ACSL3 is a novel GABARAPL2 interactor that links ufmylation and lipid droplet biogenesis

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MS TITLE: ACSL3 is a novel GABARAPL2 interactor that links ufmylation and lipid droplet biogenesis

AUTHORS: Franziska Eck, Manuel Kaulich, and Christian Behrends
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. In particular, they find some aspects of the data to be unconvincing which undermines the conclusions drawn.

They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

In this manuscript from Eck et al, the authors use gene-editing to tag the Atg8 family member protein GABARAP-L2 at its endogenous locus and then explore the localization and protein-interactions of this protein. SILAC results reveal a lipid-conjugation independent binding of GABARAP-L2 to the ER resident protein ASCL3. The authors then work to establish this interaction biochemically and to describe a possible functional consequence. Their major conclusions are that GABARAP-L2, UBA5 and ACSL3 form a complex with the principle function of delivering the ordinarily cytosolic UBA5 to the ER where is can initiate the modification of substrate proteins. Intriguingly, a very recent paper (Huber et al., Autophagy, 2020) described a role for GABARAP-L2 in trafficking UBA5, but the mechanism of ER-association was unknown.

The authors then extend their analysis, showing that activation of LD biogenesis (which depends upon ASCL3 function) suppresses UBA5 (and related complex component) expression levels, thus they conclude that the GABARAP-L2/UBA5/ASCL3 axis is likely regulated at the level of ER-remodeling including during LD production.

Comments for the author

Their model (and discussion as a whole) is enlightening and the timing of their discovery provides a very nice explanation for the key missing detail in Huber et al. However, as it stands now, the quality of the data and a missing key experimental detail suggests that the paper is incomplete. In particular, the evidence supporting that ASCL3 and GABARAPL2 form a complex is weak and unquantified, and the same problems are evident for the larger tripartite complex. Also critically, there is no direct evidence that the interaction between GABARAPL2 and ASCL3 is necessary to support UBA5 activity. The strongest link is the result that knockout of ACSL3 leads to a dramatic reduction in various UBA5 complex components (including UBA5 itself) by what the authors suspect is a transcriptional regulation, however these effects are independent of the presence of GABARAPL2.

Key Necessary Experiments/corrections: 1) -- The authors speculate on a mode of direct interaction between ACSL3 and GABARAPL2, involving a UIM/UDS motif. Ideally, they would establish the role of this motif directly, and then create mutants to block the ACSL3/GABARAPL2 interaction with which they could then test whether UBA5 recruitment to ER is lost.
2) The bulk of the paper is IPs, expression level changes, and colocalization, but outside of figure 3 none of these experiments are quantified. The immunofluorescent data supporting colocalization is very hard to interpret given the dense and mostly poorly resolved ER, and the IPs suffer from a number of background bands that migrate very close to the protein under investigation. Thus the evidence for a direct complex is limited.

Minor points:

Figure 1G-I – the HA-IPs suggest very limited pull-down. Please indicate the IP efficiencies in the figure legend. Is the lane “HA-beads”, just beads without any lysate? If so, it is very challenging to believe any pull-down of the positive control, p62, as the bands on the beads before incubation with lysate are as intense as anything following the IP. Also, please indicate directly which bands are background and which are the expected size for each protein of interest. Figure 4A HA-beads is also a concern as most of the IP signal looks like this background.

Figure 2A – Which band is the ACSL3? The strongest band on the blot only appears in the no ACSL3 lane (lanes 1 and 3), but is remarkably absent from the ACSL3-endoneongreen lane. In that lane a very faint band is observed.

Figure 2B – colocalization is not obvious. The staining of NeonGreen appears very faint and not particularly similar to Calnexin.

Figure 2D colocalization with GABARAPL2 is not apparent. Its plausible that most of the GL2 puncta are ER associated and that ACSL3 is broadly distributed across the ER. But actual colocalization is

not obvious in these pictures. As GABARAPL2 puncta will form in the course of autophagy, and these puncta will necessarily originate at the ER, the overlap of a GABARAPL2 punctum with disperse ER markers (as ACSL3 appears) is not evidence of a complex so the key condition is with their ATG7 inhibitor. Under these conditions, they do not appear to detect puncta of ACSL3 – would this not be expected if ACSL3 is the anchoring mechanism of the GABARAPL2 structures they observe? Can the authors quantify an enrichment of ACSL3 at these puncta?

The authors describe ACSL3 as a transmembrane protein and make mention of a transmembrane helix on its N-terminus which penetrates only part-way into the ER bilayer. I believe there are studies suggesting this motif is a hairpin and others suggesting an amphipathic helix. Neither is likely to actually span the whole membrane and so use of the word "transmembrane" should be avoided.

Line 134 – "we also detected lipidated GABARAPL1…" The referenced figure (4C) seems to be incorrect and is missing.

Line 199 – Are the HCS LipidTox reagents antibodies? The methods section, the figure legend and this line seem to indicate as much, but the literature refers to them only as lipid stains, which classically are dyes.

Line 354 – "Induction of… (Figure 6A)". There is no figure 6. I believe this refers to Fig. 5C.

Reviewer 2

Advance summary and potential significance to field

The discovery of a potential interaction between ACSL3 and GABARPL2 is novel. It is not clear what the significance of this finding is, however, to either the autophagy or the lipid droplet fields

Comments for the author

The manuscript of Eck et al. aims to identify binding partners of ATG8 family members in response to autophagy modulation by interaction proteomics. The authors used CRISPR/Cas9 gene editing to knock in an HA tag into the endogenous locus of four ATG8 family members (i.e., LC3B, GABARAP, GABARAPL1 and GABRAPL2) in HeLa cell lines. Although ATG8 proteins are critical for macroautophagy and their interactors previously characterized by the authors' and other groups, such interactome studies have not been well characterized at endogenous expression levels. Moreover, the specific functions of the 5 ATG8 family members in autophagy and in non-autophagic processes remain poorly understood. functions are largely uncharacterized. Therefore, the development of these cell lines to study individual ATG8 proteins in the absence of overexpression is of considerable potential importance.

The manuscript reports the following observations:
1) Affinity purification of HA-GABARAPL2 followed by mass spectrometry identified known interactors and a previously unidentified interaction with ACSL3 an ER-resident transmembrane protein that regulates lipid droplet biogenesis.
2) The authors immunoprecipitated HA-GABARAPL2 from cells overexpressing myc-ACSL3 and blotted with an anti-myc antibody to identify a band that is absent from control cells not expressing HA-GABARAPL2.
3) They generated a double KI cell line expressing GABARAPL2-HA and NeonGreen-ACSL3 and examined the localization of both proteins by fluorescence microscopy. The authors report "partial colocalization" of the two proteins, however the images do not provide convincing evidence of colocalization. NeonGreen-
ACSL3 fluorescence at best produces a diffused ER-like pattern, while GABARAPL2 is predominantly localized to discrete puncta which do not colocalize with the NeonGreen reporter. The authors use this result to support their conclusion of an interaction between ACSL3 and GABARAPL2. However, because the limitation of the resolution of light microscopy is <200nm, co-localization of fluorescent proteins cannot be used to assess physical interactions between macromolecules.

4) RNAi knockdown of ACSL3 results in partial reduction of GABARAPL2 levels but not of other ATG8 proteins; this reduction could be abrogated by treatment with bafilomycin A1.
5) The authors used immunoprecipitation of HA-GABARAPL2 from cells overexpressing myc-UBA5 to observed a weak myc-reactive band, a result they interpret as confirming a previously reported interaction between these two proteins. The necessity for overexpression of both proteins and the very weak signal obtained raise concerns about the significance of this interaction.
6) The authos also report that overexpressed HA-ACSL3 can be co-IP'd with overexpressed myc-UBA5, which they interpret as confirming the interaction between these two proteins. No attempt was made to ask if the co-IP of HA-ACSL3 and myc-UBA5 was mediated through a ternary interaction wth GABARAP. Further they report that fluorescence microscopy of cells expressing the KI- tagged versions of ACSL3 and GABARAPL2 shows colocalization of these two proteins together with endogenous UBA5; however, as with point #3 above, the images provided do not reveal any meaningful colocalization of these three markers and certainly do not provide any additional support for the purported interaction, even if the colocalization had been convincing, for the reasons stated in point #3 above.
7) In Fig. 5 they performed knockdown of ACSL3 and report decreased levels of two out of the three components of the UFM1 conjugation pathway that they tested. However, the interpretation of the data is confounded by the absence of any quantification and the fact that the loading controls also decrease under these conditions.
From these observations the authors reach the conclusion that ACSL3 is a novel regulator of the UFMylation pathway.

The primary novel finding of this study is an association between GABARAPL2 and ACSL3. However, they fail to confirm this association in cells at endogenous levels and instead used overexpression of ASCL3. Moreover, the western blot in figure 1I is so overexposed that one questions the validity of this validation experiment.

The other main conclusions regarding the physical association of ACSL3 with UBA5 and its functional role in the UFMylation pathway are too weak for the reasons stated above to be credible.

Overall evaluation: while the authors are to be commended for seeking to conduct a comprehensive study of the ATG8 family interactome using baits at endogenous expression levels, the poor overall quality of the data (other than the mass spectrometry), the naïve assumptions in interpreting the immunofluorescence images, the need for overexpression and massive overexposure of the immunoblot data, and the lack of quantification of the critical experiments detract from the impact of the work. While a regulatory interaction among ATG8 proteins, lipid droplet biogenesis and UFMylation is of potential interest, the manuscript fails to provide mechanistic insights into this phenomenology.

First revision

Author response to reviewers' comments

We are truly grateful to both reviewers for their helpful comments, valuable suggestions and insightful questions. On the basis of the reviewers' guidance we performed a series of additional experiments, which enabled us to carefully revise and significantly improve our manuscript. This revised version contains vital changes in both content and structure. Notably, we also moved some data from the supplements to the main figures as we felt that these include important additional information (now shown as new Fig. 1F, 1G, 3A and 3B).

Reviewer 1 Comments for the author
Their model (and discussion as a whole) is enlightening and the timing of their discovery provides a very nice explanation for the key missing detail in Huber et al. However, as it stands now, the quality of the data and a missing key experimental detail suggests that the paper is incomplete. In particular, the evidence supporting that ASCL3 and GABARAPL2 form a complex is weak and unquantified, and the same problems are evident for the larger tripartite complex. Also critically,

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>We performed GST pulldown assays with purified wild-typ and LIR-binding deficient GABARAPL2 as well as lysates from cells expressing either full-length ACSL3 or N- or C-terminal fragments thereof. This analysis unveiled that ACSL3 and GABARAPL2 bind in a manner dependent on a LIR and one other - possibly UIM - binding motif. Indeed, we found a candidate UIM in ACSL3' N-terminus but since the LIR-binding deficient GABARAPL2 mutant failed to cofractionate with ER markers and therefore supported the relevance of a LIR-mediated ACSL3-GABARAPL2 binding, we did not further prioritized experiments under the current Corona circumstances to unveil whether and how ACSL3's candidate UIM binds to GABARAPL2. Unfortunately, we were not able to extend our recruitment analysis to UBA5 as this would have required reconstitution of GABARAPL2 (or GABARAP triple) knockout cells with our GABARAPL2 variants. Due to working and air traffic restriction in the Corona crisis we were unable to generate GABARAPL2 knockout cells or receive established GABARAP triple knockout cells from abroad in time.

2) The bulk of the paper is IPs, expression level changes, and colocalization, but outside of figure 3 none of these experiments are quantified. The immunofluorescent data supporting colocalization is very hard to interpret given the dense and mostly poorly resolved ER, and the IPs suffer from a number of background bands that migrate very close to the protein under investigation. Thus, the evidence for a direct complex is limited.

>Firstly, we performed the missing quantification of the protein abundance analyses from the old Fig. 3E and 5A-C. This data is now included as new Fig. 3G, 7B, 7D, S4C and S4D. Together, this analysis shows significant alteration of ufmylation components in response to ACSL3 depletion and induction of LD formation. Given that ufmylation has recently been shown to be required for ER-phagy, we extended our analysis and now show that the downregulation of UFM1 conjugation pathway in response to induction of LD formation blocks ER-phagy (new Fig. 8A,B). Secondly, we performed new rounds of IP experiments (new Fig. 2C and 6B) which demonstrate binding between GABARAPL2 and ATG7, p62, ACSL3 and UBA5 at endogenous levels. Thirdly, we improved the IPs of GABARAPL2 with overexpressed ATG7, p62, ACSL3 and UBA5 (shown as new Fig. 2B and 6A). Fourthly, using overexpression conditions and RNAi we now show that the binding of UBA5 and ACSL3 requires GABARAPL2 (new Fig 6E). Lastly, we employed confocal microscopy and super-resolution radial fluctuations (SRRF) imaging to determine the subcellular distribution of ACSL3, Calnexin, GABARAPL2 and UBA5. The results of this analysis are displayed in the new Fig. 4B, 4D, 6C and 6D and show partial but clear ACSL3-Calnexin, ACSL3-GABARAPL2, ACSL3-UBA5 and ACSL3-UBA5-GABARAPL2 colocalization.

Minor points:
Figure 1G-I – the HA-IPs suggest very limited pull-down. Please indicate the IP efficiencies in the figure legend. Is the lane "HA-beads", just beads without any lysate? If so, it is very challenging to believe any pull-down of the positive control, p62, as the bands on the beads before incubation with lysate are as intense as anything following the IP. Also, please indicate directly which bands are background and which are the expected size for each protein of interest. Figure 4A HA-beads is also a concern as most of the IP signal looks like this background.

>We revisited the IP experiments of parental and endogenously HA-tagged GABARAPL2 cells expressing myc-tagged p62, ATG7 and ACSL3 (old Fig. 1G-I) as well as UBA5 (old Fig. 4A) and assembled revised panels for this interaction data in the new Fig. 2B and 6A. To corroborate our interaction data, we performed a new set of IPs with parental and endogenously HA-tagged GABARAPL2 cells and blotted for endogenous p62, ATG7, ACSL3 and UBA5. This new data is

provided as new Fig. 2C and 6B. Together, our findings now show that endogenous GABARAPL2 associates with over-expressed and endogenous p62, ATG7, ACSL3 and UBA5.

Figure 2A – Which band is the ACSL3? The strongest band on the blot only appears in the no ACSL3 lane (lanes 1 and 3), but is remarkably absent from the ACSL3-endoneongreen lane. In that lane a very faint band is observed.

>In the revised version, Fig. 2A became Fig. 4A. Arrows now indicate the bands corresponding to endogenous C-terminally NeonGreen-tagged ACSL3 (approx. 100 kDa) and untagged ACSL3 (just above 75 kDa) on the anti-ACSL3 immunoblot as well as endogenous NeonGreen tagged ACSL3 and overexpressed NeonGreen-tagged TOMM20 on the anti-NeonGreen immunoblot below. Notably, the CRIPSR-edited cells are likely homozygous for the introduced NeonGreen tag as we do not observe untagged ACSL3 in these cells. The reduced anti-ACSL3 immunoreactivity of ACSL3-NeonGreen might be explained by the fact that the epitope of the anti-ACSL3 antibody is located at the C-terminus of ACSL3 and that the C-terminal NeonGreen tag might interfere with the antibody binding in this region.

Figure 2B – colocalization is not obvious. The staining of NeonGreen appears very faint and not particularly similar to Calnexin.

>We repeated the colocalization analysis of endogenous NeonGreen-tagged ACSL3 with Calnexin using SRRF imaging and observed a similar and overlapping subcellular distribution of both proteins. This data is shown in the new Fig. 4B in which examples for clear colocalization events are shown next to a representative overview image. Notably, while ACSL3 and Calnexin are both known ER membrane proteins, it is not surprising that both proteins do not show a complete overlapping colocalization as the ER - and its membrane resident proteins - are organized in specialized subdomains.

Figure 2D colocalization with GABARAPL2 is not apparent. Its plausible that most of the GL2 puncta are ER associated and that ACSL3 is broadly distributed across the ER. But actual colocalization is not obvious in these pictures. As GABARAPL2 puncta will form in the course of autophagy, and these puncta will necessarily originate at the ER, the overlap of a GABARAPL2 punctum with disperse ER markers (as ACSL3 appears) is not evidence of a complex so the key condition is with their ATG7 inhibitor. Under these conditions, they do not appear to detect puncta of ACSL3 – would this not be expected if ACSL3 is the
anchoring mechanism of the GABARAPL2 structures they observe? Can the authors quantify an enrichment of ACSL3 at these puncta?

>We apologize for the poor quality of our initial colocalization analysis. We likewise used now SRRF imaging to re-examine the distribution of ACSL3 and GABARAPL2 and detected partial colocalization of both proteins. This data is shown in the new Fig. 4D. Since we did not observe a disperse overlap of GABARAPL2 and ACSL3 but rather specific colocalization of both proteins in a few locations, we did not extend this new experimental series to autophagy-modifying compounds. In particular, since ACSL3 does not seem to be an autophagy substrate as evident from the new Fig. 3A.

The authors describe ACSL3 as a transmembrane protein and make mention of a transmembrane helix on its N-terminus which penetrates only part-way into the ER bilayer. I believe there are studies suggesting this motif is a hairpin and others suggesting an amphipathic helix. Neither is likely to actually span the whole membrane and so use of the word “transmembrane” should be avoided.

>We greatly appreciated this comment and replaced “transmembrane” with “ER-associated”.

Line 134 – “we also detected lipidated GABARAPL1…” The referenced figure (4C) seems to be incorrect and is missing.

>We corrected this mistake. The correct reference is now Fig. 3E.

Line 199 – Are the HCS LipidTox reagents antibodies? The methods section, the figure legend and this line seem to indicate as much, but the literature refers to them only as lipid stains, which classically are dyes.

>Many thanks for pointing this out. We now refer to the HCS LipidTox reagents as lipid stains in the legend of Fig. 4C and in the methods.

Line 354 – "Induction of… (Figure 6A)". There is no figure 6. I believe this refers to Fig. 5C.

>We corrected this mistake. The correct reference for our working model is now Fig. 8.

Reviewer 2 Comments for the author
The manuscript of Eck et al. aims to identify binding partners of ATG8 family members in response to autophagy modulation by interaction proteomics. The authors used CRISPR/Cas9 gene editing to knock in an HA tag into the endogenous locus of four ATG8 family members (i.e., LC3B, GABARAP, GABARAPL1 and GABRAPL2) in HeLa cell lines. Although ATG8 proteins are critical for macroautophagy and their interactors previously characterized by the authors' and other groups, such interactome studies have not been well characterized at endogenous expression levels. Moreover, the specific functions of the 5 ATG8 family members in autophagy and in non-autophagic processes remain poorly understood. functions are largely uncharacterized. Therefore, the development of these cell lines to study individual ATG8 proteins in the absence of overexpression is of considerable potential importance.

The manuscript reports the following observations:

1) Affinity purification of HA-GABARAPL2 followed by mass spectrometry identified known interactors and a previously unidentified interaction with ACSL3, an ER-resident transmembrane protein that regulates lipid droplet biogenesis.

>We would like to point out that this interaction proteomics was done in the absence of bait overexpression.

2) The authors immunoprecipitated HA-GABARAPL2 from cells overexpressing myc-ACSL3 and blotted with an anti-myc antibody to identify a band that is absent from control cells not expressing HA-GABARAPL2.

>We improved the quality of the IPs of parental and endogenously HA-tagged GABARAPL2 cells expressing myc-tagged p62, ATG7 and ACSL3 (old Fig. 1G-I) and assembled revised panels for this interaction data in the new Fig. 2B. In addition, we performed a new set of IPs with parental and endogenously HA-tagged GABARAPL2 cells and blotted for endogenous p62, ATG7 and ACSL3. This new data is provided as new Fig. 2C. Together, our findings now show that endogenous GABARAPL2 associates with over-expressed and endogenous p62, ATG7 and ACSL3.

3) They generated a double KI cell line expressing GABARAPL2-HA and NeonGreen-ACSL3 and examined the localization of both proteins by fluorescence microscopy. The authors report "partial colocalization" of the two proteins, however the images do not provide convincing evidence of colocalization. NeonGreen-ACSL3 fluorescence at best produces a diffused ER-like pattern, while GABARAPL2 is predominantly localized to discrete puncta which do not colocalize with the NeonGreen reporter. The authors use this result to support their conclusion of an interaction between ACSL3 and GABARAPL2. However, because the limitation of the resolution of light microscopy is <200nm, co-localization of fluorescent proteins cannot be used to assess physical interactions between macromolecules.

>We employed confocal microscopy and super-resolution radial fluctuations (SRRF) imaging to determine the subcellular distribution of ACSL3, GABARAPL2 and Calnexin. The results of this analysis are included as new Fig. 4B and 4D and show partial but clear ACSL3-Calnexin and ACSL3-GABARAPL2 colocalization. Please note that GABARAPL2 and ACSL3 were tagged at the N- and C-terminus, respectively (HA-GABARAPL2 and ACSL3-NeonGreen). This is important as the proteins are otherwise not functional.

4) RNAi knockdown of ACSL3 results in partial reduction of GABARAPL2 levels but not of other ATG8 proteins; this reduction could be abrogated by treatment with bafilomycin A1.

>We now include a quantification of this data (new Fig. 3G).

5) The authors used immunoprecipitation of HA-GABARAPL2 from cells overexpressing myc-UBA5 to observed a weak myc-reactive band, a result they interpret as confirming a previously reported interaction between these two proteins. The necessity for overexpression of both proteins and the very weak signal obtained raise concerns about the significance of this interaction.

>We would like to point out that HA-GABARAPL2 was not overexpressed in this experiment but that we used cells in which endogenous GABARAPL2 carried an HA tag. Nevertheless, we improved the quality of this IP (old Fig. 4A) and assembled a revised panel for this interaction data in the new Fig. 6A. In addition, we performed an additional IP with parental and endogenously HA-tagged GABARAPL2 cells and blotted for endogenous UBA5. This data is provided as new Fig. 6B. Together, our findings now show that endogenous GABARAPL2 associates with over-expressed and endogenous UBA5.

6) The authors also report that overexpressed HA-ACSL3 can be co-IP'd with overexpressed myc-UBA5, which they interpret as confirming the interaction between these two proteins. No attempt was made to ask if the co-IP of HA-ACSL3 and myc-UBA5 was mediated through a ternary interaction with GABARAP. Further they report that fluorescence microscopy of cells expressing the KI- tagged versions of ACSL3 and GABARAPL2 shows colocalization of these two proteins together with endogenous UBA5; however, as with point #3 above, the images provided do not reveal any meaningful colocalization of these three markers and certainly do not provide any additional support for the purported interaction, even if the colocalization had been convincing, for the reasons stated in point #3 above.

>We are very thankful for this comment and performed IPs in cells overexpressing UBA5 and ACSL3 but differentially lacking GABARAPL2. This experiment revealed that the binding of UBA5 and ACSL3 requires GABARAPL2 (new Fig 6E). Moreover, we used SRRF imaging to re-examine the distribution of ACSL3, GABARAPL2 and UBA5 and detected partial pairwise colocalization of ACSL3 and UBA5 as well as triple colocalization of ACSL3, GABARAPL2 and UBA5. This data is shown in the new Fig. 6C and 6D.

7) In Fig. 5 they performed knockdown of ACSL3 and report decreased levels of two out of the three components of the UFM1 conjugation pathway that they tested. However, the interpretation of the data is confounded by the absence of any quantification and the fact that the loading controls also decrease under these conditions. From these observations the authors reach the conclusion that ACSL3 is a novel regulator of the UFMylation pathway.

>We performed new rounds of knockdown experiments and added the missing quantification of the protein abundance analyses from the old Fig. 3E and 5A,B. This data is now included as new Fig. 3G, 7A, 7B and S4C. Together, this analysis shows significant alteration of ufmylation components in response to ACSL3 depletion. Notably, we also repeated the LD induction experiments shown in the old Fig. 5C and now provide quantification of the abundance changes (new Fig. 7C, 7D and S4D).

The primary novel finding of this study is an association between GABARAPL2 and ACSL3. However, they fail to confirm this association in cells at endogenous levels and instead used overexpression of ASCL3. Moreover, the western blot in figure 1I is so overexposed that one questions the validity of this validation experiment.

>As stated in our response to 2) we now provide evidence that endogenous GABARAPL2 associates with endogenous p62, ATG7, ACSL3 and UBA5 (shown in the new Fig. 2C and 6B). Moreover, we added mechanistic insights into the GABARAPL2-ACLS3 interaction by performing GST pulldown assays with purified wild-type and LIR-binding deficient GABARAPL2 as well as lysates from cells expressing either full-length ACSL3 or N- or C-terminal fragments thereof. This analysis unveiled that ACSL3 and GABARAPL2 bind in a manner dependent on a LIR and one other - possibly UIM - binding motif. Indeed, we found a candidate UIM in ACSL3' N-terminus but since the LIR-binding

deficient GABARAPL2 mutant failed to cofractionate with ER markers and therefore supported the relevance of a LIR-mediated ACSL3-GABARAPL2 binding, we did not further prioritized experiments given the Corona circumcenters to unveil whether and how ACSL3's candidate UIM binds to GABARAPL2.

The other main conclusions regarding the physical association of ACSL3 with UBA5 and its functional role in the UFMylation pathway are too weak for the reasons stated above to be credible.

>As stated above we provided additional experiments that ACSL3 and UBA5 bind at endogenous levels and that GABARAPL2 is required for the interaction of ACSL3 and UBA5 (shown in new Fig. 6B and 6E).

Overall evaluation: while the authors are to be commended for seeking to conduct a comprehensive study of the ATG8 family interactome using baits at endogenous expression levels, the poor overall quality of the data (other than the mass spectrometry), the naïve assumptions in interpreting the immunofluorescence images, the need for overexpression and massive overexposure of the immunoblot data, and the lack of quantification of the critical experiments detract from the impact of the work. While a regulatory interaction among ATG8 proteins, lipid droplet biogenesis and UFMylation is of potential interest, the manuscript fails to provide mechanistic insights into this phenomenology.

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Second decision letter

MS ID#: JOCES/2020/243477

MS TITLE: ACSL3 is a novel GABARAPL2 interactor that links ufmylation and lipid droplet biogenesis

AUTHORS: Franziska Eck, Santosh Phuyal, Matthew D. Smith, Manuel Kaulich, Simon Wilkinson, Hesso Farhan, and Christian Behrends
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

This revised manuscript is dramatically improved over the original submission. The relatively extensive quantification (except for new LIR figure) and the much sharper fluorescence microscopy via SRRF revealing individual puncta (for example Figure 4B/D) make it much easier to evaluate the major conclusions the authors draw from their experiments. The findings that GABARAPL2 influences ASCL3 levels and localization are well established. I have only a few minor comments detailed below.

Comments for the author

Minor points:

Figure 2C, it is really not very apparent whether ACSL is even pulled down, while other gabarapl2 interactors are easy to see in the available blots. This is somewhat of a minor point in that Figure 5B (with overexpressed recombinant proteins) reveals a direct interaction fairly convincingly.

Confusingly, the inhibition of autophagy is said to stabilize levels while inhibiting the proteasome does not but actually inhibition of proteasome massively increased levels in the ASCL3 resting state and treatment with siRNA against ASCL3 simply “restored” levels to normal WT conditions. Does this reflect increased levels of ASCL3 in the presence of Btz? Do GABARAPL2 levels correlate with ASCL3 expression level even as its increased beyond ordinary endogenous levels? Some discussion about these varying levels across experiments would be helpful.

New experiment detailing a potential LIR-dependent interaction between GL2 and ASCL3 is a strong new direction. The strategy makes sense, but as with the original submission, the absence of quantification makes this challenging to interpret. Figure 5B, for example, appears to show a loss of IP with COOH-terminal fragment when the GRL2 LBS is mutated. However, this fragment also appears to be expressed at much lower levels and close inspection of the IP looks to me to have pulled down something. Is the poor IP just a function of the low protein expression level? Likewise, in 5C, conditions which lead to a loss of GRL2 at the “ER fraction” also lead to a significant loss of ASCL3 from the same compartment which is very cool but should be quantified.

The microscopy is much improved, though some discussion about the extent of colocalization is probably warranted. For example, Fig 6D appears to show at least 2 examples (out of 5 total pink spots) of UBA5 localization that does not correlate with any HA signal.

The new experiment connecting lipid droplet biogenesis to ER-phagy/reticulolysosome formation is great.