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Lipid Droplet Biogenesis Is spatially coordinated at ER-vacuole contacts under Nutritional Stress

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 13 July 2017

Thank you for the transfer of your research manuscript from The EMBO Journal to EMBO reports.

Given the potential interest of your findings, which was also recognized by the referees and given their support for a potential publication of your data in our journal, I would like to invite you to revise your study for EMBO reports. As my colleague Andrea Leibfried has already outlined, such a revised version should include a significant rewriting and toning down of the conclusions to focus on the main findings, as also outlined by referee 1. Moreover, the claim that the enzymes and Mdm1 localize to NVJs should be substantiated by co-labeling assays as suggested by the referees. Finally, point 2 raised by referee #2 should either be addressed experimentally or the conclusions should be toned down. Importantly, all necessary controls should be provided (copper acetate assay, ref #2, point 4).

Please revise the manuscript along these lines and also 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 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.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further.

Supplementary/additional data: The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix includes a table of content on the first page, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature. For more details please refer to our guide to authors.

Regarding data quantification, please ensure to specify the number "n" for how many experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in all respective figure legends. Please also include scale bars in all microscopy images.

We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure or per figure panel.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

1st Revision - authors' response 13 August 2017

Responses to reviewers' comments:

Referee #1:

In this manuscript, Hariri et al. explore the link between nuclear vacuole junctions (NVJ) and lipid droplet biogenesis (LD). They find that LD form around NVJs. Their data suggest that NVJs are important for TAG production from fatty acids and hence, LD biogenesis.
This paper collects several very exciting observations, which suggest a metabolic link between stress, neutral lipid biogenesis, and organelle contact sites. Unfortunately, the paper drifts into several different directions, very often without going the distance to convincingly support the main claims.
Below is a collection of major points that need addressing to support the claims of the paper.

First, the authors discover that NVJ1 gene expression is regulated redundantly by the Msn and Gcn factors.

1. **How is the gene regulated in absence of both Msn/Gcn pathways? Is there indeed a cumulative/synergistic effect on NVJ1 transcription?**

We thank the reviewer for this question, and have revised the transcriptional analysis section of the manuscript substantially. In particular, we have simplified this section to both tone-down the conclusions made, as well as conducted several new experiments to better understand the transcriptional regulation of NVJ1.
In the revision, we have focused on the MSN2/4 pathway. This was chosen mainly by reductionist strategy, since the GCN pathway is itself regulated in numerous ways during nutritional stress, including transcriptionally by GCN4 mRNA levels, as well as by Gcn2 enzymatic activity, which is stimulated by binding to uncharged tRNAs (Hinnebusch, et al, Eurkaryotic Cell, 2002).
In the revised manuscript, we observe that both MSN2 and MSN4 are required for full stress-induced up-regulation of NVJ1 transcripts, as deletion of either MSN2 or MSN4 significantly decreases NVJ1 transcript levels in stationary phase yeast (Sfigure 1G). These deletions also impact actual NVJ expansion in both stationary phase yeast grown in dextrose, as well as in yeast grown in

non-fermentable acetate (Sfigure 1H). Thus, we conclude that MSN2 and MSN4 are not fully redundant for NVJ1 transcriptional regulation, as each appears required for full NVJ1 up-regulation, as well as full NVJ expansion.

2. NVJ1 transcriptional regulation is claimed to be important for NVJ expansion upon stress. What happens to NVJ expansion in cells lacking Msn/Gcn-mediated regulation?

As mentioned in the previous comment, we have now directly examined NVJ expansion in MSN2 and MSN4-deficient yeast by tagging them with Nvj1-mNeonGreen, and imaging them in yeast growing in stationary phase and in acetate-containing media. We find that, consistent with their reduced NVJ1 transcript levels, both MSN2 and MSN4-deficient strains have smaller NVJs compared to wildtype yeast (Sfigure 1H).

3. The authors claim that an important stress response element (STRE) plays an important role in the upregulation, but do not go as far as to mutate it to see if Msn2/4-mediated regulation is indeed due that motif.

It is well known that NVJ1 is upregulated in starvation, and that the promoter region contains a STRE to which Msn2/4 can putatively bind. We were careful in the paper not to claim that discovery, and we referenced the supporting literature: From Kvam et al., 2005; "The sizes of NV junctions increase proportionally to the expression level of Nvj1p, which is up-regulated though nutrient depletion following the diauxic shift or acute carbon or nitrogen starvation (Gasch et al., 2000; Roberts et al., 2003). The upstream promoter region of NVJ1 contains stress response elements that control expression in response to nutrient stress (Moskvina et al., 1998)." While interesting, we don't think that mutating the Msn binding motif is necessary for the scope of this study, and given the literature, we don't anticipate that it would change the conclusions of our study.

4. In Figure S2C and S2D, the authors find that LD-deficient yeast cannot form NVJs properly. Is it due to failure of NVJ1 transcriptional upregulation? Or is Nvj1 still upregulated and these cells still fail to form NVJs?

We thank the reviewer for asking this, and have now done this experiment. The results are now included in the revised manuscript in figure S2E. Interestingly, we find that LD-deficient yeast still transcriptionally up-regulate NVJ1 mRNAs in response to stationary phase growth, yet do not exhibit extended NVJs as observed by Nvj1-GFP. Therefore, we suspect that physical NVJ expansion and NVJ1 transcript up-regulation can be uncoupled in LD-deficient yeast.

So in general, the phenomenon of transcriptional upregulation is interesting but it is unclear if it is causal to NVJ expansion. Is it necessary, sufficient, both, or is it a correlated epiphenomenon?

We agree with the reviewer that the relationship between NVJ expansion and NVJ1 transcriptional regulation was not sufficiently addressed, and may, in principle, be complex. Altogether, our revised manuscript indicates that a fully functional MSN2/4 pathway is required for full NVJ1 transcriptional up-regulation in stationary phase. Partial loss of this NVJ1 transcriptional up-regulation in the MSN2 or MSN4-deficient yeast results in a substantial decrease in NVJ size in both stationary phase dextrose-cultured yeast, as well as acetate-cultured yeast (Sfigure 1H).
Collectively, this implies that NVJ1 transcriptional up-regulation is at least partially required for full NVJ expansion. Consistent with this, over-expression of NVJ1 is sufficient to create ER-vacuole hyper-tethering (Pan, MBC, 2000).
Interestingly, we also find that NVJ1 transcriptional up-regulation can be uncoupled from physical NVJ expansion in yeast that cannot produce Lipid Droplets. Indeed, although we find that NVJ1 mRNA levels increase in LD-deficient yeast grown into stationary phase (Sfigure 2E), we find these yeast have significantly smaller NVJs compared to wildtype (Sfigure 2C and 2D). Thus, although NVJ1 transcriptional up-regulation appears to be required for NVJ expansion, it is not always sufficient (as in LD-deficient yeast).
Finally, we examined published microarray datasets of yeast over-expressing MSN2 and MSN4. In both conditions, NVJ1 transcripts were up-regulated ~1-fold (Gasch, MBoC, 2000).

Second, the authors make a visual screen of 30 candidate GFP-tagged protein for relocalization to the NVJ. They find indeed that some enzymes accumulate at NVJs, however the functional relevance of this localization is unclear.

We acknowledge this comment, and have made numerous changes to the text to soften several of our conclusions pertaining to the GFP screen to make more precise conclusions. Indeed, in the revised version of the manuscript, we have moved the GFP screen data to the final figure, as it represents a broader aspect of this study that we are continuing to investigate.
In addition to text changes, we have also added several new imaging experiments to the revision, which provide more direct evidence that enzymes detected in our screen associate with the NVJ. For example, in addition to the already existing data showing that Faa1-GFP and Hmg1-GFP colocalize with Nvj1 (figure 4C, S4A, 7A), we have now added images of Fas1-GFP foci accumulating at the NVJ periphery of Nvj1-mCh labeled yeast (figure S3E).

5. Comments on unpublished data not shown.

Moreover, several proteins claimed to accumulate at NVJs, might in fact accumulate at LDs (since LDs appear at the periphery of NVJs).
Indeed, we agree with this conclusion, and had actually written this conclusion in the earlier version of the manuscript. However, we acknowledge that the way the text was written probably made our conclusions unclear, and have changed the text substantially to rectify this ambiguity.

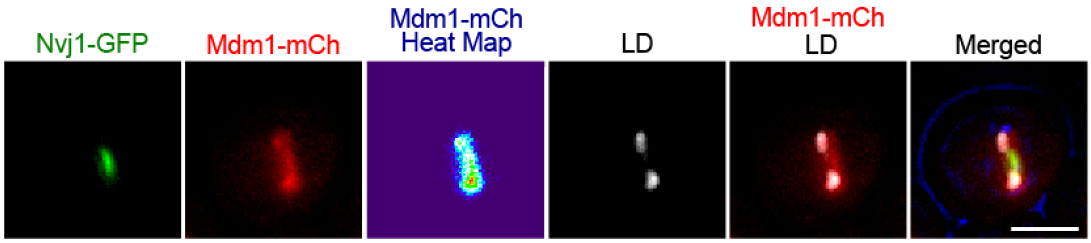
6. For instance, In Figure 3B, the authors might want to co-localize Fas1/Fas2/Fat1-GFP with a LD marker. If those foci are indeed LDs, then the interpretation is straightforward instead of the conclusion that these are "visitor" enzymes, which move to NVJs upon stress.
We agree with this straightforward conclusion, and we have now done this co-localization experiment with Fas1-GFP and a lipid droplet marker (Sfigure 3E). We have also revised the text to better clarify the observations.

7. Indeed, in Fig 3G, the re-localization of Faa1 seems to be on LDs rather than NVJs. It is certainly not similar to Hmg1/2-GFP which clearly move to NVJs upon stress.
We again agree that we did not make this point clear, and have substantially changed the text to clearly state that the Faa1-GFP appears to localize at the NVJ periphery where it colocalizes with LDs (figure 4C).

8. To label Elo1/3 as "visitor" enzymes is a bit stretched since by this logic any ER protein or a protein enriched along the nuclear ER should be a "visitor" of NVJs. Again, the physiological relevance of a "visitor" at NVJ and its contribution to LD biogenesis is correlative.
Our choice of the terms “clients” and “visitors” was intended to describe the selective architectural organization of proteins at the NVJ, rather than the functional relevance of proteins that accumulate there. We understand how this could be misunderstood, so we changed the wording to better clarify this, and omitted the “visitor” and “clients” nomenclature from our revised manuscript.

There are some questions about the localization of Mdm1.
1. In Fig. 4 B, the Mdm1 enrichment zone (NVJ edge) is represented as a ring but in Fig. 4 C, it is clear that the localization of Mdm1 is "dotty" and does not surround the NVJ "core". This representation is misleading.
We agree, and Mdm1-GFP does indeed tend to form distinct foci at the NVJ periphery. We have adjusted the model figure 7F and the text to better represent the data.

2. In Figure 4, the authors conclude that Mdm1 is enriched on dotted structures at the NVJ "edge". I am surprised that they did not do a triple-labeling experiment along with LD marker to address if Mdm1 foci (in Fig 4A) are LDs, especially since overexpressed Mdm1 accumulates at LDs (Fig. 7AB). This would significantly change the interpretation of the data.
We agree, and have conducted several triple-labeling experiment examining Mdm1, LDs, and Nvj1. We find that Mdm1 colocalizes with LDs at the NVJ (figures 5D, 5H, Sfigure 3C), which supports our conclusion that Mdm1 defines sites for LD accumulation. In particular, we find that yeast expressing Nvj1-GFP and mildly over-expressing Mdm1-mCherry show very obvious Mdm1 co-localization with LDs at the NVJ periphery. This data below has also been added to SFig 5C.



Finally, the role of Mdm1 in LD biogenesis is paradoxical. Both absence and overexpression of Mdm1 cause increased TAG accumulation, and the effect of abrogating all NVJ is, at best, modest. It is therefore not advised to claim that "NVJ provides a micro-compartment that is protected from cytoplasmic changes, and exhibit unique physicochemical properties amenable to lipid metabolism".
We agree that the effect of abrogating the NVJ is modest, and have substantially revised the text to more accurately reflect this. We have also added a section to the Discussion focusing on the ostensibly paradoxical observation that both loss and over-expression of MDM1 cause increases in TAG. Indeed, understanding this is a major focus of the lab right now, and we believe represents a dual function of Mdm1 at ER-vacuole contacts: Mdm1 functions BOTH as an ER-vacuole tether, as well as a regulator of LD dynamics. When it is over-expressed, it causes the NVJ to expand, which generates a large site for LD production. Conversely, when MDM1 is deleted, it appears to cause defects in LD dynamics and possibly LD turnover, the details of which we are continuing to investigate.

In summary, this paper conclusively shows that NVJ1 is transcriptionally regulated, that LDs appear in the periphery of NVJs, and that Mdm1 is somehow involved with LDs (either through interaction with fatty acid biosynthesis enzymes, or through its association with LDs). However, strong functional links between these observations are missing and the paper should therefore be significantly toned down.
We have substantially toned-down and rephrased our conclusions to accurately describe the results we present in this manuscript. In addition, we have also attempted to strengthen the functional links in this study. In particular, we present data that indicate:

1. *That an intact MSN2/4 pathway is necessary for full NVJ1 transcriptional up-regulation in stationary phase,*
2. *That NVJ1 transcriptional up-regulation is functionally linked to actual NVJ expansion, but may be uncoupled from it in yeast that cannot produce LDs (thus, transcriptional up-regulation is necessary, but not always sufficient, to expand the NVJ),*
3. *Mdm1 appears to demarcate sites at the NVJ (and other regions of the ER) where LDs bud.*
4. *Consistent with this, modulating Mdm1 expression level and over-expressing Mdm1 is sufficient to drive both NVJ expansion and NVJ-associated LDs*

Minor points

1. Line 163, reference should be (figure 1A, 1B and 1C).
2. Line 623, "pervious" should read "previous".
Thank you. We have fixed both minor comments in the revised version attached.

Referee #2:

This study investigates the relationship between a yeast specific contact site, the nuclear-vacuole junction (NVJ), lipid metabolism, and lipid droplet (LD) biogenesis. As the previous reviews point out, there are many interesting observations here but the story remains largely descriptive and there is little mechanistic insight. The same is true of the revised version of this manuscript. Although there could be an important story here, much more work is necessary before this work would be appropriate for EMBO or a similar journal. Overall, the work is well done but the findings are over interpreted and do not support the conclusions drawn from them.

Major points:

1. The phrase "metabolic platform" is not carefully defined. The idea seems to be that enzymes in various lipid synthesis pathways are close to each other in the platform and therefore work more efficiently or perhaps regulate one another. No evidence is presented that being in or near the platform (the NVJ) has a direct effect on the activity of any lipid metabolism protein. It is true that lipid metabolism changes in delta-NVJ cells but the question is why. Is it because the enzymes in the platform are no longer near each other and, if so, how does this this affect lipid synthesis or degradation? In addition, many of the enzymes involved in ergosterol, fatty acid, and neutral lipid synthesis are not enriched in the platform, so how does the platform work to alter lipid metabolism? It is certainly interesting that some lipid metabolism proteins are in or near the NVJ and some LDs seem to originate near these sites but it remains unproven that there is any link between these two observations. In summary, it is necessary to demonstrate that localization of lipid synthesis enzymes in the NVJ (or out of it) significantly affects their activity, the rate of neutral lipid biosynthesis, and/or LD biogenesis site selection.
We agree with this comment, and have actually removed the phrase "metabolic platform" from the revised manuscript. In general, the point of proposing that the NVJ is a "metabolic platform" was meant to build upon existing literature on the NVJ—that it recruits several proteins and enzymes from various metabolic pathways (Osh1, Tsc13, Lro1, Pah1 Ltc1, Vps13, etc) in various cellular states.* Comments on unpublished data not shown.
In our revision, we have significantly toned down the conclusions of the manuscript to accurately reflect that we do not, at this point, know if NVJ-localized enzymes exhibit changes in their enzymatic activity. However, it has been reported that, in yeast, reversible sequestration of numerous metabolic enzymes as a consequence of nutrient depletion may be a general adaptation strategy that allows the cells to conserve energy (Petrovska et al., 2014; Suresh et al., 2015). Whether NVJ-mediated sequestrations represent similar inactive storage assemblies, or serve to enhance the local efficiency of certain metabolic processes, remains a matter of debate and future studies.

2. It is premature to claim that Mdm1 regulates LD biogenesis or neutral lipid metabolism. Levels of neutral lipids change in many yeast mutants. To claim that Mdm1 directly regulates lipid metabolism it is necessary to know mechanistically how this occurs.
In our manuscript, we were careful not to claim that Mdm1 directly regulates a specific stage of lipid droplet metabolism, for the same reason that the reviewer mentioned: it would be necessary to show mechanistically how this occurs. We have revised the text further to ensure that our conclusions represent the results accurately.
Here, we conclude only that Mdm1 appears to demarcate sites at the NVJ periphery (or other ER regions) where LDs can be observed (figure 5D, 5H). Consistent with this, modulating Mdm1 expression, and over-expressing it causes LDs to accumulate at the NVJ (Sfigure 5A-C). We also provide evidence that Mdm1 may interact (by co-immuno-precipitation) with Faa1 (figure 4A). Consistent with this, it also co-localizes with Faa1, and with LDs at the NVJ periphery (Sfigure 4A, Sfigure 3C). Collectively, these observations suggest a role for Mdm1 in LD dynamics, but we are careful to go beyond this correlative observation at this time.
We are continuing to try to understand the specific role for Mdm1 in LD dynamics. Interestingly, we know that the recombinant PXA domain of Mdm1 is capable of binding lipids, and particularly free fatty acids, as detected by numerous biochemical assays. We are cautiously pursuing the meaning of this observation.

In addition, to make claims about rates of lipid conversion (for example, that FA to TAG conversion slows in cells lacking Mdm1 or the NVJ) it is necessary to actually measure rates of conversion and not just steady-state levels of lipids. Typically, this is done with labeled lipids.
We agree that in order to make conclusions on the rates of lipid metabolism we need to use labelled lipids. We have modified the text to more accurately describe our experiments.

3. The localization of Mdm1-GFP at the edges of the NVJ is interesting. Previous studies have shown that the association of the inner and outer nuclear membrane at the NVJ is so close that many ER luminal proteins are excluded. Could it be that Mdm1 has a large enough luminal domain that it is also excluded from the NVJ? Does exclusion of Mdm1 from the core portion

of the NVJ affect Mdm1 function? Does luminal domain size affect the ability of other integral membrane proteins to access the NVJ?
This is an interesting idea, but we think that the general exclusion of Mdm1 from the center of the NVJ is not due to the size of the Mdm1 luminal domain. Indeed, over-expressing Mdm1 causes it to enter the NVJ core region, indicating it is not sterically blocked from that region (figure 5D). Although we do not fully understand why Mdm1 enriches a the NVJ periphery, we can speculate on at least two mechanisms: 1) one is that Mdm1 may be part of a protein complex that form distinct foci at the NVJ periphery. This is supported by imaging evidence showing that, even in the absence of a proper NVJ (NVJ1-deficient yeast), Mdm1 still forms distinct foci along the nuclear and cortical ER network, rather than elongated patches like Nvj1 (Henne, JCB, 2015; figure 3B,C). 2) Second, much of our imaging suggests that Mdm1 foci are nearly always associated with LDs, and this LD association may dictate the focal distribution of Mdm1. Indeed, yeast fed oleic acid exhibit Mdm1-GFP "cups" that partially surround LDs, suggesting a tight coupling of Mdm1 to the LD surface (figure 6D).
However, all this does not exclude the possibility that, as the reviewer mentioned, luminal domain size could be one mechanism that regulates protein position at the NVJ.

4. Using copper acetate to quantitate lipid levels is challenging since this method is often difficult to reproduce. Please provide examples of the standard curves and indicate over what range of concentrations the quantifications are linear. Also, the results are presented as ug lipid but it is not clear if this is per protein or per cell or is normalized in some other way.
This is a fair point, although this procedure has been widely used by the field to look at changes in lipid levels. We agree that this method can be difficult to reproduce; however, our conclusions still stand when we consider multiple separate TLC plates, and the trends are reproducible. Regarding the quantification, we generally run many dilutions of a standard lipid mix of known concentration on each TLC plate, and we use that to generate a standard curve. The equations deduced from the standard curve are used to extrapolate the mass of lipids corresponding to the intensity of band measured using ImageJ. We normalize our data by cell weight. Here is an example:

2nd Editorial Decision 08 September 2017

Thank you for the submission of your revised manuscript to EMBO reports. It has been sent back to the same referees who also evaluated your study for The EMBO Journal and you will find their reports below.

As you will see, referee 1 is more positive and supports publication in EMBO reports after further revision while referee 2 still considers the conceptual advance of the study, as it stands, rather modest and indicates that the data remain preliminary in his/her opinion. I have discussed this further with the referees and both consider the finding that lipid droplets form at the NVJ or move there the most important aspect and conceptual advance of your study. Both referees suggested to focus the study on this aspect and to further strengthen these data with time course and live imaging as outlined in their reports and both referees consider such a focused story potentially suitable for EMBO reports.

Based on the referee reports and their further comments I suggest the following revision for EMBO reports:

1) Focus the story on LD biogenesis at the NVJ and strengthen this observation with further time-lapse imaging, TEM and quantification as outlined in the referee reports. Distinguish LD biogenesis at the NVJ from LDs migrating there.

2) Expand the discussion and take earlier literature into account.

3) Provide a clearer presentation of the lipid data.

4) Since the transcriptional regulation of Nvj1 by Msn2/4 appears not to be convincing I suggest to remove the data altogether from the manuscript. If you focus on the aspect of LD biogenesis at the NVJ then this information might not be so relevant at this point.

5) I further suggest removing the data on the enrichment of lipid metabolism enzymes at the NVJ since the functional consequence is not clear. This might be the subject of a further study. Alternatively, the conclusions should be toned down and the limitations clearly discussed.

If you decide to embark on this revision, please submit the revised manuscript within three months, it will otherwise be treated as new submission. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revision further.

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

REFEREE REPORTS

Referee #1:

In the revised manuscript, Hariri et al. have toned down their previous conclusions and modified the text to match the data better. They have also experimentally addressed many of the issues raised. I am satisfied and, in principle, fine with the publication of this manuscript in EMBO Reports based on the fact that the authors shed new light into the functional link between Mdm1 and LD biogenesis, although the regulation seems to be complicated. However, few issues need be addressed.

Major points

1. The relationship between Nvj1 levels and NVJ expansion is still unclear, and the data herein fails at clarifying it. To sum up what was known before:
Overexpressing Nvj1 leads to NVJ expansion (thus Nvj1 upregulation is sufficient for expansion, Pan et al, 2000).
Stress leads to increase Nvj1 expression (Gasch et al., 2000; Roberts et al., 2003) and NVJ expansion (Roberts et al. 2003). The novel aspect that is implicated here is the causality of nutritional stress leading to Nvj1 transcriptional up-regulation, which in turn leads to NVJ expansion. This is tested by ablating Msn2/4, blunting Nvj1 induction and preventing NVJ expansion (to show that Nvj1 induction is necessary). There are two comments here. First, ablating Msn2 or 4 is not the best way to test the necessity of Nvj1 induction for NVJ expansion. In principle, since in the absence of Msn2/4, the global stress response pathway is shut down, the effect on NVJ might be indirect and not via Nvj1. A cleaner test would be to ablate the Stress-response element in NVJ1's promoter, in order to selectively blunt Nvj1 upregulation, while keeping the rest of the stress response pathway intact. Second, the simple sufficiency relationship between Nvj1 expression and NVJ expansion is lost in LD-deficient cells, showing that other factors are necessary for the expansion of NVJs during stress. These yet unknown factors might also be regulated by Msn2/4, and be as important as Nvj1 upregulation for NVJ expansion. So the relationship between Nvj1 upregulation and NVJ expansion is complicated. The LD-deficient experiment shows that it is not sufficient, and the Msn2 and 4 ablation experiments are not convincing to say it is necessary. This should be acknowledged thoroughly.

2. The authors claim the appearance of LD "lenses" upon Mdm1 overexpression (Figure 5F). Higher magnifications of those images are needed to clearly show that the lenses are surrounded by a ER membrane. At the current magnification, it appears as if LDs bud off the vacuole. If this were indeed true, the interpretation would change significantly.

3. On similar lines, Figure 2K aims at showing that LDs appear in proximity of NVJs. Unfortunately, the first time point (1h) is already quite late, and LDs are already fully formed. It would also be relevant to repeat these experiments by doing a time course (for example, imaging every 5 minutes) after cerulenin washout. This could clarify better whether the LDs indeed bud proximal to NVJs, rather than migrate there following their biogenesis at an alternate site.

4. *Comments on unpublished data not shown.*

Minor points

1. In line 163, it would be more accurate if it reads "...yeast grown in metabolically challenging conditions tend to exhibit both expanded NVJs and elevated neutral lipid levels" to make sure that no causality is inferred.
2. Movie S2 seems to be missing timescale
3. In line 240, the Fas1-GFP sub-population is not well defined. What approximate percentage associates at the vacuole surface?
4. Line 313, LD "lenses" may need to be re-defined if they actually bud off from the vacuole
5. Line 361, "ambient" conditions should be replaced with "normal" conditions
6. The Discussion section could already start from Line 380

Referee #2:

The revised version of this manuscript is improved but the findings are still over interpreted. This study investigates cells during nutrient deprivation, particularly the role of the membrane contact site formed between the vacuole and nucleus (the NVJ). Some of the most notable findings are that during nutrient deprivation: (1) the NVJ expands, (2) NVJ expansion fails to occur in cells that cannot synthesize neutral lipids, (3) LDs often cluster near the NVJ, (4) LD biogenesis may occur on regions of the nucleus near the NVJ, (5) lipid homeostasis changes somewhat in cells lacking the NVJ and/or Mdm1 and (6) a few lipid synthesis enzymes become enriched on the NVJ to varying degrees. Some of these conclusions (1 and 6) are not entirely new and the others are interesting but still only add up to a modest advance over what has been shown before. To me, the most interesting is point #4. The authors suggest that the NVJ plays some sort of role in lipid metabolism (which has been proposed before) but it is not clear how. I continue to think that there are some interesting findings in this study but the story remains preliminary and requires more insight into how, specifically, the NVJ participates in lipid metabolism and/or LD biogenesis. The manuscript also needs to appropriately discuss the literature to put the findings into context.
1. Most of the first part of the manuscript is not novel but is presented as though it is. The results in Fig. 1 and Fig. 2 A-G are well presented and clearly explained but the authors fail to mention that similar results have been presented in previous studies. To take just one example, Pan et al 2000 showed that the NVJ expands as cells reach stationary phase and others have shown this as well. It is true that others have not quantified NVJ size as carefully as this study but that does not mean that previous studies should not be mentioned. The manuscript needs to appropriately cite and discuss previous studies.
2. The experiment shown in Fig. 2K is, arguably, the most interesting in this study and shows that new LD biogenesis may occur at the NVJ in stationary phase. The manuscript would be stronger if this result were expanded and built upon. Times earlier than 1 hour after cerulenin washout should be shown. Also, the results should be quantified. More importantly, in the example shown in the inset the LDs do not seem to be at the edges of the NVJ but are instead about 0.5 um away from the NVJ. When quantifying the results, it is important to clearly define which LDs count as near the edges of the NVJ. In rapidly growing cells, LDs are distributed thorough out the cell. Do they move to the NVJ? Time-lapse imaging could answer this question.
3. The lipid data is often confusingly presented. Many figures indicate that lipid levels are normalized (e.g., Fig. 3E) but normalized to what? The normalization should be clearly stated in the figure. Other figures give the ratio of FFA to TAG. The idea seems to be that this ratio is an indication of rates of TAG synthesis since FFA is a precursor of TAG. However, no evidence is cited or presented that FFA levels always increase when TAG synthesis rates decrease (not to mention that FFA is not a direct precursor of TAG). It is better to just present total TAG and FFA levels.
4. Fig. 7B shows Lro1 in the NVJ but there is no evidence provided or cited to support this claim. This is important since there is currently no evidence that neutral lipids are synthesized at the NVJ and, if they are not, how would producing precursors at the NVJ affect LD biogenesis at these sites? Plausible models should be proposed.

5. Other studies have suggested that LD biogenesis occurs at specialized sites in the ER in both yeast and higher eukaryotes. None of these are cited or discussed. Is Nem1 near the edges of the NVJ when LD biogenesis occurs?

6. The manuscript still seems assume that enrichment of enzymes in the NVJ somehow affects their function. Enrichment is also taken to suggest that the NVJ plays a role in lipid metabolism. This is

an attractive idea (and the manuscript should make clear that this idea has been suggested by others) but there is currently no evidence to support it. The addition Faa1 and Hmg1 to the list of enzymes that are moderately enriched at the NVJ does not shed any new light on how the NVJ might affect lipid metabolism and it is not clear what these findings add to the story.

2nd Revision - authors' response 21 September 2017

Response to Referee #1:

In the revised manuscript, Hariri et al. have toned down their previous conclusions and modified the text to match the data better. They have also experimentally addressed many of the issues raised. I am satisfied and, in principle, fine with the publication of this manuscript in EMBO Reports based on the fact that the authors shed new light into the functional link between Mdm1 and LD biogenesis, although the regulation seems to be complicated. However, few issues need be addressed.

Major points:

1. The relationship between Nvj1 levels and NVJ expansion is still unclear, and the data herein fails at clarifying it. To sum up what was known before:
Overexpressing Nvj1 leads to NVJ expansion (thus Nvj1 upregulation is sufficient for expansion, Pan et al, 2000). Stress leads to increase Nvj1 expression (Gasch et al., 2000; Roberts et al., 2003) and NVJ expansion (Roberts et al. 2003). The novel aspect that is implicated here is the causality of nutritional stress leading to Nvj1 transcriptional up-regulation, which in turn leads to NVJ expansion. This is tested by ablating Msn2/4, blunting Nvj1 induction and preventing NVJ expansion (to show that Nvj1 induction is necessary). There are two comments here. First, ablating Msn2 or 4 is not the best way to test the necessity of Nvj1 induction for NVJ expansion. In principle, since in the absence of Msn2/4, the global stress response pathway is shut down, the effect on NVJ might be indirect and not via Nvj1. A cleaner test would be to ablate the Stress-response element in NVJ1's promoter, in order to selectively blunt Nvj1 upregulation, while keeping the rest of the stress response pathway intact. Second, the simple sufficiency relationship between Nvj1 expression and NVJ expansion is lost in LD-deficient cells, showing that other factors are necessary for the expansion of NVJs during stress. These yet unknown factors might also be regulated by Msn2/4, and be as important as Nvj1 upregulation for NVJ expansion. So the relationship between Nvj1 upregulation and NVJ expansion is complicated. The LD-deficient experiment shows that it is not sufficient, and the Msn2 and 4 ablation experiments are not convincing to say it is necessary. This should be acknowledged thoroughly.
We agree with this comment, and indeed there are probably multiple factors that influence NVJ expansion. Indeed, as we show in this revised manuscript, NVJ1 mRNA up-regulation is not sufficient to expand the NVJ, a significant finding. In our revision, we now state this. Based on the editor's suggestions, we have now removed the Msn2/4 data from the revised manuscript to keep the story focused on the aspect of LD biogenesis at the NVJ.

2. The authors claim the appearance of LD "lenses" upon Mdm1 overexpression (Figure 5F). Higher magnifications of those images are needed to clearly show that the lenses are surrounded by a ER membrane. At the current magnification, it appears as if LDs bud off the vacuole. If this were indeed true, the interpretation would change significantly.

Thank you for this comment. We have done several things to try to address this point. First, we have added more TEM imaging in Fig. EV4G and H to clarify our point, mainly that we observe more LDs associated with ER tubules near the vacuole in cells over-expressing Mdm1. This is consistent with the light microscopy experiments we show in Fig. 6D and H. Second, we have adjusted the text to more accurately reflect the TEM images. Third, we want to comment that although we do not always observe obvious ER tubules adjacent to all LDs, we think this may be due to the thin-sectioning of the TEM slice itself, as the ER association may be occurring out-of-plane. Fourth, in the revised manuscript we have replaced the phrase "LD lenses" with "nascent LDs" to help clarity. Finally, understanding whether the nascent LDs that appear next to the vacuole are actually

budding there, or alternatively being engulfed by the vacuole, would be beyond the scope of this study.

3. On similar lines, Figure 2K aims at showing that LDs appear in proximity of NVJs. Unfortunately, the first time point (1h) is already quite late, and LDs are already fully formed. It would also be relevant to repeat these experiments by doing a time course (for example, imaging every 5 minutes) after cerulenin washout. This could clarify better whether the LDs indeed bud proximal to NVJs, rather than migrate there following their biogenesis at an alternate site.
Thank you for this comment. As we mentioned above, we agree that examining LD biogenesis at the NVJ is one of the most significant conceptual advances of this study. As such, we have expanded upon the observation made in Fig 2K, and have now conducted new time-lapse imaging experiments (Fig 3A-E, EV2B), and quantified these (Fig 3D), as well added additional new TEM that further show LDs associated with ER-vacuole contacts (EV4G and H). As Nvj1-GFP photo-bleaches rather quickly, we opted to use 10-minute imaging intervals to collect the data.

4. *Comments on unpublished data not shown.*

Minor points:

1. In line 163, it would be more accurate if it reads "...yeast grown in metabolically challenging conditions tend to exhibit both expanded NVJs and elevated neutral lipid levels" to make sure that no causality is inferred.
We adjusted the wording as suggested.

2. Movie S2 seems to be missing timescale
We have now added Figure Legends for both SMovies that describe the timescales and methodologies utilized.

3. In line 240, the Fas1-GFP sub-population is not well defined. What approximate percentage associates at the vacuole surface?
This data has been removed from the revised manuscript as per the Editor's suggestion.

4. Line 313, LD "lenses" may need to be re-defined if they actually bud off from the vacuole
As we mention above, we have done several things to address this concern. In the revised manuscript we have replaced the phrase "LD lenses" with "nascent LDs."

5. Line 361, "ambient" conditions should be replaced with "normal" conditions
This data has been removed from the revised manuscript as per the Editor's suggestion.

6. The Discussion section could already start from Line 380.
The text has been adjusted.

Response to Referee #2:

The revised version of this manuscript is improved but the findings are still over interpreted. This study investigates cells during nutrient deprivation, particularly the role of the membrane contact site formed between the vacuole and nucleus (the NVJ). Some of the most notable findings are that during nutrient deprivation: (1) the NVJ expands, (2) NVJ expansion fails to occur in cells that cannot synthesize neutral lipids, (3) LDs often cluster near the NVJ, (4) LD biogenesis may occur on regions of the nucleus near the NVJ, (5) lipid homeostasis changes somewhat in cells lacking the NVJ and/or Mdm1 and (6) a few lipid synthesis enzymes become enriched on the NVJ to varying degrees. Some of these conclusions (1 and 6) are not entirely new and the others are interesting but still only add up to a modest advance over what has been shown before. To me, the most interesting is point #4. The authors suggest that the NVJ plays some sort of role in lipid metabolism (which has been proposed before) but it is not clear how. I continue to think that there are some interesting findings in this study but the story remains preliminary and requires more insight into how, specifically, the NVJ

participates in lipid metabolism and/or LD biogenesis. The manuscript also needs to appropriately discuss the literature to put the findings into context.

1. Most of the first part of the manuscript is not novel but is presented as though it is. The results in Fig. 1 and Fig. 2 A-G are well presented and clearly explained but the authors fail to mention that similar results have been presented in previous studies. To take just one example, Pan et al 2000 showed that the NVJ expands as cells reach stationary phase and others have shown this as well.
It is true that others have not quantified NVJ size as carefully as this study but that does not mean that previous studies should not be mentioned. The manuscript needs to appropriately cite and discuss previous studies.
We appreciate this comment, and want to ensure that we properly reference past literature. However, we do reference Pan et al 2000 several times in the manuscript, including in the first sentence of the first paragraph of the Results section (line 99 of previous version). Indeed, we agree that Pan, et al observed longer NVJs in dense cultures, although they did not carefully quantify this, nor was it a focus of their study. We have added an additional reference for Pan, et al in the revision, and clearly state this.
In our present study, we thoroughly monitor and quantitatively assess NVJ expansion in a variety of stress conditions (9 conditions in total, Fig 1, and EV 1E), not just stationary phase. Furthermore, we demonstrate that the transcriptional up-regulation of NVJ1 is not sufficient to drive NVJ expansion, contrary to what was initially suggested by Pan et al. Instead, we provide new evidence that NVJ expansion requires an aspect of LD budding (Fig 2H-I).
Regarding Fig 2 A-G, we have now added three references to acknowledge that previously it has been reported that as cells go into stationary phase, they accumulate TAG.

- ***Klose et al., 2012**, (this group used mass spectrometry-based shotgun lipidomics to analyze lipid composition of yeast grown in different conditions, including stationary phase and in glycerol).*
- ***Kurat et al., 2006**, and **Kohlwein, 2010** (these studies show that in stationary phase, yeast cells accumulate TAG, which serves as a reservoir for FFAs and help yeast survive prolonged starvation).*

2. The experiment shown in Fig. 2K is, arguably, the most interesting in this study and shows that new LD biogenesis may occur at the NVJ in stationary phase. The manuscript would be stronger if this result were expanded and built upon. Times earlier than 1 hour after cerulenin washout should be shown. Also, the results should be quantified.
We thank the Referee for this comment, and have done new, quantitative time-lapse imaging experiments as discussed above (Fig 3 A-E, EV2B).

More importantly, in the example shown in the inset the LDs do not seem to be at the edges of the NVJ but are instead about 0.5 um away from the NVJ. When quantifying the results, it is important to clearly define which LDs count as near the edges of the NVJ. In rapidly growing cells, LDs are distributed thorough out the cell. Do they move to the NVJ? Time-lapse imaging could answer this question.

We appreciate this comment. Indeed, we observe a distribution of LDs both directly at the NVJ (eg. directly over-lapping with the Nvj1-GFP signal), as well as immediately "flanking" the NVJ within ~0.5um as suggested. We think this may indicate a migration of mature LDs away from the NVJ center as they grow, which has been implied in previous works examining Pah1-GFP (Barbosa, MBOC, 2015). In our revision, we add many more images of LDs at the NVJ, and carefully quantify these (Fig 3D).

3. The lipid data is often confusingly presented. Many figures indicate that lipid levels are normalized (e.g., Fig. 3E) but normalized to what? The normalization should be clearly stated in the figure. Other figures give the ratio of FFA to TAG. The idea seems to be that this ratio is an indication of rates of TAG synthesis since FFA is a precursor of TAG. However, no evidence is cited or presented that FFA levels always increase when TAG synthesis rates decrease (not to mention that FFA is not a direct precursor of TAG). It is better to just present total TAG and FFA levels.
Thank you. As we state above in the Editor's comments, we have modified the figure panels, figure legends, and Methods section to further clarify how the lipid data were generated.

In Fig. 2D, 5G, and Fig. 7C, we chose to represent the data as a ratio of FFA/TAG. This was done for simplicity, and indeed other studies in the field have represented lipid data in a ratio form (for example: Elbaz-Alon, et al. Dev Cell, 2014). Indeed, displaying the lipid data in ratio form allowed us to reduce the number of bars in our histograms, which were quite numerous for several figures. For example, in Figure 2D, the actual TAG and FFA data are still present in Fig 2B, C, and E. We feel the ratio panel in Fig 2D provides greater clarity when we discuss this data in the Results text. We have also adjusted the text in this section to ensure we have accurately represented the data (eg. we comment only on "ratio" of TAG and FFA, and not "rate" of FFA to TAG conversion).

4. Fig. 7B shows Lro1 in the NVJ but there is no evidence provided or cited to support this claim. This is important since there is currently no evidence that neutral lipids are synthesized at the NVJ and, if they are not, how would producing precursors at the NVJ affect LD biogenesis at these sites? Plausible models should be proposed.
We had actually cited Wang and Lee, J Cell Science, 2012 in a previous version of the manuscript, which demonstrated this, but this data has been removed in our revision per the editor's suggestion.

5. Other studies have suggested that LD biogenesis occurs at specialized sites in the ER in both yeast and higher eukaryotes. None of these are cited or discussed. Is Nem1 near the edges of the NVJ when LD biogenesis occurs?
We appreciate this comment, and have actually cited literature that support this idea. Choudhary et al., 2015 and Jacquier et al., 2011 were cited in the earlier version of the manuscript for their work on ER-LD budding. We also reference Barbosa, et al, 2015, which examined Pah1-GFP, which is closely coupled to Nem1 function on the nuclear ER.
We also discussed the possibility that, despite the fact that our data suggest a model whereby LDs bud at the NVJ in response to starvation, "we cannot exclude the possibility that a sub-population of LDs observed at the NVJ in diauxic shift also come from pre-existing LDs, which travel to the NVJ following their biogenesis." However, to further satisfy this concern, in the revision, we have added more references that mention this, such as Kassan et al JCB, 2013.

6. The manuscript still seems assume that enrichment of enzymes in the NVJ somehow affects their function. Enrichment is also taken to suggest that the NVJ plays a role in lipid metabolism. This is an attractive idea (and the manuscript should make clear that this idea has been suggested by others) but there is currently no evidence to support it. The addition Faa1 and Hmg1 to the list of enzymes that are moderately enriched at the NVJ does not shed any new light on how the NVJ might affect lipid metabolism and it is not clear what these findings add to the story.
We appreciate this comment, and are further developing this idea in a subsequent study. The corresponding Figure was removed from the manuscript.

3rd Editorial Decision 10 October 2017

Thank you for the submission of your revised manuscript to our journal. We have meanwhile received a complete set of reviews from all referees, which I include below for your information.

As you will see, the referees are very positive about the study and request only minor changes. Referee 1 is concerned that the resolution of the EM images does not allow to deduce if the observed structures are indeed nascent LDs. Please either provide higher resolution EM images or discuss the limitations of the current data and possible alternative interpretations in the most appropriate manner. Referee 2 suggests to quantify LDs not forming at the NVJ and to provide the absolute levels of FFA and TAG in the Appendix. If the latter are large excel files you can also supply them as source data. You could display the original measurements in an excel file that will then be linked to the respective figure displaying the calculated ratio. Please contact me, if you need further information.

From the editorial side, there are also a few things that we need before we can proceed.

- Please review the statistics in the paper. Many panels display error bars and p-values calculated over 2 replicates. Please note that statistical tests are only meaningful if at least three independent biological replicates have been performed. If n < 3 please display the individual data points in a

scatter blot rather than the mean and remove the p-values from these panels.

- Please shorten the title to 100 characters (incl. spaces)

- Please provide a running title (max. 40 characters incl. spaces)

- Please remove the synopsis and bullet points from the manuscript and provide them as separate word file.

- There is no reference to Table S1 and Table S2 in the main text.

- Please provide a legend for each movie in a separate text file and then zip this text file together with the respective movie file and upload this zipped file. Please also rename the movies to Movie EV1/2 and change the callouts in the text accordingly.

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

REFEREE REPORTS

Referee #1:

I am in general satisfied with the numerous edits from the authors. The time-lapse imaging that the authors have performed now nicely show LD biogenesis at NVJs (Figure 3C, D).

It is however still not clear why the authors do not want to show higher magnifications of EM images in Figures 6 F and EV4 G, H. Are these data simply not available (I have a hard time thinking that no high-res pictures were taken at the EM) or are they not conclusive? The authors have changed their terminology from "lenses" to "nascent LDs". This has, however, not alleviated the problem; it is still unclear whether these are indeed nascent LDs or some sort of aberrant accumulation of triglycerides between the outer and inner leaflet of the vacuole, or, as stated by the authors themselves in their rebuttal letter, intermediates in the engulfment of LDs by the vacuole. Taken together, at the current resolution, the evidence is too weak to suggest that LDs bud from the ER at the NVJs, in Mdm1-overexpression conditions, and the structures shown herein can only be reported to as "aberrant structures" and definitely not as "nascent LDs".

Referee #2:

The authors have done a good job of responding to the concerns of the reviewers. I have two minor concerns.

1. The time lapse images of LD formation in Fig 3 are a nice addition. It would be good if the percent of LDs NOT formed at the NVJ were also quantitated. How frequently do LD form away from the NVJ? Please also define what is meant by away from the NVJ.

2. I continue to think that it is not a good idea to present TAG levels as a ratio of TAG to FFA. I am not sure why this was done and the authors might want to explain it in the text. If the authors still want to present the data as a ratio they should also include the absolute levels of TAG and FFA in the supplement (normalized to total lipid phosphate, cell number, total protein, or cell weight). Without this, it is not possible to understand what the ratios mean.

3rd Revision - authors' response 12 October 2017

Response to referees:

Referee #1:

I am in general satisfied with the numerous edits from the authors. The time-lapse imaging that the authors have performed now nicely show LD biogenesis at NVJs (Figure 3C, D).
Thank you.

It is however still not clear why the authors do not want to show higher magnifications of EM images in Figures 6 F and EV4 G, H. Are these data simply not available (I have a hard time thinking that no high-res pictures were taken at the EM) or are they not conclusive? The authors have changed their terminology from "lenses" to "nascent LDs". This has, however, not alleviated the problem; it is still unclear whether these are indeed nascent LDs or some sort of aberrant accumulation of triglycerides between the outer and inner leaflet of the vacuole, or, as stated by the authors themselves in their rebuttal letter, intermediates in the engulfment of LDs by the vacuole. Taken together, at the current resolution, the evidence is too weak to suggest that LDs bud from the ER at the NVJs, in Mdm1-overexpression conditions, and the structures shown herein can only be reported to as "aberrant structures" and definitely not as "nascent LDs".
It is very challenging to answer this using thin sectioning TEM because the resolution is indeed limited. We did however collect high magnification images that were inconclusive. We have now adjusted our description of this result to satisfy the referee's comment and better represent the data generated and take into account the technical limitations.

Referee #2:

The authors have done a good job of responding to the concerns of the reviewers.
Thank you.

I have two minor concerns.

1. The time lapse images of LD formation in Fig 3 are a nice addition. It would be good if the percent of LDs NOT formed at the NVJ were also quantitated. How frequently do LD form away from the NVJ? Please also define what is meant by away from the NVJ.
This is indeed an interesting suggestion. However, we believe that quantifying non-NVJ LDs will not change the conclusions of this study. Furthermore, these and other related experiments are part of an ongoing separate study in the lab, so we respectfully will omit them from this NVJ-focused study.

Regarding defining what is meant by NVJ-associated LDs, we have adjusted the text to now state that NVJ-associated LDs are those which appear within ~0.5 microns of the Nvj1-GFP signal, as imaged by our fluorescence microscopy experiments.

2. I continue to think that it is not a good idea to present TAG levels as a ratio of TAG to FFA. I am not sure why this was done and the authors might want to explain it in the text. If the authors still want to present the data as a ratio they should also include the absolute levels of TAG and FFA in the supplement (normalized to total lipid phosphate, cell number, total protein, or cell weight). Without this, it is not possible to understand what the ratios mean.
We have adjusted the lipid data representation. Please see new Fig 2C, D, and F, and Fig 5E. We have also provided the absolute values for the lipid data in Fig 7C in separate scatter plots to satisfy this comment. Please see Fig EV5B.

EMBO PRESS

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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Mike Henne
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2017-44815-T

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

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

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	For imaging of living yeast, we selected images that represented several yeast cells that accurately represented the population. This is consistent with standard practices in the field.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	No animals were utilized in this study.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	In general, we included all samples in the experimental results of discussion of experiments in this study.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	For quantification of images, we employed a semi-automated quantification procedure that allows for the unbiased quantification of pixel intensities and pixel areas. This approach mimimizes any bias in the interpretation of experiments such as NVJ size, or colocalization of two fluorescently tagged proteins.
For animal studies, include a statement about randomization even if no randomization was used.	no animals were used in this study
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	In general, experiments were conducted and quantified by members of the lab observing the highest levels of ethical standard. Lab members, or the PI, often also show data sets to one another in a blind manner, so as to allow them to give their honest accessment of the data and its trends.
4.b. For animal studies, include a statement about blinding even if no blinding was done	no animals were used in this study
5. For every figure, are statistical tests justified as appropriate?	Yes. For each figure, we employ the appropriate statistical analysis as is standard in the field.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	In general, we utilize the field standard of p<0.05 to indicate statistical significance. Where data are indicated as statistically significant, they satisfy this criteria.

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Is there an estimate of variation within each group of data?	We employ box plots and error bars to indicate sample variation in the experiments, as is standard in the field.
Is the variance similar between the groups that are being statistically compared?	Yes.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	We have indicated reagent sources where appripriate in the Methods section.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	We note the source of our yeast cells as appropriate in the Methods section.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	No animals were utilized in this study.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	No vertebrates were utilized in this study.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	confirmed

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N/A
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	N/A
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	N/A

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	To our knowledge, our data sets fall outside the mandatory public repository standards, but are happy to upload and data as requested.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	Thank you.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	N/A
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	N/A

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	Not to our knowledge.