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COPI-TRAPPII activates Rab18 and regulates its lipid droplet association

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Transaction Report:

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

1st Editorial Decision 29 June 2016

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are shown below.

As you will see, the referees appreciate your work. However, they also raise numerous concerns and do not think that your conclusions are sufficiently supported by the data provided. Importantly, the proposed GEF activity of TRAPPII towards Rab18 and the proposed regulatory role for LDs needs much further reaching support and insight.
Should you be able to address all concerns of the referees and to add such insight, I would be happy to consider a revised version of the manuscript. I would however need strong support from the referees on the revised version, and it is thus in your own interest to seek publication elsewhere in case you cannot address the issues raised. Please contact me in case you would like to discuss the revision further. I should also add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

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

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the

conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

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

REFEREE REPORTS

Referee #1:

The TRAPP complex is a very well conserved multisubunit complex that has a well established role in membrane traffic through the Golgi apparatus by acting as an exchange factor (GEF) for the small GTPase Rab1. However there are at least two different TRAPP complexes with different subunit compositions and it is at present unclear what roles these serve.

This paper examines the role of TRAPPII which in mammalian cells has two specific subunits called TRAPPC9 and TRAPPC10 that are not present in the other major version of TRAPP (TRAPPIII). The authors find that TRAPPII subunits co-ip a different Rab, Rab18, and go one to show that TRAPPII acts as a GEF for both Rab1 and Rab18. Rab18 already has a well described GEF called Rab3GAP, but the authors produce evidence that TRAPPII activates a sub-population of Rab18 that is present on lipid droplets which form when cells are fed with lipid.

Overall there is a lot of recent interest in lipid droplet biogenesis and there is some evidence that Rab18 has a role in this process. Thus a specific GEF for activating Rab18 only on lipid droplets would be of considerable interest.
However, there are several aspects of this work that need to be strengthened for the conclusions to be reliable. These are described below, along with some more minor issues concerning the text.

a) Figure 1. The authors use co-ip of Rab18 in the presence of EDTA to make a case for TRAPPII activating this Rab. This experiment needs a positive control (Rab1) and a negative control (an irrelevant Rab). Also the authors should probe the GST-Rab18 pulldown in Figure 1C with antibodies to TRAPPIII subunits.

b) Figure 2. The acceleration of nucleotide exchange on Rab18 by TRAPPII is quite small compared to that with Rab1 (Figure 2E and 2F). Have the authors checked that TRAPPII does not associate with Rab3GAP? Have they tried optimising the exchange reaction to improve it, or simply added more TRAPPII? A more minor point is that the authors should state the n numbers for all experiments and not just those for panels H and I.

c) Figure 2. Why does TRAPPIII not act on Rab1?

d) Figure 3. The siRNA knockdown of TRAPPC9 is not very efficient (c 50%) and it is surprising that the authors not only see a phenotype, but also that the phenotype is stronger than the TRAPPC9 gene deletion. Given that the authors have made a CRISPR knockout which is a much less ambiguous approach than siRNA, it would seem best to remove all of the RNAi data and simply show the results based on the CRISPR knockouts.

e) Expanded Figure 3. The authors show a sequencing file for a TRAPPC9 CRISPR mutant. They should state which mutant line this corresponds to, and since it is clearly a mixed sequence file they should state how the two alleles are mutated.

f) Figure 4. This figure uses an antibody to examine the effect of TRAPPII deletion on the distribution of endogenous Rab18 and these are key data for the paper. However two important issues must be addressed. Firstly the authors should validate the commercial antibody that they use and prove that the staining they see goes away when Rab18 is deleted. Secondly they must show the distribution of Rab18 in both wild-type and TRAPPII mutant cells that have not been treated with

oleic acid. Is Rab18 on the Golgi in both cases? This also raises the question of why Rab18 does not show Golgi staining in TRAPPII deleted cells as Rab3GAP should still be present, and this point also needs to be discussed. Finally, the authors repeatedly refer to staining seen around lipid droplets as indicating a lipid droplet localization. How can they exclude the possibility that the labelled proteins are instead on ER that is associated with lipid droplets?

g) Figure 6. The authors examine an interaction between TRAPPC9, Rab18 and coatomer. However this is done with over-expressed tagged proteins, an approach that is prone to artefact, especially for proteins that are normally subunits of larger complexes (and the authors have already stated that TRAPPC9 is unstable without TRAPPC10). Since the authors have antibodies to endogenous TRAPPC9 and Rab18, and there are antibodies that will recognise native coatomer, then these co-ip experiments should be done with these antibodies to see if the endogenous proteins are interacting.

h) Minor points.

i) The authors should explain more clearly the structure of mammalian TRAPPII and TRAPPIII as these are different to the yeast complexes, and they should cite the work of Bassik et al 2013 (PubMed ID 23394947).

ii) Throughout the paper the authors refer to "Expended View Figures" when they mean "Expanded"

iii) Page 4, first paragraph. The authors refer to EV Figure 3 when they mean EV Figure 4.

iv) The legend to EV Figure 7 refers to EV Figure 8 and vice versa.

v) Methods: pmole should be pmol

Referee #2:

The manuscript by Li et al. is a very interesting and thorough study providing evidence that TRAPPII acts as a GEF for Rab18 on lipid droplets (LDs), regulating their morphology and function. The authors shown that compromising TRAPPII or Rab18 function inhibits lipolysis and causes an increase in the size of lipid droplets in cells. These results were obtained using siRNA depletion, CRISPR-Cas9-mediated deletions and cell lines from patients carrying TRAPP subunit mutations. The authors show that both TRAPPII and COPI, also known to regulate lipid droplet homeostasis, are required to recruit Rab18 to LDs. The data in this manuscript are excellent, and the results are new and significant. I have some comments that should be addressed prior to publication, listed below.

Detailed comments

1. Figure 2 F. The kinetics of activation on Rab18 is not typical: compare panel F to the activation of Rab1 in panel E, which shows typical activation with a rapid initial rate, then slowing to a plateau. Why is there a slowing in the rate of activation of Rab18 then an increase after 40 minutes?

2. A recent publication has shown that fibroblasts from patients with a TRAPPC11 subunit mutation, have larger LDs than control cells:
DeRossi et al. 2016. trappc11 is required for protein glycosylation in zebrafish and humans. Mol Biol Cell. 27(8):1220-34.

However, TRAPPC11 is not a subunit of TRAPPII, but rather of TRAPPIII. Li et al. in the current manuscript found that siRNA depletion of another TRAPPIII subunit, TRAPPC8, did not affect lipid droplet size. How to the authors explain this discrepancy?

3. Page 4, 3rd paragraph. The authors state that "These results demonstrated that defective TRAPPII complex caused the same aberrantly large LDs as reduced activity of Rab18, suggesting TRAPPII and Rab18 functioned in the same pathway that regulates LD homeostasis." As far as I can tell, the conclusion that TRAPPII and Rab18 function in the same pathway is based on accumulation of

larger LDs to a similar extent when function of TRAPPII or Rab18 are compromised. This deduction is not valid, and much more data is required to draw such a conclusion. As a first step, analysis of double mutants (or double knockdown) must be carried out to determine whether the phenotype is the same as each single mutant situation, less severe or more severe. However, since the mechanisms determining the size of lipid droplets is not a simple linear pathway, this is only a first step, and other approaches must be used to address the question. A similar remark applies to the statement at the end of the third paragraph, Page 5. Other data than simply the same phenotypes of inactivation must be invoked to suggest function in a similar pathway.

4. Page 5, 3rd paragraph. COPI is not exactly inactivated by brefeldin A, it is released from membranes by treatment with this drug.

5. Page 6, first paragraph of discussion. What is the evidence that Rab18 interacts with NRZ to connect LDs with the ER, contributing to the formation of LD-ER membrane bridges? The results obtained in the Munro lab study, showing that the GTP-locked Rab18-QL interacts with NRZ, are not sufficient to make this conclusion. Since there is no data in this manuscript to support these speculations, NRZ and its possible role in tethering the ER and LDs should be removed from the figure and discussion if no other data is available.

6. In some cases, information is missing from the figure legends, for example
- Figure 2H-I, what temperature were the incubations carried out at?
- Figure 3, Figure 4C, etc., were live or fixed cells used? LDs can fuse during fixation for immunofluorescence, so care must be taken to use exactly the same conditions for control and treatment conditions.

Referee #3:

The manuscript by Li et al. reports a role for TRAPPC9 in controlling the size of lipid droplets (LD) and in controlling the activation of Rab18. The Authors propose that the role of TRAPPC9 in controlling LDs is mediated by its activity as a GEF for Rab18. The observations are potentially interesting, but there are a number of issues that need further clarification.

1. The activity of TRAPPC9 as a GEF for Rab18. The authors report that TRAPPC9 from Hek293 cells co-IPs with components that are common to all of the TRAPP complexes, such as TRAPPC2 and TRAPPC3, and with TRAPPII (but not TRAPPPIII) specific components. This IP material exhibits nucleotide exchange activity towards Rab1 and Rab18, but not Rab2. However, the comparison between Rab1 and Rab18 activation leads to the conclusion that the GEF activity present in the IP is mainly towards Rab1 and very minor for Rab18. This might be due to the presence in the IP of the TRAPPI complex. In addition, the exchange activity measured in the IP of cells transfected with myc-TRAPPC9 (which has not been characterized for TRAPPC6, C10, 12) does not mirror the IP obtained with the anti-TRAPPC9 Ab in non-transfected cells, since the activity towards Rab18 of mycTRAPPC9, as compared to the endogenous TRAPPC9, seems to be faster (measurable activity at 20 min) and more comparable with that towards Rab1. How do the Authors explain this discrepancy?

2. The LD phenotype. The Authors report that the deletion of TRAPPC9 induces an enlargement of the LDs after 24h of oleic acid (OA) loading. Fig. 3 shows the efficiency of the KD of TRAPPC9 in Hela and the LD staining in Hek293. Why? The authors performed a quantitative analysis measuring the average size of the LDs. Since there are no data on the average number of LDs or the number of cells presenting the phenotype it is difficult to judge the actual penetrance and relevance of the imbalance in LD biogenesis.

The authors take the reduced release of NEFA in triacsin treated cells as an indication of decreased lipolysis. However these are conditions that do not stimulate lipolysis but impair the synthesis of FFA and as a consequence the formation of LDs. In fact, it would have been interesting to follow the formation of LDs in IF in TRAPPC9-deleted cells treated with triacsin. Another unaddressed issue is whether the phenotype of larger LDs in TRAPPC9-depleted cells emerges only after very long loading times with oleic acid. This might imply that TRAPPII plays a role only under extreme conditions of fat loading, but not in the regular biogenesis/cycle of LDs.

3. The role of Rab18 in the TRAPPC9-induced phenotype. The authors propose that the role of TRAPPC9 in LD biogenesis is exerted through its ability to activate Rab18. However, the association of Rab18 in the cells analyzed by the authors is extremely variable with a negligible association in COS and HeLa cells (0.3 %) and a low association (11%) in Hek293. The association of Rab18 with LDs is more convincing in HuH7 cells (37%). An explanation for such a striking variability is not provided. An interesting explanation might be that the TRAPPII complex is more active/abundant in HuH7 cells compared to the others. Unfortunately, the authors neither investigate this possibility nor explore the consequences of deleting TRAPPC9 on LDs in these cells.

4. The association of TRAPPII with LDs. Unfortunately, the images shown do not show any convincing association of TRAPPII with LDs. It is hard to agree with the authors in their conclusion that TRAPPC10 (together with COPI) associates with LDs after 8h of OA loading (EV Fig.11). This lack of evident association of TRAPPII with LDs casts further doubt on the proposed mechanism of action of TRAPPII in LD biogenesis that would involve Rab18 activation/recruitment to LDs. Since the association of Rab18 with LDs has been reported to be stimulated under conditions that induce lipolysis, the Authors could explore this condition to assess whether they could get a more convincing signal of TRAPPII recruitment to LDs.

Specific points:
Fig. 4A: The phenotype of larger LDs in this image is not consistent with that shown in similarly treated cells in Fig. 3A.

Fig 4D: Were the cells loaded with OA? I suppose so. The Rab18 pattern of bands in wt is generally different from that of TRAPPII-depleted cells - I am not referring to the absence of Rab18 in the LD fraction but to the presence of multiple bands in wt but not in TRAPPII-depleted cells. It is however surprising that the association of Rab18 with just 11% of LDs (and the very partial redistribution observed in IF in Hek293 cells) results in a total shift of Rab18 to the LDs. Also surprising is the practically complete "depletion" of TRAPPC9 from the LD fractions. What about other TRAPP components? Can the authors see a difference in TRAPPII-specific and non-specific components in their fractionation pattern between steady state and OA loading conditions?

There is a mislabeling in the legends for EV Figures 7 and 8.

1st Revision - authors' response 28 September 2016

Point-by-point response to the reviewers' comments

Referee #1:
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However, there are several aspects of this work that need to be strengthened for the conclusions to be reliable. These are described below, along with some more minor issues concerning the text.

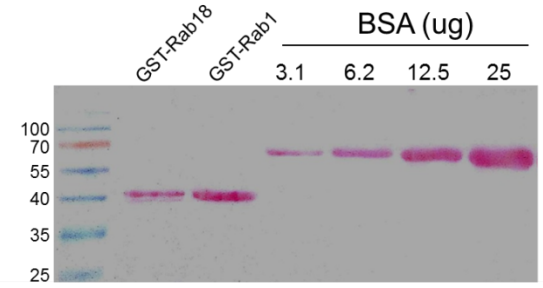
a) Figure 1. The authors use co-ip of Rab18 in the presence of EDTA to make a case for TRAPPII activating this Rab. This experiment needs a positive control (Rab1) and a negative control (an

irrelevant Rab). Also the authors should probe the GST-Rab18 pulldown in Figure 1C with antibodies to TRAPPIII subunits.

Response 1a: Following the reviewer's suggestion, we did the co-IP experiment with positive control (Rab1) and negative control (Rab2) and presented the result in the revised Figure 1C. In this experiment, HA-tagged Rab1, Rab18 and Rab2 were overexpressed and precipitated with anti-HA antibody, various endogenous TRAPP subunits were investigated from the immunoprecipitates by immunoblotting. TRAPPII, but not TRAPPIII specific subunits were present in the Rab1 and Rab18 IP. Second, we had actually blotted TRAPPIII specific subunits in the GST-Rab18 pulldown experiment and found that neither TRAPPC8 nor TRAPPC12 was present. These data has now been included in the figure (Figure 1D in revision).

b) Figure 2. The acceleration of nucleotide exchange on Rab18 by TRAPPII is quite small compared to that with Rab1 (Figure 2E and 2F). Have the authors checked that TRAPPII does not associate with Rab3GAP? Have they tried optimising the exchange reaction to improve it, or simply added more TRAPPII? A more minor point is that the authors should state the n numbers for all experiments and not just those for panels H and I.

Response 1b: We thank the reviewer for this question. After we got this comment from the reviewer, we consulted a number of colleagues who had experience in nucleotide exchange assay for small GTPases on how we could improve the Rab18 nucleotide exchange. Among the advices we had received, one suggested that the amount of Rab18 input was apparently less than that of Rab1. This may happen when the Rab18 protein purified from bacterial overexpression system was not very active (i.e. low specific activity with respect to nucleotide binding). It may happen simply because the estimated protein concentration was inaccurate. As substrate availability was reduced, the reaction did not proceed to plateau phase for a 60 minute incubation. It turns out when we went back and carefully checked the protein concentration of Rab18 and Rab1 and found that the protein concentration of Rab18 was indeed less than Rab1 (Figure below).



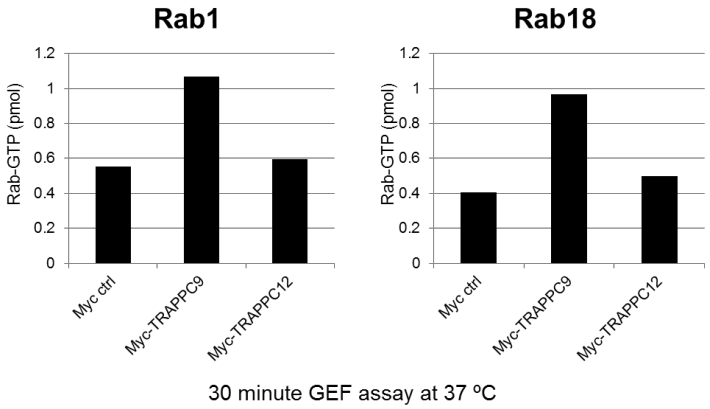
GST-Rab18 and GST-Rab1 were loaded with 10 ug according to protein concentration determined by BCA protein assay.

As a result, the amount of Rab18 used in the assay in Figure 2F was about half the quantity we used for the Rab1 exchange assay. This was a technical mistake in our part but it explains why the Rab18 curve is still in the linear range at 60 minutes time point. We repeated the experiments in Figure 2E-G with the same amount of Rab1, Rab2 and Rab18 in each assay. The experiment was done all at once, with each data point in triplicate. The results are shown in the Figure 2E-G (replacing the old data) in the revised manuscript. As shown in the new Figure 2F, the GTP-g-S-bound Rab18 started to plateau at 40 minutes of incubation. Compared to Rab1, the GEF activity toward Rab18 by TRAPPII is still weaker but is now comparable with Rab1.

The last panel in Figure 2A shows Rab3GAP1 was not associated with TRAPPII IP. This information should relieve the reviewer's concern of Rab3GAP contamination.
All experiments shown in Figure 2 were performed at least 3 times. It was our mistake that the statistical information was misplaced in panel H to I in Figure 2.

c) Figure 2. Why does TRAPPIII not act on Rab1?
Response 1c: At this stage we do not know why TRAPPIII no longer serves as Rab1 GEF. We think the mammalian TRAPPIII-specific subunits either blocked the substrate access or blocked certain

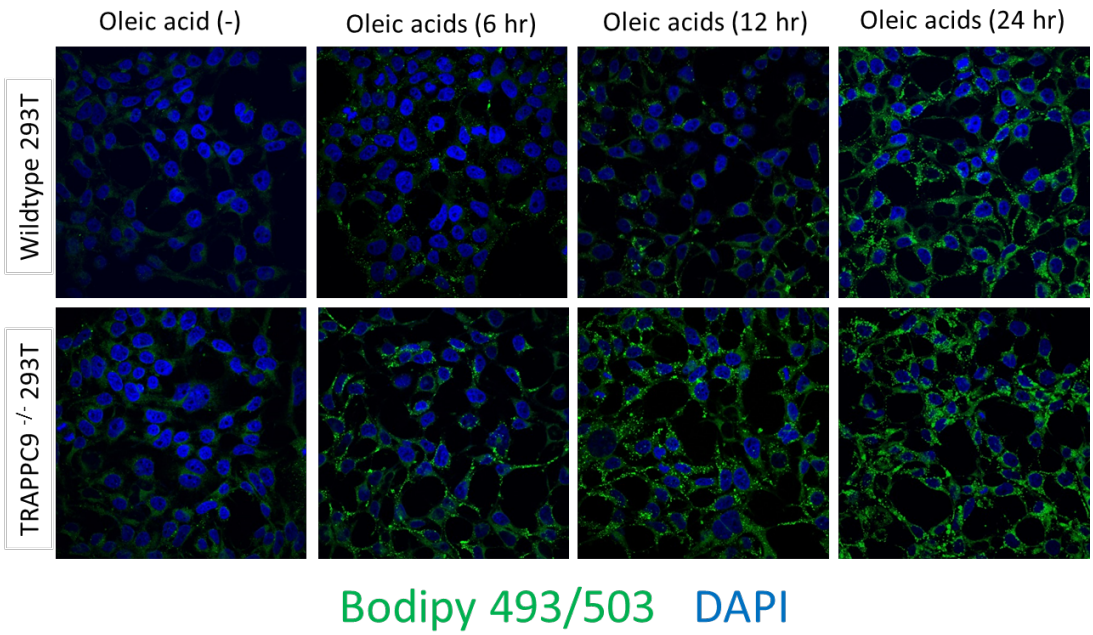
mechanistic aspects of nucleotide exchange, and therefore, TRAPPIII was no longer capable of serving as Rab1 or Rab18 GEF. The first notion seems to be supported by the new co-IP result shown in revised Figure 1C: Neither Rab1 nor Rab18 could pull down TRAPPIII complex. We could not completely eliminate the possibility that the anti-TRAPPC12 antibody used for isolating TRAPPIII blocked the GEF catalytic activity, causing a lack of observable GEF activity by TRAPPIII in our experimental system. However, this possibility seems small because TRAPIII complex isolated from immunoprecipitation of Myc-tagged TRAPPC12 (instead of immunoprecipitation using anti-TRAPPC12 antibody) still contained no Rab1 or Rab18 GEF activity (Figure below).



It is likely that mammalian TRAPPIII may have inherited some aspects of the TRAPP conversion model proposed by Nava Segev. This model suggests that when TRAPPI is converted to TRAPPII, the substrate specificity also switches from Ypt1 to Ypt31/32. This means after the conversion, TRAPPII is the GEF for Ypt31/32 and no longer act on Ypt1. So far, we observe mammalian TRAPPIII is a poor activator of Rab1, just as the TRAPP conversion model predicts. However, we have no evidence which Rab protein can now be activated by TRAPPIII. An obvious candidate is Rab11, the mammalian orthologue of Ypt31/32, but we found that TRAPPIII could not activate this Rab (data not shown).

d) Figure 3. The siRNA knockdown of TRAPPC9 is not very efficient (c 50%) and it is surprising that the authors not only see a phenotype, but also that the phenotype is stronger than the TRAPPC9 gene deletion. Given that the authors have made a CRISPR knockout which is a much less ambiguous approach than siRNA, it would seem best to remove all of the RNAi data and simply show the results based on the CRISPR knockouts.

Response 1d: Like the reviewer, we also noticed stronger phenotype in the TRAPPC9 siRNA depletion than in the Crsipr deletion. At the time when these experiments were first performed, we had concern that the TRAPPC9 deleted cells have been adapted to the loss of TRAPPC9 function during the isolation of the single cell clones. The siRNA depletion experiment is a confirmation of the phenotype observed in Crispr-mediated deletion and human genetic mutation of TRAPPC9. It is possible that some degree of adaptation may have occurred in the TRAPPC9 deletion in view of the weaker phenotype in LD accumulation when compared to siRNA depleted TRAPPC9 cells. This was one of the reasons we went further and deleted TRAPPC10 in TRAPPC9 background. Of note, TRAPPC9 deleted cells have drastically more LD accumulated compared to wildtype cells after 6 hours of oleic acid loading (Figure below). After 24 hours of oleic acid loading, the wildtype cells seemed to catch up a bit with LD accumulation and therefore, the difference became smaller. The weaker phenotype could also be caused by the fact that TRAPPC9 deletion only caused partial defect to the TRAPPII complex.

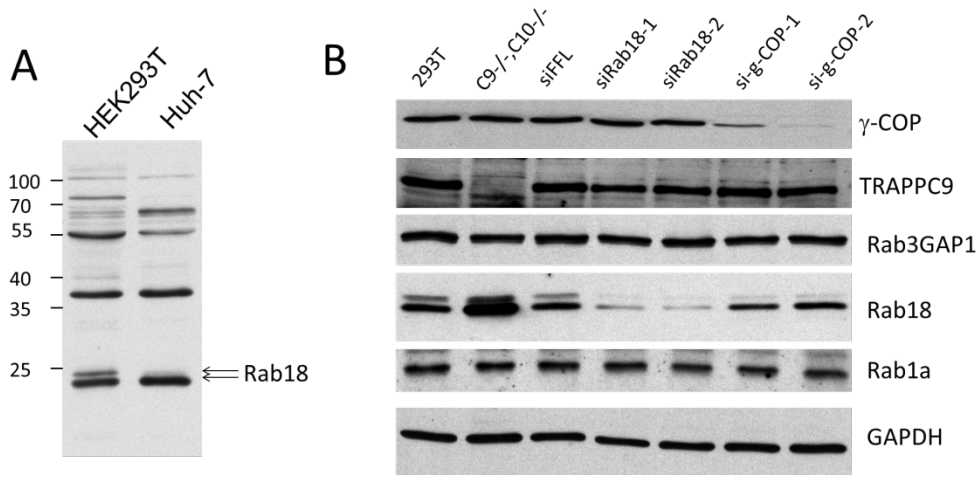


e) Expanded Figure 3. The authors show a sequencing file for a TRAPPC9 CRISPR mutant. They should state which mutant line this corresponds to, and since it is clearly a mixed sequence file they should state how the two alleles are mutated.

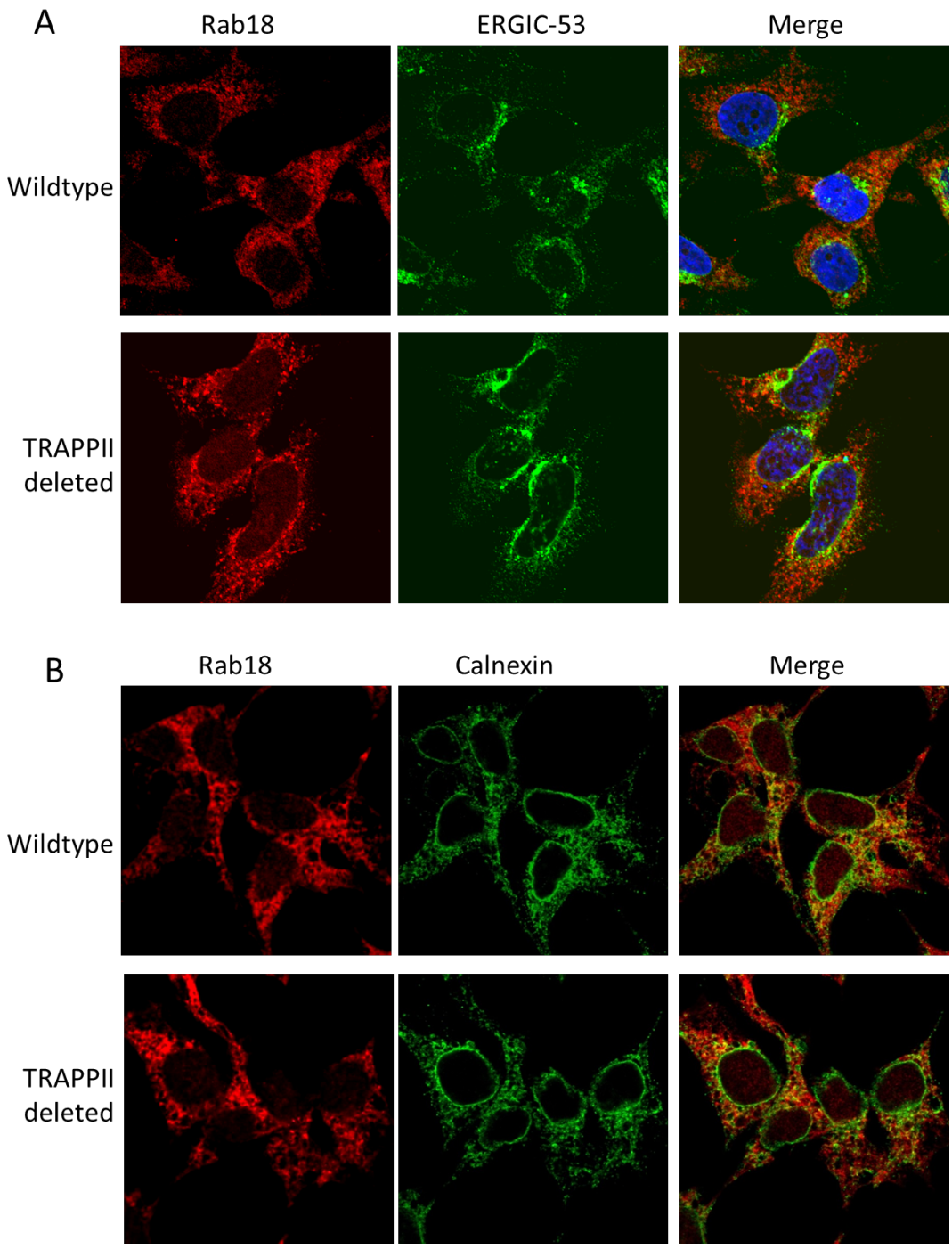
Response 1e: We thank the reviewer for pointing this out. The sequencing was done on genomic PCR product of the intended TRAPPC9 indel. We amplified this region again and subcloned the genomic fragments. 10 such genomic fragments were subjected to sequencing again. We indeed obtained two different indels representing two deleted alleles of TRAPPC9 in this clone. The DNA sequencing and the corresponding indel sites has been included in the revised EV figure 3.

f) Figure 4. This figure uses an antibody to examine the effect of TRAPPII deletion on the distribution of endogenous Rab18 and these are key data for the paper. However two important issues must be addressed. Firstly the authors should validate the commercial antibody that they use and prove that the staining they see goes away when Rab18 is deleted. Secondly they must show the distribution of Rab18 in both wild-type and TRAPPII mutant cells that have not been treated with oleic acid. Is Rab18 on the Golgi in both cases? This also raises the question of why Rab18 does not show Golgi staining in TRAPPII deleted cells as Rab3GAP should still be present, and this point also needs to be discussed. Finally, the authors repeatedly refer to staining seen around lipid droplets as indicating a lipid droplet localization. How can they exclude the possibility that the labelled proteins are instead on ER that is associated with lipid droplets?

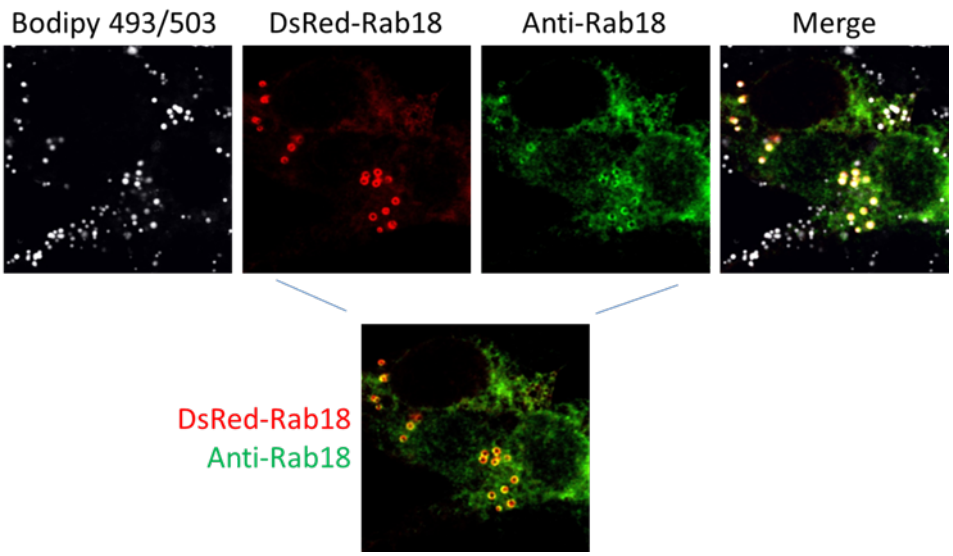
Response 1f: We did substantial amount of characterization of the Rab18 antibody after we purchased it. The Rab18 antibody is fairly specific by several experiments shown below:
First, the Rab18 antibody recognizes endogenous Rab18 as doublet at 23 kD but it does recognize a few non-specific bands by immunoblotting (panel A, figure below). siRNA depletion of Rab18 could reduce the protein expression of Rab18 as shown (panel B, figure below). It should be noted that Rab18 protein expression is elevated in TRAPPII deleted lysate (the lane labelled C9-/-;C10-/-), suggesting a possible compensatory mechanism for the inactivation of TRAPPII.



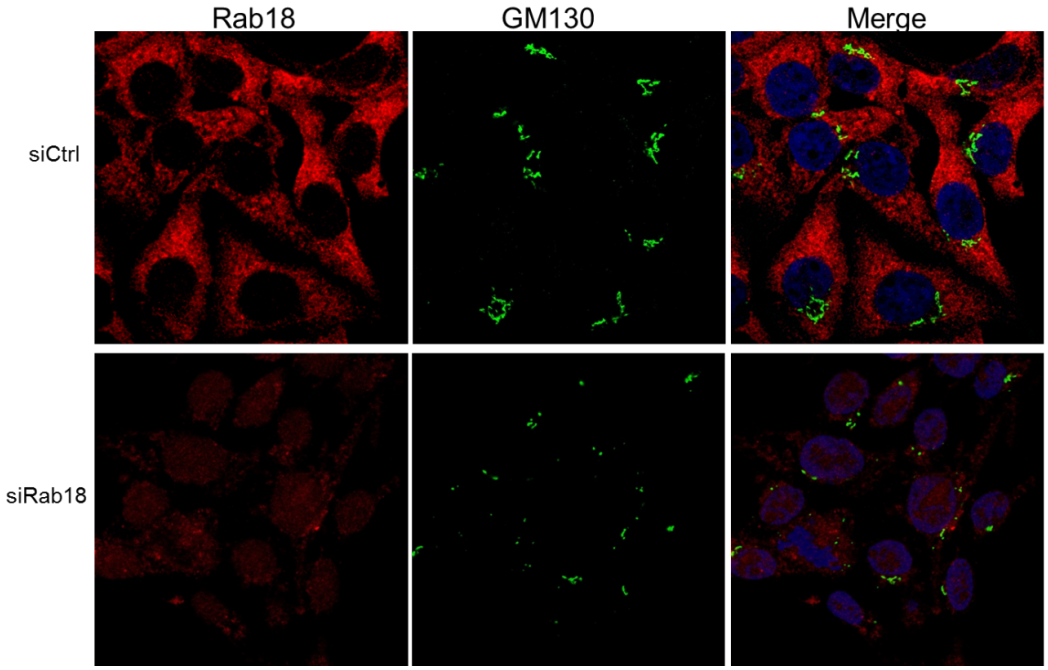
Second, endogenous Rab18 is localized mainly to ER and the Golgi in COS, Hela, and Huh-7 cells in culture condition with no added oleic acid (Expended view figure 7A). In HEK293T cells, Rab18 is in cytosol and on the ER (See figure below). Colocalized signal between Rab18 and ERGIC-53 is very limited (Panel A, figure below). Since ERGIC and cis-Golgi significantly overlap in immunofluorescence staining, we don't think Rab18 is enriched in the Golgi in HEK293T cells. Rab18 signal significantly overlapped with ER marker Calnexin (panel B, figure below). There is no gross change in the subcellular distribution of endogenous Rab18 between wildtype and TRAPPII deleted HEK293T cells. These results are in agreement with Gerondopoulos et al that the activation of Rab18 by Rab3GAP is at ER membrane (Gerondopoulos et al 2014 JCB), even if Rab3GAP has a strong presence on the Golgi (EV Figure 12).



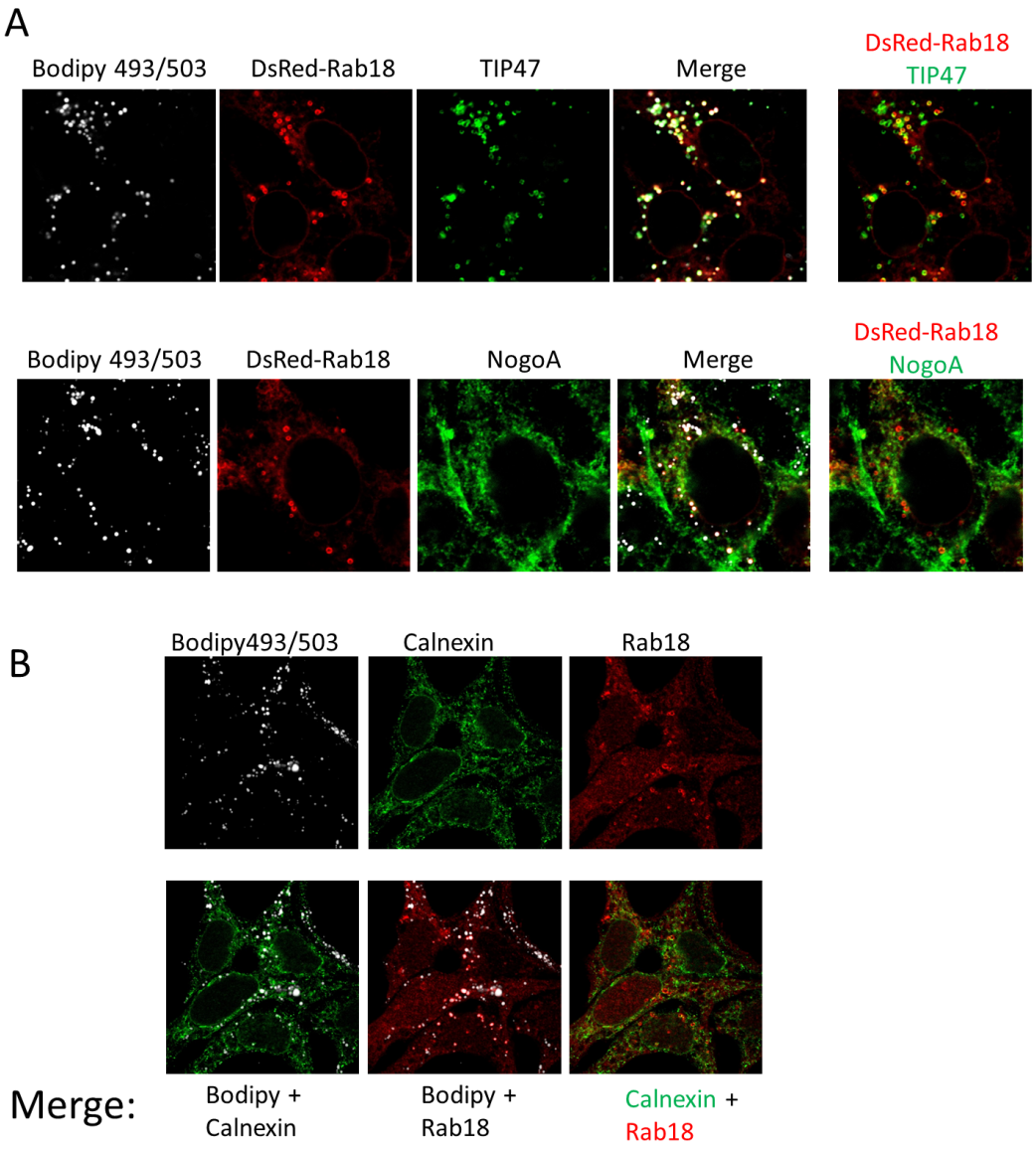
Third, by immunofluorescence, it recognizes specifically the overexpressed DsRed-Rab18 on LD surface as shown in the figure below. We find this property is particularly relevant to the present study concerning the recruitment of Rab18 onto LD surface.



Fourth, when we depleted Rab18 in HEK293T cells, the immunofluorescence signal of Rab18 was drastically reduced (Figure below). This experiment further supports that the immunofluorescence signal of Rab18 in HEK293T cells is specific. Of note, Golgi marker GM130 became highly fragmented in Rab18 depleted cells. This observation has been previously reported (Dejgaard et al 2008, Journal of Cell Science 121, 2768-81).



On the reviewer's concern that ER tubules (or ER associated proteins) surrounding the LD surface and causing artefactual staining of Rab18 on LD surface, we found that the intense DsRed-Rab18 signal on LD surface colocalized with a well-documented LD marker TIP47 (top panel of A, Figure below). However, these LD-bound DsRed-Rab18 signals were not colocalized with a known ER protein, NogoA (bottom panel of A, Figure below). We also co-stained endogenous Rab18 with another ER marker Calnexin in HEK293T cells and found that a fraction of Calnexin signal was indeed in close proximity to LDs and weakly colocalized with Rab18 signals (panel B, figure below), suggesting that ER tubules are in close association with LDs. However, the intensity of the Calnexin signal juxtaposed to LD was nowhere near the extent we observed of Rab18. Therefore, we think the Rab18 signal on LD surface is not a result of bring ER tubule or ER-localized Rab18 to close proximity of LDs. Rather, Rab18 is genuinely recruited to the LD surface by COPI-TRAPPII.



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Response 1g: We performed co-IP using antibodies to isolate native complexes and the result is consistent with the results shown in Figure 6. The native IP result is included in the revised Figure 6. We agree with the reviewer's concern that protein interactions detected in overexpression system are prone to artifact. However, the interaction between g-COP and TRAPPII was discovered by this approach (Yamasaki et al 2009 MBC). Here we confirmed the interaction between TRAPPII and COPI, and then characterized the exact interaction between TRAPPII, COPI and Rab18 based on a similar approach. The conclusion drawn from Figure 6 was further tested by experiments shown in Figures 7-9. From this perspective, the conclusion reached in Figure 6 is less likely prone to overexpression artifact.

h) Minor points.
Response 1h: We thank the reviewer for pointing out these mistakes and we have corrected them as suggested.

i) The authors should explain more clearly the structure of mammalian TRAPPII and TRAPPIII as these are different to the yeast complexes, and they should cite the work of Bassik et al 2013 (PubMed ID 23394947).

Response 1i: We have revised the first paragraph of the Introduction and cited the work of Bassik et al in appropriate places.

ii) Throughout the paper the authors refer to "Expended View Figures" when they mean "Expanded"

iii) Page 4, first paragraph. The authors refer to EV Figure 3 when they mean EV Figure 4.

iv) The legend to EV Figure 7 refers to EV Figure 8 and vice versa.

v) Methods: pmole should be pmol

Response: All have been corrected.

Referee #2:
The manuscript by Li et al. is a very interesting and thorough study providing evidence that TRAPPII acts as a GEF for Rab18 on lipid droplets (LDs), regulating their morphology and function. The authors shown that compromising TRAPPII or Rab18 function inhibits lipolysis and causes an increase in the size of lipid droplets in cells. These results were obtained using siRNA depletion, CRISPR-Cas9-mediated deletions and cell lines from patients carrying TRAPP subunit mutations. The authors show that both TRAPPII and COPI, also known to regulate lipid droplet homeostasis, are required to recruit Rab18 to LDs. The data in this manuscript are excellent, and the results are new and significant. I have some comments that should be addressed prior to publication, listed below.

Detailed comments

1. Figure 2 F. The kinetics of activation on Rab18 is not typical: compare panel F to the activation of Rab1 in panel E, which shows typical activation with a rapid initial rate, then slowing to a plateau. Why is there a slowing in the rate of activation of Rab18 then an increase after 40 minutes?
Response 2.1: Please refer to Response 1b.

2. A recent publication has shown that fibroblasts from patients with a TRAPPC11 subunit mutation, have larger LDs than control cells:
DeRossi et al. 2016. trappc11 is required for protein glycosylation in zebrafish and humans. Mol Biol Cell. 27(8):1220-34.
However, TRAPPC11 is not a subunit of TRAPPII, but rather of TRAPPIII. Li et al. in the current manuscript found that siRNA depletion of another TRAPPIII subunit, TRAPPC8, did not affect lipid droplet size. How to the authors explain this discrepancy?

Response 2.2: We have unpublished data that shows CRISPR deletion of TRAPPC12 does not increase the lipid droplet size. This result is consistent with effect of siRNA depletion of TRAPPC8. As for why TRAPPC11 defect may cause large LD and the discrepancy with our lack of such observation on TRAPPIII depletion/deletion, there are too many possibilities at this stage that we feel the workload will be too much for us to address all. First, it is possible that TRAPPC11 may act alone in regulating lipid droplet size. In fact there is precedence of a TRAPPIII subunit acting as monomer for a specific function: TRAPPC12, reportedly, regulates mitosis as a monomer (Milev et al 2015 JCB). Another possibility is that TRAPPC11 may affect lipid droplet size by affecting autophagy and endosomes/lysosomes. In fact, human skin fibroblasts containing TRAPPC11 mutation has reduced Lamp1 protein expression (Bogershausen et al 2013 Am. J. Hum. Genet.), and regulation of autophagy by TRAPPIII has been well documented. A third possibility could be mutation/deletion of TRAPPC11 fortuitously causes a dominant negative effect on TRAPPII, since TRAPPII and TRAPPIII share a common core (TRAPPI).

In addition to the possibilities listed above, apparently contradictory phenotypes from siRNA depletion of different TRAPPIII have been reported previously. For example, siRNA depletion of TRAPPC8 reduced the number of autophagosomes. However, siRNA depletion of TRAPPC12/TTC15, another TRAPPIII specific subunit, increased the number of autophagosomes (Behrends et al 2010 Nature). This discrepancy has been partially explained with the specific interaction between TRAPPC8 and TBC1D14 in a subsequent study (Lamb et al 2016 EMBO J), and with future advancement in this area of research, we fully expect the discrepancy will be understood and resolved. From the status in autophagy, it is not difficult for us to see inactivating different TRAPPIII subunits may result in different phenotype in lipid droplet homeostasis.

3. Page 4, 3rd paragraph. The authors state that "These results demonstrated that defective TRAPPII complex caused the same aberrantly large LDs as reduced activity of Rab18, suggesting TRAPPII and Rab18 functioned in the same pathway that regulates LD homeostasis." As far as I can tell, the conclusion that TRAPPII and Rab18 function in the same pathway is based on accumulation of larger LDs to a similar extent when function of TRAPPII or Rab18 are compromised. This deduction is not valid, and much more data is required to draw such a conclusion. As a first step, analysis of double mutants (or double knockdown) must be carried out to determine whether the phenotype is the same as each single mutant situation, less severe or more severe. However, since the mechanisms determining the size of lipid droplets is not a simple linear pathway, this is only a first step, and other approaches must be used to address the question. A similar remark applies to the statement at the end of the third paragraph, Page 5. Other data than simply the same phenotypes of inactivation must be invoked to suggest function in a similar pathway.

Response 2.3: We have modified these sentences in way that avoids the problem raised by the reviewer as follows:
"suggesting TRAPPII and Rab18 could be functionally related in the regulation of LD homeostasis"
"suggesting potential relationship between COPI, TRAPPII and Rab18 in LD lipolysis".

4. Page 5, 3rd paragraph. COPI is not exactly inactivated by brefeldin A, it is released from membranes by treatment with this drug.

Response 2.4: This is revised according to the reviewer's suggestion.

5. Page 6, first paragraph of discussion. What is the evidence that Rab18 interacts with NRZ to connect LDs with the ER, contributing to the formation of LD-ER membrane bridges? The results obtained in the Munro lab study, showing that the GTP-locked Rab18-QL interacts with NRZ, are not sufficient to make this conclusion. Since there is no data in this manuscript to support these speculations, NRZ and its possible role in tethering the ER and LDs should be removed from the figure and discussion if no other data is available.
Response 2.5: We have removed any reference to the NRZ complex and ER-LD bridge from the discussion and the graphical model (Figure 10 in revised manuscript).

6. In some cases, information is missing from the figure legends, for example
- Figure 2H-I, what temperature were the incubations carried out at?
- Figure 3, Figure 4C, etc., were live or fixed cells used? LDs can fuse during fixation for immunofluorescence, so care must be taken to use exactly the same conditions for control and treatment conditions.

Response 2.6: We really want to thank the reviewer for the attention to details. The incubating temperatures were all 37°C for the data we have presented in this manuscript and this has been corrected in the revision.

Figures 3 and 4C were fixed cells. We performed these experiments with wildtype control and TRAPPII deleted cells (or various indicated siRNA depleted cells) in parallel and with a common stock of Bodipy 493/503 staining solution.

Referee #3:

The manuscript by Li et al. reports a role for TRAPPC9 in controlling the size of lipid droplets (LD) and in controlling the activation of Rab18. The Authors propose that the role of TRAPPC9 in controlling LDs is mediated by its activity as a GEF for Rab18. The observations are potentially interesting, but there are a number of issues that need further clarification.

1. The activity of TRAPPC9 as a GEF for Rab18. The authors report that TRAPPC9 from Hek293 cells co-IPs with components that are common to all of the TRAPP complexes, such as TRAPPC2 and TRAPPC3, and with TRAPPII (but not TRAPPPIII) specific components. This IP material exhibits nucleotide exchange activity towards Rab1 and Rab18, but not Rab2. However, the comparison between Rab1 and Rab18 activation leads to the conclusion that the GEF activity present in the IP is mainly towards Rab1 and very minor for Rab18. This might be due to the presence in the IP of the TRAPPI complex. In addition, the exchange activity measured in the IP of cells transfected with myc-TRAPPC9 (which has not been characterized for TRAPPC6, C10, 12) does not mirror the IP obtained with the anti-TRAPPC9 Ab in non-transfected cells, since the activity towards Rab18 of mycTRAPPC9, as compared to the endogenous TRAPPC9, seems to be faster (measurable activity at 20 min) and more comparable with that towards Rab1. How do the Authors explain this discrepancy?

Response 3.1: Regarding the reviewer's concern that Rab18 represents a weak/minor substrate of TRAPPII, we eventually realized the amount of Rab18 protein used in the assay was about half of that used in the Rab1 exchange. This mistake has been corrected in the revised figure 2E-G. Please also refer to Response 1b for details.
We could detect the presence of endogenous TRAPPC2 and TRAPPC3 in Myc-TRAPPC9 IP (Figure 1B). We did not blot for other TRAPP subunits because it has also been fairly well-established that mammalian TRAPPII contains TRAPPC9 and TRAPPC10, and TRAPPIII contains TRAPPC8 and TRAPPC12 (Bassik et al 2013, Cell, Zong et al 2011, PLoS ONE). Regarding the reviewer's concern that the GEF activity toward Rab18 was stronger with Myc-TRAPPC9 IP as catalyst than with endogenous TRAPPC9 IP as catalyst, we think that this discrepancy may due to higher efficiency of IP by the anti-c-Myc antibody (clone 9E10). In other words, there were more enzymes in the Myc-TRAPPC9 IP to catalyze the nucleotide exchange reaction.

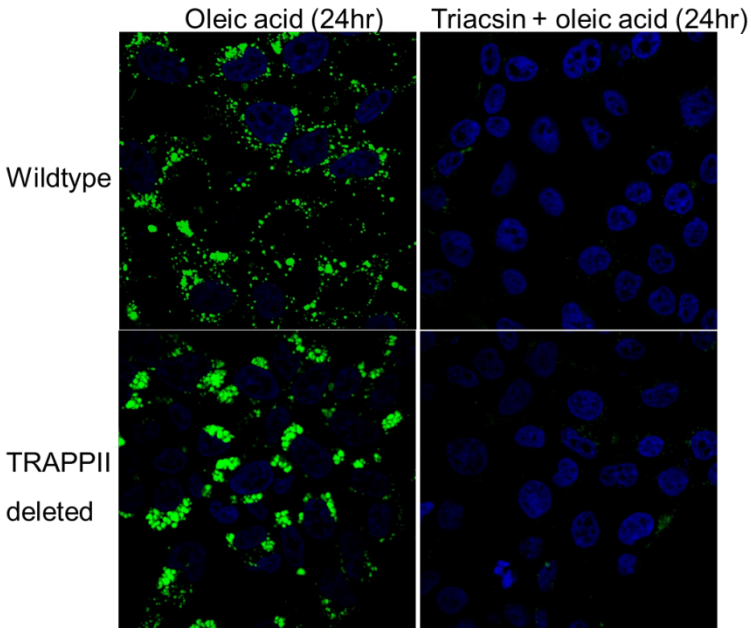
2. The LD phenotype. The Authors report that the deletion of TRAPPC9 induces an enlargement of the LDs after 24h of oleic acid (OA) loading. Fig. 3 shows the efficiency of the KD of TRAPPC9 in Hela and the LD staining in Hek293. Why? The authors performed a quantitative analysis measuring the average size of the LDs. Since there are no data on the average number of LDs or the number of cells presenting the phenotype it is difficult to judge the actual penetrance and relevance of the imbalance in LD biogenesis.

Response 3.2a: We thank the reviewer for catching this mistake for us. The legend for Figure 3 was incorrect. The cells were HEK293T. The same experiment done on Hela cells is shown in Expanded View Figure 1. The mistake was made during the last minute shuffling of the figures while forgetting to update the figure legends accordingly.
The quantification of LD size follows the method described by Ryan et al (Am. J. Hum. Genet. 93(6): 1001-14). Specifically, we used the Analysis particle in the ImageJ software to quantify the size of LDs. LDs from more than 50 cells from five confocal images were quantified for each group.

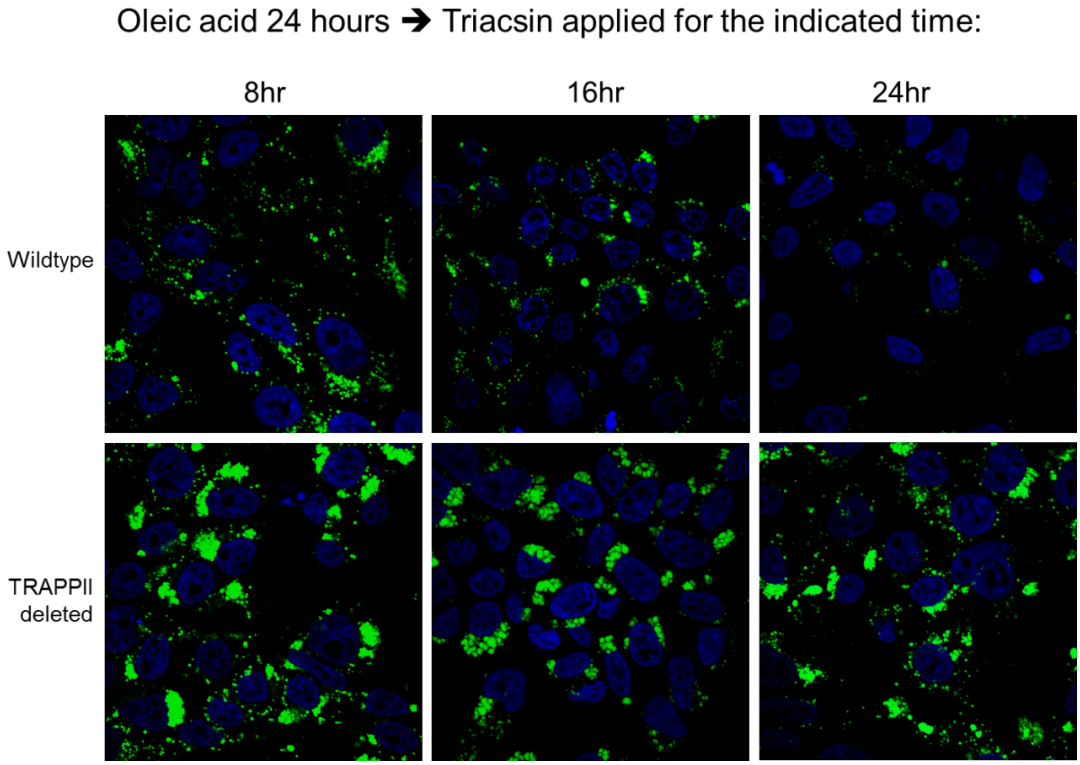
The authors take the reduced release of NEFA in triacsin treated cells as an indication of decreased lipolysis. However these are conditions that do not stimulate lipolysis but impair the synthesis of FFA and as a consequence the formation of LDs. In fact, it would have been interesting to follow the formation of LDs in IF in TRAPPC9-deleted cells treated with triacsin. Another unaddressed issue is whether the phenotype of larger LDs in TRAPPC9-depleted cells emerges only after very long loading times with oleic acid. This might imply that TRAPPII plays a role only under extreme conditions of fat loading, but not in the regular biogenesis/cycle of LDs.

Response 3.2b: We agree with the reviewer's view that triacsin inhibits acyl coA synthetase (Igal et al 1997, Biochemical Journal). In the experiment shown in Figure 5, triacsin was applied after the cells were loaded with radiolabeled oleic acid so that any FFA synthesis was stopped because the

drug prevented re-esterification of fatty acids (Please refer to the NEFA release experiments described in Beller et al 2008, PLoS Biology 6:e292).The release of radiolabeled FFA to the cell culture medium attributed to lipolysis could be measured. From the experiment shown in Figure 5, we observed lipolysis was reduced in TRAPPII deleted cells when compared to wildtype cells. We applied triacsin to cells and followed LD formation as suggested by the reviewer. We did not observe any LD formation in both wildtype and TRAPPII deleted cells, as predicted by the effect of triacsin (Figure below).



We thought the reviewer was asking for an experiment in which triacsin would be applied after the cells were allowed to form LDs, so that lipolysis could be measured indirectly as reduction of LD fluorescence signal by immunofluorescence. We did this experiment and the results are shown in the figure below.

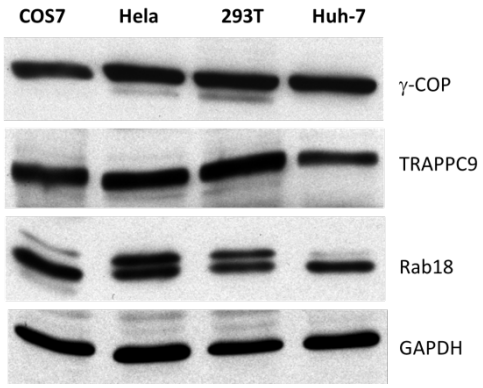


It was obvious that LD signals remained very strong in TRAPPII deleted cells 24 hours after triacsin treatment, and by comparison, most of the LD signals in wildtype HEK293T reduced to background level at this time point. Although this result apparently indicates lipolysis is defective in TRAPPII deleted cells, we are cautious in such interpretation because TRAPPII deleted cells had a lot more LD signals than wildtype cells. Any comparison of fluorescence signals between wildtype and TRAPPII deleted cells can hardly be quantitative in a situation such as this. In Figure 5, however, we measured the release of NEFA as an indication of the rate of lipolysis. This measurement is less likely affected by larger LDs in TRAPPII deleted cells.

On concern that the large LDs in TRAPPII deleted cells are caused by long oleic acid loading time, we know that for HEK293T cells, 24 hour oleic acid incubation is necessary because these cells have poor efficiency to recruit Rab18 onto LD surface. Others have also found 16-24 hour OA incubation is necessary for experiments involved in detecting Rab18 on LD (Ozeki et al 2005, JCS 118:2601-11 and Marin et al 2005 JBC 280: 42325-35). While we cannot exclude the possibility that TRAPPII may play a role only under extreme conditions of fat loading, we detected increase LD labelling in TRAPPC9 deleted cells (compared with wildtype cells) with only 6 hours of oleic acid incubation. This result favors a direct role TRAPPII as a regulator of LD homeostasis (refer to the figure in Response 1d).

3. The role of Rab18 in the TRAPPC9-induced phenotype. The authors propose that the role of TRAPPC9 in LD biogenesis is exerted through its ability to activate Rab18. However, the association of Rab18 in the cells analyzed by the authors is extremely variable with a negligible association in COS and HeLa cells (0.3 %) and a low association (11%) in Hek293. The association of Rab18 with LDs is more convincing in HuH7 cells (37%). An explanation for such a striking variability is not provided. An interesting explanation might be that the TRAPPII complex is more active/abundant in HuH7 cells compared to the others. Unfortunately, the authors neither investigate this possibility nor explore the consequences of deleting TRAPPC9 on LDs in these cells.

Response 3.3: We commented in the discussion on the variability of LD-association of Rab18 among the cell lines we tested. The following sentences are directly copied from the discussion section: "The efficiency of Rab18 association with LDs varied among the cell lines we investigated. Since TRAPPII and COPI are functional in these cell lines and the protein expression levels for TRAPPII and COPI subunits were very similar (data not shown), we think factor(s) other than the components of the COPI-TRAPPII-Rab18 pathway must also regulate the ability of Rab18 to be recruited to LDs. Such factor(s) may be very limiting or missing in COS cells, making regulation of ER morphology by Rab18 a prominent function in this cell line (Gerondopoulos et al, 2014a)." We thought about this problem alone the same line as the reviewer and also suspected varying level of TRAPPII or COPI in these cell lines might explain the variability among cell lines but this did not seem to be the case as we detected no drastic difference in the protein levels of γ-COP, TRAPPC9 or Rab18 in the lysates of these cell lines (Figure below).



Variability of LD-association of Rab18 has been an outstanding problem in this research field and this phenomenon has led to contradicting conclusions among the investigators. For example, Rab18 has been well documented to be on LD surface in many cell types, but Gerondopoulos and colleagues found that Rab18 was solely on ER tubules and concluded that it had no function in regulating LD homeostasis using COS cells as their experimental platform (Gerondopoulos et al 2014 JCB). Though we did not have good explanation for such variability at present, EV Figure 7

provided very useful information to the researchers of Rab18 and LD cell biology so that they will take into account of cell line variability in future experiments.

4. The association of TRAPPII with LDs. Unfortunately, the images shown do not show any convincing association of TRAPPII with LDs. It is hard to agree with the authors in their conclusion that TRAPPC10 (together with COPI) associates with LDs after 8h of OA loading (EV Fig.11). This lack of evident association of TRAPPII with LDs casts further doubt on the proposed mechanism of action of TRAPPII in LD biogenesis that would involve Rab18 activation/recruitment to LDs. Since the association of Rab18 with LDs has been reported to be stimulated under conditions that induce lipolysis, the Authors could explore this condition to assess whether they could get a more convincing signal of TRAPPII recruitment to LDs.

Response 3.4: We have found an improved staining method for TRAPPC9 since our last submission. In EV Figures 9 and 10 and Figure 8A of the revised manuscript, we found that TRAPPC9 immunofluorescence presented largely cytosolic staining pattern in Huh-7 cells with typical staining protocol (i.e. PFA fixation and triton or digitonin permeabilization). However, if we depleted the cytosolic pool of TRAPPC9 with digitonin prior to fixation, the TRAPPC9 signal enriched on Golgi was revealed in Huh-7 cells grown in growth medium. This method was also used to reveal the ER exit sites staining of another TRAPP subunit (TRAPPC3/mBet3) in Hela cells (Yu et al 2006 JCB). Based on this technique, we were able to show that TRAPPC9 was localized to LD surface most abundantly after serum-starved Huh-7 cells were incubated with oleic acid for 8-12 hours (Figure 8A). After these time points, TRAPPC9 signal gradually returned to the Golgi. Overall, the TRAPPC9 staining in response to oleic acid incubation follows similar kinetics as g-COP. It should be noted that COPI and Rab18 were largely depleted by digitonin permeabilization prior to fixation, and therefore, we could not co-stain TRAPPC9 with these proteins.

Specific points:
Fig. 4A: The phenotype of larger LDs in this image is not consistent with that shown in similarly treated cells in Fig. 3A.

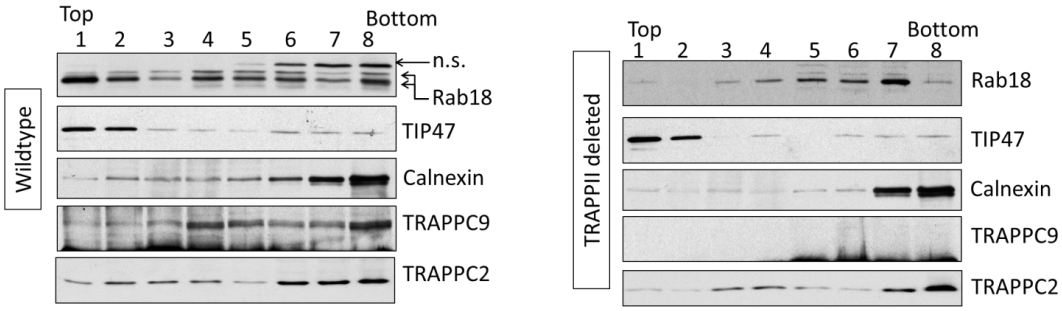
Response: Technically, the images were taken at different confocal settings. It is obvious from the images that the basal level of LD signals (i.e. the siFFL sample in Figure 3) was stronger than image of wildtype 293T in Figure 4A. Therefore, the increased in LD signal in siC9-2 cells in Figure 3A was somewhat "overexposed" and appeared to be clustered. Since we also wanted to repeat this experiment with a different cell line, we tried Hela and the result was shown in EV Figure 1.

Fig 4D: Were the cells loaded with OA? I suppose so. The Rab18 pattern of bands in wt is generally different from that of TRAPPII-depleted cells - I am not referring to the absence of Rab18 in the LD fraction but to the presence of multiple bands in wt but not in TRAPPII-depleted cells. It is however surprising that the association of Rab18 with just 11% of LDs (and the very partial redistribution observed in IF in Hek293 cells) results in a total shift of Rab18 to the LDs. Also surprising is the practically complete "depletion" of TRAPPC9 from the LD fractions. What about other TRAPP components? Can the authors see a difference in TRAPPII-specific and non-specific components in their fractionation pattern between steady state and OA loading conditions?

Response: In the experiment shown in Figure 4D, the TRAPPII deleted cells were HEK293T cells doubly deleted with TRAPPC9 and TRAPPC10 by CRISPR. From the comments, we think the reviewer may have mistaken the cells as siRNA depletion of TRAPPC9. This explains the reviewer's concern of the "depletion" of TRAPPC9 being too complete. The cells were loaded with OA for 24 hours before subjected to sucrose gradient fractionation (Method section of the manuscript). We re-ran the samples and got result that showed a clearer status of Rab18 in each fraction. This has been included in the revised figure. Rab18 shows doublet on an immunoblot, indicated by two arrows in the revised figure 4D (also in figure below). The Rab18 antibody also recognizes a non-specific protein right above the Rab18 doublets, indicated as "n.s." in the figure. We think this band is non-specific because it rarely shows up in our other Rab18 immunoblots. It is present in this experiment because the amount and composition of the proteins in these sucrose gradient fractions are different from a typical cell homogenate for IP experiments.

From the sucrose gradient experiment, it is also apparent that Rab18 signals are associated with other subcellular organelles. However, we need to emphasize that when HEK293T cells were loaded

with oleic acid, a very significant portion of the Rab18 signal was relocated onto LD surface as determined by immunofluorescence and sucrose gradient experiments. It is not necessarily a discrepancy that majority of Rab18 relocated to just 11% of the LD population, if the cells (in this case, HEK293) have the capacity to mobilize only 11% of their LD population to undergo Rab18-mediated lipolysis. For loading oleic acids for 24 hours, only a minor fraction of the TRAPPC9 signals is present in the LD enriched fractions, in agreement with the observation that TRAPPC9 had returned to the Golgi at this time point based on our immunofluorescence study on Huh-7 cells (EV Figure 10 and Figure 8). Per the reviewer's request, we have also added the immunoblots of TRAPPC2, a common subunit of TRAPP complexes, and ER marker Calnexin to the sucrose gradient experiment in the revised figure 4D.



There is a mislabeling in the legends for EV Figures 7 and 8.
Response: This has been corrected.

2nd Editorial Decision 28 October 2016

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are enclosed. As you will see, all three referees now support publication, pending satisfactory minor revision.

I would thus like to invite you to submit a final version of your manuscript, addressing the suggestions made by referee #1 and #2.

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

REFEREE REPORTS

Referee #1:

This is a revised version of a paper reporting the unexpected finding that one form of the TRAPP complex (TRAPPII), is able to activate a pool of Rab18 on lipid droplets in addition to its well established role in activating Rab1. This is potentially a very interesting finding given the widespread interest in lipid droplet biology, but I had a number of concerns about the technical quality of the data and suggested areas where further experiments or controls seemed required for ensuring the conclusion is well supported.

In revising the paper the authors have made an impressive effort to engage with my suggestions and to incorporate new data. In particular the in vitro evidence that TRAPPII can act as a GEF on Rab18 is now much more convincing, and also many of the reagents for the in vivo work are now properly validated. Overall the authors have dealt with all of my concerns satisfactorily, and although some issues remain enigmatic (like why TRAPPIII acts on neither Rab1 or Rab18), there are outside of the scope of the paper and thus better left for future studies.

There is only one minor issue that has arisen from one of the extra experiments that the authors

should address in the text (see below). Otherwise I am happy to recommend publication of this interesting paper in the EMBO Journal.

Minor issue: In figure 6D the authors show an IP of native Rab18 that demonstrates that it brings down coatomer, and that that this interaction is lost if TRAPPC9 or TRAPPC10 are missing. However the data clearly shows that TRAPPC2 remains associated with Rab18 even when these other TRAPP subunits are absent. This should be commented on in the text as it suggests that either TRAPPC9 or TRAPPC10 makes the link between TRAPPII and coatomer.

Referee #2:

The authors have done an excellent job of responding to the reviewers' comments. I am satisfied that they have responded to the points raised, and only have some minor points that need to be addressed in this revised version.

1. Page 3, second paragraph. The statement "COPI complex may positively regulate lipolysis through removing TIP47 (perillipin3) from LD surfaces and in turn promote ATGL migrating to LDs (Beller et al, 2008)" is not correct. COPI depletion (or Arf1 inactivation) does not affect TIP47 binding to LDs (Soni et al., J Cell Sci 2009, 122:1834-41). COPI is required for ADRP/Plin2 binding to LDs, so the sentence could be changed to "The COPI complex may positively regulate lipolysis through inhibition of ADRP (perillipin2) binding to LD surfaces, and in turn promote ATGL migrating to LDs (Soni et al., 2009).

2. Page 3, 3rd paragraph. The following statement is possibly true, but has not been demonstrated: "Together Rab18 and NRZ establish a connection between the LDs and ER and form LD-ER membrane bridges." The authors should rather state that it is possible that a Rab18 - NRZ interaction could mediate interaction between ER and LDs, as proposed by Munro and colleagues (Gillingham et al, 2014).

3. Page 7, 2nd paragraph. The following statement should be corrected, as yeast does not have an orthologue of Rab18: "Unlike yeast TRAPPIII, we did not observe GEF activity of mammalian TRAPPIII toward Rab1 or Rab18." It is better to say: "Unlike yeast TRAPPIII, we did not observe GEF activity of mammalian TRAPPIII toward Rab1. The reason that mammalian TRAPPIII fails to activate either Rab1 or Rab18 is not clear...."

4. Page 7, 4th paragraph. The statement "....or that the observed ER-to-Golgi traffic mediated by TRAPPII in COS cells might not be ubiquitous to all mammalian cells" could be misleading. Another explanation is that there is a redundant pathway with a function similar to that of TRAPPII in some mammalian cells such as HEK293T. This redundant function could be Rab1 activation, and the pathway or component (eg. a second GEF for Rab1) may not exist or be expressed in yeast or in COS cells.

Referee #3:

The Authors have satisfactorily addressed the concerns raised in my previous review.

2nd Revision - authors' response 11 November 2016

Point-by-point response to reviewers’ comments

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ensuring the conclusion is well supported.

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Response: We showed in Figure 6A that TRAPPC9 was likely the subunit that directly interacted with COPI. The TRAPPC2 result in Figure 6D implicates that the core TRAPPI complex may bind to Rab18 the same way as the TRAPPI-Rab1 interaction. After all, it is very likely that TRAPPII acts as GEF for Rab1 and Rab18 by the same mechanism. This point has been added to the text concerning Figure 6D in the revised manuscript.

Referee #2:

The authors have done an excellent job of responding to the reviewers' comments. I am satisfied that they have responded to the points raised, and only have some minor points that need to be addressed in this revised version.

1. Page 3, second paragraph. The statement "COPI complex may positively regulate lipolysis through removing TIP47 (perillipin3) from LD surfaces and in turn promote ATGL migrating to LDs (Beller et al, 2008)" is not correct. COPI depletion (or Arf1 inactivation) does not affect TIP47 binding to LDs (Soni et al., J Cell Sci 2009, 122:1834-41). COPI is required for ADRP/Plin2 binding to LDs, so the sentence could be changed to "The COPI complex may positively regulate lipolysis through inhibition of ADRP (perillipin2) binding to LD surfaces, and in turn promote ATGL migrating to LDs (Soni et al., 2009).
Response: We have modified the sentence according to the reviewer's suggestion.

2. Page 3, 3rd paragraph. The following statement is possibly true, but has not been demonstrated: "Together Rab18 and NRZ establish a connection between the LDs and ER and form LD-ER membrane bridges." The authors should rather state that it is possible that a Rab18 - NRZ interaction could mediate interaction between ER and LDs, as proposed by Munro and colleagues (Gillingham et al, 2014).
Response: We have changed the sentence to reflect a possibility rather than a factual statement as suggested by the reviewer:
"It is possible that a Rab18-NRZ interaction could mediate interaction between ER and LDs."

3. Page 7, 2nd paragraph. The following statement should be corrected, as yeast does not have an orthologue of Rab18: "Unlike yeast TRAPPIII, we did not observe GEF activity of mammalian TRAPPIII toward Rab1 or Rab18." It is better to say: "Unlike yeast TRAPPIII, we did not observe GEF activity of mammalian TRAPPIII toward Rab1. The reason that mammalian TRAPPIII fails to activate either Rab1 or Rab18 is not clear...."
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Response: We have revised this according to the reviewer's suggestion.

Referee #3:

The Authors have satisfactorily addressed the concerns raised in my previous review.

3rd Editorial Decision 16 November 2016

Thank you for submitting your revised manuscript to us. I appreciate the introduced changes and I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Sidney Yu
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2016-94866R1

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

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

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

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ^2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	NA
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	For Figures 3A and 7A, comparison between wildtype and TRAPPII depleted cells were made with blinding of which samples was control and which one were siRNA depleted when the images were taken. Again, for Figure 4A, EV1-2, EV6B, the identities of the geneotype of indicated cell lines were concealed when the confocal images were taken. For Figure EV3D, at least 384 fluorescence dots representing lipid droplets were counted in each cell samples. Biochemical experiments were done three times. The results shown in this manuscript are representative results.
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	NA
Is there an estimate of variation within each group of data?	Yes, data presented in Figure 2 and Figure 5 came from triplicate experiments. Error bars indicate standard deviation.
Is the variance similar between the groups that are being statistically compared?	NA

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

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http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jji.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
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C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	The sources for various antibodies used in this study are described in the methods section.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	The source of cell lines is from ATCC

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

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

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

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	NA
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No