EMBO reports

The nucleolar protein GNL3 prevents resection of stalled replication forks.

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DOI: 10.15252/embr.202357585

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Review Timeline:

Transfer from Review Commons:	2nd Jun 23
Editorial Decision:	20th Jun 23
Revision Received:	20th Sep 23
Editorial Decision:	16th Oct 23
Revision Received:	25th Oct 23
Accepted:	27th Oct 23

Editor: Esther Schnapp

Transaction Report: This manuscript was transferred to EMBO reports following peer review at Review Commons.

Review COMMONS

Review #1

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

In this manuscript Lebdy et al. describe a new role of GNL3 in DNA replication. They show that GNL3 controls replication fork stability in response to replication stress and they propose this is due to the regulation of ORC2 and the licensing of origins of replication. Their data suggest that GNL3 regulates the sub nuclear localization of ORC2 to limit the number of licensed origins of replication and to prevent resection of DNA at stalled forks in the presence of replication stress.

While many of the points of the manuscript are proven and well supported by the results, there are some experiments that could improve the quality and impact of the manuscript. The main issue is that the connection between the role of GNL3 in controlling ORC2, the firing of new origins and the protection of replication forks is not clearly established. At the moment the model relies on mainly correlative data. In order to further substantiate the model, we propose to address some of the following issues:

1. The authors indicate that RPA and RAD51 accumulation at stalled forks is not affected by GNL3 depletion. These data should be included and other proteins should be analysed. In addition, the role of helicases could be explored through the depletion of the main helicases involved in the remodelling of the forks.
2. The proposed model implies that GNL3 depletion leads to increased origin licensing. FThe authors should address if the primary effect of GNL3 depletion is on origin firing by using CDC7 inhibition in the absence of stress (Rodríguez-Acebes et al., JBC 2018).
3. A way to prove that origin firing mediates the effect of GNL3 on fork protection would be to reduce the number of available origins. The depletion of MCM complexes has been shown to limit the number of back-up origins that are licensed and leads to sensitivity to replication stress (Ibarra et al., PNAS 2008). If GNL3 depletion results in increased number of origins, this effect should be prevented by the partial depletion of MCM complexes.
4. Alternatively, the authors could try to modulate the depletion of GNL3. Origin licensing takes place in the G1 phase and thus the depletion of GNL3 by siRNA could affect the following S phase. Using an inducible degron for GNL3 depletion would allow to deplete GNL3 in G1 or S phase specifically. If the model is correct, the removal of GNL3 in S phase should not affect fork protection but removing GNL3 in

the previous G2/M phase should reduce the number of licensed origins and lead to impaired fork protection.
In addition to the connection GNL3-origin firing-fork protection, it is unclear how the lack of GNL3 in the nucleolus and the change in the sub nuclear localization of ORC2 controls origin firing and resection. The strong interaction observed between GNL3-dB and ORC2, and the subsequent change in ORC2 localization does not explain how origin licensing can be affected. In this sense, the authors could address:
1. Does the depletion of GNL3 and the expression of GNL3-dB affect the formation of the ORC complex, its subnuclear localization or its binding to chromatin? The authors have not explored if the interaction of GNL3 with ORC2 is established in the context of the ORC complex. An IF showing NOP1 with PLA data from GNL3-dB and ORC2 is needed to analyse how the expression of increasing amounts of GNL3-dB affects ORC2.
2. In order to confirm if the mislocalization of ORC2 by the expression of GNL3-dB increases origin firing and mediates the effects on fork protection the authors could check DNA resection levels inhibiting CDC7 in high GNL3-dB conditions. Also, the levels of MCM2, phosphor-MCM2, CDC45, have not been analysed upon expression of GNL3-dB.
3. The data in the paper suggest that GNL3 may affect the role of ORC2 in centromeres. Since depletion of GNL3 leads to increased levels of γH2AX, it would be interesting to address if this damage is due to incomplete replication in centromeres by analysing the co-localization of γH2AX and centromeric markers both in unstressed conditions and upon the induction of replication stress.

Minor points:

1. In the initial esiRNA screen the basal levels of γH2AX should also be shown.
2. Figure EV1B: I think the rank needs another RS mark to see better the effect of each esiRNA on DNA lesions (high variability in all the conditions showed).
3. Figure 1C and Figure EV1D/E: the quantification of the pCHK1/CHK1 levels could be included to show that there are no changes in phosphorylation upon GNL3 depletion.
4. In the first section of the results, at the end Figure 4B is incorrectly called for.
5. The levels of GLN3 expression in 293 cells should be already included in section GNL3 interacts with ORC2.
6. The full MS data needs to be included for both GNL3 and ORC2.
7. Figure 4B should be improved, since there is a faint band in the IgG mouse control.

2. Significance:

Significance (Required)

The work is nicely written, the figures are well presented and the experiments have the necessary controls. It provides relevant information to understand how replication stress is controlled and linked to replication fork protection through origin firing. These results are relevant to the field, linking GNL3 to origin firing and with potential to help understand the role of GNL3 in cancer. They provide new information and can give rise to new studies in the future. Many of the conclusions of the manuscript are well supported. Additional support for some of the main claims would strengthen the results and also increase the impact providing a bigger conceptual advance by performing some of the suggested experiments.

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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Review #2

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

This manuscript explores the role of GNL3/nucleostemin in DNA replication and specifically in the response of DNA replication to DNA damage. GNL3 is a predominantly nucleolar protein, previously characterised as a GTP-binding protein and shown to be necessary for effective recruitment of the RAD51 recombinase to DNA breaks. The entry point for this report is a mini screen, based on proteins identified previously by the authors to associate with replication forks by iPOND, for factors that increase gamma-H2Ax (an indicator of DNA damage) after treatment with the Top1 inhibitor camptothecin (CPT). In this mini-screen GNL3 emerged as the top hit.

The authors put forward the hypothesis that GNL3 is able to sequester the replication licensing factor ORC2 in the nucleolus and that failure of this mechanism leads to excessive origin firing and DNA resection following CPT treatment.

The model put forward is interesting, but currently rather confusing. However, for the reasons upon which I expand below, I do not believe that the data provide a compelling mechanistic explanation for the effects that are reported and I am left not being certain about some of the links that are made between the various parts of the study, even though individual observations appear to be of good quality.

Specific points:

The knockdown of GNL3 is very incomplete. In this regard, the complementation experiments are welcome and important. However, is it an essential protein? Can it be simply deleted with CRISPR-Cas9?

Global instant fork density is not quite the same as actually measuring origin firing. Ideally, it would be good to see some more direct evidence of addition origin firing e.g. by EdU-seq (Macheret & Halazonetis Nature 2018) but this would be quite a significant additional undertaking. However, given the authors have performed DNA combing with DNA counterstain, they should be able to provide accurate measurements of origin density and inter-origin distance.

'Replication stress' is induced with CPT. This term is frequently used to describe events that lead to helicase-polymerase uncoupling (e.g. O'Connor Mol Cell 2015) but that is not the case with CPT, which causes fork collapse and breaks. Are similar effects seen with e.g. UV or cisplatin? Additionally, a clear statement of the authors definition of replication stress would be welcome.

It is really not clear how the authors explain the link between potential changes in

origin firing and resection. i.e. What is the relationship between global origin firing and resection at a particular fork, presumably broken by encounter with a CPT-arrested TOP1 complex.
What is the link mechanistically? This link needs elaborating experimentally or clearly explaining based on prior literature.

Related to this, I remain unconvinced that the experiments in Figure 3 show that the effects of ATRi and Wee1i on origin firing and on resection are contingent on each other. I do not believe that the authors have adequately supported the statement (end of pg 9) 'We conclude that the enhanced resection observed upon GNL3 depletion is a consequence of increased origin firing.' The link between origin firing and resection needs really needs further substantiation and / or explanation.

It is not clear whether the binding of ORC2 to GNL3 also sequesters other components of the origin recognition complex? Does loss of the ability of GNL3 to bind ORC2 actually lead to more ORC bound to chromatin? How does GNL3 contribute to regulation of origin firing under normal conditions? Is it a quantitatively significant sink for ORC2 and what regulates ORC2 release?

Minor points:

All blots should include size markers

Some use of language is not sufficiently precise. For instance:

- the meaning of 'DNA lesions' at the end of the first paragraph of the introduction needs to be more explicit.
- the approach to measurement of these 'lesions' (monitoring gamma-H2Ax) needs to be spelled out explicitly, e.g. line 4 of the last paragraph of the introduction.
- 'we observed that the interaction between GNL3-dB and ORC2 was stronger' ... I do not see how number of foci indicates necessarily the strength of an interaction.
- in many places throughout 'replication origins firing' should be 'replication origin firing' (or 'firing of replication origins').

2. Significance:

Significance (Required)

The model put forward here has the potential to shed light on an important facet of the cellular response to DNA damage, namely the control of origin firing in response to replication stress that will certainly be of interest to the DNA repair / replication community and possibly more widely. The roles of GNL3 are poorly understood and this study could improve this state of affairs. However, the gaps in the mechanism outlined above and somewhat confusing conclusions do limit the ability of the paper to achieve this at present.

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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No

Review #3

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

In this study, Lebdy et al propose a new mechanism to regulate the resection of nascent DNA at stalled replication forks. The central element of this mechanism is

nucleolar protein GNL3, whose downregulation with siRNA stimulates DNA resection in the presence of stress induced by HU (Figure 1). Resection depends on the activity of nucleases MRE11 and CtIP, and can be rescued by reintroducing exogenous GNL3 protein in the cells (Figure 1G). GNL3 downregulation decreases fork speed and increases origin activity, without any strong effect on replication timing (Figure 2). Inhibition of Dbf4-dependent kinase CDC7 (a known origin-activating factor) also restricts fork resection (Figure 3). GNL3 interacts with ORC2, one of the subunits of the origin recognition complex, preferentially in nucleolar structures (Figure 4). A mutant version of GNL3 (GNL3-dB) that is not sufficiently retained in the nucleoli fails to prevent fork resection as the WT protein (Figure 5). In the final model, the authors propose that GNL3 controls the levels of origin activity (and indirectly, stalled fork resection) by maintaining a fraction of ORC2 in the nucleoli (Figure 6).

This model is interesting and provocative, but it also relies on a significant degree of speculation. The authors are not trying to "oversell" their observations, because the Discussion section entertains different interpretations and possibilities, and the model itself contains several interrogative statements (e.g. "ORC2-dependent?"; "exhaustion of factors?").

While the article is honest about its own limitations, the major concern remains about its highly speculative nature. I have some questions and suggestions for the authors to consider that could contribute to test (and hopefully support) their model.

1. If GNL3 downregulation induces an excess of licensed origins and mild replicative stress resulting in some G2/M accumulation (Figure 2), what is the consequence of longer-term GNL3 ablation? Do the cells adapt, or do they accumulate signs of chromosomal instability? (micronuclei, chromosome breaks and fusions, etc)
2. The model relies on the link between origin activity and stalled fork resection that is almost exclusively based on the results obtained with CDC7i (Figure 3). But CDC7 has other targets besides pre-RC components at the origins, such as Exo1 (from the Weinreich lab, cited in the study), MERIT40 and PDS5B (from the Jallepalli lab, also cited). The effect of CDC7i could be exerted through these factors, which are linked to fork stability and DNA resection. The loss of BRCA1 (Figure 3F) could somehow entail the loss of control over these factors. Could the authors check the possible participation of these proteins?
3. The model also relies on the fact that GNL3-dB mutant (not retained in the nucleoli) is not sufficient to counteract fork resection induced by HU (Figure 5G). The authors should test directly whether GNL3-dB induces extra origin activation, using their available DNA fibers-based technique.
4. Finally, the model implies an exquisite regulation of the amount of ORC2 protein,

which could influence the number of active origins and the extent of fork resection in case of stress. In this scenario, one could predict that ORC2 ectopic expression would have similar, or even stronger effects, than GNL3 downregulation. Is this the case?
5. Even if the connection between origins and fork resection could be firmly established, the molecular link between them remains enigmatic. The authors hint (as "data not shown") that it is neither mediated by RPA nor RAD51. Unfortunately, the reader is left without a clear hypothesis about this point.

Referees cross-commenting

In addition to each reviewer's more specific comments, the three reviews share a main criticism: the lack of mechanistic information about the proposed link between origin activity and resection of nascent DNA at stalled forks.

2. Significance:

Significance (Required)

In principle, this study would appeal to the readership interested in fundamental mechanisms of DNA replication and the cellular responses to replicative stress.

For the reasons outlined in the previous section, I believe that in its current version the study is not strong enough to provide a new paradigm about origins being regulated by partial ORC2 sequestering at nucleoli. The other potentially interesting advance is the connection between frequency of origin activity and the extent of nascent DNA resection at stalled forks, but the molecular link between both remains unknown.

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

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

More than 6 months

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Revision Plan

Manuscript number: RC-2023-01920
Corresponding author(s): Cyil Ribeyre

[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.

Reviewers highlight the quality of our experiments and our interpretation of the data (examples below). The goal of our study was to report a previously uncharacterized role of GNL3 in response to replication stress. We wish to highlight that very little was known about the role of GNL3 during replication and replication stress, therefore the novelty of our work is high. Of course, questions are remaining and will be exciting challenges for the future.

Reviewer 1 (R1):
The work is nicely written, the figures are well presented and the experiments have the necessary controls. It provides relevant information to understand how replication stress is controlled and linked to replication fork protection through origin firing. These results are relevant to the field, linking GNL3 to origin firing and with potential to help understand the role of GNL3 in cancer.
Reviewer 2 (R2):
The model put forward here has the potential to shed light on an important facet of the cellular response to DNA damage, namely the control of origin firing in response to replication stress that will certainly be of interest to the DNA repair / replication community and possibly more widely. The roles of GNL3 are poorly understood and this study could improve this state of affairs.
Reviewer 3 (R3):
This model is interesting and provocative, but it also relies on a significant degree of speculation. The authors are not trying to "oversell" their observations, because the Discussion section entertains different interpretations and possibilities, and the model itself contains several interrogative statements (e.g. "ORC2-dependent?"; "exhaustion of factors?").

Revision Plan

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.

Based on reviewers comments the two points that we need to improve are how GNL3 limits excessive origins activity and how this is important for preventing nascent DNA resection. In addition to our point-by-point responses to reviewers (see below) this is the summary of the experiments that we already have done and the experiments we propose to do based on reviewers' comments.

Role of GNL3 in limiting origins firing:

We now show that:
-GIFD (global Instant Fork Density) is increased upon GNL3-dB expression, a mutant that is diffusing in the nucleolus. This important result indicates that origin firing is increased upon GNL3-dB expression, providing a link between aberrant localization and increased origin firing. Since replication origin firing is increasing upon GNL3 depletion, we believe that the accumulation of GNL3 in the nucleolus limits replication origin firing likely via ORC2 sequestration since GNL3-dB interacts mainly with ORC2 in the nucleoplasm.

We will test if:
-CDC7 inhibition increases forks speed upon GNL3 depletion. This experiment should tell us if the primary defect of GNL3 depletion is excessive origin firing.

Mechanism of increased resection upon GNL3 depletion:

We now show that:
-the recruitment of RAD51 and RPA on chromatin is not affected by GNL3 depletion.
-DNA resection in absence of GNL3 is suppressed by roscovitine. This confirms that excessive firing upon GNL3 depletion is responsible for increased resection in response to replication stress. We may extent this observation by depleting MCM.
-Resection in absence of GNL3 is dependent on Exo1. This experiment extends our knowledge of the mechanisms of resection in absence of GNL3.

We will test if:
-interaction of GNL3-dB with ORC2 is dependent on the level of GNL3-dB. This experiment should link resection, GNL3-dB expression and regulation of ORC2 subnuclear localization.
-resection upon GNL3-dB expression is dependent on CDC7. This experiment should strengthen the link between resection and the control of the number of origins.
-overexpression of RPA reduces resection in GNL3-depleted cells. This experiment should tell us if RPA exhaustion is responsible for increased resection upon GNL3 depletion and HU treatment.

Revision Plan

Reply to reviewers.

We deeply thank the reviewers for the time spent on evaluating our manuscript as well as providing comments and suggestions to improve our study.

Reviewer #1 (Evidence, reproducibility and clarity (Required)):

In this manuscript Lebdy et al. describe a new role of GNL3 in DNA replication. They show that GNL3 controls replication fork stability in response to replication stress and they propose this is due to the regulation of ORC2 and the licensing of origins of replication. Their data suggest that GNL3 regulates the sub nuclear localization of ORC2 to limit the number of licensed origins of replication and to prevent resection of DNA at stalled forks in the presence of replication stress.

While many of the points of the manuscript are proven and well supported by the results, there are some experiments that could improve the quality and impact of the manuscript. The main issue is that the connection between the role of GNL3 in controlling ORC2, the firing of new origins and the protection of replication forks is not clearly established. At the moment the model relies on mainly correlative data. In order to further substantiate the model, we propose to address some of the following issues:

1. The authors indicate that RPA and RAD51 accumulation at stalled forks is not affected by GNL3 depletion. These data should be included and other proteins should be analysed. In addition, the role of helicases could be explored through the depletion of the main helicases involved in the remodelling of the forks.

Response: As asked by the reviewer we will add the fractionation experiments that show that the level of RAD51 and RPA on chromatin is not affected by GNL3 depletion. So far, the other proteins we checked (RIF1 and BRCA1), both involved in nascent strand protection, did not show clear differences. Therefore, we concluded that depletion of GNL3 does not seem to affect the recruitment of major proteins required for protection of nascent DNA. Of course, we cannot exclude that other proteins may be affected by GNL3 depletion, but testing all the possible candidates would be time consuming with a very low chance of success. In addition, fractionation experiments are possibly not quantitative enough to uncover small differences and may be not that informative. Thus it remains possible that RPA exhaustion may be the cause of resection in absence of GNL3 as suggested by the work conducted in Lukas' lab (Toledo et al. 2013. https://pubmed.ncbi.nlm.nih.gov/24267891/). To test this hypothesis, we will analyze if resection in absence of GNL3 is still occurring in a well-characterized cell line that overexpress the three RPA subunits that we obtained from Lukas' lab.

To our knowledge not many helicases have been shown to be involved in remodeling of stalled forks. The best example is RECQ1, however we feel that testing RECQ1 involvement in resection upon GNL3 depletion will complicate our story without adding much regarding the mechanism. We hope the reviewer understands our concern.

2. The proposed model implies that GNL3 depletion leads to increased origin licensing. FThe authors should address if the primary effect of GNL3 depletion is on origin firing by using CDC7 inhibition in the absence of stress (Rodríguez-Acebes et al., JBC 2018).

Response: This is an excellent point raised by the reviewer. To test if the primary effect of GNL3 depletion in on origin firing we will test if the defect in replication fork progression is dependent on CDC7 using DNA fibers experiments and CDC7 inhibitor.

3. A way to prove that origin firing mediates the effect of GNL3 on fork protection would be to reduce the number of available origins. The depletion of MCM complexes has been shown to limit the number of back-up origins that are licensed and leads to sensitivity to replication stress (Ibarra et al., PNAS 2008). If GNL3 depletion results in increased number of origins, this effect should be prevented by the partial depletion of MCM complexes.

Response: This is also an excellent point. We will test if MCM depletion decreases resection upon GNL3 depletion and treatment with HU. In addition, we will integrate in the manuscript experiments that we have done recently that show that treatment with roscovitine, a CDK inhibitor that impairs origin firing, decreases the level of resection observed in absence of GNL3. We think this experiment strengthens the results obtained with CDC7 inhibitors.

4. Alternatively, the authors could try to modulate the depletion of GNL3. Origin licensing takes place in the G1 phase and thus the depletion of GNL3 by siRNA could affect the following S phase. Using an inducible degron for GNL3 depletion would allow to deplete GNL3 in G1 or S phase specifically. If the model is correct, the removal of GNL3 in S phase should not affect fork protection but removing GNL3 in the previous G2/M phase should reduce the number of licensed origins and lead to impaired fork protection.

Response: This is obviously a good point given the fact that GNL3 deletion is not viable (see responses to reviewer 2). We tried to develop an auxin induced degron of GNL3, but we could not obtain homozygous clones, meaning that our clones had always an untagged GNL3 allele. Since GNL3 is essential its tagging may impair its function, explaining why we could not obtain homozygous clones. However, we are planning to optimize the design using other degrons system (for instance Halo-tag) to address the role of GNL3 specifically during S-phase. But we think this is above the scope of the present study.

In addition to the connection GNL3-origin firing-fork protection, it is unclear how the lack of GNL3 in the nucleolus and the change in the sub nuclear localization of ORC2 controls origin firing and resection. The strong interaction observed between GNL3-dB and ORC2, and the subsequent change in ORC2 localization does not explain how origin licensing can be affected. In this sense, the authors could address:

1. Does the depletion of GNL3 and the expression of GNL3-dB affect the formation of the ORC

complex, its subnuclear localization or its binding to chromatin? The authors have not explored if the interaction of GNL3 with ORC2 is established in the context of the ORC complex. An IF showing NOP1 with PLA data from GNL3-dB and ORC2 is needed to analyse how the expression of increasing amounts of GNL3-dB affects ORC2.

Response: We tested if GNL3 depletion impacts ORC2 and ORC1 recruitment on chromatin, but we could not observe significant differences. No clear differences were observed upon GNL3-dB expression either. One reason for this may be due to the excess of ORC complex on the chromatin, in addition chromatin fractionation is likely not sensitive enough to observe small differences. We think that quantitative ChIP-seq of ORC2 or other ORC subunits upon GNL3 depletion is required to visualize such differences, but this is above the scope of the study, and this constitutes the following of this project. We also tried to look at subnuclear localization of ORC2 using immunofluorescence, but the signal was not specific enough to observe differences. We think that the increased interaction (PLA) of ORC2 with GNL3-dB (Figure 5E) demonstrates a change in ORC2 subnuclear localization. To confirm this, we will perform the excellent experiment proposed by the reviewer to test if increasing level of GNL3-dB affects its interaction with ORC2 using PLA.

We do not think that the interaction between ORC2 and GNL3 is established in the context of the ORC complex since only ORC2 (and not the other ORC) was significantly enriched in the GNL3 Bio-ID experiment. The full list of proteins from the Bio-ID experiment (Figure 4A) will be provided in the revised version. Therefore, we think that either GNL3 regulates ORC2 subnuclear localization that in turns impact the ORC complex or GNL3 regulates ORC2-specific functions. More and more evidences show that ORC2 plays roles possibly independently of the ORC complex (see Huang et al. 2016 https://doi.org/10.1016/j.celrep.2016.02.091 or Richards et al. 2022 https://doi.org/10.1016/j.celrep.2022.111590 for instance). Future work should uncover how these ORC2 functions may regulate origins activity.

2. In order to confirm if the mislocalization of ORC2 by the expression of GNL3-dB increases origin firing and mediates the effects on fork protection the authors could check DNA resection levels inhibiting CDC7 in high GNL3-dB conditions. Also, the levels of MCM2, phosphor-MCM2, CDC45, have not been analysed upon expression of GNL3-dB.

Response: This is a good point; we will test if the resection observed upon expression of GNL3-dB is dependent on origin firing using CDC7 inhibitor. We have not measured the level of the cited proteins but instead we performed DNA combing to measure Global Instant Fork Density. We now show that expression of GNL3-WT suppresses the increased origin firing observed upon GNL3 depletion, in contrast expression of GNL3-dB does not suppress it. This important result indicates that origin firing is increased upon GNL3-dB expression, providing a link between aberrant localization and increased firing. These data will be part of the revised version of the manuscript.

3. The data in the paper suggest that GNL3 may affect the role of ORC2 in centromeres. Since

depletion of GNL3 leads to increased levels of γH2AX, it would be interesting to address if this damage is due to incomplete replication in centromeres by analysing the co-localization of γH2AX and centromeric markers both in unstressed conditions and upon the induction of replication stress.

Response: This is indeed and interesting comment, however since it has been previously shown that γH2AX signal is rather strong upon GNL3 depletion (see Lin et al. 2013. https://pubmed.ncbi.nlm.nih.gov/24610951/ ; Meng et al. 2013. https://pubmed.ncbi.nlm.nih.gov/23798389/) we do not think that co-localization experiments with CENP-A for instance will be informative given the high number of γH2AX foci.

Minor points:

1. In the initial esiRNA screen the basal levels of γH2AX should also be shown.

Response: Our negative control is the transfection of an esiRNAs that targets EGFP (a gene that is not expressed in the tested cell line). This esiRNAs is ranked at the end of the list and therefore constitutes the basal level of γH2AX signal. In any case it is well-established that GNL3 depletion increases γH2AX signal (see Lin et al. 2013. https://pubmed.ncbi.nlm.nih.gov/24610951/ ; Meng et al. 2013. https://pubmed.ncbi.nlm.nih.gov/23798389/).

2. Figure EV1B: I think the rank needs another RS mark to see better the effect of each esiRNA on DNA lesions (high variability in all the conditions showed).

Response: We understand this issue, but we cannot repeat this set of experiments for technical reasons (reagents and cost mainly). Anyway, we believe that the role of GNL3 is response to replication stress is extensively addressed by other experiments of this manuscript.

3. Figure 1C and Figure EV1D/E: the quantification of the pCHK1/CHK1 levels could be included to show that there are no changes in phosphorylation upon GNL3 depletion.

Response: it is a good point; we will put quantification in the revised version.

4. In the first section of the results, at the end Figure 4B is incorrectly called for.

Response: Thanks for the comment, we will modify accordingly.

5. The levels of GLN3 expression in 293 cells should be already included in section GNL3 interacts with ORC2.

Response: We will add a figure that shows the level of expression in 293 cells.

6. The full MS data needs to be included for both GNL3 and ORC2.

Response: This will be integrated in the revised version.

7. Figure 4B should be improved, since there is a faint band in the IgG mouse control.

Response: it is true that the figure is not perfect, but we believed that our Bio-ID and PLA experiments fully demonstrate the interaction between GNL3 and ORC2.

Reviewer #1 (Significance (Required)):

The work is nicely written, the figures are well presented and the experiments have the necessary controls. It provides relevant information to understand how replication stress is controlled and linked to replication fork protection through origin firing. These results are relevant to the field, linking GNL3 to origin firing and with potential to help understand the role of GNL3 in cancer. They provide new information and can give rise to new studies in the future. Many of the conclusions of the manuscript are well supported. Additional support for some of the main claims would strengthen the results and also increase the impact providing a bigger conceptual advance by performing some of the suggested experiments.

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

This manuscript explores the role of GNL3/nucleostemin in DNA replication and specifically in the response of DNA replication to DNA damage. GNL3 is a predominantly nucleolar protein, previously characterised as a GTP-binding protein and shown to be necessary for effective recruitment of the RAD51 recombinase to DNA breaks. The entry point for this report is a mini screen, based on proteins identified previously by the authors to associate with replication forks by iPOND, for factors that increase gamma-H2Ax (an indicator of DNA damage) after treatment with the Top1 inhibitor camptothecin (CPT). In this mini-screen GNL3 emerged as the top hit.

The authors put forward the hypothesis that GNL3 is able to sequester the replication licensing factor ORC2 in the nucleolus and that failure of this mechanism leads to excessive origin firing and DNA resection following CPT treatment.

The model put forward is interesting, but currently rather confusing. However, for the reasons upon which I expand below, I do not believe that the data provide a compelling mechanistic explanation for the effects that are reported and I am left not being certain about some of the links that are made between the various parts of the study, even though individual observations appear to be of good quality.

Specific points:

The knockdown of GNL3 is very incomplete. In this regard, the complementation experiments are welcome and important. However, is it an essential protein? Can it be simply deleted with CRISPR-Cas9?

Response: There are obviously variations between experiments but overall, the depletion of GNL3 using siRNA seems good in our opinion. Deletion of GNL3/nucleostemin leads to embryonic lethality in mouse (Beekman et al. 2006. https://pubmed.ncbi.nlm.nih.gov/17000755/ ; Zhu et al. 2006. https://pubmed.ncbi.nlm.nih.gov/17000763/). ES cells deleted for GNL3 can be obtain but do not proliferate probably because of their inability to enter in S-phase (Beekman et al. 2006. https://pubmed.ncbi.nlm.nih.gov/17000755/). We wanted to test if it was the case in our cellular model and we tried to delete it using CRISPR-Cas9. We managed to obtain few clones deleted for GNL3, but they grow really poorly prevented us to do experiments. To bypass this, and as suggested by the reviewer 1, we tried to make an auxin-induced degron of GNL3. Unfortunately, we did not manage to obtain homozygous clones, only heterozygous. One possibility could be that the tagging induced a partial loss of function of GNL3, and since GNL3 is essential, it may explain why we did not obtain homozygous clones. We may also want to use alternative degron systems such as Halo-Tag, but we believe this is out of the scope of the study.

Global instant fork density is not quite the same as actually measuring origin firing. Ideally, it would be good to see some more direct evidence of addition origin firing e.g. by EdU-seq (Macheret & Halazonetis Nature 2018) but this would be quite a significant additional undertaking. However, given the authors have performed DNA combing with DNA counterstain, they should be able to provide accurate measurements of origin density and inter-origin distance.

Response: As indicated by the reviewer EdU-seq would need a lot of development since we are not using this approach in our team. In addition, this method can detect replication origins only if performed in the beginning of S-phase, meaning that only the early firing origins will be detected and not the others. GIFD measurement is actually directly linked with origin firing since it is counting the forks to duplicate the genome. The measurements of IODs have at least two main limitations: (1) there is a bias for short IODs due to the length of analyzed fibers and (2) it focuses only on origins within a cluster not globally. Overall, we believe that GIFD is the method of choice to measures origins firing. In addition, these experiments have been done by the lab of Etienne Schwob (see acknowledgments), a leader in the field.

'Replication stress' is induced with CPT. This term is frequently used to describe events that lead to helicase-polymerase uncoupling (e.g. O'Connor Mol Cell 2015) but that is not the case with CPT, which causes fork collapse and breaks. Are similar effects seen with e.g. UV or cisplatin? Additionally, a clear statement of the authors definition of replication stress would be welcome.

Response: We will better define the term ‘replication stress’ in the revised version of the manuscript. It should be understood, in our case, that any impediment that leads to replication fork stalling and measurable by DNA combing or Chk1 phosphorylation. We have not performed experiments using UV and cisplatin.

It is really not clear how the authors explain the link between potential changes in origin firing and resection. i.e. What is the relationship between global origin firing and resection at a particular fork, presumably broken by encounter with a CPT-arrested TOP1 complex. What is the link mechanistically? This link needs elaborating experimentally or clearly explaining based on prior literature.

Response: Most of our results on resection has been performed with hydroxyurea, but it is true that we saw resection in absence of GNL3 in response to CPT. Treatment with HU or CPT reduces fork speed and activates additional replication origins (see Ge et al. 2007 https://pubmed.ncbi.nlm.nih.gov/18079179/ for HU or Hayakawa et al. 2021 https://pubmed.ncbi.nlm.nih.gov/34818230/ for CPT). When GNL3 is depleted, more forks are active, meaning more targets for HU and CPT. In addition, it is likely that the firing of additional origins in response to HU and CPT is stronger in absence of GNL3. Because of this we believe that factors required to protect stalled forks may be exhausted explaining why resection is observed. This is inspired by the work of Lukas’ lab (Toledo et al. 2013 https://pubmed.ncbi.nlm.nih.gov/24267891/) and is described in the figure 6. One obvious candidate that may be exhausted is RPA, to test this we will check if resection upon GNL3 depletion and treatment with HU is still occurring in cell lines provided by Lukas’ lab that overexpress RPA complex (described in Toledo et al.). We will explain our model more carefully in the revised version.

Related to this, I remain unconvinced that the experiments in Figure 3 show that the effects of ATRi and Wee1i on origin firing and on resection are contingent on each other. I do not believe that the authors have adequately supported the statement (end of pg 9) 'We conclude that the enhanced resection observed upon GNL3 depletion is a consequence of increased origin firing.' The link between origin firing and resection needs really needs further substantiation and / or explanation.

Response: Our rational was the following. Inhibition of ATR or WEE1 increase replication origin firing, a situation that may be like the one observed for GNL3 depletion. In Toledo et al, they show that inhibition of WEE1 or ATR induces exhaustion of RPA. This exhaustion is reduced in presence of CDC7 inhibitor, roscovitine (a CDK inhibitor that inhibits origin firing) or depletion of CDC45, indicating that this is due to excessive origin activation. In our case we show that the resection observed upon WEE1 or ATR inhibition is reduced upon treatment with CDC7 inhibitor. We conclude that excessive replication origin firing induces DNA resection. Since we observed the same thing upon GNL3 depletion (but not upon BRCA1 depletion) we conclude that excessive origin firing favors DNA resection likely through exhaustion of RPA. As indicated above we will test this hypothesis by overexpressing RPA. In addition, we now show that

treatment with roscovitine decreases resection upon GNL3 depletion (this will be part of the revised manuscript), an experiment that we believe confirms that excessive replication origins firing is responsible for resection upon GNL3 depletion. As suggested by reviewer 1, we will also test if depletion of MCM also reduces resection observed in absence of GNL3.

It is not clear whether the binding of ORC2 to GNL3 also sequesters other components of the origin recognition complex? Does loss of the ability of GNL3 to bind ORC2 actually lead to more ORC bound to chromatin? How does GNL3 contribute to regulation of origin firing under normal conditions? Is it a quantitatively significant sink for ORC2 and what regulates ORC2 release?

Response: The results of GNL3 Bio-ID were extremely clear, we could not significantly detect any other ORC subunits than ORC2 (these data were not present in the manuscript but will be added in the revised version), therefore we believe that GNL3 may sequester/regulate only ORC2. We tried to see if GNL3 depletion was changing the binding of ORC1 and ORC2 to the chromatin, but we could not see any difference, one possibility may be that small differences are not detectable by chromatin fractionation. We believe that ChIP-seq or ORC2 or other ORC subunits in absence of GNL3 is required but this it out of the scope of the study. GNL3 may regulates the stability of the ORC complex on chromatin via ORC2 but GNL3 may also regulates other ORC2 functions, at centromeres for instance. It has been shown indeed that ORC2 plays roles possibly independently of the ORC complex (see Huang et al. 2016 https://doi.org/10.1016/j.celrep.2016.02.091 or Richards et al. 2022 https://doi.org/10.1016/j.celrep.2022.111590 for instance). How exactly this is affecting origin firing is still mysterious. This is something we are planning to address in the future.

We do not know if it is a quantitatively sink for ORC2 or how this is regulated, however we believe that the ability of GNL3 to accumulate in the nucleolus may sequester ORC2. Consistent with this, we show that a mutant of GNL3 (GNL3-dB) that diffuses in the nucleoplasm interacts more with ORC2 in the nucleoplasm suggesting a release. As suggested by reviewer 1 we will now test if the interaction between ORC2 and GNL3-dB is dependent on the level of expression of GNL3-dB. In addition, we now show that expression of GNL3-dB increases replication origin firing like GNL3 depletion (data that will be added in the revised version), suggesting that regulation of ORC2 is the major cause of increased firing upon GNL3 depletion.

Minor points:

All blots should include size markers

Response: We will add them

Some use of language is not sufficiently precise. For instance:

- the meaning of 'DNA lesions' at the end of the first paragraph of the introduction needs to be more explicit.

- the approach to measurement of these 'lesions' (monitoring gamma-H2Ax) needs to be spelled out explicitly, e.g. line 4 of the last paragraph of the introduction.

- 'we observed that the interaction between GNL3-dB and ORC2 was stronger' ... I do not see how number of foci indicates necessarily the strength of an interaction.

- in many places throughout 'replication origins firing' should be 'replication origin firing' (or 'firing of replication origins').

Response: We will correct these language mistakes.

Reviewer #2 (Significance (Required)):

The model put forward here has the potential to shed light on an important facet of the cellular response to DNA damage, namely the control of origin firing in response to replication stress that will certainly be of interest to the DNA repair / replication community and possibly more widely. The roles of GNL3 are poorly understood and this study could improve this state of affairs. However, the gaps in the mechanism outlined above and somewhat confusing conclusions do limit the ability of the paper to achieve this at present.

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

In this study, Lebdy et al propose a new mechanism to regulate the resection of nascent DNA at stalled replication forks. The central element of this mechanism is nucleolar protein GNL3, whose downregulation with siRNA stimulates DNA resection in the presence of stress induced by HU (Figure 1). Resection depends on the activity of nucleases MRE11 and CtIP, and can be rescued by reintroducing exogenous GNL3 protein in the cells (Figure 1G). GNL3 downregulation decreases fork speed and increases origin activity, without any strong effect on replication timing (Figure 2). Inhibition of Dbf4-dependent kinase CDC7 (a known origin-activating factor) also restricts fork resection (Figure 3). GNL3 interacts with ORC2, one of the subunits of the origin recognition complex, preferentially in nucleolar structures (Figure 4). A mutant version of GNL3 (GNL3-dB) that is not sufficiently retained in the nucleoli fails to prevent fork resection as the WT protein (Figure 5). In the final model, the authors propose that GNL3 controls the levels of origin activity (and indirectly, stalled fork resection) by maintaining a fraction of ORC2 in the nucleoli (Figure 6).

This model is interesting and provocative, but it also relies on a significant degree of speculation. The authors are not trying to "oversell" their observations, because the Discussion section entertains different interpretations and possibilities, and the model itself contains several interrogative statements (e.g. "ORC2-dependent?"; "exhaustion of factors?").

While the article is honest about its own limitations, the major concern remains about its highly speculative nature. I have some questions and suggestions for the authors to consider that could contribute to test (and hopefully support) their model.

1. If GNL3 downregulation induces an excess of licensed origins and mild replicative stress resulting in some G2/M accumulation (Figure 2), what is the consequence of longer-term GNL3 ablation? Do the cells adapt, or do they accumulate signs of chromosomal instability? (micronuclei, chromosome breaks and fusions, etc)

Response: This is an important point also raised by Reviewer 2: deletion of GNL3 leads to embryonic lethality in mouse and ES cells deleted for GNL3 do not proliferate and fail to enter into S-phase. Consistent with this, the clones deleted for GNL3 that we obtained using CRISPR-Cas9 grow poorly, thus preventing us to do experiments. To our knowledge micronuclei and chromosome breaks have never been analyzed upon transient depletion of GNL3 using siRNA. However, it is well established that depletion of GNL3 induces phosphorylation of H2A.X) and the formation of ATR, RPA32 and 53BP1 foci due to S-phase arrest (Lin et al. 2013. https://pubmed.ncbi.nlm.nih.gov/24610951/ ; Meng et al. 2013. https://pubmed.ncbi.nlm.nih.gov/23798389/). DNA lesions have also been visualized by comet assay (Lin et al. 2019. https://pubmed.ncbi.nlm.nih.gov/30692636/). Consistent with this we observed a weak increased of DNA double-strand breaks upon GNL3 depletion using pulse-field gel electrophoresis as well as mitotic DNA synthesis (MiDAS). We can integrate this data in the revised version of the manuscript if required. To sum up, it is clear that GNL3 depletion is inducing problems during S-phase that may lead to possible genomic rearrangements.

2. The model relies on the link between origin activity and stalled fork resection that is almost exclusively based on the results obtained with CDC7i (Figure 3). But CDC7 has other targets besides pre-RC components at the origins, such as Exo1 (from the Weinreich lab, cited in the study), MERIT40 and PDS5B (from the Jallepalli lab, also cited). The effect of CDC7i could be exerted through these factors, which are linked to fork stability and DNA resection. The loss of BRCA1 (Figure 3F) could somehow entail the loss of control over these factors. Could the authors check the possible participation of these proteins?

Response: It is true that CDC7 has other targets than pre-RC components. We therefore decided to inhibit origin firing using roscovitine, a broad CDK inhibitor, a strategy previously used in Lukas lab (Toledo et al. 2013. https://pubmed.ncbi.nlm.nih.gov/24267891/). We observed that treatment with roscovitine decreased significantly resection observed upon GNL3 depletion, confirming the link between origin activity and stalled fork resection. This will be integrated in the revised version of the manuscript. As asked by Reviewer 1, we will also perform depletion of MCM to strength our model.

Exo1 is indeed a target of CDC7 as shown by the Weinreich lab (Sasi et al. 2018. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6111017/) however the authors do not formally

demonstrate that Exo1 phosphorylation is required for its activity. We observed that depletion of Exo1 significantly reduced resection upon GNL3 depletion (data that will be added in the revised version), indicating that the effect of CDC7 inhibitor could be exerted via the control of Exo1. This is why our BRCA1 control is important, it is well stablished that Exo1 is required for nascent strand degradation upon BRCA1 depletion (Lemaçon et al. 2017. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5643552/) but CDC7 inhibition has no effect on resection upon BRCA1 depletion suggesting that resection by Exo1 may not be regulated by CDC7 in our context.

As stated by the reviewer MERIT40 and PDS5B are targets of DDK kinases (Jones et al. 2021 https://doi-org.insb.bib.cnrs.fr/10.1016/j.molcel.2021.01.004) and seem to be required for protection of nascent DNA and in response to HU. However, little is known about the role(s) of these proteins and we think that adding them will complicate message. We hope the reviewer understands this.

3. The model also relies on the fact that GNL3-dB mutant (not retained in the nucleoli) is not sufficient to counteract fork resection induced by HU (Figure 5G). The authors should test directly whether GNL3-dB induces extra origin activation, using their available DNA fibers-based technique.

Response: This is an excellent point. We have now GIFD (Global Instant Fork Density) data that shows that the number of active forks is increased upon dB GNL3-dB expression. It demonstrates that when GNL3 is no longer retained in the nucleolus more origins are active. These data will be integrated in the revised version of the manuscript, and we believe further support the regulation of ORC2 by GNL3.

4. Finally, the model implies an exquisite regulation of the amount of ORC2 protein, which could influence the number of active origins and the extent of fork resection in case of stress. In this scenario, one could predict that ORC2 ectopic expression would have similar, or even stronger effects, than GNL3 downregulation. Is this the case?

Response: We completely agree with this prediction. However, we are afraid that overexpression of ORC2 may have indirect effects due to the many described functions of ORC2, therefore it may be difficult to interpret the data. We will give a try anyway.

5. Even if the connection between origins and fork resection could be firmly established, the molecular link between them remains enigmatic. The authors hint (as "data not shown") that it is neither mediated by RPA nor RAD51. Unfortunately, the reader is left without a clear hypothesis about this point.

Response: We will add data that show that RPA and RAD51 recruitment is not affected by GNL3 depletion. However, the sensitivity of chromatin fractionation approach may be too weak to detect low differences. Based on the work of Lukas Lab (Toledo et al. 2013

https://pubmed.ncbi.nlm.nih.gov/24267891/) one possible mechanism may be exhaustion of the pool of RPA. This may link the excessive activation of origins observed upon GNL3 depletion and resection. To test this, we will check if resection upon GNL3 depletion and treatment with HU is still occurring in cell lines that overexpress RPA complex (described in Toledo et al.) that we obtained from Lukas' lab.

****Referees cross-commenting****

In addition to each reviewer's more specific comments, the three reviews share a main criticism: the lack of mechanistic information about the proposed link between origin activity and resection of nascent DNA at stalled forks.

Reviewer #3 (Significance (Required)):

In principle, this study would appeal to the readership interested in fundamental mechanisms of DNA replication and the cellular responses to replicative stress.

For the reasons outlined in the previous section, I believe that in its current version the study is not strong enough to provide a new paradigm about origins being regulated by partial ORC2 sequestering at nucleoli. The other potentially interesting advance is the connection between frequency of origin activity and the extent of nascent DNA resection at stalled forks, but the molecular link between both remains unknown.

2. 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.

3. 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.

This is the summary of the experiments that we chose not to conduct:

Revision Plan

Roles of helicases in resection upon GNL3 depletion (Reviewer 1). We think this will complicate our message without adding much. Moreover, it will require to buy siRNA and antibodies to test them,

Colocalization between centromeric markers and gammaH2.AX (Reviewer 1). Given the strong gammaH2A.X signal upon GNL3 depletion (see references in the responses to reviewers), colocalization with centromeric markers like CENP-A will not be informative.

Roles of MERIT4 and PDS5B (Reviewer 3). The roles of these factors in resection of stalled forks is still mysterious. We feel that testing them will not be informative regarding the main message of our work.

Dear Dr. Ribeyre,

Thank you for the submission of your manuscript and your proposed revision plan to EMBO reports, and I am sorry for my delayed reply. I had contacted referee 2 for comments on your revision plan, and her/his comments are now pasted below.

We agree that your study is potentially interesting, and that your proposed revisions might render it suitable for publication here. We would thus like to invite you to revise your manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. Please also consider and address referee 2's additional comments pasted below. Feel free to contact me if you have any comments or questions regarding the revisions.

Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of major revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

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 (20th Sep 2023). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions.

IMPORTANT NOTE: we perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:
1) A data availability section providing access to data deposited in public databases is missing. If you have not deposited any data, please add a sentence to the data availability section that explains that.
2) Your manuscript contains statistics and error bars based on n=2. Please use scatter blots in these cases. No statistics should be calculated if n=2.

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure). See https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf for more info on how to prepare your figures.

3) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here:

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

4) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

5) a complete author checklist, which you can download from our author guidelines . Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

6) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines

7) Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database (see https://www.embopress.org/page/journal/14693178/authorguide#datadeposition). Please remember to provide a reviewer password if the datasets are not yet public. The accession numbers and database should be listed in a formal "Data

Availability" section placed after Materials & Method (see also https://www.embopress.org/page/journal/14693178/authorguide#datadeposition). Please note that the Data Availability Section is restricted to new primary data that are part of this study. * Note - All links should resolve to a page where the data can be accessed. *
If your study has not produced novel datasets, please mention this fact in the Data Availability Section.

8) At EMBO Press we ask authors to provide source data for the main manuscript figures. Our source data coordinator will contact you to discuss which figure panels we would need source data for and will also provide you with helpful tips on how to upload and organize the files.

9) Our journal also encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at https://www.embopress.org/page/journal/14693178/authorguide#referencesformat

10) Regarding data quantification (see Figure Legends: https://www.embopress.org/page/journal/14693178/authorguide#figureformat)

The following points must be specified in each figure legend:

- the name of the statistical test used to generate error bars and P values,

- the number (n) of independent experiments (please specify technical or biological replicates) underlying each data point,

- the nature of the bars and error bars (s.d., s.e.m.),

- If the data are obtained from n Program fragment delivered error ``Can't locate object method "less" via package "than" (perhaps you forgot to load "than"?) at //ejpvfs23/sites23b/embor_www/letters/embor_decision_rc_revise_and_rereview.txt line 56.' 2, use scatter blots showing the individual data points.

Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.

- Please also include scale bars in all microscopy images.

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File (RPF) to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

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I look forward to seeing a revised form of your manuscript when it is ready.

Best regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee 2's additional comments:

Looking at the authors' responses to my comments I think they are generally fine, but some further explanation of some points in the revision may help the reader.

Point 1. Level of knockdown and use of CRISPR. Fine, as GNL3 is essential. Make sure this is clear in the discussion. I think it would be valuable to mention somewhere the attempts to create a regulatable version and CRISPR ko even though negative as this may be valuable for those pursuing these observations further.

Point 2. OK. I think it would be useful to clearly explain the rationale for this approach. I have no doubt about the observations, it's just the interpretation where the general reader might need some help.

Point 3. Replication stress. Fine.

Point 4. Relation between origin firing and resection. I think really this primarily just needs more clear explanation. The 'exhaustion' hypothesis is interesting, but needs clear discussion in terms of how this leads to greater resection. The additional experiment with the cell lines from the Toledo paper could indeed be valuable.

Point 5. Effects of ATRi/Wee1i on origin firing and resection. The model is clearer, but in the revision do take the space to explain really clearly and emphasise that this is not really a direct link i.e. the act of origin firing per se induces resection. The experiments proposed in response to the other reviewers should also strengthen the model.

Point 6. Sequestration of other ORC components. This is fine for the present paper.

I think the responses to the other reviewers comments will also strengthen the paper. Much can be done to improve it also by careful explanation of the model and suitable caveats to the limitations of the authors preferred interpretation.

Response to reviewers

We deeply thank the reviewers for the time spent on evaluating our manuscript as well as providing comments and suggestions to improve our study. We performed most of the changes and new experiments proposed and we think it greatly increases the quality of our study. We hope the reviewers will agree with us.

The major changes in the text to clarify the message and describe the new data are highlighted in yellow. This is a summary of changes and additions that has been made in the revised version for figures:

Figure 1:
Depletion of EXO1 was added in panel 1E
Figure EV1:
Modification of 1I and 1J to show depletion of EXO1
Addition of 1M: chromatin fractionation experiment
Figure 2:
Addition of 2F: analysis of IdU tracts length GNL3 depletion in presence of CDC7 inhibitor
Figure EV2:
Addition of 2E: biological replicates of the experiment of Figure 2F
Figure 3:
Addition of 3G: level of resection upon GNL3 depletion and treatment with roscovitine
Addition of 3H: level of resection upon GNL3 depletion and MCM3 depletion
Addition of 3I: level of resection upon GNL3 depletion in U-2 OS cells control and SuperRPA
Figure EV3:
Addition of 3H: biological replicates of the experiment of Figure 3G
Addition of 3I: Western-blot to show MCM3 depletion
Addition of 3J: biological replicates of the experiment of Figure 3H
Addition of 3K: Western-blot to show the depletion of GNL3
Addition of 3L: biological replicates of the experiment of Figure 3I
Figure 4: no change
Figure EV4: no change
Figure 5
5G and 5H moved to new figure 6
Addition of 5G: measurement of GIFD upon GNL3-dB expression
Figure 6 (new figure)
6B: level of resection upon expression of GNL3-dB and treatment with CDC7 inhibitor
6D: analysis of interaction between GNL3-dB and ORC2 with increasing amount of doxycycline
6E: level of resection upon expression of FLAG-ORC2
Figure EV5
5C: biological replicate of the experiment of Figure 5G
5E: biological replicates of the experiment of Figure 6B
5I: immunofluorescence analysis to show ectopic expression of ORC2
5J: biological replicates of the experiment of Figure 6E
Figure 7: was figure 6 before, a scheme to explain the putative role of GNL3 in ORC2 sequestration has been added

Reviewer #1 (Evidence, reproducibility and clarity (Required)):

In this manuscript Lebdy et al. describe a new role of GNL3 in DNA replication. They show that GNL3 controls replication fork stability in response to replication stress and they propose this is due to the regulation of ORC2 and the licensing of origins of replication. Their data suggest that GNL3 regulates the sub nuclear localization of ORC2 to limit the number of licensed origins of replication and to prevent resection of DNA at stalled forks in the presence of replication stress.

While many of the points of the manuscript are proven and well supported by the results, there are some experiments that could improve the quality and impact of the manuscript. The main issue is that the connection between the role of GNL3 in controlling ORC2, the firing of new origins and the protection of replication forks is not clearly established. At the moment the model relies on mainly correlative data. In order to further substantiate the model, we propose to address some of the following issues:

1. The authors indicate that RPA and RAD51 accumulation at stalled forks is not affected by GNL3 depletion. These data should be included and other proteins should be analysed. In addition, the role of helicases could be explored through the depletion of the main helicases involved in the remodelling of the forks.

Response: We now added fractionation experiments made in presence of HU that show that the level of RAD51 on chromatin is not strongly affected by GNL3 depletion (Fig EV1M), RPA recruitment was slightly increased which is consistent with its increased level of phosphorylation. So far, the other proteins we checked (RIF1 and BRCA1), both involved in nascent strand protection, did not show major differences (Fig EV1M). Therefore, we concluded that depletion of GNL3 does not seem to affect the recruitment of major proteins required for protection of nascent DNA. Of course, we cannot exclude that other proteins may be affected by GNL3 depletion, but testing all the possible candidates would be time consuming with a very low chance of success. In addition, fractionation experiments are possibly not quantitative enough to uncover small differences and may be not that informative. Thus, it remained possible that RPA exhaustion may be the cause of resection in absence of GNL3 as suggested by the work conducted in Jiri Lukas' lab (Toledo et al. 2013. https://pubmed.ncbi.nlm.nih.gov/24267891/). To test this hypothesis, we analyzed if resection in absence of GNL3 is still occurring in a well-characterized cell line (SuperRPA) that overexpresses the three RPA subunits that we obtained from Jiri Lukas' lab. We now show that, in contrast to the control, overexpression of RPA strongly decreased the resection observed upon GNL3 depletion (Fig3I, EV3K, EV3L). From this experiment we conclude that the resection that occurs upon GNL3 depletion is caused by RPA exhaustion due to excessive number of fired replication origins that titrate RPA. This model is inspired by the work of Jiri Lukas and is now detailed in the manuscript.

To our knowledge not many helicases have been shown to be involved in remodeling of stalled forks. The best example is RECQ1, however we feel that testing RECQ1 involvement in resection upon GNL3 depletion will complicate our story without adding much regarding the mechanism. We hope the reviewer understands our concern.

2. The proposed model implies that GNL3 depletion leads to increased origin licensing. FThe authors should address if the primary effect of GNL3 depletion is on origin firing by using CDC7 inhibition in the absence of stress (Rodríguez-Acebes et al., JBC 2018).

Response: This is an excellent point raised by the reviewer. To test if the primary effect of GNL3 depletion in on origin firing we analyzed forks velocity in GNL3-depleted cells upon CDC7 inhibition in basal conditions (Fig 2F, EV2E). We could show that the decreased in fork speed observed upon GNL3 depletion is suppressed by CDC7 pre-treatment. We conclude that GNL3 depletion primary increases the number of origins that fire, that in turn reduces replication forks speed.

3. A way to prove that origin firing mediates the effect of GNL3 on fork protection would be to reduce the number of available origins. The depletion of MCM complexes has been shown to limit the number of back-up origins that are licensed and leads to sensitivity to replication stress (Ibarra et al., PNAS 2008). If GNL3 depletion results in increased number of origins, this effect should be prevented by the partial depletion of MCM complexes.

Response: This is also an excellent point. We partially depleted MCM3 (Fig 3H, EV3I, EV3J) and observed that its depletion decreases DNA resection in absence of GNL3. This result was also confirmed using roscovitine, a DDK inhibitor that reduces replication origin firing (Fig 3G, EV3H). To our opinion these new set of experiments strengthens the results obtained with CDC7 inhibitors and indicate that excessive activation of replication origins in presence of replication stress induces DNA resection.

4. Alternatively, the authors could try to modulate the depletion of GNL3. Origin licensing takes place in the G1 phase and thus the depletion of GNL3 by siRNA could affect the following S phase. Using an inducible degron for GNL3 depletion would allow to deplete GNL3 in G1 or S phase specifically. If the model is correct, the removal of GNL3 in S phase should not affect fork protection but removing GNL3 in the previous G2/M phase should reduce the number of licensed origins and lead to impaired fork protection.

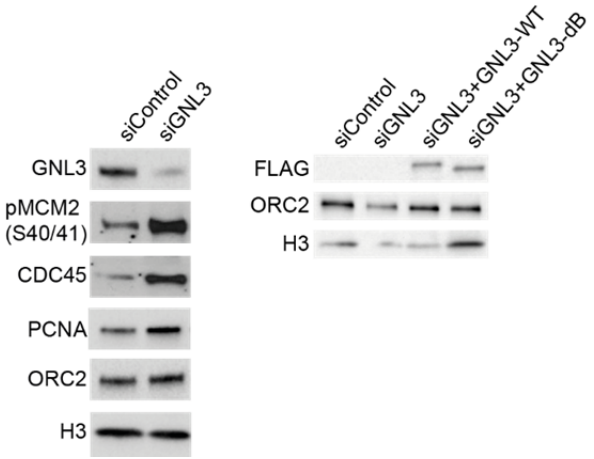
Response: This is obviously a good point given the fact that *GNL3* deletion is not viable (see responses to reviewer 2). We tried to develop an auxin induced degron of GNL3, but we could not obtain homozygous clones, meaning that our clones had always an untagged *GNL3* allele. Since *GNL3* is essential its tagging may impair its function, explaining why we could not obtain homozygous clones. However, we are planning to try again with an optimized design using other degrons system for instance to address the role of GNL3 specifically during S-phase. But we think this is above the scope of the present study. We hope the reviewer will agree with us.

In addition to the connection GNL3-origin firing-fork protection, it is unclear how the lack of GNL3 in the nucleolus and the change in the sub nuclear localization of ORC2 controls origin firing and resection. The strong interaction observed between GNL3-dB and ORC2, and the subsequent

change in ORC2 localization does not explain how origin licensing can be affected. In this sense, the authors could address:

1. Does the depletion of GNL3 and the expression of GNL3-dB affect the formation of the ORC complex, its subnuclear localization or its binding to chromatin? The authors have not explored if the interaction of GNL3 with ORC2 is established in the context of the ORC complex. An IF showing NOP1 with PLA data from GNL3-dB and ORC2 is needed to analyse how the expression of increasing amounts of GNL3-dB affects ORC2.

Response: We tested if GNL3 depletion impacts ORC2 recruitment on chromatin, but we could not observe significant and reproducible differences (figure below). No clear difference on ORC2 recruitment on chromatin was observed upon GNL3-dB expression either (figure below). One reason for this may be due to the excess of ORC complex on the chromatin, in addition chromatin fractionation is likely not sensitive enough to observe small differences. We think that quantitative ChIP-seq of ORC2 or other ORC subunits upon GNL3 depletion is required to visualize such differences, but this is above the scope of the study, and this constitutes the following of this project.



We also tried to look at subnuclear localization of ORC2 using immunofluorescence, but the signal was not specific enough to observe differences. We think, however, that the increased interaction (PLA) of ORC2 with GNL3-dB (Fig 5E) demonstrates a change in ORC2 subnuclear localization. To confirm this, we performed the excellent experiment proposed by the reviewer and observed that the PLA signal between ORC2 and GNL3-dB tends to increase with the level of expression of GNL3-dB, this result reinforce the idea that the expression of GNL3-dB induces the release of ORC2 on the nucleoplasm (Fig 6D). We now added a model to show how the accumulation of GNL3 in the nucleolus may sequester ORC2 to limit the number of licensed origins (Fig 7B).

We do not think that the interaction between GNL3 and ORC2 is occurring in the context of the ORC complex for 3 main reasons: **(1)** Our GNL3 BioID experiment coupled with mass spectrometry identifies only ORC2 (Fig 4A), the other subunits were never identified in 4 independent experiments. The full list of proteins from the GNL3 Bio-ID experiment is now

provided in the revised version (Table EV2). **(2)** The interaction of ORC2 and GNL3 is occurring mainly in the nucleolus and not in the nucleoplasm where ORC complex is localized on chromatin (Fig 4D). **(3)** No overlap between GNL3 binding regions and ORC2 binding sites was observed (Fig 4C). Based on this, we think that either GNL3 regulates ORC2 subnuclear localization that in turns impact the ORC complex or GNL3 regulates ORC2-specific functions. More and more evidences show that ORC2 plays roles possibly independently of the ORC complex (see Huang et al. 2016 https://doi.org/10.1016/j.celrep.2016.02.091 or Richards et al. 2022 https://doi.org/10.1016/j.celrep.2022.111590 for instance). Future work should uncover how these ORC2 functions may regulate origins activity.

2. In order to confirm if the mislocalization of ORC2 by the expression of GNL3-dB increases origin firing and mediates the effects on fork protection the authors could check DNA resection levels inhibiting CDC7 in high GNL3-dB conditions. Also, the levels of MCM2, phosphor-MCM2, CDC45, have not been analysed upon expression of GNL3-dB.

Response: This is a good point, we analyzed if the resection observed upon expression of GNL3-dB is dependent on origin firing using the CDC7 inhibitor and found that this is indeed the case (Fig 6B, EV5E), this result suggests that increased origin firing occurs upon GNL3-dB expression. We tested this hypothesis by performing DNA combing to measure Global Instant Fork Density, an assay that is more robust that measuring the level of the cited proteins. We now show that expression of GNL3-WT suppresses the increased origin firing observed upon GNL3 depletion, in contrast expression of GNL3-dB does not suppress it (Fig 5G). This important result confirms that origin firing is increased upon GNL3-dB expression, providing a link between aberrant localization and increased firing.

3. The data in the paper suggest that GNL3 may affect the role of ORC2 in centromeres. Since depletion of GNL3 leads to increased levels of γH2AX, it would be interesting to address if this damage is due to incomplete replication in centromeres by analysing the co-localization of γH2AX and centromeric markers both in unstressed conditions and upon the induction of replication stress.

Response: This is indeed and interesting comment, however since it has been previously shown that γH2AX signal is rather strong upon GNL3 depletion (see Lin et al. 2013. https://pubmed.ncbi.nlm.nih.gov/24610951/ ; Meng et al. 2013. https://pubmed.ncbi.nlm.nih.gov/23798389/) we do not think that co-localization experiments with CENP-A for instance will be informative given the high number of γH2AX foci. We hope the reviewer understand this issue.

Minor points:

1. In the initial esiRNA screen the basal levels of γH2AX should also be shown.

Response: Our negative control is the transfection of an esiRNAs that targets EGFP (a gene that is not expressed in the tested cell line). This esiRNAs is ranked at the end of the list and

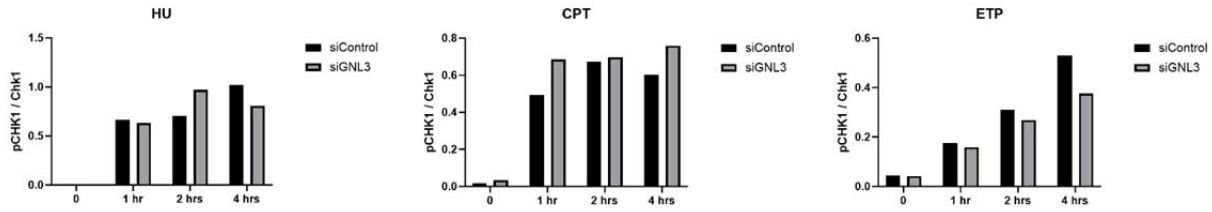
therefore constitutes the basal level of γH2AX signal. In any case it is well-established that GNL3 depletion increases γH2AX signal (see Lin et al. 2013. https://pubmed.ncbi.nlm.nih.gov/24610951/ ; Meng et al. 2013. https://pubmed.ncbi.nlm.nih.gov/23798389/).

2. Figure EV1B: I think the rank needs another RS mark to see better the effect of each esiRNA on DNA lesions (high variability in all the conditions showed).

Response: We understand this issue, but we cannot repeat this set of experiments for technical reasons (reagents and cost mainly). Anyway, we believe that the role of GNL3 is response to replication stress is extensively addressed by other experiments of this manuscript.

3. Figure 1C and Figure EV1D/E: the quantification of the pCHK1/CHK1 levels could be included to show that there are no changes in phosphorylation upon GNL3 depletion.

Response: We performed this quantification but we could see not major differences (Figure Below) so we decided to not integrate these data in the manuscript.

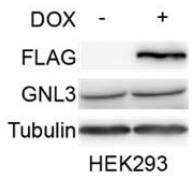


4. In the first section of the results, at the end Figure 4B is incorrectly called for.

Response: Thanks for the comment, we modified accordingly.

5. The levels of GLN3 expression in 293 cells should be already included in section GNL3 interacts with ORC2.

Response: We used only HEK293 flp-in cells for the Bio-ID experiment (Fig 4A). These cells have an integrated GNL3-BirA-FLAG copy inducible with doxycycline that biotinylates proteins in vicinity (Fig EV4A). They express the endogenous GNL3 at expected level (Figure below). We can integrate this figure if the reviewer thinks it is necessary.



6. The full MS data needs to be included for both GNL3 and ORC2.

Response: This is now integrated in Table EV2.

7. Figure 4B should be improved, since there is a faint band in the IgG mouse control.

Response: it is true that the figure is not perfect, but we believed that our Bio-ID and PLA experiments fully demonstrate the interaction between GNL3 and ORC2.

Reviewer #1 (Significance (Required)):

The work is nicely written, the figures are well presented and the experiments have the necessary controls. It provides relevant information to understand how replication stress is controlled and linked to replication fork protection through origin firing. These results are relevant to the field, linking GNL3 to origin firing and with potential to help understand the role of GNL3 in cancer. They provide new information and can give rise to new studies in the future. Many of the conclusions of the manuscript are well supported. Additional support for some of the main claims would strengthen the results and also increase the impact providing a bigger conceptual advance by performing some of the suggested experiments.

Response: We thank the reviewer for his/her positive feedback as well as the time spent on reading our manuscript**.** We believe that the new experiments we added strengthen our model, we hope the reviewer will agree on this and we thank her/him again for its suggestions.

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

This manuscript explores the role of GNL3/nucleostemin in DNA replication and specifically in the response of DNA replication to DNA damage. GNL3 is a predominantly nucleolar protein, previously characterised as a GTP-binding protein and shown to be necessary for effective recruitment of the RAD51 recombinase to DNA breaks. The entry point for this report is a mini screen, based on proteins identified previously by the authors to associate with replication forks by iPOND, for factors that increase gamma-H2Ax (an indicator of DNA damage) after treatment with the Top1 inhibitor camptothecin (CPT). In this mini-screen GNL3 emerged as the top hit.

The authors put forward the hypothesis that GNL3 is able to sequester the replication licensing factor ORC2 in the nucleolus and that failure of this mechanism leads to excessive origin firing and DNA resection following CPT treatment.

The model put forward is interesting, but currently rather confusing. However, for the reasons upon which I expand below, I do not believe that the data provide a compelling mechanistic explanation for the effects that are reported and I am left not being certain about some of the links that are made between the various parts of the study, even though individual observations appear to be of good quality.

Specific points:

The knockdown of GNL3 is very incomplete. In this regard, the complementation experiments are welcome and important. However, is it an essential protein? Can it be simply deleted with CRISPR-Cas9?

Response: There are obviously variations between experiments but overall, the depletion of GNL3 using siRNA seems good in our opinion. Deletion of GNL3/nucleostemin leads to embryonic lethality in mouse (Beekman et al. 2006. https://pubmed.ncbi.nlm.nih.gov/17000755/ ; Zhu et al. 2006. https://pubmed.ncbi.nlm.nih.gov/17000763/). ES cells deleted for GNL3 can be obtain but do not proliferate probably because of their inability to enter in S-phase (Beekman et al. 2006. https://pubmed.ncbi.nlm.nih.gov/17000755/). We wanted to test if it was the case in our cellular model and we tried to delete it using CRISPR-Cas9. We managed to obtain few clones deleted for GNL3, but they grow poorly prevented us to do experiments. To bypass this, and as suggested by the reviewer 1, we tried to make an auxin-induced degron of GNL3. Unfortunately, we did not manage to obtain homozygous clones, only heterozygous. One possibility could be that the tagging induced a partial loss of function of GNL3, and since GNL3 is essential, it may explain why we did not obtain homozygous clones. We may also want to use alternative degron systems such as Halo-Tag, but we believe this is out of the scope of the study.

Response from reviewer:

Point 1. Level of knockdown and use of CRISPR. Fine, as GNL3 is essential. Make sure this is clear in the discussion. I think it would be valuable to mention somewhere the attempts to create

a regulatable version and CRISPR ko even though negative as this may be valuable for those pursuing these observations further.

Response: We added few sentences at the very end of the discussion to mention these issues.

Global instant fork density is not quite the same as actually measuring origin firing. Ideally, it would be good to see some more direct evidence of addition origin firing e.g. by EdU-seq (Macheret & Halazonetis Nature 2018) but this would be quite a significant additional undertaking. However, given the authors have performed DNA combing with DNA counterstain, they should be able to provide accurate measurements of origin density and inter-origin distance.

Response: As indicated by the reviewer EdU-seq would need a lot of development since we are not using this approach in our team. In addition, this method can detect replication origins only if performed in the beginning of S-phase, meaning that only the early firing origins will be detected and not the others. GIFD measurement is actually directly linked with origin firing since it is counting the forks to duplicate the genome. The measurements of IODs have at least two main limitations: (1) there is a bias for short IODs due to the length of analyzed fibers and (2) it focuses only on origins within a cluster not globally. Overall, we believe that GIFD is the method of choice to measures origins firing. In addition, these experiments have been done by the lab of Etienne Schwob (see acknowledgments), a leader in the field.

Response from reviewer: *Point 2. OK. I think it would be useful to clearly explain the rationale for this approach. I have no doubt about the observations, it's just the interpretation where the general reader might need some help.*

Response: We provide more explanations on the method when we are describing these results.

'Replication stress' is induced with CPT. This term is frequently used to describe events that lead to helicase-polymerase uncoupling (e.g. O'Connor Mol Cell 2015) but that is not the case with CPT, which causes fork collapse and breaks. Are similar effects seen with e.g. UV or cisplatin? Additionally, a clear statement of the authors definition of replication stress would be welcome.

Response: We will better define the term ‘replication stress’ in the revised version of the manuscript. It should be understood, in our case, that any impediment that leads to replication fork stalling and measurable by DNA combing or Chk1 phosphorylation. We have not performed experiments using UV and cisplatin.

Response from reviewer: *Point 3. Replication stress. Fine.*

Response: We modified our definition of replication stress in the introduction (3rd paragraph).

It is really not clear how the authors explain the link between potential changes in origin firing

and resection. i.e. What is the relationship between global origin firing and resection at a particular fork, presumably broken by encounter with a CPT-arrested TOP1 complex. What is the link mechanistically? This link needs elaborating experimentally or clearly explaining based on prior literature.

Response: Most of our results on resection has been performed with hydroxyurea, but it is true that we saw resection in absence of GNL3 in response to CPT. Treatment with HU or CPT reduces fork speed and activates additional replication origins (see Ge et al. 2007 https://pubmed.ncbi.nlm.nih.gov/18079179/ for HU or Hayakawa et al. 2021 https://pubmed.ncbi.nlm.nih.gov/34818230/ for CPT). When GNL3 is depleted, more forks are active, meaning more targets for HU and CPT. In addition, it is likely that the firing of additional origins in response to HU and CPT is stronger in absence of GNL3. Because of this we believe that factors required to protect stalled forks may be exhausted explaining why resection is observed. This is inspired by the work of Lukas' lab (Toledo et al. 2013 https://pubmed.ncbi.nlm.nih.gov/24267891/) and is described in the figure 6. One obvious candidate that may be exhausted is RPA, to test this we will check if resection upon GNL3 depletion and treatment with HU is still occurring in cell lines provided by Lukas' lab that overexpress RPA complex (described in Toledo et al.). We will explain our model more carefully in the revised version.

Response from reviewer: *Point 4. Relation between origin firing and resection. I think really this primarily just needs more clear explanation. The 'exhaustion' hypothesis is interesting, but needs clear discussion in terms of how this leads to greater resection. The additional experiment with the cell lines from the Toledo paper could indeed be valuable.*

Response: In the revised version we added new experiments suggested by the other reviewers where we inhibit origin firing using roscovitine (CDK inhibitor) or MCM3 depletion (Fig 3G, 3H, EV3H, EV3I, EV3J). Both conditions decreased the increased resection upon GNL3 depletion, thus confirming the link between origin firing and resection. We also performed experiments in the SuperRPA cell line, and we show that resection upon GNL3 depletion was strongly decreased compared to the control (Fig 3I, EV3K, EV3L). We conclude that excessive firing upon GNL3 depletion and HU treatment inducing RPA exhaustion that leads to increased resection. These new data are now described and discussed in the manuscript.

Related to this, I remain unconvinced that the experiments in Figure 3 show that the effects of ATRi and Wee1i on origin firing and on resection are contingent on each other. I do not believe that the authors have adequately supported the statement (end of pg 9) 'We conclude that the enhanced resection observed upon GNL3 depletion is a consequence of increased origin firing.' The link between origin firing and resection needs really needs further substantiation and / or explanation.

Response: Our rational was the following. Inhibition of ATR or WEE1 increase replication origin firing, a situation that may be like the one observed for GNL3 depletion. In Toledo et al, they show that inhibition of WEE1 or ATR induces exhaustion of RPA. This exhaustion is reduced in

presence of CDC7 inhibitor, roscovitine (a CDK inhibitor that inhibits origin firing) or depletion of CDC45, indicating that this is due to excessive origin activation. In our case we show that the resection observed upon WEE1 or ATR inhibition is reduced upon treatment with CDC7 inhibitor. We conclude that excessive replication origin firing induces DNA resection. Since we observed the same thing upon GNL3 depletion (but not upon BRCA1 depletion) we conclude that excessive origin firing favors DNA resection likely through exhaustion of RPA. As indicated above we will test this hypothesis by overexpressing RPA. In addition, we now show that treatment with roscovitine decreases resection upon GNL3 depletion (this will be part of the revised manuscript), an experiment that we believe confirms that excessive replication origins firing is responsible for resection upon GNL3 depletion. As suggested by reviewer 1, we will also test if depletion of MCM also reduces resection observed in absence of GNL3.

Response from reviewer: *Point 5. Effects of ATRi/Wee1i on origin firing and resection. The model is clearer, but in the revision do take the space to explain really clearly and emphasise that this is not really a direct link i.e. the act of origin firing per se induces resection. The experiments proposed in response to the other reviewers should also strengthen the model.*

Reponse: As stated in the answer before, we now have different pieces of evidence that links excessive origin firing and resection through RPA exhaustion. This is now explained in the discussion. This is true for resection occurring upon GNL3 depletion but also for resection upon ATR or WEE1 inhibition since it is established that exhaustion of RPA is occurring in these conditions (Toledo et al. 2013 https://pubmed.ncbi.nlm.nih.gov/24267891/).

It is not clear whether the binding of ORC2 to GNL3 also sequesters other components of the origin recognition complex? Does loss of the ability of GNL3 to bind ORC2 actually lead to more ORC bound to chromatin? How does GNL3 contribute to regulation of origin firing under normal conditions? Is it a quantitatively significant sink for ORC2 and what regulates ORC2 release?

Response: The results of GNL3 Bio-ID were extremely clear, we could not significantly detect any other ORC subunits than ORC2 (these data were not present in the manuscript but will be added in the revised version), therefore we believe that GNL3 may sequester/regulate only ORC2. We tried to see if GNL3 depletion was changing the binding of ORC2 to the chromatin, but we could not see any difference, one possibility may be that small differences are not detectable by chromatin fractionation. We believe that ChIP-seq or ORC2 or other ORC subunits in absence of GNL3 is required but this it out of the scope of the study. GNL3 may regulates the stability of the ORC complex on chromatin via ORC2 but GNL3 may also regulates other ORC2 functions, at centromeres for instance. It has been shown indeed that ORC2 plays roles possibly independently of the ORC complex (see Huang et al. 2016 https://doi.org/10.1016/j.celrep.2016.02.091 or Richards et al. 2022 https://doi.org/10.1016/j.celrep.2022.111590 for instance). How exactly this is affecting origin firing is still mysterious. This is something we are planning to address in the future.

We do not know if it is a quantitatively sink for ORC2 or how this is regulated, however we believe that the ability of GNL3 to accumulate in the nucleolus may sequester ORC2. Consistent

with this, we show that a mutant of GNL3 (GNL3-dB) that diffuses in the nucleoplasm interacts more with ORC2 in the nucleoplasm suggesting a release. As suggested by reviewer 1 we will now test if the interaction between ORC2 and GNL3-dB is dependent on the level of expression of GNL3-dB. In addition, we now show that expression of GNL3-dB increases replication origin firing like GNL3 depletion (data that will be added in the revised version), suggesting that regulation of ORC2 is the major cause of increased firing upon GNL3 depletion.

Response from reviewer: Point 6. Sequestration of other ORC components. This is fine for the present paper.

Response: We now show that the level of interaction between ORC2 and GNL3-dB is largely dependent of its level of expression (Fig 6D). This experiment suggested by reviewer 1 support the possibility that GNL3 sequesters ORC2 in the nucleolus. In addition to this, we performed an experiment suggested by reviewer 3 that show that expression of an ectopic version of ORC2 induces resection upon HU treatment (Figure 6E, EV5I, EV5J). We believe this experiment highlights the importance of the regulation of ORC2 level in the nucleoplasm to limit replication stress in response to HU. We added a putative model (Fig 7B) to try to explain how GNL3 may regulate ORC2 sub nuclear localization.

Minor points:

All blots should include size markers

Response: We usually not add them because our panels and made of different blots and we find it quite confusing. In addition, the antibodies we are using are commercially available and fully validated.

Some use of language is not sufficiently precise. For instance:

- the meaning of 'DNA lesions' at the end of the first paragraph of the introduction needs to be more explicit.

Response: This has been moved to the third paragraph and we added “DNA lesions such as single-strand gaps or DNA double-strand breaks (DSBs).”

- the approach to measurement of these 'lesions' (monitoring gamma-H2Ax) needs to be spelled out explicitly, e.g. line 4 of the last paragraph of the introduction.

Response: We better explained our approach “Here, we performed a small siRNA screen to identify those novel factors whose depletion increases the number of DNA lesions, such as DSBs or single-strand gaps, in response to exogenous replication stress using H2A.X phosphorylation (γH2A.X) as a readout.”

- 'we observed that the interaction between GNL3-dB and ORC2 was stronger' ... I do not see how number of foci indicates necessarily the strength of an interaction.

Response: We agree with the reviewer and changed the sentence by "*'we observed that the interaction between GNL3-dB and ORC2 occurred more frequently"*

- in many places throughout 'replication origins firing' should be 'replication origin firing' (or 'firing of replication origins').

Response: We changed accordingly in the text.

Reviewer #2 (Significance (Required)):

The model put forward here has the potential to shed light on an important facet of the cellular response to DNA damage, namely the control of origin firing in response to replication stress that will certainly be of interest to the DNA repair / replication community and possibly more widely. The roles of GNL3 are poorly understood and this study could improve this state of affairs. However, the gaps in the mechanism outlined above and somewhat confusing conclusions do limit the ability of the paper to achieve this at present.

Response: We are grateful to the reviewer for his/her positive outcome and on the time spent on evaluating the manuscript. We also thank the reviewer for his/her comments and suggestions that we believe enriched deeply our manuscript. We hope that the reviewer will agree that the modifications of the manuscript and the new experiments performed strongly our model.

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

In this study, Lebdy et al propose a new mechanism to regulate the resection of nascent DNA at stalled replication forks. The central element of this mechanism is nucleolar protein GNL3, whose downregulation with siRNA stimulates DNA resection in the presence of stress induced by HU (Figure 1). Resection depends on the activity of nucleases MRE11 and CtIP, and can be rescued by reintroducing exogenous GNL3 protein in the cells (Figure 1G). GNL3 downregulation decreases fork speed and increases origin activity, without any strong effect on replication timing (Figure 2). Inhibition of Dbf4-dependent kinase CDC7 (a known origin-activating factor) also restricts fork resection (Figure 3). GNL3 interacts with ORC2, one of the subunits of the origin recognition complex, preferentially in nucleolar structures (Figure 4). A mutant version of GNL3 (GNL3-dB) that is not sufficiently retained in the nucleoli fails to prevent fork resection as the WT protein (Figure 5). In the final model, the authors propose that GNL3 controls the levels of origin activity (and indirectly, stalled fork resection) by maintaining a fraction of ORC2 in the nucleoli (Figure 6).

This model is interesting and provocative, but it also relies on a significant degree of speculation. The authors are not trying to "oversell" their observations, because the Discussion section entertains different interpretations and possibilities, and the model itself contains several interrogative statements (e.g. "ORC2-dependent?"; "exhaustion of factors?").

While the article is honest about its own limitations, the major concern remains about its highly speculative nature. I have some questions and suggestions for the authors to consider that could contribute to test (and hopefully support) their model.

1. If GNL3 downregulation induces an excess of licensed origins and mild replicative stress resulting in some G2/M accumulation (Figure 2), what is the consequence of longer-term GNL3 ablation? Do the cells adapt, or do they accumulate signs of chromosomal instability? (micronuclei, chromosome breaks and fusions, etc)

Response: This is an important point also raised by Reviewer 2: deletion of *GNL3* leads to embryonic lethality in mouse and ES cells deleted for *GNL3* do not proliferate and fail to enter S-phase. Consistent with this, the clones deleted for *GNL3* that we obtained using CRISPR-Cas9 grow poorly, thus preventing us to do experiments. To our knowledge micronuclei and chromosome breaks have never been analyzed upon transient depletion of GNL3 using siRNA. However, it is well established that depletion of GNL3 induces phosphorylation of H2A.X) and the formation of ATR, RPA32 and 53BP1 foci due to S-phase arrest (Lin et al. 2013. https://pubmed.ncbi.nlm.nih.gov/24610951/ ; Meng et al. 2013. https://pubmed.ncbi.nlm.nih.gov/23798389/). DNA lesions have also been visualized by comet assay (Lin et al. 2019. https://pubmed.ncbi.nlm.nih.gov/30692636/). Consistent with this, we observed a weak increased of DNA double-strand breaks upon GNL3 depletion using pulse-field gel electrophoresis as well as mitotic DNA synthesis (MiDAS). We chose to not integrate these data because they are not convincing enough to our opinion, stronger inactivation of GNL3

(using a degron for instance) would be required. To sum up, it is clear that GNL3 depletion is inducing problems during S-phase that may lead to possible genomic rearrangements.

2. The model relies on the link between origin activity and stalled fork resection that is almost exclusively based on the results obtained with CDC7i (Figure 3). But CDC7 has other targets besides pre-RC components at the origins, such as Exo1 (from the Weinreich lab, cited in the study), MERIT40 and PDS5B (from the Jallepalli lab, also cited). The effect of CDC7i could be exerted through these factors, which are linked to fork stability and DNA resection. The loss of BRCA1 (Figure 3F) could somehow entail the loss of control over these factors. Could the authors check the possible participation of these proteins?

Response: It is true that CDC7 has other targets than pre-RC components. We therefore inhibited origin firing using roscovitine, a broad CDK inhibitor, a strategy previously used in Lukas lab (Toledo et al. 2013. https://pubmed.ncbi.nlm.nih.gov/24267891/). We observed that treatment with roscovitine decreased significantly resection observed upon GNL3 depletion, confirming the link between origin activity and stalled fork resection (Fig 3G, EV3H). As asked by Reviewer 1, we partially depleted MCM3 (Fig 3H, EV3I, EV3J) and observed a decrease of DNA resection in absence of GNL3. We think that this data strengthens the impact on origin firing on resection upon GNL3 depletion.

EXO1 is indeed a target of CDC7 as shown by the Weinreich lab (Sasi et al. 2018. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6111017/) however the authors do not formally demonstrate that EXO1 phosphorylation is required for its activity. We now show that depletion of EXO1 significantly reduced resection upon GNL3 depletion (Fig 1E, EV1I, EV1J), indicating that the effect of CDC7 inhibitor could be exerted via the control of EXO1. However, as indicated above, we now show that both addition of roscovitine and depletion of MCM3 also reduced DNA resection upon GNL3 depletion, suggesting that the potential inhibition of EXO1 by CDC7 inhibition may not play a major role in this context. Our BRCA1 control is also supporting this idea. It is established that EXO1 is required for nascent strand degradation upon BRCA1 depletion (Lemaçon et al. 2017. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5643552/) but CDC7 inhibition has no effect on resection upon BRCA1 depletion suggesting that resection by EXO1 may not be regulated by CDC7 in our situation.

As stated by the reviewer MERIT40 and PDS5B are targets of DDK kinases (Jones et al. 2021 https://doi-org.insb.bib.cnrs.fr/10.1016/j.molcel.2021.01.004) and seem to be required for protection of nascent DNA and in response to HU. However, little is known about the role(s) of these proteins and we think that adding them will complicate message. We hope the reviewer understands this.

3. The model also relies on the fact that GNL3-dB mutant (not retained in the nucleoli) is not sufficient to counteract fork resection induced by HU (Figure 5G). The authors should test directly whether GNL3-dB induces extra origin activation, using their available DNA fibers-based technique.

Response: This is an excellent point also raised by Reviewer 1. We have now measured GIFD (Global Instant Fork Density) and show that the number of active forks is increased upon GNL3-dB expression (Fig 5G). It demonstrates that when GNL3 is no longer retained in the nucleolus more origins are fired. We showed that resection in response to HU is increased upon expression of GNL3-dB, a result that is consistent with the increased origin firing upon GNL3-dB expression. To confirm this, we performed an experiment that reviewer 1 suggested that demonstrates that resection upon GNL3-dB expression is also reduced when CDC7 is inhibited (Fig 6B, EV5E).

4. Finally, the model implies an exquisite regulation of the amount of ORC2 protein, which could influence the number of active origins and the extent of fork resection in case of stress. In this scenario, one could predict that ORC2 ectopic expression would have similar, or even stronger effects, than GNL3 downregulation. Is this the case?

Response: We completely agree with this prediction and performed the proposed experiment. We transiently transfected HeLa cells with a plasmid that expresses a FLAG-ORC2 construct that increases ORC2 expression (Fig EV5I). We could see that ORC2 expression is sufficient to induces DNA resection in response to HU (Fig 6E, Figure EV5J). We believe that this model strongly supports the fact that the precise regulation of ORC2 level is important to limit resection in response to replication stress.

5. Even if the connection between origins and fork resection could be firmly established, the molecular link between them remains enigmatic. The authors hint (as "data not shown") that it is neither mediated by RPA nor RAD51. Unfortunately, the reader is left without a clear hypothesis about this point.

Response: We now added a chromatin fractionation experiment that shows that the recruitment of RAD51 and RPA (and also RIF1 and BRCA1) is not strongly affected by GNL3 depletion (Fig EV1M). Based on the work of Lukas Lab (Toledo et al. 2013 https://pubmed.ncbi.nlm.nih.gov/24267891/) one possible hypothesis to link origins and fork resection may be exhaustion of the pool of RPA. To test this hypothesis, we took advantage of cell lines from the Lukas' lab that overexpress the three subunits of the RPA complex (SuperRPA). As expected, the depletion of GNL3 in the control U-2 OS cell line induces nascent strand resection in response to HU (Fig 3I, EV3K, EV3L). In contrast depletion of GNL3 in the SuperRPA cell line has no significant impact on resection. We now believe that the excessive origin activation upon GNL3 depletion and treatment with HU induces the exhaustion of RPA that leads to nascent DNA resection. This is now discussed in the manuscript.

Reviewer #3 (Significance (Required)):

In principle, this study would appeal to the readership interested in fundamental mechanisms of DNA replication and the cellular responses to replicative stress.

For the reasons outlined in the previous section, I believe that in its current version the study is

not strong enough to provide a new paradigm about origins being regulated by partial ORC2 sequestering at nucleoli. The other potentially interesting advance is the connection between frequency of origin activity and the extent of nascent DNA resection at stalled forks, but the molecular link between both remains unknown.

Response: We thank the reviewer and we believe that the new data we provide show that excessive origin firing due to GNL3 depletion and exogenous replication stress induces DNA resection due to RPA exhaustion. In addition, we think that we provide new data that that GNL3 may regulate ORC2 functions.

****Referees cross-commenting****

In addition to each reviewer's more specific comments, the three reviews share a main criticism: the lack of mechanistic information about the proposed link between origin activity and resection of nascent DNA at stalled forks.

Response: With the new data we provide we believe that we now have more mechanistic information regarding the link between origin activity and resection of nascent DNA at stalled forks.

Dear Dr. Ribeyre,

Thank you for the submission of your revised manuscript. We have now received the enclosed reports from the referees. Referee 1 and 2 still have a few more suggestions that I would like you to address and incorporate before we can proceed with the official acceptance of your manuscript.

Please let me know in case you would not like to or cannot address these last comments so that we can discuss this further.

A few editorial requests will also need to be addressed:

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- Please answer all questions on statistics in the author checklist and send us a new, completed checklist.

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- The pixelation for Figure EV1-I, MRE11 looks unusual. Please send us the Figure EV1-I source data.

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2. Please note that legend for figure EV2F is incorrectly labeled as 'E'.
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5. Please note that the box plots need to be defined in terms of minima, maxima, centre, bounds of box and whiskers, and percentile in the legend of figure EV1b.

I would like to suggest that you re-write this difficult-to-understand sentence in the abstract:
"We demonstrate that inhibition of origin firing decreases resection, indicating that the increased replication origin firing seen upon GNL3 depletion mainly accounts for the observed DNA resection likely due to exhaustion of the pool of RPA."

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I look forward to seeing a final version of your manuscript as soon as possible.

Kind regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #1:

The revised version of the manuscript by Lebdy et al. provides a fuller view of the role of GNL3 in the control of origin firing and its impact in the stability of stalled forks. Although the mechanism behind this process is not completely determined in the manuscript, the new experiments demonstrate some of the missing links in the initial work. Most importantly, the connection between GNL3 and origin firing is now supported by different lines of evidence, and the relevance of nucleolar localization of GNL3 and ORC2 is better explained with the more detailed analysis of the dB mutant. There are only two minor issues which we feel might further strengthen the new results:

1. Figure EV1M shows that GNL3 depletion does not change Rad51 levels on chromatin. A positive control in the presence of damage, such as HU (as provided in iPOND experiments in Figure 1H) is necessary to show that Rad51 accumulation can be effectively identified in these experiments.
2. Interestingly, Figure EV1M shows an accumulation of RPA32 on chromatin upon GNL3 depletion. Can the authors detect the exhaustion of the soluble pool of RPA32? The experiments with the super RPA2 cells are very compelling but they would be stronger if the authors show a difference in RPA32 levels in the soluble and chromatin fractions upon GNL3 depletion. Alternatively, concomitant knockdown of RPA32 and GNL3 in super RPA2 cells would also show the direct involvement of RPA in this process.

Together, we feel the new version of the manuscript is very solid and provides enough insights into the mechanisms of action of GNL3 to support its publication in EMBO Reports.

Referee #2:

The authors have implemented the points that I raised earlier and I feel the manuscript will be an interesting addition to the field that I am sure will stimulate further work. I have only one minor point that should be addressed, which I did not pick up earlier. The presented Western blots really should have molecular weight markers indicated.

Referee #3:

In this revised manuscript, Lebdy et al have included additional data in support of their model that nucleolar protein GNL3 regulates the resection of nascent DNA at stalled replication forks. In the previous version, the model was not convincing as most of it mechanistic aspects remained speculative.

Several of my comments and suggestions to the first version have been addressed, and the results incorporated in the revision generally fit with the authors´ hypothesis. Combined with the responses provided to other reviewers, the mechanistic gaps have been covered at least in part, and I think the manuscript is now ready to be published. As it provides new insights into the intriguing link between origin activity and fork resection in situations of replicative stress, it will be interesting to a wide readership that follows the DNA replication field.

Referee #1:

The revised version of the manuscript by Lebdy et al. provides a fuller view of the role of GNL3 in the control of origin firing and its impact in the stability of stalled forks. Although the mechanism behind this process is not completely determined in the manuscript, the new experiments demonstrate some of the missing links in the initial work. Most importantly, the connection between GNL3 and origin firing is now supported by different lines of evidence, and the relevance of nucleolar localization of GNL3 and ORC2 is better explained with the more detailed analysis of the dB mutant. There are only two minor issues which we feel might further strengthen the new results:

1. Figure EV1M shows that GNL3 depletion does not change Rad51 levels on chromatin. A positive control in the presence of damage, such as HU (as provided in iPOND experiments in Figure 1H) is necessary to show that Rad51 accumulation can be effectively identified in these experiments.

This experiment has actually been done in presence of 5mM HU for 4 hours, it is indicated in the legend. We conclude that the recruitment of RAD51 on chromatin in presence of HU is not affected by GNL3

2. Interestingly, Figure EV1M shows an accumulation of RPA32 on chromatin upon GNL3 depletion. Can the authors detect the exhaustion of the soluble pool of RPA32?

We think that the accumulation of RPA32 simply corresponds to the recruitment to single-stranded DNA generated by resection in absence of GNL3 and HU treatment. We have not check for an exhaustion of the soluble pool of RPA32 using Western-blot in this particular experiment but we could not observe such difference previously.

The experiments with the super RPA2 cells are very compelling but they would be stronger if the authors show a difference in RPA32 levels in the soluble and chromatin fractions upon GNL3 depletion.

As indicated before we could not see exhaustion of RPA in soluble fraction. We previously looked for simultaneous recruitment of RPA and gammaH2A.X using flow cytometry to check for RPA exhaustion. We could clearly reproduce the results (i.e. RPA exhaustion) of Toledo et al. in presence of ATR inhibitor and HU. However, comparing siControl and siGNL3 did not provide significant difference in term of RPA exhaustion. We think that this assay may be not sensitive enough to detect exhaustion is situation less drastic than ATR inhibition. This is why we decided to test this hypothesis this by overexpressing RPA inside.

Alternatively, concomitant knockdown of RPA32 and GNL3 in super RPA2 cells would also show the direct involvement of RPA in this process.

We understand reviewer point but we do not think this experiment will add much since the only difference with the normal cell line is overexpression of the three RPA subunits (as shown in the original publication Toledo 2013 Cell), therefore depletion of RPA32 would simply make the cell line back to normal with the risk of having not enough RPA anymore. In addition to do things perfectly right we should deplete the three RPA subunits which is certainly a tricky experiment.

Together, we feel the new version of the manuscript is very solid and provides enough insights into the mechanisms of action of GNL3 to support its publication in EMBO Reports.

We thank the reviewer for his/her suggestions and comment that increase greatly our manuscript.

Referee #2:

The authors have implemented the points that I raised earlier and I feel the manuscript will be an interesting addition to the field that I am sure will stimulate further work. I have only one minor point that should be addressed, which I did not pick up earlier. The presented Western blots really should have molecular weight markers indicated.

We now have added the molecular weights in the Western-blots.

We are grateful to the reviewer for his/her comments and suggestions.

Referee #3:

In this revised manuscript, Lebdy et al have included additional data in support of their model that nucleolar protein GNL3 regulates the resection of nascent DNA at stalled replication forks. In the previous version, the model was not convincing as most of it mechanistic aspects remained speculative.

Several of my comments and suggestions to the first version have been addressed, and the results incorporated in the revision generally fit with the authors´ hypothesis. Combined with the responses provided to other reviewers, the mechanistic gaps have been covered at least in part, and I think the manuscript is now ready to be published. As it provides new insights into the intriguing link between origin activity and fork resection in situations of replicative stress, it will be interesting to a wide readership that follows the DNA replication field.

We are grateful to the reviewer for his/her invaluable suggestions and comments, which have significantly enhanced the quality of our manuscript.

Cyril Ribeyre
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Montpellier 34396
France

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The data shown in figures should satisfy the following conditions:

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- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
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- an explicit mention of the biological and chemical entity(ies) that are being measured.
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- a statement of how many times the experiment shown was independently replicated in the laboratory.
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  - definition of 'center values' as median or average;
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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	Material and Methods

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Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

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