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Unsaturated fatty acids induce non-canonical autophagy

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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.)

Editor: Andrea Leibfried

Additional Editorial Correspondence 27 July 2014

Thank you for submitting your manuscript entitled 'Unsaturated fatty acids induce non-canonical autophagy'. I have now received reports of all three referees, which are enclosed below. As you will see, while the referees consider that your study is potentially interesting, referees 1 and 2 conclude that the work is too preliminary at this stage. More substantive controls are required to assess Golgi and PLD function upon oleate addition and a comparison between equal carbon-containing fatty acids should be included. The referees also note that the study is currently not well developed at the mechanistic level and referee #1 points out that a differentiation from other 'noncanonical autophagies' would strengthen your manuscript tremendously. Therefore, before taking a decision, I think it would be most productive if you could provide me upfront with a point-by-point response to the raised criticisms in order to see how you would address them. I don't expect you to solve the mechanism underlying the reported UFA induced autophagy pathway in full, but as the EMBO Journal emphasizes broad interest and mechanism, it would be important to develop the depth of the analysis as suggested by referee #1 in terms of molecular mechanism in order to place the described pathway more clearly in the context of the literature.

Please send me the point-by-point draft by reply email - I am looking forward to receiving it before making a formal editorial decision. Thank you very much.

REFEREE REPORTS:

Referee #1:

1. The study is good in its main observations. It is definitely intriguing. I am not a fan of asking for more experiments, so I will not do that, or at least insist on some experiments that would interest me but may not be on the authors' list of things to do. Instead, I did make some general suggestions below. I find the study as very promising but also very, very preliminary. It appears to me as a good starting point for something potentially interesting.
2. I am also not a big fan for asking for more when the authors decided this is what they are interested in reporting. However , there are too many "noncanonical autophagies" described in the literature at the moment. I don't know whether it is appropriate to ask the authors to differentiate theirs form other "noncanonical autophagies", but that would be one reasonable thing to do and within the reach of a revision.
3. Is the choice of comparing oleate (C-18) and palmytate (C16) a good comparison? Do they incorporate into the same lipids (e.g. triglycerides, phospholipids etc). Would not C18 unsatrated vs C18 saturated (stearate) FA be a fairer comparison (barring solubility issues, which may plague other FAs screened)? Or comparing cis vs trans unsaturated FA of the same length?
4. Oleic acid is also a well known inducer of PLD. Have the authors tested the role of this important signaling pathway. This definitely needs to be addressed - use t-butanol, vs n-butanol and/or genetic means to see (or rule out) whether it is PLD activation that is behind their observations with oleic acid.
5. The authors are to be congratulated on the use of carnitine palmitoyltransferase 1A (CPT1A) knockdown (an enzyme that is required for import to mitochondrial long-chain FAs destined to oxidation into the matrix to document inverse inhibition of GFP-LC3+ triggered puncta by palmitate vs oleate. Great control. Makes this reviewer lament over why other parts of the manuscript are not replete with such elegant experiments...
6. The discussion of all various ways to induce noncanonical autophagy (often admixed with various cell death pathways) is a bit too nebulous (my apologies for the word here) for my taste. Perhaps a better, more discriminating analysis that critiques (not just lists) the often contradictory and confusing publications would be far more useful to grasp the significance of the work under consideration here.
7. The BFA result with oleate would be a good starting point to generate a study with the beginning (like what is now presented) and an end (what might represent a definition of a specific pathway). Given that this is a stellar team of investigators with lots of firepower and resources, it would indeed be good to invest a bit more in the definition of the pathway, beyond its initial recognition (as presented here).
8. Overall, as a reader I am intrigued but as a reviewer I am left without understanding what exactly is going on, and what exactly would I be endorsing. Perhaps this reflects my bottom-line views: as a reader I endorse this study for publication, but as a reviewer, I feel that more due diligence and in depth analysis is needed. In summary, I recommend extensive revision (of authors' choice). Provided that the authors take upon themselves the task of revision seriously, it would be interesting to see how this story unfolds, even if the ending is only partially resolved, but with some firmer conclusions than just having a yet another undefined noncanonical autophagy. It would be enough to define whether it is as a true pathway or show (equally acceptable) that it is an epiphenomenon (as many of the cited observational studies may be).

Additional points:

9. p. 3 The correct reference for p62 turn-over should be one of T. Johansen's references.

10. p. 4 The discussion of lipid droplets and TG utilization for or by autophagy is not complete - there is one improtant missing reference and description of a key concept whereby TGs serve as

neutral lipid precursors for phospholipids to build autophagosomes - they also used oleic acid (Dupont et al, 2014; Current Biology).

11. Is LC3 dots (LC3 on vesicles) a measure of autophagy under all circumstances? In this case, having LC3 on Golgi membranes may not be an autophagy at all, but a completely different process. Many things have been recently termed "noncanonical autophagy". Perhaps it is time to define what autophagy means as a pathway and as a physiologically defined entity. Other manifestations whereby LC3 and other proteins can be seen 'elsewhere" not necessarily represent autophagy.

12. Minor spelling etc. For example: "co-straining" is probably "co-staininig"

13. Fig. 1. Y axes. "LC3 dots" is a dimensionless definition. What's missing is : Number, number per cell, area per cell, etc.

Referee #2:

The effect of fatty acid load on autophagy has been extensively studied in recent years. The present study provides new insights into the relationship between fatty acids and autophagy utilizing both in vitro and in vivo systems. A panel of different length saturated and unsaturated fatty acids was screened for GFP-LC3 positive puncta formation in U2OS cancer cells. The authors provide evidence that on palmitate (16:0) and oleate (18:1) act via different mechanism to activate autophagy. Autophagy triggered by palmitate depends on signaling factors such as AMPK, PKR, JNK and BECN1/PIK3C complex known to activate the canonical autophagic pathway. Conversely, oleate induces autophagy was found independent of BECN1/PIK3C but led to redistribution of LC3 and p62 to the Golgi apparatus.
Altogether, the authors show substantial evidence to support the model whereby LC3 accumulation and different signaling pathways mediate p62 degradation when palmitate or oleate are given. However, the authors' main claim that oleate leads to the induction of non-canonical autophagy is not well based. The authors should consider the possibility that oleate treatment might lead to Golgi dysfunction that by itself indirectly affects autophagy. The data presented could be interpreted as a general traffic jam at this organelle. The authors should therefore determine the effect of their treatment on both endocytosis and exocytosis. Moreover, the effect of oleate on lysosomal degradation should be determined by combining IF and WB analysis.

Additional comments:

Induction of autophagy both by palmitate and by oleate should be better established. Autophagic flux with or without lysosomal inhibitors in U2OS cells is presented only by accumulation of GFP-LC3 dots (Fig. 1B). WB analysis under these conditions should be determined measuring both LC3 and p62. The autophagic flux presented in Fig. 4B is missing the WT mice and another autophagy degradation marker.
The authors should address the fact that in data presented in Fig. 1F oleate treatment leads to higher levels of phosphorylated AMPK whereas Fig. 3A shows that KD of AMPK is not abolishing the GFP-LC3 dots accumulation.
All colocalization panels should include the red and green channels separately. The quantification of colocalization values needs to be explained (what was the method/software used for the calculation?).
Fig. 2A+B and Fig. E1 A-C the BFA+ fatty-acids treatment should be presented as in Fig. 2C-E. Additionally, Fig. E1 B+C should be moved to Fig. 2.
In Fig. 2F the rational for the data analysis is not fully clear. Testing the distance between the two proteins do not necessarily indicate autophagic activity. Moreover, the control image is missing, there is no indication what are the arrows pointing at, the gold particles labeling p62 and LC3 are not specified and no explanation how the calculation of the distance between the dots is measured.
The TEM images (Fig. 2 G+H should be analyzed with Golgi marker to solidify the authors' conclusion.
AMPK, PKR and JNK function and the relation to autophagy should be clarified in the text.

The accumulation of neutral lipid droplets in the Golgi under oleate treatment presented in Fig. 2B and the data presented in Fig. 3B should be removed as they do not contribute to the main manuscript message.

Referee #3:

Summary:

This study describes a series of experiments that investigate the ever more complicated lipid signaling events that accompany autophagic responses in cells, specifically the differential autophagic responses of cells to saturated vs unsaturated fatty acids. However, how these signaling events are regulated is not well understood. What recommends this MS is that the study makes interesting observations, in several different contexts, regarding the differential (and conserved) mechanisms of inducing autophagy in response to palmitate vs oleate challenge. It is from that perspective that the EMBO J readership (and the cell biology community in general) will find this work of interest. The authors raise some interesting points, these points are well supported experimentally, and are pleasantly unexpected. While these studies do not address the actual mechanism by which oleate induces non-canonical autophagy, it is this reviewer's opinion that those types of mechanistic studies appropriately lay in future work guided by the important results reported here.

Comments:

Some suggestions for the authors for how this MS might be strengthened are listed below.

(i) Does involvement of the Golgi complex in oleate-induced autophagy involve accelerated autophagic degradation of Golgi markers (GALT, LMAN1, etc) relative to mitochondrial or ER markers?
(ii) By the same token, is lipophagy differentially affected upon palmitate vs oleate challenge?
(iii) Why was arachidonate used as UFA in the worm beclin knockdown experiments rather than oleate, especially given that oleate challenge stimulates non-canonical autophagy in the worm? Consistency would suggest presenting oleate data here.

1st Editorial Decision 29 July 2014

Thank you for submitting your manuscript for consideration by the EMBO Journal. I attach once more the referee comments below.

Given the referees' comments and the outline you have already provided on how to address the concerns, I would like to invite you to submit a revised version of the manuscript. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version. We generally allow three months as standard revision time, but as discussed before, a resubmission in November/December is fine.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website:
http://emboj.embopress.org/about#Transparent_Process

As a matter of policy, competing manuscripts published during the revision period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting the revision deadline, please let us know in advance and we may be able to grant an extension.

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

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1st Revision - authors' response 28 November 2014

Reviewer n° 1 commented:

The study is good in its main observations. It is definitely intriguing. I am not a fan of asking for more experiments, so I will not do that, or at least insist on some experiments that would interest me but may not be on the authors' list of things to do. Instead, I did make some general suggestions below. I find the study as very promising but also very, very preliminary. It appears to me as a good starting point for something potentially interesting.

Our response: We thank the reviewer for appreciating the potential interest of our manuscript. We have done our best to specifically address all the points raised by the reviewer, as detailed below.

Then raised the following major points:

1. *I am also not a big fan for asking for more when the authors decided this is what they are interested in reporting. However, there are too many "noncanonical autophagies" described in the literature at the moment. I don't know whether it is appropriate to ask the authors to differentiate theirs form other "noncanonical autophagies", but that would be one reasonable thing to do and within the reach of a revision.*

Our response: The following proteins belong to the canonical autophagic machinery, but have been shown to be dispensable for autophagy induction in specific settings (see Codogno et al. 2012 Nat Rev Mol Cell Biol): ULK1, AMPK, BECN1, PIK3C3, ATG5, ATG7. We have already tested the dependence of oleate and palmitate-induced autophagy on all these factors, *in vitro*, by individually downregulating each of them in U2OS cells and assessing the ability of the latter to mount an autophagic response upon exposure to FAs. These results were incorporated in the previous version of the paper. To obtain further insights into this issue, we have downregulated several other factors involved in non-canonical autophagy (i.e., AMBRA1, ATG14, BIF1,HMGB1, RUBICON, PINK1, UVRAG and VMP1) and tested their requirement for oleate-induced autophagy. We found that none of these factors interferes with the ability of oleate to increase the autophagic flux. These results have been incorporated in the revised version of Figure E3. Moreover, since not all forms of autophagy respond to MTORCI signaling, we tested how modulating the MTORCI signal transduction cascade impacts on palmitate- versus oleate-induced autophagy. To this aim, we altered the expression levels of upstream MTORCI inhibitors (i.e., TSC2, PTEN) and monitored autophagic responses to palmitate versus oleate. We found that the hyperactivation of MTORCI resulting from the absence (in MEFs) or the downregulation (in U2OS cells) of TSC2 or PTEN inhibits indeed oleate- as well as palmitate-induced autophagy, which indeed appears to involve the inhibition of MTORCI. These results have been incorporated in the revised version of Figures 6 and E3.

2. *Is the choice of comparing oleate (C18) and palmytate (C16) a good comparison? Do they incorporate into the same lipids (e.g. triglycerides, phospholipids etc). Would not C18 unsaturated vs C18 saturated (stearate) FA be a fairer comparison (barring solubility issues, which may plague other FAs screened)? Or comparing cis vs trans unsaturated FA of the same length?.*

Our response: We performed a systematic comparison of saturated versus non-saturated fatty acids of the same length in several instances. For instance, oleate and stearate (octadecanoic acid) were compared side-by-side in experiments shown in the original versions of Fig. 1 (autophagic responses measured in terms of GFP-LC3$^+$), E1 (colocalization of Golgi markers with LC3 dots) and E2 (dependence of autophagic responses measured in terms of GFP-LC3$^+$ and FYVE-RFP$^+$ dots on beclin 1 and VPS34). We chose to perform the majority of our experiments with palmitate over stearate for two reasons: (1) solubility issues (as correctly guessed by the reviewer), and (2) the fact that oleate and palmitate are the most abundant FAs contained in mammalian tissues. We know explicitly motivate our choice in the revised version of the manuscript. To confirm that the autophagy-inducing potential of palmitate is very similar – if not identical – to that of stearate, we performed additional flux experiments with stearate, which we integrated in the revised version of Figure 1.

3. *Oleic acid is also a well-known inducer of PLD. Have the authors tested the role of this important signaling pathway. This definitely needs to be addressed - use t-butanol, vs n-butanol and/or genetic means to see (or rule out) whether it is PLD activation that is behind their observations with oleic acid.*

Our response: Driven by the constructive remark from the reviewer, we tested the implication of PLD in the autophagic response of osteosarcoma U2OS cells to oleate (versus palmitate). Oleate is an established PLD activator, but the role of PLD in autophagy remains controversial (see for instance PMID 21266992, 24802400 and 24632948, pointing to PLD as an autophagy-stimulatory factor, versus PMID 24009738 suggesting that PLD inhibits autophagy). To get insights into this issue, we (1) downregulated PLD1 in U2OS cells or (2) exposed these cells to several PLD inhibitors (i.e., VU015, BML-280 and BML-279), followed by the assessment of autophagy upon treatment with oleate or palmitate. As shown in **Figure 1** of this rebuttal letter, the inhibition of PLD by chemical agents *per se* promotes LC3 lipidation, and this effect appears to add up to, rather than interfering with, the pro-autophagic activity of oleate (and palmitate). It therefore seems that PLDs are not involved in oleate-induced autophagy. The effects of a partial PLD1 depletion were mild.

Nonetheless, the downregulation of PLD1 failed to inhibit, if not exacerbate to some extent, LC3 lipidation induced by oleate. We feel that including these data in the revised version of the manuscript would not add to the take-home message but would complicate understanding.

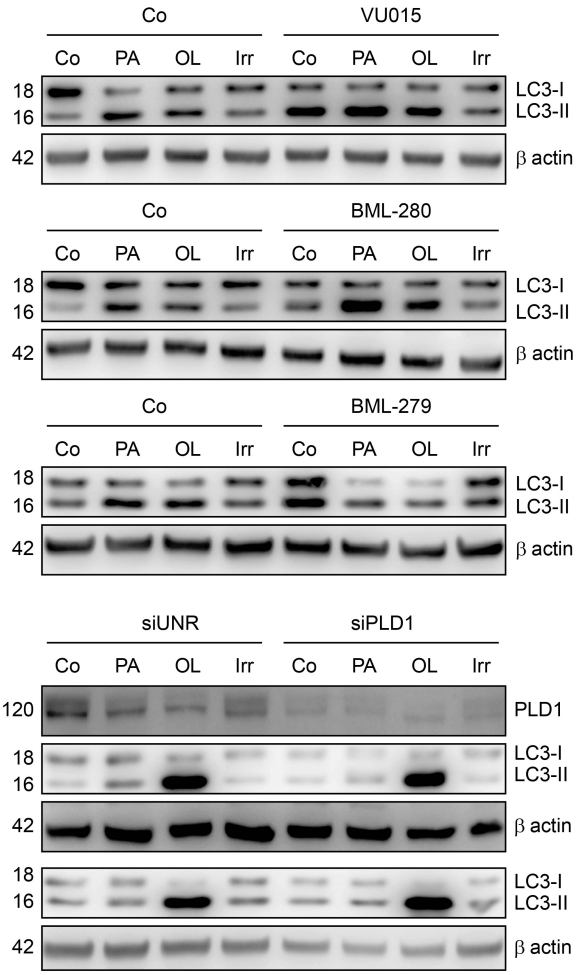

Figure 1. Implication of PLD1 and PLD2 in autophagy induced by oleate and palmitate. *WT U2OS cells were transfected with a control siRNA (siUNR) or with validated siRNAs specific for PLD1 (siPLD1) for 48 hrs, then maintained in control (Co) conditions or treated with 500 μM oleate (OL) or 500 μM palmitate (PA) for additional 6 hrs. Alternatively, WT U2OS cells were maintained in control conditions or treated with 500 μM OL or 500 μM PA, alone or in the presence of 10 μM VU015, 10 μM BML-280 or 10 μM BML-279, for 6 hrs. In both settings, the lipidation of LC3 was assessed by immunoblotting, and β actin levels were monitored to ensure equal loading of lanes. Irr, irrelevant sample.*

4. *The authors are to be congratulated on the use of carnitine palmitoyltransferase 1A (CPT1A) knockdown (an enzyme that is required for import to mitochondrial long-chain FAs destined to β oxidation into the matrix) to document inverse inhibition of GFP-LC3$^+$ puncta triggered by palmitate vs oleate. Great control. Makes this reviewer lament over why other parts of the manuscript are not replete with such elegant experiments...*

Our response: We thank the reviewer for the positive comment. As the reviewer will surely appreciated, we have performed several additional experiments to strengthen the conclusions of our paper, as detailed in this rebuttal letter.

5. *The discussion of all various ways to induce non-canonical autophagy (often admixed with various cell death pathways) is a bit too nebulous (my apologies for the word here) for my taste. Perhaps a better, more discriminating analysis that critiques (not just lists) the often contradictory and confusing publications would be far more useful to grasp the significance of the work under consideration here.*

Our response: We apologize for this. We have done our best to provide a clear and concise discussion on the interrelationship between non-canonical autophagy and cell death. We are positive that this will help the reader to fully grasp the significance of our work.

6. *The BFA result with oleate would be a good starting point to generate a study with the beginning (like what is now presented) and an end (what might represent a definition of a specific pathway). Given that this is a stellar team of investigators with lots of firepower and resources, it would indeed be good to invest a bit more in the definition of the pathway, beyond its initial recognition (as presented here).*

Our response: Inspired by the constructive critique from the reviewer, we have performed several experiments to address the implication of the Golgi apparatus in oleate-induced autophagy. In particular, we performed flux experiments as well as long-lived protein degradation assays in the presence or absence of BFA. We found that the disruption of the Golgi apparatus remarkably inhibits the ability of oleate, but not palmitate, to promote LC3 lipidation, p62 degradation of and the autophagic catabolism of long-lived proteins. These results have been included in the revised version of Figure 4. We concluded that the Golgi apparatus is not only involved, but also required for, oleate-induced autophagy.

7. *Overall, as a reader I am intrigued but as a reviewer I am left without understanding what exactly is going on, and what exactly would I be endorsing. Perhaps this reflects my bottom-line views: as a reader I endorse this study for publication, but as a reviewer, I feel that more due diligence and in depth analysis is needed. In summary, I recommend extensive revision (of authors' choice). Provided that the authors take upon themselves the task of revision seriously, it would be interesting to see how this story unfolds, even if the ending is only partially resolved, but with some firmer conclusions than just having a yet another undefined noncanonical autophagy. It would be enough to define whether it is as a true pathway or show (equally acceptable) that it is an epiphenomenon (as many of the cited observational studies may be).*

Our response: Inspired by the recommendations of this reviewer as well as by the critiques raised by reviewers n° 2 and n° 3 (see below), we have tested several hypothesis and extensively revised the manuscript according to the results of this experimental effort. First, we excluded the role of several proteins involved in non-canonical form of autophagy in the autophagic response of U2OS cells to oleate. Second, we demonstrated that oleate induces BECN1-independent autophagy not only in mammalian cells, but also in mice, nematodes and yeast. Third, we demonstrated that oleate does not compromise the ability of the Golgi apparatus to glycosylate proteins (as BFA does) and that Golgi markers are not decreased in the course of oleate-induced autophagy, proving that the Golgi apparatus is involved, but not a substrate for this process. Fourth, we demonstrated that oleate does not interfere with the exocytic/endocytic pathways. Finally, we demonstrated that BFA compromises the ability of oleate (but not palmitate) to accelerate the degradation of long-lived proteins, leading us to conclude that the functional and structural integrity of the Golgi apparatus is required for oleate-induced autophagy.

As well as these minor issues:

8. *p. 3 The correct reference for p62 turn-over should be one of T. Johansen's references.*

Our response: We have substituted this reference, as indicated by the reviewer.

9. *p. 4 The discussion of lipid droplets and TG utilization for or by autophagy is not complete - there is one important missing reference and description of a key concept whereby TGs serve as neutral lipid precursors for phospholipids to build autophagosomes - they also used oleic acid (Dupont et al, 2014; Current Biology).*

Our response: We thank the reviewer for this constructive critique. We now mention in the Introduction of the paper that “neutral lipid droplets have recently been shown to contribute to autophagic responses by providing substrates for the formation of autophagosomes”, citing the work of Dupont et al. (*Curr Biol* 2014).

10. *Is LC3 dots (LC3 on vesicles) a measure of autophagy under all circumstances? In this case, having LC3 on Golgi membranes may not be an autophagy at all, but a completely different process. Many things have been recently termed "non-canonical autophagy". Perhaps it is time to define what autophagy means as a pathway and as a physiologically defined entity.*

Other manifestations whereby LC3 and other proteins can be seen 'elsewhere" not necessarily represent autophagy.

Our response: We agree with the reviewer that the accumulation of $LC3^+$ dots to cytoplasmic structures may not be an indication of autophagy in all circumstances. This is why we measured several parameters related linked to bona fide autophagic responses, including LC3 lipidation, p62 degradation and the catabolism of long-lived proteins both in the absence and in the presence of protease inhibitors. Altogether, these data corroborate the notion that oleate and palmitate stimulate the autophagic flux rather than simply promoting the accumulation of $LC3^+$ dots in cytoplasmic vacuoles.

11. *Minor spelling etc. For example: "co-straining" is probably "co-staining".*

Our response: We thank the reviewer for spotting this type, which has now been corrected. We have also double-checked the article for other spelling issues.

12. *Fig. 1. Y axes. "LC3 dots" is a dimensionless definition. What's missing is : Number, number per cell, area per cell, etc...*

Our response: Following the reviewer's indications, we have added a dimension to the Y-axis labels in all figures where this was an issue.

Referee n° 2 made the following general comments to the manuscript:

The effect of fatty acid load on autophagy has been extensively studied in recent years. The present study provides new insights into the relationship between fatty acids and autophagy utilizing both in vitro and in vivo systems. A panel of different length saturated and unsaturated fatty acids was screened for GFP-LC3 positive puncta formation in U2OS cancer cells. The authors provide evidence that on palmitate (16:0) and oleate (18:1) act via different mechanism to activate autophagy. Autophagy triggered by palmitate depends on signaling factors such as AMPK, PKR, JNK and BECN1/PIK3C complex known to activate the canonical autophagic pathway. Conversely, oleate induces autophagy was found independent of BECN1/PIK3C but led to redistribution of LC3 and p62 to the Golgi apparatus. Altogether, the authors show substantial evidence to support the model whereby LC3 accumulation and different signaling pathways mediate p62 degradation when palmitate or oleate are given. However, the authors' main claim that oleate leads to the induction of non-canonical autophagy is not well based. The authors should consider the possibility that oleate treatment might lead to Golgi dysfunction that by itself indirectly affects autophagy. The data presented could be interpreted as a general traffic jam at this organelle. The authors should therefore determine the effect of their treatment on both endocytosis and exocytosis. Moreover, the effect of oleate on lysosomal degradation should be determined by combining IF and WB analysis.

Our response: We thank the reviewer for acknowledging that we show "substantial evidence" that the mechanisms of LC3-II accumulation and p62 degradation induced by palmitate or oleate are different. In response to the constructive critique from this reviewer, we have now assessed whether the Golgi apparatus is disrupted/inhibited by oleate by several means. In particular, (1) we tested the ability of the Golgi apparatus to properly glycosylate proteins for secretion in the presence of oleate; (2) we checked whether proteins of the Golgi apparatus are degraded in the course of oleate-induced autophagy; and (3) we monitored protein secretion and endocytosis in cells exposed to oleate. In all these experimental settings, we employed BFA (a known inducer of Golgi apparatus dysfunction) as a positive control. We found that oleate, as opposed to BFA, does not impair the Golgi apparatus, neither structurally, nor functionally. These results have been incorporated in the revised version of Figure E2.

Then raised the following points:

1. *Induction of autophagy both by palmitate and by oleate should be better established. Autophagic flux with or without lysosomal inhibitors in U2OS cells is presented only by accumulation of GFP-LC3 dots (Fig. 1B). WB analysis under these conditions should be determined measuring both LC3 and p62. The autophagic flux presented in Fig. 4B is missing the WT mice and another autophagy degradation marker.*

Our response: Following the reviewers' recommendations, we monitored autophagic flux in U2OS or HeLa cells exposed to palmitate and oleate by immunoblotting for p62 degradation and LC3 conversion, as well as by monitoring the catabolism of long-lived proteins. Moreover, to substantiate the validity of palmitate as a SFA control for oleate (see also our response to point n° 2 raised by reviewer n° 1), we have monitored the autophagic flux in U2OS exposed to stearate, by immunoblotting for p62 degradation and LC3 conversion as well as by immunofluorescence microscopy, quantifying the accumulation of GFP-LC3 dots. As expected, all these FAs induce a bona fide increase in autophagic flux. These results have been incorporated in the revised version of Figure 1. We have also added the data on WT mice treated with palmitate and oleate, as requested by the reviewer, in the revised version of Figure 7.

2. *The authors should address the fact that in data presented in Fig. 1F oleate treatment leads to higher levels of phosphorylated AMPK whereas Fig. 3A shows that KD of AMPK is not abolishing the GFP-LC3 dots accumulation.*

Our response: We repeated these experiments several times, reproducing our initial findings. This may reflect the difference in experimental models (mice in old Fig. 1F, cultured GFP-LC3-expressing U2OS cells in old Fig. 3A). Alternatively, AMPK activation may occur along with, but not be responsible for, oleate-triggered autophagy.

3. *All colocalization panels should include the red and green channels separately. The quantification of colocalization values needs to be explained (what was the method/software used for the calculation?).*

Our response: As requested by the reviewer, we provided images corresponding to separate channels for all colocalization panels. Moreover, we precisely described how the percentage of colocalization was calculated in the revised version of the "Materials and Methods" section, under the heading "Automated fluorescence microscopy".

4. *Fig. 2A+B and Fig. E1 A-C the BFA+ fatty-acids treatment should be presented as in Fig. 2C-E.*
5. *Additionally, Fig. E1 B+C should be moved to Fig. 2.*

Our response: The entire figure set has been reshuffled to accommodate results obtained during the revision process. Alongside, as suggested by the reviewer in these comments, the data presented in old Fig. E1A-C have been moved to the main figure set. Now we conceptually and visually separate the findings pointing to a differential implication of the Golgi apparatus in palmitate- versus oleate-induced autophagy (Figures 2-3) from the results indicating that the Golgi apparatus is required for autophagic responses elicited by oleate (Figure 4 and E3)

6. *In Fig. 2F the rational for the data analysis is not fully clear. Testing the distance between the two proteins do not necessarily indicate autophagic activity. Moreover, the control image is missing, there is no indication what are the arrows pointing at, the gold particles labeling p62 and LC3 are not specified and no explanation how the calculation of the distance between the dots is measured.*

Our response: To address these concerns, we have (1) improved the explanation of the data presented in the old version of Figure 2F, explicitly mentioning that these results corroborate (but not indicate *per se*) the activation of an autophagic response; (2) included the control image; (3)

explained, in the figure legend, what arrows are pointing to, and (4) specified how distance between dots was calculated.

7. *The TEM images (Fig. 2 G+H) should be analyzed with Golgi marker to solidify the authors' conclusion.*

Our response: Owing to technical issues, we were unable to perform the experiments requested by the reviewer here, and we apologize for this. In substitution, we collected several additional lines of evidence that causally implicate the Golgi apparatus in oleate-induced autophagy. In particular, we demonstrated that while oleate does not interfere with the integrity and the functions of the Golgi apparatus (as monitored in U2OS cells exposed to oleate by immunoblotting for Golgi apparatus markers, by colocalization studies on immunofluorescence microscopy, by an exocytosis/endocytosis assays, and by a protein glycosylation test, see **Figure E3**), interventions that do compromise the Golgi apparatus structurally and functionally, such as the administration of BFA, inhibits oleate-induced autophagy (see **Figure 4**). As the reviewer will surely appreciate, these results corroborate our previous findings, solidify our conclusion and add value to the take-home message of the paper.

8. *AMPK, PKR and JNK function and the relation to autophagy should be clarified in the text.*

Our response: Driven by the constructive critique from the reviewer, we have now explicitly mentioned in the Introduction of the paper how AMPK, PKR and JNK are linked to (palmitate-induced) autophagy.

9. *The accumulation of neutral lipid droplets in the Golgi under oleate treatment presented in Fig. 2B and the data presented in Fig. 3B should be removed as they do not contribute to the main manuscript message.*

Our response: As requested by the reviewer, we removed the data originally presented in Figure 2B from the revised version of the manuscript. Conversely, we preferred to keep the data shown in the original version of Figure 2B as part of the main Figure set, mainly because reviewer n° 1 found this experiment very elegant (see also point n° 4 raised by reviewer n° 1)

Referee n° 3 said:

This study describes a series of experiments that investigate the ever more complicated lipid signaling events that accompany autophagic responses in cells, specifically the differential autophagic responses of cells to saturated vs unsaturated fatty acids. However, how these signaling events are regulated is not well understood. What recommends this MS is that the study makes interesting observations, in several different contexts, regarding the differential (and conserved) mechanisms of inducing autophagy in response to palmitate vs oleate challenge. It is from that perspective that the EMBO J readership (and the cell biology community in general) will find this work of interest. The authors raise some interesting points, these points are well supported experimentally, and are pleasantly unexpected. While these studies do not address the actual mechanism by which oleate induces non-canonical autophagy, it is this reviewer's opinion that those types of mechanistic studies appropriately lay in future work guided by the important results reported here.

Our response: We thank the reviewer for the positive appraisal of our article.

Then noted three points for improving the paper:

1. *Does involvement of the Golgi complex in oleate-induced autophagy involve accelerated autophagic degradation of Golgi markers (GALT, LMAN1, etc..) relative to mitochondrial or ER markers?*

Our response: Driven by the constructive criticism from this reviewer and reviewer n° 2, we have extensively investigated whether the Golgi apparatus is functionally impaired and/or degraded in the course of oleate-induced autophagy. Among other approaches, (see also our response to the general comments made by reviewer n° 2) we tested whether three distinct markers of the Golgi apparatus, namely, GM130, ERGIC and GBF1, are degraded in U2OS cells exposed to palmitate or oleate. We found that palmitate and oleate fail to affect the abundance of Golgi apparatus proteins in conditions in which they efficiently induce autophagy. These results have been incorporated in the revised version of Figure E2.

2. *By the same token, is lipophagy differentially affected upon palmitate vs oleate challenge?*

Our response: We agree with the reviewer that it would be interesting to test whether lipophagy is influenced by the administration of palmitate versus oleate. However, we feel that these results would not add to the current take-home message of the paper, i.e., that oleate induces a non-canonical form of autophagy that rely on the Golgi apparatus. Our point of view is shared by reviewer n° 2, who explicitly requested to remove from the paper the only data on lipids we had in the original version of the paper.

3. *Why was arachidonate used as UFA in the worm beclin knockdown experiments rather than oleate, especially given that oleate challenge stimulates non-canonical autophagy in the worm? Consistency would suggest presenting oleate data here.*

Our response: Following the reviewer's indications, we repeated the work experiments involving bec-1 downregulation with oleate. Consistent with data from mammalian cells, we found that the autophagic response of *C. elegans* to oleate is not inhibited by the absence of the worm ortholog of Beclin 1. We have investigated the same issue in yeast, finding comparable results. These results are now displayed in Figure 8.

2nd Editorial Decision 15 December 2014

Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by the original referees (see comments below), who all now support publication pending minor revision.

I would therefore like to ask you to address the remaining concerns in a point-by-point response and by providing a final version of your manuscript. Most if not all of the concerns can be addressed by amending the text. Please contact me in case of questions regarding the remaining issues to be addressed.

A few editorial points need to be taken care of at this stage as well:

- Please check whether all figure files are of adequate resolution and quality for production, and upload improved versions if necessary.
- Please suggest (in a cover letter) 2-5 one-sentence 'bullet points', containing brief factual statements that summarize key aspects of the paper - they will form the basis for an editor-drafted 'synopsis' accompanying the online version of the article. Please see the latest research articles on our website (emboj.embopress.org) for examples - I am happy to offer further guidance on this if necessary.
- As you might know, we encourage our authors to provide original source data, particularly uncropped/-processed electrophoretic blots for the main figures of your manuscript. If you would like to add source data, we would welcome one PDF-file per figure for this information. These will be linked online as supplementary "Source Data" files.

- Finally, it would also be great if you could provide a very basic model figure to be used for the synopsis. It would have to be rearranged to fit best within the format restrictions of 550 pixels (width) x 150-400 pixels (height).

I am therefore formally returning the manuscript to you for a final round of minor revision. Once we should have received them, we should then be able to swiftly proceed with formal acceptance and production of the manuscript!

REFEREE REPORTS:

Referee #1:

The authors have beautifully addressed all my concerns. In reading response to other reviewer, I feel that the revision is a substantial step forward from the initial submission and fully endorse this study.
The authors, however, may consider one minor modification:
I am not sure why the authors did not include PLD analysis in the revision - they show the data only in the rebuttal letter. They could easily place this in the supplement, and have a two sentence description + references. This may be important to do, as it is really quite well appreciated in the biochemistry community as well as among general cell biologists that PLD is robustly stimulated by oleic acid, and both the authors and the reviewers would be remiss not to have addressed that (no matter what the outcome was) - since PLD is implicated in autophagy by independent studies.

Referee #2:

The revised manuscript addresses most of my concerns and the manuscript is now much improved. Below are few minor suggestions that should be addressed to clarify it further.
Fig. 1C - While the LC3 flux is evident in these experiments p62 flux is not - particularly in the right panel. The authors should consider adding quantification and statistical information to solidify their statement.
Fig. 4D and E - The effect of BFA on autophagy is marginal at best and not too convincing. The authors may choose to remove this experiment or relate in the text to the fact that the observed inhibition of autophagy by this drug is limited.
Fig. 6 - It is hard to through a clear conclusion regarding autophagic activity if autophagic flux is not tested. Moreover, it seems that in the presence of PA LC3II is accumulated in both WT and Tsc2-/- cells whereas upon the OL treatment inhibits LC3II is only accumulated in the WT but not the Tsc2-/- Cells. This may be in contrast to the authors' main argument.
Fig. 8A - It is not clear what exactly the authors are measuring when looking for Bodipy and VPH1. There is no evidence that the yellow staining actually indicate autophagy. There are better ways to visualize autophagy in yeast and therefore the authors should consider use them or simply remove this figure from the manuscript.

Referee #3:

The authors have addressed the criticisms of this reviewer and, in my opinion, have invested the effort to adequately address the concerns of the other Reviewers.

2nd Revision - authors' response 17 December 2014

Reviewer n° 1 commented:

The authors have beautifully addressed all my concerns. In reading response to other reviewer, I feel that the revision is a substantial step forward from the initial submission and fully endorse this study.

Our response: We thank the reviewer of the positive evaluation of the revised version of our paper.

Then raised the following minor point:

1. *I am not sure why the authors did not include PLD analysis in the revision - they show the data only in the rebuttal letter. They could easily place this in the supplement, and have a two sentence description + references. This may be important to do, as it is really quite well appreciated in the biochemistry community as well as among general cell biologists that PLD is robustly stimulated by oleic acid, and both the authors and the reviewers would be remiss not to have addressed that (no matter what the outcome was) - since PLD is implicated in autophagy by independent studies.*

Our response: We followed the reviewer's suggestions and included the (negative) results on PLD in the paper. These are shown in the revised version of Figure E4.

Referee n° 2 made the following general comments to the manuscript:

The revised manuscript addresses most of my concerns and the manuscript is now much improved. Below are few minor suggestions that should be addressed to clarify it further.

Our response: We thank the reviewer for appreciating how we addressed the majority of his/her concerns. We have further improved the manuscript following the reviewer's indications, as detailed below.

Then raised the following points:

10. *Fig. 1C - While the LC3 flux is evident in these experiments p62 flux is not - particularly in the right panel. The authors should consider adding quantification and statistical information to solidify their statement.*

Our response: As requested by the reviewer, we have quantified p62 degradation and LC3 lipidation by densitometry and added these data to Figure 1C.

11. *Fig. 4D and E - The effect of BFA on autophagy is marginal at best and not too convincing. The authors may choose to remove this experiment or relate in the text to the fact that the observed inhibition of autophagy by this drug is limited.*

Our response: We respectfully disagree with the reviewer on this point. BFA reduced the levels of lipidated LC3 as induced by oleate in U2OS cells by 50% at least (please compare lane 3 to lane 6 in Fig. 4D). Along similar lines, p62 could not be degraded when oleate was administered in the presence of BFA (again, please compare lane 3 to lane 6 in Fig. 4D). Conversely, the autophagic response to palmitate was completely unaffected by BFA. Similarly, BFA only affected long-lived protein degradation as induced by oleate (by 20-25%), but not by nutrient deprivation or palmitate.

12. *Fig. 6 - It is hard to through a clear conclusion regarding autophagic activity if autophagic flux is not tested. Moreover, it seems that in the presence of PA LC3II is accumulated in both WT and Tsc2-/- cells whereas upon the OL treatment inhibits LC3II is only accumulated in the WT but not the Tsc2-/- Cells. This may be in contrast to the authors' main argument.*

Our response: To clarify this point, we have quantified LC3 lipidation and p62 degradation by densitometry, and included these data in the revised version of figure Fig. 6 (normalized to β actin levels). It is now clear that the absence of TSC2 limits the accumulation of LC3-II as induced by both palmitate (in Tsc2 cells, 2.75/2.22 = 1.24 fold incease; in WT cells, 3.35/1.15 = 2.91 fold) and oleate (in Tsc2 cells, 1,34/2.22 = 0.66 fold increase; in WT cells, 2.96/1.15 = 2.57 fold increase). Of note, this is not in contrast with our main argument.

13. *Fig. 8A - It is not clear what exactly the authors are measuring when looking for Bodipy and VPH1. There is no evidence that the yellow staining actually indicate autophagy. There are better ways to visualize autophagy in yeast and therefore the authors should consider use them or simply remove this figure from the manuscript.*

Our response: We have clarified in the manuscript, figure and figure legend what was measured in Fig. 8A and how this relates to autophagy. Of note, the fact that the accumulation of BODIPY-containing $VPH1^+$ vacuoles as induced by palmitate is inhibited by the absence of atg6 strongly supports the idea that the localization of BODIPY within $VPH1^+$ vacuoles results from the activation of autophagy.

Referee n° 3 said:

The authors have addressed the criticisms of this reviewer and, in my opinion, have invested the effort to adequately address the concerns of the other Reviewers.

Our response: We thank the reviewer for the appreciating the efforts that we invested in addressing the concerns of all reviewers.