EMBO reports

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TBC1D5 and the AP2 complex regulate ATG9 trafficking and initiation of autophagy

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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.)

Editor: Nonia Pariente

1st Editorial Decision 01 October 2013

Thank you for your patience while your study has been under peer-review at EMBO reports. As you will see from the reports pasted below, the are disparate opinions among the referees. Referee 2 is rather negative about the advance provided, whereas referees 1 and 3 consider that a strengthened manuscript would be suitable for publication here.

As the reports are pasted below, I will not detail them here. Overall, I think the study is a good candidate for consideration in EMBO reports after appropriate revision. Please note that the following issues would have to be addressed during revision for the study to be successful here:

- all the technical concerns brought up by referee 1 need to be addressed, and the data tightened up with the new controls, image analysis and text rewriting

- additional evidence of the role of TBC1D5, following the suggestions of referee 3, would also be necessary (which would also alleviate some of referee 2's concerns)

As you may remember, it is EMBO reports policy to undergo one round of revision only and thus, acceptance of your study will depend on the outcome of the next, final round of peer-review, which will involve referees 1 and 3.

Given the recent publication of a study showing AP2-dependent trafficking of ATG9 from the plasma membrane, it would be ideal if your revision could be submitted in the shortest time frame possible, and always within our standard three months of revision.

Revised manuscript length must be a maximum of 28,500 characters (including spaces). When submitting your revised manuscript, please also include editable TIFF or EPS-formatted figure files, a separate PDF file of any Supplementary information (in its final format) and a letter detailing your responses to the referees.

We also welcome the submission of cover suggestions or motifs that might be used by our Graphics Illustrator in designing a cover.

I look forward to seeing a revised form of your manuscript when it is ready. In the meantime, do not hesitate to get in touch with me if I can be of any assistance.

REFEREE REPORTS:

Referee #1 (Report):

Popovic and Dikic here continue their investigation into the role of TBC1D5 in endocytic trafficking and autophagy. As they have shown TBC1D5 is interacts with retromer as well as LC3 they explore the relationship between TBC1D5-directed sorting with other endocytic processes, and look at the function of TBC1D5 in the context of sorting Atg9 in this process, and in particular between the plasma membrane and the retromer-positive endosomal compartment. In addition, they examine the role of AP2 in this process and interactions. Their conclusions are the Atg9 trafficking is regulated under starved conditions by a dynamic interaction with TBC1D5 and AP2. While a few of the data are convincing enough to support their interesting hypothesis, the manuscript is far below the standard expected for EMBO Reports. Much of the data is not presented or explained well enough to be convincing and the manuscript is poorly written and the text at times confusing and contradictory. Some points are listed below, (please note this is not an extensive list), to provide some guidance for improvement of all the data presented.

Major points:
1. Many of the immunoprecipitations are done with M2 beads and the control is untransfected or uninduced lysates. The additional control of transfected lysates with a non-relevant antibody and beads without antibody is needed. When agarose is used (Fig. 3F and S7B there is no input shown and the condition is not specificed).
2. The use false coloring, the contrast and presentation of the immunofluorscence, and the choice of fluorophores (cy5 and mCherry) for instance make the images very unconvincing. See for example Fig. 1G right, S1B where the 2 or 3 channels are impossible to identify in the merge, and the contrast seems to be enhanced in the merge.
3. The Atg9, LC3 and AP2 stainings vary widely and appear different in several images. Compare for instance for Atg9 2B and 3D, for AP2 3D and E, 5A. LC3 in many images appears vey diffuse even under conditions where one would expect discrete spots (see 1C, S3 lower, 6B).
Other points:
4. The areas magnified in immunofluorescence need to be boxed in the main figures.
5. It is not clear how the quantification was done with the Threshold Pearson's coefficient and should be better explained.
6. The text is in places confusing and contradictory, or doesn't support the data, of is unclear. For instance
-page 3 What is the "Atg9-mediated growth phase"?
-page 3 references 14, 18 and 19 seem to be cited incorrectly
-page 4 "stabilized Atg9" is Atg9 degraded?

-page 4 "mislocalized to late endosomes", Atg9 is found on late endosomes so it cant be mislocalized
-page 5 "it is possible that Atg9....CCVs[25]. This is not at all clear.
-page 5 "large patches" this is not clear what these are.
7. Fig. S7 appears to contradict much of the other data, and doesn't show extended starvation periods, they have used standard conditions.
8. Fig. 1I and 5C are unlabelled.
9. Other comments: DFCP-1 is not an Atg protein; autophagyc should be autophagic.

Referee #2 (Report):

This paper reports the following observations:
1. TBC1D5 associates with ATG9 and ULK1
2. TBC1D5 is required for ATG9 trafficking - in the absence of TBC1D5, cells have decreased numbers of ATG9 vesicles and these are mislocalised to late endosomes when autophagy is induced.
3. TBC1D5 and ATG9 interact with the AP2 complex - the authors suggest that since TBC1D5 depletion reduced the ATG9- AP2 interaction, TBC1D5 is acting as an adaptor
4. AP2 and clathrin-mediated endocytosis is required for ATG9 sorting and autophagy.

The timing of this paper is unfortunate as Rubinsztein's group have recently shown that ATG9 is in clathrin-coated pits and is endocytosed in an AP2- and dynamin-dependent manner (Cell 2013 154: 1285-1299). So the novelty of the last part of the paper, which may be the most interesting component, is lost. Furthermore, Dikic's group have previously described that TBC1D5 is involved in autophagosome formation.

In order for the current paper to have impact now, we need to understand what TBC1D5 is doing. It is not clear if it is really acting as an adaptor - if ATG9 vesicle numbers are decreased and ATG9 is mislocalised after TBC1D5 knockdown, then this may explain why there is less ATG-AP2 interaction in the knockdown cells - maybe the ATG9 is mislocalised and less is at the plasma membrane. This may be additionally explained as the knockdown cells have less AP2, so this in itself could explain the data in 3G - the AP2 may not bind less effectively molecule-by-molecule but there may be simply less of it to bind the ATG9. Much more work will be required to show that TBC1D5 is an adaptor for ATG and AP2. The data presented also do not explain how the TBC1D5 knockdown causes the mislocalisation of the ATG9 in late endosomes.

Thus, I do not think that the current study has sufficient novelty and depth for EMBO Reports.

Referee #3 (Report):

In this manuscript, the authors reported that TBC1D5 and AP2 regulate Atg9 trafficking. The authors found that TBC1D5 can interact with Ap2 and Atg9, and depletion of TBC1D5 leads to missorting Atg9 into late endosome.

The membrane source of autophagosome is one of fundamental questions in autophagy field. Plasma membrane has been identified as one potential source for autophagosome membrane. Very recently, Atg9 has been reported to trafficking from plasma membrane to early endosome through clathrin-coated structure. Thus identifying TBC1D5 and Ap2 as new components regulating Atg9 trafficking is a timely and important discovery.

I have a few suggestions on this manuscript.

1) Could the authors elaborate more on why Atg9 colocalizes with TGN in Ap2 depleted cells upon autophagy induction? One will expect that Atg9 stays at plasma membrane under this condition.
2) Could the authors explain why TBC1D5 depletion causes Atg9 missorting into late endosome? Does it imply that TBC1D5 regulates sorting of Atg9 from early endosome to autophagosome,

rather than plasma membrane to early endosome? In this scenario, TBC1D5 depletion will cause the defection of sorting of Atg9 from early endosome to autophagsosome, thus cause the missorting Atg9 to late endosome.
3) The authors proposed TBC1D5 as the adaptor linking Atg9 to AP2. The authors should test whether knockdown TBC1D5 can reduce the colocalization between Ap2 /clathrin and Atg9.
4) Does TBC1D5 affect endocytosis in general?

Minor points:
1) LC3 puncta formation in TBC1D5 depleted cells should be shown.
2) The conclusion drawn from Dynasore need backing by the data from a dominant negative mutant of Dynamin 2.

1st Revision - authors' response 16 December 2013

Response to the reviewer #1:

Popovic and Dikic here continue their investigation into the role of TBC1D5 in endocytic trafficking and autophagy. As they have shown TBC1D5 is interacts with retromer as well as LC3 they explore the relationship between TBC1D5-directed sorting with other endocytic processes, and look at the function of TBC1D5 in the context of sorting Atg9 in this process, and in particular between the plasma membrane and the retromer-positive endosomal compartment. In addition, they examine the role of AP2 in this process and interactions. Their conclusions are the Atg9 trafficking is regulated under starved conditions by a dynamic interaction with TBC1D5 and AP2. While a few of the data are convincing enough to support their interesting hypothesis, the manuscript is far below the standard expected for EMBO Reports. Much of the data is not presented or explained well enough to be convincing and the manuscript is poorly written and the text at times confusing and contradictory. Some points are listed below, (please note this is not an extensive list), to provide some guidance for improvement of all the data presented.

We appreciate the reviewer´s concerns, comments and suggestions for the improvement of the manuscript. We have introduced several changes and provided additional figures to better indicate a critical role for TBC1D5 in controlling the co-localization and functional interplay between AP2 and ATG9. This new evidence strengthens our conclusion that TBC1D5 regulates ATG9 trafficking via AP2 complex and we believe that these new findings contribute to better understanding of the ATG9 trafficking pathways.

Major points:
1. Many of the immunoprecipitations are done with M2 beads and the control is untransfected or uninduced lysates. The additional control of transfected lysates with a non-relevant antibody and beads without antibody is needed. When agarose is used (Fig. 3F and S7B there is no input shown and the condition is not specificed).

We have repeated the immunoprecipitation experiment from Fig. 3F, and Fig S7B (presented now as Fig. 5F) and we included the controls suggested by reviewer. We did not observe any non-specific binding.

2. The use false coloring, the contrast and presentation of the immunofluorscence, and the choice of fluorophores (cy5 and mCherry) for instance make the images very unconvincing. See for example Fig. 1G right, S1B where the 2 or 3 channels are impossible to identify in the merge, and the contrast seems to be enhanced in the merge.

We provide in Fig. S1B 2D plot showing intensity of fluorescence for

each channel. Additionally, we repeated all live-cell imaging experiments exclusively on confocal spinning disc microscope, and not on epifluorescent microscope, and subsequently we provide 4 new movies, as well as new Fig 1I (as the reviewer was concerned about the intensity in figure 1). Upon confocal imaging we did not observe any more background or fluctuations in intensity of fluorescence signal, while colocalization of mCherry-TBC1D5 and GFP-ATG9 was more obvious.
Additionally, we described better the settings that were used for the acquisition of confocal images in our Supplementary Material and Methods.

3. The Atg9, LC3 and AP2 stainings vary widely and appear different in several images. Compare for instance for Atg9 2B and 3D, for AP2 3D and E, 5A. LC3 in many images appears vey diffuse even under conditions where one would expect discrete spots (see 1C, S3 lower, 6B).

We prefered staining of endogenous proteins whenever it was possible. However, as the reviewer is probably aware, it is way more challenging to get the same antibody performance in each staining when non-tagged, endogenous proteins are inspected. We avoided harsh cytoplasmic extractions prior to staining (as we observed loss of proteins, particularly TBC1D5, and AP2), therefore the LC3 puncta may appear less strong due to the presence of cytosolic LC3. Moreover, the cells presented in Figures S1B and S1C have been stained with different LC3 antibodies. As our ATG9 antibody is a rabbit antibody we were forced to use the LC3 antibody from mouse (Novus Biologicals) for ATG9/LC3 colocalization experiments (Fig. S1B). Despite the weaker performance of the LC3 mouse antibody (NanoTools) compared to the rabbit antibody (MBL) (Fig. S1C) in immunofluorescence, we hope that the reviewer can still appreciate the co-localizations of the stained proteins. We provide 2D plots showing intensity of all 3 fluorophores in depicted puncta in Fig. S1B.
Also we provide better quality live cell imaging (Supplementary Movies 1, 2, 3 and 5 and Fig. 1I) and immunofluorescence picture of endogenous ATG9, TBC1D5 and AP2 (Fig S6A). Under both conditions we observed co-localization.

Other points:
4. The areas magnified in immunofluorescence need to be boxed in the main figures.

We boxed all magnified regions in each figure as suggested.

5. It is not clear how the quantification was done with the Threshold Pearson's coefficient and should be better explained.

We explained better our quantification and statistical analysis in Supplementary Material and Methods, under paragraph Confocal microscopy and live cell imaging, as suggested.

6. The text is in places confusing and contradictory, or doesn't support the data, of is unclear. For instance

-page 3 What is the "Atg9-mediated growth phase"?
-page 3 references 14, 18 and 19 seem to be cited incorrectly
-page 4 "stabilized Atg9" is Atg9 degraded?
-page 4 "mislocalized to late endosomes", Atg9 is found on late endosomes so it cant be mislocalized
-page 5 "it is possible that Atg9....CCVs[25]. This is not at all clear.
-page 5 "large patches" this is not clear what these are.

We included all corrections in text that are suggested above, and we have checked references 14, 18 and 19:

Page3: Text "ATG9-mediated growth phase" is corrected to: "ATG9 labeled early autophagosomes".

Page3: Reference 14 is a study published by Orsi et al. (2012), where the authors report that knock-down of retromer subunit VPS26 does not affect autophagy, reference 18 is a study published by Dengjel et al. (2012) where authors report association of retromer with autophagosome enriched fraction, subsequently subjected to mass spectrometry based proteomic analysis, suggesting its relevance in autophagosome biogenesis. Reference 19 on page 4 of manuscript is report from Yamamoto at al. (2012) where authors measured the size of ATG9 traffic carriers by electron microscopy.

Page 4: "stabilized ATG9" – we wanted to stress the fact that the level of ATG9 is increased, which could be due to the defect in degradation of ATG9.

Page 4: "mislocalized to late endosomes"- We agree with the reviewer, it has been shown that ATG9 can traffic through various membrane compartments (including the endosomes), however, upon autophagy ATG9 pools are primarily targeted to the site of nascent autophagosomal membranes. Upon depletion of TBC1D5, ATG9 is primarily localized in endosomes during autophagy induction, which is not the case in control cells. We corrected the text and now it stands "ATG9 was enriched in late endosomes" instead of "mislocalized".

Page 5*:* "it is possible that Atg9….CCVs[25]. – We have removed this sentence from the text, in order to avoid a lack of clarity.

Page5: "large patches"- We intended to refer to the domains on plasma membrane that were enriched in AP2, TBC1D5 and ATG9 proteins. As suggested, we changed the text and use "plasma membrane regions enriched with AP2/ATG9/TBC1D5" instead of "large patches".

7. Fig. S7 appears to contradict much of the other data, and doesn't show extended starvation periods, they have used standard conditions.

In order to emphasize the general message of the manuscript, we provide a new blot showing that dynamin2 inhibitor Dynasore enhances interaction of TBC1D5 and ATG9, which can be additionally increased by starvation or abolished upon knock-down of AP2 (Fig. 5F). Therefore we excluded previous Fig. S7.

8. Fig. 1I and 5C are unlabelled.

We provided new figure 1I and we labeled both, Fig. 1I and Fig. 5C as suggested.

9. Other comments: DFCP-1 is not an Atg protein; autophagyc should be autophagic.

We corrected the text as suggested: "autophagosome precursors initiated by autophagyc protein DFCP1" has been replaced by "autophagosome precursors initiated by DFCP1".

Response to the reviewer #2:

This paper reports the following observations:

1. TBC1D5 associates with ATG9 and ULK1
2. TBC1D5 is required for ATG9 trafficking - in the absence of TBC1D5, cells have decreased numbers of ATG9 vesicles and these are mislocalised to late endosomes when autophagy is induced.
3. TBC1D5 and ATG9 interact with the AP2 complex - the authors suggest that since TBC1D5 depletion reduced the ATG9- AP2 interaction, TBC1D5 is acting as an adaptor
4. AP2 and clathrin-mediated endocytosis is required for ATG9 sorting and autophagy.

The timing of this paper is unfortunate as Rubinsztein's group have recently shown that ATG9 is in clathrin-coated pits and is endocytosed in an AP2- and dynamindependent manner (Cell 2013 154: 1285-1299). So the novelty of the last part of the paper, which may be the most interesting component, is lost. Furthermore, Dikic's group have previously described that TBC1D5 is involved in autophagosome formation.

In order for the current paper to have impact now, we need to understand what TBC1D5 is doing. It is not clear if it is really acting as an adaptor - if ATG9 vesicle numbers are decreased and ATG9 is mislocalised after TBC1D5 knockdown, then this may explain why there is less ATG-AP2 interaction in the knockdown cells - maybe the ATG9 is mislocalised and less is at the plasma membrane. This may be additionally explained as the knockdown cells have less AP2, so this in itself could explain the data in 3G - the AP2 may not bind less effectively molecule-by-molecule but there may be simply less of it to bind the ATG9. Much more work will be required to show that TBC1D5 is an adaptor for ATG and AP2. The data presented also do not explain how the TBC1D5 knockdown causes the mislocalisation of the ATG9 in late endosomes.

Thus, I do not think that the current study has sufficient novelty and depth for EMBO Reports.

We appreciate reviewer's comments and suggestions. It was indeed unfortunate that upon submission of our manuscript, the paper from the Rubinzstein lab appeared in Cell. We have not been aware of their findings prior to the publication and we have now referenced their work.
The Puri et al. manuscript shows that ATG9 localizes on the plasma membrane in clathrin-coated structures and is internalized following a classical endocytic pathway through early and then recycling endosomes. They have also shown that these vesicles fuse with the ATG16-positive pool at the site of autophagosomal formation. We feel that our studies are complementary to this report as we identify the molecular machinery that underlies the basis of ATG9 trafficking from the plasma membrane toward the autophagosome formation. In particularly, we demonstrate that TBC1D5 links AP2 endocytic complex with the ATG9 trafficking route. We provide evidence that clathrin mediated endocytosis is essential for trafficking of ATG9 and extend these findings by showing that this process is also dependent on the AP2 complex as well as on the RabGAP protein TBC1D5. We also show that upon depletion of TBC1D5, co-localization of ATG9 and AP2 is decreased (Fig. S6B), which additionally supports our hypothesis that TBC1D5 regulates autophagy via direct binding to LC3 and subsequent recruitment of AP2-clathrin vesicles that contain ATG9, and that effect is specific for TBC1D5 depletion and not only caused by decrease in total AP2. We did not intend to make a general statement that TBC1D5 is an AP2 adaptor, despite the fact that it binds to AP2. We rather propose that via direct binding to both, LC3 and AP2, TBC1D5 is able to regulate ATG9 trafficking to the site of phagophore formation, as depletion of AP2 abolishes their interaction and affects autophagy flux, similarly to depletion of TBC1D5.

Response to the reviewer #3:

In this manuscript, the authors reported that TBC1D5 and AP2 regulate Atg9 trafficking. The authors found that TBC1D5 can interact with Ap2 and Atg9, and depletion of TBC1D5 leads to missorting Atg9 into late endosome.

The membrane source of autophagosome is one of fundamental questions in autophagy field. Plasma membrane has been identified as one potential source for autophagosome membrane. Very recently, Atg9 has been reported to trafficking from plasma membrane to early endosome through clathrin-coated structure. Thus identifying TBC1D5 and Ap2 as new components regulating Atg9 trafficking is a timely and important discovery.

I have a few suggestions on this manuscript.

We appreciate the reviewer´s positive comments and helpful suggestions.

1) Could the authors elaborate more on why Atg9 colocalizes with TGN in Ap2 depleted cells upon autophagy induction? One will expect that Atg9 stays at plasma membrane under this condition.

We also expected that ATG9 would be localized on plasma membrane in case of AP2 depletion, but our data suggest that internalization of ATG9 could be regulated via multiple mechanisms (such as it is the case for EGFR). Upon induction of autophagy ATG9 shows enhanced colocalization with AP2, suggesting that the rate of internalization and secretion from TGN are increased. AP2 depletion affects internalization of ATG9, but also secretion, and we are not sure what would be the exact mechanism that contributes to a block in secretion. ATG9 could potentially have multiple internalization signals within its N and C termini, and there are also two ubiquitination sites within its C terminus, published by two independent studies of human ubiquitinome (Kim et al. 2010, Mol Cell; Wagner et al. 2011, Mol Cell Proteomics), that could possibly regulate ubiquitin dependent mechanism of ATG9 endocytosis (similar to EGFR).

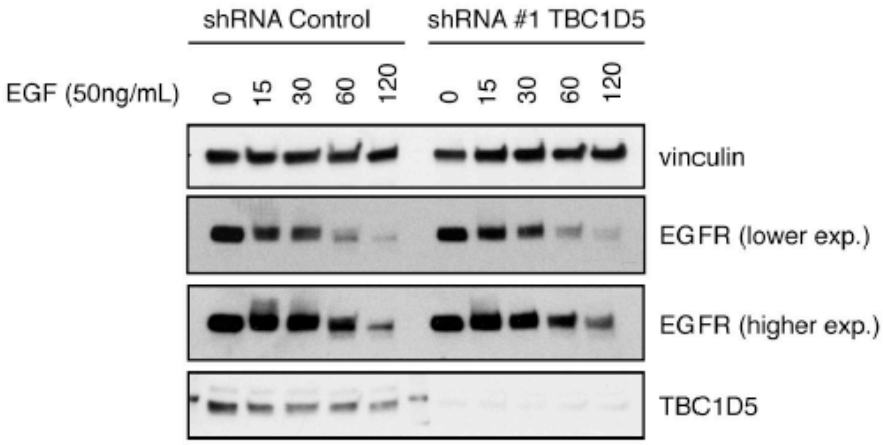
Alternatively, a small amount of residual AP2 (after knock-down) could still mediate internalization (as it has been shown to be the case for other receptors), or ATG9 could be internalized in a caveolin dependent manner, and instead of being sorted towards autophagosomes, ends up in endosomes and eventually in lysosomes. Different aspects of endocytosis of ATG9 require further biochemical dissection and thorough analysis by high resolution live-cell imaging techniques.

2) Could the authors explain why TBC1D5 depletion causes Atg9 missorting into late endosome? Does it imply that TBC1D5 regulates sorting of Atg9 from early endosome to autophagosome, rather than plasma membrane to early endosome? In this scenario, TBC1D5 depletion will cause the defection of sorting of Atg9 from early endosome to autophagsosome, thus cause the missorting Atg9 to late endosome.

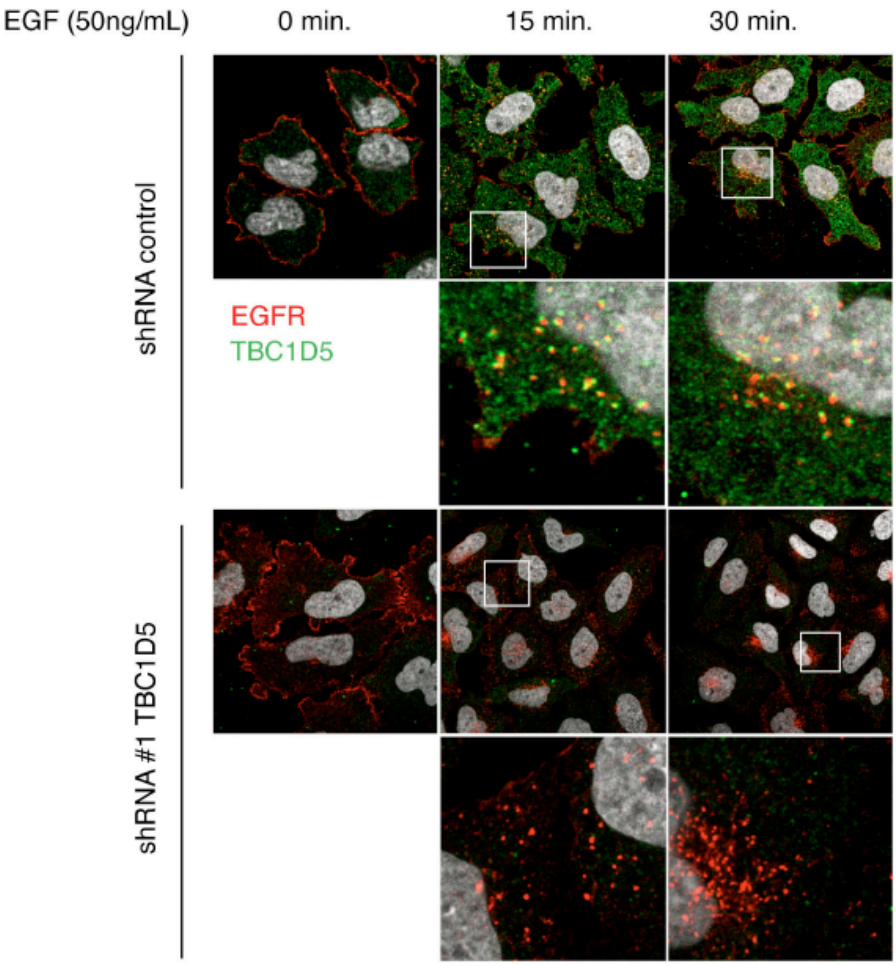
This is very good point. We believe that TBC1D5 could contribute to sorting of ATG9 vesicles that are clathrin-AP2 positive, however this does not imply that TBC1D5 directly regulates formation and budding of vesicles on the plasma membrane, since ATG9 is still able to localize with endosomes in TBC1D5 deficient cells. AP2 depletion and Dynasore treatment affect ATG9 internalization suggesting that source of vesicles is of plasma membrane origin. Importantly, AP2 depletion leads to loss of ATG9 and TBC1D5 interaction (Fig 5F). In order to completely dissect internalization mechanisms of ATG9 one would need to deplete several known AP2 accessory proteins, and to systematically dissect sorting motifs within N

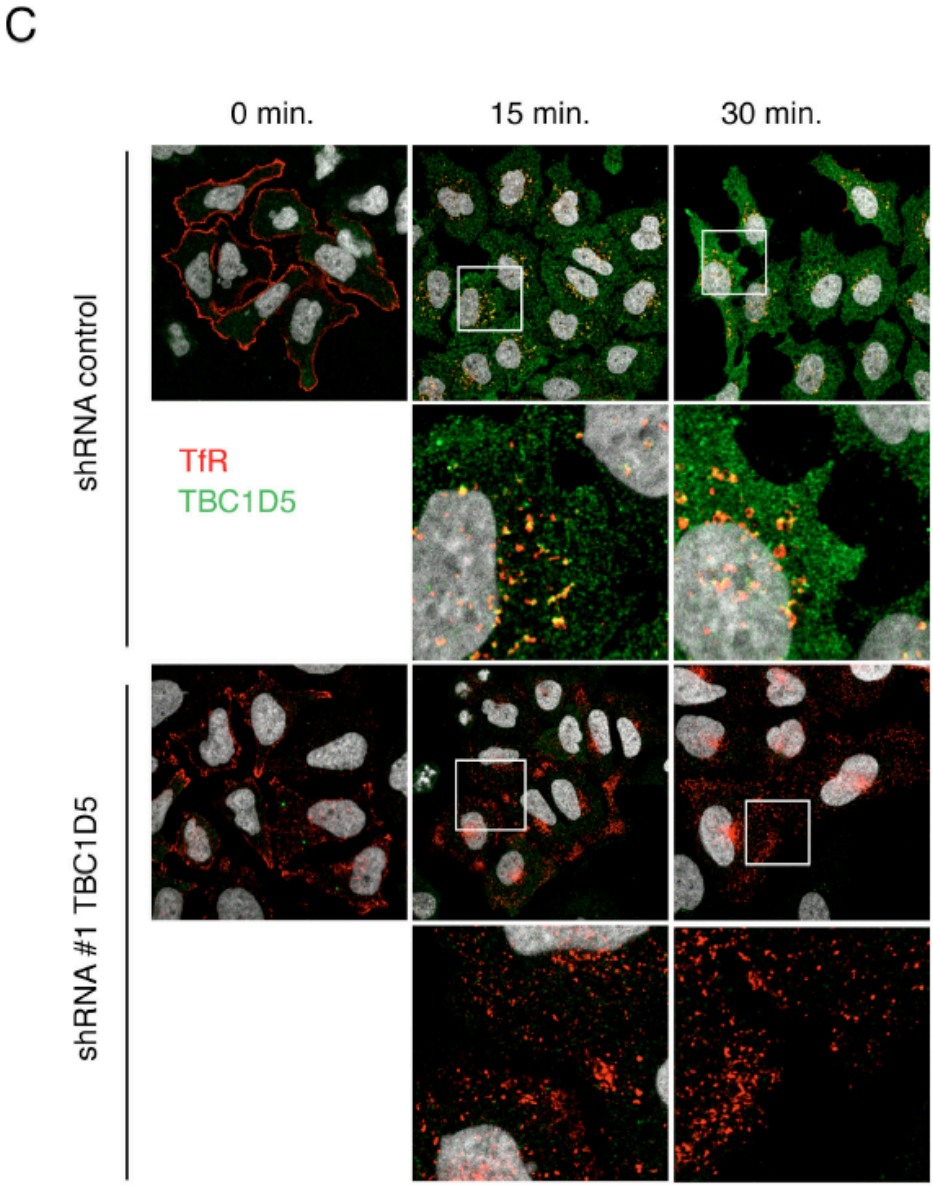
and C termini of ATG9.
We performed antibody feeding assays in order to tackle internalization of EGFR and TfR in TBC1D5 deficient cells, where we did not observe any significant defect in EGFR internalization neither degradation, while TfR appeared to be internalized with slower dynamics, and to reside in smaller vesicles. We plan to focus on this aspect of TBC1D5 function in our future studies.

A



B





(Control and TBC1D5 deficient HeLa cells were starved over night in 1%FBS, DMEM, blocked in 1xPBS, 2%BSA for 30 min. and subsequently labeled with EGFR antibody (13GB, Mouse monoclonal, Santa Cruz) or TfR antibody (Gene-Tex MEM-75, Mouse monoclonal) for 20 minutes on 37C. Antibody was dissolved in 1xPBS, 2%BSA, and cells stimulated with human EGF (50ng/mL) BD Biosciences in DMEM (A,B) or full media (10%FBS, DMEM) (C), and fixed at timepoints 15 and 30 minutes. 0 timepoint cells were not stimulated, but fixed and stained without permeablization.

3) The authors proposed TBC1D5 as the adaptor linking Atg9 to AP2. The authors should test whether knockdown TBC1D5 can reduce the colocalization between Ap2 /clathrin and Atg9.

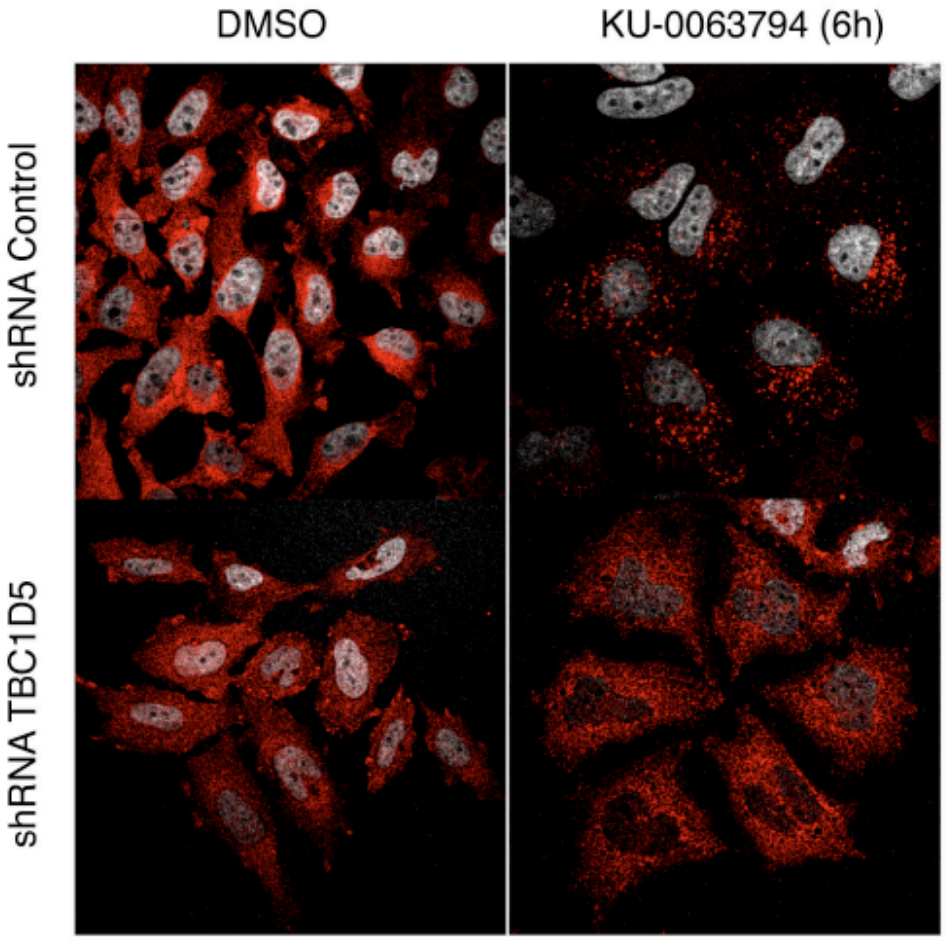
Upon depletion of TBC1D5 we observed that ATG9 is less co-localized with AP2, also during autophagy (Fig. S6B).

4) Does TBC1D5 affect endocytosis in general?

Currently we believe that TBC1D5 regulates AP2-clathrin dependent sorting, via direct binding to AP2, however, depletion of TBC1D5 does not necessarily affect endocytosis in general. Other regulatory mechanisms of internalization could still be functional, as we do not observe defects in EGFR degradation.

Minor points:
1) LC3 puncta formation in TBC1D5 depleted cells should be shown.

We provide staining of LC3B in control and TBC1D5 depleted HeLa cells. TBC1D5 deficient cells had less LC3 puncta upon autophagy stimulation by KU0063794, mTOR inhibitor in comparison with control cells.



2) The conclusion drawn from Dynasore need backing by the data from a dominant negative mutant of Dynamin 2.

We observe that ATG9 and TBC1D5 are dispersed upon expression of

dominant negative Dynamin 2 (K44A) (Fig. 4D), similarly as inhibition with Dynasore.

We also performed co-immunoprecipitation experiments, upon overexpression of Dynamin2 WT or Dynamin2 K44A, but we were not able to get reproducible results in our co-precipitation assays possibly due to the different transfection efficiency and expression level of transfected Dynamin 2 constructs (in comparison with precise inhibition using chemical inhibitor Dynasore), therefore we only included binding assays with Dynasore based inhibition of clathrin endocytosis.

2nd Editorial Decision 29 January 2014

Thank you for your patience while we have reviewed your revised manuscript. As you will see from the reports below, the referees are now all positive about its publication in EMBO reports. I have now also had time to go through your file in detail in preparation for acceptance and I am happy to write with an 'accept in principle' decision, which means that we will formally accept your manuscript for publication once the following minor issues/corrections have been addressed.

- There are a few missing details in the figure legends regarding the analyses performed. Please ensure that all relevant figure legends have information regarding what is represented by the bar (mean, median?) and error bars, as well as the number of independent experiments and statistical test used. This will allow the readers to better interpret the figure.

- I have noticed that the Materials & Methods section is very succinct. Please note that basic Materials and Methods required for understanding the experiments performed must be included in the main text, although additional detailed information may be included as Supplementary Material. In this regard, I think it would be useful to include a "Statistical analysis" subheading in the main text .

- We now encourage the publication of original source data for the key experiments in a study - particularly for electrophoretic gels and blots, but also for graphs- with the aim of making primary data more accessible and transparent to the reader. If you agree, you would need to provide one PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figure and an Excel sheet or similar with the data behind the graphs. The files should be labeled with the appropriate figure/panel number, and the gels should have molecular weight markers; further annotation could be useful but is not essential. The source files will be published online with the article as supplementary "Source Data" files and should be uploaded when you submit your final version.

After all remaining corrections have been attended to, you will receive an official decision letter from the journal accepting your manuscript for publication in the next available issue of EMBO reports. This letter will also include details of the further steps you need to take for the prompt inclusion of your manuscript in our next available issue.

Very many thanks for your contribution to EMBO reports.

REFEREE REPORTS:

Referee #1:

The authors have addressed all my points and substantially improved the manuscript.

Referee #3:

The authors have done an adequate job with the revision, and now make a more convincing case. I therefore recommend publication of this revised manuscript.

2nd Revision - authors' response 06 February 2014

Thank you very much for the final reviews of our manuscript. We are pleased with the positive comments by the Reviewers and the Editorial Board. We have incorporated all of the suggested corrections, including the changes in the title and abstract. Also there is a minor change in the proposed model:

- Proposed model in the Fig. 5.: we added additional arrow pointing to the secretion from TGN to endosomes (since this is critical route of the CI-M6PR and potentialy ATG9 secretion, and has been neglected in previous picture).

We hope that the current manuscript contains all necessary corrections and is suitable for the publication in EMBO Reports.

Looking forward to hearing from you.

3rd Editorial Decision 10 February 2014

I am very happy to accept your manuscript for publication in the next available issue of EMBO reports.

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