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Lipid droplets and their component triglycerides and steryl esters regulate autophagosome biogenesis

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Transaction Report:

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

1st Editorial Decision

14 November 2014

Thank you for submitting your manuscript entitled 'Lipid droplets and their component triglycerides and steryl esters regulate autophagosome biogenesis'. I have now received reports of all three referees, which are enclosed below.

As you will see, while the referees consider that your work is well-performed and potentially interesting, referees 1 and 3 are not fully supportive of publication here. They both think that the study does not offer at this stage the sufficient insight into how lipid droplets regulate autophagosome biogenesis. Referee number 2 is more supportive, but also thinks that the work could be further improved by additional experiments and amendments.

Given the interest into the topic and the constructive comments provided by the referees, I can offer to consider a revised version should you be able to substantiate your model along the lines suggested by the referees. This might demand a lot of work, and I will be happy to extend the revision time should that be useful. I am certain that adding more insight into the described process will make your paper an outstanding one. However I would also like to note that it is important to add such further insight in order to consider publication here. I think it would be productive in this case to discuss the revisions further, and I would be grateful if you could send me a point-by-point response upfront detailing what type of experiments you can undertake to address the concerns raised. Please also note our scooping protection; we will not reject your manuscript based on novelty issues in case similar work is published elsewhere while yours is under consideration here.

Thank you for the opportunity to consider your work for publication. I look forward to your

revision.

REFEREE REPORTS

Referee #1:

This manuscript investigates the effects of lipid droplet and fatty acid deprivation on autophagy in general and autophagosome biogenesis in particular. The motivation for studying these particular questions comes from the fact that, despite a number of studies and claims, it still remains uncertain which cellular membrane compartments provide the membrane components required for autophagosome production. As this process is central to autophagy, and a number of human diseases are associated with autophagic processes, this is of general interest topic. In this study, the authors exploit the yeast system to investigate the relationship between LD metabolism and autophagy from the perspective of execution of the autophagic program. Previous studies had established LDs as autophagic substrates, so this work approaches the LD/autophagy relationship from a different perspective. The authors report several lines of evidence to indicate that LDs are required for early steps in the autophagy program, and that formation of the autophagosome is disturbed in the absence of LDs.

In general, the MS describes a large body of observations to support the concept that LDs are required for autophagosome biogenesis. Particularly, the various experiments clearly indicate there is some sort of essential requirement for Fatty acids, TAGs and steryl esters in LD stores for efficient passage through the committed step in autophagy (identified by Atg8 lipidation). The work is basically well-done and ultimately deserving of publication. However, as it stands, it is largely descriptive. The authors do not address interesting possibilities their experiments raise. In sum, it is not clear to this referee that the MS breaks much new ground in its present form.

Specific comments:

- (i) The major criticism of the submitted MS is that it does not even attempt to address how LDs might regulate autophagosome biogenesis. One obvious model is that mobilization of fatty acids from LD stores contributes to autophagosome biogenesis. One would expect that some resident TAG lipase or SE esterase, or some combination of such activities, might be required for autophagy. Demonstration of such a requirement would support the idea that LDs directly donate material to the growing autophagosome. This is a particularly important point given the recent Dupont et al (2014) report, cited by the authors, that LD lipase activity contributes to autophagosome formation.
- (ii) In a related vein, are fatty acids mobilized from LDs during the period of time that the autophagosome is forming? If one labels LD components (for example, fatty acids or proteins) with a fluorescence tracer, does the label become incorporated into the autophagosome?
- (iii) What happens to PE pools under conditions of nitrogen starvation in LD-sufficient and LD-insufficient conditions? Perhaps reduction of PE pools (whether bulk pools or LD pools) forms the basis for the defect in Atg8 lipidation. LD and bulk PE analyses would be informative. This is particularly the case for *tagΔ* cells that show defects in Atg8 lipidation.
- (iv) How do the authors explain that Atg8 is lipidated in *steΔ* cells, is unlipidated in *tagΔ* cells, yet is lipidated in *steΔ tagΔ* double mutants with a massive accumulation of lipidated Atg8 at that? I would expect Atg8 to be un-lipidated in the double mutants. Perhaps inappropriate de-lipidation occurs in *tagΔ* cells.
- (v) The statistics in Fig 2A are from only two independent experiments. At least three independent experiments, please.

Referee #2:

This is a very nice and important study from one of the most respected autophagy investigators and

a strong collaborative group. The study addresses the interplay between autophagy and neutral lipid stores, commonly known as lipid droplets. The relationships described in the literature before this study indicate that lipid droplets can be substrate for autophagic degradation (Singh et al) or source of lipid precursors for autophagic membrane (Dupont et al). This study uses the power of genetics in yeast as well as nice experimental follow-ups to demonstrate that neutral lipids (by manipulating their biosynthesis) play an essential role in autophagic capacity of the cell. The key strength of this work is the strength of the mutant phenotypes that without doubt place lipid droplets (with both neutral lipid components - triglycerides and sterol-esters) upstream of autophagosome biogenesis. This is an important study replete with elegant demonstrations and should be published after some necessary adjustments.

1. General: The authors state that because triglyceride synthesis and sterol ester (esters) synthesis show different effects on LC3 lipidation that this represents an unexpected (novel) angle. This is interesting, since if TGs from LDs are precursors for phospholipids contributing to autophagic membranes (as shown by Dupont et al), does this reveal an opposing role for cholesterol/sterols? LDs can vary in composition and are either dominated by TGS or by SEs. Would it be possible for the authors to physiologically manipulate the composition of LDs (for their abundance and TG:SE ratios) to get at the physiology and pathways of this - beyond the LC3 lipidation (which, granted, is a convenient assay). This is not absolutely necessary but would be of interest and might strengthen the overall study.
2. The experiment with Atg1 distribution (Fig. 1C) is very interesting as it indicates an upstream (regulatory?) role of FA on the regulation of the top protein kinase responsible for autophagy cascade. Is this metabolic or involves some specific signaling event?
3. Fig. 1E. The assay for Cvt pathway is very convincing. Is cerulenin (and by extension FA) affecting mitophagy, pexophagy or other targets of autophagy (beyond the reporter substrate GFP-Atg8, shown in the study)?
4. Fig. 2. The authors state: "We found that the decrease in autophagic activity upon cerulenin treatment correlated with a reduction in the amount of LDs". Is lipolysis (other forms, independent of autophagy) continuing under these conditions or this is purely biosynthetic (i.e. LDs are simply not formed due to TGs not being synthesized)?
5. Whereas the experiments with Δtag and Δare are intriguing and informative, the findings that they differentially affect LC3 lipidation may require some more mechanistic follow-up and understanding.
6. Fig. 6. The Ems are very convincing and quantification are provided. However, any statistics is absent. This should be remedied.
7. Fig. 6. I am surprised that the authors do not comment (or at least this reviewer missed it - my apologies if this is the case) on the state of the vacuole, which presents very striking phenotypic differences.
8. Fig. 6. ER proliferation and mitochondrial enlargement and morphology - can this be expressed in quantitative terms? Can any of these phenotypes be "fixed" by using different metabolic pathways (substrate changes) in yeast? The question to be addressed is - are these phenotypes due to autophagy loss (e.g. mitophagy, ER-phagy) or due to effects of lipids on these organelles independently of autophagy or an altered ability of these organelles to handle metabolic or stress processes.
9. Discussion. I think that in the discussion, the author's choice to focus on van Manen's interpretations (with arachidonic acids) instead of acknowledging the DAG and phospholipid donor pathway for building the autophagic membrane (shown by Dupont et al) is a bit dubious. The authors may have valid reasons for that, so it would be good to get/read their fuller interpretations of the literature. This would not only be fair (which is less important) but also in keeping with the authors' conclusion that LDs (or rather their lipids) contribute to making larger phagosomes. This is precisely what the conclusions were in the Dupont et al study in mammalian systems (not complete shut down, but enhancing the capacity of autophagy). Of course, this should be authors' choice, but from this reviewer's perspective it seems logical to elaborate on this parallel especially since there is now corroborative evidence. Again, I would leave this up to authors' discretion, and it is not mandatory to acknowledge similar conclusions by Dupont et al.

In conclusion, this is a very powerful demonstration (using yeast genetics) of LD-autophagy relationships, and is important to publish. The comments above are rather minor and less important. The core of the study is as definitive as one might expect, and given the controversy (and significance) of autophagy biogenesis pathways this will be a important and highly cited

contribution.

Referee #3:

General summary and opinion about the principle significance of the study, its questions and findings

Recent studies suggest the existence of a complex interplay between autophagy and LDs. In this study, the authors aimed to elucidate the roles of LDs in autophagy. They found that addition of cerulenin that binds and inhibits the activity of fatty acid synthase, blocked the Cvt pathway and autophagy, that free fatty acids were essential for autophagy using fatty acid synthase mutants, and that inhibition of fatty acid synthesis lead to disappearance of LDs. In the strain lacking LDs, autophagic activity was severely impaired. These results suggest that LDs are crucial for autophagy. They speculated that in the absence of LDs, the cells might encounter a shortage of fatty acids that would result in autophagy blockage. However, addition of fatty acids to the LD-deficient strain failed to rescue autophagy. They concluded that LDs but not free fatty acids are important for autophagy. Finally, they found that the lack of LDs inhibited autophagosome formation. Although the data are convincing and sound, I think that the advancement achieved in this study is not sufficient for publication in this high-profile journal. Most importantly, the mechanisms that link between LDs and autophagosome formation are mostly unknown.

Specific major concerns essential to be addressed to support the conclusions

I feel that the data are sufficient and convincing, and the conclusions are valid. As stated above, the authors need further investigation to obtain insights into how LDs are involved in autophagosome formation.

Minor concerns that should be addressed

1. In the abstract, the authors describe "from deletion of the Are1 and Are2 enzymes". Is this 'from' needed?
2. In Figure 2B, LDs were diminished by treatment with cerulenin. Do the amounts of TAG and STE also decrease?
3. In Figure 2E, multiple puncta were observed in LD-deficient cells. These puncta might reflect the site of action of LDs. Where do these puncta localize?
4. Figure 4D indicates that high FAS activity in LD-deficient cells does not contribute to rescue autophagic activity. To further investigate the involvement of LDs in free fatty acids, it is interesting to examine autophagic activity in mutants of triacylglycerol lipases (tg13, tg14, and tg15) and those of steryl ester hydrolases (tg11, yeh1, and yeh2).

Any additional non-essential suggestions for improving the study (which will be at the author's/editor's discretion)

1st Revision - authors' response

02 April 2015

Referee #1:

In general, the MS describes a large body of observations to support the concept that LDs are required for autophagosome biogenesis. Particularly, the various experiments clearly indicate there is some sort of essential requirement for Fatty acids, TAGs and steryl esters in LD stores for efficient passage through the committed step in autophagy (identified by Atg8 lipidation. The work is basically well-done and ultimately deserving of publication.

We would like to thank the referee for these positive comments about our work. We have revised the manuscript and have added important new information, which provides a better mechanistic view of the requirement for lipid droplets (LDs) in the autophagic process. We believe that in its

present form, with the valuable new information added, our study contributes to an improved understanding of the mechanism of autophagosome biogenesis. Below please find a point-by-point response to this reviewer's comments.

Specific comments:

- (i) *The major criticism of the submitted MS is that it does not even attempt to address how LDs might regulate autophagosome biogenesis. One obvious model is that mobilization of fatty acids from LD stores contributes to autophagosome biogenesis. One would expect that some resident TAG lipase or SE esterase, or some combination of such activities, might be required for autophagy. Demonstration of such a requirement would support the idea that LDs directly donate material to the growing autophagosome. This is a particularly important point given the recent Dupont et al (2014) report, cited by the authors, that LD lipase activity contributes to autophagosome formation.*

We thank the reviewer for this pertinent criticism and the excellent suggestions. We have now tested the autophagic activity in all known TAG-lipase and STE-hydrolase deletion strains, in the triple-deletion strain of *tgl3Δtg14Δtg15Δ*, and in the double-deletion strain of *ldh1Δayr1Δ*. We identified the TAG lipases Ayr1 and Ldh1 (which possess both esterase and TAG-lipase activities) as important for the autophagy process. Whereas single deletion of AYR1 or LDH1 led to some inhibition of the autophagic process, double deletion of these lipases significantly inhibited the processing of GFP-Atg8 and its delivery to the vacuole. Having shown (for the first time) that LD lipases are important for autophagy in yeasts, we now show that the STE hydrolase Yeh1 is also important for autophagy. Deletion of YEH1 significantly inhibited GFP-Atg8 processing and its delivery to the vacuole. This is the first report that points to STE hydrolysis as a requirement for the autophagic process. Our new results strongly suggest that LDs contribute lipids that are needed for the autophagic process (Fig 7 panels A–D, F, and E5 panels A–C of the revised manuscript). Moreover, we identified the ER–LD contact-site proteins Ice2 and Ldb16 as requirements for the autophagic process. Ice2, which seems to be the more important of the two in this regard, was recently shown to be essential for the utilization of LD-derived lipids for phospholipid synthesis in the ER (Markgraf, D. F. *et al. Cell Rep.* 2014). Together, these results strongly support the notion that LDs contribute lipids to the autophagosome.

- (ii) *In a related vein, are fatty acids mobilized from LDs during the period of time that the autophagosome is forming? If one labels LD components (for example, fatty acids or proteins) with a fluorescence tracer, does the label become incorporated into the autophagosome?*

The reviewer raises an important question. Whereas labeling of fatty acids is successfully used in mammalian cell cultures (Rambold, A. S., Cohen, S. & Lippincott-Schwartz, J. *Dev Cell.* 2015), it is hardly utilized in the yeast system. In attempting to address this issue we utilized different BODIPY-tagged fatty acids (BODIPY 558/568 C12 and C1-BODIPY500/510 C12). Under our experimental conditions, however, some of these reagents did not label LDs while others did not change their distribution over time. As these assays are not yet established in yeasts, their feasibility is uncertain and a substantial effort will be needed to establish them. We therefore feel that this issue is beyond the scope of the present study, particularly because the results provided in the revised manuscript (new figure 7 and E5), strongly support functional mobilization of lipids to the autophagosome.

- (iii) *What happens to PE pools under conditions of nitrogen starvation in LD-sufficient and LD-insufficient conditions? Perhaps reduction of PE pools (whether bulk pools or LD pools) forms the basis for the defect in Atg8 lipidation. LD and bulk PE analyses would be informative. This is particularly the case for tagD cells that show defects in Atg8 lipidation.*

This is another important question. We have now carried out a TLC analysis of the different LD mutants, and the results do not demonstrate significant differences between the amounts of PE under conditions of LD sufficiency and under nitrogen starvation. The slightly lower amounts of PC/PS

that the analysis revealed in the LD mutants than in the WT may imply that phospholipid synthesis is impeded in these strains. Further studies are needed, however, to better elucidate this question, and we therefore decided **not** to include this in the current manuscript. In an attempt to address this question we also isolated LDs and tested for the presence of the conjugation machinery, but were unable to detect Atg8 or Atg3 in LDs (new Fig E3D). Although our results do not show a correlation between autophagy defects and the amounts of PE, we are currently attempting to analyze lipid biosynthesis during nitrogen starvation as well as the local distribution of newly synthesized phospholipids. This line of experiments will be the subject of future studies.

(iv) How do the authors explain that Atg8 is lipidated in steD cells, is unlipidated in tagD cells, yet is lipidated in steD tagD double mutants with a massive accumulation of lipidated Atg8 at that? I would expect Atg8 to be un-lipidated in the double mutants. Perhaps inappropriate de-lipidation occurs in tagD cells.

We thank the referee for raising this intriguing question. Both *steΔ* and *steΔtagΔ* are defective in their ability to store steryl esters. Our new data indicate that defects in STE hydrolysis (*yeh1Δ* cells) lead to inhibition of autophagy and the accumulation of non-lipidated Atg8 (new Fig E5C). Accordingly, we propose that the inability of Atg8 to conjugate to PE in the *tagΔ* strain may indicate that this process is inhibited by the imbalance in STE accumulation.

(v) The statistics in Fig 2A are from only two independent experiments. At least three independent experiments, please.

Results from three independent experiments are now presented.

Referee #2:

This is a very nice and important study from one of the most respected autophagy investigators and a strong collaborative group. The study addresses the interplay between autophagy and neutral lipid stores, commonly known as lipid droplets. The relationships described in the literature before this study indicate that lipid droplets can be substrate for autophagic degradation (Singh et al) or source of lipid precursors for autophagic membrane (Dupont et al). This study uses the power of genetics in yeast as well as nice experimental follow-ups to demonstrate that neutral lipids (by manipulating their biosynthesis) play an essential role in autophagic capacity of the cell. The key strength of this work is the strength of the mutant phenotypes that without doubt place lipid droplets (with both neutral lipid components - triglycerides and sterol-esters) upstream of autophagosome biogenesis. This is an important study replete with elegant demonstrations and should be published after some necessary adjustments.

We would like to thank the referee for the complimentary remarks. We have made a strong effort to address all of the listed concerns and those of the other referees.

1. General: The authors state that because triglyceride synthesis and sterol ester (esters) synthesis show different effects on LC3 lipidation that this represents an unexpected (novel) angle. This is interesting, since if TGs from LDs are precursors for phospholipids contributing to autophagic membranes (as shown by Dupont et al), does this reveal an opposing role for cholesterol/sterols? LDs can vary in composition and are either dominated by TGS or by SEs. Would it be possible for the authors to physiologically manipulate the composition of LDs (for their abundance and TG:SE ratios) to get at the physiology and pathways of this - beyond the LC3 lipidation (which, granted, is a convenient assay). This is not absolutely necessary but would be of interest and might strengthen the overall study.

We thank the referee for this excellent suggestion. We now provide evidence, by using different deletion strains of STE and TAG lipases, that lipolysis of TAG and STE is essential for autophagosome biogenesis (new Fig 7 panels A–D, F and new Supplementary Fig 5 A–C) (please also see response to Referee #1, comment 1). Defects in STE hydrolysis (*yeh1Δ* cells) led to inhibition of autophagy and accumulation of non-lipidated Atg8, suggesting that both STE and sterols play a role in the lipidation of Atg8 (new Fig E5C).

2. *The experiment with Atg1 distribution (Fig. 1C) is very interesting as it indicates an upstream (regulatory?) role of FA on the regulation of the top protein kinase responsible for autophagy cascade. Is this metabolic or involves some specific signaling event?*

Intriguing though it is, we feel that this issue is beyond the scope of the present manuscript to pursue it. Nevertheless, we argue that the accumulation of Atg8 and Atg1 in puncta structures suggests that the signals for autophagy initiation are active under these conditions.

3. *Fig. 1E. The assay for Cvt pathway is very convincing. Is cerulenin (and by extension FA) affecting mitophagy, pexophagy or other targets of autophagy (beyond the reporter substrate GFP-Atg8, shown in the study)?*

Our results indicate that ER proteins are not properly targeted for degradation in cerulenin-treated cells. These results may imply that ER-phagy is also defective under these conditions (see new Fig E1A-B).

4. *Fig. 2. The authors state: "We found that the decrease in autophagic activity upon cerulenin treatment correlated with a reduction in the amount of LDs". Is lipolysis (other forms, independent of autophagy) continuing under these conditions or this is purely biosynthetic (i.e. LDs are simply not formed due to TGs not being synthesized)?*

Cerulenin is accepted as a drug that prevents the synthesis of fatty acid and is widely used to promote lipolysis of TAG and STE (Koffel, R., Tiwari, R., Falquet, L. & Schneider, R. *Mol Cell Biol* .2005. and Athenstaedt, K. & Daum, G. *J Biol Chem*. 2003). We have evidence that support lipolysis under nitrogen starvation. First, both TAG lipases and STE hydrolases are essential requirements for the autophagic process (new Fig 7 and new Figure E5), and second, we observe a reduction in the amount of LDs in cerulenin-treated cells over time (Fig 2B and S2A-B). Together, these results indicate that LDs are consumed during nitrogen starvation and upon cerulenin treatment.

5. *Whereas the experiments with Δtag and Δare are intriguing and informative, the findings that they differentially affect LC3 lipidation may require some more mechanistic follow-up and understanding.*

Both *ste Δ* and *ste $\Delta tag\Delta$* are defective in the ability to store steryl esters. Our new data indicate that defects in STE hydrolysis (*yeh1 Δ* cells) lead to inhibition of autophagy and accumulation of non-lipidated Atg8 (see new Fig E5C). Accordingly, we propose that the inability of Atg8 to conjugate to PE in the *tag Δ* strain may indicate that such conjugation is inhibited by the imbalance in STE accumulation. We are currently attempting to perform mass spectrometric analysis and TLC, as well as pulse-chase analysis with radiolabeled fatty acids, to gain a better understanding of the contribution of lipids by TAG and STE. We feel sure that the referee will agree, however, that this investigation is beyond the scope of the present manuscript.

6. *Fig. 6. The Ems are very convincing and quantification are provided. However, any statistics is absent. This should be remedied.*

We have now included statistical analyses and moved this figure to the supplementary data (Fig E4). In addition, we performed EM analysis on the LD mutants in the background of *pep4 Δ* (see new Fig 6). Our new data strongly support the inability of these strains to accumulate autophagic bodies in the vacuole.

7. *Fig. 6. I am surprised that the authors do not comment (or at least this reviewer missed it - my apologies if this is the case) on the state of the vacuole, which presents very striking phenotypic differences.*

We thank the referee for pointing out this issue. We have now addressed the fragmented state of the vacuole, which is most probably due to aberrant sterol synthesis (Kato, M. & Wickner, W. *EMBO J* 20, 4035-4040. Jones, L., Tedrick, K., Baier, A., Logan, M. R. & Eitzen, G. *J Biol Chem* 285, 4298-4306. Tedrick, K., Trischuk, T., Lehner, R. & Eitzen, G. *Mol Biol Cell* 15, 4609-4621) (see page 9 of the revised manuscript)

8. Fig. 6. ER proliferation and mitochondrial enlargement and morphology - can this be expressed in quantitative terms? Can any of these phenotypes be "fixed" by using different metabolic pathways (substrate changes) in yeast? The question to be addressed is - are these phenotypes due to autophagy loss (e.g. mitophagy, ER-phagy) or due to effects of lipids on these organelles independently of autophagy or an altered ability of these organelles to handle metabolic or stress processes.

We thank the referee for this excellent question. By using fluorescence-tagged proteins we observed aberrant morphology of the ER in autophagy-deficient strains under nitrogen starvation (see new Fig E1A). This aberrant morphology could be rescued by the use of cerulenin, suggesting that new synthesis of lipids need to be maintained by LD formation and by the autophagic machinery.

9. Discussion. I think that in the discussion, the author's choice to focus on van Manen's interpretations (with arachidonic acids) instead of acknowledging the DAG and phospholipid donor pathway for building the autophagic membrane (shown by Dupont et al) is a bit dubious. The authors may have valid reasons for that, so it would be good to get/read their fuller interpretations of the literature. This would not only be fair (which is less important) but also in keeping with the authors' conclusion that LDs (or rather their lipids) contribute to making larger phagosomes. This is precisely what the conclusions were in the Dupont et al study in mammalian systems (not complete shut down, but enhancing the capacity of autophagy). Of course, this should be authors' choice, but from this reviewer's perspective it seems logical to elaborate on this parallel especially since there is now corroborative evidence. Again, I would leave this up to authors' discretion, and it is not mandatory to acknowledge similar conclusions by Dupont et al.

We thank the reviewer for this suggestion. In accord with our new data we have changed the Discussion section and included a discussion on the roles of TAG and STE lipases in the autophagic process.

In conclusion, this is a very powerful demonstration (using yeast genetics) of LD-autophagy relationships, and is important to publish. The comments above are rather minor and less important. The core of the study is as definitive as one might expect, and given the controversy (and significance) of autophagy biogenesis pathways this will be a important and highly cited contribution.

We greatly appreciate the strong support of the referee for this study. We feel confident that the revised manuscript now meets the standards set by EMBO J in that it sheds new light and uncovers novel aspects with regard to the role of LDs in autophagy.

Referee #3:

Although the data are convincing and sound, I think that the advancement achieved in this study is not sufficient for publication in this high-profile journal. Most importantly, the mechanisms that link between LDs and autophagosome formation are mostly unknown.

We take it as a compliment that referee finds our data convincing and sound. In the revised version we have made a great effort to acquire further knowledge of the mechanism by which LDs regulate autophagy. Below please find our detailed response to the listed comments.

Specific major concerns essential to be addressed to support the conclusions

I feel that the data are sufficient and convincing, and the conclusions are valid. As stated above, the authors need further investigation to obtain insights into how LDs are involved in autophagosome formation.

We have identified three LD-resident lipases as well as two LD-ER contact site proteins that are required for the autophagic process. Our study shows that both the formation and the lipolysis of LDs are essential for the autophagic process and we propose that lipids channeled from LDs to the ER promote autophagosome biogenesis.

Minor concerns that should be addressed

1. In the abstract, the authors describe "from deletion of the *Are1* and *Are2* enzymes". Is this 'from' needed?

We have modified the abstract according to the referee's suggestion and according to the new data presented in this version.

2. In Figure 2B, LDs were diminished by treatment with cerulenin. Do the amounts of TAG and STE also decrease?

Cerulenin is accepted as a drug that prevents fatty acid synthesis and is widely used to promote the lipolysis of TAG and STE (Koffel, R., Tiwari, R., Falquet, L. & Schneider, R. *Mol Cell Biol* .2005. and Athenstaedt, K. & Daum, G. *J Biol Chem*. 2003). We now show that both TAG lipases and STE hydrolases are active during nitrogen starvation (see new Fig 7). In view of this, and given the reduction in amounts of LDs, we have no reason to believe that TAG and STE levels are not also diminished, though we did not measure the rates of their consumption.

3. In Figure 2E, multiple puncta were observed in LD-deficient cells. These puncta might reflect the site of action of LDs. Where do these puncta localize?

We thank the reviewer for this excellent suggestion. To address this question we performed subcellular fractionations of the LD-deficient and the WT strains. Our new data indicated that while Atg8 levels in the LD-deficient strain are higher, this protein is predominantly localized to the ER fraction (new Fig E2C). As the ER and LDs are functionally connected and the LD-ER contact-site protein Ice2 (responsible for lipid flow from LDs to the ER) is important for the autophagic process, we suggest that LDs contribute lipids to the ER, and that this is essential for biogenesis of the autophagosome.

4. Figure 4D indicates that high FAS activity in LD-deficient cells does not contribute to rescue autophagic activity. To further investigate the involvement of LDs in free fatty acids, it is interesting to examine autophagic activity in mutants of triacylglycerol lipases (*tgl3*, *tgl4*, and *tgl5*) and those of steryl ester hydrolases (*tgl1*, *yeh1*, and *yeh2*).

To check out this excellent suggestion we tested the autophagic activities of all known TAG lipases and STE hydrolases. We identified the TAG lipases *Ayr1* and *Ldh1* (which possess both esterase and TAG lipase activities) as important for the autophagy process. Single deletion of these lipases resulted in a small reduction in the autophagic process, while their double deletion significantly inhibited the processing of GFP-Atg8 and its delivery to the vacuole. This, to our knowledge, is the first study showing that TAG lipases are important for autophagy in yeasts. Interestingly, the TAG lipases *Tgl3*, *Tgl4* and *Tgl5* were not found to be necessary for the autophagic process. Moreover, we now showed that the STE hydrolase *Yeh1* is important for autophagy, and that its deletion significantly inhibits GFP-Atg8 processing and delivery to the vacuole. So this is also the first report that shows a need for STE hydrolysis in the autophagic process. Our new results thus strongly suggest that LDs contribute lipids that are essential for the autophagic process (see new Fig 7 A-D, F and new Supplementary Fig 5 A,B).

Any additional non-essential suggestions for improving the study (which will be at the author's/editor's discretion)

We identified the ER-LD contact-site proteins *Ldb16* and *Ice2* the latter recently shown to be essential for the utilization of lipids derived from LDs for phospholipid synthesis in the ER) as vital for the autophagic process (Markgraf, D. F. *et al. Cell Rep*. 2014). These findings strongly support the idea that LDs contribute lipids to the autophagosome.

Thank you for submitting your revised manuscript for our consideration.
Your manuscript has now been seen once more by two of the original referees (see comments

below), and I am happy to inform you that they are both in favor of publication, pending satisfactory minor revision.

I would therefore like to ask you to address referee #1's suggestion and to provide a final version of your manuscript.

I am therefore formally returning the manuscript to you for a final round of minor revision, only to allow you to easily modify/replace the files. Once we should have received the revised version, we should then be able to swiftly proceed with formal acceptance and production of the manuscript!

REFEREE REPORTS

Referee #1:

The authors have come back with a strong and positive response to the reviewers' criticisms and the response satisfies this reviewer's major concerns. one small editorial issue remains. The authors have strengthened the MS by showing LD sterol ester hydrolysis is important for biogenesis of the autophagosomal membrane system, and they emphasize this point as a matter of impact as a first demonstration to that effect.

While this may be true for autophagy, it was previously demonstrated that LD ergosterol ester hydrolysis is required for formation of prospore membranes in sporulating diploid yeast cells even though the signaling pathways for initiating assembly of this membrane during meiosis remain intact (Ren, J. et al 2014 MBOC 25, 712-727).

In principle, the results in this submission describe an entirely analogous process, that is, LD-dependent biogenesis of a complex membrane system in cells undergoing nutrient limitation. This issue should be explicitly identified and referenced in the MS as it demonstrates a broader cellular strategy for investing LD stores in the formation of membranes in nutrient-deprived cells.

Referee #2:

This is an outstanding revision of an already highly interesting and significant work that required some adjustments, including experimental ones. The authors have responded in full and very successfully to all criticisms, not just from this reviewer but in general. Whereas the initial submission was already suitable for publication, the revised version gives three new elements each representing a discovery in their own right. The authors demonstrated that both triglycerides and sterols esters are required for autophagy and have identified specific lipases of these neutral lipid stores as required to mobilize them into lipids that may be required for autophagy. Moreover, the authors report a novel finding (an important discovery made in the process of revision!) that ER-lipid droplet contacts directed by Ice2 and Ldb16 are necessary for autophagy. Thus, the authors provide convergent evidence that lipolysis of neutral lipids results in delivery of lipids essential for autophagosomal biogenesis and function. This study is superb and should be published without further delay.

2nd Revision - authors' response

08 June 2015

Referee #1:

The authors have come back with a strong and positive response to the reviewers' criticisms and the response satisfies this reviewer's major concerns. one small editorial issue remains. The authors have strengthened the MS by showing LD sterol ester hydrolysis is important for biogenesis of the autophagosomal membrane system, and they emphasize this point as a matter of impact as a first demonstration to that effect. While this may be true for autophagy, it was previously demonstrated that LD ergosterol ester hydrolysis is required for formation of prospore membranes in sporulating diploid yeast cells even though the signaling pathways for initiating assembly of this membrane during meiosis remain intact (Ren, J. et al 2014 MBOC 25, 712-727). In principle, the results in this submission describe an entirely analogous process, that is, LD-dependent biogenesis of a complex

membrane system in cells undergoing nutrient limitation. This issue should be explicitly identified and referenced in the MS as it demonstrates a broader cellular strategy for investing LD stores in the formation of membranes in nutrient-deprived cells.

We would like to thank the reviewer for this suggestion. We agree that this reference should be included in this manuscript and that the role of LDs under nutrient limiting conditions can very well extend beyond their role in autophagy. We now included this reference in the manuscript and addressed it in the discussion (page 11).