nature portfolio

Peer Review File

AAA+ ATPase chaperone p97/VCP^{FAF2} governs basal pexophagy

Corresponding Author: Professor Noriyuki Matsuda

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The maintenance of peroxisome homeostasis in mammalian cells is partly regulated by the processes of peroxisome biogenesis and degradation. The process of peroxisome degradation through pexophagy adheres to several fundamental principles of autophagy-mediated organelle degradation. Pexophagy in mammalian cells typically involves the ubiquitination of surface proteins (PEX5, PEX3, PMP70) regulated by ubiquitin ligases (PEX2/10/12, MITOL) and the de-ubiquitin enzyme (USP30). Additionally, the recognition of ubiquitinated proteins by autophagy adaptors (p62 and NBR1) plays a crucial role in this process.

In this study, Koyano et al. discovered that FAF2-/- cells contain fewer peroxisomes than WT cells. They characterized that this reduction is caused by the enhanced pexophagy. USP30 suppression further increased pexophagy in FAF2-deficient cells. Additionally, they found that the ubiquitination of PMP70 is increased in FAF2-deficient cells. They identified the FAF2 domains responsible for inhibiting basal pexophagy. Finally, they examined various autophagy adaptors and determined that OPTN is required for pexophagy in FAF2-deficient cells. Overall, this study suggests a working model, in which FAF2 associates with peroxisome to extract ubiquitinated PMP70 at basal level. In the absence of FAF2, the ubiquitinated PMP70 recruits the autophagy adaptor OPTN to mediate pexophagy.

This study is novel as it establishes a connection between FAF2 and pexophagy, possibly contributing to a better understanding of the diverse mechanisms governing pexophagy in mammalian cells. Given the well-established function of USP30 in pexophagy, it is not surprising that it plays the same role in FAF2-deficiency-induced pexophagy. However, the most significant and intriguing conclusion that "PMP70 is the genuine substrate of FAF2 for extraction from peroxisomal membrane" is not adequately supported. Since no direct experiments were conducted to investigate the function of p97 in basal pexophagy, the title of this manuscript is also somewhat misleading.

Specific points:
1. FAF2 is the adaptor, the actual working enzyme is the ATPase p97. It is shown that overexpressed HA-FAF2 partially co-localizes with peroxisomes (Fig 1C). How about the enzyme p97 itself? Does p97 localize to peroxisomes? Is the co-localization (if it exists) between p97 and peroxisomes dependent on FAF2? The "extraction" model would be more persuasive if ATPase localization could be demonstrated.

2. To determine whether "extraction" can be inhibited to induce pexophagy, p97 or proteasome inhibitors should be used. In fact, FAF2(delta UBX), which cannot bind p97 for "extraction", also inhibits pexophagy (Fig. 4G), suggesting perhaps there are other mechanisms.

3. PMP70 Knockdown restored peroxisome numbers in FAF2 knockout cells (Fig. 3K). Why would a knockdown of PMP70 be beneficial if it is the ubiquitinated PMP70 that initiates pexophagy? According to Figs. 3A and 3B, only a small fraction of PMP70 is ubiquitinated. The knockdown efficiency was only shown in WT cells but not in FAF2 KO cells (Fig 3J).

4. For the interaction study, IP of FAF2 identified PMP70, PEX16 and USP30 (Fig 4A), but only PMP70 is considered as the substrate for FAF2. Why isn't PEX16 considered?

5. Lines 205-207, “collectively, these results suggest that PEX16 and ….” Not sure how this conclusion was reached given

that PEX16 was not investigated in Fig 3.

6. Lines 195-197, the data shows PMP70 is ubiquitinated in FAF2-deficient cells, but this is not by default equal to PMP70 is the target of FAF2. This connection needs to be established as suggested in points 1-2.

Reviewer #2

(Remarks to the Author)
In this manuscript, Koyano F et al., report that FAF2, a membrane-anchored VCP adaptor known to localize to ER, lipid droplet and mitochondria, also localizes to peroxisomes. FAF2-KO causes peroxisome loss due to excessive pexophagy. The authors next identify ubiquitylated PMP70 as FAF2 substrate. In FAF2-KO cells, ubiquitylated PMP70 initiates pexophagy by recruiting autophagy receptor Optineurin, P62 etc. The initial finding of pexophagy suppression by FAF2 (Figs 1 and 2) is quite interesting. However, I have major concerns about the PMP70 part (Figs 3-5) as elaborated below, and cannot recommend publication at this stage.

Major questions:
1. In Figure 1C, the authors overexpressed HA-FAF2 and examined its colocalization with catalase. Because overexpression often causes protein mislocalization, it is important to examine the localization of endogenous FAF2 using antibody (use FAF2-KO cells as the negative control) or knockin an HA tag to label endogenous FAF2.
2. If PMP70 is a dual-substrate for FAF2 and autophagy as the authors proposed, why FAF2-KO cells treated with Baf.A1 (lysosome inhibitor) do not accumulate PMP70 to higher levels than WT cells (Fig 3A, lane 4)? In contrast, many reported substrates of FAF2, such as Insig1 (PMID:18835813) and Noxa (PMID:35979733), accumulate in FAF2-KO cells. Please note that PMP70 ubiquitylation is so weak in Baf.A1-treated FAF2-KO cells (Fig 3A, lane 4; Fig 3B, lane 8) that ubiquitylated PMP70 unlikely constitute a large fraction of total PMP70. This unusual result need extra experimental evidence or reasoning to justify authors' claim. Otherwise, it is highly possible that ubiquitylated PMP70 is just one of many pexophagy initiators or a passenger of pexophagy.
3. The only evidence that may support that PMP70 has a special role in pexophagy is that siPMP70 restores peroxisome amount in FAF2-KO cells (Figs 3K and 3L). Because this data is so critical, I would suggest the authors do rescue experiment with siRNA-resistant PMP70 and perform a complete set of analysis of peroxisome content (IF staining and western blot). The Torin experiment (Fig 2H and 2I) is not critical for the main conclusion and can be moved to supplement.
4. The authors propose FAF2's role in PMP70 degradation is recruiting VCP complex to peroxisome (Fig 6). However, the FAF2 truncation results in Fig 4 suggest FAF2's role in pexophagy is more complex than proposed. Notable, as many previous FAF2 papers showed, UBX mutant of FAF2 disrupts FAF2 association with VCP, it should behave like FAF2-KO and HP mutant (mislocalized to cytosol). This is true in PMP70 protein level regulation (Fig 4H and 4I). However, UBX is half-functional in mitophagy (Fig 4F and 4G). It is strong evidence arguing that FAF2's role at peroxisome is more than recruiting VCP and arguing that PMP70 regulation is not a key step in pexophagy regulation.
5. If FAF2 facilitates recruitment of ubiquitylated PMP70 to VCP, the authors can express FLAG-VCP-QQ to trap substrates, and examine the interaction between FLAG-VCP-QQ and ubiquitylated PMP70 (FLAG-VCP-QQ IP) in the presence or absence of FAF2. This is a critical experiment that the authors may consider.
6. It is nice that optineurin plays a key role in pexophagy in FAF2-KO cells (Fig 5). However, the evidence in Fig 6 remains preliminary. Because the authors propose ubiquitylated PMP70 as the major pexophagy initiator, and considering that there are many ubiquitylated proteins at peroxisome as shown in Fig S3D-H, it is important to perform optineurin pull down to see if it preferentially binds uniquitylated PMP70. It is also critical to test if siPMP70 reduces/blocks optineurin recruitment to peroxisome in FAF2-KO cells.

Minor question:
7. In Figure 1A, the authors generated FAF2-/- cells. These cells are presumably derived from single clone, but the staining showed that catalase content is highly heterogeneous among FAF2-/- cells. This is different from other figures shown in Fig2H, 3E, 3H, 3K etc. The authors may want to discuss the underlying reason.

Reviewer #3

(Remarks to the Author)
Peroxisome quality control is essential for maintaining functional peroxisomes. Recent studies have shown that genetic mutations or stress conditions can induce pexophagy. In most of these systems, the accumulation of ubiquitinated proteins on the peroxisomal membrane acted to signal pexophagy. Other work has shown that peroxisome quality control is mediated by systems other than autophagy. The quality control of peroxisomal matrix proteins is monitored by Lonp2, a protease that degrades damaged matrix proteins. However, the mechanism by which peroxisomal membrane proteins are maintained is unknown. It is known that in culture cells such as CHO cells, the half-life of a membrane protein ranges between 2-6 hrs (PEX3) to up to 36 hrs for PEX16 (PMID: 19719477), which are shorter than the 2-3 days of a peroxisome half-life, suggesting that a mechanism exists for extracting damaged membrane proteins from the peroxisomal membrane. However, the mechanism by which peroxisomal membrane proteins are extracted is unknown.

Here, in this manuscript by Koyano et al., the authors present data that supports UBDX8, a protein associated with the VCP/p97 complex, is required to remove ubiquitinated peroxisomal membrane protein. The authors show that HCT116 cells knockout of FAF2/UBDX8 show decreased peroxisomes and increased ubiquitinated peroxisomal membrane proteins. To

show that the increase in ubiquitinated peroxisomal membrane protein is associated with pexophagy, they used ratiometric imaging of a pH-sensitive fluorescent probe that showed that in cells without FAF2, results in an increased localization of a peroxisomal marker in lysosomes. Based on these findings, the authors suggest that the FAF2-linked p97 complex prevents pexophagy.

The model that the loss of FAF2 induces pexophagy by preventing p97 removal of ubiquitinated peroxisomal membrane protein is feasible and attractive given the role of p97 in the quality control of membrane proteins of other organelles, including the ER and mitochondria. However, the authors do not provide convincing data that FAF2-p97 "represses" pexophagy. For one, the authors do not demonstrate that p97 is required to prevent pexophagy, or that the loss of p97 results in pexophagy. They only show that the loss of FAF2 is associated with an increased loss of peroxisomes and an increase in ubiquitinated PEX16, PMP70 and PEX14. Further, does FAF2-p97 repress pexophagy? Instead of repressing pexophagy, wouldn't the most likely interpretation (if p97 was required) be that FAF2-p97 prevents a peroxisome from being targeted for pexophagy by removing ubiquitinated membrane proteins? Although it may sound like an argument of semantics, it is nevertheless an important point as there are mechanistic consequences for a pathway that represses a pathway vs preventing a substrate from entering the pathway. Repression suggests a mechanism by which a pathway is inhibited by activity preventing a component or components of a pathway. In the case of FAF2-p97, it does not appear that pexophagy is inhibited but instead that the consequence of not removing ubiquitinated protein from peroxisomes signals the individual peroxisomes for degradation by pexophagy. Others have shown similar mechanism, including targeting ubiquitin-tagged peroxisomal membrane protein or mutations that induces the accumulation of ubiquitinated PEX5 on the membrane. Still, others have shown that targeting E3 ligases to peroxisomes also induces pexophagy by causing an accumulation of ubiquitinated membrane protein. If the author wants to support a model where FAF2-p97 is repressing pexophagy, the authors need to demonstrate that FAF2-p97 is actually inhibiting the autophagy-mediated degradation of peroxisomes. Currently, there is no evidence in the manuscript that FAF2-p97 inhibits pexophagy.

However, their data can easily be interpreted to suggest that FAF2-p97 regulates peroxisome quality control by removing ubiquitinated peroxisomal membrane protein. This model is novel as it provides the first mechanism by which old or damaged peroxisomal membrane proteins are removed from the peroxisomal membrane. As mentioned above, evidence suggests that different peroxisomal membrane proteins have different half-life. However, how these proteins are removed from the peroxisomal membrane is unknown. In this manuscript, the authors have provided data that supports FAF2-p97 as a regulator of peroxisome membrane protein quality control. However, to support this model, the authors must show that p97 directly removes ubiquitinated peroxisomal membrane proteins.

There are also other concerns with the data that need to be addressed.

1) To support a model that FAF2-p97 removes ubiquitinated peroxisomes (either to repress pexophagy or prevent the activation of pexophagy of a given peroxisomes), the authors performed colocalization studies in HeLa cells of HA-FAF2 and catalase. Using line-scanning, they show that HA-FAF2 and Catalase are colocalized. However, the problem with this experiment is that FAF2 (UBDX8) has already been shown to not localize on peroxisomes but to be juxtaposed to peroxisomes. More specifically, Schrul & Kopito (ref20 in manuscript) showed that in vitro translated FAF2/UBDX8 targets structures juxtaposition to mature peroxisomes, which was most evident using the super-resolution SIM imaging system. Further, FAF2 was not reported in peroxisomes but in mitochondria, lipid droplets and ER. Given these previous studies, authors must clearly demonstrate that FAF2 targets peroxisomes. Subcellular fractionation of peroxisomes may help demonstrate the subcellular localization of FAF2.

2) To test whether a defect in FAF2 results in a peroxisome biogenesis defect, the authors compared the stability of peroxisomal membrane protein in FAF2 knockout cells to cells depleted of PEX19 expression. However, since PEX19 is essential for targeting peroxisomal membrane protein, this experiment demonstrates that FAF2 is not required for the targeting or stability of peroxisomal membrane protein and not that FAF2 is not required for peroxisome biogenesis. Therefore, the authors cannot rule out the possibility that FAF2 is involved in the biogenesis of peroxisomes. In fact, the heterogeneous phenotype of the FAF2 knockout cells showing some cells with peroxisomes while others show the absence of peroxisomes appears to be similar to VPS13D knockout cell lines recently described by Richard Youle's group (PMID: 33891012). Youle's group found that the knockout of VPS13D results in a heterogenous cell phenotype. Together with Pietro De Camilli's group work showed that VPS13D links peroxisomes to the ER, suggesting that VPS13D regulates peroxisome biogenesis. The author should further explore whether FAF2 also regulates peroxisome biogenesis.

3) In Figure 1, it is not clear why the authors selected "less than 20 peroxisomes" as a means to measure the loss of peroxisomes. It would be more meaningful to quantify peroxisomal structures as they did in Figures 2I, 3F, 3L, etc. Further, in their images in Figure 1A, there is a group of cells with peroxisomes (Catalase and PEX14) and others with no catalase. Based on the quantification in Figure 1B, 30% of the cells have peroxisomes. Do they have full peroxisomes as wild type, but have they lost peroxisomes? Quantifying the number of peroxisomes in each cell may give a more accurate and full view of the consequences of FAF2 in HCT116 cells.

4) it is also unclear whether PEX14 in the FAF2 knockout HCT116 cells is located in mitochondria. It appears to be peroxisomes that collapsed with mitochondria toward the perinuclear area. It would be better to use PMP70 or another membrane protein marker for peroxisomes since PEX14 is known to target mitochondria. PMP70 is likely a better choice in counting peroxisomes given that it does not readily target mitochondria, whereas PEX14 does. Nevertheless, the authors should comment on the lack of uniformity in peroxisome numbers.

5) Also, it is not clear whether PEX14 in the FAF2 knockout HCT116 cells is located on mitochondria. It appears to be

peroxisomes that collapsed with mitochondria toward the perinuclear area. It would be better to use PMP70 or another membrane protein marker for peroxisomes since PEX14 is known to target mitochondria. PMP70 is likely a better choice in counting peroxisomes given that it does not readily target mitochondria, whereas PEX14 does. Nevertheless, the authors should comment on the lack of uniformity in peroxisome numbers.

6) The Keima-skl data is confusing in the context of Figure 1. If the majority of FAF2 knockout cells do not have peroxisomes, then how is Keima-skl getting targeted to lysosomes? There is some evidence that peroxisomal matrix proteins can target mitochondria in peroxisome-deficient cells (PMID: 31129117). Given that the mitochondria appear to be collapsed in the FAF2 deficient cells, could it be possible that the Keima-skl in lysosomes is a result of mitophagy? This could explain the OPTN experiment. The author should deplete an autophagy factor (e.g. FIP200) to test whether the loss of peroxisomes is due to autophagy using the Keima-skl and a count of peroxisomal structures.

7) The PMP70 ubiquitination assay is not entirely convincing. To determine whether FAF2 increases ubiquitinated PMP70, the authors IP FLAG in cells expressing PMP70-3xFLAG and probe immunoblots for FLAG and ubiquitin. The most convincing evidence is increased molecular bands in the FAF2 KO cells treated with BAF. A1. The FLAG blots are difficult to assess, given the high levels of PMP70-FLAG. However, the larger issue is that the experiment may not have been done correctly. For ubiquitination experiments, the lysates need to be heat dissociated with other proteins before immunoprecipitation to ensure that other proteins associated with the protein of interest are not co-precipitated. Otherwise, one cannot be sure whether the streaks in the ubiquitinated blots are not ubiquitinated proteins associated with PMP70, PEX16 or PEX14 instead of being a streak of their protein of interest.

8) Figure 3E-F. It is surprising how many new peroxisomes are formed within 24 hours of treating the cells with Baf.A1. There appears to be a 3-fold increase in peroxisomes in the FAF2 KO cells but no significant increase in the wild-type cells. Also, the peroxisomes appear to be evenly disrupted. If there is an increase in ubiquitinated proteins on these peroxisomes (Fig 3a-d), wouldn't they become sequestered in autophagosomes? Also, why are the peroxisomes in 3E FAF2 -/- HCT116 BAF.A1 blue?

9) Figure 3G: It is unclear why inhibiting mTOR would increase pexophagy in the FAF2 KO and not in the WT cells. Several studies, including one of the co-authors of this manuscript, clearly demonstrated that inhibiting mTOR induces pexophagy. For this reason, one would expect to see a loss of peroxisomes after 24 hrs of Torin1 also in the WT cells. In Figure 3H, there appears to be a decreased catalase staining in the wild-type cells treated with Torin1. The issue is in the method of peroxisome quantification. The authors should quantify peroxisomes per area of the cell to demonstrate changes in peroxisome numbers and not % of cells with less than 20 peroxisomes.

10) To demonstrate that PMP70 is ubiquitinated and degraded by pexophagy, one must inhibit autophagy by either silencing an autophagy factor such as FIP200 or ATG5 for both their ubiquitination and peroxisome quantification assay.
11) Why isn't there a decrease in peroxisomal proteins in the WT cells treated with Torin1? One would expect to see a decrease in peroxisomal protein if
12) Figure 4A: Does FAF2 interact with ubiquitinated or non-ubiquitinated PMP70 and PEX16?

13) To demonstrate an increase in ubiquitinated and LC3B peroxisomes, the authors co-stained their cells with antibodies against ubiquitin and LC3B. They show an increase in cells with ubiquitinated and LC3B-positive structures. There are several issues with this study. The main issue is their quantification of the % of cells with LC3-positive structures. It is not clear what is being counted. Does this mean cells with at least one GFP-SKL positive for LC3B are considered positive cells? This may be appropriate, but what is missing are control experiments. First is a positive control such as amino acid starved cells and Baf.A1 treated cells. Both treatments should show increased LC3B in both WT and knockout cells. The Baf.A1 is especially important as it will demonstrate whether inhibiting lysosomal fusion with autophagosomes results in an increase in ubiquitinated peroxisomes that are not localized in autophagosomes.

14) Finally, in Figure 5A, why does the FAF2 knockout cell not show a decrease in GFP-SKL positive structure?

15) Figure 4- the authors demonstrate that p97/VCP interacts with FAF2 but should also provide evidence that the lack of p97 at the peroxisome leads to increased pexophagy.

16) Figure 5A- While LC3B does appear to be colocalized with GFP-SKL puncta, the text implies that all puncta were LC3B positive, which does not appear to be the case based on the images. The text should be changed (lines 269-270)

17) Figure 5D- OPTN and p62 stained images were co-stained with PMP70 as the peroxisome marker, but NBR1 stained images were co-stained with PEX14 (as labelled within the figure), although the authors discuss only PMP70 labelled structures. Why change the peroxisome marker midway through the figure? Additionally, a colocalization coefficient quantification should be included to claim "clear colocalization between peroxisomes and OPTN (line 285)".

18) Figure 5E, F- in this specific trial, how does the repression of pexophagy by OPTN depletion compare to pexophagy levels in WT cells? Compared to flow cytometry data from other figures (i.e. Figure 4F), it would appear that pexophagy is still occurring in these depleted cells. Therefore, OPTN is not the only receptor for pexophagy in FAF2-/- cells. A co-depletion with the other receptors should be performed.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The revisions have adequately addressed the concerns raised in my initial review. However, there's one more thing that needs to be discussed. In line 154, the author stated, "Torin1 treatment also enhanced the Halo bands, in particular in FAF2 -/- cells (Supplementary Figs. 4c lane 6 and Fig. 4d)". In fact, the enhancement is also significant in wild-type cells (Fig. S4c lane 3, and Fig. S4e lane 3). It was previously well-established that Torin1 induces the ubiquitination of PMP70 in wild-type cells (PMID 34747980). This point should be added, as it helps to explain why pexophagy is accelerated in FAF2-/- cells.

Reviewer #2

(Remarks to the Author)
The authors have addressed most of my comments and improved other parts of the manuscript. I am happy to recommend acceptance for publication.

Reviewer #3

(Remarks to the Author)
This reviewer would like to thank the authors for a very concise description of their response to the reviewers' comments. They did an outstanding job addressing this reviewer's concerns. One minor comment is that the number of cells quantified for some of the assays is not clear. For example, for the number of catalase structures in Figure 1, the authors state a range of cells quantifed. However, it is not clear whether this is the total number of cells quantified over three independent experiments or whether it is the number of cells quantified for each trial. Based on the graph, the number appears to be a total over three trials. If this is the case, the authors should also state the number of cells per trial. The number of cells per trial should also be stated for other similar quantifications.

Responses to Reviewer comments

Aug 20, 2024

MS #: NCOMMS-23-28541

Title: AAA+ ATPase chaperone p97/VCP^{FAF2} governs basal pexophagy

Authors: *Fumika Koyano, Koji Yamano, Tomoyuki Hoshina, Hidetaka Kosako, Yukio Fujiki, Keiji Tanaka, and *Noriyuki Matsuda

Correspondence to: koyano-fm.biom@tmd.ac.jp and nr-matsuda.biom@tmd.ac.jp

We would like to thank the editor and three reviewers for their comments and the opportunity to address the concerns raised. We believe that the inclusion of these new experiments has improved our manuscript. Our point-by-point responses to the reviewer comments are listed below.

Referee #1:

The maintenance of peroxisome homeostasis in mammalian cells is partly regulated by the processes of peroxisome biogenesis and degradation. The process of peroxisome degradation through pexophagy adheres to several fundamental principles of autophagy-mediated organelle degradation. Pexophagy in mammalian cells typically involves the ubiquitination of surface proteins (PEX5, PEX3, PMP70) regulated by ubiquitin ligases (PEX2/10/12, MITOL) and the de-ubiquitin enzyme (USP30). Additionally, the recognition of ubiquitinated proteins by autophagy adaptors (p62 and NBR1) plays a crucial role in this process.

In this study, Koyano et al. discovered that FAF2-/- cells contain fewer peroxisomes than WT cells. They characterized that this reduction is caused by the enhanced pexophagy. USP30 suppression further increased pexophagy in FAF2-deficient cells. Additionally, they found that the ubiquitination of PMP70 is increased in FAF2-deficient cells. They identified the FAF2 domains responsible for inhibiting basal pexophagy. Finally, they examined various autophagy adaptors and determined that OPTN is required for pexophagy in FAF2-deficient cells. Overall, this study suggests a

working model, in which FAF2 associates with peroxisome to extract ubiquitinated PMP70 at basal level. In the absence of FAF2, the ubiquitinated PMP70 recruits the autophagy adaptor OPTN to mediate pexophagy.

This study is novel as it establishes a connection between FAF2 and pexophagy, possibly contributing to a better understanding of the diverse mechanisms governing pexophagy in mammalian cells. Given the well-established function of USP30 in pexophagy, it is not surprising that it plays the same role in FAF2-deficiency-induced pexophagy. However, the most significant and intriguing conclusion that "PMP70 is the genuine substrate of FAF2 for extraction from peroxisomal membrane" is not adequately supported. Since no direct experiments were conducted to investigate the function of p97 in basal pexophagy, the title of this manuscript is also somewhat misleading.

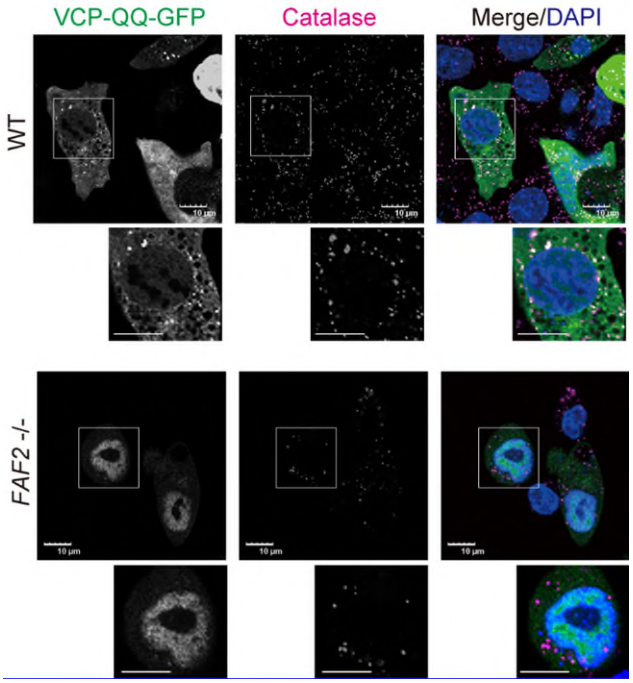
We appreciate the overall positive evaluation of our manuscript. To address the reviewers' concerns, additional experiments (elaborated in detail below) were performed and those results have been incorporated into the revised manuscript as new Figs. 1, 2, 3, 4, 6, 7, S2, S4, S5, S6, S8, S9, S12, and S13. We believe that these new findings sufficiently satisfy the criticism raised.

Specific points:
1. FAF2 is the adaptor, the actual working enzyme is the ATPase p97. It is shown that overexpressed HA-FAF2 partially co-localizes with peroxisomes (Fig 1C). How about the enzyme p97 itself? Does p97 localize to peroxisomes? Is the co-localization (if it exists) between p97 and peroxisomes dependent on FAF2? The "extraction" model would be more persuasive if ATPase localization could be demonstrated.

We thank the reviewer for this constructive comment. As indicated, FAF2 is well known as a p97/VCP cofactor, and since membrane protein extraction is energy-depending, it is reasonable to consider that the extraction process depends on the ATPase VCP/p97. Since ATP-hydrolysis deficient VCP stably binds to FAF2 (Fig. 5c), we performed immunofluorescence staining to examine the peroxisomal localization of VCP in the presence of FAF2. WT and *FAF2* -/- cells transiently expressing ATPase-deficient VCP-QQ (E305Q/E578Q)-GFP were treated with Baf.A1 and immunostained with a peroxisomal marker (catalase). In the presence of FAF2 (WT cells), VCP-QQ-GFP partially localized to peroxisomes (*Figure 1 for Reviewer #1*). This localization, however,

was not observed in *FAF2* -/- cells. We thus concluded that VCP is recruited to peroxisomes in a FAF2-dependent manner. Moreover, data shown in *Figure 2B and 2C for Reviewer #1*, which address comment #2, support the involvement of VCP in the prevention of pexophagy. These results indicate that ubiquitylated proteins (e.g., PMP70) in cells with dysfunctional FAF2 cannot be extracted by VCP and that their subsequent accumulation on peroxisomes induces pexophagy. These data have been incorporated into the revised manuscript.

Figure 1 for Reviewer #1



2. To determine whether "extraction" can be inhibited to induce pexophagy, p97 or proteasome inhibitors should be used. In fact, FAF2(delta UBX), which cannot bind p97 for "extraction", also inhibits pexophagy (Fig. 4G), suggesting perhaps there are other mechanisms.

We agree with the reviewer's comment on the importance of determining the role of p97/VCP on accelerated pexophagy in *FAF2* -/- cells and thus sought to determine if pexophagy was induced following inhibition of p97/VCP-mediated protein extraction. To monitor pexophagy flux in *FAF2* -/- cells, we exploited HaloTag (Halo)-based reporter assay[1]. Unlike ligand-free Halo, which undergoes autophagy-induced lysosomal

degradation, ligand-bound Halo is resistant to lysosomal degradation. Consequently, immunoblots with a Halo-positive band would be indicative of autophagic activity. Please see *Figure 2A for Reviewer #1* below. Immunoblots of *FAF2* -/- cells stably expressing Halo-mGFP-SKL (peroxisome-targeted Halo-mGFP) had a robust Halo band when treated with a ligand (lane 5), whereas WT cells yielded a less intense Halo band (lane 2), indicating that pexophagy is enhanced in *FAF2* -/- cells (*Figure 2A for Reviewer #1*). However, contrary to our expectations, treatment with the p97/VCP inhibitor NMS-873 resulted in a notable reduction of the Halo band in *FAF2* -/- cells (lane6). Since Murakami *et al.* reported that the import of PTS1-containing proteins (e.g., catalase) into the peroxisomal matrix is inhibited by p97/VCP depletion[2], the decreased Halo signal in the NMS-873-treated *FAF2* -/- cells (lane 6) may simply reflect impaired peroxisomal translocation of Halo-mGFP-SKL. We subsequently switched the peroxisome targeting protein from SKL to PMP34, an integral membrane protein of peroxisomes[3] as integration of PMP34 into a peroxisomal membrane is thought to occur independently of p97/VCP. As before, a Halo-mGFP fusion was generated and cells stably expressing PMP34-Halo-mGFP were established. As shown in *Figure 2B and 2C for Reviewer #1,* the Halo band was more clearly detected in *FAF2* -/- cells (lane 5) than in WT cells (lane 2), suggesting pexophagy-induced lysosomal accumulation of PMP34-Halo-mGFP in *FAF2* -/- cells. Importantly, NMS-873 treatment enhanced the Halo band even in WT cells (lane 2 vs 3). As with FAF2 depletion, this result clearly demonstrates that p97/VCP inhibition enhances pexophagy. In FAF2-deficient cells, an effect of p97/VCP inhibition on pexophagy induction is not observed because FAF2 is absent.

Since deletion of FAF2 UBX domain dramatically reduced interactions with p97/VCP, we speculated that the disruption disturbs pexophagy recovery (*Figure 2D for Reviewer #1*). However, FAF2 ΔUBX partially recovered pexophagy (Figs. 5f and 5g). These results might be caused by a strong interaction between FAF2 ΔUBX and USP30 which reduces peroxisomal poly-ubiquitin chains via deubiquitylation.

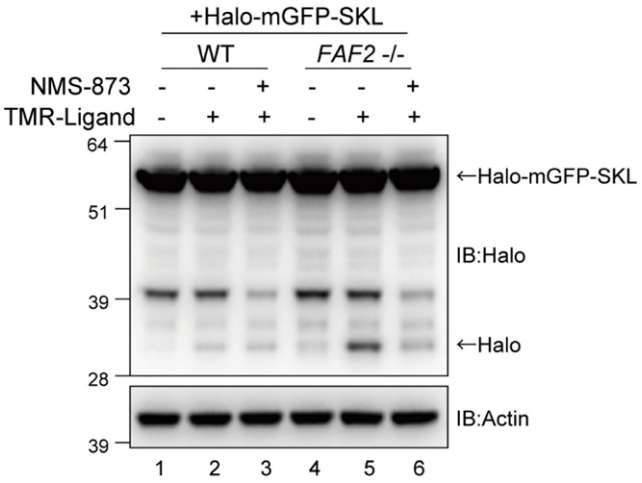
Finally, we used HaloTag assay to determine if pexophagy is affected by proteasome inhibition. The Halo band was not enhanced with MG-132 (proteasome inhibitor) treatment, suggesting that the accumulation of ubiquitylated proteins in response to proteasome inhibition are not initiators of pexophagy in *FAF2* -/- cells (*Figure 2E for Reviewer #1*).

Using the HaloTag method for monitoring pexophagy, we were able to pleiotropically

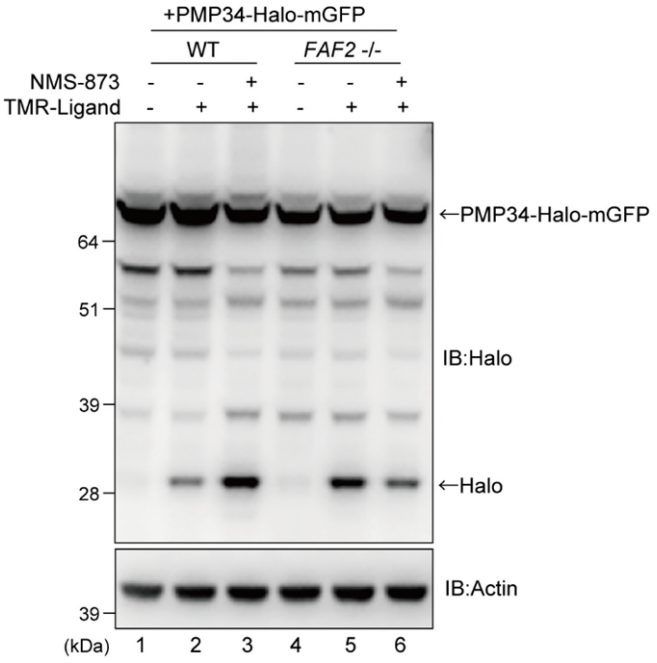
evaluate pexophagy in FAF2-deficient cells. These new results have been incorporated into the revised manuscript.

Figure 2 for Reviewer #1

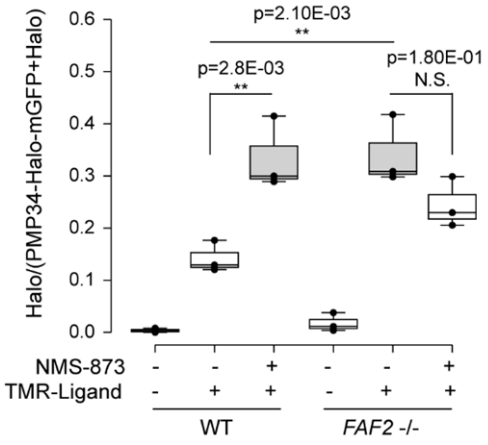
A



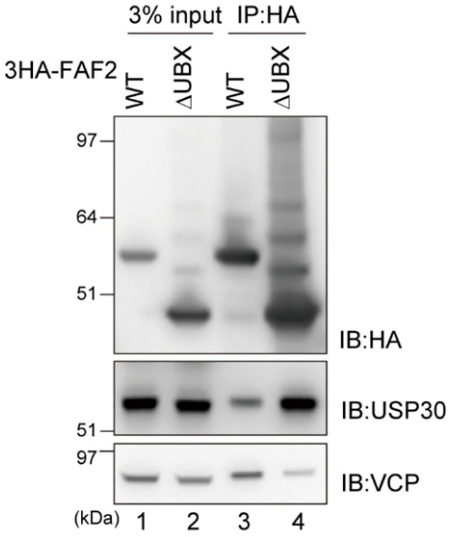
B



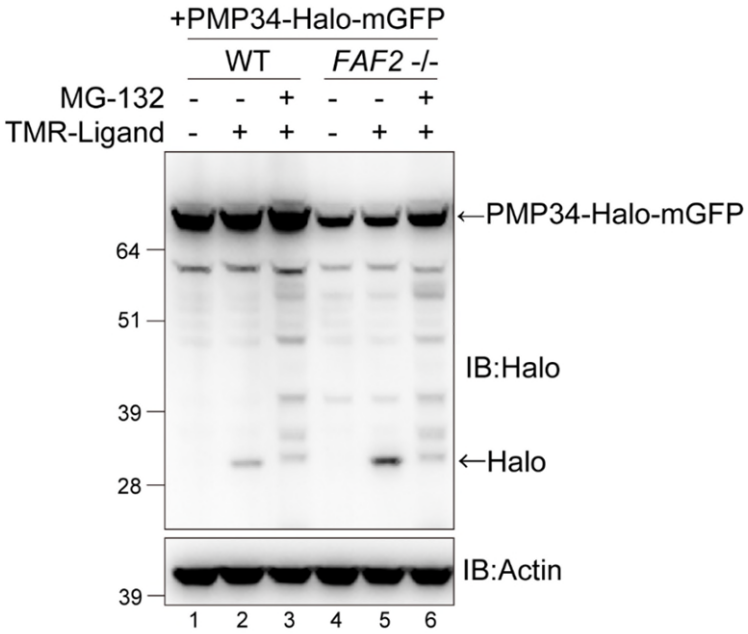
C


p=2.10E-03
**
p=2.8E-03
**
p=1.80E-01
N.S.
Halo/(PMP34-Halo-mGFP+Halo)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
NMS-873 - - + - - +
TMR-Ligand - + + - + +
WT
FAF2 -/-

D


3% input
IP:HA
3HA-FAF2
WT
ΔUBX
WT
ΔUBX
97
64
51
IB:HA
51
IB:USP30
97
IB:VCP
(kDa)
1
2
3
4

E

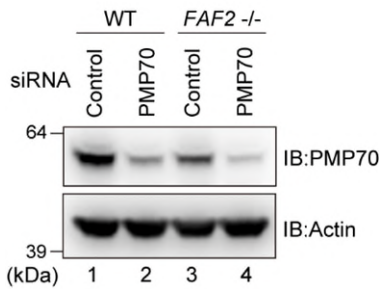


3. PMP70 Knockdown restored peroxisome numbers in FAF2 knockout cells (Fig. 3K). Why would a knockdown of PMP70 be beneficial if it is the ubiquitinated PMP70 that initiates pexophagy? According to Figs. 3A and 3B, only a small fraction of PMP70 is ubiquitinated. The knockdown efficiency was only shown in WT cells but not in FAF2 KO cells (Fig 3J).

We confirmed that the efficiency of PMP70 knockdown does not vary between WT cells and *FAF2* -/- cells (*Figure 3 for Reviewer #1* below). The data have been incorporated into the revised manuscript as new Fig. 4g.

If PMP70 is a genuine substrate, pexophagy in *FAF2* -/- cells might not be induced by forced inhibition of PMP70 expression. When PMP70 expression was reduced by siRNA, the number of peroxisomes in *FAF2* -/- cells was comparable to that of WT cells. This suggests that PMP70 is a substrate of FAF2 and that it is an important trigger of pexophagy. Indeed, it is likely that ubiquitylated forms of PMP70 comprise only a small portion of the total PMP70 (Fig. 4a). However, given the proteomics-based determination that PMP70 is abundant relative to average matrix proteins[4], we speculate that even a relatively limited portion of the total protein is sufficient to initiate pexophagy.

Figure 3 for Reviewer #1



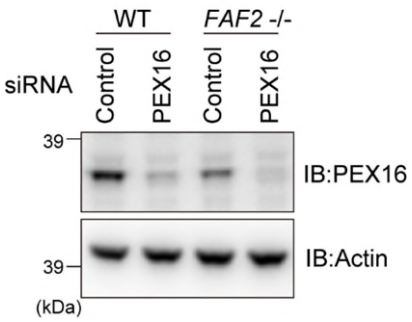
4. For the interaction study, IP of FAF2 identified PMP70, PEX16 and USP30 (Fig 4A), but only PMP70 is considered as the substrate for FAF2. Why isn't PEX16 considered?

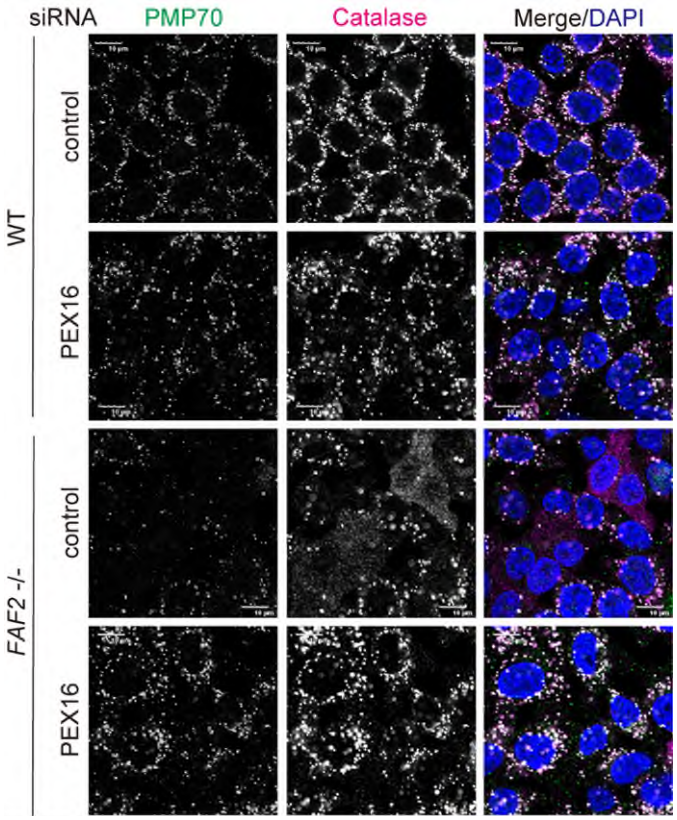
5. Lines 205-207, "collectively, these results suggest that PEX16 and …." Not sure how this conclusion was reached given that PEX16 was not investigated in Fig 3.

In addition to PMP70, PEX16 may be a FAF2 substrate. Immunoprecipitation experiments showed that PEX16 interacts with FAF2 (Fig. 5a) and PEX16 is ubiquitylated in *FAF2* -/- cells (Fig. S7c). Further, peroxisome abundance increased following PEX16 knockdown in *FAF2* -/- cells (*Figure 4A, 4B, and 4C for Reviewer #1*). However, unlike PMP70, PEX16 did not accumulate in response to Baf.A1 treatment in *FAF2* -/- cells (Fig. S7c). Based on these results, we focused this study on the role of PMP70 as a peroxisomal substrate of FAF2.

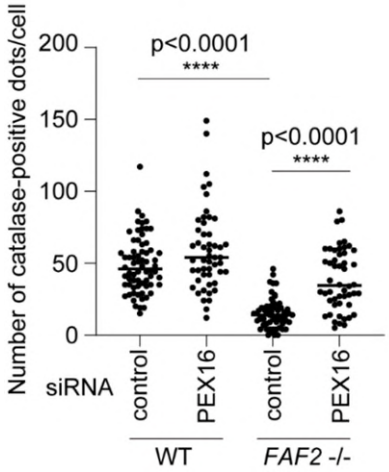
Figure 4 for Reviewer #1

A





B



6. Lines 195-197, the data shows PMP70 is ubiquitinated in FAF2-deficient cells, but this is not by default equal to PMP70 is the target of FAF2. This connection needs to be established as suggested in points 1-2.

We consider PMP70 to be a constitutive substrate of FAF2 because PMP70 coimmunoprecipitates with FAF2 (Fig. 5a), and ubiquitylated forms of PMP70 accumulate in *FAF2* -/- cells in response to Baf.A1-mediated inhibition of pexophagy

(Figs. 4a and 4b). Further, as pexophagy increases in *FAF2* -/- cells, the amount of PMP70 decreases (Fig. 1e). In addition, we show that forced inhibition of PMP70 expression by siRNA impairs FAF2-dependent pexophagy (Fig. 4h). Taken together, these results strongly suggest that ubiquitylated PMP70 is extracted from peroxisomes by the p97/VCP-FAF2 complex.

We also show *in vitro* that the FAF2 UBA domain can interact with poly-ubiquitin (Fig. S10a). Although FAF2 ΔUBA rescued pexophagy (Figs. 5f and 5g), FAF2 can also interact with additional cofactors, such as UFD1 and NPLOC4 (Fig. 5d)[5]. This suggests that poly-ubiquitin chains conjugated to PMP70 are recognized by UFD1-NPLOC4 as well, which in turn are associated with p97/VCP-FAF2 complex. Since FAF2 recruits the UFD1-NPLOC4-VCP complex to peroxisomes, FAF2 may enhance the recognition of ubiquitylated PMP70 by the UFD1-NPLOC4-VCP complex on peroxisomes. Indeed, it has been recently reported that multiple UBX proteins are important for efficient unfolding of ubiquitylated proteins by the UFD1-NPLOC4-VCP complex[6]. We thus speculate that FAF2 functions as a hub for the recruitment of the UFD1-NPLOC4-VCP complex to peroxisomes.

Referee #2:

In this manuscript, Koyano F et al., report that FAF2, a membrane-anchored VCP adaptor known to localize to ER, lipid droplet and mitochondria, also localizes to peroxisomes. FAF2-KO causes peroxisome loss due to excessive pexophagy. The authors next identify ubiquitylated PMP70 as FAF2 substrate. In FAF2-KO cells, ubiquitylated PMP70 initiates pexophagy by recruiting autophagy receptor Optineurin, P62 etc. The initial finding of pexophagy suppression by FAF2 (Figs 1 and 2) is quite interesting. However, I have major concerns about the PMP70 part (Figs 3-5) as elaborated below, and cannot recommend publication at this stage.

We greatly appreciate the reviewer's insightful comments as they provided insights into additional experiments that strengthened our study. In particular, new experimental data were incorporated into the revised manuscript to dispel concerns about the importance of FAF2 and PMP70. For example, *in situ* proximity assays revealed that endogenous FAF2 localizes to peroxisomes. Furthermore, we found that pexophagy initiation in FAF2-deficient cells is dependent on PMP70-mediated recruitment of OPTN to peroxisomes. These findings are elaborated in more detail in our point-by-point responses below. We

trust that the reviewer agrees that these new data have significantly improved our manuscript.

Major questions:

1. In Figure 1C, the authors overexpressed HA-FAF2 and examined its colocalization with catalase. Because overexpression often causes protein mislocalization, it is important to examine the localization of endogenous FAF2 using antibody (use FAF2-KO cells as the negative control) or knockin an HA tag to label endogenous FAF2.

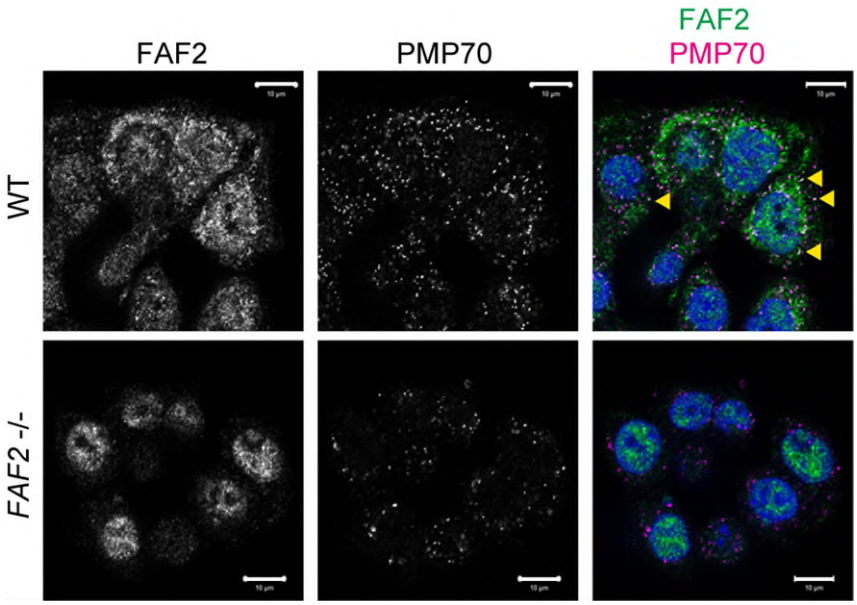
To address the reviewer's comments regarding the subcellular localization of endogenous FAF2, we used an antibody that recognizes endogenous FAF2. *FAF2* -/- HCT116 cells were used as a negative control (*Figure 1A for Reviewer #2*). Although non-specific signals were detected in nuclei, cytosolic signals were specifically detected in WT cells, but not in *FAF2* -/- cells. Further, these signals partially colocalized with a peroxisome marker PMP70. During immunostaining experiments, *FAF2* -/- cells which had remained peroxisomes were selected to show the representative images of PMP70-positive but FAF2-negative peroxisomes in *FAF2* -/- cells. FAF2-staining signals other than on peroxisomes we observed may reflect that FAF2 also localizes on the other organelles including ER and mitochondria, which was described in the previous reports[7-9].

Since FAF2 also localizes to other organelles, we felt that conventional immunocytochemistry against endogenous FAF2 was not suitable for examining its peroxisomal localization. Therefore, as an alternative approach, we utilized an *in situ* proximity assay (PLA assay) to show peroxisomal localization of FAF2 as being an index with a presence/absence of interactions between FAF2 and PMP70.

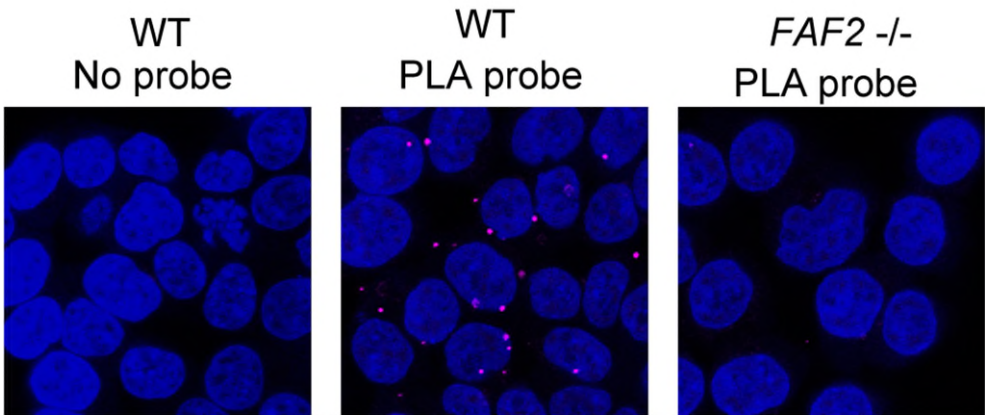
For this assay, rabbit anti-FAF2 and mouse anti-PMP70 antibodies were used as primary antibodies, whereas secondary antibodies were oligonucleotide-conjugated antibodies (PLA probes). Following the incubation of DNA polymerase with fluorescence-labeled oligonucleotides, PLA dots would be generated if the probes were in close proximity (< 40 nm). As shown in *Figure 1B for Reviewer #2*, red dots were observed in WT cells in a PLA probe-dependent manner, but not in *FAF2* -/- cells. The data clarified that endogenous FAF2 localizes on peroxisomes. *Figure 1B for Reviewer #2* has been incorporated into the revised manuscript.

Figure 1 for Reviewer #2

A



B



2. If PMP70 is a dual-substrate for FAF2 and autophagy as the authors proposed, why FAF2-KO cells treated with Baf.A1 (lysosome inhibitor) do not accumulate PMP70 to higher levels than WT cells (Fig 3A, lane 4)? In contrast, many reported substrates of FAF2, such as Insig1 (PMID:18835813) and Noxa (PMID:35979733), accumulate in FAF2-KO cells. Please note that PMP70 ubiquitylation is so weak in Baf.A1-treated FAF2-KO cells (Fig 3A, lane 4; Fig 3B, lane 8) that ubiquitylated PMP70 unlikely constitute a large fraction of total PMP70. This unusual result need extra experimental evidence or reasoning to justify authors' claim. Otherwise, it is highly possible that ubiquitylated PMP70 is just one of many pexophagy initiators or a passenger of pexophagy.

While this comment is based on data shown in Fig. 3A (lane 4) and Fig. 3B (lane 8), it should be noted that Fig. 3A was generated following a long exposure and lane 8 in Fig. 3B is an IP product. More appropriate depictions of PMP70 levels are shown in Figs. 3C, 3D, and 3I. As such, we will focus our response to the reviewer comment on the data shown in Figs. 3C, 3D, and 3I.

As indicated by the reviewer, Noxa accumulates in *FAF2* KO cells and decreases in response to overexpression of exogenous FAF2 (PMID:35979733). On the other hand, as shown in Figs. 3C, 3D, and 3I, PMP70 decreased in *FAF2* -/- cells (e.g., Fig. 3C, compare lane 1 with lane 3). This is because PMP70 can be eliminated by two paths. Even if PMP70 escapes VCP-dependent extraction followed by proteasomal degradation in *FAF2* -/- cells, it will still be degraded in lysosomes due to accelerated pexophagy. Therefore, PMP70 will decrease following *FAF2* knockout rather than increase. Furthermore, as shown in Figs. 3C and 3D, PMP70 did increase in *FAF2* -/- cells following Baf.A1 treatment (e.g., Fig. 3C, compare lane 3 with lane 4). Because the mechanism underlying PMP70 degradation differs from that of Noxa, we think that PMP70 levels do not need to exceed those in wild type cells. In fact, Fig. 3D shows that PMP70 that has undergone pexophagic degradation in *FAF2* -/- cells is restored to the same level as that in the wild type cells following Baf.A1 treatment.

As suggested by the reviewer, it is possible that PMP70 is one of multiple FAF2 substrates on peroxisomes. However, our finding that siRNA-mediated knockdown of PMP70 recovers peroxisomes demonstrates that PMP70 is an important and necessary initiator of pexophagy in FAF2-deficient cells.

3. The only evidence that may support that PMP70 has a special role in pexophagy is that siPMP70 restores peroxisome amount in FAF2-KO cells (Figs 3K and 3L). Because this data is so critical, I would suggest the authors do rescue experiment with siRNA-resistant PMP70 and perform a complete set of analysis of peroxisome content (IF staining and western blot). The Torin experiment (Fig 2H and 2I) is not critical for the main conclusion and can be moved to supplement.

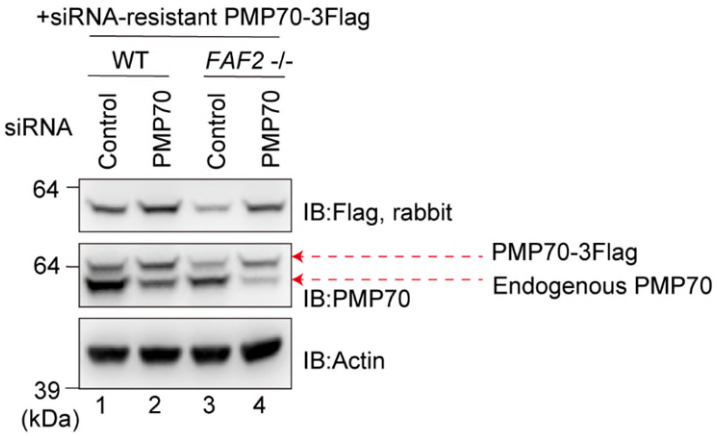
To address the reviewer's concern, we made *FAF2* -/- cells expressing siRNA-resistant PMP70-3Flag (Fig. S8). Knockdown of endogenous PMP70 was confirmed by immunoblotting (see the lower band in the middle panel, lane 1 vs 2 or 3 vs 4 of *Figure*

2A for Reviewer #2), whereas siRNA-resistant PMP70-3Flag did not decrease in siPMP70 sample (see the upper band in the middle panel, lane 1 vs 2 or 3 vs 4 of *Figure 2A for Reviewer #2*).

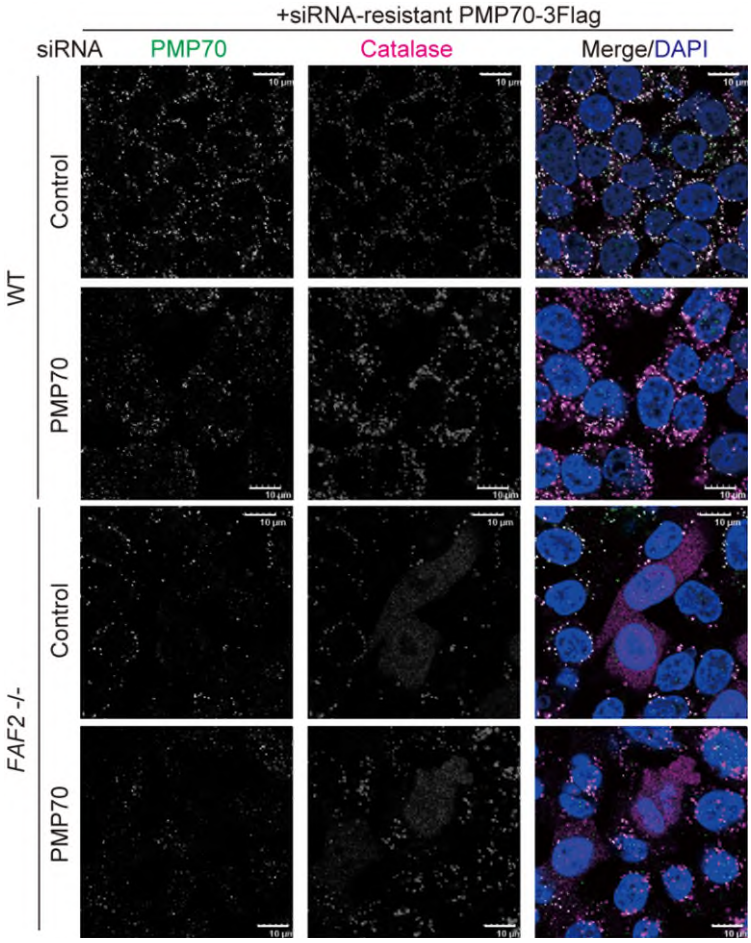
Figs. 3K and 3L in the original manuscript (Figs. 4h and 4i in the revised manuscript) show that the number of peroxisomes in *FAF2* -/- cells are increased following PMP70-knockdown. Conversely, peroxisome abundance did not change in response to siPMP70-treatment in *FAF2* -/- cells expressing siRNA-resistant PMP70-3Flag (*Figure 2B and 2C for Reviewer #2*) but did change in cells lacking siRNA-resistant PMP70-3Flag (*Figure 2D for Reviewer #2*, *Fig. 4h and 4i*). Furthermore, PMP70 knockdown reduces OPTN recruitment to peroxisomes (*Figure 5B and 5C for Reviewer #2*). The rescue experiment using siRNA-resistant PMP70 and associated analyses suggested by the reviewer clearly indicate that PMP70 accumulation initiates pexophagy. We have incorporated the new data into the revised manuscript and moved the Torin1 data (Fig. 3h and 3i) to the supplemental section as Fig. S4a and S4b.

Figure 2 for Reviewer #2

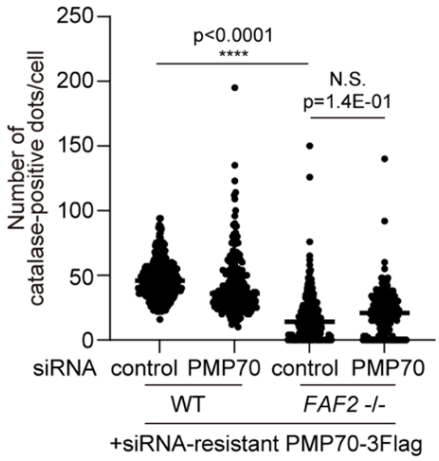
A



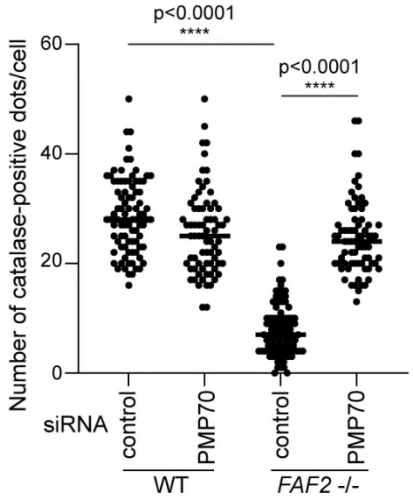
B



C



D (Fig. 3K in the revised manuscript)



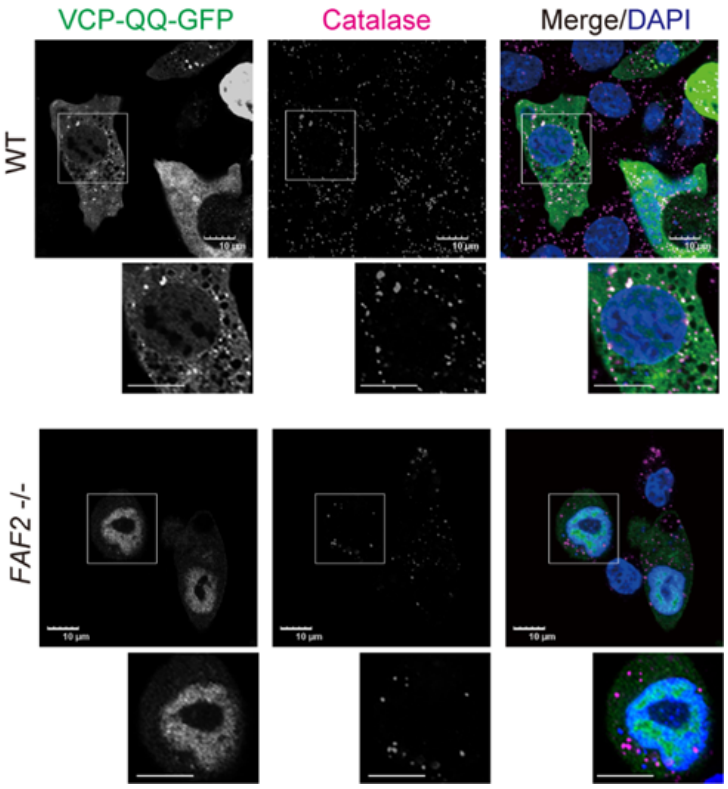
4. The authors propose FAF2's role in PMP70 degradation is recruiting VCP complex to peroxisome (Fig 6). However, the FAF2 truncation results in Fig 4 suggest FAF2's role in pexophagy is more complex than proposed. Notable, as many previous FAF2 papers showed, ΔUBX mutant of FAF2 disrupts FAF2 association with VCP, it should behave like FAF2-KO and ΔHP mutant (mislocalized to cytosol). This is true in PMP70 protein level regulation (Fig 4H and 4I). However, ΔUBX is half-functional in mitophagy (Fig 4F and 4G). It is strong evidence arguing that FAF2's role at peroxisome is more than recruiting VCP and arguing that PMP70 regulation is not a key step in pexophagy regulation.

Regarding the role of VCP during pexophagy, we found that VCP recruitment to peroxisomes is dependent on FAF2 since ATP hydrolysis-deficient VCP mutant (VCP-QQ-GFP) does not translocate to peroxisomes in the absence of FAF2 (*Figure 3A for Reviewer #2*). Nevertheless, as indicated by the reviewer, FAF2 ΔUBX recovered pexophagy to some extent (Figs. 5f and 5g) even though the FAF2 ΔUBX mutation disrupted the association between FAF2 and VCP (*Figure 3B for Reviewer #2*). We explain this result as follows.

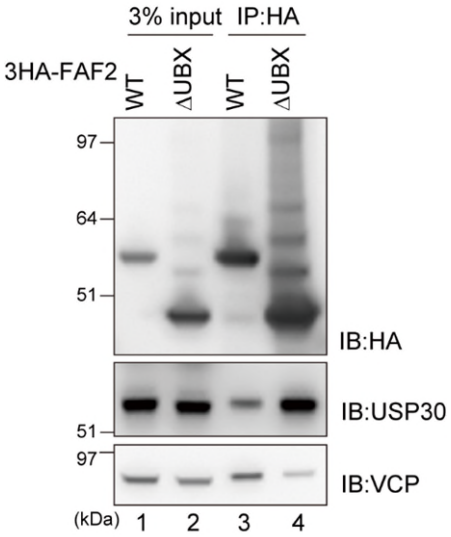
We speculate that de-ubiquitylation is the key. Even though FAF2 ΔUBX interactions with VCP were reduced, FAF2 ΔUBX interactions with USP30 were increased (*Figure 3B for Reviewer #2*). USP30 may reduce the number of poly-ubiquitin chains on peroxisomes and inhibit pexophagy, but in FAF2 ΔUBX-expressing cells would lead to partial recovery of pexophagy.

Figure 3 for Reviewer #2

A.



B.

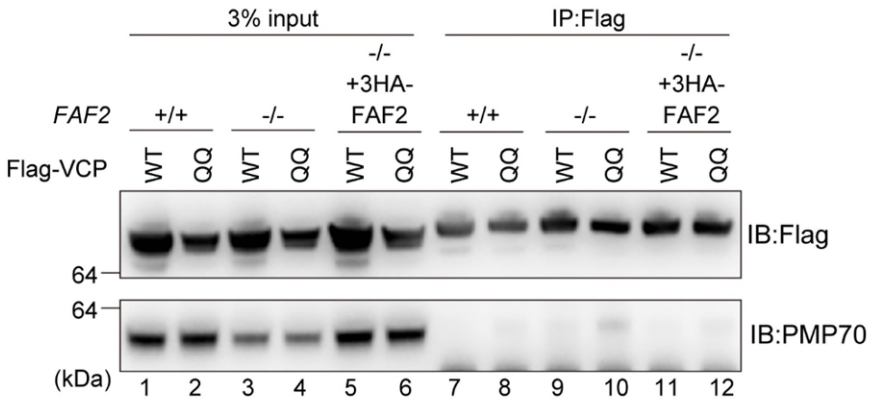


5. If FAF2 facilitates recruitment of ubiquitylated PMP70 to VCP, the authors can express FLAG-VCP-QQ to trap substrates, and examine the interaction between FLAG-

VCP-QQ and ubiquitylated PMP70 (FLAG-VCP-QQ IP) in the presence or absence of FAF2. This is a critical experiment that the authors may consider.

In response to the reviewer's suggestion, we examined if FAF2 recruits VCP to peroxisomes, and if VCP interacts with ubiquitylated PMP70. Flag-VCP WT or QQ were expressed in WT cells, *FAF2* -/- cells, and 3HA-FAF2-expressing *FAF2* -/- cells, and immunoprecipitated using an anti-Flag antibody (*Figure 4 for Reviewer #2*).
Unfortunately, PMP70 was not coimmunoprecipitated under any of the conditions we tested (*Figure 4 for Reviewer #2*). This may be due to low levels of PMP70 ubiquitylation. VCP interacts with multiple protein substrates[10]. Since the ubiquitylated form of PMP70 represents a small fraction of the total VCP substrate, it is likely difficult to detect interactions between PMP70 and VCP.

Figure 4 for Reviewer #2



6. It is nice that optineurin plays a key role in pexophagy in FAF2-KO cells (Fig 5). However, the evidence in Fig 6 remains preliminary. Because the authors propose ubiquitylated PMP70 as the major pexophagy initiator, and considering that there are many ubiquitylated proteins at peroxisome as shown in Fig S3D-H, it is important to perform optineurin pull down to see if it preferentially binds uniquitylated PMP70. It is also critical to test if siPMP70 reduces/blocks optineurin recruitment to peroxisome in FAF2-KO cells.

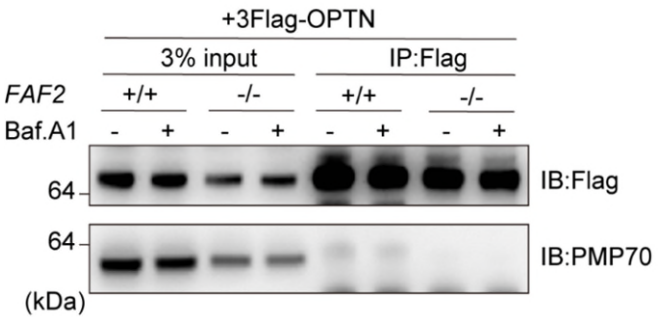
This constructive comment is much appreciated. 3Flag-OPTN was immunoprecipitated and the IP products were subjected to SDS-PAGE. As shown in *Figure 5A for Reviewer #2*, the predominant interaction between 3Flag-OPTN and ubiquitylated or unmodified

PMP70 did not reach a detectable level. The interaction between OPTN and PMP70 is assumed to be quite weak or transient. Indeed, a recent study demonstrated that the binding affinity between OPTN UBAN domain and K63-linked di-ubiquitin is very low [11].

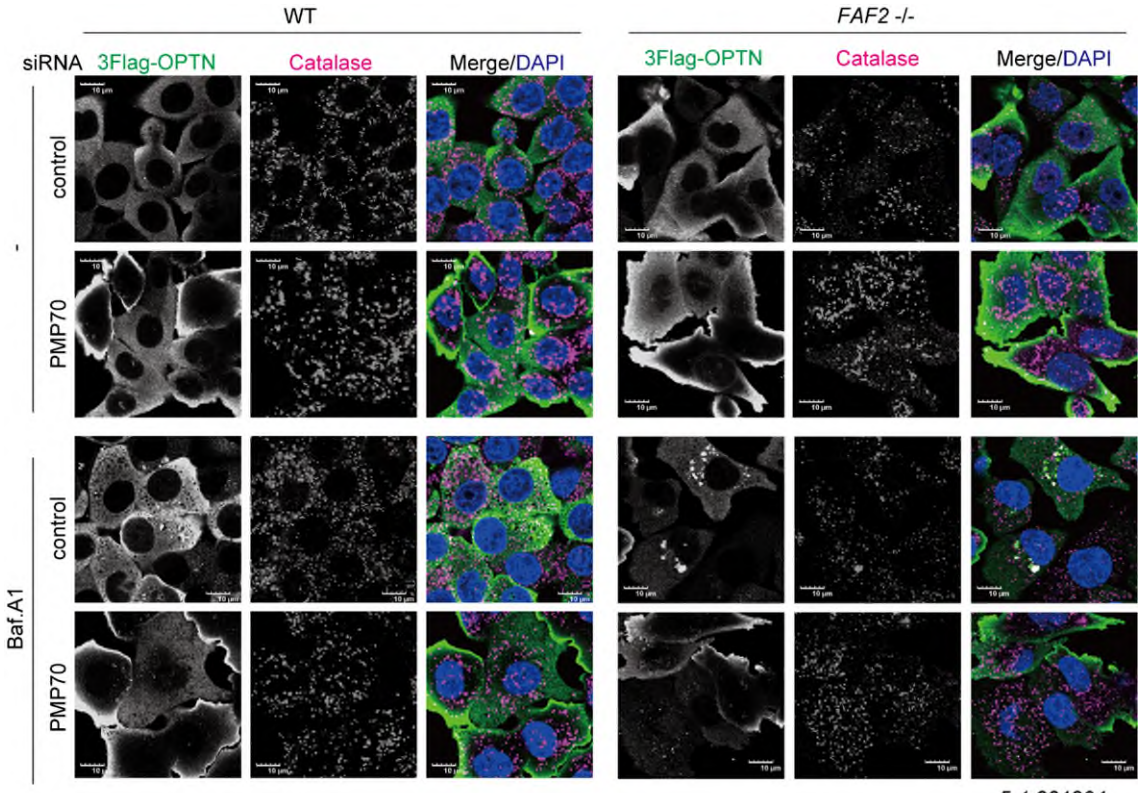
In contrast, the second experiment suggested by the reviewer (i.e., examine the effects of PMP70 knockdown on OPTN recruitment to peroxisomes) yielded valuable data. As shown in *Figure 5B and 5C for Reviewer #2*, after Baf.A1 treatment, the percentage of *FAF2* -/- cells with peroxisomal OPTN was significantly reduced by PMP70 knockdown as compared to the corresponding control (reduced from 55.4 to 15.1 %). This result strongly suggests that PMP70 is required for OPTN recruitment to peroxisomes for pexophagy. We have included these data in the revised manuscript.

Figure 5 for Reviewer #2

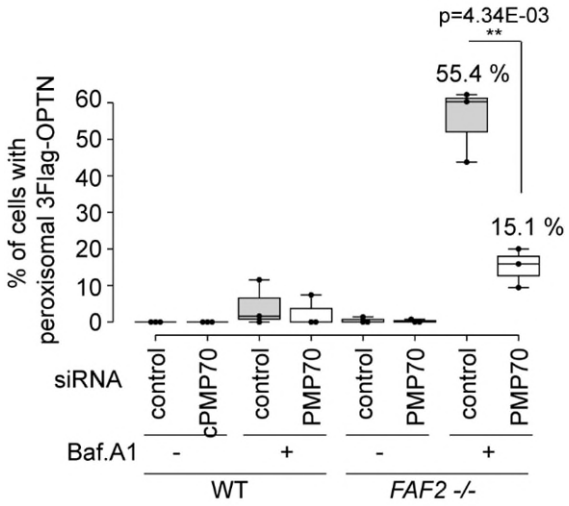
A



B



C



Minor question:

7. In Figure 1A, the authors generated FAF2-/- cells. These cells are presumably derived from single clone, but the staining showed that catalase content is highly heterogeneous among FAF2-/- cells. This is different from other figures shown in Fig2H, 3E, 3H, 3K etc. The authors may want to discuss the underlying reason.

We thank the reviewer for the comment. As shown in Fig. 1, the number of cells with either reduced or missing peroxisomes was clearly higher in *FAF2*-/- cells as compared with WT cells. Furthermore, peroxisomal proteins such as PMP70, PEX14, PEX16, and catalase were significantly reduced in *FAF2* -/- cells.
After transfecting the gRNA, a single clone was isolated to make *FAF2* -/- cells. The *FAF2* -/- cells used in this study were derived from the same clone. However, as indicated, there are variations in the number of peroxisomes. This heterogeneity in peroxisome abundance in *FAF2* -/- cells could indicate an unknown complementation system (such as USP30) for FAF2-deficiency, which is uncontrollable at this stage. *VDAC2* -/- and *VPS13D* -/- similarly give rise to heterogenously deficient peroxisomes[12,13].

Because the aim of the experiment in Figs. 6a and 6c was to assess whether ubiquitin and autophagy adaptors were localized to peroxisomes, we were unable to obtain data for cells without peroxisomes. In these cases, the *FAF2* -/- cells with residual amount of peroxisomes were selected.

Referee #3:

Peroxisome quality control is essential for maintaining functional peroxisomes. Recent studies have shown that genetic mutations or stress conditions can induce pexophagy. In most of these systems, the accumulation of ubiquitinated proteins on the peroxisomal membrane acted to signal pexophagy. Other work has shown that peroxisome quality control is mediated by systems other than autophagy. The quality control of peroxisomal matrix proteins is monitored by Lonp2, a protease that degrades damaged matrix proteins. However, the mechanism by which peroxisomal membrane proteins are maintained is unknown. It is known that in culture cells such as CHO cells, the half-life of a membrane protein ranges between 2-6 hrs (PEX3) to up to 36 hrs for PEX16 (PMID: 19719477), which are shorter than the 2-3 days of a peroxisome half-life, suggesting that a mechanism exists for extracting damaged membrane proteins from the peroxisomal membrane. However, the mechanism by which peroxisomal membrane proteins are extracted is unknown.

Here, in this manuscript by Koyano et al., the authors present data that supports UBDX8, a protein associated with the VCP/p97 complex, is required to remove ubiquitinated peroxisomal membrane protein. The authors show that HCT116 cells knockout of FAF2/UBDX8 show decreased peroxisomes and increased ubiquitinated peroxisomal

membrane proteins. To show that the increase in ubiquitinated peroxisomal membrane protein is associated with pexophagy, they used ratiometric imaging of a pH-sensitive fluorescent probe that showed that in cells without FAF2, results in an increased localization of a peroxisomal marker in lysosomes. Based on these findings, the authors suggest that the FAF2-linked p97 complex prevents pexophagy.

The model that the loss of FAF2 induces pexophagy by preventing p97 removal of ubiquitinated peroxisomal membrane protein is feasible and attractive given the role of p97 in the quality control of membrane proteins of other organelles, including the ER and mitochondria. However, the authors do not provide convincing data that FAF2-p97 "represses" pexophagy. For one, the authors do not demonstrate that p97 is required to prevent pexophagy, or that the loss of p97 results in pexophagy. They only show that the loss of FAF2 is associated with an increased loss of peroxisomes and an increase in ubiquitinated PEX16, PMP70 and PEX14. Further, does FAF2-p97 repress pexophagy? Instead of repressing pexophagy, wouldn't the most likely interpretation (if p97 was required) be that FAF2-p97 prevents a peroxisome from being targeted for pexophagy by removing ubiquitinated membrane proteins? Although it may sound like an argument of semantics, it is nevertheless an important point as there are mechanistic consequences for a pathway that represses a pathway vs preventing a substrate from entering the pathway. Repression suggests a mechanism by which a pathway is inhibited by activity preventing a component or components of a pathway. In the case of FAF2-p97, it does not appear that pexophagy is inhibited but instead that the consequence of not removing ubiquitinated protein from peroxisomes signals the individual peroxisomes for degradation by pexophagy. Others have shown similar mechanism, including targeting ubiquitin-tagged peroxisomal membrane protein or mutations that induces the accumulation of ubiquitinated PEX5 on the membrane. Still, others have shown that targeting E3 ligases to peroxisomes also induces pexophagy by causing an accumulation of ubiquitinated membrane protein. If the author wants to support a model where FAF2-p97 is repressing pexophagy, the authors need to demonstrate that FAF2-p97 is actually inhibiting the autophagy-mediated degradation of peroxisomes. Currently, there is no evidence in the manuscript that FAF2-p97 inhibits pexophagy.

However, their data can easily be interpreted to suggest that FAF2-p97 regulates peroxisome quality control by removing ubiquitinated peroxisomal membrane protein. This model is novel as it provides the first mechanism by which old or damaged peroxisomal membrane proteins are removed from the peroxisomal membrane. As

mentioned above, evidence suggests that different peroxisomal membrane proteins have different half-life. However, how these proteins are removed from the peroxisomal membrane is unknown. In this manuscript, the authors have provided data that supports FAF2-p97 as a regulator of peroxisome membrane protein quality control. However, to support this model, the authors must show that p97 directly removes ubiquitinated peroxisomal membrane proteins.

There are also other concerns with the data that need to be addressed.

We appreciate the reviewer's recognition that our work highlights a new role for FAF2/UBXD8 in peroxisomal quality control.
As the reviewer mentioned, we think that FAF2/VCP "prevents" peroxisomes from being targeted for pexophagy by removing ubiquitylated membrane proteins. In our model, the FAF2-p97/VCP axis does not participate in pexophagy midway through the pathway as an inhibitory (negative) regulator. Instead, the loss of FAF2-p97/VCP accelerates pexophagy as a mechanistic consequence of not removing ubiquitylated proteins from peroxisomes. To avoid misinterpretation, we have changed the corresponding text in the revised manuscript.

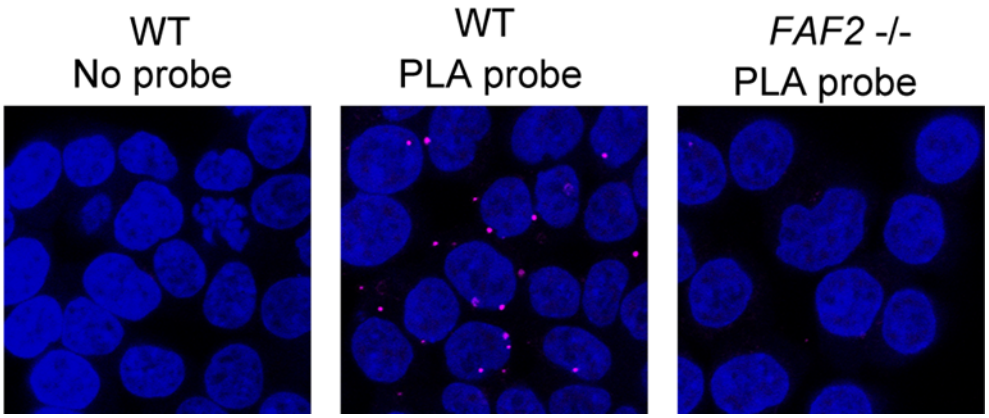
To address reviewer comments, we performed a number of additional experiments. We believe that these new results, which are detailed in our point-by-point responses, adequately address concerns raised by the reviewers.

1) To support a model that FAF2-p97 removes ubiquitinated peroxisomes (either to repress pexophagy or prevent the activation of pexophagy of a given peroxisomes), the authors performed colocalization studies in HeLa cells of HA-FAF2 and catalase. Using line-scanning, they show that HA-FAF2 and Catalase are colocalized. However, the problem with this experiment is that FAF2 (UBDX8) has already been shown to not localize on peroxisomes but to be juxtaposed to peroxisomes. More specifically, Schrul & Kopito (ref20 in manuscript) showed that in vitro translated FAF2/UBDX8 targets structures juxtaposition to mature peroxisomes, which was most evident using the super-resolution SIM imaging system. Further, FAF2 was not reported in peroxisomes but in mitochondria, lipid droplets and ER. Given these previous studies, authors must clearly demonstrate that FAF2 targets peroxisomes. Subcellular fractionation of peroxisomes may help demonstrate the subcellular localization of FAF2.

We agree that endogenous FAF2 localizes to multiple membrane organelles including mitochondria. However, we are confident that FAF2 localizes to peroxisome as well. To provide more convincing data, we performed an *in situ* proximity assay (PLA) to determine if FAF2 interacts with the peroxisomal marker, PMP70. For this assay, rabbit anti-FAF2 and mouse anti-PMP70 antibodies were used as primary antibodies, whereas secondary antibodies were oligonucleotide-conjugated antibodies (PLA probes). Following the inclusion of a DNA polymerase and fluorescence-labeled oligonucleotides, PLA dots would be generated if the probes were in close proximity (< 40 nm). Red dots indicating peroxisome-localized FAF2 were observed in WT cells in a PLA probe-dependent manner, but not in *FAF2* -/- cells (*Figure 1 for Reviewer #3*). The data clarified that a part of endogenous FAF2 localizes on peroxisomes. *Figure 1A for Reviewer #3* has been incorporated into the revised manuscript.

As indicated in *Figure 1 for Reviewer #2*, immunocytochemistry methods also demonstrated that endogenous FAF2 colocalized with PMP70 (*Figure 1A for Reviewer #2)*.

Figure 1 for Reviewer #3



2) To test whether a defect in FAF2 results in a peroxisome biogenesis defect, the authors compared the stability of peroxisomal membrane protein in FAF2 knockout cells to cells depleted of PEX19 expression. However, since PEX19 is essential for targeting peroxisomal membrane protein, this experiment demonstrates that FAF2 is not required for the targeting or stability of peroxisomal membrane protein and not that FAF2 is not required for peroxisome biogenesis. Therefore, the authors cannot rule out the possibility that FAF2 is involved in the biogenesis of peroxisomes. In fact, the heterogeneous phenotype of the FAF2 knockout cells showing some cells with peroxisomes while others show the absence of peroxisomes appears to be similar to

VPS13D knockout cell lines recently described by Richard Youle's group (PMID: 33891012). Youle's group found that the knockout of VPS13D results in a heterogenous cell phenotype. Together with Pietro De Camilli's group work showed that VPS13D links peroxisomes to the ER, suggesting that VPS13D regulates peroxisome biogenesis. The author should further explore whether FAF2 also regulates peroxisome biogenesis.

We thank the reviewer for the constructive comments. To determine if peroxisome biogenesis is affected by FAF2 depletion, we performed several experiments including those suggested by the reviewer.

Initially, we based our experimental approach on the method used by the Dr. Youle's group that the reviewer referenced (Baldwin *et al* JCB 2021)[13]. PEX19 was stably expressed as PEX19-P2A-GFP-SKL in both *PEX19* -/- cells and *PEX19*/*FAF2* double KO (*PEX19* -/- *FAF2* -/-) cell lines (*Figure 2A for Reviewer #3*). Within a day of PEX19-P2A-GFP-SKL expression, *de novo* peroxisome synthesis that persisted over time was observed in both cell lines. Differences in the rate of GFP-positive peroxisome formation, however, were observed between the two lines (*Figure 2B and 2C for Reviewer #3*). Although these results seemingly suggest that peroxisomal biogenesis was delayed in *FAF2* -/- cells, as is the case with *VPS13D -/-* cells (see Fig. 8C in Baldwin *et al* JCB 2021), this experiment alone is insufficient to conclude that FAF2 is involved in peroxisome biogenesis (see the discussion below for details). Therefore, to more conclusively determine which peroxisomal event (biogenesis or degradation) FAF2 impacts, we conducted additional experiments.

In WT cells, the peroxisome population is balanced between biogenesis and degradation via pexophagy (*Figure 2D for Reviewer #3*). To explain the significant peroxisome reduction in *FAF2* -/- cells, we hypothesized that the absence of FAF2 promotes pexophagy by de-suppression (Model A). As suggested by Reviewer #3, we cannot rule out the possibility that FAF2 is involved in *de novo* peroxisome biosynthesis (Model B).

To test these two hypotheses, we made *FAF2*/*FIP200* double KO cells (*Figure 2E for Reviewer #3*). FIP200 is essential for autophagy initiation. Consequently, peroxisome levels should be restored in the *FIP200*/*FAF2* double KO cells if Model A is correct. Conversely, if Model B is correct, peroxisome levels should remain depressed in the *FIP200*/*FAF2* double KO line as compared to those in *FAF2* -/- cells (*Figure 2D for Reviewer #3*).

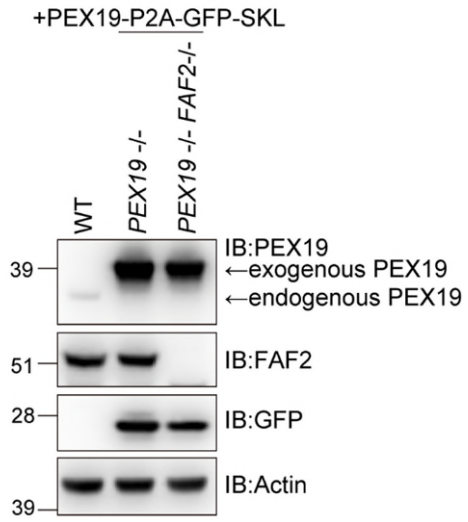
To compare molecular functions of FAF2 and PEX19, we also made *PEX19* -/- cell line and *PEX19*/*FIP200* double KO cell line, since PEX19 was reported to be involved in *de novo* synthesis of peroxisomes. Please see *Figure 2F and 2G for Reviewer #3*. The loss of peroxisomes observed in *PEX19* -/- cells were not recovered when FIP200 was further knocked out, which is consistent of a previous finding in which PEX19 is involved in peroxisome biogenesis. In sharp contrast, peroxisome abundance in *FAF2* -/- cells was increased by FIP200 knockout. Furthermore, if FAF2 is assumed to participate in peroxisome biogenesis, treatment of cells with autophagy inhibitor would have no effect on the peroxisomal number in *FAF2* -/- cells. However, Baf.A1 treatment significantly elevated both peroxisome abundance and PMP70 levels in *FAF2* -/- cells but not in *PEX19* -/- cells (*Figure 2H, 2I, 2J, and 2K for Reviewer #3*). These data again suggest that FAF2 is involved in peroxisome degradation (note that signals in the nuclei of *PEX19* -/- cells stained by our anti-PMP70 antibody in *Figure 2H for Reviewer #3* are non-specific signals). Similarly, FIP200 knockdown resulted in increased peroxisome abundance in *FAF2* -/- cells but not in *PEX19* -/- cells (*Figure 2L, and 2M for Reviewer #3*). These data are consistent with the above results.

Given these findings, we can consider the differences between the *VPS13D* KO cells and the *FAF2* KO cells. Importantly, Dr. Youle's group reported that the number of peroxisomes in *VPS13D*/*FIP200* DKO cells did not exceed that of *VPS13D* single KO cells (see Fig. 8A in Baldwin *et al* JCB 2021). By combining this observation with the peroxisome recovery triggered by the reintroduction of PEX19 into *PEX19* KO cells, they concluded that VPS13D is involved in peroxisome biogenesis.
In contrast, peroxisome abundance in *FAF2* -/- cells increased following FIP200 depletion (described above). This indicates that FAF2 is involved in autophagic degradation of peroxisomes, which is clearly different from the role of VPS13D.

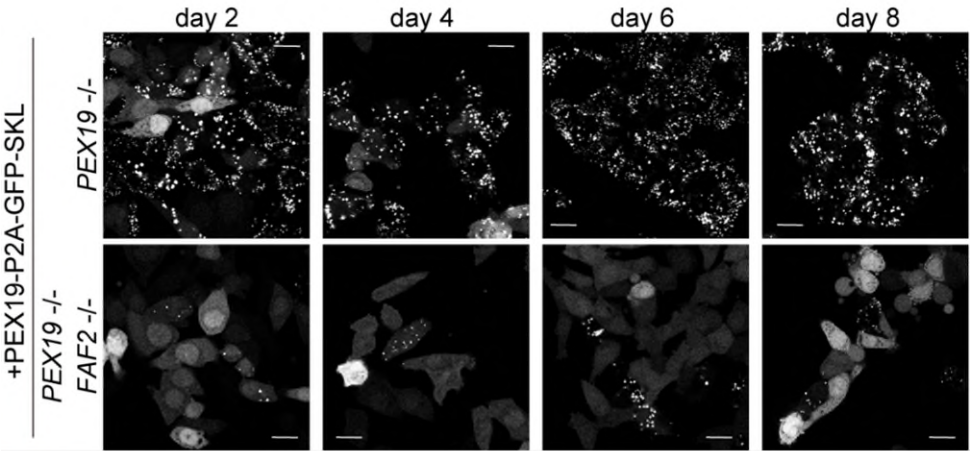
Collectively, our results again revealed that a FAF2 function aligns with Model A and is involved in pexophagy rather than *de novo* synthesis of peroxisomes (*Figure 2D for Reviewer #3*). *Figure 2E-2M for Reviewer #3* have been incorporated into the revised manuscript.

Figure 2 for Reviewer #3

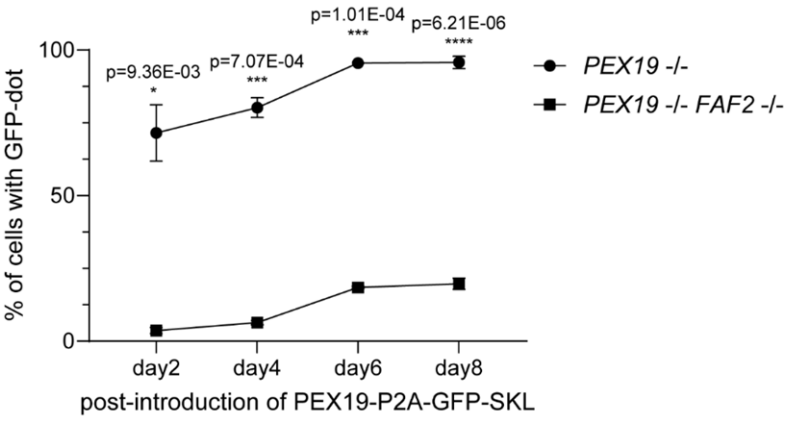
A


+PEX19-P2A-GFP-SKL
WT
PEX19 -/-
PEX19 -/- FAF2-/-
39
IB:PEX19
←exogenous PEX19
←endogenous PEX19
51
IB:FAF2
28
IB:GFP
39
IB:Actin

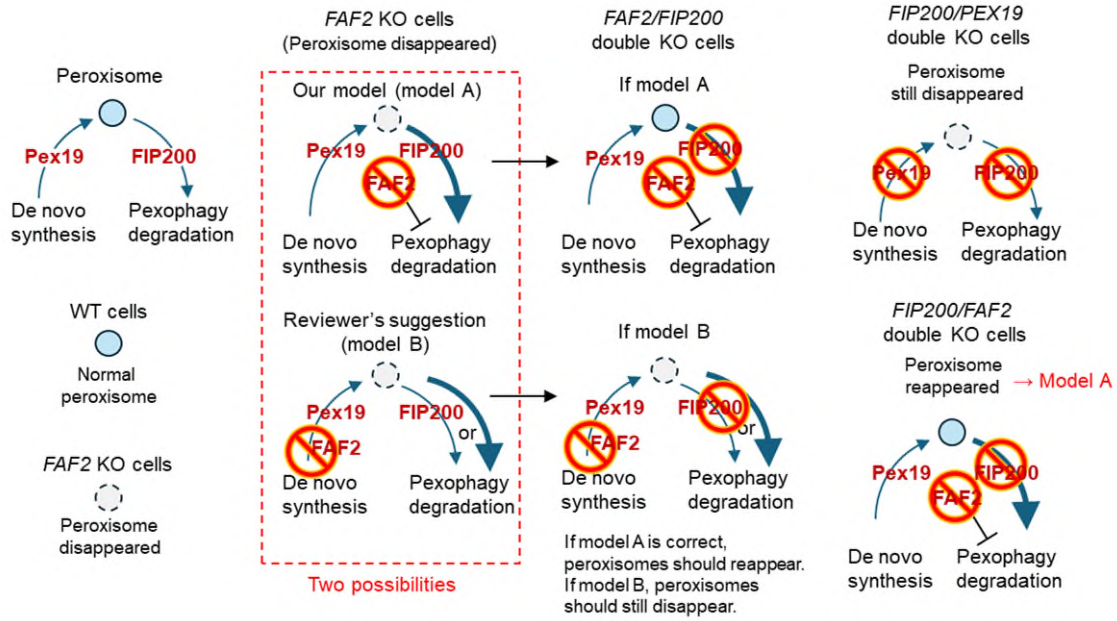
B


day 2
day 4
day 6
day 8
+PEX19-P2A-GFP-SKL
PEX19 -/-
PEX19 -/- FAF2 -/-

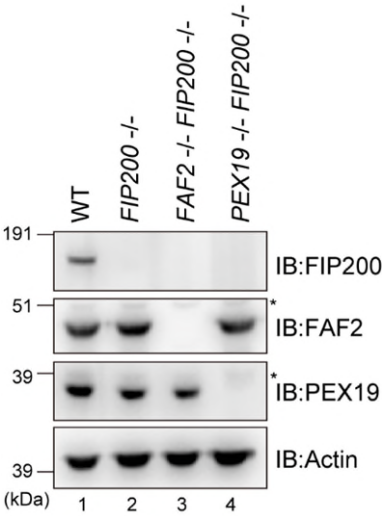
C


p=9.36E-03
*
p=7.07E-04
***
p=1.01E-04
***
p=6.21E-06
****
100
50
0
% of cells with GFP-dot
day2
day4
day6
day8
post-introduction of PEX19-P2A-GFP-SKL
PEX19 -/-
PEX19 -/- FAF2 -/-

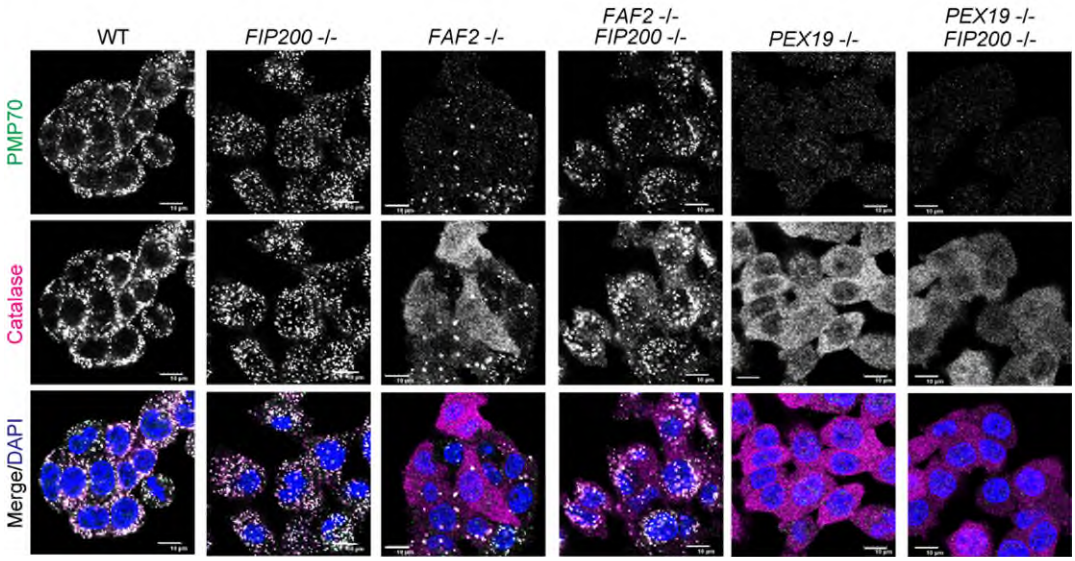
D


Peroxisome
Pex19
FIP200
De novo synthesis
Pexophagy degradation
WT cells
Normal peroxisome
FAF2 KO cells
Peroxisome disappeared
FAF2 KO cells
(Peroxisome disappeared)
Our model (model A)
Pex19
FIP200
FAF2
De novo synthesis
Pexophagy degradation
Reviewer's suggestion (model B)
Pex19
FIP200
FAF2
or
De novo synthesis
Pexophagy degradation
Two possibilities
FAF2/FIP200 double KO cells
If model A
Pex19
FIP200
FAF2
De novo synthesis
Pexophagy degradation
If model B
Pex19
FIP200
FAF2
or
De novo synthesis
Pexophagy degradation
If model A is correct, peroxisomes should reappear.
If model B, peroxisomes should still disappear.
FIP200/PEX19 double KO cells
Peroxisome still disappeared
Pex19
FIP200
De novo synthesis
Pexophagy degradation
FIP200/FAF2 double KO cells
Peroxisome reappeared
→ Model A
Pex19
FIP200
FAF2
De novo synthesis
Pexophagy degradation

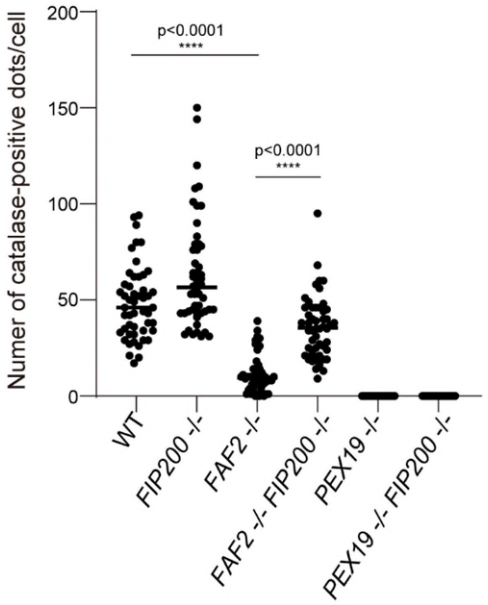
E


WT
FIP200 -/-
FAF2 -/- FIP200 -/-
PEX19 -/- FIP200 -/-
191
51
39
39
(kDa)
1
2
3
4
IB:FIP200
IB:FAF2
IB:PEX19
IB:Actin
*
*

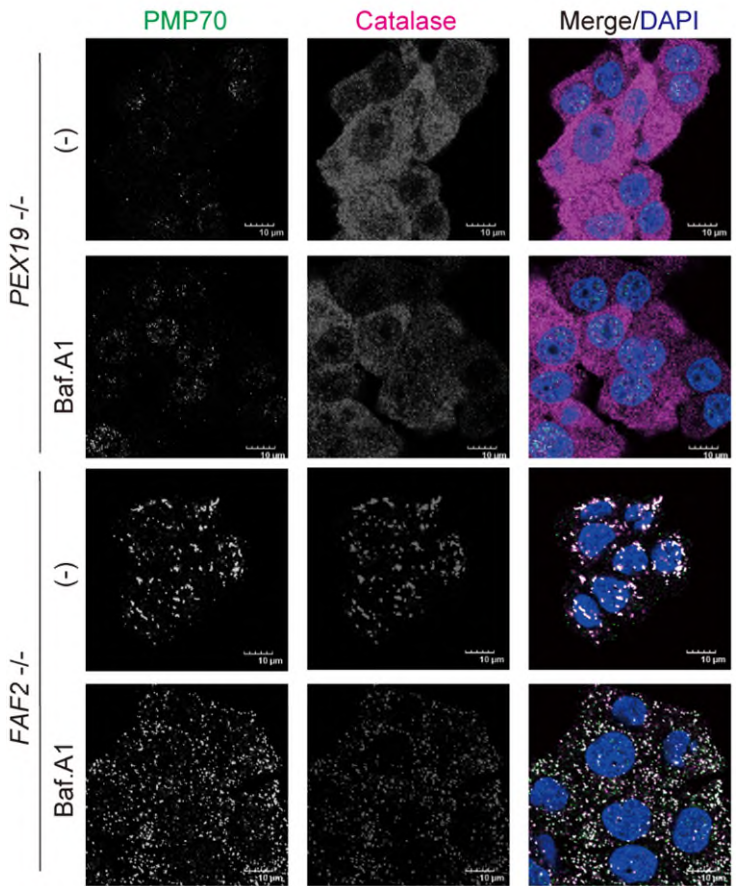
F


WT
FIP200 -/-
FAF2 -/-
FAF2 -/-
FIP200 -/-
PEX19 -/-
PEX19 -/-
FIP200 -/-
PMP70
Catalase
Merge/DAPI

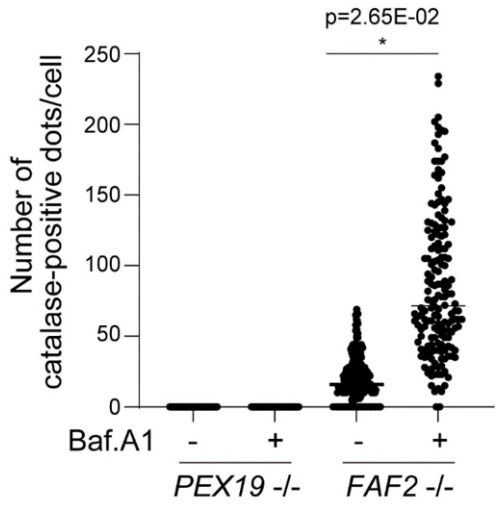
G


Numer of catalase-positive dots/cell
200
150
100
50
0
p<0.0001
****
p<0.0001
****
WT
FIP200 -/-
FAF2 -/-
FAF2 -/- FIP200 -/-
PEX19 -/-
PEX19 -/- FIP200 -/-

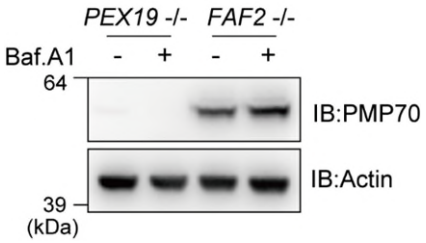
H


PMP70
Catalase
Merge/DAPI
PEX19 -/-
(-)
Baf.A1
FAF2 -/-
(-)
Baf.A1
10 µm

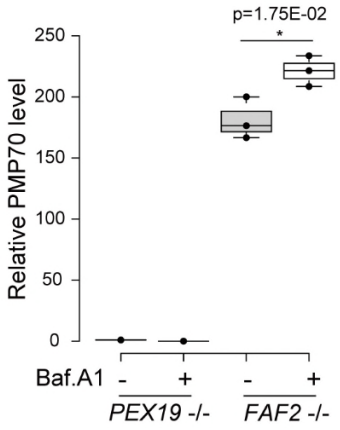
I


p=2.65E-02
*
Number of catalase-positive dots/cell
250
200
150
100
50
0
Baf.A1
-
+
-
+
PEX19 -/-
FAF2 -/-

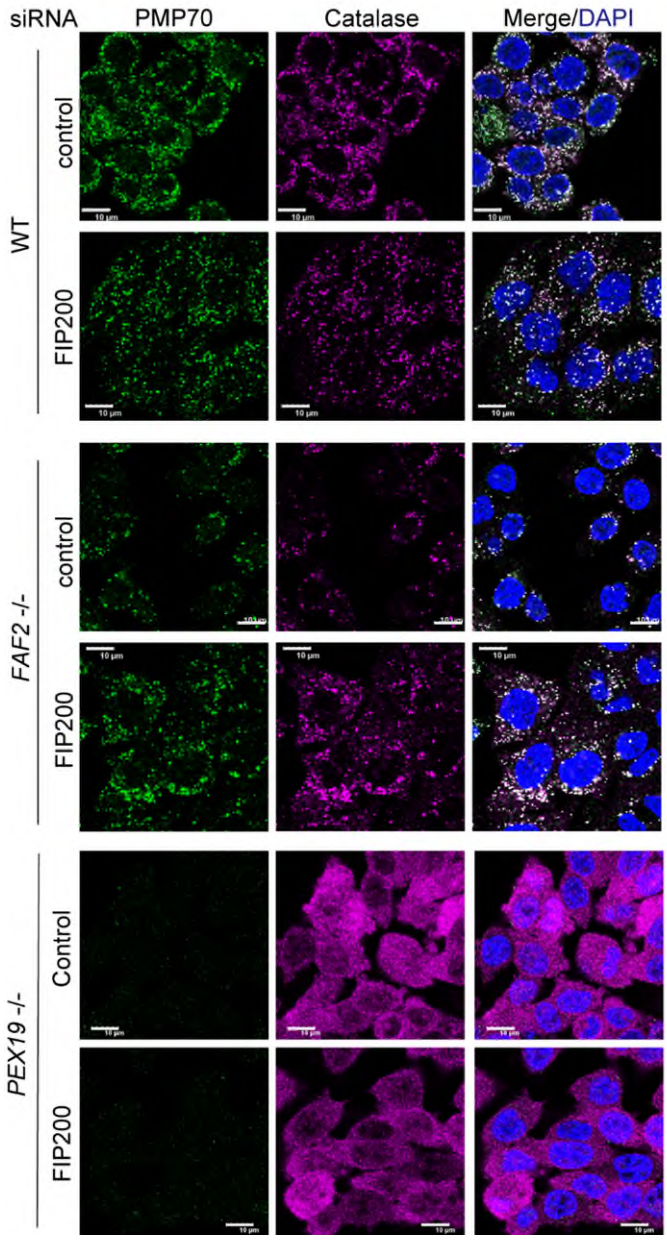
J


PEX19 -/-
FAF2 -/-
Baf.A1
- + - +
64
IB:PMP70
IB:Actin
39
(kDa)

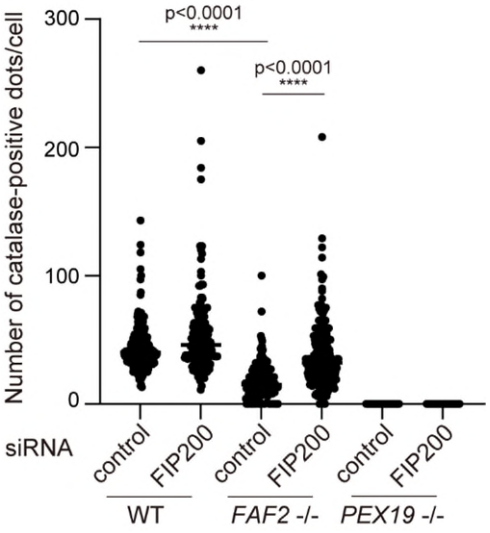
K


p=1.75E-02
*
Relative PMP70 level
250
200
150
100
50
0
Baf.A1
- + - +
PEX19 -/-
FAF2 -/-

L


siRNA
PMP70
Catalase
Merge/DAPI
WT
control
FIP200
FAF2 -/-
control
FIP200
PEX19 -/-
Control
FIP200

M



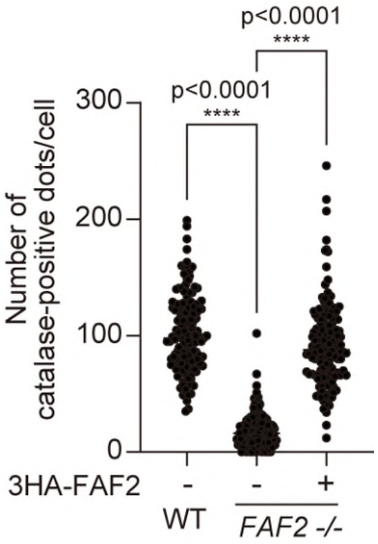
3) In Figure 1, it is not clear why the authors selected "less than 20 peroxisomes" as a means to measure the loss of peroxisomes. It would be more meaningful to quantify peroxisomal structures as they did in Figures 2I, 3F, 3L, etc. Further, in their images in Figure 1A, there is a group of cells with peroxisomes (Catalase and PEX14) and others with no catalase. Based on the quantification in Figure 1B, 30% of the cells have peroxisomes. Do they have full peroxisomes as wild type, but have they lost peroxisomes? Quantifying the number of peroxisomes in each cell may give a more accurate and full view of the consequences of FAF2 in HCT116 cells.

We thank the reviewer for this suggestion. The number of peroxisomes per cell was determined (*Figure 3A for Reviewer #3* below; previous Fig. 1B and *Figure 3B for Reviewer #3* below; previous Fig. 3I) and new figures have been incorporated into the revised manuscript.

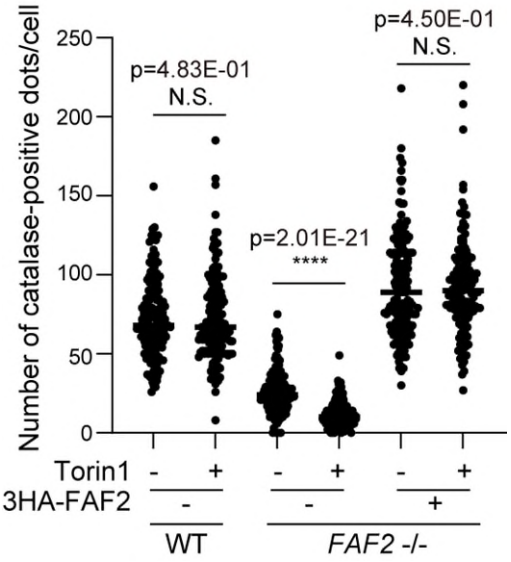
The original Fig. 1B showed that 30% of *FAF2* -/- cells had more than 20 peroxisomes. *Figure 3A and 3B for Reviewer #3* have been incorporated into the revised manuscript.

Figure 3 for Reviewer #3

A



B



4) it is also unclear whether PEX14 in the FAF2 knockout HCT116 cells is located in mitochondria. It appears to be peroxisomes that collapsed with mitochondria toward the perinuclear area. It would be better to use PMP70 or another membrane protein marker for peroxisomes since PEX14 is known to target mitochondria. PMP70 is likely a better choice in counting peroxisomes given that it does not readily target mitochondria, whereas PEX14 does. Nevertheless, the authors should comment on the lack of uniformity in peroxisome numbers.

5) Also, it is not clear whether PEX14 in the FAF2 knockout HCT116 cells is located on mitochondria. It appears to be peroxisomes that collapsed with mitochondria toward the perinuclear area. It would be better to use PMP70 or another membrane protein marker for peroxisomes since PEX14 is known to target mitochondria. PMP70 is likely a better choice in counting peroxisomes given that it does not readily target mitochondria, whereas PEX14 does. Nevertheless, the authors should comment on the lack of uniformity in peroxisome numbers.

We agree that "PMP70 is likely a better choice in counting peroxisomes" and have predominantly utilized PMP70 for peroxisome detection. For figures in which PMP70 was not used, we have detailed the reasons for the change.

In Fig. 1A, we used a mouse anti-catalase antibody to detect mature peroxisomes, which precluded our use of the mouse anti-PMP70 antibody. In Fig. 4h, catalase and PEX14 were used as peroxisome markers because PMP70 had been knocked down. In Fig. 6c and S12b, we used a mouse anti-PMP70 antibody as a peroxisome marker to examine the subcellular localization of p62, OPTN, NDP52, and TAX1BP1. However, we could not use this antibody with the mouse anti-NBR1 antibody. Instead, a rabbit anti-PEX14 antibody was used as the peroxisome marker (Fig. 6c).
Cells in Fig. 2E were immunostained using anti-PMP70 and anti-catalase antibodies and cells in Fig. 3E were immunostained using anti-PMP70 instead of an anti-PEX14 antibody.

Our interpretation of the PEX14 mitochondrial localization suggests mislocalization of the protein occurs under conditions when peroxisomes are absent. In Fig. 1a, catalase was used as the marker of mature peroxisomes. In *FAF2* -/- cells, catalase was dispersed throughout the cytosol and PEX14 was localized on mitochondria (Fig. 1a). This mislocalization in the absence of peroxisomes is consistent with a previous report[14]. Simply, if PEX14 is found on mitochondria, it could be evidence that peroxisomes are absent.

The heterogeneity in peroxisome abundance in *FAF2* -/- cells might be due to an intrinsic factor (e.g., USP30) that compensates for the FAF2 deficiency such that fluctuations in the expression levels of the factor affect the number of peroxisomes present.

6) The Keima-skl data is confusing in the context of Figure 1. If the majority of FAF2 knockout cells do not have peroxisomes, then how is Keima-skl getting targeted to lysosomes? There is some evidence that peroxisomal matrix proteins can target mitochondria in peroxisome-deficient cells (PMID: 31129117). Given that the mitochondria appear to be collapsed in the FAF2 deficient cells, could it be possible that the Keima-skl in lysosomes is a result of mitophagy? This could explain the OPTN experiment. The author should deplete an autophagy factor (e.g. FIP200) to test whether the loss of peroxisomes is due to autophagy using the Keima-skl and a count of peroxisomal structures.

We appreciate the insightful comments. Although the data are not shown, we observed that mKeima-SKL is precisely targeted to peroxisomes even in *FAF2* -/- cells if they retain a few peroxisomes. If *FAF2* -/- cells do not harbor peroxisomes, mKeima-SKL was found diffusely in the cytosol, but not in mitochondria. Actually, we showed that, in the original manuscript, catalase that possesses a PTS1 signal accumulated in the cytosol in *FAF2* -/- cells (Fig. 1a). Therefore, it is unlikely that the mKeima-SKL signal upon FACS analysis is derived from mitophagy.

We think that pexophagy can be observed even cells have few to no peroxisomes present. For instance, if a cytosolic protein undergoes autophagic degradation, whose speed is the same as the speed of biosynthesis, the presence under steady state conditions might not be detectable. On the other hand, its degradation-derived signal would be detected. When flow process proceeds, it is reasonable to observe mKeima-SKL signals (pexophagy signals) in *FAF2* -/- cells.

Nevertheless, in response to the reviewer's comments, we sought to determine if the peroxisomal loss is an autophagy-dependent.

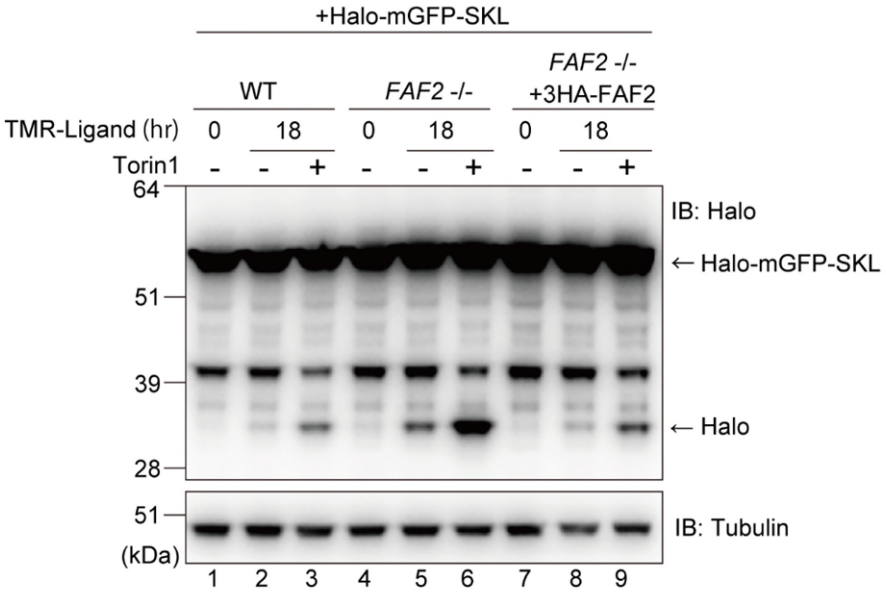
SKL was fused with Halo-mGFP-tag (For more details on the HaloTag processing assay, please refer to our reply for Reviewer #1, comment #2) and stably expressed in WT or *FAF2* -/- cells. In the presence of the TMR-ligand, the Halo fragment in *FAF2* -/- cells (lane 5) was more intense than in WT cells (lane 2, *Figure 4A and 4B for Reviewer #3*). The Halo fragment band was less pronounced in *FAF2* -/- cells stably expressing 3HA-FAF2 (lane 8). In addition, Torin1, an mTOR inhibitor, enhanced the Halo fragment bands (lane 3, 6, and 9). Therefore, this result indicated that we can monitor pexophagy using HaloTag assay.

When the autophagy essential FIP200 protein was knocked down, the intensity of the Halo band was reduced in *FAF2* -/- cells, suggesting that the more intense Halo band in *FAF2* -/- cells was the result of autophagy (*Figure 4C and 4D for Reviewer #3*). Similarly, when the Halo-mGFP-tag was fused with the peroxisomal membrane protein PMP34, the HaloTag processing assay revealed that the Halo fragment band was more intense in *FAF2* -/- cells following Torin1 treatment (*Figure 4E and 4F for Reviewer #3*). These results strongly suggest that pexophagy is accelerated in *FAF2* -/- cells.

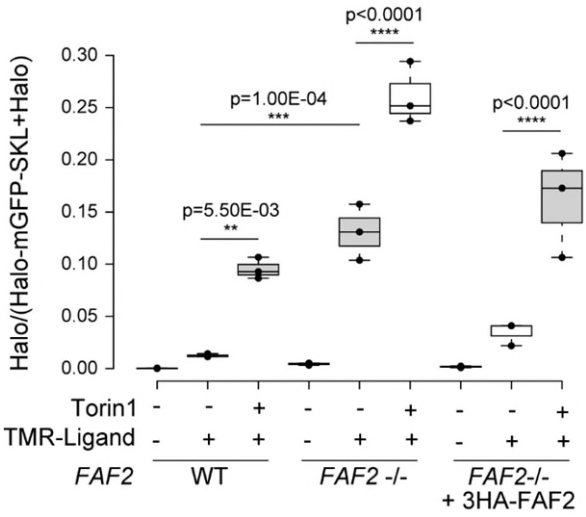
As suggested by the reviewer, we also found that FIP200 knocked down increased PMP70 levels in *FAF2* -/- cells (*Figure 4G and 4H for Reviewer #3*). Furthermore, catalase-positive peroxisomes were elevated in *FAF2* -/- cells treated with FIP200 siRNA (*Figure 4I and 4J for Reviewer #3*). Since these results are significant, they have been incorporated into the revised manuscript.

Figure 4 for Reviewer #3

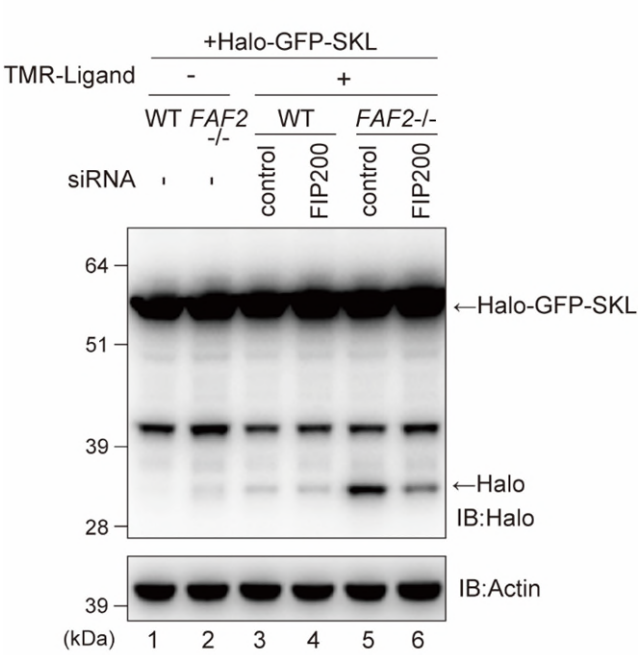
A



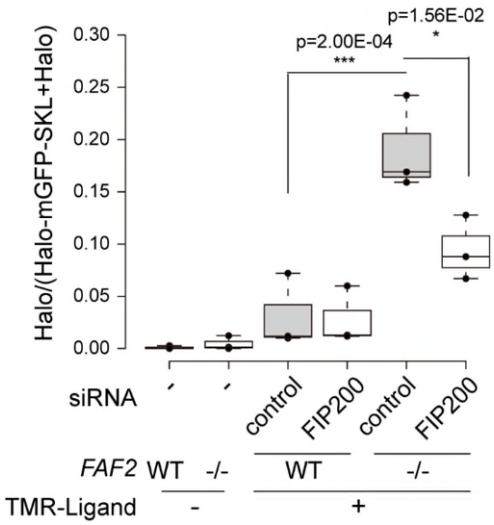
B


p<0.0001
****
p=1.00E-04
***
p<0.0001
****
p=5.50E-03
**
Halo/(Halo-mGFP-SKL+Halo)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Torin1 - - + - - + - - +
TMR-Ligand - + + - + + - + +
FAF2 WT FAF2 -/- FAF2-/- + 3HA-FAF2

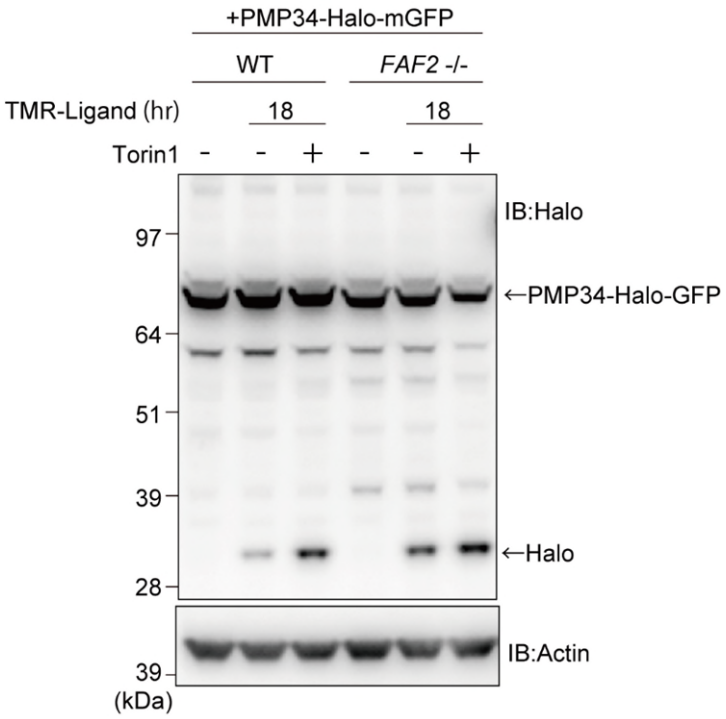
C


+Halo-GFP-SKL
TMR-Ligand - +
WT FAF2 -/- WT FAF2-/-
siRNA control FIP200 control FIP200
64
51
39
28
39
←Halo-GFP-SKL
←Halo
IB:Halo
IB:Actin
(kDa) 1 2 3 4 5 6

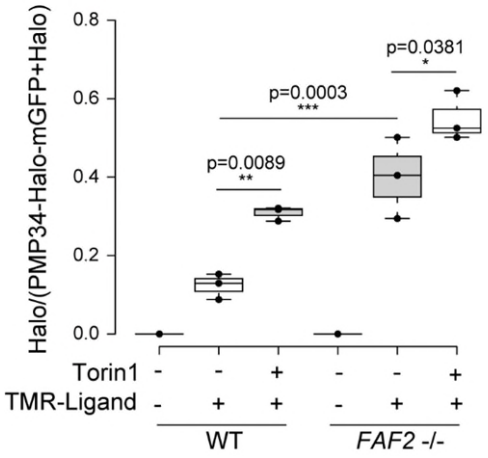
D


p=1.56E-02
p=2.00E-04
***
*
Halo/(Halo-mGFP-SKL+Halo)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
siRNA
control
FIP200
control
FIP200
FAF2 WT -/- WT -/-
TMR-Ligand - +

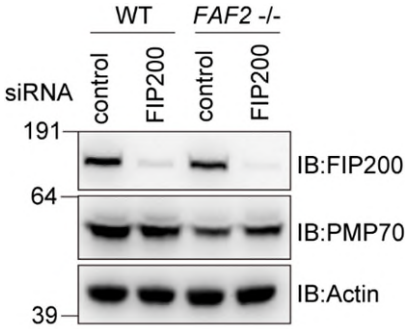
E


+PMP34-Halo-mGFP
WT
FAF2 -/-
TMR-Ligand (hr)
18
18
Torin1 - - + - - +
97
64
51
39
28
39
(kDa)
IB:Halo
←PMP34-Halo-GFP
←Halo
IB:Actin

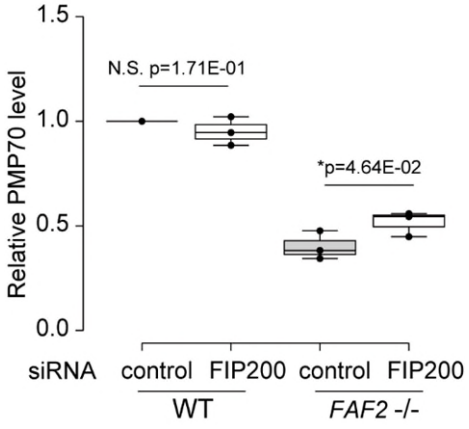
F


Halo/(PMP34-Halo-mGFP+Halo)
0.8
0.6
0.4
0.2
0.0
p=0.0381
*
p=0.0003
***
p=0.0089
**
Torin1 - - + - - +
TMR-Ligand - + + - + +
WT
FAF2 -/-

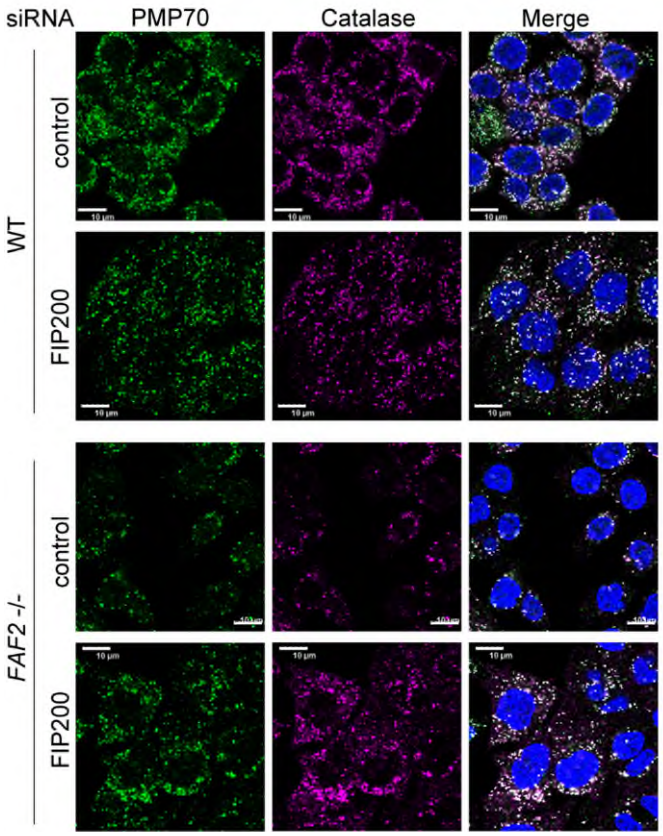
G


WT
FAF2 -/-
siRNA
control
FIP200
control
FIP200
191
64
39
IB:FIP200
IB:PMP70
IB:Actin

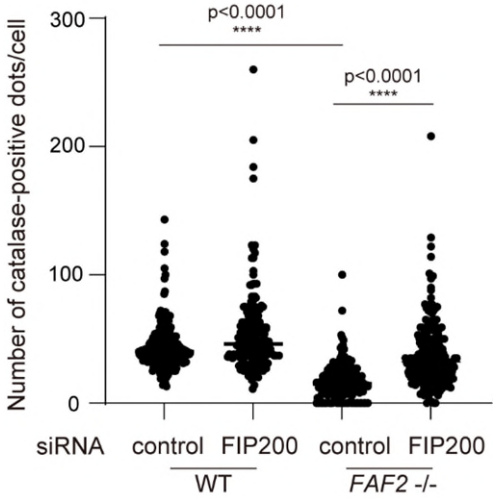
H


Relative PMP70 level
1.5
1.0
0.5
0.0
N.S. p=1.71E-01
*p=4.64E-02
siRNA control FIP200 control FIP200
WT
FAF2 -/-

I



J



7) The PMP70 ubiquitination assay is not entirely convincing. To determine whether FAF2 increases ubiquitinated PMP70, the authors IP FLAG in cells expressing PMP70-3xFLAG and probe immunoblots for FLAG and ubiquitin. The most convincing evidence is increased molecular bands in the FAF2 KO cells treated with BAF. A1. The

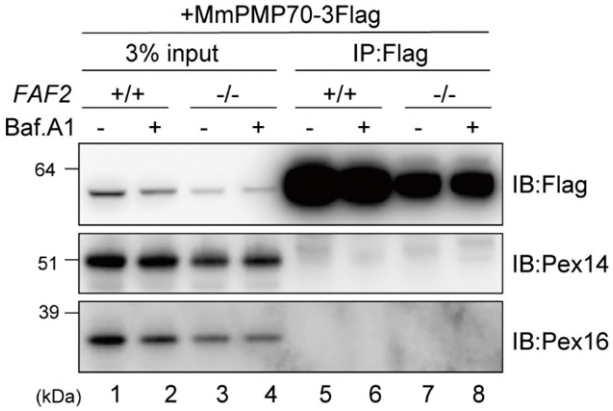
FLAG blots are difficult to assess, given the high levels of PMP70-FLAG. However, the larger issue is that the experiment may not have been done correctly. For ubiquitination experiments, the lysates need to be heat dissociated with other proteins before immunoprecipitation to ensure that other proteins associated with the protein of interest are not co-precipitated. Otherwise, one cannot be sure whether the streaks in the ubiquitinated blots are not ubiquitinated proteins associated with PMP70, PEX16 or PEX14 instead of being a streak of their protein of interest.

To evaluate the possibility that the smear in the high molecular weight region of the PMP70-3Flag immunoblots was due to ubiquitylation of other peroxisomal proteins, PMP70-3Flag IP products were electrophoresed and immunoblotted with anti-PEX16 and anti-PEX14 antibodies. However, no interactions between the PMP70-3Flag IP product and either PEX16 or PEX14 were detected (*Figure 5A for Reviewer #3*).

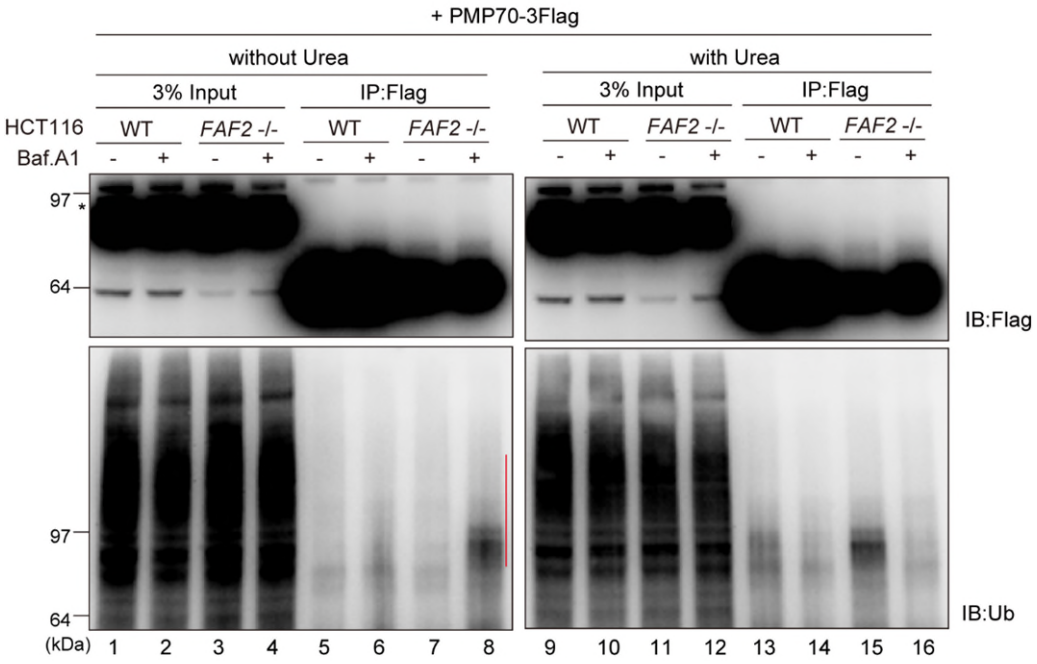
We next examined if PMP70 was directly ubiquitylated. Collected cell lysates were treated with urea prior to immunoprecipitation and then subsequently immunoblotted (*Figure 5B for Reviewer #3*). The high molecular weight smeared immunoreactivity was not observed in the urea-treated lysate samples (*Figure 5B for Reviewer #3, lane 16*). While we found no evidence that ubiquitin was directly conjugated to PMP70 in our experimental method, we speculate that either PMP70 or PMP70 interacting protein is ubiquitylated in *FAF2* -/- cells. We have indicated this in the manuscript.

Figure 5 for Reviewer #3

A



B



8) Figure 3E-F. It is surprising how many new peroxisomes are formed within 24 hours of treating the cells with Baf.A1. There appears to be a 3-fold increase in peroxisomes in the FAF2 KO cells but no significant increase in the wild-type cells. Also, the peroxisomes appear to be evenly disrupted. If there is an increase in ubiquitinated proteins on these peroxisomes (Fig 3a-d), wouldn't they become sequestered in autophagosomes? Also, why are the peroxisomes in 3E FAF2 -/- HCT116 BAF.A1 blue?

Thank you for the insightful comments. In the initial version of our manuscript, the number of peroxisomes was determined across three independent experiments and the average value from each experiment was plotted. However, as suggested by the reviewer, the data have been converted to scatter plots to show the counted peroxisome number rather than the average value. This allowed us to more accurately evaluate the variation in the number of peroxisomes per cell. As a result, we have concluded that peroxisomes were slightly elevated in WT cells following Baf.A1 treatment (*Figure 6A for Reviewer #3*). The new scatter plot has been incorporated into the revised manuscript.

Further, we think the increase in peroxisome number following 24 h Baf.A1 is reasonable given that the reduction in peroxisomes in *FAF2* -/- cells is due to accelerated degradation. The increase in peroxisomes would thus correspond to newly synthesized peroxisomes

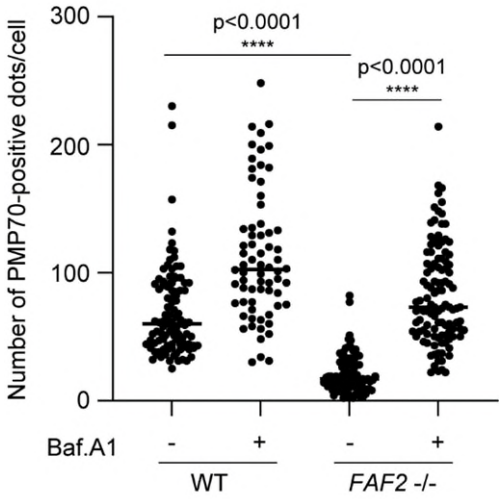
that accumulated during the 24 h Baf.A1 treatment period. Data presented in our response to the reviewer comment #2 show peroxisome abundance recovered in *PEX19* -/- cells within 48 h of PEX19 restoration. Moreover, it might be possible that new peroxisome synthesis is accelerated in *FAF2* -/- cells due to feedback mechanisms involving factors such as PPAR, which actively stimulates peroxisome biogenesis[15].

To determine if autophagosome sequestration occurs, we performed a series of immunocytochemistry experiments. In Fig. 6a, WT or *FAF2* -/- cells stably expressing GFP-SKL were immunostained with an anti-LC3B antibody. Similarly, cells in *Figure 6B for Reviewer #3* (below) were immunostained with anti-LC3B and anti-PMP70 antibodies. Both sets of data demonstrate that LC3B-positive dots partially colocalize with GFP-SKL-positive or PMP70-positive peroxisomes, suggesting that a portion of ubiquitylated peroxisomes are sequestrated in autophagosomes in *FAF2* -/- cells.

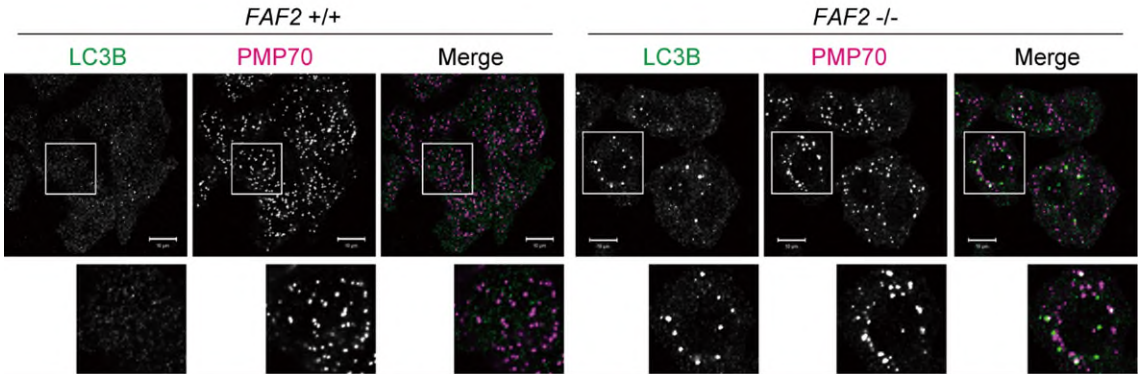
The "blue peroxisomes" that the reviewer noticed in Fig. 3E correspond to uneven DAPI staining of nuclei. To demonstrate that the blue coloration was not overlaid, we have shown the PMP70 signals as white dots in the images below (*Figure 6C for Reviewer #3).* Please note that *Figure 6B for Reviewer #3* has been included in the revised manuscript.

Figure 6 for Reviewer #3

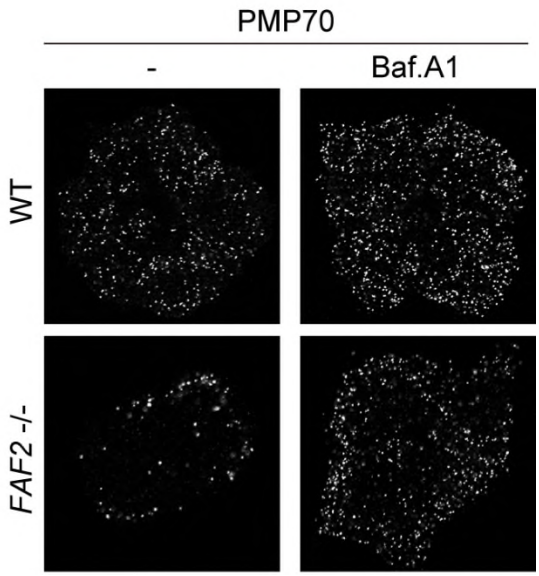
A



B



C



9) Figure 3G: It is unclear why inhibiting mTOR would increase pexophagy in the FAF2 KO and not in the WT cells. Several studies, including one of the co-authors of this manuscript, clearly demonstrated that inhibiting mTOR induces pexophagy. For this reason, one would expect to see a loss of peroxisomes after 24 hrs of Torin1 also in the WT cells. In Figure 3H, there appears to be a decreased catalase staining in the wild-type cells treated with Torin1. The issue is in the method of peroxisome quantification. The authors should quantify peroxisomes per area of the cell to demonstrate changes in peroxisome numbers and not % of cells with less than 20 peroxisomes.

As indicated by the reviewer, our approach for quantifying peroxisomes in the original manuscript may not have been optimized. We thus switch to the Halo-mGFP-SKL assay

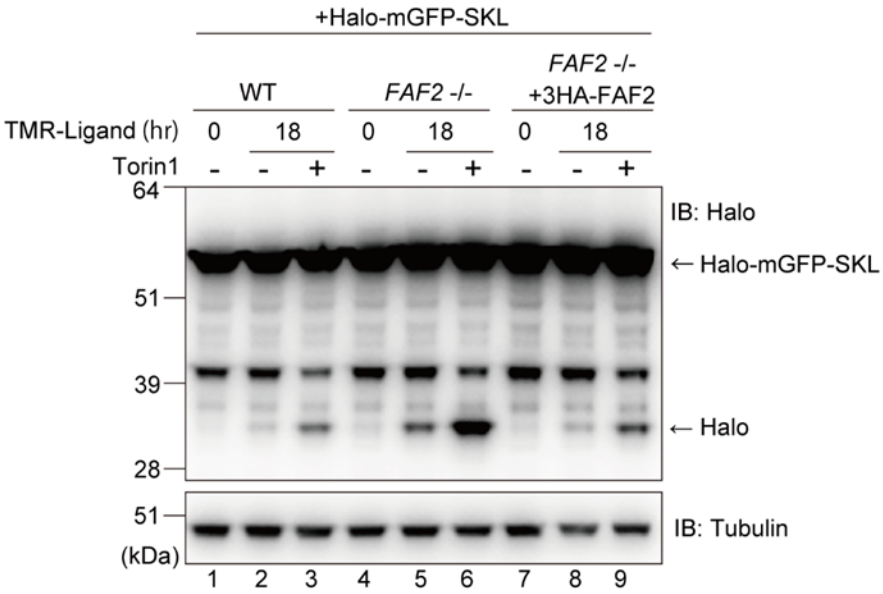
to directly quantify the progression of pexophagy (for detail on the HaloTag processing assay please refer to our reply to comment #1 by Reviewer #1 and comment #6 by Reviewer #3). As shown in *Figure 7A and 7B for Reviewer #3* below, we confirmed that pexophagy was clearly enhanced by Torin1 treatment.

The number of peroxisomes per cell were re-counted by ImageJ and a new scatter plot (*Figure 7C for Reviewer #3)* has been incorporated into the revised manuscript to replace for the original Fig. 3I. Despite using the experimental method suggested by the reviewer #3, we were unable to observe a reduction in the number of peroxisomes in WT cells following mTOR inhibition. However, as described above, the Halo-mGFP-SKL assay clearly demonstrated that pexophagy was accelerated following mTOR inhibition. A potential reason for this discrepancy is provided in our response to comment #11, which raised this specific question.

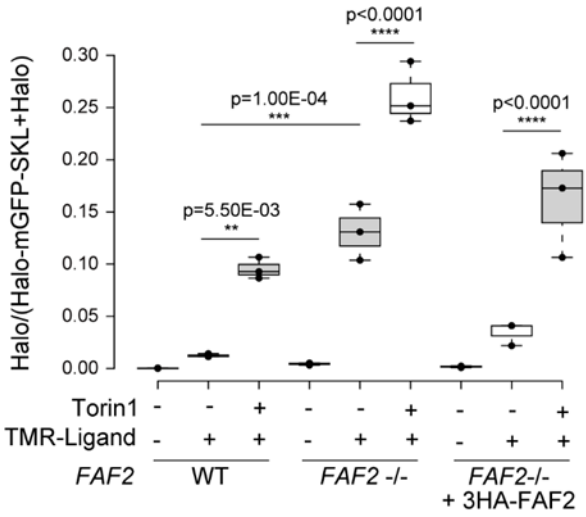
These new experimental results revealed that the data shown in the original Figs. 3G and 3I were no longer valid and we decided to remove the two figures from our manuscript.

Figure 7 for Reviewer #3

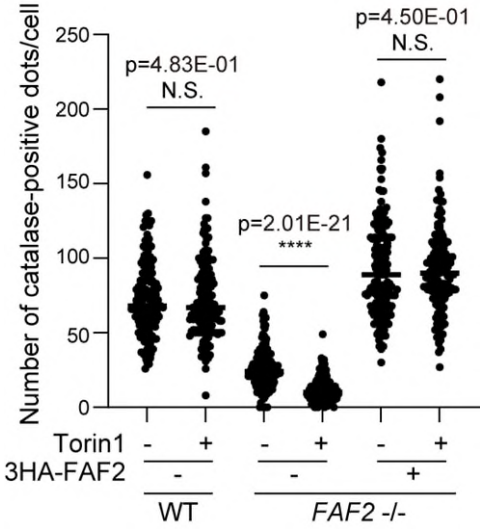
A



B



C



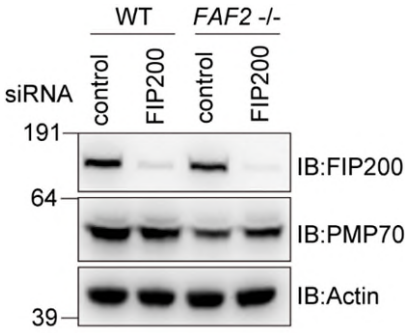
10) To demonstrate that PMP70 is ubiquitinated and degraded by pexophagy, one must inhibit autophagy by either silencing an autophagy factor such as FIP200 or ATG5 for both their ubiquitination and peroxisome quantification assay.

We appreciate the keen insight with this comment. To determine if pexophagy is inhibited in *FAF2* -/- cells in response to autophagy inhibition, we used siRNA to knockdown FIP200, a protein that is essential for autophagosome formation. Immunoblotting confirmed FIP200 knockdown *(Figure 8A for Reviewer #3*). In *FAF2* -/- cells, FIP200 knockdown increased the relative level of PMP70 *(Figure 8A and 8B for Reviewer #3*) with peroxisome abundance comparable to that of WT cells. *(Figure 8C and 8D for*

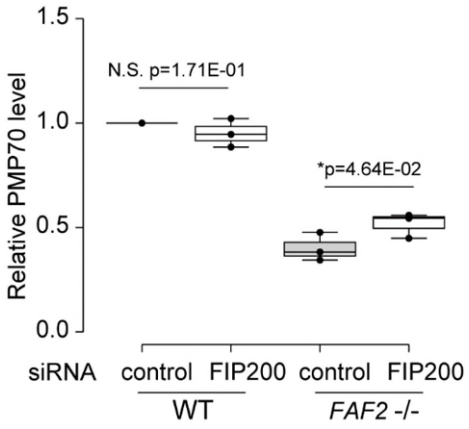
Reviewer #3). Since these data are key for our claim, *Figure 8A-D for Reviewer #3* have been incorporated into the revised manuscript.

Figure 8 for Reviewer #3

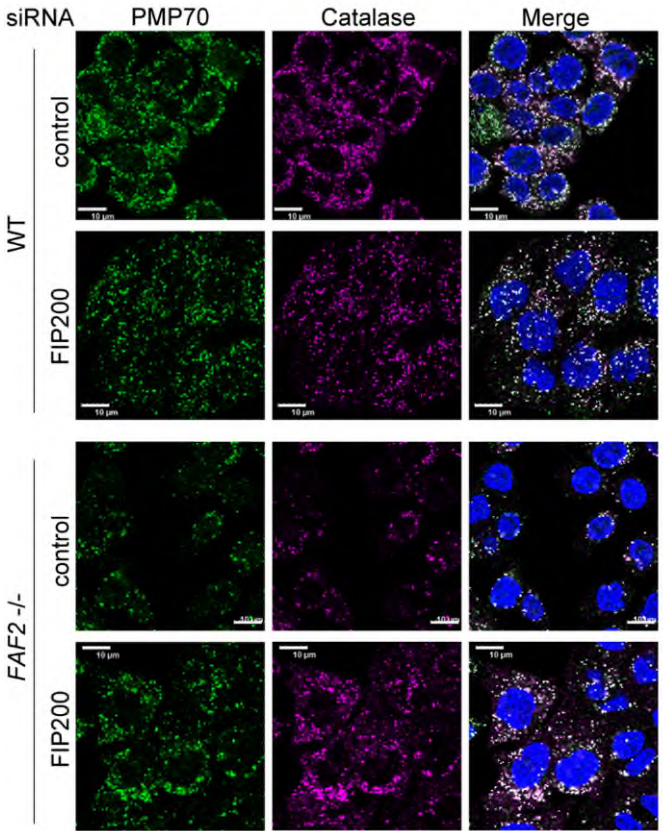
A



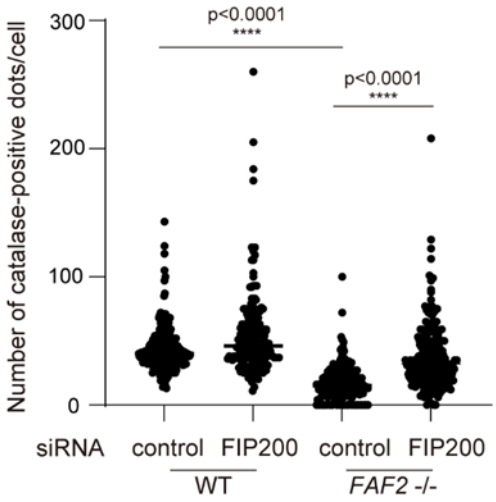
B



C



D



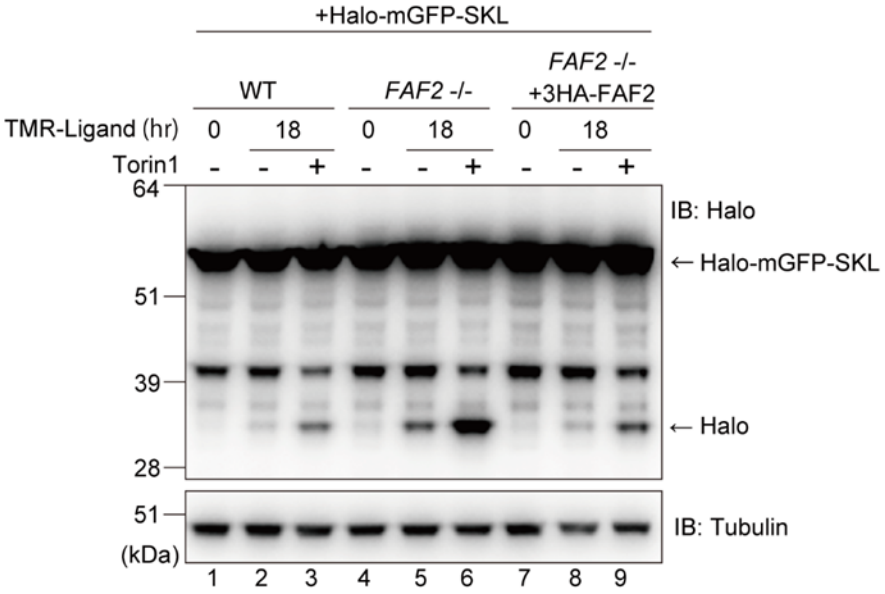
11) Why isn't there a decrease in peroxisomal proteins in the WT cells treated with Torin1? One would expect to see a decrease in peroxisomal protein if

Although the comment is incomplete, we surmise that the reviewer was questioning why

peroxisomal proteins are largely unaffected in WT cells when pexophagy is promoted by TOR inhibition, but are reduced in pexophagy-accelerated *FAF2* -/- cells.

Pexophagy acceleration in *FAF2* -/- cells is caused by ubiquitin-dependent autophagy and thus requires both ubiquitin and autophagy. Torin1 activates autophagy. However, Torin1 alone has no effect on pexophagy in WT cells because ubiquitylation on peroxisomes in WT cells is not changed upon Torin1 treatment. On the other hand, ubiquitin accumulates on peroxisomes in *FAF2* -/- cells. Under this condition, Torin1 treatment activates autophagic degradation of ubiquitylated peroxisomes which had accumulated in *FAF2* -/- cells and this cause intense pexophagy signal. We added the according sentence in the revised manuscript. In addition, we performed HaloTag processing assay. SKL was fused with Halo-mGFP-tag and stably expressed in WT or *FAF2* -/- cells. In the presence of TMR-ligand, the Halo fragment in *FAF2* -/- cells (lane 5, *Figure 9 for Reviewer #3*) was more intense than in WT cells (lane 2). The Halo fragment band was less pronounced in *FAF2* -/- cells stably expressing 3HA-FAF2 (lane 8). Consistent with above model, the Halo band in Torin1-treated WT cells (lane 3) was less pronounced than Torin1-treated *FAF2* -/- cells (lane 6). As a side note, the Halo fragment indicates the number of peroxisomes which had been engulfed by lysosomes. WT cells contain many peroxisomes but *FAF2* -/- cells have only a few peroxisomes. It is thus worth noting that pexophagy efficiency in *FAF2* -/- cells differs from that in WT cells.

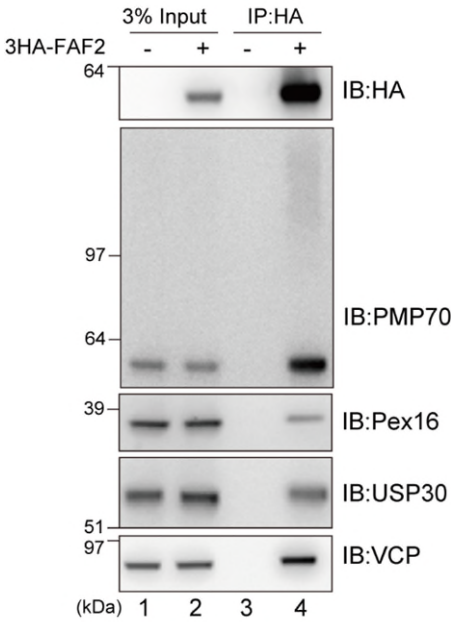
Figure 9 for Reviewer #3



12) Figure 4A: Does FAF2 interact with ubiquitinated or non-ubiquitinated PMP70 and PEX16?

We think that FAF2 recognizes PMP70 as we show that FAF2 interacts with both unmodified PMP70 and ubiquitylated PMP70 (*Figure 10 for Reviewer #3*).
On the other hand, we observed that FAF2 interacts with unmodified PEX16 but not ubiquitylated PEX16. However, we cannot eliminate the possibility that the amount of ubiquitylated PEX16 did not reach a detectable level (Fig. 5a).

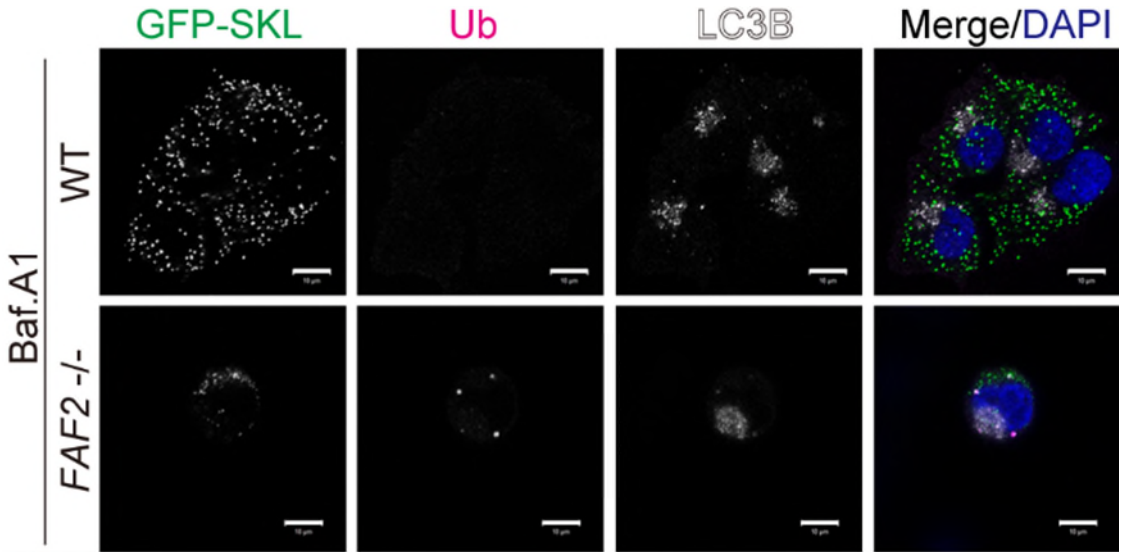
Figure 10 for Reviewer #3



13) To demonstrate an increase in ubiquitinated and LC3B peroxisomes, the authors co-stained their cells with antibodies against ubiquitin and LC3B. They show an increase in cells with ubiquitinated and LC3B-positive structures. There are several issues with this study. The main issue is their quantification of the % of cells with LC3-positive structures. It is not clear what is being counted. Does this mean cells with at least one GFP-SKL positive for LC3B are considered positive cells? This may be appropriate, but what is missing are control experiments. First is a positive control such as amino acid starved cells and Baf.A1 treated cells. Both treatments should show increased LC3B in both WT and knockout cells. The Baf.A1 is especially important as it will demonstrate whether inhibiting lysosomal fusion with autophagosomes results in an increase in ubiquitinated peroxisomes that are not localized in autophagosomes.

In Fig. 5A and 5B, cells in which at least one GFP-SKL signal was also positive for LC3B were counted as a positive cell. We included how to quantify LC3-positive structures in the figure legends. For positive control, we treated WT cells and *FAF2* -/- cells with Baf.A1 as suggested by the reviewer. We confirmed that a lot of large, LC3B-positive structures formed in both WT and *FAF2* -/- cells in response to Baf.A1 treatment (*Figure 11 for Reviewer #3*). Furthermore, after inhibiting lysosomal acidification, ubiquitylated peroxisomes positive for GFP-SKL were observed near LC3B-positive structures.

Figure 11 for Reviewer #3



14) Finally, in Figure 5A, why does the FAF2 knockout cell not show a decrease in GFP-

SKL positive structure?

In Fig. 6a, cells with the limited number of peroxisomes were used to observe the subcellular localization of ubiquitins, LC3B, and peroxisomes.

Because it would be impractical to use peroxisome-null cells to examine colocalization of LC3 and peroxisomes, cells retaining peroxisomes were selected from the heterogenous *FAF2* -/- line for immunocytochemistry. It should be stressed that the number of cells with either reduced or missing peroxisomes was clearly higher in *FAF2* -/- cells than WT cells as shown in Fig. 1. Please also refer to our reply to comment #7 by Reviewer #2.

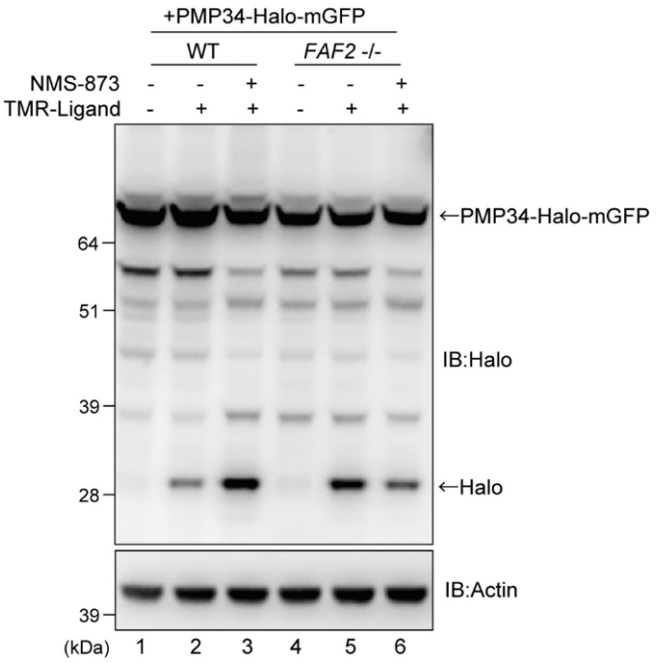
15) Figure 4- the authors demonstrate that p97/VCP interacts with FAF2 but should also provide evidence that the lack of p97 at the peroxisome leads to increased pexophagy.

We appreciate the insightful suggestion. Because Reviewer #1 had similar comments, please refer to our reply to comment #2 by Reviewer #1.

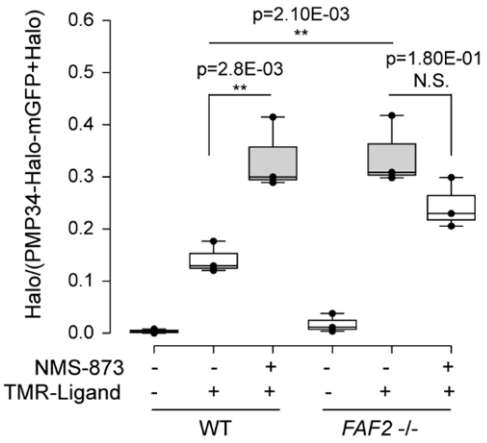
Since our model proposes that FAF2 governs pexophagy in concert with p97/VCP, p97/VCP inhibition would be expected to induce pexophagy. To examine pexophagy flux in cells treated with the p97/VCP inhibitor (NMS-873), we used a HaloTag-based reporter processing assay (PMP34-Halo-mGFP). In *Figure 12A for Reviewer #3*, the Halo fragment band is pronounced in NMS-873 treated WT cells (see *lane 2 vs 3* and *Figure 12B for Reviewer #3).* This implies that the p97/VCP impairment facilitates pexophagy even in WT cells. This is a key result for our proposed model and the associated data have been incorporated into the revised manuscript.

Figure 12 for Reviewer #3

A



B



16) Figure 5A- While LC3B does appear to be colocalized with GFP-SKL puncta, the text implies that all puncta were LC3B positive, which does not appear to be the case based on the images. The text should be changed (lines 269-270)

We have modified the sentence in the revised manuscript.

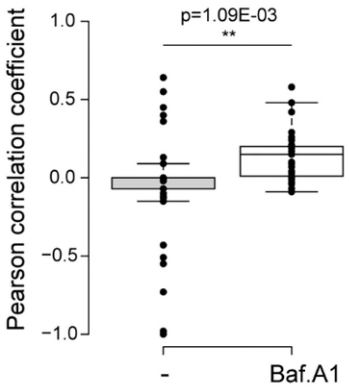
17) Figure 5D- OPTN and p62 stained images were co-stained with PMP70 as the peroxisome marker, but NBR1 stained images were co-stained with PEX14 (as labelled within the figure), although the authors discuss only PMP70 labelled structures. Why

change the peroxisome marker midway through the figure? Additionally, a colocalization coefficient quantification should be included to claim "clear colocalization between peroxisomes and OPTN (line 285)".

In Fig. 6c, a mouse anti-PMP70 antibody could not be used because NBR1 was detected using a mouse anti-NBR1 antibody. For this reason, we chose PEX14 as the peroxisome marker. Our description of NBR1 and PEX14 colocalization has been changed accordingly in the revised manuscript (please see our reply to comment #5 by Reviewer #3).

To show co-localization between PMP70 and OPTN, the Pearson correlation coefficient was measured (*Figure 13 for Reviewer #3*). Although there was a variation in the correlation between PMP70- and OPTN-localization across cells, the correlation for the two proteins was weakly positive in Baf.A1-treated cells.

Figure 13 for Reviewer #3



18) Figure 5E, F- in this specific trial, how does the repression of pexophagy by OPTN depletion compare to pexophagy levels in WT cells? Compared to flow cytometry data from other figures (i.e. Figure 4F), it would appear that pexophagy is still occurring in these depleted cells. Therefore, OPTN is not the only receptor for pexophagy in FAF2-/- cells. A co-depletion with the other receptors should be performed.

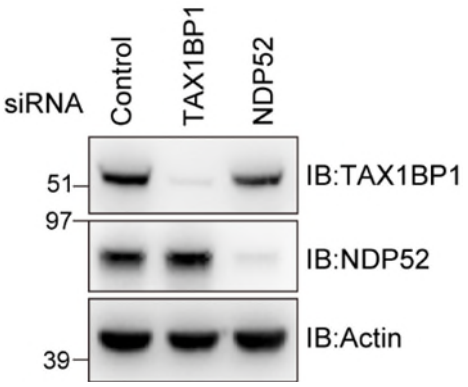
In our original manuscript, TAX1BP1 and NDP52 had not been tested for FACS-based pexophagy flux because they were not recruited to peroxisomes in *FAF2* -/- cells (Fig.

S12b). However, for revised experiment, we did perform FACS to determine if the pexophagy flux was changed in TAX1BP1- or NDP52-knockdown cells. Although we confirmed that TAX1BP1 and NDP52 were depleted (*Figure 14A for Reviewer #3*), their knockdown in *FAF2* -/- cells failed to have a significant impact on the pexophagy flux (*Figure 14B and 14C for Reviewer #3*). This result implies that TAX1BP1 and NDP52 do not mediate pexophagy in FAF2-deficient cells.

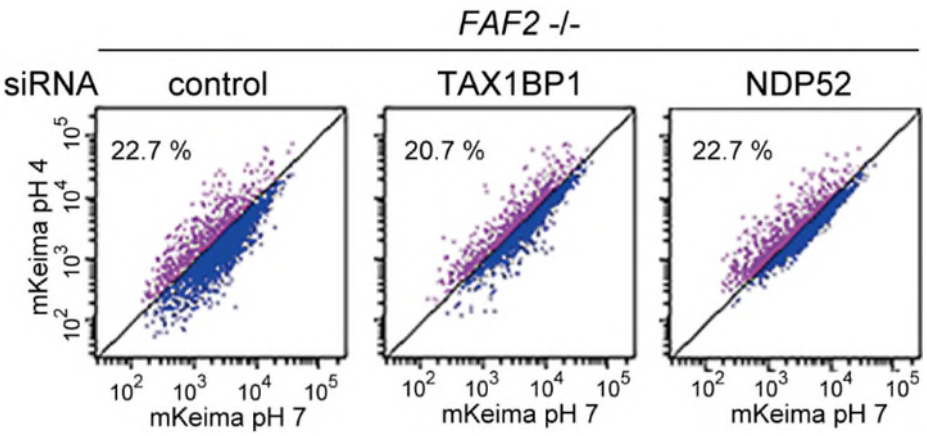
The reason why the pexophagy flux in siOPTN-treated *FAF2* -/- cells have not reduced as much as the siControl WT cells may be due to insufficient depletion of endogenous OPTN by the siRNA.

Figure 14 for Reviewer #3

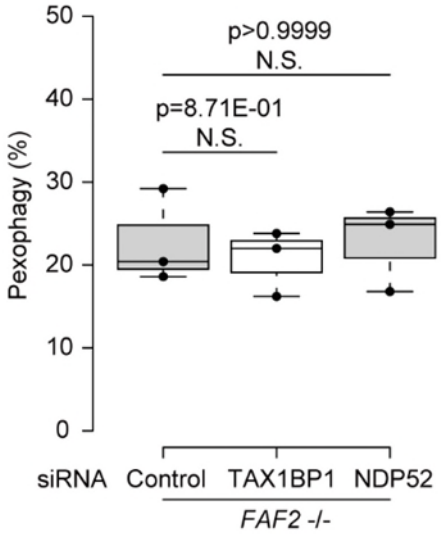
A



B



C



References

1 Yim, W. W., Yamamoto, H. & Mizushima, N. A pulse-chasable reporter processing assay for mammalian autophagic flux with HaloTag. *Elife* **11** (2022). https://doi.org/10.7554/eLife.78923

2 Murakami, K. *et al.* VCP Is an integral component of a novel feedback mechanism that controls intracellular localization of catalase and H2O2 Levels. *PLoS One* **8**, e56012 (2013). https://doi.org/10.1371/journal.pone.0056012

3 Honsho, M. & Fujiki, Y. Topogenesis of peroxisomal membrane protein requires a short, positively charged intervening-loop sequence and flanking hydrophobic segments. study using human membrane protein PMP34. *J Biol Chem* **276**, 9375-9382 (2001). https://doi.org/10.1074/jbc.M003304200

4 Kikuchi, M. *et al.* Proteomic analysis of rat liver peroxisome: presence of peroxisome-specific isozyme of Lon protease. *J Biol Chem* **279**, 421-428 (2004). https://doi.org/10.1074/jbc.M305623200

5 Raman, M. *et al.* Systematic proteomics of the VCP-UBXD adaptor network identifies a role for UBXN10 in regulating ciliogenesis. *Nat Cell Biol* **17**, 1356-1369 (2015). https://doi.org/10.1038/ncb3238

6 Fujisawa, R., Polo Rivera, C. & Labib, K. P. M. Multiple UBX proteins reduce the ubiquitin threshold of the mammalian p97-UFD1-NPL4 unfoldase. *Elife* **11** (2022). https://doi.org/10.7554/eLife.76763

7 Mueller, B., Klemm, E. J., Spooner, E., Claessen, J. H. & Ploegh, H. L. SEL1L nucleates a protein complex required for dislocation of misfolded glycoproteins. *Proc Natl Acad Sci U S A* **105**, 12325-12330 (2008). https://doi.org/10.1073/pnas.0805371105

8 Zheng, J., Cao, Y., Yang, J. & Jiang, H. UBXD8 mediates mitochondria-associated degradation to restrain apoptosis and mitophagy. *EMBO Rep* **23**, e54859 (2022). https://doi.org/10.15252/embr.202254859

9 Ganji, R. *et al.* The p97-UBXD8 complex regulates ER-Mitochondria contact sites by altering membrane lipid saturation and composition. *Nat Commun* **14**, 638 (2023). https://doi.org/10.1038/s41467-023-36298-2

10 Chu, S., Xie, X., Payan, C. & Stochaj, U. Valosin containing protein (VCP): initiator, modifier, and potential drug target for neurodegenerative diseases. *Mol Neurodegener* **18**, 52 (2023). https://doi.org/10.1186/s13024-023-00639-y

11 Li, F. *et al.* Structural insights into the ubiquitin recognition by OPTN (optineurin) and its regulation by TBK1-mediated phosphorylation. *Autophagy* **14**, 66-79 (2018). https://doi.org/10.1080/15548627.2017.1391970

12 Hosoi, K. I. *et al.* The VDAC2-BAK axis regulates peroxisomal membrane permeability. *J Cell Biol* **216**, 709-722 (2017). https://doi.org/10.1083/jcb.201605002

13 Baldwin, H. A. *et al.* VPS13D promotes peroxisome biogenesis. *J Cell Biol* **220** (2021). https://doi.org/10.1083/jcb.202001188

14 Sugiura, A., Mattie, S., Prudent, J. & McBride, H. M. Newly born peroxisomes are a hybrid of mitochondrial and ER-derived pre-peroxisomes. *Nature* **542**, 251-254 (2017). https://doi.org/10.1038/nature21375

15 Lauer, C., Völkl, A., Riedl, S., Fahimi, H. D. & Beier, K. Impairment of peroxisomal biogenesis in human colon carcinoma. *Carcinogenesis* **20**, 985-989 (1999). https://doi.org/10.1093/carcin/20.6.985

Responses to Reviewer comments

Sep. 27th, 2024

MS #: NCOMMS-23-28541A

Title: AAA+ ATPase chaperone p97/VCP^{FAF2} governs basal pexophagy

Authors: *Fumika Koyano, Koji Yamano, Tomoyuki Hoshina, Hidetaka Kosako, Yukio Fujiki, Keiji Tanaka, and *Noriyuki Matsuda

Correspondence to: koyano-fm.biom@tmd.ac.jp and nr-matsuda.biom@tmd.ac.jp

We would like to thank the editor and three reviewers for their comments and the opportunity to address the additional concerns raised. We believe that our manuscript has been completed. Our point-by-point responses to the reviewer comments are listed below.

Referee #1:

The revisions have adequately addressed the concerns raised in my initial review. However, there's one more thing that needs to be discussed. In line 154, the author stated, "Torin1 treatment also enhanced the Halo bands, in particular in FAF2 -/- cells (Supplementary Figs. 4c lane 6 and Fig. 4d)". In fact, the enhancement is also significant in wild-type cells (Fig. S4c lane 3, and Fig. S4e lane 3). It was previously well-established that Torin1 induces the ubiquitination of PMP70 in wild-type cells (PMID 34747980). This point should be added, as it helps to explain why pexophagy is accelerated in FAF2-/- cells.

We appreciate the positive evaluation of our manuscript. To address the reviewer's concern, following sentence has been added to lane 159 in the revised manuscript. "It has been reported that Torin1 induces the ubiquitylation of PMP70 in WT cells (PMID 34747980), explaining why pexophagy is accelerated in FAF2 -/- cells (Supplementary Fig. 4)."

Referee #2:

The authors have addressed most of my comments and improved other parts of the manuscript. I am happy to recommend acceptance for publication.

We greatly appreciate the reviewer's comments.

Referee #3:

This reviewer would like to thank the authors for a very concise description of their response to the reviewers' comments. They did an outstanding job addressing this reviewer's concerns. One minor comment is that the number of cells quantified for some of the assays is not clear. For example, for the number of catalase structures in Figure 1, the authors state a range of cells quantifed. However, it is not clear whether this is the total number of cells quantified over three independent experiments or whether it is the number of cells quantified for each trial. Based on the graph, the number appears to be a total over three trials. If this is the case, the authors should also state the number of cells per trial. The number of cells per trial should also be stated for other similar quantifications.

We thank the reviewer for this positive evaluation of our manuscript. To avoid misinterpretation, we have added the experimental information (including the number of cells/experiments) in the revised manuscript.