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Rab12 regulates mTORC1 activity and autophagy through controlling the degradation of amino acid transporter PAT4

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Transaction Report:

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

1st Editorial Decision 29 October 2012

Thank you very much for the submission of your research manuscript to our editorial office. We have now received the full set of reviews on your manuscript.

As the detailed reports are pasted below I will only repeat the main points here. You will see that all reviewers acknowledge the potential interest of the findings and support publication of your study once their concerns have been addressed. Referee 1 recommends strengthening the link between Rab12 and PAT4 by measuring the amino acid levels in Rab12-deficient cells and by testing whether the addition of amino acids can rescue the effects of Rab12 overexpression. This reviewer also feels that the potential role of Rab11A should at least be further discussed, or ideally be supplemented with additional experimental data. Both referees 2 and 3 point out several technical issues (including missing controls) and additional clarifications that would need to be addressed.

Overall, given these positive evaluations, the reviewers constructive comments and the potential interest of the study, I would like to give you the opportunity to revise your manuscript, with the understanding that the main concerns of the reviewers should be addressed. Acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is EMBO reports policy to allow a single round of revision only and that therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. If you feel that this period is insufficient for a successful submission of your revised manuscript I can potentially extend this period slightly. Also, the length of the revised manuscript should not exceed roughly 28,000 characters (including spaces). Should you find the length constraints to be a problem, you may consider including some peripheral data in the form of Supplementary information. However, materials and methods essential for the repetition of the key experiments should be described in the main body of the text and may not be displayed as supplemental information only.

We have also started encouraging authors to submit the raw data for western blots (i.e. original scans) to our editorial office. These data will be published online as part of the supplementary information. This is voluntary at the moment, but if you agree that this would be useful for readers I would like to invite you to supply these files when submitting the revised version of your study.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

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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. Should you in the meantime have any questions, please do not hesitate to contact me.

REFEREE REPORTS:

Referee #1:

General opinion and remarks:
The authors present a new model for Rab-mediated regulation of mTORC1 activity and autophagy. They follow a two-step screening procedure using siRNA-knockdown of each mouse Rab isoform and use different established read-outs for autophagy. Thereby, the authors identify Rab12 as a regulator of autophagy as a result of altered mTORC1 activity. They further show that Rab12 colocalizes with the amino acid transporter PAT4 on recycling endosomes, and that absence of Rab12 increases PAT4 levels on endosomes and the plasma membrane and causes increased mTORC1 activity (Sch9-phosphorylation).
They conclude that the effect of Rab12 knock down on PAT4 trafficking results in an increase mTORC1 activation possibly by modulating intracellular amino acids levels. Finally, they present a model in which amino acid transporter recycling by Rab proteins provides a means to regulate mTORC1 activity.
In summary, the data is convincing and well presented. Several points should be adressed before publication, however:

Experimental issues:
1) The authors argue that Rab12 knockdown does not affect autophagic flux as measured by LC3 turnover. However, the data presented in Fig 1F is missing the control "starvation 1-2 h without bafilomycin". Only if the amount of LC3-II is higher in "starvation + bafilomycin" vs "starvation - bafilomycin" can a conclusion be made about autophagic flux.
2) A measure of the intracellular amino acid levels in Rab12-knockdown cells would be of great value as it would provide a functional link between trafficking of PAT4 and mTORC1 activation.
3) In this context, it should be studied whether addition of amino acids or of membrane-permeable

amino acid analogs in a Rab12-overexpression mutant can restore normal S6K phosphorylation. Alternatively, Rab12-knockdown cells might be less sensitive to low amounts of amino acids in the media.
4) In the context of recycling of PAT4, the authors should use knockdown of Rab11A to block recycling of PAT4, which should lead to a decrease of PAT4 amounts at the plasma membrane. It should be studied whether this correlates with decreased Sch9 phosphorylation . (See also comment 6) below).

Discussion:
5) The authors should cite existing information from other groups appropriately and include some major publications addressing TOR localization on vacuoles/lysosomes in yeast/mammals and the effect of Rab proteins on TOR (e.g. Sturgill et al, Eukaryotic Cell, 2008, p1819-1830; Bridges et al, 2012, JBC).
6) In their model, the authors show that PAT4 is recycled via recycling endosomes. They do not comment on the role of Rab11 in this recycling step, although their data (figure1A) show that knockdown of Rab11A has a significant effect on LC3-II accumulation. This would be in good agreement with their model. Indeed, knockdown of Rab11A could block recycling of PAT4 and therefore lead to a decrease of PAT4 amounts at the plasma membrane. Furthermore, it would be interesting to know if this correlates with lower Sch9 phosphorylation. This is particularly interesting because it would provide an attractive model that explains some of the previously observed effect on S6K phosphorylation of several Rabs - especially the study of Li et al. (JBC, VOL. 285, NO. 26, pp. 19705-19709) where it was shown that knockdown of Rab5 or Rab11 decreased the amount of phosphorylated S6K.
7) In view of their hypothesis that PAT4 might modulate mTORC1 activity via amino intracellular acid levels, it should be discussed what are the amino acid specificities (if any) of PAT4, and whether there could be a link towards recent findings of mTORC1 activation by Gln and Leu (Durán et al, Mol Cell, 2012, p349-358).
8) It should also be addressed whether there could be other mechanisms of PAT4-mediated signaling towards TOR besides amino acid influx, e.g. PAT4 acting as amino acid sensor on the plasma membrane (as was proposed by, e.g. Goberdhan et al., 2005, Development, p2365-2375).

Minor comments:
9) Throughout the paper: mTOR should be denoted as "mammalian target of rapamycin", not "mechanistic target of rapamycin".

Referee #2:

The manuscript "Rab12 regulates mTORC1 activity and autophagy through controlling the degradation of amino acid transporter PAT4" by Matsui and Fukuda describes a novel regulation mechanism of mTORC1 via Rab12 with the identification of the new actor, PAT4.
In my opinion, this manuscript is accurate, well written and interesting. The experiments are clearly presented and discussed.
The paper deals with a topic that will undoubtedly be of interest for the readership of EMBO Reports.
Minor points should be addressed:
1) Figures 3C, D and G: the control western blot (such tubulin or actin or GADPH) is missing.
2) A statistic paragraph should be added to the manuscript.

Referee #3:

The article "Rab12 regulates mTORC1 activity and autophagy through controlling the degradation of amino acid transporter PAT4" is a short report describing a knockdown screen of Rab proteins and their effect on autophagy. The authors identify Rab12 depletion as having the greatest effect on autophagy activity as measured by LC3II and p62 accumulation. They further characterised the effect on autophagy using LC3 and Atg16L punctum formation, and found that Rab12 depletion resulted in accumulation of the amino acid transporter PAT4 at the plasma membrane, leading to an

increase in mTORC1 activity and an inhibition of autophagy. This study builds on the group's previous findings that Rab12 controls constitutive degradation of the transferrin receptor.
The study is carried out and written well. It gives interesting and timely mechanistic insight into how regulators of membrane traffic may impact on the activity of mTORC1 by regulating cytosolic nutrient availability. The study would be improved if several points listed below were addressed:
Major points
1. While the western blot data in figures 1E and F are convincing, quantification of the levels of LC3II and p62 from several experiments would reinforce the point.
2. The first paragraph of the section "Rab12 regulates the efficiency of autophagy..." is somewhat confusing. The authors conclude from their findings about autophagy markers that "Rab12 regulates the efficiency of autophagy rather than regulation of formation or maturation". This statement does not make much sense as inhibiting formation or maturation would also reduce the "efficiency" of autophagy. Do the authors mean that the data suggest Rab12 modulates the signals involved in initiation of autophagy?
3. The authors imply that PAT4 follows the same degradative pathway as the transferrin receptor (Tfr). As internalisation of Tfr does not require ubiquitination, it would be informative to look at the ubiquitination status of PAT4 in Rab12 depleted and control cells. Does Rab12 depletion increase or decrease the amount of ubiquitinated PAT4? If not this would indicate that PAT4 follows a similar pathway to Tfr. This could be easily done by immunoprecipitating the HA-tagged PAT4 and blotting for ubiquitin.
4. In figure 3G, while there is an increase in transferrin and PAT4 at the cell surface on Rab12 knockdown which is consistent with the hypotheses presented, there is a reduction in the levels of EGFR at the cell surface and indeed total EGFR levels under the same conditions. Could the authors speculate as to why this is?
5. A control that could be included in the supplemental data is to treat control and Rab12 knockdown cells with Rapamycin as a specific TOR inhibitor working independently of amino acid levels. Given the model the authors present in figure 4, Rapamycin treatment would be expected to lessen the effect of Rab12 depletion and promote autophagy.
Minor points
1. In figures 1G, 3A,B and S3 A,B please show the whole merged image. This would give the reader a better overview of the level of colocalisation.
2. Also, representative images for figure 1C and D would be useful.

1st Revision - authors' response 23 January 2013

[Response to the reviewer #1]

The authors present a new model for Rab-mediated regulation of mTORC1 activity and autophagy. They follow a two-step screening procedure using siRNA-knockdown of each mouse Rab isoform and use different established read-outs for autophagy. Thereby, the authors identify Rab12 as a regulator of autophagy as a result of altered mTORC1 activity. They further show that Rab12 colocalizes with the amino acid transporter PAT4 on recycling endosomes, and that absence of Rab12 increases PAT4 levels on endosomes and the plasma membrane and causes increased mTORC1 activity (Sch9-phosphorylation). They conclude that the effect of Rab12 knock down on PAT4 trafficking results in an increase mTORC1 activation possibly by modulating intracellular amino acids levels. Finally, they present a model in which amino acid transporter recycling by Rab proteins provides a means to regulate mTORC1 activity.

In summary, the data is convincing and well presented. Several points should be addressed before publication.

We greatly appreciated the reviewer's positive comments and helpful suggestions.

1. The authors argue that Rab12 knockdown does not affect autophagic flux as measured by LC3 turnover. However, the data presented in Fig 1F is missing the control "starvation 1-2 h without bafilomycin". Only if the amount of LC3-II is higher in "starvation + bafilomycin" vs "starvation - bafilomycin" can a conclusion be made about autophagic flux.

We agreed with the reviewer. We have included the starvation 1 hr without bafilomycin control in new Fig. 1G.

2. A measure of the intracellular amino acid levels in Rab12-knockdown cells would be of great value as it would provide a functional link between trafficking of PAT4 and mTORC1 activation.

As suggested by the reviewer, we have measured the intracellular L-amino acid concentration in Rab12-depleted cells (Supplementary Fig. S5). As we expected, the intracellular L-amino acid concentration was much higher in the Rab12-depleted cells than in the control cells.

3. In this context, it should be studied whether addition of amino acids or of membrane-permeable amino acid analogs in a Rab12-overexpression mutant can restore normal S6K phosphorylation. Alternatively, Rab12-knockdown cells might be less sensitive to low amounts of amino acids in the media.

Yes. Addition of certain L-amino acids, e.g., Pro and Trp, both of which have been found to be high-affinity substrates of PAT4 when expressed in *Xenopus* oocytes (ref. 32), to Rab12(QL)-overexpressing cells was able to restore phosphorylation of S6K (Supplementary Fig. S4C and S4D).

4. In the context of recycling of PAT4, the authors should use knockdown of Rab11A to block recycling of PAT4, which should lead to a decrease of PAT4 amounts at the plasma membrane. It should be studied whether this correlates with decreased Sch9 phosphorylation. (See also comment 6) below).

As suggested, we have investigated the effect of Rab11A knockdown on PAT4 recycling. However, the amount of plasma membrane-localized PAT4 protein was unaltered by Rab11A knockdown, although the amount of plasma membrane-localized TfR protein was clearly reduced under the same experimental conditions (Supplementary Fig. S7). Thus, PAT4 is not actively recycled back to the plasma membrane by Rab11 unlike TfR protein. Moreover, Rab11A (or 11A/B) knockdown had virtually no effect on the phosphorylation of S6K (Supplementary Fig. S9A).

5. The authors should cite existing information from other groups appropriately and include some major publications addressing TOR localization on vacuoles/lysosomes in yeast/mammals and the effect of Rab proteins on TOR (e.g. Sturgill et al, Eukaryotic Cell, 2008, p1819-1830; Bridges et al, 2012, JBC).

As suggested, we have cited these references in the revised manuscript.

6. In their model, the authors show that PAT4 is recycled via recycling endosomes. They do not comment on the role of Rab11 in this recycling step, although their data (figure1A) show that knockdown of Rab11A has a significant effect on LC3-II accumulation. This would be in good agreement with their model. Indeed, knockdown of Rab11A could block recycling of PAT4 and therefore lead to a decrease of PAT4 amounts at the plasma membrane. Furthermore, it would be interesting to know if this correlates with lower Sch9 phosphorylation. This is particularly interesting because it would provide an attractive model that explains some of the previously observed effect on S6K phosphorylation of several Rabs - especially the study of Li et al. (JBC, VOL. 285, NO. 26, pp. 19705-19709) where it was shown that knockdown of Rab5 or Rab11 decreased the amount of phosphorylated S6K.

As noted above (response to query #4), Rab11 seems not to be involved in PAT4 recycling or S6K phosphorylation at least in our MEF cells (Supplementary Figs. S7 and S9A). We speculate

that PAT4 is recycled back to the plasma membrane in a Rab11-independent fashion, and this mechanism should be investigated in a different project. Since Rab11A has been reported to regulate autophagosome formation (*J. Cell Biol*. 2012;197:659-675) or fusion between autophagosome and MVBs (*Traffic* 2008;9:230-250), LC3-II accumulation observed in Rab11A-knockdown cells may be caused by defects in autophagosome formation/maturation.

7. In view of their hypothesis that PAT4 might modulate mTORC1 activity via intracellular amino acid levels, it should be discussed what are the amino acid specificities (if any) of PAT4, and whether there could be a link towards recent findings of mTORC1 activation by Gln and Leu (Durán et al, Mol Cell, 2012, p349-358).

We also think that functional relationship between amino acid specificity of PAT4 and mTORC1 activation is worth investigating. Currently, however, since we do not know whether L-amino acids, e.g, Pro and Trp, transported into cells directly (or indirectly) affect mTORC1 activity, we do not want to discuss this point at the present manuscript. We would like to investigate the functional relationship between amino acid specificity of PAT4 and mTORC1 activation in more detail in our future study.

8. It should also be addressed whether there could be other mechanisms of PAT4-mediated signaling towards TOR besides amino acid influx, e.g. PAT4 acting as amino acid sensor on the plasma membrane (as was proposed by, e.g. Goberdhan et al., 2005, Development, p2365-2375).

Although we cannot rule out the possibility that PAT4 act as an amino acid sensor, because the intracellular amount of L-amino acids in Rab12-depleted cells was dramatically increased (Supplementary Fig. S5), the most straightforward explanation for the increased mTOR activity is that PAT4 acts as an amino acid transporter as shown in Fig. 4D.

9. Throughout the paper: mTOR should be denoted as "mammalian target of rapamycin", not "mechanistic target of rapamycin".

Since the official symbol of mTOR in the NCBI is now mechanistic target of rapamycin, we would like to describe mTORC1 as mammalian/mechanistic target of rapamycin complex 1 in the revised manuscript.

[Response to the reviewer #2]

The manuscript "Rab12 regulates mTORC1 activity and autophagy through controlling the degradation of amino acid transporter PAT4" by Matsui and Fukuda describes a novel regulation mechanism of mTORC1 via Rab12 with the identification of the new actor, PAT4.

In my opinion, this manuscript is accurate, well written and interesting. The experiments are clearly presented and discussed. The paper deals with a topic that will undoubtedly be of interest for the readership of EMBO Reports.

We greatly appreciated the reviewer's very positive comments and helpful suggestions.

1. Figures 3C, D and G: the control western blot (such tubulin or actin or GADPH) is missing.

As suggested, we have included control actin blots in Fig. 3C, D, and G.

2. A statistic paragraph should be added to the manuscript.

A statistic paragraph has been included in the Methods section.

[Response to the reviewer #3]

The article "Rab12 regulates mTORC1 activity and autophagy through controlling the degradation of amino acid transporter PAT4" is a short report describing a knockdown screen of Rab proteins and their effect on autophagy. The authors identify Rab12 depletion as having the greatest effect on autophagy activity as measured by LC3II and p62 accumulation. They further characterised the effect on autophagy using LC3 and Atg16L punctum formation, and found that Rab12 depletion resulted in accumulation of the amino acid transporter PAT4 at the plasma membrane, leading to an increase in mTORC1 activity and an inhibition of autophagy. This study builds on the group's previous findings that Rab12 controls constitutive degradation of the transferrin receptor.

The study is carried out and written well. It gives interesting and timely mechanistic insight into how regulators of membrane traffic may impact on the activity of mTORC1 by regulating cytosolic nutrient availability. The study would be improved if several points listed below were addressed:

We greatly appreciated the reviewer's positive comments and helpful suggestions.

1. While the western blot data in figures 1E and F are convincing, quantification of the levels of LC3II and p62 from several experiments would reinforce the point.

According to the reviewer's suggestion, quantification data was provided in new Fig. 1F and Fig. 1G legend.

2. The first paragraph of the section "Rab12 regulates the efficiency of autophagy..." is somewhat confusing. The authors conclude from their findings about autophagy markers that "Rab12 regulates the efficiency of autophagy rather than regulation of formation or maturation". This statement does not make much sense as inhibiting formation or maturation would also reduce the "efficiency" of autophagy. Do the authors mean that the data suggest Rab12 modulates the signals involved in initiation of autophagy?

We agreed with the reviewer. We have corrected this sentence as suggested by the reviewer.

3. The authors imply that PAT4 follows the same degradative pathway as the transferrin receptor (Tfr). As internalisation of Tfr does not require ubiquitination, it would be informative to look at the ubiquitination status of PAT4 in Rab12 depleted and control cells. Does Rab12 depletion increase or decrease the amount of ubiquitinated PAT4? If not this would indicate that PAT4 follows a similar pathway to Tfr. This could be easily done by immunoprecipitating the HA-tagged PAT4 and blotting for ubiquitin.

As shown in Supplementary Fig. S9B and C, we have confirmed that HA-PAT4 was not ubiquitinated in Rab12-depleted cells.

4. In figure 3G, while there is an increase in transferrin and PAT4 at the cell surface on Rab12 knockdown which is consistent with the hypotheses presented, there is a reduction in the levels of EGFR at the cell surface and indeed total EGFR levels under the same conditions. Could the authors speculate as to why this is?

As pointed out by the reviewer, a slight reduction in the levels of EGFR at the cell surface and of total EGFR was observed in three independent experiments, suggesting that Rab12 is involved in the negative regulation of EGFR degradation. At this stage, however, we have no idea about this mechanism, and we would like to investigate it in our future study. We have just described the fact in the text as follows: "the total amounts of EGFR protein and plasma membrane-

localized EGFR protein in these cells were slightly lower, but the mechanisms responsible for these changes are unknown".

5. A control that could be included in the supplemental data is to treat control and Rab12 knockdown cells with Rapamycin as a specific TOR inhibitor working independently of amino acid levels. Given the model the authors present in figure 4, Rapamycin treatment would be expected to lessen the effect of Rab12 depletion and promote autophagy.

As suggested, we have treated Rab12-depleted cells with rapamycin and observed its effect on autophagy (Supplementary Fig. S3). As we expected, rapamycin treatment reduced mTORC1 activity and induced autophagy (i.e., p62 degradation and LC3-dot formation) even in the Rab12-depleted cells.

Minor points:
6. In figures 1G, 3A,B and S3 A,B please show the whole merged image. This would give the reader a better overview of the level of colocalisation.

We have supplied the whole merged images in the revised manuscript.

7. Also, representative images for figure 1C and D would be useful.

Representative images for Fig. 1C and D were shown in new Supplementary Fig. S2.

2nd Editorial Decision 15 February 2013

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 am therefore writing with an 'accept in principle' decision, which means that I will be happy to accept your manuscript for publication once the minor issue with the citation of the Rab11 work (please see referee 3's comment below) has been corrected.

If all remaining corrections have been attended to, you will then 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.

Thank you for your contribution to EMBO reports.

REFEREE REPORTS:

Referee #1:

The authors have adequately addressed the points raised in our initial review

Referee #3:

The authors have addressed all the experimental concerns very nicely. However, as pointed out by another referee they but still have not cited the primary data properly in particular about Rab11 as requested. The review they have chosen is not open access and doesn't in fact mention Rab11 in the abstract. As the authors have included Rab11 in their data it seems reasonable to include a sentence

or two about the role of Rab11 in autophagy and recycling endosome function and include appropriate references.

2nd Revision - authors' response 16 February 2013

[Response to the reviewer #3]

The authors have addressed all the experimental concerns very nicely. However, as pointed out by another referee they but still have not cited the primary data properly in particular about Rab11 as requested. The review they have chosen is not open access and doesn't in fact mention Rab11 in the abstract. As the authors have included Rab11 in their data it seems reasonable to include a sentence or two about the role of Rab11 in autophagy and recycling endosome function and include appropriate references.

According to the reviewer's suggestion, we have described the involvement of Rab11 and recycling endosomes in autophagy (page 3) and cited appropriate references (Fader *et al*., 2008; Longatti *et al*., 2012) in the revised the manuscript.

3rd Editorial Decision 19 February 2013

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.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. As you are aware, this File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

If you do NOT want this File to be published, please inform the editorial office within 2 days, if you have not done so already, otherwise the File will be published by default [contact: emboreports@embo.org]. If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

