Coordinated activities of Myosin Vb isoforms and mTOR signaling regulate epithelial cell morphology during development

Kirti Gupta, Sudipta Mukherjee, Sumit Sen and Mahendra Sonawane
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MS ID#: DEVELOP/2020/199363

MS TITLE: Coordinated activities of Myosin Vb isoforms and mTOR signaling regulate epithelial cell morphology during development

AUTHORS: Kirti Gupta, Sudipta Mukherjee, Sumit Sen, and Mahendra Sonawane

Dear Mahendra,

I have now received two referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPressand click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but have some significant criticisms suggestions for improvements to your manuscript. For instance, given concerns that morpholinos often give non-specific phenotypes, the journal requires very robust controls to ensure specificity for any newly described morpholino-induced phenotypes (as described in more detail in the Stainier et al paper on morpholino usage). If you are able to revise the manuscript along the lines suggested, I will be happy receive a revised version of the manuscript. Please also note that Development will normally permit only one round of major revision.

We are aware that you may be experiencing disruption to the normal running of your lab that make experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The manuscript by Gupta et al. describes the role of atypical Myosin Vb isoforms in modulating the cell size in the simple squamous epithelium periderm, which is the outer layer of the epidermis of zebrafish larvae. The authors show the two distinct roles of Myosin Vb: the isoform that interacts with Rab11 regulates recycling of apical endosomes maintaining cell size whereas the other that binds to Rab10 primarily mediates vesicular transport from the trans-golgi to the plasma membrane. Furthermore, the authors demonstrate that the Rab10-interacting form is required for the expansion of the apical surface area in hypertrophy, which is caused by inhibition of cell proliferation. In this process, Myosin Vb acts downstream of mTOR that regulates fatty acid synthase (FASN) and trans-golgi morphology.

This is an important work that nicely demonstrates the role of myosin Vb in modulating the apical surface area through the mTOR-FASN axis and trans-golgi morphology in compensatory growth conditions, as to how epithelial cells control their morphology in compensatory growth conditions remains elusive. Even though focused on compensatory growth conditions, the authors ought to clearly state its role in normal conditions and incorporate it into the working model (Figure 7G). Overall, this work would be worth publishing in this journal if the major points are clarified.

Comments for the author

(Major points)
1) In principle, the basolateral surface area would correlate with the cell height. However, this is not always the case (e.g. Fig3D in the case of deltaNp63 MO or PD168393). Please clarify in what context the basolateral surface area correlates with the cell height. Please quantitate the cell height in Fig 7CD.

2) It ought to add the y-z views of all the experimental groups in Fig 5, like Fig 7C, and to quantitate the cell height.

3) The authors mention that 'the basolateral membrane growth in Exon D morphants is a consequence of the compensatory synthesis of membrane components and their delivery independent of Myosin Vb mediated transport' based on Rapamysin treatment in Exon D morphants. The authors need to strengthen this by treating Exon D morphants with Cerulenin (FASN inhibition), as the Rapamysin phenotype in WT embryos is different from the Cerulenin phenotype.

4) In Fig 7D, it needs to be clearly described in the text that the apical surface area is regulated cooperatively by mTOR and Myosin Vb in the normal state (wild-type embryos), reflecting the fact that either single inhibition does not show the apical surface area phenotype but the simultaneous inhibition does so.

5) Figure 7G is too complicated and confusing. It would be better to separate it into two parts (one in normal development and the other in the compensatory cell growth state). This diagram does not reflect the fact that Rab10-interacting myosin Vb is able to regulate trans-golgi morphology. It is also unclear what indicates the arrow from 'cell size maintenance' to 'compensatory cell growth'.

(Minor point)
P16 L4, 'when treated with Rapamysin and Cerulenin' should be when treated with Rapamysin or Cerulenin'.

Reviewer 2

Advance summary and potential significance to field

This manuscript investigates the mechanisms by which Myosin Vb interactions with rab proteins regulate epithelial membrane trafficking, and reveals that TOR signaling and MyoV/rab protein interactions are required for homeostatic changes in plasma membrane size. The study uses a

combination of beautiful in vivo microscopy and elegant genetics, and the results have broad implications for the regulation of membrane homeostasis in epithelial cells.

Comments for the author

Moderate/major issues for revision:

1. The study creates Myosin V mutant zebrafish cDNAs that are expected to be defective either in Rab10 or Rab11 binding. These mutations were based on well characterized mutations in the human Myosin V homolog, but not validated in zebrafish. Biochemical binding experiments or a yeast 2-hybrid assay would be ideal to validate these mutants, but if these methods would be too burdensome, it may be sufficient to characterize rab10 and rab11 localization in each of the mutants. The prediction would be that rab10, but not rab11, would mislocalize in myovb-rab10-/rab11+-expressing cells and vice versa. This is only a moderate issue, since these mutants are only used for a few experiments in the paper.

2. The study relies heavily on the phenotypes of a myoVb Exon D morpholino, which the authors show can specifically reduce the isoform containing the rab10-binding site. However, since most of the manuscript's conclusions depend on this morpholino reagent, the authors should confirm that phenotypes caused by this morpholino result specifically from the loss of rab10 binding, rather than simply a reduction in the amount of myoVb protein, or a non-specific morpholino effect. The most reassuring experiment would be to rescue the phenotype with a cDNA containing Exon D, as well as an attempted rescue with a cDNA containing a specific mutation in the rab10-binding site. The authors seem to have all the tools to attempt these experiments, and they would address a critical aspect of the manuscript's conclusions.

Additional Minor comments:

3. The beginning of the results section is a bit difficult to understand since the authors seem to assume that the reader is already familiar with details of their previous paper. Further explanation should be provided so that the reader can understand the experiments here without referring to the previous paper. For example, no explanation is provided of the "balling" or "rounding up" phenotype, making it difficult to understand the rationale for this experiment.

4. The golgi vesicle phenotypes in figure 1C and supplemental figure 2C are hard to appreciate in the images, and the description was vague. A clearer description of this phenotype and the criteria for scoring it should be provided.

5. Why are the phenotypes quantified in Figure 1D as % animals instead of % cells? Does this mean that if even 1 cell in the animals showed the phenotype, the phenotype was recorded? Is this phenotype seen in the no rescue condition (MyoVb morpholino alone)?

6. This is a very minor issues, but how can the authors tell that the large vesicles in the morphants shown in supplemental figure 2D are "exclusively in the periGolgi region" if no Golgi reporter is shown here?

7. The experiment in Figure 2D is important because it attributes an identity to these Rab10+ blobs, so it should be supplemented with a quantitation of 620/515 fluorescence intensity ratios of these endosomes. For comparison, the 620/515 ratio of the general Golgi compartment should also be measured. If these structures are truly post-Golgi compartments, then their 620/515 ratios should be significantly higher than the 620/515 ratio of the general Golgi compartment.

8. It would be helpful if the authors provided some quantification for the data shown in supplemental Figure 3.

9. It would be helpful if the FASN staining were quantified in some way (Figure 4A)--total FASN fluorescence per cell, or average fluorescence intensity per cell. Some interpretation of the significance of increased cortical recruitment would also be appreciated. If the fatty acids are incorporated into the plasma membrane by ER-Golgi transport pathways, what is the significance of increased FASN cortical signal?

10. The increase in the Golgi compartment (Figure 6) is observed both in cases where the apical domain is expanded (proliferation inhibition, mTOR activation) and in cases where machinery required for that expansion is impaired (exon D morphants). This makes the link between changes in Golgi size and apical hypertrophy a bit difficult to interpret. It may be interesting to see BODIPY-Ceramide staining in one of the compensatory hypertrophy conditions. If the change in Golgi size reflects increased biosynthetic output, then one may expect to observe more sphingolipid-enriched carriers in the hypertrophic conditions than in WT periderm cells.

First revision

Author response to reviewers' comments

We thank reviewers for their constructive criticism. Here is our point-by-point response to reviewers' comments.

Reviewer 1 Advance Summary and Potential Significance to Field:
The manuscript by Gupta et al. describes the role of atypical Myosin Vb isoforms in modulating the cell size in the simple squamous epithelium periderm, which is the outer layer of the epidermis of zebrafish larvae. The authors show the two distinct roles of Myosin Vb: the isoform that interacts with Rab11 regulates recycling of apical endosomes maintaining cell size whereas the other that binds to Rab10 primarily mediates vesicular transport from the trans-golgi to the plasma membrane. Furthermore, the authors demonstrate that the Rab10-interacting form is required for the expansion of the apical surface area in hypertrophy, which is caused by inhibition of cell proliferation. In this process, Myosin Vb acts downstream of mTOR that regulates fatty acid synthase (FASN) and trans-golgi morphology.

This is an important work that nicely demonstrates the role of myosin Vb in modulating the apical surface area through the mTOR-FASN axis and trans-golgi morphology in compensatory growth conditions, as to how epithelial cells control their morphology in compensatory growth conditions remains elusive. Even though focused on compensatory growth conditions, the authors ought to clearly state its role in normal conditions and incorporate it into the working model (Figure 7G). Overall, this work would be worth publishing in this journal if the major points are clarified.

Reviewer 1 Comments for the Author:

(Major points)
1) In principle, the basolateral surface area would correlate with the cell height. However, this is not always the case (e.g. Fig3D in the case of deltaNp63MO or PD168393). Please clarify in what context the basolateral surface area correlates with the cell height. Please quantitate the cell height in Fig 7CD.

Authors' response: The increase in basolateral area will increase the height only if the apical area (or cell cross-sectional area) does not grow (or expand) concomitantly. The isometric growth in both the domain may not necessarily increase the cell height. We have added this explanation in the results section in the context of figure 3F wherein the increase in basolateral domain doesn't yield an increase in the cell height.
b. Cell height data is included in the Fig. 7C and D.

2) It ought to add the y-z views of all the experimental groups in Fig 5, like Fig7C, and to quantitate the cell height.
Authors' response: We have included the orthogonal views in Figure 5 and cell height data is also included.

3) The authors mention that 'the basolateral membrane growth in Exon D morphants is a consequence of the compensatory synthesis of membrane components and their delivery independent of Myosin Vb mediated transport' based on Rapamysin treatment in Exon D morphants.

The authors need to strengthen this by treating Exon D morphants with Cerulenin (FASN inhibition), as the Rapamysin phenotype in WT embryos is different from the Cerulenin phenotype.

Authors' response: We have performed the experiment suggested by the reviewer. The results are consistent with the Rapamycin experiments. We observed that FASN inhibition leads to a decrease in basolateral domain in ExonD morphants (Please refer to the figure in the PDF version submitted as supplementary information). However, unlike Rapamycin, Cerulenin treatment results in reduced apical surface area as well as basolateral area shifting the baseline. Therefore, when we combine ExonD MO with cerulenin inhibition, the results – although consistent with Rapamycin treatment – are difficult to interpret as that FASN inhibition counters the "increased synthesis of membrane components and their delivery independent of Exon D isoform". Hence, we have decided not to include this data in the revised version. We hope that the reviewer will support our stand.

4) In Fig 7D, it needs to be clearly described in the text that the apical surface area is regulated cooperatively by mTOR and Myosin Vb in the normal state (wild-type embryos), reflecting the fact that either single inhibition does not show the apical surface area phenotype but the simultaneous inhibition does so.

Authors' response: Thank you for pointing this out. We have included this and a related argument in the manuscript.

5) Figure 7G is too complicated and confusing. It would be better to separate it into two parts (one in normal development and the other in the compensatory cell growth state). This diagram does not reflect the fact that Rab10-interacting myosin Vb is able to regulate trans-golgi morphology. It is also unclear what indicates the arrow from 'cell size maintenance' to 'compensatory cell growth'.

Authors' response: We have made modifications in the schematic and we hope that the figure is now easy to understand.

(Minor point)
P16 L4, 'when treated with Rapamysin and Cerulenin' should be when treated with Rapamysin or Cerulenin'.

Authors' response: Thank you for pointing this out. We have corrected this.

Reviewer 2 Advance Summary and Potential Significance to Field:
This manuscript investigates the mechanisms by which Myosin Vb interactions with rab proteins regulate epithelial membrane trafficking, and reveals that TOR signaling and MyoV/rab protein interactions are required for homeostatic changes in plasma membrane size. The study uses a combination of beautiful in vivo microscopy and elegant genetics, and the results have broad implications for the regulation of membrane homeostasis in epithelial cells.

Reviewer 2 Comments for the Author:

Moderate/major issues for revision:

1. The study creates Myosin V mutant zebrafish cDNAs that are expected to be defective either in Rab10 or Rab11 binding. These mutations were based on well characterized mutations in the human Myosin V homolog, but not validated in zebrafish. Biochemical binding experiments or a yeast 2-hybrid assay would be ideal to validate these mutants, but if these methods would be too burdensome, it may be sufficient to characterize rab10 and rab11 localization in each of the mutants. The prediction would be that rab10, but not rab11, would mislocalize in myovb-rab10-/rab11+-expressing cells and vice versa. This is only a moderate issue, since these mutants are only used for a few experiments in the paper.

Authors' response: Thank you. This was a very good suggestion. We have performed these experiments and the data is included as Supplementary Figure 2. The quantification for Rab10 (number of embryos showing the presence of Rab10 vesicles) was done as per the method used for 1D whereas for Rab11 we counted the number of vesicles / cell.

2. The study relies heavily on the phenotypes of a myoVb Exon D morpholino, which the authors show can specifically reduce the isoform containing the rab10-binding site. However, since most of the manuscript's conclusions depend on this morpholino reagent, the authors should confirm that phenotypes caused by this morpholino result specifically from the loss of rab10 binding, rather than simply a reduction in the amount of myoVb protein, or a non-specific morpholino effect. The most reassuring experiment would be to rescue the phenotype with a cDNA containing Exon D, as well as an attempted rescue with a cDNA containing a specific mutation in the rab10-binding site. The authors seem to have all the tools to attempt these experiments, and they would address a critical aspect of the manuscript's conclusions.

Authors' response: We have performed the rescue experiments. The injection of mRNAs while tolerated in the MyoVb MO background, these injections resulted in early embryonic lethality in WT and ExonD morpholino background possibly due to overexpression of Myosin Vb isoforms. Therefore, we attempted rescues in clones. Our data shows that the Golgi phenotype in ExonD morphants is rescued by mRNA for MyoVb-Rab10+/Rab11+ but not by MyoVb-Rab10-/Rab11+. This data is included in Supplementary Figure 3.

Additional Minor comments:

3. The beginning of the results section is a bit difficult to understand since the authors seem to assume that the reader is already familiar with details of their previous paper. Further explanation should be provided so that the reader can understand the experiments here without referring to the previous paper. For example, no explanation is provided of the "balling" or "rounding up" phenotype, making it difficult to understand the rationale for this experiment.

Authors' response: Thank you for pointing this out. We have included the explanation for cell rounding up phenotype in the revised version.

4. The golgi vesicle phenotypes in figure 1C and supplemental figure 2C are hard to appreciate in the images, and the description was vague. A clearer description of this phenotype and the criteria for scoring it should be provided.

Authors' response: We have included a better description of the Golgi phenotype in the revised version. The Golgi phenotype is typically characterized by the increase in number and length of TGN branches in most of peridermal cells in the Exon D morphants. In addition, some cells also show the presence of large post-TGN vesicles. These morphological changes do get reflected in the surface area and volume quantifications that we have included wherever applicable.

5. a) Why are the phenotypes quantified in Figure 1D as % animals instead of % cells?Does this mean that if even 1 cell in the animals showed the phenotype, the phenotype was recorded? b) Is this phenotype seen in the no rescue condition (MyoVb morpholino alone)?

Authors' response: The Golgi phenotype in the Myosin Vb morphants rescued with mRNA for MyoVb-Rab10-/11+ RNA is characterized by increased branching of TGN in the majority of cells in the periderm along with the presence of TGN associated vesicles in some cells of each of embryos. When an embryo showed phenotype in a larger proportion of cells (more than approximately 40-50% cells), it was counted as the embryo showing Rab10 binding deficiency phenotype. This statement is now included in the revised version of the manuscript.
b) MyoVb deficient embryos and the MyoVb deficient embryos injected with MyoVb-Rab10+/Rab11- mRNA did not exhibit a higher number of Golgi associated Rab10 vesicles. In both these conditions, there is a massive accumulation of other large vesicles, which disrupts Golgi morphology. We have previously shown that these large vesicles are late endosomes and lysosomes (in MyoVb deficient animals; Sonal et al 2014). We think that the process of biogenesis is altered in these two conditions given the disruption of Golgi morphology or there is a faster clearance of post-TGN vesicles due to highly enhanced lysosomal activity in Myosin Vb morphants. We have included these observations and the explanations in the revised version in the context of the Rab10 vesicle analysis (Supplementary figure 2A).

6. This is a very minor issues, but how can the authors tell that the large vesicles in the morphants shown in supplemental figure 2D are “exclusively in the periGolgi region” if no Golgi reporter is shown here? -
Authors’ response: Thank you for pointing this out. We have modified our statement.

7. The experiment in Figure 2D is important because it attributes an identity to these Rab10+ blobs, so it should be supplemented with a quantitation of 620/515 fluorescence intensity ratios of these endosomes. For comparison, the 620/515 ratio of the general Golgi compartment should also be measured. If these structures are truly post-Golgi compartments, then their 620/515 ratios should be significantly higher than the 620/515 ratio of the general Golgi compartment.

Authors’ response: We have performed the suggested analysis and the data is included in Figure 2H.

8. It would be helpful if the authors provided some quantification for the data shown in supplemental Figure 3.

Authors’ response: We have performed the suggested analysis. The data is included in supplementary Figure 4 of the revised manuscript. We quantified the vesicles showing only WGA staining and those showing co-localization with LAMP1. Our analysis suggests that most of the WGA vesicles are also LAMP1 positive.

9. a) It would be helpful if the FASN staining were quantified in some way (Figure4A)--total FASN fluorescence per cell, or average fluorescence intensity per cell.
b) Some interpretation of the significance of increased cortical recruitment would also be appreciated. If the fatty acids are incorporated into the plasma membrane by ER-Golgi transport pathways, what is the significance of increased FASN cortical signal?

Authors’ response: a) The FASN intensity has been quantified for PD 168393 treated embryos as well ΔNp63 morphants in Figure 4A and also for Exon D MO condition in supplementary figure 8 (Supp Fig 8B).
b) At the moment we do not understand the importance of membrane localization and we do not wish to speculate as we do not have any basis for such speculation.

10. a) The increase in the Golgi compartment (Figure 6) is observed both in cases where the apical domain is expanded (proliferation inhibition, mTOR activation) and in cases where machinery required for that expansion is impaired (exon D morphants). This makes the link between changes in Golgi size and apical hypertrophy a bit difficult to interpret.
b) It may be interesting to see BODIPY-Ceramide staining in one of the compensatory hypertrophy conditions. If the change in Golgi size reflects increased biosynthetic output, then one may expect to observe more sphingolipid-enriched carriers in the hypertrophic conditions than in WT periderm cells.

Authors’ response: a) We have discussed this aspect in detail in the discussion part. Briefly, In the Exon-D morphants, the increase in Golgi is due to feedback activation of the mTOR-FASN axis. We think this is due to the housekeeping function of Exon-D isoform in recycling and replenishment of plasma membrane components as part of membrane surveillance mechanisms, which gets altered in the Exon-D morphants.
b) We performed preliminary BODIPY-Ceramide staining on both the compensatory hypertrophy conditions. To begin with, the untreated (control) wild type cells have very few sphingolipid-enriched vesicles and this number increases marginally under hypertrophy conditions (Please refer to the figure in the PDF version submitted as supplementary information). We reasoned that these low numbers and a small increase could be due to the following - a) we did not target the appropriate time window in which the transport of sphingolipid-enriched vesicles is maximum under hypertrophy conditions b) although sphingolipids are important components, they do not form the bulk of the transport to the plasma membrane in these two conditions at this stage and c) even though the biogenesis increases under hypertrophy conditions, the number of sphingolipid-enriched TGN vesicles may not increase if the rate of the detachment of the vesicles (from Golgi) and their transport remains rapid like in controls. It is important to keep in mind that the15-20-fold increase in these vesicles in ExonD morphants is possibly due to their accumulation over time.

We already have strong evidence (based on mTOR and FASN inhibition studies), that increased Golgi size indicates the biosynthetic output. Because of this, and due to the fact that the increase in the number of sphingolipid-enriched vesicles was too small under hypertrophy conditions, we did not pursue these experiments further.

Second decision letter

MS ID#: DEVELOP/2020/199363

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AUTHORS: Kirti Gupta, Sudipta Mukherjee, Sumit Sen, and Mahendra Sonawane
ARTICLE TYPE: Research Article

I am happy to tell you that the referees are happy with your revisions and your manuscript has been accepted for publication in Development, pending our standard ethics checks. The referee reports are appended below.

Reviewer 1

Advance summary and potential significance to field

The same as I mentioned in the previous review.

Comments for the author

The revised manuscript by Gupta et al. has been improved to a satisfactory degree. Now I recommend this for publication in the current form.

Reviewer 2

Advance summary and potential significance to field

This manuscript investigates the mechanisms by which Myosin Vb interactions with rab proteins regulate epithelial membrane trafficking, and reveals that TOR signaling and MyoV/rab protein interactions are required for homeostatic changes in plasma membrane size. The study uses a combination of beautiful in vivo microscopy and elegant genetics, and the results have broad implications for the regulation of membrane homeostasis in epithelial cells.

Comments for the author

This interesting manuscript has been improved by revision. The authors have been exceptionally responsive to reviewers, adding considerable new data that strengthen their conclusions. I was particularly impressed to see the validation of the MyoV mutants (supp Fig 2), and rescue experiments to validate the morpholino (supp Fig 3). These were not trivial experiments, but they were important, since they add confidence in critical tools. In addition to these new experiments, the authors have revised the text for clarity and added data quantification for several experiment. This manuscript is rigorous, thorough, and makes important advances in understanding molecular mechanisms of membrane homeostasis in epithelial cells.