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TFG binds LC3C to regulate ULK1 localization and autophagosome formation

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1st Editorial Decision 2nd Oct 2019

Thank you for submitting your manuscript (EMBOJ-2019-103563) to The EMBO Journal. Please accept my apologies for the delay in getting back to you with our decision. I have now read your study carefully and discussed the work with the other members of the editorial team. In addition, I have sought external advice on it from a good expert in the field. The outcome of these proceedings is that we have decided not to pursue publication of this manuscript.

We appreciate that you identify TRK-fused gene (TFG) as an ULK-1-interacting protein, which regulates ULK1 stability and localization in mammalian cells, independently of its established role as a COPII-vesicle regulatory factor. Furthermore, you report that TFG possesses a LIR motif, through which it binds LC3B and promotes the maturation of autophagosomes.

We recognize that you are the first to show that TFG acts as a molecular scaffold to control ULK-1 subcellular localization during autophagy. However, we find that the mechanism through which TFG regulates ULK-1 localization and autophagosome formation remains unclear. This view was also shared by our external advisor, who states that the work is of good quality but also premature for publication in The EMBO Journal.

Nevertheless, given the clear relevance of your data to the more immediate field, I feel that your study would be a good match with our sister journal EMBO reports that mainly focuses on the novel physiological/functional insights and put less emphasis on molecular mechanisms. I could not directly discuss your manuscript with my colleague Martina Rembold at EMBO reports. However, I would invite to contact her (martina.rembold@embo.org) if you are interested in the transfer option. Alternatively, I would be happy to talk to Martina as soon as she is back in the office and then let you know her thoughts on the work.

I thank you for giving us the opportunity to consider this manuscript and look forward to hearing from you.

We added a number of experiments, finally proving that the TFG interaction with LC3C is mandatory for favouring the formation of the ULK1 complex at the ER, via a TFG LIR domain and upon anchoring ULK1 to the omeagasome via LC3C.

This is an excellent finding in order to unravel autophagosome formation dynamics in our context of studies (see also the novel importance of LC3C in mitophagy, EMBO Rep. 2019 Nov 11:e48317. doi: 10.15252/embr.201948317. [Epub ahead of print]). The 3 of us, myself, Chris Behrends and Nick Ktistakis, are fully convinced that this is an extremely important information and that what we found is key for unravelling upstream autophagy regulation (revised Figure 6). Also, we added a part on human patient fibroblasts representative of one of the TFG-related diseases, clearly pointing to a completely novel autophagy dysfunction in that case (revised Figure 4) and making our model of the highest interest in human medicine.

For these reasons, I dare to ask you to reconsider our paper, that now contains novel key molecular mechanistic insights and more ample scientific perspectives, for peer reviewing.

Can you give a sight at the manuscript and provide us with your valuable and updated opinion?

2nd Editorial Decision 11th Jan 2020

Thank you for submitting your manuscript entitled "TFG (TRK-fused gene) regulates ULK1 to promote autophagosome formation through LC3C binding" [EMBOJ-2019-103563R-Q] to The EMBO Journal. Please accept my apologies for the delay in communicating our decision due to the recent seasonal holidays. The study has now been seen by three referees, whose comments are provided below. In light of these reports, I am afraid that the manuscript is not a sufficiently strong candidate for publication here.

As you can see, while the referees find the study potentially interesting, referee #1 and #2 also stress that the work lacks mechanistic insight into the role of TFG during the early phase of autophagy. In addition, referee #2 states that the biochemical characterization of the LIR-LC3C interaction would need to be strengthened and requests you to analyze it at the endogenous level. Given these opinions from trusted experts in the field and the large amount of new experiments required to substantiate the proposed mechanism of TFG-mediated autophagy, we are regretfully unable to invite a revised version of your manuscript.

However, considering the potential interest of your findings, I would be willing to reconsider your study as a new submission at a later time if the referees' concerns would be fully addressed and their suggestions implemented. Please note that the novelty of the manuscript will be assessed at the time of re-submission.

I would like to mention that EMBO reports remains interested in your study and you can swiftly transfer your manuscript to our sister journal using the link below, if interested in this option. No reformatting is required.

While I am sorry that I cannot communicate more positive news for The EMBO Journal, I hope that the referees' comments will be helpful for you to improve the study.

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Referee #1:

Carinci et al investigate the role of an ULK1 interaction partner termed TFG. The authors find that TFG plays a role in correct ULK1 localisation by preventing the accumulation of ULK1 at the ERGIC. This function of TFG is proposed to play a role in autophagy by promoting omegasome and

autophagosome maturation. A LIR motif within TFG interacts preferentially with LC3C (although mutation of this motif reduces but does not abolish LC3C interaction). TFG binding to LC3C was reported to be important for correct ULK1 distribution. The manuscript would benefit with proof reading by a native English speaker since parts of the manuscript were difficult to understand and interpret. Overall there is the basis of a very interesting study here but in its current form the manuscript is predominantly observational and would benefit with more mechanistic insight. For example, why is puncta turnover of ULK1 important for autophagosome formation? Is it because ULK1 puncta that have initiated phagophore formation must subsequently be recycled through trafficking or are the puncta failing to be correctly trafficked to phagophore initiation sites? Live imaging of ULK1 with trafficking and autophagy initiation markers (e.g Atg13, FIP200) along with WT TFG and disease or LIR mutant TFG would provide some insight into why and how ULK1 puncta are mislocalised by the loss of TFG. The localisation of TFG with ULK1 was only shown under what I assume are fed conditions (Fig 1B, it was not clear in the text). It would be interesting to assess TFG (WT and mutant) and ULK1 dynamics during autophagy induced conditions. This is important since the authors argue that TFG plays a role in correct trafficking of ULK1 despite showing that ULK1 substrates are correctly phosphorylated (which raises the question why is autophagosome formation defective?). The live imaging analyses will go some way to better understand what is going wrong in the absence of TFG.

The manuscript would also benefit with using more than one marker of autophagy turnover. The authors currently focus on LC3 lipidation as a readout, but this does not directly measure impaired autophagy i.e. lysosomal turnover of substrates. The manuscript would therefore be strengthened with the use of p62 blotting and/or Keima tagged autophagy substrates or the GFP-mCherry constructs that many researchers are now utilising to measure autophagic turnover.

Specific comments:
1. Is ULK1 normally turned over by proteasomes or by the endolysosomal system (MG132 or Baf treatment) and why might TFG be required for ULK1 turnover but not other ULK1 complex subunits? One possibility is that TFG traffics ULK1 independently of the ULK1 complex.

2. Figure 1A: Does TFG co-IP with other ULK1 complex subunits or is the ULK1-TFG interaction specific for these proteins and independent of other ULK1 complex subunits? It would be helpful to show whether other Ulk1 complex subunits are pulled down. This will also help in partly addressing point 1 above.

3. Figure 1B: Are these fed conditions (also please clarify for Figure S1A)? It is surprising to see so many ULK1 puncta given that under fed conditions in other experiments within the manuscript there are very few ULK1 puncta. Is it possible that TFG overexpression increases ULK1 puncta? In addition, do the puncta in this figure co-localise with Atg13, FIP200 as well?

4. To ensure that there is no off-target TFG RNAi effect can the authors conduct rescue experiments to show that ULK1 is no longer retained at the ERGIC and that Atg13 foci numbers are brought back down to normal levels?

5. Figure 5: What is the main conclusion from the live imaging (slightly increased Atg13 lifespan, slight delay in LC3B appearance) with respect to TFG function and ULK1 trafficking? Further clarification in the text would be helpful. It is unclear why the live imaging was conducted with Atg13 but not ULK1 which is the focus of TFG related function. In addition, given the focus on LC3C in TFG and ULK1 function, can the authors clarify why Atg13 and LC3B were analysed?

6. Figure 5E: The electron microscopy is low quality and appears to be out of focus. These are unconvincing examples of autophagosomes in control conditions and the 'abnormal vacuoles' in RNAi conditions look like normal multivesicular bodies. Scale bars are missing. Can the authors provide higher quality images?

7. On Page 10 the authors cite a xenophagy paper which reports that LC3C is required for recruiting other Atg8s. However, that work was in the context to xenophagy and cannot be extrapolated for starvation autophagy in which LC3s have been shown to be dispensable (Nguyen et al (2016) J Cell Biol, Vaites et al (2017) Mol Cell Biol, Grunwald et al (2019) Autophagy). The study by Grunwald et al argues that LC3 family members negatively regulate ULK1.
Given that authors argue that TFG-LC3C is important, does depletion of LC3C inhibit autophagy? Do cells lacking LC3C phenocopy the retention of ULK1 at ERGIC sites and decrease in autophagic activity?

Referee #2:

The manuscript by Carinci et al. entitled "TFG (TRK-fused gene) regulates ULK1 to promote autophagosome formation through LC3C binding" reports on a role for TFG in the early steps of autophagosome formation in mammalian cells that is independent of its role in the COPII vesicle pathway. Notably, siRNA-mediated knockdown (KD) of TFG1 stabilizes ULK1 and leads to more of the ULK1-positive puncta being juxtaposed to the ERGIC relative to the control situation. The autophagic flux, measured as LC3B puncta formation and p62 dots on immunofluorescence stainings of endogenous proteins and western blots of LC3B-II (lipidated form) and p62, was also reduced both in full medium and under starvation conditions upon TFG KD. Excellent live cell imaging show a delay in the appearance of LC3B on ATG13-positive punctae and an increased lifespan of ULK1, ATG13 and DFCP1-positive structures upon KD of TFG. TFG has three putative LIR motifs and one of these is implicated in an interaction with LC3C and the TFG-LC3C interaction is suggested to impact strongly on the role of TFG in autophagy and be involved in the TFG-ULK1 interaction.

This is in principle an interesting study as it presents a completely novel role for TFG in autophagy induction and autophagosome formation. The fundamental findings of a negative effect on ULK1 complex and proper autophagosome formation with negative consequences for autophagic flux are clear, and the experimental strategies and data produced to show this give a very solid impression. However, the biochemical part with LIR and LC3C interaction does not hold the same high standard as the imaging and cell biology parts. This part lacks consistency in some of the experimental strategy and needs more controls. Also, some of the conclusions made are not supported by the data shown. Although interesting, this study falls short in providing any mechanistic insight into the role of TFG in the early phases of macroautophagy. The authors themselves rightly state in the Discussion "the mechanisms underlining how TFG-LC3C interaction affects LC3B recruitment and early-autophagy markers remain elusive and additional structural studies on LC3C should be performed in high details to fully understand this process."

Major concerns
1. Almost the entire study is based on TFG overexpression (OE) with no decisive data on endogenous TFG or endogenous LC3C.

2. The authors do not show any IP with endogenous TFG and experiments are most often based on transient transfection and hence overexpression (OE) of TFG and interaction partners like LC3C. This is a clear weakness of the study, as is the lack of use of reconstituted KO cells of TFG to analyze the effects of the LIR mutant.

3. Fig. 1. From the immunoprecipitation (IP) results it is impossible to know if there is a direct interaction between ULK1 and TFG1. It is also important to test ATG13 and FIP200 to see if they also coIP as ATG13 and TFG colocalizes.
It also looks like overexpression (OE) of HA-TFG leads to higher levels of ULK1 similar to siRNA-mediated knockdown of TFG. Is there a dominant negative effect of OE TFG?

4. FIG 3A. I think it is not correct to use Mander's correlation coefficient with dots of TFG on a massive background of ER staining.

5. Fig 3D and E: I do not see a direct colocalization but a juxtaposition. Is what is measured and reported as increased coloc just due to an increase of ULK1 dots as such, so that more ULK1 dots will be juxtaposed to ERGIC-positive structures?

6. Fig 4C: The quantification of LC3 puncta in full medium plus CLQ does not correspond to what is seen on the photomicrograph where there is clearly fewer puncta than in the image of cells treated with CTRL siRNA. Also, the WB data in Fig 4D show less LC3-II in full medium plus CLQ relative to the extracts from cells treated with CTRL siRNA.

7. Fig 4E and F. The authors have not used CLQ in their analyses of p62 and cannot say that p62 is not degraded when an inhibitor of lysosomal degradation is not used.

8. In relation to the WB data in Fig 4H a LC3B puncta formation analysis should be performed on the R106C fibroblasts relative to WT to accompany the WB data on LC3 lipidation.

9. Fig 5E. The EM images do not look very good with poor ultrastructure of mitochondrial structures and dubious assignment of double membranes. The quality of the images shown need to be much improved as this is Epon-embedded sections analyzed by TEM which should yield much higher quality than seen here.

10. Fig 6A. GABARAP, GABARAPL1 and GABARAPL2 need to be included in the GST-pulldown assay. The accumulated data on LIR-ATG8 interactions makes it very unlikely that there is no binding to GABARAP subfamily members. The IP in Fig S6A also lacks a positive control to show that the IP of a GABARAP interactor actually works. Anyway, it is absolutely imperative to include the GABARAPs in the GST-pulldown shown in Fig, 6A.

11. Fig 6C. The LIR3 mutant clearly shows still quite significant binding to LC3C and LC3A (LIR3 mutant does not affect LC3A binding) suggesting that there may be other interactions as well with the ATG8s. The authors should also test LIR2 with the appropriate mutants. The possibility of an interaction not involving canonical LIRs should also be controlled for using LDS mutants.

12. Fig6. E-J. The experiments conducted here with transient transfections and siRNA-mediated knockdown of TFG need to be backed up by experiments where the authors have reconstituted TFG KO cells with WT and LIR3 mutant TFG stably expressed at near endogenous levels. The TFG blot showing the effect of siRNA is missing in Fig. 6E.

13. In Fig. 6I the effect of TFG KD on the endogenous ULK1-GABARAP interaction should be analyzed, not only the effect on OE LC3C. In fact OE may be rather perturbing to the relevant complexes analyzed.

14. Why is there no effect on the ULK1 level of TFG KD in Fig 6I?

15. The bias towards a role for LC3C with TFG is not warranted as long as endogenous LC3C is not implicated experimentally and the GABARAP subfamily proteins are not properly examined for interaction with TFG.

16. Fig S6F: The authors need to knock down GABARAP and LC3B to compare with knock down of LC3C on ULK1 levels.

Minor concerns

17. The use of the term "maturation" is a bit misleading from what is the usual use in the research field. At the end of the first paragraph of the Introduction it is stated:... "LC3 incorporation represents the maturation step of autophagosomes"... and bottom of p4:... "proper distribution of ULK1 and the maturation of autophagosomes"... Heading on p7:
"Loss of TFG impairs autophagosome maturation" and p11 bottom of page: ..."autophagosome maturation during autophagy induction"... to mention the most misleading ones from the usual understanding of autophagosome maturation as culminating with fusion to late endosomes giving amphisomes or fusion with lysosomes giving autolysosomes. The authors should use another term to avoid confusion here as what they are referring to is a defect at some stage after nucleation and initiation of phagophore (isolation membrane) formation but before closure of autophagosomes, perhaps at the transition from initiation to elongation.

18. Fig 2 is basically control experiments with no effect of siRNA-mediated knockdown (KD) of the COP-II components SEC23A and SEC16 on ULK1 puncta numbers and ULK1 protein levels that can be shown as supplemental figures (EV) and not as main figures.

19. Fig. 6I: The label "TFG-IP" must be replaced with "HA-IP".
Fig S2: SEC23A KD has a negative effect on autophagic flux during starvation as read off from LC3B lipidation levels.

Referee #3:

The submitted manuscript concerns the identification of a role of the "TRK-fused gene" protein (TFG) in macroautophagy (autophagy). In previous work, the lab identified TFG as binding to the autophagy protein ULK by proteomics, and this interaction is verified biochemically. Loss of function analysis is performed by gene knockdown and reveal a reduction in overall autophagy flux. As TFG is known to act in the secretory pathway, they investigated whether the COPII secretory pathway is involved, but rule this out. Instead, the authors demonstrate using quantitative and live microscopy accumulation a delay in early autophagosome maturation and resulting accumulation of autophagy cargo. A direct LC3 binding domain (LIR) is identified in TFG, and TFG binds LC3C. They demonstrate further that this domain is functionally important, possibly underlying the delayed

dynamics in autophagosome maturation. Ultrastructural analysis in TFG knockdown cells display abnormal vesicular structures, but how, and if at all this relates to the defect on autophagy is unclear. Primary fibroblasts from a patient suffering from Hereditary Spastic Paraplegia disease with a defined TFG mutation (R106C) also display reduced autophagy flux, suggesting that autophagy defects may underlie neural dysfunction in this patient. Rescue or sufficiency experiments for this mutation was not performed. In sum the manuscript defines a novel role of TFG in autophagy flux not explained by its secretory pathway role, and suggests that this is clinically informative. The manuscript is thus interesting to a wider audience; to the autophagy field, cell biology and clinical field. Overall, the manuscript well written, sound and experiments well performed.
Comments to the authors:
1. Page 8. The TFG mutation (R106C) is believed to underlie the defect in autophagy flux, but no rescue experiment is provided. Presumably as this is a dominant mutation. To strengthen the conclusion that the TFG mutation is responsible, the athuors should express an exogeneous TFG (R106C) in primary fibroblasts or other cell types to show that this mutation is causative of autophagy defects.
2. Figure 5E. Ultrastrucural analysis is described to reveal multivesicular structures, potentially representing the underlying defects in TFG cells in autophagy flux. This link is currently weak. This point should be improved by Correlative Light and Electron Microscopy to show that indeed the observed structures are positive for the described autophagy marker(s).
If ultrastructural data are available from the patient fibroblast line,
a related interesting question is whether these structures is also observed in TFG R106C fibroblasts?
3. I was unable to readily identifly the number of experimental independent repeats, number of measurements and source data underlying all graphs in the figure legends nor the materials and methods or uploaded files. This has to be presented in an easily available form in the manuscript itself as well as in source data format (Excel files or similar). I am therefore unable to judge the quantifications and graphs in this submitted version and my comments are based on the assumption that that experiments are carefully and correctly carried out. I need to see this in a potential revised version of the mansucript.

POINT-BY-POINT REBUTTAL TO THE REFEREES' COMMENTS

Before addressing each and every comment raised by the three expert reviewers, we wish to express our gratitude for their insightful and stimulating criticisms and suggestions, which allowed us to clarify some issues and further support and strengthen our results and the concept that we propose. Indeed, we were asked to perform more diverse experiments, and therefore the revision has been really demanding, especially in this COVID-time. Also, we would like to mention that our author team has been in a good position to postulate and validate the concepts presented in our manuscript, as 1) we were the first to study in details, and by breakthrough time-lapse imaging, the early phases of autophagosome formation at the ER via the omegasomes (Axe *et al.,* J Cell Biol. 2008 Aug 25; 182(4): 685–701. doi: 10.1083/jcb.200803137), 2) we were the first to report the existing link between LC3C, functional ERGIC/ERES, COPII vesicles and autophagosome formation (Stadel *et al.,* 2015 Oct 1;60(1):89-104. doi: 10.1016/j.molcel.2015.09.010); 3) we were among the first to describe the dynamic of ULK1 early regulation and its stability (Di Bartolomeo et al. 2010 Oct 4;191(1):155-68. doi: 10.1083/jcb.201002100; Nazio *et al.,* 2016 Dec 19;215(6):841-856. doi: 10.1083/jcb.201605089). These 'historical notes' are not mentioned here to impress the reviewers, but simply to point out that we do have the broad expertise required to address these mechanistically and biologically linked pathways and concepts.

Referee #1:

Carinci et al investigate the role of an ULK1 interaction partner termed TFG. The authors find that TFG plays a role in correct ULK1 localisation by preventing the accumulation of ULK1 at the ERGIC. This function of TFG is proposed to play a role in autophagy by promoting omegasome and autophagosome maturation. A LIR motif within TFG interacts preferentially with LC3C (although mutation of this motif reduces but does not abolish LC3C interaction). TFG binding to LC3C was reported to be important for correct ULK1 distribution. The manuscript would benefit with proof reading by a native English speaker since parts of the manuscript were difficult to understand and interpret. Overall, there is the basis of a very interesting study here but in its current form the manuscript is predominantly observational and would benefit with more mechanistic insight.

Answer: We are grateful to this Reviewer for the interest in our work and for outlining its relevance. Thanks to his/her comments, we have significantly ameliorated the paper from a mechanistic point of view, by means of a number of approaches, as described below.

For example, why is puncta turnover of ULK1 important for autophagosome formation? Is it because ULK1 puncta that have initiated phagophore formation must subsequently be recycled through trafficking or are the puncta failing to be correctly trafficked to phagophore initiation sites? Live imaging of ULK1 with trafficking and autophagy initiation markers (e.g Atg13, FIP200) along with WT TFG and disease or LIR mutant TFG would provide some insight into why and how ULK1 puncta are mislocalised by the loss of TFG. The localisation of TFG with ULK1 was only shown under what I assume are fed conditions (Fig 1B, it was not clear in the text). It would be interesting to assess TFG (WT and mutant) and ULK1 dynamics during autophagy induced conditions. This is important since the authors argue that TFG plays a role in correct trafficking of ULK1 despite showing that ULK1 substrates are correctly phosphorylated (which raises the question why is autophagosome formation defective?). Live imaging analyses will go some way to better understand what is going wrong in the absence of TFG.

Answer: We are grateful to this Reviewer for this important suggestion. Indeed, we have used live imaging to first analyze the dynamic interaction existing between ATG13 (a *bona fide* marker of the ULK1 complex) and the ERES/ERGIC component SEC16. Interestingly, this interaction is severely impacted during time upon starvation and in TFG-mutant conditions, with ATG13 being more stably associated to the ERES/ERGIC compartments in the time frame of the analysis. Second, we have analyzed the dynamic interaction existing between TFG wt/mutant and ULK1 complex and we found that TFG-LC3C interaction is also crucial for TFG-ULK1 complex association. These results are now shown in Figures 5I-J and EV5H-I and discussed in the related Figure legend and text.

The manuscript would also benefit with using more than one marker of autophagy turnover. The authors currently focus on LC3 lipidation as a readout, but this does not directly measure impaired autophagy i.e. lysosomal turnover of substrates. The manuscript would therefore be strengthened with the use of p62 blotting and/or Keima tagged autophagy substrates or the GFP-mCherry constructs that many researchers are now utilising to measure autophagic turnover.

Answer: We thank the Referee for this important concern. However, we preferred to use a novel LAMP1 lysosomal staining to implement our analysis instead of p62, which turned out in many instances to be less reliable, due to its multiple level of regulation upon stress. Indeed, in order to evaluate whether TFG knockdown had an impact on autophagy flux, we analysed LC3B by three different approaches: First, a significant reduction in the number of LC3B puncta, which measure early LC3BII recruitment to autophagosomes, is visible only upon starvation (+STV/+CLQ), when comparing control with TFG-depleted cells (Figure 3A). Second, WB analyses reveal a significant decrease in LC3BII protein levels after both starvation and TFG depletion, in the presence of CLQ respect to the control (Figure EV3A). Third, the colocalization between LC3B *puncta* and LAMP1, an endosomal and lysosomal marker, show that TFG depletion impacts on autophagosome clearance compared to control cells (Figure 3B). Collectively these results support an impairment of the autophagy process.

Specific comments:
1. Is ULK1 normally turned over by proteasomes or by the endolysosomal system (MG132 or Baf treatment) and why might TFG be required for ULK1 turnover but not other ULK1 complex subunits? One possibility is that TFG traffics ULK1 independently of the ULK1 complex.

Answer: Levels and activity of ULK1 are explicitly controlled during autophagic flux via multiple mechanisms. Yeast Atg1 is transported to and degraded in the vacuole via its direct interaction with autophagosome-associated Atg8 (Kraft *et al.,* 2012), whereas mammalian ULK1 is mainly degraded by the proteasome (Alemu *et al.,* 2012). Different E3 ligases mediate ubiquitination and degradation of ULK1 under different conditions (Nazio *et al.,* 2016; Liu *et al.,* 2016). Of the highest importance, degradation of ULK1, a quite stable protein in steady-state conditions, mostly depends on the need to limit autophagy activation (Nazio *et al.,* 2016; Liu *et al.,* 2016, Allavena *et al.,* 2016). In steady state conditions and in our context, we found that ULK1 degradation may depend in part by the proteasome and in part by the lysosomal system (see below Figure for the Referee, results obtained by Bafilomycin and MG132 treatments, respectively). Further, ULK1 degradation is quite specific and does not imply simultaneous regulation of other complex members stability (Allavena *et al.,* 2016). In line with this, in our experimental set up, downregulation of TFG does not affect ATG13 and FIP200 basal levels (Figure EV1C). The reason of this ULK1 specificity may rely on the importance of the ULK1 kinase as an initial hub for this complex formation (Itakura and Mizushima, 2010), or on the specific need for a proper localization and function of ULK1 to maintain its stability, with both features being regulated by TFG.

Figure for Referee #1.1

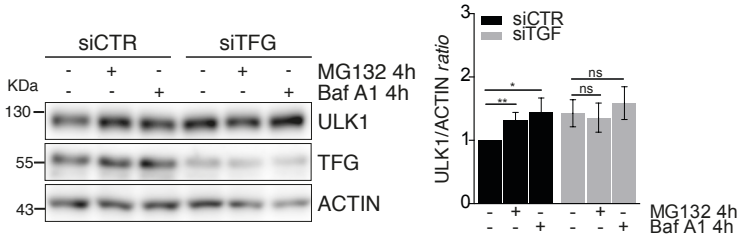

Figure 1.1. Hela cells were transfected with TFG or control siRNA, after 48h cells were treated with 3uM of MG132 or 10nm of Bafilomycin A1 for indicated time period. Untreated cells were used as control condition. ULK1, TFG and ACTIN were analysed by WB. Densitometry analysis of ULK1 protein levels over actin is reported in the right graph. Values are mean ± SEM (n=4). Statistics analysis were performed by unpaired t-test. *,P<0,05; **, P<0,01.

2. Figure 1A: Does TFG co-IP with other ULK1 complex subunits or is the ULK1-TFG interaction specific for these proteins and independent of other ULK1 complex subunits? It would be helpful to show whether

other Ulk1 complex subunits are pulled down. This will also help in partly addressing point 1 above.

Answer: We agree with the Referee, who is absolutely right. As requested, we immunoprecipitated endogenous TFG, and we are able to detect all the endogenous ULK1 complex members (ATG13-FIP200) as shown in the novel Figure 1A.

3. Figure 1B: Are these fed conditions (also please clarify for Figure S1A)? It is surprising to see so many ULK1 puncta given that under fed conditions in other experiments within the manuscript there are very few ULK1 puncta. Is it possible that TFG overexpression increases ULK1 puncta? In addition, do the puncta in this figure co-localise with Atg13, FIP200 as well?

Answer: Admittedly, previous Figure 1B-EV1A could turn out to be confusing. Since TFG overexpression induces the formation of enlarged foci with respect to the endogenous TFG signal (as also reported by Johnson *et al.,* 2015), we hypothesize that this could increase ULK1 *puncta*. To overcome this point, we performed IF to detect ULK1/ATG13 complex and TFG at endogenous levels, as reported in the novel Figures 1B and EV1A. We found a strict association between TFG and ULK1 complex; this point is supported also by co-IP assay as described above.

4. To ensure that there is no off-target TFG RNAi effect can the authors conduct rescue experiments to show that ULK1 is no longer retained at the ERGIC and that Atg13 foci numbers are brought back down to normal levels?

Answer: We agree with the referee. We checked for ULK1-ERGIC colocalization and ATG13 *puncta* number in a rescue experiment. As shown in the novel Figures 2F and EV1F, we are able to restore ULK1-ERGIC colocalization and ATG13 *puncta* numbers by re-expression TFG.

5. Figure 5: What is the main conclusion from the live imaging (slightly increased Atg13 lifespan, slight delay in LC3B appearance) with respect to TFG function and ULK1 trafficking? Further clarification in the text would be helpful. It is unclear why the live imaging was conducted with Atg13 but not ULK1 which is the focus of TFG related function. In addition, given the focus on LC3C in TFG and ULK1 function, can the authors clarify why Atg13 and LC3B were analysed?

Answer: We thank the Referee for this comment that helps us clarify here this key point. As reported also by others (see Karanasios *et al.,* 2013 and Karanasios *et al.,* 2016), since ATG13 is a stable component of the ULK1 complex and is involved exclusively in autophagy, it could be used as a surrogate for monitoring the ULK1 complex. Indeed, as discussed above, we also found that ULK1 and ATG13 are in the same complex also after TFG downregulation and upon starvation conditions. The reasons for using ATG13 to monitor ULK1 complex dynamics are now briefly reported in the manuscript (see lanes 143-145).
About LC3B, we analysed LC3B appearance just as a marker to monitor autophagy progression after TFG downregulation. This was done to get insight into the role of TFG in the phases of autophagosome biogenesis between initiation and elongation, and in full agreement with the literature. This point is now better explained in the manuscript (see lanes 195-197; 217-220).

6. Figure 5E: The electron microscopy is low quality and appears to be out of focus. These are unconvincing examples of autophagosomes in control conditions and the 'abnormal vacuoles' in RNAi conditions look like normal multivesicular bodies. Scale bars are missing. Can the authors provide higher quality images?

Answer: Admittedly, the images have low quality. To improve this point, we repeated EM analyses and novel images are now shown in Figure 4D. As expected, "abnormal structures" (As) are found in TFG-interfered conditions. Moreover, by performing Correlative Light and Electron Microscopy (CLEM) after TFG downregulation and autophagy induction by starvation (as requested by Referee #3), we found that the abnormal structures (As) are positive for ATG13, ATG16L1 and Rab7 (Figure 4E). We could now hypothesize that As are generated as a consequence of defective autophagosome sprouting from omegasomes, together with an impaired formation of multivesicular bodies, as demonstrated by co-staining with ATG16L, Rab7 and ATG13.

7. On Page 10 the authors cite a xenophagy paper which reports that LC3C is required for recruiting other Atg8s. However, that work was in the context to xenophagy and cannot be extrapolated for starvation autophagy in which LC3s have been shown to be dispensable (Nguyen et al (2016) J Cell Biol, Vaites et al (2017) Mol Cell Biol, Grunwald et al (2019) Autophagy). The study by Grunwald et al argues that LC3 family members negatively regulate ULK1. Given that authors argue that TFG-LC3C is important, does depletion of LC3C inhibit autophagy? Do cells lacking LC3C phenocopy the retention of ULK1 at ERGIC sites and decrease in autophagic activity?

Answer: We thank the Reviewer for this nice catch. Although a handful of reports now describe LC3C as a key factor in regulating various selective autophagy processes (Muhlinen *et al.,* 2012; Liu *et al.,* 2017; Le Guerroué *et al.,* 2017; Wetzel *et al.,* 2020), we performed a number of experiments in our cell systems that undoubtedly demonstrate a role for the axis TFG-LC3C in regulating starvation-dependent autophagy. Primed by the Reviewer suggestion, we corroborate this conclusion in the revised version of the manuscripts, by downregulating LC3C (RNAi) in HeLa cells, and analyzing the autophagy flux by WB. As shown in the novel Figure EV5O, LC3C downregulation is able to impair autophagy flux upon autophagy induction by starvation. This said, we cannot rule out that in other cell systems, such in degenerating neurons, the same molecule may play specific roles in more selective autophagy, i.e. aggrephagy or mitophagy. As for the opinion found in Grunwald *et al.* about the possibility that LC3 family members negatively regulate ULK1, the authors there speculate that this phenomenon could be the consequence of a renewed availability of ULK1 (when all LC3s are absent) for alternative mATG8 factors, such as the GABARAPs. Indeed, the condition of triple LC3 depletion on one side is not physiological, while -in the other side, LC3B and LC3C overexpression may saturate ULK1 LIRs and cells may counteract the given modifications, as the authors admittedly recognize in their conclusion. In our opinion, a role for LC3s in ULK1 negative regulation is thus far from being established. Of note, by assessing point (13) from Reviewer #2, we were able to prove that also ULK1-GABARAP interaction is impaired after TFG depletion (see novel Figure EV5P), with this further strengthening our findings about a crucial role for TFG in globally regulating ULK1 during autophagy.
As for the last issue, i.e. LC3C phenocopying ULK1 retention at ERGIC sites, we could not conclude that this is the case (see Figure Referee #1.2); by contrast, we proved that LC3C impairment impacts ULK1 protein levels and autophagy (see Figure 5K-EV5O). Due to our novel finding that -upon TFG depletion, the interaction between ULK1 and GABARAP is also decreased by yet unknown mechanisms (see above), we could speculate that TFG activity on ULK1 may have additional roles on ULK1 besides that mediated by LC3C. However, the investigation of this aspect requires significant additional experiments that we believe go fairly beyond the scope of this paper; we hope the Referee will share with us this view.

Figure for Referee #1.2

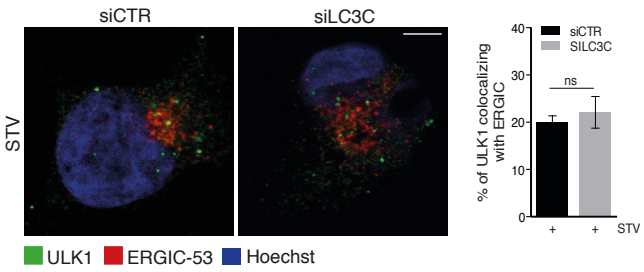

Figure 1.2. HeLa cells were transfected with specific RNAi oligonucleotides (siLC3C) or unrelated oligonucleotides as a negative control (siCTR) and gro wn starved conditions for 1h. Cells were fixed and immunolabelled with ULK1, ERGIC-53 and Hoechst as reported. Analysis of ULK1 localization with ERGIC compartment is reported in the right graph. Co-localization analyses were performed by ImageJ plugin Jacop. Values of Mander's coefficient for ULK1 are expressed as mean ± SEM (n=3). Significance was assigned by unpaired t-test. ns, not significant.

Referee #2:

The manuscript by Carinci et al. entitled "TFG (TRK-fused gene) regulates ULK1 to promote autophagosome formation through LC3C binding" reports on a role for TFG in the early steps of autophagosome formation in mammalian cells that is independent of its role in the COPII vesicle pathway. Notably, siRNA-mediated knockdown (KD) of TFG1 stabilizes ULK1 and leads to more of the ULK1-positive puncta being juxtaposed to the ERGIC relative to the control situation. The autophagic flux, measured as LC3B puncta formation and p62 dots on immunofluorescence stainings of endogenous proteins and western blots of LC3B-II (lipidated form) and p62, was also reduced both in full medium and under starvation conditions upon TFG KD. Excellent live cell imaging show a delay in the appearance of LC3B on ATG13-positive punctae and an increased lifespan of ULK1, ATG13 and DFCP1-positive structures upon KD of TFG. TFG has three putative LIR motifs and one of these is implicated in an interaction with LC3C and the TFG-LC3C interaction is suggested to impact strongly on the role of TFG in autophagy and be involved in the TFG-ULK1 interaction.

This is in principle an interesting study as it presents a completely novel role for TFG in autophagy induction and autophagosome formation. The fundamental findings of a negative effect on ULK1 complex and proper autophagosome formation with negative consequences for autophagic flux are clear, and the experimental strategies and data produced to show this give a very solid impression. However, the biochemical part with LIR and LC3C interaction does not hold the same high standard as the imaging and cell biology parts. This part lacks consistency in some of the experimental strategy and needs more controls. Also, some of the conclusions made are not supported by the data shown. Although interesting, this study falls short in providing any mechanistic insight into the role of TFG in the early phases of macroautophagy. The authors themselves rightly state in the Discussion "the mechanisms underlining how TFG-LC3C interaction affects LC3B recruitment and early-autophagy markers remain elusive and additional structural studies on LC3C should be performed in high details to fully understand this process."

Major concerns

1. Almost the entire study is based on TFG overexpression (OE) with no decisive data on endogenous TFG or endogenous LC3C.

Answer: We share this concern, and performed new experiments (both IP and IF) in endogenous conditions:

1. **IP TFG-ULK1-ATG13-FIP200 (see novel Figure 1A)**
2. **IP TFG-LC3C (see novel Figure EV5J)**
3. **IP ULK1-LC3C (see novel Figure 5L EV5J)**
4. **IP ULK1- GABARAP (see novel Figure EV5P)**
5. **IF TFG-ULK1 and TFG-ATG13 (see novel Figure 1B-EV1A)**

2. The authors do not show any IP with endogenous TFG and experiments are most often based on transient transfection and hence overexpression (OE) of TFG and interaction partners like LC3C. This is a clear weakness of the study,

Answer: As stated above, we have performed IPs in endogenous conditions. As shown in the novel Fig. 1A and EV5J, endogenous TFG interacts with both endogenous ULK1/ATG13/FIP200 complex and LC3C.

as is the lack of use of reconstituted KO cells of TFG to analyze the effects of the LIR mutant.

Answer: About this point, unfortunately we (and others, see McCaughey *et al.,* 2016 and Johnson *et al.,* 2015) are not able to stably downregulate TFG. To perform rescue experiments, we need to use transient downregulation obtained by means of oligonucleotides against 3'UTR-TFG. See answer (12).

3. Fig. 1. From the immunoprecipitation (IP) results it is impossible to know if there is a direct interaction between ULK1 and TFG1. It is also important to test ATG13 and FIP200 to see if they also coIP as ATG13 and TFG colocalizes.

Answer: We thank the Referee for this comment that helps us clarify here this key point. By co-IP experiments, we found that TFG co-immunoprecipitates with all ULK1 complex members (see novel Figure 1A). Moreover, we have also performed IF analyses in endogenous conditions as shown in the novel Figure 1B-EV1A. Endogenous TFG is strictly associated with ULK1 and ATG13 *puncta* under starvation conditions.

It also looks like overexpression (OE) of HA-TFG leads to higher levels of ULK1 similar to siRNA-mediated knockdown of TFG. Is there a dominant negative effect of OE TFG?

Answer: We absolutely agree. Since TFG overexpression induces the formation of enlarged foci with respect to the endogenous TFG signal (as also reported by Johnson *et al.,* 2015), we hypothesize that this could increase ULK1 *puncta*. To overcome this point, we performed IF to detect ULK1/ATG13 complex and TFG at endogenous levels, as reported in novel Figure 1B and EV1A. We found a strict association between TFG and ULK1 complex; this point is supported also by co-IP assay as described above.

4. FIG 3A. I think it is not correct to use Mander's correlation coefficient with dots of TFG on a massive background of ER staining.

Answer: We thank the Referee for this important comment that let us explain how we proceeded. To generate Figure 2A (showing ULK1 dots on ER, and not TFG), although we show the original image, we equally removed the background without affecting the original signal, in order to do a Mander's analysis. Indeed, our finding of the ULK1-ER association is in line with published results, indicating that upon starvation there is an increase of ULK1-ER co-localization (Itakura *et al.,* 2010).

5. Fig 3D and E: I do not see a direct colocalization but a juxtaposition. Is what is measured and reported as increased coloc just due to an increase of ULK1 dots as such, so that more ULK1 dots will be juxtaposed to ERGIC-positive structures?

Answer: We thank the Referee for these nice suggestions, and we agree with him/her. Of course, We also believe that ULK1 puncta are juxtaposed (and not directly colocalized) to ERGIC-positive structure, as reported in the manuscript (see lanes 181-187). Moreover, by using Mander's correlation coefficient we can exclude that the increased juxtaposition between ULK1 and ERIGIC-positive structures is not due to the increased ULK1 puncta numbers in TFG-depleted cells. This point is also supported by no changes in either ULK1-CALNEXIN, ULK1-GOLGIN97 or ULK1-SEC31A in the same conditions (see Figure 2).

6. Fig 4C: The quantification of LC3 puncta in full medium plus CLQ does not correspond to what is seen on the photomicrograph where there is clearly fewer puncta than in the image of cells treated with CTRL siRNA. Also, the WB data in Fig 4D show less LC3-II in full medium plus CLQ relative to the extracts from cells treated with CTRL siRNA.

Answer: We thank the Referee for this remark. Admittedly, the WB image we chose was not representative of the quantification. A more representative image is now shown in the novel Figure EV3A.

7. Fig 4E and F. The authors have not used CLQ in their analyses of p62 and cannot say that p62 is not degraded when an inhibitor of lysosomal degradation is not used.

Answer: We thank the Referee for this important concern. However, we preferred to use a novel LAMP1 lysosomal staining to implement our analysis instead of p62, which turned out in many instances to be less reliable, due to its multiple levels of regulation upon stress. Indeed, in order to

evaluate whether TFG knockdown had an impact on autophagy flux, we analysed LC3B by three different approaches: First, a significant reduction in the number of LC3B puncta, which measure early LC3BII recruitment to autophagosomes (Kabeya *et al.,* 2000) is visible only upon starvation (+STV/+CLQ), when comparing control with TFG-depleted cells (Figure 3A). Second, WB analyses reveal a significant decrease in LC3BII protein levels after both starvation and TFG depletion, in the presence of CLQ, respect to the control (Figure EV3A). Third, the colocalization between LC3B puncta and LAMP1, an endosomal and lysosomal marker, shows that TFG depletion impacts on autophagosome clearance when compared with control cells (Figure 3B). Collectively, these results support an impairment of the autophagy process.

8. In relation to the WB data in Fig 4H a LC3B puncta formation analysis should be performed on the R106C fibroblasts relative to WT to accompany the WB data on LC3 lipidation.

Answer: In full agreement with this Referee's request, we analysed LC3B *puncta* in R106C fibroblasts (see novel Figure EV4D). Our results are fully reproducible and coherent with the hypotheses.

9. Fig 5E. The EM images do not look very good with poor ultrastructure of mitochondrial structures and dubious assignment of double membranes. The quality of the images shown need to be much improved as this is Epon-embedded sections analyzed by TEM which should yield much higher quality than seen here.

Answer: Admittedly, the images were of low quality. In full agreement with this and other Referees' request, we repeated EM analyses and novel images are now shown in Figure EV4D. As expected, "Abnormal structures" (As) are found in TFG-interfered conditions.

10. Fig 6A. GABARAP, GABARAPL1 and GABARAPL2 need to be included in the GST-pulldown assay. The accumulated data on LIR-ATG8 interactions makes it very unlikely that there is no binding to GABARAP subfamily members. The IP in Fig S6A also lacks a positive control to show that the IP of a GABARAP interactor actually works. Anyway, it is absolutely imperative to include the GABARAPs in the GST-pulldown shown in Fig, 6A.

Answer: We thank the referee for this excellent suggestion. We have performed GST-pulldown assays by using GST GABARAP, GABARAPL1 and GABARAPL2 constructs, respectively. As shown in the novel Figure EV5E, TFG interacts with all GABARAP family members, even though these interactions are independent of the LIR3 motif of TFG. We have now included ULK1 as a positive control (a well-known GABARAP family members interactor, see Alemu *et al.* 2012). The table here below summarizes the interactions between the GABARAPs and TFG, detected by the two different approaches.

		Pull-down		IP	
		TFG^{wtLIR}	$TFG^{mutLIR3}$	TFG^{wtLIR}	$TFG^{mutLIR3}$
LC3	A	✓	✓	✗	
	B	✓	✗	✗	
	C	✓	✗	✓	✗
GABARAP	-	✓	✓	✗	
	-L1	✓	✓	✗	
	-L2	✓	✓	✗	

11. Fig 6C. The LIR3 mutant clearly shows still quite significant binding to LC3C and LC3A (LIR3 mutant does not affect LC3A binding) suggesting that there may be other interactions as well with the ATG8s. The authors should also test LIR2 with the appropriate mutants. The possibility of an interaction not involving canonical LIRs should also be controlled for using LDS mutants.

Answer: To address this question, we have checked LIR2 mutant capability to interact with all LC3 family members (LC3A-C) by GST-pulldown assay, whereas only with LC3C by co-IP. As shown in the novel Supp. Fig. EV5F-G, no changes whatsoever are found in this interaction, suggesting that LIR2 is not necessary for TFG-LC3s binding.
This said, as for the use of LDS mutants, we have performed a number of preliminary structural *in silico* studies, by predicting the putative effects of swapping mutations between LC3C and LC3B LDSs (K32Q, F33H, L64V, F69L) (see Figure for Referee #2.1 here below).
In more details, we calculated whether or not mutated LC3C residues (K32, F33, L64, F69) and corresponding residues in LC3B (Q26, H27, V58, L63) would establish the same intermolecular interactions with TFG LIR residues or, in alternative, whether or not specific interactions could exist exclusively between TFG and LC3C or between TFG and LC3B. Among such residues, we detected only contacts of the "proximal" type (indicating two residues in close physical contact within the structure), or "hydrophobic interactions". This analysis does not show any major differences, with the exception of the TFG residue L366 that may form a hydrophobic interaction with the single L64 residue of LC3C, or with the single L63 residue in LC3B. The fact that both LDS in LC3C and LC3B are quite similar and that their swapping does not change their ability to interact with TFG canonical LIRs, despite the observation that only LIR3 seems to be crucial *per se* for this protein-protein interaction, argues against the existence of additional LIR-like domains in the TFG sequence, enabling LDS binding. For this reason, we decided to not proceed with the identification of additional TFG/ATG8s interacting domains, and rather focus on LIR3.

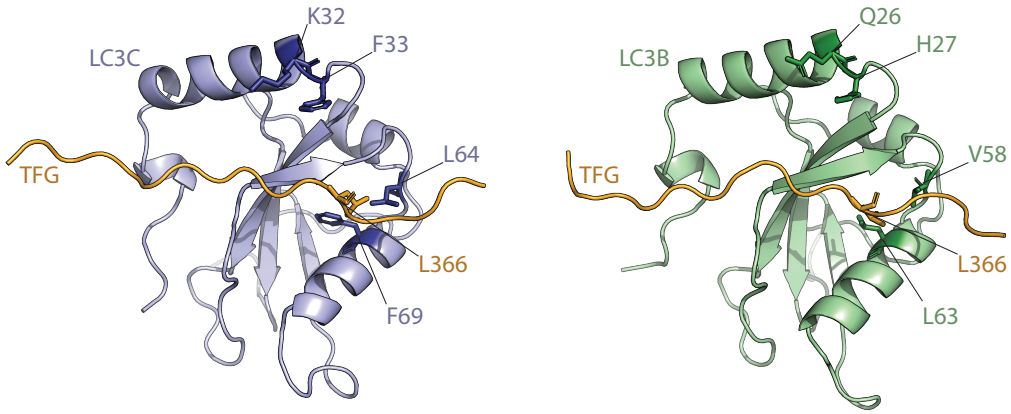

Figure for Referee #2.1.

Figure 2.1. Models of complex structures: (left) LC3C LDS interaction complex with TFG LIR, and (right) LC3B LDS interaction complex with TFG. The two models were obtained by *Arpeggio*, a webserver for calculating interatomic interactions in protein structures (http://biosig.unimelb.edu.au/arpeggioweb).

12. Fig6. E-J. The experiments conducted here with transient transfections and siRNA-mediated knockdown of TFG need to be backed up by experiments where the authors have reconstituted TFG KO cells with WT and LIR3 mutant TFG stably expressed at near endogenous levels. The TFG blot showing the effect of siRNA is missing in Fig. 6E.

Answer: We understand this point of the Referee. However, as reported by others (Johnson *et al.*, 2015) and also in our hands, extended periods of TFG depletion (~72 h after siRNA treatment) resulted in an inhibition of cellular proliferation. These data are consistent with other findings, which demonstrated that inhibition of COPII vesicle secretion results in cell death as a consequence of elevated ER stress (Xu *et al*, 2005). For this reason, we chose the 48-60h timepoint of siRNA treatment for all subsequent experiments. We have now added this explanation also in the text (see lanes 131-133). However, to answer this Referee's comment, we performed a new experiment expressing TFG^{wt} and TFG^{mutLIR3} constructs at near endogenous levels (see novel Figure 5E-F). Our results are fully

reproducible and coherent with the hypotheses. Fig. 6E has been replaced by the novel experiments (see Figure 5E).

13. In Fig. 6I the effect of TFG KD on the endogenous ULK1-GABARAP interaction should be analyzed, not only the effect on OE LC3C. In fact OE may be rather perturbing to the relevant complexes analyzed.

Answer: Important point! We have performed both endogenous ULK1-GABARAP and ULK1-LC3C co-IPs as suggested. As shown in the novel Figure 5L, we confirm that ULK1-LC3C interaction decreases after TFG downregulation, suggesting that overexpression did not perturb the binding in the previous experiments. Moreover, thanks to this Referee's suggestion, we found that also ULK1-GABARAP interaction is impaired after TFG depletion (see novel Figure EV5P), suggesting a crucial role for TFG in comprehensively regulating ULK1 during autophagy.

14. Why is there no effect on the ULK1 level of TFG KD in Fig 6I?

Answer: Nice catch! We believe that this could be due to ULK1 overexpression. We have now performed the same co-IP in endogenous conditions (see novel Figure 5L), and it is possible to appreciate an increase in ULK1 protein levels in TFG KD, as expected. The previous experiment has been removed.

15. The bias towards a role for LC3C with TFG is not warranted as long as endogenous LC3C is not implicated experimentally and the GABARAP subfamily proteins are not properly examined for interaction with TFG.

Answer: As explained above, we have now performed endogenous TFG-LC3C co-IP (see Figure EV5J) and we have also tested TFG-GABARAP family members interaction by pulldown assays as requested (see answer 10).

16. Fig S6F: The authors need to knock down GABARAP and LC3B to compare with knock down of LC3C on ULK1 levels.

Answer: Absolutely agree. We analysed ULK1 protein levels after knocking down GABARAP, L1, L2 and LC3B and LC3C. As shown in the novel Figures 5K and EV5K-N, we are now able to appreciate a significant increase in ULK1 protein levels only after LC3C downregulation, supporting the importance of both TFG and LC3C in regulating ULK1 levels.

Minor concerns

17. The use of the term "maturation" is a bit misleading from what is the usual use in the research field. At the end of the first paragraph of the Introduction it is stated:... "LC3 incorporation represents the maturation step of autophagosomes"... and bottom of p4:... "proper distribution of ULK1 and the maturation of autophagosomes"... Heading on p7:
"Loss of TFG impairs autophagosome maturation" and p11 bottom of page: ..."autophagosome maturation during autophagy induction"... to mention the most misleading ones from the usual understanding of autophagosome maturation as culminating with fusion to late endosomes giving amphisomes or fusion with lysosomes giving autolysosomes. The authors should use another term to avoid confusion here as what they are referring to is a defect at some stage after nucleation and initiation of phagophore (isolation membrane) formation but before closure of autophagosomes, perhaps at the transition from initiation to elongation.

Answer: Thank you for your suggestion. We have now modified the text.

18. Fig 2 is basically control experiments with no effect of siRNA-mediated knockdown (KD) of the COP-II components SEC23A and SEC16 on ULK1 puncta numbers and ULK1 protein levels that can be shown as supplemental figures (EV) and not as main figures.

Answer: Thank you; We have now moved Figure 2 as a supplementary figure (see EV1G-K).

19. Fig. 6I: The label "TFG-IP" must be replaced with "HA-IP".
Fig S2: SEC23A KD has a negative effect on autophagic flux during starvation as read off from LC3B lipidation levels.

Answer: We corrected this mistake.

Referee #3:

The submitted manuscript concerns the identification of a role of the "TRK-fused gene" protein (TFG) in macroautophagy (autophagy). In previous work, the lab identified TFG as binding to the autophagy protein ULK by proteomics, and this interaction is verified biochemically. Loss of function analysis is performed by gene knockdown and reveal a reduction in overall autophagy flux. As TFG is known to act in the secretory pathway, they investigated whether the COPII secretory pathway is involved, but rule this out. Instead, the authors demonstrate using quantitative and live microscopy accumulation a delay in early autophagosome maturation and resulting accumulation of autophagy cargo. A direct LC3 binding domain (LIR) is identified in TFG, and TFG binds LC3C. They demonstrate further that this domain is functionally important, possibly underlying the delayed dynamics in autophagosome maturation. Ultrastructural analysis in TFG knockdown cells display abnormal vesicular structures, but how, and if at all this relates to the defect on autophagy is unclear. Primary fibroblasts from a patient suffering from Hereditary Spastic Paraplegia disease with a defined TFG mutation (R106C) also display reduced autophagy flux, suggesting that autophagy defects may underlie neural dysfunction in this patient. Rescue or sufficiency experiments for this mutation was not performed. In sum the manuscript defines a novel role of TFG in autophagy flux not explained by its secretory pathway role, and suggests that this is clinically informative. The manuscript is thus interesting to a wider audience; to the autophagy field, cell biology and clinical field. Overall, the manuscript well written, sound and experiments well performed.

Answer: Many thanks to this Reviewer for the appreciation of our work and the many insightful comments, that we have addressed in full, as follows:

Comments to the authors:
1. Page 8. The TFG mutation (R106C) is believed to underlie the defect in autophagy flux, but no rescue experiment is provided. Presumably as this is a dominant mutation. To strengthen the conclusion that the TFG mutation is responsible, the athuors should express an exogeneous TFG (R106C) in primary fibroblasts or other cell types to show that this mutation is causative of autophagy defects.

Answer: In full agreement with this Referee's request, we analyzed autophagy flux in HeLa cells after overexpression of HA-TFG^{R106C} mutant. As shown in Figure EV4G, autophagy is impaired after HA-TFG^{R106C} mutant overexpression with respect to the wild-type form.

2. Figure 5E. Ultrastructural analysis is described to reveal multivesicular structures, potentially representing the underlying defects in TFG cells in autophagy flux. This link is currently weak. This point should be improved by Correlative Light and Electron Microscopy to show that indeed the observed structures are positive for the described autophagy marker(s).

Answer: In full agreement with this Referee's request, we performed Correlative Light and Electron Microscopy (CLEM) after TFG downregulation and autophagy induction by starvation. As shown in novel Figure 4E, the "abnormal structures" (As) are positive for ATG13, ATG16L1 and Rab7. Thanks to this Referee's request, we could now hypothesize that *As* are generated as a consequence of defective autophagosome sprouting from omegasomes together with an impaired formation of multivesicular bodies, as demonstrated by co-staining with ATG16L, Rab7 and ATG13. This is now reported in the Result paragraph and discussed in the Discussion paragraph.

If ultrastructural data are available from the patient fibroblast line, a related interesting question is whether these structures is also observed in TFG R106C fibroblasts?

Answer: To assess this interesting issue, we have performed EM analyses on both patient (TFG R106C) and control fibroblast lines after autophagy induction by starvation. In the novel Figure EV4E representative images of patient's fibroblasts show the presence of As.

3. I was unable to readily identify the number of experimental independent repeats, number of measurements and source data underlying all graphs in the figure legends nor the materials and methods or uploaded files. This has to be presented in an easily available form in the manuscript itself as well as in source data format

(Excel files or similar). I am therefore unable to judge the quantifications and graphs in this submitted version and my comments are based on the assumption that that experiments are carefully and correctly carried out. I need to see this in a potential revised version of the manuscript.

Answer: The required informations are now added. The source data are now available on the supplementary information page.

1st Revision - Editorial Decision 2nd Feb 2021

Thank you for submitting your revised study. The manuscript has now been sent back to referee #2 and #3, whose comments are appended below. As you will see, both reviewers find that their criticisms have been sufficiently addressed and recommend the work for publication. However, referee #2 asks you to show LC3C mRNA expression data in siTFG cells.

Our editorial assistants and production team are currently looking at your manuscript. As soon as this process is completed, I will send you a separate e-mail with a list of editorial requests concerning the text and the figures, which should be addressed before we can officially accept your manuscript.

Referee #2:

The authors have in this revised manuscript added many new experiments with important new data strengthening their conclusions.
In doing so they have also addresses the majority of my comments/criticism in a satisfactory manner. In such an exhaustive revision exercise addressing a relative complex biological problem I realize that it is hard to also approach a detailed mechanistic explanation for the role TFG has on autophagosome formation. However, the information coming through here is in my view interesting enough.
I do have a problem with LC3C which the authors implicate to be the ATG8 doing the job here together with TFG. Looking at mRNA expression data (https://portals.broadinstitute.org/ccle/page?gene=MAP1LC3C) or proteomics data (PAXdb) LC3C is hardly expressed at all in most tissues and cell lines. As far as I know there is presently not available a good antibody specifically recognizing LC3C hampering progress on the expression issue here. The authors use knock-in cell with HA knocked in at the endogenous locus in HeLa cells. However, this is not clear that this particular cell line does not have increased copy number or other genetic changes that increases the expression of HA-LC3C above the relevant endogenous level.
It would be reassuring if the authors could show mRNA expression data for LC3C by sequencing or qPCR showing that the cells (not the HA-LC3C cell line) they use for experiments where siTFG has great effect do express mRNA for LC3C and that the level is reduced by siTFG treatment.

Referee #3:

The revised manuscript is much improved in response to the reviewers' comments.

My own concerns and suggestions have been satisfactorily addressed.
In particular, source data, experimental repeats and statistics are described and deposited.

Point-by-point response to Editors and Reviewers

Referee #2:
The authors have in this revised manuscript added many new experiments with important new data strengthening their conclusions.
In doing so they have also addresses the majority of my comments/criticism in a satisfactory manner. In such an exhaustive revision exercise addressing a relative complex biological problem I realize that it is hard to also approach a detailed mechanistic explanation for the role TFG has on autophagosome formation. However, the information coming through here is in my view interesting enough.
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It would be reassuring if the authors could show mRNA expression data for LC3C by sequencing or qPCR showing that the cells (not the HA-LC3C cell line) they use for experiments where siTFG has great effect do express mRNA for LC3C and that the level is reduced by siTFG treatment.

Answer: We thank this Referee for the useful request to analyze *LC3C* mRNA expression levels in HeLa cells, in the absence of a good antibody specifically recognizing LC3C.

In general, low or even undetectable *LC3C* mRNA levels in expression studies should not represent a problem *per se*, since it is well accepted that in many instances there is a clear disconnection between mRNA and protein expression levels. In line with this, we believe that the analysis of mRNA has limited explanatory power as far as LC3C protein levels are concerned. However, also proteomics data are not fully informative in this case: indeed, such data show that LC3C shares tryptic peptides with LC3A and LC3B, due to the high sequence similarity, making difficult to obtain conclusive results by this approach. In addition, all mATG8 proteins are known to be post-translationally modified, which could further complicate the correct detection of their peptides. In sum, overall LC3C peptides might be unrepresentative in MS databases, and -in other words, for these reasons, the fact that LC3C is hardly found in *broadinstitute.org* or *Paxdb* data, does not argue for a lack of expression of LC3C in real tissues or cells.

Thus, to address the Referee's question, and since we agree that this would be quite reassuring, we performed qPCR analysis by using two different pairs of primers for LC3C on HeLa cells after LC3C downregulation by RNAi. As shown now in the revised version of the manuscript (see new Figure EV5F). We are able to detect *LC3C* mRNA levels in control conditions and a significant decrease of such levels after siRNA silencing. Also, the related LC3C amplification plots and the Ct values are shown below (see below Figure #1 for the Referee), demonstrate the presence of detectable *LC3C* mRNA levels in HeLa cells. We further analyzed *LC3C* mRNA levels in the presence or absence of TFG (as requested by this Reviewer, see below Figure #2 for the Referee) and observed no significant differences when comparing the two conditions. This is in line with our

hypothesis about a role for TFG on LC3C, at post-translation level, in regulating LC3C-ULK1 interaction.

Figure #1

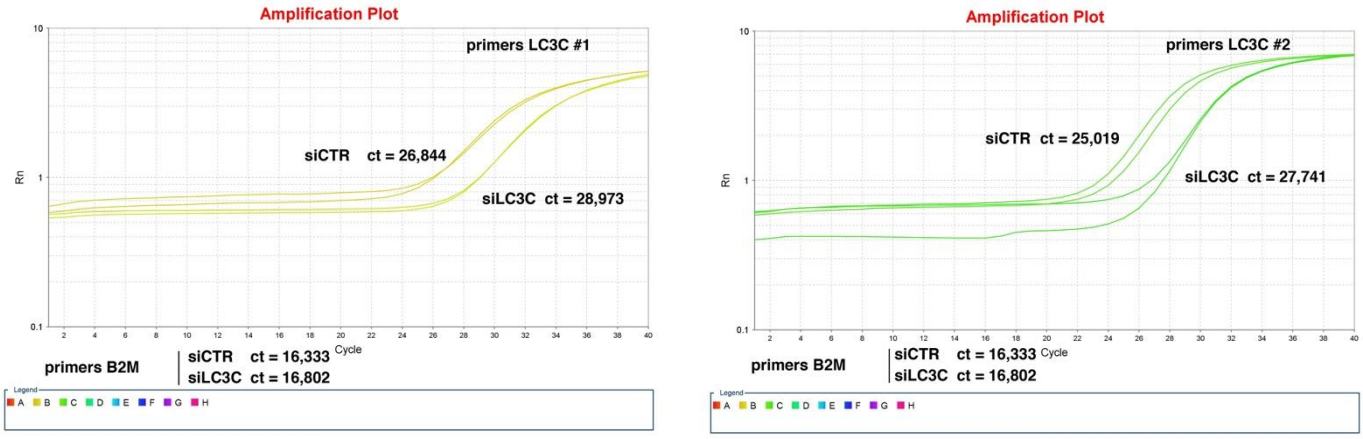

Figure 1. Amplification plots of LC3C primers. Plots show ct of LC3C in the control and in siLC3C samples by using primers LC3C#1 (left) or LC3C#2 (right). B2M ct for the analysed conditions are reported (bottom).

Figure #2

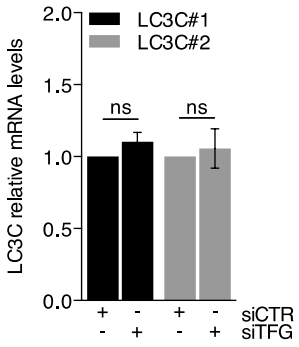

Figure 2. HeLa cells were transfected with the indicated siRNA, and LC3C mRNA relative levels were analysed by qPCR by using two different LC3C primers. Values are expressed as mean ± SEM. Statistical analysis was performed by unpaired t test. (n=5 independent experiments).

About the HA-LC3C HeLa cell line used in this study, it is a clonal cell line which was tested by PCR and SANGER sequencing for correct knock-in (see Table1). The 5'UTR has not been altered by the knock-in approach; however, the insertion of a Blasticidine resistant gene followed by a P2A site and a 1xHA tag could influence mRNA stability or processing. This said, most likely this would rather decrease or delay transcript translation.

Table 1: Sequencing of HA-LC3C.

LC3C^{endoHA} :
AGGGGAGGGAGAGGAGAGGCCTGATGTCACTCAGCCCTACATAAGGGCCTCCTTCA GGCTCCTGCAGGCAGTTTGGAAGCAGCTGGAGGAATGAGTTAGGTTCCCGGTTGCG GGACAGTTTTTTTTTCTTTTTTAAAACAGACACAGCTACTGAGTGCAATG**CCGGCCAA** **GCCTTTGTCTCAAGAAGAATCCACCCTCATTGAAAGAGCAACGGCTACAATCAACA**

GCATCCACATCTCTGAAGACTACAGCGTCGCCAGCGCAGCTCTCTCTAGCGACGGC
CGCATCTTCACTGGTGTCAATGTATATCATTTTACTGGGGGACCTTGTGCAGAACTC
GTGGTGCTGGGCACTGCTGCTGCTGCGGCAGCTGGCAACCTGACTTGTATCGTCGC
GATCGGAAATGAGAACAGGGGCATCTTGAGCCCCTGCGGACGGTGCCGACAGGTG
CTTCTCGATCTGCATCCTGGGATCAAAGCCATAGTGAAGGACAGTGATGGACAGCA
GACGGCAGTTGGGATTCGTGAATTGCTGCCCTCTGGTTATGTGTGGGAGGGC

2nd Revision - Editorial Decision 1st Mar 2021

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The data shown in figures should satisfy the following conditions:

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- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
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Each figure caption should contain the following information, for each panel where they are relevant:

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- 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;
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  - 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.

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Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Statistical analysis was performed using unpaired Student's t test, assuming a two-tailed distribution, one-way and two-way analysis of variance (ANOVA) followed by Tukey's post hoc test or followed by Dunnett's multiple comparison test, or multiple t-test, or Kolmogorov-Smirnov test using GraphPad Prism program as reported in the figure legends.
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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.	All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards as reported in the manuscript.
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