studies, that the storage of sterols within LD (yellow line) occurs at later stages than the activation of the DDR by Tel1 (purple line) and Rad53 phosphorylation (red line). Tel1 foci decline is necessary for subsequent engagement of downstream DNA long-range resection (green line). Since we propose that sterol storage within LD is a means to attenuate Tel1 engagement at DSBs, it is thus logical (and thus compatible with the data we show) that LD number increase occurs simultaneously with Tel1 foci decrease, at late stages of the reaction:



We will include this explanation and graph in the revised version of the work.

In addition, evidence is not provided on the mechanisms by which PI(4)P metabolism would be controlled, which would be expected to be DDR-independent as they are placed upstream of this signaling pathway in the author's model.

> The key mechanism through which, in the end, PI(4)P metabolism will be controlled, is the esterification of sterols within LD. Given that, as clarified above, LD formation in response to DSBs occurs “late” (i.e., after 120 min), it is not excluded that the DDR itself can instruct, through phosphorylation of some effector(s), LD formation. In other words, by ordering LD formation, the DDR would be launching a self-limiting mechanism. In support, we now know, although we do not show in this work, that eliminating key DDR proteins prevents the formation of LD in response to DNA damage. Because of this, we have undertaken an educated-guess approach and chosen critical or rate-limiting enzymes in LD biology either possessing an S/T-Q cluster domain (predicted to be a phosphorylation substrate for the DNA Damage Response kinases (1), and/or retrieved in phospho-proteomic screens as specific DDR targets (2,3). This adds up to 28 proteins in *S. cerevisiae* and 45 proteins in *Homo sapiens*. Importantly, the emergent candidates fall into two identical categories in both organisms. To provide initial support for their pertinence, we have generated a point mutant in the putative S/T-Q cluster of one of the yeast candidates. Of high relevance, we find that the concerned mutant is impaired in correctly triggering LD formation in response to DNA damage:



We have now obtained a specific funding to pursue this characterization that, as such, constitutes a different work from the one presented in this manuscript. We hope that the reviewer is now convinced yet that she/he agrees in keeping this information for subsequent manuscript(s).

The damaging agents used have been suggested to alter the redox metabolism and even lipid peroxidation (Kitanovic 2009, Mizumoto 1993, Krol 2015, Todorova 2015, Ren 2019, Singh 2014). Hence it is possible that PI(4)P changes are not due to DSBs, but an indirect though relevant effect. In absence of direct evidence supporting an active regulation of PI(4)P dynamics in response to DNA breaks, this conclusion remains speculative and this should be noted in the manuscript.

> We fully agree with the reviewer that the used genotoxins are triggering a myriad of effects which could elicit the same phenomenon by indirect means. Yet, we want to stress that the use of camptothecin, which elicits a very robust LD formation phenotype (Figure 1C), is very likely specific, as it is proven as a potent and direct trapper of Top1 onto DNA after having cleaved it. Nevertheless, we propose in the next paragraph two specific experiments to dismiss this problem, please see immediately below.

Authors conclude that LD is specific to DSB induction. This seems an overstatement as they just reported LD increases in response to two agents that also induce other kinds of DNA damage. To also strengthen the link between DSBs and PI(4)P modulation of Tel1 function, authors should analyze LD numbers, Rad53 phosphorylation and Tel1 nuclear re-localization in response to HO-induced DNA breaks (e.g., using the system employed in Figure 3C).

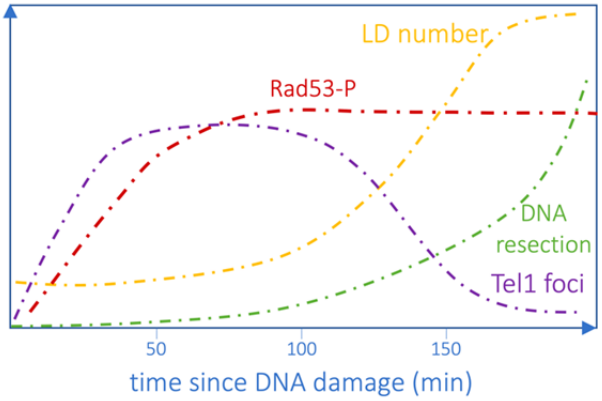
> We humbly think that enzymatically-induced DNA breaks will both activate Rad53 phosphorylation and Tel1 nuclear concentration, as this has already been established, thus requiring no further exploration. Yet, it is very important to assess the reviewer's suggestion concerning whether enzymatically-induced DNA breaks also trigger the formation of LD. To this end, we will perform two complementary studies in which, instead of using HO, which cuts only a few times in the genome, we will:

a) exploit the naturally DSB-accumulating mutant *rad3-102*, which we previously characterized in the past (4), and which we already exploit in this work for recombination analyses (Figure S4A), to evaluate whether it endogenously harbors more LD in comparison with the WT.

b) we have recently created a tool in which gRNAs targeted to different subsets of transposons in the genome can drive Cas9 to create DSB in a dose-dependent manner ((9), under revision in ***Genetics***). We will use this system to monitor the LD formation in response to Cas9-triggered cuts.

In addition, on figure 5A, significant differences in GFP-Tel1 foci abundance between WT and steD or yeh2D cells are only observed after 210', way after the slight effect on Rad53 phosphorylation is observed. This is at odds with the conclusion that Tel1 association to STEs modulates DDR signaling.

As evoked already above, our kinetic studies show that the storage of sterols within LD (yellow line) occurs at later stages than the activation of the DDR by Tel1 (purple line) and Rad53 phosphorylation (red line). Tel1 foci decline is necessary for subsequent engagement of downstream DNA long-range resection (green line). Since we propose that sterol storage within LD is a means to attenuate Tel1 engagement at DSBs, there is no inconsistency if LD number increase occurs simultaneously with Tel1 foci number decrease at late stages of the reaction:



Minor comments:

Figure S1D and E, experiments should be carried out to include time points in which LD accumulation and cell cycle arrest are observed upon zeocin treatment (i.e., up to 210' as in Figure 1A)

> We will provide cytometry profiles of cells at 210 min. These data exist already in our laboratory.

How do authors explain increased single strand annealing recombination frequencies in steD and oleate-treated wild type cells (Figure 4A). Should it not be expected that increased STEs also impair recombination induced by endogenous damage?

> Only *steΔ* (and not +oleate) indeed manifests an increase in basal recombination frequencies, likely arising from endogenous damage. Although the increase is observed, it is not significant. We agree anyway with the reviewer that, was the experiment to be repeated more times, the increase may be found significantly different. We do not have any honest proposal to explain this.

Revision Plan

Data presented in figure 4B and 4C are not fully convincing. Performing time course experiments might help concluding if the differences observed represent a relevant defect in DSB processing.

> We will perform a Pulsed Field Gel Electrophoresis (PFGE) kinetcis in response to zeocin with or without oleate pre-loading to reinforce the conclusion.

Is Figure 5B referring to Flag-tagged Tel1 or GFP-tagged Tel1 as stated in the figure legend?

> There is a misunderstanding here, as the mentioned Figure 5B corresponds to P-ATM immunofluorescences in human cells, not to any tagged Tel1 experiment.

Treatment with the ATM inhibitor AZD0156 increased PI(4)P-ATM PLA signals. From these authors conclude that "association of ATM and PI(4)P inversely correlated with the need for ATM within the nucleus. Do they imply that treatment with ATM-inhibitors reduces the requirement for ATM function in the nucleus? The interpretation of this result should be further elaborated to sustain this conclusion.

> We may have conveyed a wrong notion at this point. We do not imply at all that ATM inhibitors reduce the need for ATM in the nucleus. Instead, we imply that, by reinforcing ATM attachment to Golgi-resident PI(4)P, ATM inhibitors end up titrating ATM away from the nucleus. We will clarify our explanation to avoid misunderstandings.

An increase of GFP-Tel1 foci upon OSH4 overexpression is described on Figure 7B. These are described as nuclear in the results, but no reference is made in the figure or legend as to how nucleus positions are addressed in these experiments. This should be clarified.

> We systematically combine the tagging of a nucleoplasmic protein (mCherry-Pus1) with the detection of GFP-Tel1 foci, as to unambiguously assess the nuclear position of Tel1 foci. We will include this explanation and the corresponding mCherry-Pus1 channel to clarify this.

Also, WT controls and quantifications should be included in the experiments shown on Figure 7C.

> These experiments are quantified from the moment we did them, although we did not include such quantifications in the present version for the sake of space. We will do so in the revised version.

Reviewer #1 (Significance (Required)):

While the conclusion of lipid metabolism responding to DSBs is not convincing, the observation that Tel1/ATM function is modulated by PI(4)P biding is significant and advances the understanding on the function and regulation of this key kinase in promoting genome integrity maintenance. This is an unanticipated result which is highly novel and has implications for the modulation of Tel1/ATM function through pharmacological manipulation of lipid metabolism. This finding would be of broad interest for scientists working on the response to DNA damage and the maintenance of genome integrity. This reviewer belongs to that group and has limited expertise to evaluate the lipid metabolism genetic manipulation in the manuscript.

Revision Plan

Reviewer #2 (Evidence, reproducibility and clarity (Required)):

The authors show that cytoplasmic PI4P have a regulatory role on ATM response to DNA double strand breaks. The process involves a balance between exchange of PI4P between Golgi and ER in exchange of esterified sterols. The study is of interest, however provides indirect evidences to support their conclusions.

Major comments :
1). Since the major conclusion relates to PI4P association with ATM in basal conditions to keep ATM outside nucleus and known presence of PI4P, ATM in other organelles of a cell, further experiments such as cell fractionation experimental that show golgi specific interaction would support the main conclusion.

In continuation of 1st comment, since PI4P in substrate of PI4 phosphoinositol kinases, is there a competition between PI4kinases and ATM for PI4P binding should be addressed through immunoprecipitation studies.

> First of all, we need to specify here that PI4kinases will phosphorylated PI4 to create PI(4)P. Thus, PI(4)P is the product, and not the substrate, of PI4kinases. We therefore do not expect any competition between such kinases and ATM.

> Second, we take good note of the reviewer's concern that the pool of PI(4)P at the Golgi may be shared, and also that it would be important to reinforce the notion of the relative subcellular localization of ATM under different treatments. To this end, we will perform the following integrative experiment:

Immunoprecipitation of PI(4)P could theoretically be done using our specific antibody, yet the IP efficiency of a lipid cannot be verified by western blot. Further, there are PI(4)P pools elsewhere in the cell that would mess up with interpretations. We therefore dismiss the use of anti-PI(4)P as a tool to perform immunoprecipitations.

Instead, to explore the impact of PI(4)P levels on ATM both at the Golgi and within the nucleus, we will split our cultures in two to either immunoprecipitate specific cytoplasmic Trans-Golgi Network-associated proteins (we will test separately TGN46 and GOLPH3); or the nuclear ATM-interacting factor MRE11 from nuclei, then blot for co-immunoprecipitated ATM. The relative co-immunoprecipitated ATM is expected to vary under different treatments to which the cells will be exposed, namely:

- untreated
- zeocin, to trigger ATM need in the nucleus
- OSBP inhibition (+/- zeocin), to stabilize PI(4)P at the Golgi
- PIK93, an inhibitor of PI4 kinases that prevents PI(4)P synthesis

Revision Plan

2). The authors claim that the ATM retention is the main function of PI4P in Golgi. The authors should rule out the possibility that the phenotype observed on DNA damage response is not due to non availability of PI4P substrate for PI4P kinases, that have recently been shown to participate in genome integrity maintenance.

> We want to explain that we do not intend to say that PI(4)P main function at the Golgi is ATM retention, as PI(4)P is a molecule binding and modulating multiple proteins, as for example the aforementioned GOLPH3. We will first revise our text to correct it, in case we have conveyed this incorrect notion, as it stems from the reviewer's comment.

> Second, the reviewer evokes the notion that PI(4)P can be the substrate of a second phosphorylation, which could give rise to PI(3,4)P or to PI(4,5)P, which could still undergo remodeling into PI(3)P, for example. Recent work by Dr Michael Sheetz's lab demonstrated that this set of phosphoinositides serves to drive the nucleation and activation of the ATR-Chk1 branch of the DNA Damage Response upon genotoxic stress, yet was completely inert with respect to the ATM-Chk2 branch (5). To rule out the possibility, as evoked by the reviewer, that the oleate-induced DDR phenomena we describe relate to these other events, we have now explored the response of the ATR-Chk1 branch when comparing the response of zeocin-treated cells that have been pre-loaded or not with oleate. We observe that the ATR-Chk1 branch is unaltered by oleate loading. Thus, we can now propose that the PI(4)P branch exclusively modulates the ATM-Chk2 axis.

3). Does Oleate treatment influences Rad53 protein levels in addition to its phosphorylation that affect DNA damage response may be addressed.

> Exponential cultures from three different WT, three different *steΔ* and three different *yeh2Δ* strains have now been taken and pre-loaded for 2 hours with 0.05% oleate, then total levels of Rad53 (without induction of DNA damage) assessed. We can now formally say that basal levels of Rad53 protein are not altered by this incubation. We will include this control in the revised manuscript.

4). Does Yeh2 deletion reduces LDS should be checked.

> We frequently use *yeh2Δ* cells in our studies. In particular, we have recently published work characterizing the phenotype of this strain with respect to the formation of lipid droplets in the nucleus (6). We are currently exploiting those same sets of data to quantify the total number of LD in order to satisfy the reviewer's concern.

5). Figure 4D representation should show % of phospho reduction of initial activation and a better western blot image should be shown that show equal loading of samples.

> We are currently repeating these gels and blots for the sake of clarity, as requested.

6). In immunoprecipitation experiments, kindly include isotypee IgG controls as well to rule out non-specificity.

> Of course, this important control will be included every time.

Revision Plan

Minor points:

1). Figure S1F do not show oleate treatment as presented in results section.

> We will revise the accurate naming.

2). A better gel for S4B should be presented with ponceau of the same gel.

> We are currently repeating this gel and associated blot for the sake of clarity, as requested.

3). Nuclear PI4Ps has also been previously reported, an explanation to the specific interaction of ATM and PI4P in the Golgi should be addressed/discussed.

> We take it that the reviewer is referring here to the recent work by Fáberová et al (7) in which PI(4)P and PI(4,5)P were described as very dynamic in the nucleus, and mostly related then to mRNA transcription, splicing and export. We will reinforce the connection of our phenomenon to the Golgi-associated pool of PI(4)P thanks to the co-immunoprecipitation experiments proposed above, and will timely contextualize these in light of the paper by Fáberová and co-workers in the revised version. Thank you for reminding us of this work.

Reviewer #2 (Significance (Required)):

The current work definitely adds a layer in our understanding to ATM regulation and cross-talk between different PIKK family of kinases. ATM localisation in extra nuclear regions of a cell has been described earlier with significant impact on cell physiology such as mitochondria etc., ATM retention at golgi and limiting nuclear ATM levels is significant advance at ATM activity regulation, while signifying non canonical function of PI4P.

Revision Plan

Reviewer #3 (Evidence, reproducibility and clarity (Required)):

Summary:

In this manuscript, the authors propose that ATM/Tel1 signaling is regulated in a spatiotemporal manner during genotoxic stress both in yeast and mammalian cells. They show that Lipid droplets accumulate in response to genotoxic stress. As a consequence, there is a decrease of exchange of PI4P from the Golgi to ER, thus dampening ATM/Tel1 signaling by sequestering this kinase into the Golgi. The authors combined findings in yeast and mammals showing that this mechanism is conserved throughout eukaryotes. For this purpose, they use a vast number of techniques that support their proposed model.

Major comments:

The conclusions were made based on evidence combining yeast genetics, immunofluorescence, DNA end resection analysis and pharmacological interventions. The hypothesis that ATM is kept away from the nucleus by physically interacting with PI4P at the Golgi, thus allowing processive repair is bold and contributes for a better understanding of the choreography of the DDR kinases during DSB repair. However, many of the experiments in yeast and mammals show only mild phenotypes and there is no evidence that this mode of ATM dampening impact cell viability in mammals.

> We agree with the reviewer that the effects associated to the reported phenomenon are indeed mild. This is a fact. We would like to remind that the metabolism of sterols is finely controlled, and at many different levels, in a very complex manner. For example, sterol increases in the cell will immediately be compensated by reduced synthesis, while synthesis inhibition will immediately promote uptake from the medium, and/or release from stores (for example, see (8)). As a natural consequence, the window of manipulation and, more importantly, the strength of the phenotypes we can uncover are small.

Therefore, I have some comments and suggestions of experiments that I think could improve the quality of the manuscript. I believe that most of these new experiments does not require much time and resources.

- Does oleate treatment in RPE-1/Huh-7 cells induce loss of viability? An experiment showing loss of viability like MT-assay or decreased cell proliferation would reinforce the importance of the mechanism proposed.

> This experiment **was already included in the previous version**, yet it may have escaped the attention of the reviewer. We show in Figure S2E that oleate treatment restricts viability in Huh-7 cells alone, and also worsens their tolerance to zeocin. Perhaps we should reconsider moving this result to the main figures so that it does not go unnoticed.

- In yeast there is evidence that a ste delta strain show sensitivity to zeocin/CPT, but there is no experiment showing the same effect on cells lacking Yeh2. Since both strains share similar phenotypes,

it would be interesting to show that increased kinetics of Rad53 signaling leads to sensitivity to genotoxins.

> We have now performed this experiment, we will include the matching information for *yeh2Δ* cells, which agrees with the predictions.

- The conclusion that ste delta cells exposed to zeocin leads to unproductive events due to defects in DNA-end resection could be reinforced by a decrease in Rad52 foci. It has been previously shown by the group of Dr. Marcus Smolka, that inhibition of DNA-end resection decreases Rad52 foci (https://doi.org/10.1083/jcb.201607031). Since the authors were able to monitor Rad52-YFP (Figure S1A), it shouldn't consume time and resources.

> The reviewer is right that this experiment should not be time- or resources-consuming. We will evaluate the accumulation of Rad52 foci in response to the concerned genotoxin in *steΔ* cells.

- Since the authors propose that there is a DNA repair defect due to inhibition of long-range DNA-end resection, it would be important to monitor gamma-H2A(X) signal either in yeast or mammals.

> Taking into consideration the reviewer's suggestion, we have now performed anti-γH2AX immunofluorescence of all the implied conditions (genotoxins +/- oleate pre-load) and will quantify them to answer the concern.

- How do the authors exclude the possibility that yeast mutants or oleate treatment in yeast/mammalian cells change membrane permeability allowing an increase in genotoxin concentration?

> Although this is a very reasonable criticism, we want to remind the data we present in Figure S4A in which we use the naturally DSB-bearing *rad3-102* cells for recombination analyses, showing that, in the absence of any genotoxin, the same phenotype also applies. Yet, we want to reinforce the notion that LD formation in response to DSB can also occur when the breaks are not chemically, but physically, induced. To this end, and also to match a related request by Reviewer 1, we will:

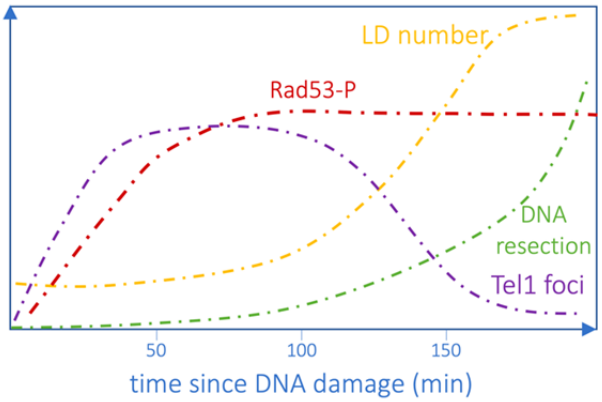
a) exploit the naturally DSB-accumulating mutant *rad3-102* (4) to evaluate whether it endogenously harbors more LD in comparison with the WT.

b) we have recently created a tool in which gRNAs targeted to different subsets of transposons in the genome can drive Cas9 to create DSB in a dose-dependent manner ((9), under revision in ***Genetics***). We will use this system to monitor the LD formation in response to Cas9-triggered cuts.

In addition, on figure 5A, significant differences in GFP-Tel1 foci abundance between WT and steD or yeh2D cells are only observed after 210', way after the slight effect on Rad53 phosphorylation is observed. This is at odds with the conclusion that Tel1 association to STEs modulates DDR signaling.

> We are afraid that we have not been clear enough in explaining the kinetics giving rise to our model. As indicated by the reviewer, our work shows, through kinetic studies, that the storage of sterols within LD (yellow line) occurs at later stages than the activation of the DDR by Tel1 (purple line) and Rad53 phosphorylation (red line). Tel1 foci decline is necessary for subsequent engagement of downstream DNA long-range resection (green line). Since we propose that sterol storage within LD is a

means to attenuate Tel1 engagement at DSBs, it is thus logical (and thus compatible with the data we show) that LD number increase occurs simultaneously with Tel1 foci decrease, at late stages of the reaction:



We will include this explanation and graph in the revised version of the work.

- It would be interesting to investigate genetic interactions between ste delta (or yeh2delta) and yeast mutants with DNA-end resection problems (exo1delta; sae2delta). For instance, it has been shown that Sae2 antagonizes checkpoint signaling by competing with Rad9 to DSB sites (https://doi.org/10.1073/pnas.1816539115). Also, cells lacking Sae2 show an increase in Rad53 signaling due to increased Tel1 Signaling. Therefore, an epistatic effect between these two pathways would reinforce the hypothesis of the manuscript.

> we will build the double mutant *sae2Δ yeh2Δ* and assess the potential epistatic behavior they may display with respect to some key phenotypes (Tel1 foci formation, Rad53 phosphorylation…).

- The authors showed that Tel1-GFP does not accumulate in the nucleus in cells lacking Sac1 (Figure 7C). Tel1 is important to cope with increased DSBs in the absence of Mec1, thus avoiding genomic instability. Cells lacking both Mec1 and Tel1 show a sick phenotype with an exponential increase in gross chromosomal rearrangements and sensitivity to genotoxins. Therefore, does deletion of Mec1 (and Sml1) in sac1 delta phenocopies a mec1tel1 delta? Alternatively, does pharmacological inhibition of ATR in the presence of the OSBP1 inhibitor causes loss of viability or chromosomal aberrations?

> We will delete *SAC1* in *mec1Δ sml1Δ* and compare the fitness, through growth drop assays, with respect to the mutant *tel1Δ mec1Δ sml1Δ*.

> We will expose cells either to OSBP1 inhibitor, ATR inhibitor, or both, and assess the phosphorylation of their downstream common effector H2AX. Additionally, we will assess the effect on cell growth of the combination of ATRi and OSBP1i using synergy matrices. We will determine if the combination of both drugs synergizes or not to impair cell proliferation and reduce cell viability.

- Finally, it seems strange to me that ATR/Mec1 signaling is not mentioned throughout the entire manuscript. Does PI4P pathway affect only ATM/Tel1? In Figure 2D, an antibody against phospho-CHK1 could be used to monitor ATR signaling. In line with that, I would like to see in the discussion how these new findings are in line with evidence from a 2019 paper showing that phophoinositides PIP2 and PIP3,

but not PI4P are important for ATR signaling (DOI: 10.1038/s41467-017-01805-9). They showed that a nuclear pool of PIP2 increases upon DNA damage induction and rapidly accumulates at DNA lesions. This event is important for the recruitment of ATR. Since PI4P is substrate for PIP2 synthesis and there is a nuclear pool of PI4P and PIP2, I think it is important to discuss if the results presented here are in line with these previous findings.

> The reviewer evokes recent work by Dr Michael Sheetz's lab demonstrating that a different set of phosphoinositides serves to drive the nucleation and activation of the ATR-Chk1 branch of the DNA Damage Response upon genotoxic stress, yet was completely inert with respect to the ATM-Chk2 branch (5). We have now explored, also to satisfy a similar concerned raised by Reviewer 2, the response of the ATR-Chk1 branch when comparing the response of zeocin-treated cells that have been pre-loaded or not with oleate. We observe that the ATR-Chk1 branch is unaltered by oleate loading. Thus, we can now propose that the PI(4)P branch exclusively modulates the ATM-Chk2 axis.

> We will of course give the needed credit to this work and contextualize our findings accordingly.

Minor comments:

- Line 124: The correct is Figure S1E, lower panel and not Figure S1F
-Lines 127-128: Figure S2A does not show zeocin treatment

> Both minor mistakes will be corrected.

Reviewer #3 (Significance (Required)):

Together, these new findings, if corroborated by others, might be important to open new lines of investigation in basic and translational research regarding human diseases as explored in the discussion section. I believe this paper will attract attention not only from the DDR field but also from other areas of research such as nutrient and lipid signaling both in yeast and mammals. I hope I was able to collaborate in this review, since my main expertise is in the area of DNA damage signaling using budding yeast as an organism model.

Reviewer #4 (Evidence, reproducibility and clarity (Required)):

This is a very interesting study where Sara et al. demonstrated a link between lipid metabolism with DNA repair response (DDR). In this study, they have proposed ATM as a novel PI4P-effector. The sterol deposition into lipid droplets impacts the Golgi PI4P level due to lipid exchange machinery facilitated by OSBP1, therefore regulating the cytosolic retention of ATM due to PI4P binding. Although how lipid droplets in the cytosol sense the DNA damage and control the initiation of DDR by regulating ATM is still unclear, this study linked lipid biology/PI signaling to DNA damage repair and showed the evolutionary conservation of PI signaling and DNA repair machinery from yeast to humans. The experiments are well designed, nicely controlled, with a high quality of data presentation. With some improvements, this work could be a very interesting study attracting a broad readership.

In their model, ATM is PI4P-bound and sequestered inside the cytosol under basal conditions. Upon genotoxic stress, activation of OSBP1 removes PI4P and free PI4P-bound ATM for nuclear translocation of DNA repair. This could also be interpreted as genotoxic stress-induced PIP-kinase activity, where PI4P is processed into PIP2 or PIP3, somehow redirecting ATM into the nucleus to initiate its activation for DDR. Those aspects should be discussed and improved.

> Both Reviewers 2 and 3 have somehow evoked a similar concern. More precisely, the work by Dr Michael Sheetz's lab demonstrating that a different set of phosphoinositides serves to drive the nucleation and activation of the ATR-Chk1 branch of the DNA Damage Response upon genotoxic stress, yet was completely inert with respect to the ATM-Chk2 branch (5). We have now explored, to satisfy all reviewers' concerns, the response of the ATR-Chk1 branch when comparing the response of zeocin-treated cells that have been pre-loaded or not with oleate. We observe that the ATR-Chk1 branch is unaltered by oleate loading. Thus, we can now propose that the PI(4)P branch exclusively modulates the ATM-Chk2 axis.

> Additionally, we will of course give the needed credit to this work and contextualize our findings accordingly.

Upon stress, there is nuclear activation of p53-phosphoinositide (PI) signalosomes and PIP-kinases. Also, there is a significant PIP2 pool inside the nucleus with an involvement in DNA damage repair. Those papers and their relevance to the current study need to be discussed. If ATM is a novel PI4P-effector, there is also nuclear PI4P formation or nuclear PI4P accumulation upon stresses based on recent studies; how the ATM interacts with PIPn in the nucleus upon translocation? A know ATM substrate p53 is PIP2/PIP3 bound in the nucleus based on recent studies. Will ATM prefer to interact with other PIPn-bound proteins in the nucleus or PIPn regulate their interaction needs to be discussed.

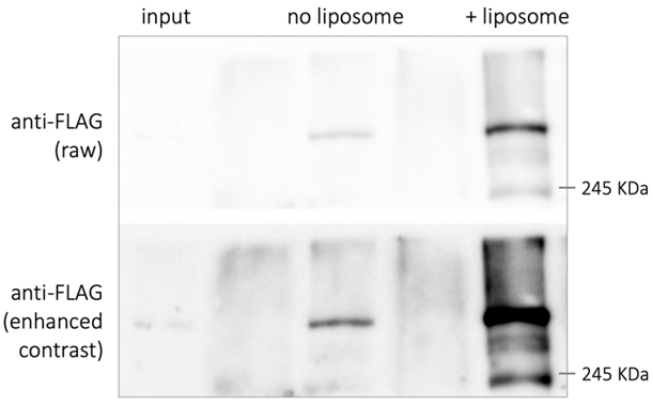
> These additional notions are in line with the previous paragraph presented by the reviewer, and our answers too. We will provide a constructive overview of all these ideas in the revised version of the manuscript.

Major points:

1. The PI4P-ATM complex is supported only by PLA and PIP strips. Need more robust biochemical characterization of the interaction: co-IP, lipid binding, and/or in vitro constitution.

> We agree with the need to perform assays in which PI(4)P is embedded in a bilayer, as to confidently assess whether Tel1 can bind it in that context. We have now performed a pilot experiment in which we have confronted purified FLAG-Tel1 to liposomes harboring PI(4)P. Western blot analysis using anti-FLAG antibody shows the encouraging result that FLAG-Tel1 can be found there. As a control, we have performed the same process but in the absence of any liposomes. We observe that a residual fraction of FLAG-Tel1 can nevertheless be found in this control, most probably because the buffer used during the liposome assay makes part of FLAG-Tel1 precipitate.

Revision Plan



To avoid this type of background and to increase our trust in the results, we propose to perform the liposome assay but on a discontinuous density gradient, so that liposomes will be retrieved in the top layer (and bound FLAG-Tel1 with them, if that is the case), while any precipitated FLAG-Tel1 will be in the bottom fraction (liposome floatation assay). As a further control, we will include the same liposomes but lacking PI(4)P. We expect to be successful in the floatation assays. If we are not, we will repeat the experiment presented above to be confident that the observed increase is reproducible.

2. The use of drug inhibitors only in the final figure is problematic. KD or KO experiments should be performed to confirm that ATM and the exchanger are the relevant targets.

> We have now used siRNAs against the exchanger protein, OSBP1, with a very high silencing rate success. We have next monitored the activation status of the chromatin-associated ATM target KAP1, in order to monitor the predicted decrease of ATM activity specifically inside the nucleus. Our results confirm the role of OSBP1, by KD experiments as requested by the reviewer, in attenuating ATM nuclear participation.

3. Poor quality of some WBs (e.g Fig. S1F).

> We have now repeated the Western Blot to detect Rad53-P in response to 20 mM HU in WT *versus steΔ* cells.

4. Lack of statistical analyses for some data (e.g. Fig. 1B-E)

> **We had already included, in the previous version, the complete statistical analyses corresponding to Figures 1B to E and evoked here by the reviewer**. They were indeed included in Figure S1C, and our brief reference to them in the text may have escaped her/his attention. We will make a clear reference to this in the revised version.

Additional clarification points:

Figure 1: No representative images were shown for quantifications in Figure 1C, D, E.

> If the reviewer / editor estimates it pertinent, we can of course include them. Yet, they will be very redundant with the images displayed in Figure 1A.

Revision Plan

Line 121: Should be Figure S1E, upper panel.
Line 124: Should be Figure S1E, lower panel.
Figure 2D-E, please show the quantification of the ratio of pCHK2/CHK2 with an N=3

> We will correct / include the requested changes.

Figure S2B: needs quantification of NileRed staining to conclude induction in LD formation

> We will quantify the LD as requested.

Figure S6A, PI4P level could be assessed by IF staining using PI4P antibody besides using PI4P sensor.

> We will use our PI(4)P antibody to monitor by immunofluorescence the behavior of this molecule in response to either MMS or zeocin, as suggested.

References

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3. Description of the revisions that have already been incorporated in the transferred manuscript

Please insert a point-by-point reply describing the revisions that were already carried out and included in the transferred manuscript. If no revisions have been carried out yet, please leave this section empty.

Revision Plan

4. Description of analyses that authors prefer not to carry out

Please include a point-by-point response explaining why some of the requested data or additional analyses might not be necessary or cannot be provided within the scope of a revision. This can be due to time or resource limitations or in case of disagreement about the necessity of such additional data given the scope of the study. Please leave empty if not applicable.

Reviewer #4:

Figure 3C, to show the selectivity of ATM-binding toward PI4P, PLA of ATM with other PIPn species should be assessed, such as PI3P, PI4,5P2, and PI3,4,5P3.

> We have provided an overview of the binding preferences of ATM with respect to the full battery of phosphoinositides in the strip-binding assay shown in Figures S5C and 6B. Other than that, we are afraid **that PLA studies as the ones we develop in the current manuscript for PI(4)P are not feasible, since no reliable antibodies exist for most of the phosphoinositide species evoked** by the reviewer.

Dr. María Moriel-Carretero
Centre de Recherche en Biologie cellulaire de Montpellier (CRBM), Université de Montpellier-Centre National de la Recherche Scientifique
1919 Route de Mende
Montpellier 34293
France

6th Oct 2022

Re: EMBOJ-2022-112684
A Sterol-PI(4)P exchanger controls the Tel1/ATM axis of the DNA Damage Response

Dear Dr. Moriel-Carretero,

Thank you for transferring your manuscript from Review Commons to The EMBO Journal. I have now had the chance to carefully read your study, as well as to go through the four referee reports and your responses to them. I realize that the findings of the study, if substantiated, are potentially interesting, and that the referees in principle share this notion, albeit with a number of caveats. Nevertheless, I appreciate that some of the key issues (including the one about the temporal order of events) can be well answered, and that you are proposing reasonable experiments to address many others. In this light, we would be interested in pursuing a revised version of the study further for EMBO Journal publication, pending positive re-review by (some of) the original referees. In this respect, I need to stress however that all relevant data should be shown, at least in response to the referees (we do allow referee-only figures to be taken out of the published "transparent peer review" transcript if they are meant to be part of a future publication) - this concerns particularly the responses to referee 1, "we now know that eliminating key DDR proteins prevents formation of LD..." and the unnamed yeast candidate whose S/TQ cluster has been mutated.

When preparing a revised manuscript, please try to adhere to the guidelines listed below and in our Guide to Authors as closely as possible, as this should greatly facilitate our assessment at the time of resubmission - in particular regarding the completion of our author checklist, the inclusion of editable text files and individual figures, the formatting references and the conversion of "supplemental" material into Expanded View and/or Appendix content. Please also note that it is our policy to allow only a single round of (major) revision, making it important to comprehensively answer all criticisms at this point - if this should require more time than our standard three-months deadline, I would be happy to discuss an extension of the revision time, during which our 'scooping protection' (meaning that competing work appearing elsewhere in the meantime will not affect our considerations of your study) would of course remain valid. Please do not hesitate to contact me should you have any further questions at this stage.

Thank you again for the opportunity to consider this study for The EMBO Journal. I look forward to receiving your revision.

Yours sincerely,

Hartmut Vodermaier

Hartmut Vodermaier, PhD
Senior Editor, The EMBO Journal
h.vodermaier@embojournal.org

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1) Every manuscript requires a Data Availability section (even if only stating that no deposited datasets are included). Primary datasets or computer code produced in the current study have to be deposited in appropriate public repositories prior to resubmission, and reviewer access details provided in case that public access is not yet allowed. Further information: embopress.org/page/journal/14602075/authorguide#dataavailability

2) Each figure legend must specify
- size of the scale bars that are mandatory for all micrograph panels
- the statistical test used to generate error bars and P-values

- the type error bars (e.g., S.E.M., S.D.)
- the number (n) and nature (biological or technical replicate) of independent experiments underlying each data point
- Figures may not include error bars for experiments with n<3; scatter plots showing individual data points should be used instead.

3) Revised manuscript text (including main tables, and figure legends for main and EV figures) has to be submitted as editable text file (e.g., .docx format). We encourage highlighting of changes (e.g., via text color) for the referees' reference.

4) Each main and each Expanded View (EV) figure should be uploaded as individual production-quality files (preferably in .eps, .tif, .jpg formats). For suggestions on figure preparation/layout, please refer to our Figure Preparation Guidelines: http://bit.ly/EMBOPressFigurePreparationGuideline

5) Point-by-point response letters should include the original referee comments in full together with your detailed responses to them (and to specific editor requests if applicable), and also be uploaded as editable (e.g., .docx) text files.

6) Please complete our Author Checklist, and make sure that information entered into the checklist is also reflected in the manuscript; the checklist will be available to readers as part of the Review Process File. A download link is found at the top of our Guide to Authors: embopress.org/page/journal/14602075/authorguide

7) All authors listed as (co-)corresponding need to deposit, in their respective author profiles in our submission system, a unique ORCiD identifier linked to their name. Please see our Guide to Authors for detailed instructions.

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9) Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be clearly noted in the figure legend and/or the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure. Finally, we generally encourage uploading of numerical as well as gel/blot image source data; for details see: embopress.org/page/journal/14602075/authorguide#sourcedata

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We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (4th Jan 2023). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions. Use the link below to submit your revision:

Link Not Available

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Rev_Com_number: RC-2022-01580
New_manu_number: EMBOJ-2022-112684
Corr_author: Moriel-Carretero
Title: A Sterol-PI(4)P exchanger controls the Tel1/ATM axis of the DNA Damage Response

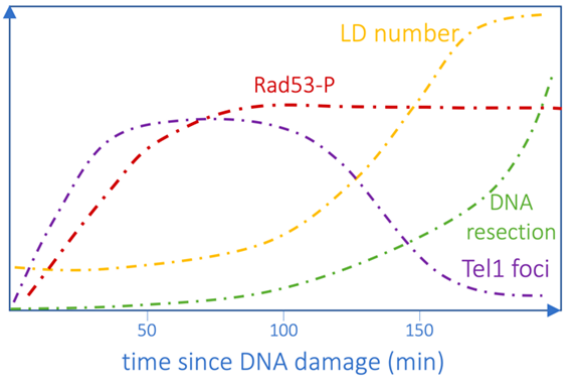
Please find our responses to the reviewers' comments preceded by the symbol ">", and in blue font.

Reviewer #1 (Evidence, reproducibility and clarity (Required)):
Ovejero et al. report an increase in lipid droplet (LD) abundance after long (from 120' on) exposure of budding yeast cells to DNA damaging agents zeocin and camptothecin (CPT). Next, they analyze DNA damage signaling in yeast mutants that impair triacylglycerol (TAGs) or sterol (STEs) esterification. They observe a slight anticipation in Rad53/CHK2 phosphorylation (indicative of DDR signaling) in yeast stem mutants, as well as in yeast cells or human cells lines pre-treated with oleate upon zeocin treatment. Yeast stem mutants are sensitive to zeocin and captothecin, but only confer sensitivity to hydroxyurea upon combination with tagD mutations. Authors relate these phenotypes to a somewhat decreases DSB resection in yeh2D mutants (expected to have reduced steryl esters pools) and RPA-foci in steD yeast cells. Next, a reduction in single strand annealing recombination repair events upon zeocin treatment is reported using a genetic reporter in steD mutants and oleate-treated cells. From these data they conclude that inability to process sterols in response to DSBs leads to an exacerbated DDR and prevents DNA repair. Next, it is shown that Flag-tagged Tel1 distinctly interacts with mono-phosphate phosphoinositides, including PI(4)P. An interaction in vivo is also inferred through Proximity Ligation Assays (PLA) using anti-PI(4)P and anti-ATM antibodies in human cell lines, which was moderately downregulated upon treatment with MMS or zeocin. Over-expression of the Osh4/OSBP1 transporter, which consumes PI(4)P, increased the number of Tel1 (nuclear) foci upon zeocin treatment. Conversely Sac1 ablation, in which accumulation of PI(4)P is expected, abrogated nuclear Tel1 foci formation and reduced telomere length (a phenotype related to lack of Tel1 function). From these results authors conclude that Tel1 availability in the nucleus is influenced by PI(4)P availability. Lastly, treatment with an OSBP1 inhibitor led to a cell line and damaging agent -variable reduction of ATM phosphorylation and a mostly non-significant reduction of DNA resection, measured by native BrdU detection, in response to CPT treatment.
Overall, authors conclude that i) biding of Tel1/ATM to PI(4)P modulates its functional availability in the nucleus, and that ii) DNA damage elicits the esterification and storage of sterols toward LDs, which contributes to tritate Tel1/ATM away from the nucleus dampening the DDR and affecting long-range resection.

Major comments:

While the conclusion that Tel1/ATM binds PI(4)P and this interaction modulates Tel1/ATM functional availability at the nucleus is convincing, the conclusion that DSBs elicit a change in the metabolism of this lipid to "control" Tel1/ATM function is not demonstrated. The notion that sterol processing occurs in response to DSBs is not sufficiently supported by the data presented, as the increase in LD numbers is observed much after activation of the DDR (Rad53 phosphorylation) in Zeozin-treated yeast cells.

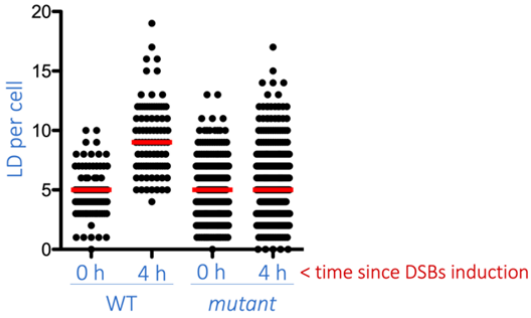
> We are afraid that we have not been clear enough in explaining the kinetics giving rise to our model. As indicated by the reviewer, our work shows, through kinetic studies, that the storage of sterols within LD (yellow line) occurs at later stages than the activation of the DDR by Tel1 (purple line) and Rad53 phosphorylation (red line). Tel1 foci decline is necessary for subsequent engagement of downstream DNA long-range resection (green line). Since we propose that sterol storage within LD is a means to attenuate Tel1 engagement at DSBs, it is thus logical (and thus compatible with the data we show) that LD number increase occurs simultaneously with Tel1 foci decrease, at late stages of the reaction:



We have now included this explanation early in the discussion and the graph in New Figure 10A.

In addition, evidence is not provided on the mechanisms by which PI(4)P metabolism would be controlled, which would be expected to be DDR-independent as they are placed upstream of this signaling pathway in the author's model.

> The key mechanism through which, in the end, PI(4)P metabolism will be controlled, is the esterification of sterols within LD. Given that, as clarified above, LD formation in response to DSBs occurs "late" (i.e., after 120 min), it is not excluded that the DDR itself can instruct, through phosphorylation of some effector(s), LD formation. In other words, by ordering LD formation, the DDR would be launching a self-limiting mechanism. In support, we now know, although we do not show in this work, that eliminating key DDR proteins prevents the formation of LD in response to DNA damage. Because of this, we have undertaken an educated-guess approach and chosen critical or rate-limiting enzymes in LD biology either possessing an S/T-Q cluster domain (predicted to be a phosphorylation substrate for the DNA Damage Response kinases (1), and/or retrieved in phospho-proteomic screens as specific DDR targets (2,3). This adds up to 28 proteins in *S. cerevisiae* and 45 proteins in *Homo sapiens*. Importantly, the emergent candidates fall into two identical categories in both organisms. To provide initial support for their pertinence, we have generated a point mutant in the putative S/T-Q cluster of one of the yeast candidates. Of high relevance, and only for the reviewer's perusal, we report that the mutant indicated in the joint figure, in which we introduce a serine-to-alanine substitution, is impaired in correctly triggering LD formation in response to DNA damage:



We have now obtained a specific funding to pursue this characterization that, as such, constitutes a different work from the one presented in this manuscript. We hope that the reviewer is now convinced yet that she/he agrees in us keeping this information for subsequent manuscript(s).

The damaging agents used have been suggested to alter the redox metabolism and even lipid peroxidation (Kitanovic 2009, Mizumoto 1993, Krol 2015, Todorova 2015, Ren 2019, Singh 2014). Hence it is possible that PI(4)P changes are not due to DSBs, but an indirect though relevant effect. In absence of direct evidence supporting an active regulation of PI(4)P dynamics in response to DNA breaks, this conclusion remains speculative and this should be noted in the manuscript.

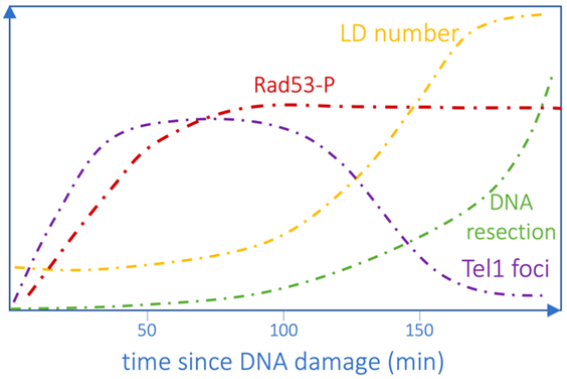
> We fully agree with the reviewer that the used genotoxins are triggering a myriad of effects which could elicit the same phenomenon by indirect means. Yet, we want to stress that the use of camptothecin, which elicits a very robust LD formation phenotype (Fig 1C), is very likely specific, as it is proven as a potent and direct trapper of Top1 onto DNA after having cleaved it. Nevertheless, we have performed two specific experiments to dismiss this criticism (please see below).

Authors conclude that LD is specific to DSB induction. This seems an overstatement as they just reported LD increases in response to two agents that also induce other kinds of DNA damage. To also strengthen the link between DSBs and PI(4)P modulation of Tel1 function, authors should analyze LD numbers, Rad53 phosphorylation and Tel1 nuclear re-localization in response to HO-induced DNA breaks (e.g., using the system employed in Figure 3C).

> We humbly think that enzymatically-induced DNA breaks will both activate Rad53 phosphorylation and Tel1 nuclear concentration, as this has already been established, thus requiring no further exploration. Yet, it is very important to assess the reviewer's suggestion concerning whether enzymatically-induced DNA breaks also trigger the formation of LD. To this end, and instead of using HO, which cuts only a few times in the genome, we have used a tool we recently created in which gRNAs targeted to different subsets of transposons in the genome can drive Cas9 to create DSB in a dose-dependent manner (Coiffard 2021 bioRxiv, under revision in ***Genetics***). We have used this system to monitor the LD formation in response to Cas9-triggered cuts. In particular, we have grown our cells overnight in a medium devoid of glucose, then induced Cas9 expression by adding galactose in presence of a gRNA instructing either 59 cuts per genome, or none. We have performed this induction from cultures grown in glycerol, or raffinose, to allow for different basal number of LD. We recurrently observe a neat, time-dependent increase of LD per cell ONLY when the cuts are provoked. These data can be now found in New Figure 1G-K.

In addition, on figure 5A, significant differences in GFP-Tel1 foci abundance between WT and steD or yeh2D cells are only observed after 210', way after the slight effect on Rad53 phosphorylation is observed. This is at odds with the conclusion that Tel1 association to STEs modulates DDR signaling.

As evoked already above, our kinetic studies show that the storage of sterols within LD (yellow line) occurs at later stages than the activation of the DDR by Tel1 (purple line) and Rad53 phosphorylation (red line). Tel1 foci decline is necessary for subsequent engagement of downstream DNA long-range resection (green line). Since we propose that sterol storage within LD is a means to attenuate Tel1 engagement at DSBs, there is no inconsistency if LD number increase occurs simultaneously with Tel1 foci number decrease at late stages of the reaction:



Minor comments:

Figure S1D and E, experiments should be carried out to include time points in which LD accumulation and cell cycle arrest are observed upon zeocin treatment (i.e., up to 210' as in Figure 1A)

> These cytometries have now been included in New Appendix Fig S1D, with unchanged conclusions.

How do authors explain increased single strand annealing recombination frequencies in steD and oleate-treated wild type cells (Figure 4A). Should it not be expected that increased STEs also impair recombination induced by endogenous damage?

> Only *steΔ* (and not +oleate) indeed manifests an increase in basal recombination frequencies, likely arising from endogenous damage. Although the increase is observed, it is not significant. We agree anyway with the reviewer that, was the experiment to be repeated more times, the increase could be found as significantly different. Perhaps basal genetic instability accumulates in the *steΔ* mutant given the reasons we present in this work. Yet, since this responds to pure speculation, we have not included this comment in the revised manuscript.

Data presented in figure 4B and 4C are not fully convincing. Performing time course experiments might help concluding if the differences observed represent a relevant defect in DSB processing.

> We have encountered technical difficulties to perform PFGE experiments since the machine to which we have access now (from Bio-RAD) is not behaving in accordance with the parameters used in our old Rotaphor. To provide an alternative experiment allowing us to perform kinetic studies in response to zeocin with or without oleate pre-loading, as to reinforce the conclusion and as requested by the reviewer, we have now performed the same analysis but at the single-cell level using comet assays. We have now preloaded the cells with oleate then induced breaks with zeocin for 1 hour, then we withdrew zeocin to allow repair. We show now in New Fig 4E,F that the kinetics of repair is slowed down if cells are preloaded with oleate, and have been able to estimate differential repair kinetic slopes between mock and oleate-loaded cells.

Is Figure 5B referring to Flag-tagged Tel1 or GFP-tagged Tel1 as stated in the figure legend?

> There is a misunderstanding here, as the mentioned Old Figure 5B corresponded to P-ATM immunofluorescences in human cells, not to any tagged Tel1 experiment.

Treatment with the ATM inhibitor AZD0156 increased PI(4)P-ATM PLA signals. From these authors conclude that "association of ATM and PI(4)P inversely correlated with the need for ATM within the nucleus. Do they imply that treatment with ATM-inhibitors reduces the requirement for ATM function in the nucleus? The interpretation of this result should be further elaborated to sustain this conclusion.

> We may have conveyed a wrong notion at this point. We do not imply at all that ATM inhibitors reduce the need for ATM in the nucleus. Instead, we imply that, by reinforcing ATM attachment to Golgi-resident PI(4)P, ATM inhibitors end up titrating ATM away from the nucleus. This notion has been pertinently developed now in the discussion, lines 451-453.

An increase of GFP-Tel1 foci upon OSH4 overexpression is described on Figure 7B. These are described as nuclear in the results, but no reference is made in the figure or legend as to how nucleus positions are addressed in these experiments. This should be clarified.

> We systematically combine the tagging of a nucleoplasmic protein (mCherry-Pus1) with the detection of GFP-Tel1 foci, as to unambiguously assess the nuclear position of Tel1 foci, as presented the time this tool was used (New Fig 6A). We have now specified this information also in the Figure Legend of the concerned Osh4 figure, namely New Fig 8D.

Also, WT controls and quantifications should be included in the experiments shown on Figure 7C.

> In New Expanded View Fig 4C, we have now included the quantifications related to the percentage of cells in the population displaying GFP-Tel1-positive structures IN THE CYTOPLASM at each timepoint in WT *versus sac1Δ* cells. This representation not only permits to see that cytoplasmic GFP-Tel1-positive structures fail to dissolve upon zeocin treatment, when Tel1 normally goes to the

nucleus, as is the case in the WT, but also that these cell demonstrate basally very high levels of GFP-Tel1 out of the nucleus, as expected for high Golgi PI(4)P pools. Thank you for suggesting plotting these data.

Additionally, please let us know explain that these data very well match another addition we have done to the work, in which we have prepared protein fractions from cytoplasm and nuclei from human RPE-1 cells and found that an ATM pool exists in the cytoplasm basally that decreases in response to zeocin, when ATM is "requested" in the nucleus. These data can be seen in New Fig 8B.

Reviewer #1 (Significance (Required)):

While the conclusion of lipid metabolism responding to DSBs is not convincing, the observation that Tel1/ATM function is modulated by PI(4)P biding is significant and advances the understanding on the function and regulation of this key kinase in promoting genome integrity maintenance. This is an unanticipated result which is highly novel and has implications for the modulation of Tel1/ATM function through pharmacological manipulation of lipid metabolism. This finding would be of broad interest for scientists working on the response to DNA damage and the maintenance of genome integrity. This reviewer belongs to that group and has limited expertise to evaluate the lipid metabolism genetic manipulation in the manuscript.

Reviewer #2 (Evidence, reproducibility and clarity (Required)):

The authors show that cytoplasmic PI4P have a regulatory role on ATM response to DNA double strand breaks. The process involves a balance between exchange of PI4P between Golgi and ER in exchange of esterified sterols. The study is of interest, however provides indirect evidences to support their conclusions.

Major comments :

1) Since the major conclusion relates to PI4P association with ATM in basal conditions to keep ATM outside nucleus and known presence of PI4P, ATM in other organelles of a cell, further experiments such as cell fractionation experimental that show golgi specific interaction would support the main conclusion. In continuation of 1st comment, since PI4P in substrate of PI4 phosphoinositol kinases, is there a competition between PI4kinases and ATM for PI4P binding should be addressed through immunoprecipitation studies.

> First of all, and in response to this latter point, we need to specify here that PI4kinases will phosphorylate PI4 to create PI(4)P. Thus, PI(4)P is the product, and not the substrate, of PI4kinases. We therefore do not expect any competition between such kinases and ATM.

> Second, to reinforce the notion of a differential subcellular localization of ATM under different treatments, we have now, using cells treated or not with zeocin, fractionated the extracts and assessed the relative presence of ATM in the cytoplasmic *versus* the nuclear fractions. Fractionation quality controls have been systematically included. Two important notions derive from these experiments: *a)* there is indeed a pool of cytoplasmic ATM; and *b)* this pool is dynamic, with ATM entering the nucleus in response to zeocin. These data are now shown in New Fig 8B. In the same line, we have now quantified that deletion of Sac1 phosphatase, which is expected to stabilize the pool of Golgi PI(4)P, leads to the formation of Tel1-positive cytoplasmic structures that are irresponsive to zeocin treatment (New Fig EV4C).

> Reciprocally, we have now also used the anti-PI(4)P antibody to detect PI(4)P main localization by immunofluorescence, and show its robust co-localization with the Golgi apparatus. Importantly, when cells are treated with MMS or zeocin, this PI(4)P pool is dismantled, suggestive of its hydrolysis. These data are shown in New Fig 8C.

2). The authors claim that the ATM retention is the main function of PI4P in Golgi. The authors should rule out the possibility that the phenotype observed on DNA damage response is not due to non availability of PI4P substrate for PI4P kinases, that have recently been shown to participate in genome integrity maintenance.

> We want to explain that we did not mean to say that PI(4)P main function at the Golgi is ATM retention, as PI(4)P is a molecule binding and modulating multiple proteins. We have specified this notion in the discussion, lines 454-456, to prevent conveying any wrong notion.

> Second, the reviewer evokes the notion that PI(4)P can be the substrate of a second phosphorylation, which could give rise to PI(3,4)P or to PI(4,5)P, which could still undergo remodeling into PI(3)P, for example. Recent work by Dr Michael Sheetz's lab demonstrated that this set of phosphoinositides serves to drive the nucleation and activation of the ATR-Chk1 branch of the DNA Damage Response upon genotoxic stress, yet was completely inert with respect to the ATM-Chk2 branch (5). To rule out the possibility, as evoked by the reviewer, that the oleate-induced DDR phenomena we describe relate to these other events, we have now explored the response of the ATR-Chk1 branch when comparing the response of zeocin-treated cells that have been pre-loaded or not with oleate. We observe that the ATR-Chk1 branch is not further stimulated by oleate pre-loading and, if anything, it would be slightly dampened, as shown now in New Expanded View Fig 1B. Thus, we can now propose that the PI(4)P branch exclusively activates the ATM-Chk2 axis. We discuss this timely in a new section of the Discussion, lines 471-488.

3). Does Oleate treatment influences Rad53 protein levels in addition to its phosphorylation that affect DNA damage response may be addressed.

> Exponential cultures from three different WT, three different *steΔ* and three different *yeh2Δ* strains have now been taken and pre-loaded for 2 hours with 0.05% oleate, then total levels of Rad53 (without induction of DNA damage) assessed. We can now formally say that basal levels of Rad53 protein are not altered by this incubation. One representative experiment of this battery of controls is now included in New Appendix Fig S2A.

4). Does Yeh2 deletion reduces LDs should be checked.

> We have now quantified the number of LD per cell in the *yeh2Δ* strain. In more detail, we have monitored the same culture at a given time 0, then 2 and 4 hours later. This experiment has been done three independent times. In all instances, the mean number of LD per cell oscillates between one and two, thus, less than a standard WT grown in the same conditions. This count agrees with the notion that steryl esters are mobilized from LD stores to release free sterols with which to replenish the plasma membrane, given the absence of Yeh2 to provide sterols from the extracellular medium (explained in lines 163-166). We have now included this information in New Appendix Fig S2E and cited it in lines 170-171.

5). Figure 4D representation should show % of phospho reduction of initial activation and a better western blot image should be shown that show equal loading of samples.

> We have repeated these migrations using more resolutive gels (new recipe detailed in M&M), presented in New Fig 5. We have kept the old gels, because the profiles are similar to those shown in the main Fig 2, but have added below the new runs, as requested by the reviewer. This includes new Ponceau staining to show improved loading quality. The plotted values in the graph have not been altered, as the input data have not been changed, yet we have included in it the values for *yeh2Δ*, previously shown in Old Fig S4B. Also, we have included plots of average pixel intensity corresponding to each phosphorylated Rad53 isoform in New Fig 5D to reinforce the message. While the conclusions are unaltered, the specific profile of resistant highly phosphorylated isoforms in *steΔ* and *yeh2Δ* cells is now more convincing. Thank you.

6). In immunoprecipitation experiments, kindly include isotype IgG controls as well to rule out non-specificity.

> As justified above, the IP experiments were not conducted.

Minor points:

1). Figure S1F do not show oleate treatment as presented in results section.

> This mistake has been amended.

2). A better gel for S4B should be presented with ponceau of the same gel.

> As explained above (see point 5), this gel has been re-migrated along with the data previously presented in Old Figure 4D, therefore the Rad53-P de-activation kinetics in *yeh2Δ* cells is now presented in an integrated manner with those from *steΔ* and *tagΔ* strains. As a consequence, the quantifications for *yehΔ2* data are also included in New Fig 5B.

3). Nuclear PI4Ps has also been previously reported, an explanation to the specific interaction of ATM and PI4P in the Golgi should be addressed/discussed.

> We take it that the reviewer is referring here to the recent work by Fáberová et al (7) in which PI(4)P and PI(4,5)P were described as very dynamic in the nucleus, and mostly related then to mRNA transcription, splicing and export. We have now reinforced the connection of our phenomenon to the Golgi-associated pool of PI(4)P thanks to the new experiments presented in New Fig 8, and have now also timely contextualized these in light of the paper by Fáberová and co-workers, as widely

contextualized in a new section of the Discussion, lines 471-488. Thank you for reminding us of this work.

Reviewer #2 (Significance (Required)):

The current work definitely adds a layer in our understanding to ATM regulation and cross-talk between different PIKK family of kinases. ATM localisation in extra nuclear regions of a cell has been described earlier with significant impact on cell physiology such as mitochondria etc., ATM retention at golgi and limiting nuclear ATM levels is significant advance at ATM activity regulation, while signifying non canonical function of PI4P.

Reviewer #3 (Evidence, reproducibility and clarity (Required)):

Summary:

In this manuscript, the authors propose that ATM/Tel1 signaling is regulated in a spatiotemporal manner during genotoxic stress both in yeast and mammalian cells. They show that Lipid droplets accumulate in response to genotoxic stress. As a consequence, there is a decrease of exchange of PI4P from the Golgi to ER, thus dampening ATM/Tel1 signaling by sequestering this kinase into the Golgi. The authors combined findings in yeast and mammals showing that this mechanism is conserved throughout eukaryotes. For this purpose, they use a vast number of techniques that support their proposed model.

Major comments:

The conclusions were made based on evidence combining yeast genetics, immunofluorescence, DNA end resection analysis and pharmacological interventions. The hypothesis that ATM is kept away from the nucleus by physically interacting with PI4P at the Golgi, thus allowing processive repair is bold and contributes for a better understanding of the choreography of the DDR kinases during DSB repair. However, many of the experiments in yeast and mammals show only mild phenotypes and there is no evidence that this mode of ATM dampening impact cell viability in mammals.

> We agree with the reviewer that the effects associated to the reported phenomenon are indeed mild. This is a fact. We would like to remind that the metabolism of sterols is finely controlled, and at many different levels, in a very complex manner. For example, sterol increases in the cell will immediately be compensated by reduced synthesis, while synthesis inhibition will immediately promote uptake from the medium, and/or release from stores (for example, see (8)). As a natural consequence, the window of manipulation and, more importantly, the strength of the phenotypes we can uncover are small. We have now included this consideration in our Discussion, namely in the final paragraph.

Therefore, I have some comments and suggestions of experiments that I think could improve the quality of the manuscript. I believe that most of these new experiments does not require much time and resources.

- Does oleate treatment in RPE-1/Huh-7 cells induce loss of viability? An experiment showing loss of viability like MT-assay or decreased cell proliferation would reinforce the importance of the mechanism proposed.

> This experiment was already included in the previous version, yet it may have escaped the attention of the reviewer. We showed in Old Figure S2E (New Expanded View Fig1D) that, in Huh-7 cells, which manage to grow (modestly) despite the presence of zeocin, oleate treatment restricts their viability.

- In yeast there is evidence that a ste delta strain show sensitivity to zeocin/CPT, but there is no experiment showing the same effect on cells lacking Yeh2. Since both strains share similar phenotypes, it would be interesting to show that increased kinetics of Rad53 signaling leads to sensitivity to genotoxins.

> We have now performed this experiment, although in a more sensitive manner. We have exposed WT, *steΔ* and *yeh2Δ* to an acute dose of zeocin, then washed it away and seeded the cells in rich medium plates without any treatment. We have monitored whether this short (though acute) exposure to the drug hampered their growth. Results, now shown in New Fig 3B, reveal that both mutants are indeed more sensitive than the WT to this challenge, in agreement with the notion that an increase of free sterols in membranes limits their tolerance to zeocin.

- The conclusion that ste delta cells exposed to zeocin leads to unproductive events due to defects in DNA-end resection could be reinforced by a decrease in Rad52 foci. It has been previously shown by the group of Dr. Marcus Smolka, that inhibition of DNA-end resection decreases Rad52 foci https://doi.org/10.1083/jcb.201607031). Since the authors were able to monitor Rad52-YFP (Figure S1A), it shouldn't consume time and resources.

> We have now evaluated the accumulation dynamics of Rad52 foci in *steΔ* mutants, and observed, in full contrast to the prediction of the reviewer, an strong accumulation of these foci. Yet, we need to remind here that the defect we propose is one in long-range resection, and showed in Old Figure 3C (now New Fig 3D) that the early tract is correctly resected. Thus, we propose that this shortly resected tracks that lead to (as already shown) an increase in Rfa1 foci accumulation are able to engage Rad52 binding, as short homology search would be possible. Yet, since repair is not efficient and in the continuous presence of the drug the damages keep on accumulating, this leads with time to an accumulation of Rad52 foci, as downstream Rad51-mediated exchange is not expected to take place efficiently. These new data have now been added in New Expanded View Fig2E and presented in lines 191-193.

- Since the authors propose that there is a DNA repair defect due to inhibition of long-range DNA-end resection, it would be important to monitor gamma-H2A(X) signal either in yeast or mammals.

> Taking into consideration the reviewer's suggestion, we have now performed anti-γH2AX immunofluorescence of all the implied conditions (genotoxins +/- oleate pre-load) and, upon quantification, we observe inconsistent variations of total γH2AX signals in response to either zeocin or MMS whether in RPE-1 or Huh-7 cells if pre-loaded with oleate. Overall, no significant pattern variation can be claimed with respect to the same genotoxin treatment without oleate pre-load (New Fig 6E). We interpret this as a similar ATM spatial activation, in agreement with the notion that the phenomenon under study is more about an extension of ATM activation in time. This has been described in lines 286-289.

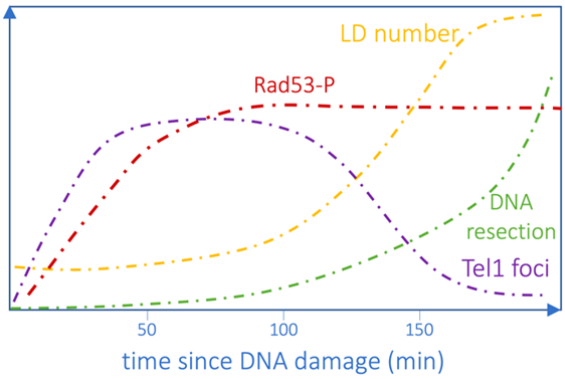
- How do the authors exclude the possibility that yeast mutants or oleate treatment in yeast/mammalian cells change membrane permeability allowing an increase in genotoxin concentration?

> Although this is a very reasonable criticism, we want to remind the data we present in Old Figure S4A, in which we use the naturally DSB-bearing *rad3-102* cells for recombination analyses, showing that, in the absence of any genotoxin, the same phenotype also applies. Yet, we want to reinforce the notion that LD formation in response to DSB can also occur when the breaks are not chemically, but physically, induced. To this end, and also to match a related request by Reviewer 1, we have recently created a tool in which gRNAs targeted to different subsets of transposons in the genome can drive Cas9 to create DSB in a dose-dependent manner (Coiffard 2021 bioRxiv, under revision in ***Genetics***). We have used this system to monitor the LD formation in response to Cas9-triggered cuts. In particular, we have grown our cells overnight in a medium devoid of glucose, then induced Cas9 expression by adding galactose in presence of a gRNA instructing either 59 cuts per genome, or none. We have performed this induction from cultures grown in glycerol, or raffinose, to allow for different basal number of LD. We recurrently observe a neat, time-dependent increase of LD per cell ONLY when the cuts are provoked. These data can be now found in New Figure 1G-K.

In addition, on figure 5A, significant differences in GFP-Tel1 foci abundance between WT and steD or yeh2D cells are only observed after 210', way after the slight effect on Rad53 phosphorylation is observed. This is at odds with the conclusion that Tel1 association to STEs modulates DDR signaling.

> We are afraid that we have not been clear enough in explaining the kinetics giving rise to our model. As indicated by the reviewer, our work shows, through kinetic studies, that the storage of sterols within LD (yellow line) occurs at later stages than the activation of the DDR by Tel1 (purple line) and Rad53 phosphorylation (red line). Tel1 foci decline is necessary for subsequent engagement of

downstream DNA long-range resection (green line). Since we propose that sterol storage within LD is a means to attenuate Tel1 engagement at DSBs, it is thus logical (and thus compatible with the data we show) that LD number increase occurs simultaneously with Tel1 foci decrease, at late stages of the reaction:



We have now included this explanation early in the discussion and the graph in New Figure 10A.

- It would be interesting to investigate genetic interactions between ste delta (or yeh2delta) and yeast mutants with DNA-end resection problems (exo1delta; sae2delta). For instance, it has been shown that Sae2 antagonizes checkpoint signaling by competing with Rad9 to DSB sites (https://doi.org/10.1073/pnas.1816539115). Also, cells lacking Sae2 show an increase in Rad53 signaling due to increased Tel1 Signaling. Therefore, an epistatic effect between these two pathways would reinforce the hypothesis of the manuscript.

> we have now built the double mutant *sae2Δ yeh2Δ* and assessed the behavior that this mutant manifests with respect to Tel1 foci formation. We show now in New Figure 6B that, in agreement with the reviewer's prediction, and with the idea that *yeh2Δ* cells display a defect in resection, the formation of GFP-Tel1 foci is *1)* increased in the *sae2Δ* mutant alone; *2)* the double mutant shows an trend to accumulate GFP-Tel1 foci in the nucleus in response to zeocin that is epistatic with any of the simple mutants.

- The authors showed that Tel1-GFP does not accumulate in the nucleus in cells lacking Sac1 (Figure 7C). Tel1 is important to cope with increased DSBs in the absence of Mec1, thus avoiding genomic instability. Cells lacking both Mec1 and Tel1 show a sick phenotype with an exponential increase in gross chromosomal rearrangements and sensitivity to genotoxins. Therefore, does deletion of Mec1 (and Sml1) in sac1 delta phenocopies a mec1tel1 delta? Alternatively, does pharmacological inhibition of ATR in the presence of the OSBP1 inhibitor causes loss of viability or chromosomal aberrations?

> We have evaluated the effect on cell growth of combining ATRi and OSBP1i using synergy matrices, which allow to estimate whether the combination of both drugs synergizes or not to impair RPE-1 cell proliferation and reduce their viability. Importantly, we show after three independent experiments that the simultaneous inhibition of ATR and OSBP1 led, as predicted by the reviewer, to a modest but recurrent synergic lethality. These data are now included in New Fig 9C.

- Finally, it seems strange to me that ATR/Mec1 signaling is not mentioned throughout the entire manuscript. Does PI4P pathway affect only ATM/Tel1? In Figure 2D, an antibody against phospho-CHK1 could be used to monitor ATR signaling. In line with that, I would like to see in the discussion how these new findings are in line with evidence from a 2019 paper showing that phophoinositides PIP2 and PIP3, but not PI4P are important for ATR signaling (DOI: 10.1038/s41467-017-01805-9). They showed that a nuclear pool of PIP2 increases upon DNA damage induction and rapidly accumulates at DNA lesions. This event is important for the recruitment of ATR. Since PI4P is substrate for PIP2 synthesis and there is a nuclear pool of PI4P and PIP2, I think it is important to discuss if the results presented here are in line with these previous findings.

> The reviewer evokes recent work by Dr Michael Sheetz's lab demonstrating that a different set of phosphoinositides serves to drive the nucleation and activation of the ATR-Chk1 branch of the DNA Damage Response upon genotoxic stress, yet was completely inert with respect to the ATM-Chk2 branch (5). We have now explored, also to satisfy a similar concerned raised by Reviewer 2, the response of the ATR-Chk1 branch when comparing the response of zeocin-treated cells that have been pre-loaded or not with oleate. We observe that the ATR-Chk1 branch is not exacerbated, contrary to the CHK2 axis, by oleate pre-loading. If anything, it is slightly downregulated. This information is now included in New Expanded View Fig 1B. Thus, we can now propose that the PI(4)P branch exclusively activates the ATM-Chk2 axis.

> We have now of course given the needed credit to this work and contextualized our findings accordingly in a new section of the Discussion, lines 471-488.

Minor comments:

- Line 124: The correct is Figure S1E, lower panel and not Figure S1F
- Lines 127-128: Figure S2A does not show zeocin treatment

> All these panels have now been reorganized.

Thank you very much for your knowledgeable help to improve our work!

Reviewer #3 (Significance (Required)):

Together, these new findings, if corroborated by others, might be important to open new lines of investigation in basic and translational research regarding human diseases as explored in the discussion section. I believe this paper will attract attention not only from the DDR field but also from other areas of research such as nutrient and lipid signaling both in yeast and mammals. I hope I was able to collaborate in this review, since my main expertise is in the area of DNA damage signaling using budding yeast as an organism model.

Reviewer #4 (Evidence, reproducibility and clarity (Required)):

This is a very interesting study where Sara et al. demonstrated a link between lipid metabolism with DNA repair response (DDR). In this study, they have proposed ATM as a novel PI4P-effector. The sterol deposition into lipid droplets impacts the Golgi PI4P level due to lipid exchange machinery facilitated by OSBP1, therefore regulating the cytosolic retention of ATM due to PI4P binding. Although how lipid droplets in the cytosol sense the DNA damage and control the initiation of DDR by regulating ATM is still unclear, this study linked lipid biology/PI signaling to DNA damage repair and showed the evolutionary conservation of PI signaling and DNA repair machinery from yeast to humans. The experiments are well designed, nicely controlled, with a high quality of data presentation. With some improvements, this work could be a very interesting study attracting a broad readership.

In their model, ATM is PI4P-bound and sequestered inside the cytosol under basal conditions. Upon genotoxic stress, activation of OSBP1 removes PI4P and free PI4P-bound ATM for nuclear translocation of DNA repair. This could also be interpreted as genotoxic stress-induced PIP-kinase activity, where PI4P is processed into PIP2 or PIP3, somehow redirecting ATM into the nucleus to initiate its activation for DDR. Those aspects should be discussed and improved.

> Both Reviewers 2 and 3 have somehow evoked a similar concern. More precisely, the work by Dr Michael Sheetz's lab demonstrating that a different set of phosphoinositides serves to drive the nucleation and activation of the ATR-Chk1 branch of the DNA Damage Response upon genotoxic stress, yet was completely inert with respect to the ATM-Chk2 branch (5). We have now explored, to satisfy all reviewers' concerns, the response of the ATR-Chk1 branch when comparing the response of zeocin-treated cells that have been pre-loaded or not with oleate. We observe that the activation of the ATR-Chk1 branch is not exacerbated by oleate pre-loading, and these new data can be now found in New Expanded View Fig 1B. Thus, we can now propose that the PI(4)P branch exclusively boosts the ATM-Chk2 axis.

Upon stress, there is nuclear activation of p53-phosphoinositide (PI) signalosomes and PIP-kinases. Also, there is a significant PIP2 pool inside the nucleus with an involvement in DNA damage repair. Those papers and their relevance to the current study need to be discussed. If ATM is a novel PI4P-effector, there is also nuclear PI4P formation or nuclear PI4P accumulation upon stresses based on recent studies; how the ATM interacts with PIPn in the nucleus upon translocation? A know ATM substrate p53 is PIP2/PIP3 bound in the nucleus based on recent studies. Will ATM prefer to interact with other PIPn-bound proteins in the nucleus or PIPn regulate their interaction needs to be discussed.

> These additional notions are in line with the previous paragraph presented by the reviewer, and our answers too. We have provided a constructive overview of all these ideas in a new section of the Discussion, lines 471-488. Thank you sincerely for bringing these works to our attention.

Major points:

1. The PI4P-ATM complex is supported only by PLA and PIP strips. Need more robust biochemical characterization of the interaction: co-IP, lipid binding, and/or in vitro constitution.

> We agree with the need to perform assays in which PI(4)P is embedded in a bilayer, as to confidently assess whether Tel1 can bind it in that context. We have now first performed experiments in which we have confronted purified FLAG-Tel1 to liposomes harboring PI(4)P. Western blot analysis using anti-FLAG antibody shows that FLAG-Tel1 can be found in the +liposome condition. Yet, the control in the absence of any liposomes showed a residual fraction of FLAG-Tel1, most probably because the buffer used during the liposome assay makes part of FLAG-Tel1 precipitate. This information has been included in Expanded View Fig 3D.

> Next, to avoid this type of background and to increase our trust in the results, we have performed the liposome assay but on a discontinuous density gradient, otherwise known as a floatation assay. In more detail, the purified FLAG-Tel1 and / or the liposomes (control or +PI(4)P) have

been deposited in the bottom of the ultracentrifugation tube and a discontinuous gradient has been poured over it (bottom 40% > 30% > 20% > 0% sucrose). After ultracentrifugation, liposomes float up to the above layers, while unbound material will stay in the bottom of the tube. This way, if FLAG-Tel1 is bound to them, it should abandon the bottom fraction. We present in New Fig 7C the ability of FLAG-Tel1 to bind the PI(4)P-containing liposomes preferentially.

2. The use of drug inhibitors only in the final figure is problematic. KD or KO experiments should be performed to confirm that ATM and the exchanger are the relevant targets.

> We have now used siRNAs against the exchanger protein, OSBP1, with a very high silencing rate success. We have next monitored the activation status of the chromatin-associated ATM target KAP-1, in order to monitor the predicted decrease of ATM activity specifically inside the nucleus. Our results confirm the role of OSBP1, by KD experiments as requested by the reviewer, in attenuating ATM nuclear participation. This set of experiments is shown in New Fig 9B.

3. Poor quality of some WBs (e.g Fig. S1F).

> We have now repeated the western blot to detect Rad53-P in response to 20 mM HU in WT *versus steΔ* cells using a more resolutive gel recipe (conveniently described in M&M). The new results can be found in New Expanded View Fig 1A.

4. Lack of statistical analyses for some data (e.g. Fig. 1B-E)

> We had already included, in the previous version, the complete statistical analyses corresponding to Old Figures 1B to E and evoked here by the reviewer. They were indeed included in Old Figure S1C, and our brief reference to them in the text may have escaped the reviewer's attention. We have made a clear reference to this in the revised version and placed these data in New Fig 1F.

Additional clarification points:

Figure 1: No representative images were shown for quantifications in Figure 1C, D, E.

> If the reviewer / editor estimates it pertinent, we can of course include them. Yet, the number of figures in the paper is now very heavy, leading us to simplify even what was shown in main Fig 1A.

Line 121: Should be Figure S1E, upper panel.

Line 124: Should be Figure S1E, lower panel.

Figure 2D-E, please show the quantification of the ratio of pCHK2/CHK2 with an N=3

> We have corrected / included the requested changes.

Figure S2B: needs quantification of NileRed staining to conclude induction in LD formation

> We have now quantified the LD as requested, and this information is displayed in New Appendix Fig S2C.

Figure 3C, to show the selectivity of ATM-binding toward PI4P, PLA of ATM with other PIPn species should be assessed, such as PI3P, PI4,5P2, and PI3,4,5P3.

> We have provided an overview of the binding preferences of ATM with respect to the full battery of phosphoinositides in the strip-binding assay shown in New EV3C and Fig 7B. Other than that, we are afraid that PLA studies as the ones we develop in the current manuscript for PI(4)P are not feasible, since no reliable antibodies exist for most of the other phosphoinositide species. Our impossibility to execute this experiment has been timely discussed with the Editor.

Figure S6A, PI4P level could be assessed by IF staining using PI4P antibody besides using PI4P sensor.

> We have now used our anti-PI(4)P antibody to monitor by immunofluorescence the behavior of this molecule in response to either MMS or zeocin, as suggested. Our direct monitoring of PI(4)P

has been combined with a specific antibody to localize GOLPH3 as a Golgi marker. We show how PI(4)P signals decrease following zeocin or MMS treatments, as anticipated if ATM release into the nucleus is coupled to PI(4)P consumption by the OSBP1 exchanger. As a comparison, we show that signal attenuation compares to a situation in which in PI(4)P synthesis was prevented using the specific inhibitor PIK93 (Fig 8C).

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9th May 2023

Re: EMBOJ-2022-112684R
A Sterol-PI(4)P exchanger controls the Tel1/ATM axis of the DNA Damage Response

Dear Dr. Moriel-Carretero,

Thank you again for submitting your revised manuscript for our consideration, and once more apologies for the delayed decision after re-review by the four original Review Commons referees. Given that all of them are largely satisfied with the revisions, we shall be happy to publish this work in The EMBO Journal after a final round of minor revision, in which I would invite you to carefully respond to the remaining comments. As discussed, please tone down certain wording as suggested by referee 1; include any data you may already have in response to referee 2; and carefully answer/discuss all other points; while I realize that adding additional experiments to address referee 1's second point and referee 2's first point would fall beyond the scope of the present revision.

In addition, please also address the following important editorial points at this stage:

- Pre-acceptance checks by our data editors have raised several queries with the data descriptors in the figure legends, which you will find as comments in the attached edited/commented Word document with activated "Track changes" option. I would appreciate if you incorporated the requested final text modifications and answered the Figure legend queries directly in this version (and modified figures where necessary), uploading the edited main text document upon resubmission with changes/additions still highlighted via the "Track changes" option, to facilitate our final checking.

- Please pre-face the Appendix Figures with an Appendix title page containing a brief Table of Contents, listing all the Appendix parts with Appendix page numbers. Also, please re-name the two "Supplementary Tables" into "Appendix Table S1/2", and move them into the Appendix PDF as well; making sure to correctly reference them at least once in the main text.

- In the abstract, please add informations as to the organismal origins of the cell systems being studied here, as this remains currently very unclear to readers.

- On the abstract page of the manuscript, please include 4-5 general keyword terms to enhance searchability.

- As we are switching from a free-text author contribution statement towards a more formal statement based on Contributor Role Taxonomy (CRediT) terms, please remove the present Author Contribution section and instead specify each author's contribution(s) directly in the Author Information page of our submission system during upload of the final manuscript. See https://casrai.org/credit/ for more information.

- Please adjust the header and the format of the Disclosure and competing interests statement (next to the Acknowledgment section) as specified in our Guide to Authors - for details, see https://www.embopress.org/competing-interests

- Please adjust the format for citation of preprints as specified in our author guidelines. The citation in the text should be: " (preprint: NAME1 et al, YEAR)"; in the reference list: "Author NAME1, Author NAME2, ... (YEAR) article title. bioRxiv doi: XXX"

- Finally, please provide suggestions for a short 'blurb' text prefacing and summing up the study in two sentences (max. 250 characters), followed by 3-5 one-sentence 'bullet points' with brief factual statements of key results of the paper; they will form the basis of an editor-written 'Synopsis' accompanying the online version of the article. Please also upload a synopsis image, which can be used as a "visual title" for the synopsis section of your paper. The image should be in PNG or JPG format with the modest dimensions of (exactly!) 550 wide and 300-600 pixel high.

I am therefore returning the manuscript to you for a final round of minor revision, to allow you to make these adjustments and upload all modified files. Once we will have received them, we should be ready to swiftly proceed with formal acceptance and production of the manuscript.

Yours sincerely,

Hartmut Vodermaier

Hartmut Vodermaier, PhD
Senior Editor, The EMBO Journal
h.vodermaier@embojournal.org

Use the link below to submit your revision:

Link Not Available

Referee #1:

In the revised version of the manuscript, authors have added a significant amount of data correlating with, or supporting, their main conclusions. However, main concerns, as some effects being of a small magnitude or correlational, have not been satisfactorily addressed.

Data indicating that Tel1/ATM function is modulated by changes in the lipid metabolism is novel and interesting to DDR scientist and general readers. However, the notion of sterol-PI(4)P exchanger "control" of the Tel1/ATM axis would require additional evidence; or should be toned down in the title and text for the study to be published in EMBO J. Evidence of DDR "control" of lipid metabolism actors, that would in turn modulate Tel1/ATM functions is not provided. In addition, the kinetics of the process still does not seem fully clear as, for instance, significant resection seems to occur prior to LD accumulation.

Referee #2:

The authors have majorly addressed the clarifications I asked during the review process through review commons. I have only one aspect that the authors did not address and I believe is required to make their claim convincing. I would suggest to confirm on whether there is a competition between kinases that further phosphorylate PI4P and ATM, since the cells do have lipid kianses in golgi that responds and use PI4P as substrate in response to different stress conditions.

Additionally, PI4P phosphorylating enzymes also localize in the nucleus of the cells, suggesting that PI4P may also be there in the nucleus and respond to genotoxic stress (cisplatin; work by Professor Anderson's lab). A selective association of PI4P and ATM in the golgi and not in the nucleus should be shown through experimental evidence.

Referee #3:

In the revised version of the manuscript, the authors carefully addressed many of my concerns that I raised in my review. Specifically, they have provided more detailed explanations and data to support their findings. I believe that this study will significantly impact the field suggesting new avenues for research into the interplay between DDR and lipid metabolism.

Minor comment: I agree with the reviewers about the conclusion that the impairment of long range resection does not affect engagement of Rad52 to short tracks. Probably a stronger inhibition of resection (as it was observed by the group of Dr. Marcus Smolka) would start to affect Rad52 binding in this model.

Minor comment: Regarding the CRISPR-Cas9 system to induce DSBs, a control with a marker of DSB after gal induction (gamma-H2A or Rad53 activation) would be important to show that Cas9 expression is working and cutting DNA.

Referee #4:

The revised manuscript by Ovejero et. al. has sufficiently addressed all of the recommended revisions and has added clarity, specificity, and robustness to the work. The manuscript has provided a mechanism to connect lipid signaling to the DNA damage response whereby lipid droplet-stabilized PI4P binds ATM to attenuate the ATM-driven DNA damage response. Given the implications this research for basic mechanistic insights to fuel further studies as well as potential translational components involving the manipulation of this pathway, we highly recommend publication in EMBO. Further, we believe that work should be emphasized with a commentary that we would be delighted to write and detailing the relevance to the field and to other distinct but related research fields.

Referee #1:

In the revised version of the manuscript, authors have added a significant amount of data correlating with, or supporting, their main conclusions. However, main concerns, as some effects being of a small magnitude or correlational, have not been satisfactorily addressed.

Data indicating that Tel1/ATM function is modulated by changes in the lipid metabolism is novel and interesting to DDR scientist and general readers. However, the notion of sterol□PI(4)P exchanger "control" of the Tel1/ATM axis would require additional evidence; or should be toned down in the title and text for the study to be published in EMBO J.

> We understand from this comment that the influence we show sterols/PI(4)P have on Tel1/ATM biology appears too mild as to justify the sentence "The sterol□PI(4)P exchanger **controls** the Tel1/ATM axis". We agree that the effects are not black and white, most probably because the metabolism of cholesterol is extremely well monitored at multiple checkpoints in the cell, leaving us with a modest window of experimental manipulation, a limitation we had already stated in the text. We have now toned down the title (and any related sentence in the main text) to a more conservative one, such as "The sterol□PI(4)P exchanger **modulates** the Tel1/ATM axis".

Evidence of DDR "control" of lipid metabolism actors, that would in turn modulate Tel1/ATM functions is not provided.

> Our work uncovers an alteration in the metabolism of sterols upon DNA double strand breaks and then goes on to decipher how this modification can end up modulating (switching off) the DDR. Of course, this raises the question of how the sterol metabolism "knew" in the first place that there were breaks, which calls for exploration of a DDR-dependent signal. Yet, this represents another full work, which is in fact ongoing with a specific funding provided for it. It has been agreed with the editor that we will develop this notion in future work, while in the current manuscript this concept has been acknowledged in the final section as a perspective.

In addition, the kinetics of the process still does not seem fully clear as, for instance, significant resection seems to occur prior to LD accumulation.

> The LD accumulation kinetics have been, at present, assessed in response to multiple breaks, as generated by zeocin or by CPT, as well as in response to 59 cuts, as created by Cas9. However, the resection experiment to which the reviewer refers, in which a resection defect starts to be seen in the *yeh2*Δ mutant only after 9 to 15 kb from the break, occurs in response to a single HO cut. It is therefore somehow logical that a single cut represents a more modest signal that takes longer to impose its attenuating feedback, thus authorizing a relatively longer resection before the DDR attenuation (or a defect in it) can manifest. We have now included in the text a small comment stating this possibility in lines 218-20.

Referee #2:

The authors have majorly addressed the clarifications I asked during the review process through review commons. I have only one aspect that the authors did not address and I believe is required to make their claim convincing. I would suggest to confirm on whether there is a competition between kinases that further phosphorylate PI4P and ATM, since the cells do have lipid kianses in golgi that responds and use PI4P as substrate in response to different stress conditions.

> To explore the competition between other Golgi-resident proteins, kinases or others, that use PI4P as a means to reinforce the putative competition between such proteins and ATM, would no doubt reinforce the link we uncover. These ideas were already evoked in the discussion of the manuscript (lines 484-8). However, a decent demonstration of those competitions would deserve heavy additional work, and it has been agreed with the editor that we will develop these notions in future work. Thank you for the ideas.

Additionally, PI4P phosphorylating enzymes also localize in the nucleus of the cells, suggesting that PI4P may also be there in the nucleus and respond to genotoxic stress (cisplatin; work by Professor Anderson's lab). A selective association of PI4P and ATM in the golgi and not in the nucleus should be shown through experimental evidence.

> This prediction is legitimate and is already confirmed in the previous version or the manuscript, as we show ATM/PI(4)P interactions by PLA, a technique that has the virtue of, not only evidencing proximity, but also revealing WHERE this proximity occurs within the cell. Our images show, but it is true that this was not openly stated in the text (in this updated version we comment on this observation), that ATM/PI(4)P signals very rarely emanate from the nucleus, but from the cytoplasm, which already answers the concern. What is more, when they apparently overlap with the nucleus, we show in this revised version that careful assessment shows these PLA signals stem from apposed perinuclear Golgi stacks, as shown now by co-localization with the Trans-Golgi marker TGN46. These additional data have been included in New Figure EV3F and commented on lines 342-6.

Referee #3:

In the revised version of the manuscript, the authors carefully addressed many of my concerns that I raised in my review. Specifically, they have provided more detailed explanations and data to support their findings. I believe that this study will significantly impact the field suggesting new avenues for research into the interplay between DDR and lipid metabolism.

Minor comment: I agree with the reviewers about the conclusion that the impairment of long range resection does not affect engagement of Rad52 to short tracks. Probably a stronger inhibition of resection (as it was observed by the group of Dr. Marcus Smolka) would start to affect Rad52 binding in this model.

> We understand from this comment that the reviewer meant "I agree with the AUTHORS about…", as it was us suggesting that the reason why Rad52 foci do not drop in the *ste*Δ mutant may be because, at shortly resected tracts, Rad52 can already load and continue accumulating. We are happy that she/he agrees with our interpretation and we agree with her/him that a stronger resection inhibition would be needed to invert this microscopy-revealed Rad52 accumulation trend.

Minor comment: Regarding the CRISPR-Cas9 system to induce DSBs, a control with a marker of DSB after gal induction (gamma-H2A or Rad53 activation) would be important to show that Cas9 expression is working and cutting DNA.

> We kindly bring the attention of the reviewer to the fact that this tool to induce 59 cuts derive from a work we have already pre-printed (and is currently under revision) in which we prove actual cuts being implemented by Southern blot, and also through Rfa1 and Rad52 foci accumulation, among others. This work is timely cited in the current manuscript (https://doi.org/10.1101/2021.10.21.465387).

Referee #4:

The revised manuscript by Ovejero et. al. has sufficiently addressed all of the recommended revisions and has added clarity, specificity, and robustness to the work. The manuscript has provided a mechanism to connect lipid signaling to the DNA damage response whereby lipid droplet-stabilized PI4P binds ATM to attenuate the ATM-driven DNA damage response. Given the implications this research for basic mechanistic insights to fuel further studies as well as potential translational components involving the manipulation of this pathway, we highly recommend publication in EMBO. Further, we believe that work should be emphasized with a commentary that we would be delighted to write and detailing the relevance to the field and to other distinct but related research fields.

> We are truly happy that the reviewer thinks the concerns have been satisfactorily assessed and we are truly honored that she/he also thinks that a commentary should be commissioned to highlight our work. Thank you very much!

Dr. María Moriel-Carretero
Centre de Recherche en Biologie cellulaire de Montpellier (CRBM), Université de Montpellier-Centre National de la Recherche Scientifique
1919 Route de Mende
Montpellier 34293
France

26th May 2023

Re: EMBOJ-2022-112684R1
A Sterol-PI(4)P exchanger modulates the Tel1/ATM axis of the DNA Damage Response

Dear Dr. Moriel-Carretero,

Thank you for submitting your final revised manuscript for our consideration. I am pleased to inform you that we have now accepted it for publication in The EMBO Journal.

Your article will be processed for publication in The EMBO Journal by EMBO Press and Wiley, who will contact you with further information regarding production/publication procedures and license requirements. You will also be provided with page proofs after copy-editing and typesetting of main manuscript and expanded view figure files.

Should you be planning a Press Release on your article, please get in contact with embojournal@wiley.com as early as possible, in order to coordinate publication and release dates.

Thank you again for this contribution to The EMBO Journal and congratulations on a successful publication! Please consider us again in the future for your most exciting work.

Yours sincerely,

Hartmut Vodermaier

Hartmut Vodermaier, PhD
Senior Editor, The EMBO Journal
h.vodermaier@embojournal.org

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Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2022-112684R1

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This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your
Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for 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.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

2. Captions
Each figure caption should contain the following information, for each panel where they are relevant:
- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

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Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Materials and Methods

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Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes	ATCC STR profiling, reported in Material and Methods

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Please detail **housing and husbandry conditions**.	Not Applicable	

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Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Not Applicable	
Include a statement about **blinding** even if no blinding was done.	Yes	Material and Methods
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Yes	We usually repeat one experiment 3 times. In rare cases, if one replicate yielded very different values from the rest, the experiment was repeated up to 7 times and, if such a replicate still remained an outlier, it was ignored from the final analysis.
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Studies involving experimental **animals**: State details of **authority granting ethics approva**l (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Not Applicable	
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