Rab7b modulates autophagic flux by interacting with Atg4B

Ingrid Kjos, Marita Borg Distefano, Frank Sætre, Urska Repnik, Petter Holland, Arwyn T. Jones, Nikolai Engedal, Anne Simonsen, Oddmund Bakke, and Cinzia Progida

Corresponding authors: Cinzia Progida and Oddmund Bakke, University of Oslo

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1st Editorial Decision 15 March 2017

Thank you for the submission of your research manuscript to our journal. We have now received the full set of referee reports that is copied below.

As you will see, the referees acknowledge the potential interest of the findings. However, referees 1 and 2 also point out several technical concerns and have a number of suggestions for how the study should be strengthened, and I think that all of them should be addressed.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as detailed above and in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

REFeree REPORTS

Referee #1:
The manuscript describes a very interesting story on an unexpected role of RAB7B in autophagy. Also unexpectedly, RAB7B is shown to interact and colocalize with ATG4B, a protease that processes LC3. The text reads well and the results are well presented and mostly convincing. The story will be of interest to a wide readership, provided that the authors add some experimental data to support their conclusions.
Major comments:

1. In all experiments the authors use just one siRNA to silence RAB7B. Thus, there is no evidence that they are not looking at off-target effects. One possibility to convince the readers is that the authors use two or three different oligos to silence RAB7B, which all show the same effects on autophagy. As a minimum requirement, the exclusion of off-target effects should be shown in one or two key experiments.

2. The conclusions are based on the proposition that autophagosomes are larger in RAB7B silenced cells. This is only shown by fluorescence microscopy, where it is not possible to separate large vesicles from several small vesicles that are clustered. If the authors wish to make this conclusion, they should show the enlarged size of autophagosomes by electron microscopy, using plastic embedded samples (since Tokuyasu cryosections are very delicate and prone to severe distortions during cutting and picking up on grids).

3. The authors show that RAB7B co-precipitates with ATG4B under starvation conditions (Fig. 1a), and interacts with ATG4B in PLA on starved cells (Fig. 1c). Are these interactions starvation dependent, i.e. do the proteins also interact in non-starved cells?

4. In Figure 3a-c, the authors use BafA as a 'control'. It would be more appropriate to add BafA to treatments, i.e. siRAB7B+BafA with and without starvation, and siControl+BafA with and without starvation. Further, the authors should also show the total area of LC3 vesicles per cell area (or per cell), as an estimate of the total amount of autophagosomes (including both number and size).

5. In Figure S1d, the authors should also show the microscopy images. BafA should be used in all treatments (as in Fig. 3).

6. In Figure 4E, it is unclear whether the sample labeled 'HA-Rab7b+Baf' is only overexpressing Rab7b, or whether it was first silenced for RAB7B, and then transfected to overexpress RAB7B. Please clarify the annotation.

Referee #2:

This manuscript entitled "Rab7b modulates autophagic flux by interacting with Atg4B" by Ingred Kjos et al., describes about the role of Rab7b, which is poorly characterized Rab protein, in autophagy. The authors first found that Rab7b interacts with Atg4B. Rab7b is involved in determining the autophagosome size by modulating Atg4B activity toward LC3 lipidation form. Thus the authors proposed that Rab7B is a negative regulator of autophagy. The data are mostly acceptable, however, some points must be confirmed to accept the conclusion.

Major points:

1. Figure1 A. The authors need to show the localization of GFP-Rab7Q67L and T22N in comparison to autophagosome. According to their model, only GFP-Rab7Q67L will be targeted to the autophagosomes.

2. Figure 1C. In order to exclude the possibility that the signals arises form Attg4B antibody, the authors should show negative control using only Atg4B antibody.

3. Figure 5A and B. The ratio of Atg4B activation may be statistically significant, but I am concerned that if this small activation is really associated with autophagy enhancement. To support the model that Rab7b activate Atg4B, the authors need to show if overexpression of Rab7B enhances LC3 processing.
We thank the referees for their constructive comments. We have added the experimental data requested as indicated below in the point-by-point answers. These new data strengthens our conclusion that Rab7b negatively regulates autophagy through its interaction with Atg4B.

Referee #1:

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Major comments:

1. In all experiments the authors use just one siRNA to silence RAB7B. Thus, there is no evidence that they are not looking at off-target effects. One possibility to convince the readers is that the authors use two or three different oligos to silence RAB7B, which all show the same effects on autophagy. As a minimum requirement, the exclusion of off-target effects should be shown in one or two key experiments.

We have now included two additional siRNAs targeting Rab7b in the experiments shown in figure 3a-c (and suppl. Figure EV2: quantification of endogenous LC3 vesicles), and figure 3f-h (and suppl. Figure EV3a-c: quantification of the levels of LC3-II and LC3-II/LC3-I ratio). The obtained results are in line with those obtained with the previously used siRNA targeting Rab7b (indicated now as siRab7b#1), indicating that we are very unlikely looking at off-target effects. In addition, the rescue experiment in figure 4e further confirms the specificity of Rab7b silencing.

2. The conclusions are based on the proposition that autophagosomes are larger in RAB7B silenced cells. This is only shown by fluorescence microscopy, where it is not possible to separate large vesicles from several small vesicles that are clustered. If the authors wish to make this conclusion, they should show the enlarged size of autophagosomes by electron microscopy, using plastic embedded samples (since Tokuyasu cryosections are very delicate and prone to severe distortions during cutting and picking up on grids).

We agree with the reviewer and we have now included in figure 3d-e the results obtained by ultrastructural analysis of autophagosomes by electron microscopy, using plastic embedded samples. Autophagosomes were identified as membrane-bound compartments containing morphologically intact cytoplasm, ribosomes, often endoplasmic reticulum as reported by Eskelinen, 2008. Quantification of the autophagosome size pointed to the same tendency that we detected by IF analysis, that is an increase in size after Rab7b silencing compared to control (fig. 3d-e). Also, IF analysis is now further confirmed using two additional siRNAs targeting Rab7b (Fig. 3b, Figure EV2).

3. The authors show that RAB7B co-precipitates with ATG4B under starvation conditions (Fig. 1a), and interacts with ATG4B in PLA on starved cells (Fig. 1c). Are these interactions starvation dependent, i.e. do the proteins also interact in non-starved cells?

We followed the referee suggestions and repeated the co-IP and PLA of Rab7b and ATG4B including non-starved cells (now Fig. EV1a and Fig.1c-d respectively). Both types of experiments show that the two proteins interact also without starvation, suggesting that the interaction between Rab7b and ATG4B is required during both basal and starvation-induced autophagy. This is not surprising as in our experiments we do see an effect of Rab7b knockdown on basal autophagy, as shown for example in figure 3b (LC3 vesicle size), and figure 3f-h (levels of LC3-II and LC3-II/LC3-I ratio). Several other interactions involved in the regulation of autophagy have also been reported to occur under both basal and starved conditions. For example, the autophagy kinase ULK1 interacts with TBC1D14 in both fed and starved conditions (Longatti et al., 2012). Also, the interaction between Atg13, ULK1, and
FIP200, which is essential for autophagosome formation, is occurring under both nutrient-rich and starvation conditions (Hosokawa et al. 2009; Hara et al., 2008).

4. In Figure 3a-c, the authors use BafA as a 'control'. It would be more appropriate to add BafA to treatments, i.e. siRAB7b+BafA with and without starvation, and siControl+BafA with and without starvation. Further, the authors should also show the total area of LC3 vesicles per cell area (or per cell), as an estimate of the total amount of autophagosomes (including both number and size).

We have now included in Figures 3a-c, 3f-h, Figures EV2 and EV3, siRab7b+BafA with and without starvation, and siControl+BafA with and without starvation. We have also quantified the total area of LC3 vesicles per cell in cells silenced for Rab7b. This is in accordance with the combination of the data relative to both the number of LC3 vesicles (which is unaltered) and size (which increases in samples silenced for Rab7b).

5. In Figure S1d, the authors should also show the microscopy images. BafA should be used in all treatments (as in Fig. 3).

The microscopy images and the data relative to BafA treatments are now shown in Fig. EV3d-e. In all the tested conditions, we did not observe any significant difference in the percentage of LC3 vesicles positive for Lamp-1 between control and Rab7b silenced samples.

6. In Figure 4E, it is unclear whether the sample labeled 'HA-Rab7b+Baf' is only overexpressing Rab7b, or whether it was first silenced for RAB7B, and then transfected to overexpress RAB7B. Please clarify the annotation.

We apologize for not being clear. We have now amended the text (in the section iRab7b depletion increases autophagic sequestration, pag.7) and explained that the sample labelled 'HA-Rab7b+Baf' is a rescue sample, where the cells were first silenced for Rab7b and then transfected with HA-Rab7b.

Referee #2:

This manuscript entitled "Rab7b modulates autophagic flux by interacting with Atg4B" by Ingred Kjos et al., describes about the role of Rab7b, which is poorly characterized Rab protein, in autophagy. The authors first found that Rab7b interacts with Atg4B. Rab7b is involved in determining the autophagosome size by modulating Atg4B activity toward LC3 lipidation form. Thus the authors proposed that Rab7b is a negative regulator of autophagy. The data are mostly acceptable, however, some points must be confirmed to accept the conclusion.

Major points:

1. Figure1 A. The authors need to show the localization of GFP-Rab7Q67L and T22N in comparison to autophagosome. According to their model, only GFP-Rab7Q67L will be targeted to the autophagosomes.

Following the referee’s suggestion, we have investigated the localization of GFP-Rab7bQ67L and T22N in comparison to autophagosomes in live cells. Similarly to Rab7b wt, Rab7bQ67L is targeted to LC3-positive vesicles (autophagosomes). On the contrary, the dominant negative mutant of Rab7b is not targeted to autophagosomes as it is cytosolic, in agreement with the literature (Wang et al., 2007; Progida et al., 2010). These results are now included in suppl. Figure EV1e-f and movie 3.

2. Figure 1C. In order to exclude the possibility that the signals arises form Atg4B antibody, the authors should show negative control using only Atg4B antibody.

To exclude that the signals arises from the Atg4B antibody in the PLA assay, we have performed the assay using only the Atg4B antibody and as shown in Fig.1E there was no signal.
3. Figure 5A and B. The ratio of Atg4B activation may be statistically significant, but I am concerned that if this small activation is really associated with autohagy enhancement. To support the model that Rab7b activate Atg4B, the authors need to show if overexpression of Rab7B enhances LC3 processing.

We thank the referee for the suggestion. We have now performed the same experiment overexpressing Rab7b. Overexpression of Rab7b indeed enhances LC3 processing of about 30%, as shown in the new fig. 5c-d, further supporting the proposed model.

References:


2nd Editorial Decision 20 June 2017

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the full set of referee reports that is copied below. As you will see, both referees are now positive about the study and support publication in EMBO reports. Referee 1 suggests using the EM data to calculate the increase in autophagosome size in Rab7b-depleted cells.

I look forward to seeing a final version of your manuscript as soon as possible.

REFEREE REPORTS

Referee #1:

The revision has improved the manuscript considerably, and all my concerns have been addressed. I have one minor suggestion left. Since the EM analysis on autophagosome size was done using point counting, the authors can use the results to estimate how much the total volume of autophagosomes is increased in the RAB7B silenced cells (considering that the number of autophagosomes is not changed). Would this volume increase be in agreement with the results of the protein degradation and LDH secretion assays?

Referee #2:

In this revised manuscript, the authors adequately responded to the concerns raised in the previous reviewing process.
Referee #1:

The revision has improved the manuscript considerably, and all my concerns have been addressed. I have one minor suggestion left. Since the EM analysis on autophagosomes size was done using point counting, the authors can use the results to estimate how much the total volume of autophagosomes is increased in the RAB7B silenced cells (considering that the number of autophagosomes is not changed). Would this volume increase be in agreement with the results of the protein degradation and LDH sequestration assays?

We thank the referee. We estimated that the autophagosomes in Rab7b silenced cells increase the volume by 16% and we have now added this in the text (page 5). This increase is in agreement with the increase measured in both protein degradation and LDH sequestration assays (fig. 4d-e) but we can't directly compare the numbers as the methods and the way the measurement are obtained are different. All together these data indicate that sequestration and degradation of content in autophagosomes in Rab7b depleted cells increases as consequence of the size increase as we discussed at page 7 of the manuscript.

Referee #2:

In this revised manuscript, the authors adequately responded to the concerns raised in the previous reviewing process.

We thank the referee for supporting the publication of our manuscript.

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.
The data shown in figures should satisfy the following conditions:

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