Rab21 in enterocytes participates in intestinal epithelium maintenance

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March 31, 2021

RE: Manuscript #E21-03-0139
TITLE: The early endosomal protein Rab21 is critical for enterocyte functions

Dear Prof. Jean:

Thank you for submitting your interesting manuscript on the role of Rab21 in adult Drosophila enterocytes to MBoC. I have looked over the reviews and your revision plan, and I feel that your responses to the reviewers' comments seem quite reasonable. I do have two additional thoughts regarding the proposed revisions:

1. In response to Reviewer comment 2, it could also be helpful to express a constitutively-active version of Rab11 to determine whether this suppresses the observed defects.

2. In response to Reviewer comment 6, it would be worth testing whether knockdowns of the candidates you selected (Tret1-1, hermes and ApoLpp) suppress any of the Rab21 phenotypes.

When revising the manuscript, it would be helpful if you would number the pages and use a different font color to indicate changes in the text.

I will endeavor to make a decision on the manuscript without re-review. However, if necessary, I may decide to consult with one or more of the previous reviewers.

I look forward to receiving your revised manuscript.

Sincerely,

Julie Brill
Monitoring Editor
Molecular Biology of the Cell

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Dear Prof. Jean,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made, as described in the Monitoring Editor's decision letter above and the reviewer comments below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

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Thank you for submitting your manuscript to Molecular Biology of the Cell. We look forward to receiving your revised paper.
Sincerely,

Eric Baker  
Journal Production Manager  
MBoC Editorial Office  
mbc@ascb.org
RESPONSES TO REFEREE COMMENTS

Reviewer #1:

**Reviewer comment 1:** While the authors assert that the RNAi constructs targeting Rab21 have been previously validated, it would be helpful to demonstrate knockdown efficiency (by qRT-PCR) for the two Rab21 RNAi constructs (in enterocytes if possible). Complementation needs to be shown for both RNAi constructs for key assays (PH3 and CC3 for example).

**Author response:** We thank the reviewer for their helpful comments, which have resulted in an improved manuscript. As suggested, qRT-PCR and western blot analyses validating the Rab21 RNAi efficiencies in enterocytes have been added to the manuscript (Supp. Fig. 2A, B).

Concerning the complementation requirements mentioned by the reviewer, we would like to emphasize that we provide rescue evidence for intestinal organization (Fig. 1G) as well as pH3 positivity for both RNAi reagents used (Fig. 3D). Moreover, we used a dominant negative construct (Rab21-DN) to further assess their specificity in various settings. Thus, we believe that the two rescue experiments and the use of two independent tools (RNAi and the DN construct) adequately demonstrates the specificity of our reagents and have not performed additional complementation experiments, electing to focus on other requested experiments that we believed are more beneficial to the manuscript. Nonetheless, we have added a sentence regarding the limitations of the RNAi approach (Lines 295 and 296).

**Reviewer comment 2:** The CC3 data in 3A mostly looks like an outlier effect. Not sure this result is even correct. Conversely the data in 3E and F looks quite compelling and the mechanisms connecting Rab21 to this change is not pursued. This is the strongest phenotype in the paper.

**Author response:** Upon close examination, although there were few individual intestines with high cCASP3 staining, we were able to support increased cell death in Rab21-depleted guts by SYTOX assays (Fig. 3B) and qRT-PCR of the caspase activator hid (Supp. Fig. 3A). Furthermore, the functional significance of apoptosis to the observed Rab21 phenotype was demonstrated by rescue experiments with the caspase inhibitor p35 (Fig. 4C, D). Importantly, additional rescue experiments with yki RNAi
also reinforced the experiment by again demonstrating that loss of RAB21, with two independent RNAi reagents, leads to increased cCASP3 staining (Fig. 4E).

We thank the reviewer for acknowledging the importance of the upd3 and JAK/STAT phenotypes. As mentioned, we did not follow up on the direct involvement of Rab21 in these cell responses, and we apologize for that. The reason why we did not pursue it further is because we believe that JAK/STAT pathway activation is a downstream effect of Rab21 depletion, due to increased upd3 secretion, which is enhanced via yki activity and cell death (Fig. 4F). We showed that apoptosis inhibition rescues JAK/STAT activation in Rab21-depleted enterocytes (Fig. 4C), supporting our hypothesis. Following the reviewer’s suggestion, we have added epistatic experiments showing that co-depletion of Rab21 and yki is sufficient to reduce JAK/STAT activation (Fig 4C), which is consistent with this hypothesis and further strengthens the link between Rab21, yki, and JAK/STAT.

**Reviewer comment 3:** The data in Fig 5 shows weak effects for many genes and does not advance the mechanism. The proportion of pH3+ cells is very small in all experiments. While some manipulations result in statistically significant changes in this measure, is this a biologically meaningful or relevant effect? Can this be normalized to the number of stem cells instead of the total number of cells?

**Author response:** The screen targeted genes involved in specific membrane trafficking steps and genes related to previously described Rab21 functions, to get insights about the precise trafficking pathways phenocopying Rab21 loss in enterocytes. It is important to note that pH3+ cell percentages were assessed as the total number of pH3+ cells in a region of interest (in the distal part of the posterior midgut) divided by the total number of cells (revealed using DAPI staining) in the same region. Hence, the small percentages result from the low number of pH3+ cells compared to total cells. We used this approach to determine if the increase in pH3+ cells was dependent or independent of increased cell density. The quantification method used has been better detailed in the materials and methods section (lines 919 to 925). To keep data representation consistent throughout the manuscript, we decided to keep the proportion of pH3+ cells. Nonetheless, we have graphed the absolute number of pH3+ cells below, as suggested by the referee. We believe that the large differences observed using this type of representation supports meaningful and relevant differences in pH3+ cells.
Proliferation is increased upon knockdown of specific membrane trafficking genes. Absolute quantification of pH3+ cells in the distal part of the posterior midgut.

Reviewer comment 4: The statistical tests used for determining significant differences between groups are often incorrect: for example a one-way ANOVA should be used for all experiments with >2 conditions.

Author response: In the revised manuscript, we have used *t*-tests and Mann Whitney U tests for pairwise comparisons as done in the literature (Chakrabarti et al., 2014; Chen et al., 2020; Du et al., 2020; Hao et al., 2020; Houtz et al., 2017; Jin et al., 2020; Li et al., 2018; Liang et al., 2017; Mundorf et al., 2019; Nagai et al., 2021; Nie et al., 2019; Xiang et al., 2017; Xu et al., 2017; You et al., 2014; Zhang et al., 2019; Zhou and Boutros, 2020). After discussing the matter with our institute statistician, we have used one-way ANOVA followed by Dunnett’s tests when multiple conditions were tested (Fig.1 F, Fig. 2C, D, E, Fig. 3A, B, C, E, Fig. 5C, D, Fig. 6A, C, Fig. 8D, Fig. 9E, Supp. Fig. 3C, Supp. Fig. 4A, B, C, D, Supp. Fig. 5A, B, Supp. Fig. 6B).
Reviewer comment 5: The survival data are compelling and would be better suited in figure 1 rather than in the supplemental material (an analysis of developmental delay and/or size/weight would further support the effects of Rab21 loss on viability).

Author response: As suggested, we incorporated the survival data in Figure 1 (Fig. 1J). Since Rab21 knockdown is only performed in adult flies, we do not expect developmental delays. Size/weight analyses would be of great interest, and represent nice follow up experiments for future studies.

Reviewer comment 6: The proteomics experiment included at the end (figure 7) seems out of place and leaves the reader wondering about the role of the differentially abundant proteins in rab21 deficient flies - strongly suggest removing these data from the manuscript unless detailed follow-up experiments are performed. In their current state, these data do not support the rest of the narrative.

Author response: We agree that without follow-up, the proteomic data leaves the reader with a lot of questions. Since these data provide exciting fodder for future studies, and are therefore a potential resource for the community, we decided to include the data in the manuscript. The proteomic experiment was initially designed to get insights into the physiological defect(s) caused by Rab21 depletion, hoping that it would uncover specific roles of Rab21 in enterocytes causing the various phenotypes. The results have been promising, as they identified defects in unexpected physiological processes (sugar transport, lipid metabolism, as well as nutrient digestion). We think these data are of interest and point to Rab21’s functions in enterocytes. To expand on these results as suggested by the referee, and solidify the manuscript, we decided to perform follow up experiments assessing the main physiological aspects highlighted by the proteomic data.

Although, it was difficult to find tools, we managed to obtain transgenic flies or antibodies for three important hits: Tret1-1 (a trehalose transporter), apolpp (an apolipoprotein) and hermes (a monocarboxylate transporter), all of which were more abundant in Rab21-depleted flies. Using transgenic flies expressing HA-tagged Tret1-1 and an antibody against apolpp, we confirmed that these proteins accumulate in the intestinal epithelium (Fig. 8B, 9B) upon Rab21 loss. Unfortunately, we were unable to generate recombinant lines (Myo1A<sup>RNAi</sup>, GFP:hermes or Rab21<sup>RNAi</sup>, GFP:hermes) allowing us
to analyze hermes abundance upon Rab21 depletion in enterocytes. Apolpp and Tret1-1 are involved in lipid and carbohydrate transport, respectively, therefore we investigated both aspects using different approaches:

For **apolpp**, based on its function (Figure 8A; manuscript lines 507-509), we evaluated lipid transport and lipid droplet morphology upon Rab21 depletion from enterocytes. Interestingly, we found decreased lipid droplet sizes upon Rab21 knockdown (Fig. 8C). To further refine these observations, we also assessed lipid droplet morphology in the main TAG storage organ: the fat body. Nile Red staining highlighted an increase in lipid droplet sizes when Rab21 was depleted from enterocytes compared to controls (Fig 8E). We also quantified circulating lipids (mono-, di-, and triacylglycerol) in the hemolymph of adult flies, and did not detect any differences (Fig. 8D). Because apolpp is mainly produced by the fat body, we also investigated the impact of apolpp knockdown in the fat body on the intestinal epithelium. Surprisingly, fat body apolpp inhibition tended to decrease proliferation (although not significantly) while tissue morphology was not affected (Suppl. Fig. 7A-B). Although apolpp is barely expressed in the gut (Palm et al. 2012), we nevertheless attempted apolpp silencing in enterocytes. Interestingly, we observed that lipid droplets sizes were decreased in apolpp-depleted enterocytes compared to controls (Suppl. Fig. 7C), while their density was increased (Suppl. Fig. 7C). Tissue morphology was also affected, while proliferation was unchanged (Suppl. Fig. 7D-E). Finally, we co-inhibited Rab21 and apolpp in enterocytes to assess if apolpp accumulation was responsible for some of the epithelial defects observed upon Rab21 knockdown. Apolpp co-depletion with Rab21 did not affect the increased proliferation or lipid droplet size modulation caused by Rab21 loss (Fig. 8F-G). Nonetheless, these findings are exciting and indicate a role for Rab21 in lipid metabolism in gut enterocytes, something previously unknown. These results also emphasize potential cross-talk and compensation between the fat body and the gut upon defective lipid absorption or mobilization in the gut. Finally, these results have potential clinical significance, as two recent manuscripts linked SNPs in human **RAB21** to improper lipid levels.

**Tret1-1** is a trehalose transporter. Confirming the mass spectrometry data, we observed higher levels of transgenic HA-tagged Tret1-1 upon Rab21 knockdown (Fig. 9B). Given the increased Tret1-1, we assessed circulating concentrations of trehalose in the hemolymph of adult flies depleted of enterocyte Rab21 (Fig. 9E). Because trehalose is composed of two glucose molecules, and previous studies
highlighted a role for it in circulating glucose transport regulation, we also quantified the glucose level in the hemolymph. This experiment highlighted a decreasing tendency of both trehalose and glucose levels upon Rab21 depletion (Fig. 9E). To assess Tret1-1 involvement in gut maintenance, we depleted Tret1-1 in enterocytes and analyzed intestinal stem cell proliferation and tissue architecture. No changes in proliferation were observed, but the tissue architecture was modified by Tret1-1 knockdown (Fig. 9C-D). Finally, to test if increased levels of Tret1-1 were linked to Rab21 phenotypes, we co-depleted Rab21 and Tret1-1 in enterocytes. These experiments did not show a rescue of Rab21 loss of function-related phenotypes (Fig. 9F).

In these experiments, we confirmed two hits from the mass spectrometry analysis. Furthermore, we highlighted a lipid uptake (and/or) mobilization defect in Rab21-depleted guts and found a role of apolpp in enterocytes. Furthermore, we confirmed that Rab21 also affects Tret1-1 levels, with slight decreases in circulating glucose and trehalose levels. Epistasis experiments demonstrated that the intestinal phenotypes observed upon Rab21 loss are not caused by either increased ApoLpp or Tret1-1. These novel experiments did not allow us to pinpoint the cellular mechanism by which enterocyte Rab21 loss causes compensatory proliferation and tissue disorganization. Nonetheless, we identified two new important functions for Rab21 linked to lipid and trehalose transport or metabolism in enterocytes. How Rab21 is involved in these specific functions in enterocytes remains to be elucidated. These findings will serve as foundation for future studies aimed at better define trafficking events in enterocytes and how they affect their absorptive functions.

**Reviewer comment 7:** How is it possible that early endosome formation is perturbed in Rab21-deficient IECs yet late endosome and lysosome formation are normal? The authors should address this more carefully and thoroughly in the discussion.

**Author response:** This is now addressed in the revised manuscript (manuscript lines 377-381).

**Reviewer comment 8:** The authors should more explicitly indicate what time point(s) were analyzed. Were all experiments performed at 10 days post-eclosion? If so, why? Analysis of the effects of cell death and proliferation on physiology is difficult to interpret from a single time point, and kinetic experiments would greatly benefit this study.
Author response: All experiments from the manuscript were performed at 10 days post-eclosion, except the proteomic experiment, which was done at 5 days to get an accurate idea of Rab21-specific contributions rather than secondary effects. We did perform a time-course analysis of proliferation and cell junction marker disruption upon Rab21 depletion (Rab21 RNAi-1 and -2, and with Rab21\textsuperscript{DN}) at earlier time points (3 and 5 days). While these phenotypes were already strong for Rab21 RNAi-1 at 3 and 5 days, Rab21 RNAi-2 and Rab21\textsuperscript{DN} did not show strong phenotypes at these time points (Suppl. Fig. 2D, 3C). In a gut autophagic-deficient context, it was recently shown (Zhang et al., 2019) that all pathways known to enhance intestinal stem cell proliferation can rescue overproliferation at an early time point (5d) but at a later time point (10d). Therefore, we opted for the 10 day time point for epistasis studies to (1) easily discriminate between a rescue or lack of one and (2) characterize specific downstream effectors. Experiments performed at earlier time points have been added to the manuscript (Suppl. Fig. 2D, 3C) as well as a discussion of the stronger impact of Rab21 RNAi-1 at earlier time points l (manuscript lines 295-299).

Reviewer comment 9: The Myo1A driver used in this study has expression restricted to enterocytes. However, in many of the experiments reported in this manuscript (eg Fig3, Fig4, Fig5, and Fig6), the assays lack sufficient cellular specificity and resolution to delineate autocrine vs paracrine effects. Moreover, the mosaicism of the knockdown is not clear. Many experiments would be more convincing if performed with dual transgenes or labeling (e.g., pH3 staining with prospero or DL:GFP; SYTOX / upd3:LacZ / 10xSTATE92E-GFP + Myo1A reporter).

Author response: As described at the beginning of the manuscript (Fig. 1A-C), Rab21 is not homogeneously expressed in enterocytes. Its expression is higher in distal enterocytes of the posterior midgut, while it is weakly expressed in the proximal part. Importantly, even in the distal region, Rab21 is not present in all enterocytes (Fig. 1B-D). Therefore, what reviewer refers to as “knockdown mosaicism” is probably a consequence of Rab21’s particular expression pattern. We would expect that Rab21 depletion would affect only enterocytes expressing it. To gain a broad overview of Rab21’s effects and pinpoint its specific functions, we initially decided not to look at the cellular level, particularly because we are unable to discriminate between enterocytes that have been depleted of Rab21 versus those that do not express it. Thus, it is difficult to get any information about autocrine vs
paracrine effects by looking at cell types. Nevertheless, we agree with the referee that dual transgenes/labeling for pH3 with Delta and Prospero is informative in this context; therefore, we performed these experiments and incorporated them into the revised manuscript (Suppl. Fig. 3B). Given the scope of the manuscript and the fact that we already showed dual-labeling for dying cells with an enterocyte reporter (Fig. 3A), we believe that although interesting, performing new dual transgenes labelling for upd3:LacZ and stat92E will not advance our mechanistic findings further. Nonetheless, we provided ‘zoomed-in’ views of upd3:LacZ stain to allow better assessment of the affected cells (Suppl. Fig. 3D). In addition, we included upd3:LacZ/cCASP3 dual staining to highlight their lack of strong overlap (Suppl. Fig. 3E).

**Reviewer comment 10:** Many signal transduction pathways converge on ERK phosphorylation and the authors over-interpret their observations: in the absence of EGFR knockdown or pharmacological blockade, it is impossible to determine whether differences in dpERK staining are in fact due to changes in EGFR signaling.

**Author response:** ERK phosphorylation is indeed the consequence of various transduction pathways. Because we could rescue the increased proliferation induced upon Rab21 depletion via overexpression of EgfrDN, which often used in the field to conclude on the requirement of EGFR signaling (Buchon et al. 2010, Gervais et al., 2019), we concluded that increased dpERK was linked to increased Egfr signaling. To support this conclusion, we have added qRT-PCR for the Egfr pathway target genes Mkp3 and argos upon knockdown of Rab21, showing increased transcript levels for these genes (Suppl. Fig. 6A).

**Reviewer comment 11:** The author's use of the term "homeostasis" is an overstatement of their findings: the data reported in this manuscript do not test enterocyte functions, and the inference of lost intestinal homeostasis is largely based on the proportions of apoptotic and proliferation cells, which is an oversimplification.

**Author response:** By “homeostasis”, we meant tissue equilibrium. Because this equilibrium is lost (cell type proportions impaired, proliferation increased, cell junctions affected, etc.) upon loss of Rab21 we
believed that it was correct to say that Rab21 was required for proper tissue homeostasis. We have modified the term “homeostasis” throughout the revised manuscript.

Reviewer comment 12: The authors should attempt to reconcile the apparent increase in apoptosis, the modest increase in pH3 staining, and the striking increase in total cellularity in the discussion.

Author response: Apart from an increase in apoptosis and pH3 staining, we also showed increases in prospero\(^+\) and delta\(^+\) cells (Fig.2D, E) which mostly account for the increase in total cellularity. This point is addressed in the revised discussion (manuscript lines 585-586).

Reviewer comment 13: In figure 3A, the cCASP3 staining in the Rab21 RNAi image seems much more than ~1.5 fold greater than the control (which is the effect size portrayed in the quantification). Are these images truly representative?

Author response: While the image is representative, we have exchanged it with one showing a weaker effect (Fig.3A). We have also provided more details regarding the image analysis pipeline in the materials and methods section.

Reviewer comment 14: The vast majority of the findings presented in this manuscript are derived from epistasis experiments, and while a summary model would help the reader synthesize the convergent and independent pathways that result in the observed phenotypes, the authors should take care when linking genes in the Rab21 pathway; moreover, cross-talk between cell types should be more carefully assessed.

Author response: We totally agree with the reviewer. We have modified the initial summary model to make it easier for the reader to understand and added a more general one including all epistatic experiments performed (Fig. 4F). Assessing crosstalk between cells is an interesting aspect; unfortunately, we believe that it represents the next step in our experiments and will be studied in more detail in follow-up studies. Nonetheless, we have mentioned this in the revised discussion (manuscript lines 632-644).
Reviewer comment 14: The title is misleading: with the exception of the barrier function assay (the results of which have been relegated to supplemental material), none of the data presented in the manuscript are from experiments which test the "functions" of enterocytes.

Author response: We agree with this comment and have modified the title accordingly.

Reviewer comment 15: Key details regarding experimental design and materials and methods are generally lacking.

Author response: We have made our best efforts to incorporate all necessary information in the revised materials and methods section (manuscript lines 777-778, 824-827, 847-856, 874-879, 896-925, 933-934, 937-938).

Reviewer comment 16: Please clarify sample sizes in figure legends and indicate what the points of scatter dot plots represent (individual flies, fields, etc).

Author response: Although this information was provided in the methods, we now have incorporated it in the figure legends as well. Dots from scatter dot plots correspond to individual flies. If an experiment has no graph, the sample size is indicated in the figure legend. The number of independent experiments corresponds to N and is incorporated in all figure legends. All quantifications/analyses were performed in the distal part of the posterior midgut. This information has been incorporated in the materials and methods section (manuscript lines 920-921, 937-938).

Reviewer comment 17: Please clarify in the materials & methods how image analysis was performed for quantification of "normalized signal intensity" (Figs 3A, 3B, 3E, 3F, 5D, 6A, 6C).

Author response: We apologize for not making this point clearer in our original submission. This point has been clarified in the revised manuscript (manuscript lines 923-925).

Reviewer comment 18: In figure 1G, why is quantification of lumen area expressed as a normalization instead of absolute area? Also, please clarify what region(s) were analyzed for this quantification.
Author response: We have included the absolute area measurement in the revised manuscript (Fig. II). Distal parts of posterior midguts were analyzed. This information has also been added to the manuscript (manuscript lines 920-921).

Reviewer comment 19: The quantification shown in figure 1H is rather coarse and could be improved: cell density, intestinal thickness, or intestinal cellularity would be more satisfying metrics.

Author response: We hope that we are not misinterpreting the comment, but cell density analyses were incorporated in the original manuscript. As suggested by the reviewer, in order to support quantification shown in Fig. 1H, we now have incorporated cell density analysis in figure 1 (Fig. 1F) from the revised manuscript.

Reviewer comment 20: In the materials and methods, please provide sequences and/or a schematic (suitable for supplemental information) for the Rab21Deg allele.

Author response: We have added this sequence information into the manuscript (Supplemental Table 1).

Reviewer comment 21: The composition of the loading buffer used for protein extraction is confusing: Tris is listed twice (at two different concentrations and pH)

Author response: We thank the reviewer for noticing this error, which has been corrected (manuscript lines 875-879).

Reviewer comment 22: Regarding TEM in the materials and methods: reference 58 is likely inappropriate as this paper did not perform TEM on drosophila. Also, please include the following details: what region(s) of the intestine were analyzed, what embedding medium was used, what fixatives and stains were used after sectioning, what was the operating keV during image acquisition.

Author response: This information has been incorporated (manuscript lines 848-856).
Reviewer comment 23: The authors should add additional detail in the materials and methods regarding in situ hybridization assays: what kit(s) were used for probe synthesis and labeling, was a sense probe used for a control, and provide catalog numbers for reagents used.

Author response: This information has been added in the materials and methods section (manuscript lines 824-827)

Reviewer #1 (Significance (Required)):
Nature and significance of advance: The study advances of the body of knowledge regarding the roles of Rab family small GTPases in intestinal epithelial cell biology using an in vivo model (i.e. Drosophila). While this protein has been studied in epithelial cell culture systems, its role(s) in intestinal epithelial biology in vivo remain poorly characterized. Moreover, the data presented suggest the possibility of evolutionarily conserved roles for this protein in the intestine.

Context: A quick google search indicates there isn't much literature investigating the role of Rab21 in vivo

Audience: This work would appeal to scientists interested in: novel in vivo models to investigate the functions of Rab GTPases, regulators of endocytic recycling and trafficking pathways

Expertise: Intestinal epithelial cell biology.

Author response: We thank the reviewer for highlighting the novelty of this study and how it will be of interest to a broad public.

Reviewer #2:

Reviewer comment 1: First, the major effect of Rab21 loss in the intestine seems to be an effect on the progenitor cells and lineage differentiation. This is interesting because previous studies in mammalian
systems suggested that levels of Rab21 were low in the proliferative crypts in the small intestine. This. Difference should be discussed.

**Author response:** We thank the reviewer for their helpful comments, which have resulted in an improved manuscript. In our study, we are assessing Rab21 function in differentiated cells—enterocytes. Although knockdown of enterocyte Rab21 affects intestinal stem cells, this effect is likely not direct, as shown by our epistasis experiments (Fig. 3 and 4). In mammals, Rab21 has low and high expression levels in intestinal stem cells and villi, respectively (Zhang et al., 2016), which is akin to what we observed in flies. This point is now discussed in the revised manuscript (manuscript lines 588-591).

**Reviewer comment 2:** The screens in Figure 5 examine autophagy and find some subtle effects and suggest an effect of Rab21 on EGFR signaling, a finding that has previously be noted in mammalian cells. It is unclear to this reader why effects of Rab21 in relation to the recycling system (Rab8 and rab11) are not examined.

**Author response:** A few studies have shown that knockdown of Rab11 in fly enterocytes leads to inflammation and increased proliferation (D’Agostino et al., 2019; Nie et al., 2019; Yu et al., 2014). Rab11 acts synergistically with the RasV12 oncogene to induce inflammation (Nie et al., 2019). To assess whether Rab21 phenotypes are associated with defects in endosomal recycling, we overexpressed Rab11 in Rab21-depleted enterocytes and monitored if increased recycling could rescue the loss of Rab21, using proliferation as a readout (Suppl. Fig. 5C). Rab11 activation in the context of Rab21 loss did not rescue the increased proliferation, suggesting that the effects of Rab21 and Rab11 are independent, or that Rab21 acts upstream of Rab11 and its loss is dominant and non-rescuable by increased Rab11 activity.

**Reviewer comment 3:** The studies show changes in the expression of some SLC proteins but there is little investigation of the importance of these changes and even their relevance to endocytosis on the apical versus basolateral membranes. Indeed this is one of the most confusing aspects of this work is there is no clarification of what the significance is for Rab21 in apical versus basolateral membrane endocytosis and how specific cargoes are altered.
Author response: We completely agree with this comment. The problem we had at the time of submission was that most SLC proteins we identified had no described localization patterns in enterocytes. Unfortunately, during our revision, we only managed to obtain constructs for Tret1-1 and Hermes (SLC16A12), and Hermes knock-in flies proved difficult to work with. Given these limitations, we were unable to test the impact of Rab21 loss on an SLC protein and study the apico-basal localization of that SLC. Since Rab21 does not show an apico-basal polarity, we would hypothesize that both types of cargos could be affected. We have addressed the point raised by the referee in greater details in the discussion. Nonetheless, we have acquired some tools that have allowed us to expand on the importance of Rab21 on Tret1-1 levels (please read the reply to Reviewer 1’s Comment #6”).

Reviewer comment 4: It is unclear to me what the authors mean by "intestinal homeostasis". Is this normal proliferation? Normal differentiation? Normal expression of transporters? In the end I am left with a number of interesting findings that are relatively correlative but do not lead to clear insights into cell physiology.

Author response: By using “homeostasis” we meant “tissue equilibrium”. Therefore, changes in tissue composition, due to modifications in intestinal cell numbers or ratios would modify this equilibrium. For easier understanding, we have replaced the term “homeostasis” throughout the manuscript. To expand on the physiological effects of Rab21, we now performed new experiments to validate two proteins modulated by its depletion in enterocytes (please see Reviewer 1-Comment #6). We have also modified the discussion for clarity and to discuss the limitations of our findings.

**Other concerns:**

Reviewer comment 5: The introduction refers to a number of papers dealing with vesicle trafficking associated with Rab11 and Rab8, these should really be referred to as Rab11a and Rab8a.

Author response: We have made this correction (manuscript lines 82, 84 and 90).
**Reviewer comment 6:** The exact mutations for dominant negative and dominant active Rab21 forms should be noted at their first introduction.

**Author response:** We added the information in the text for dominant negative Rab21 (manuscript line 740). Because we do not show data with activated Rab21 in the revised manuscript, we did not include this information.

**Reviewer comment 7:** Many of the references appear incomplete.

**Author response:** The references have been corrected.

**Reviewer comment 8:** There is no statistical analysis performed in figure 1G.

**Author response:** Because we only have two individual samples (arising from two independent experiments) from the LacZ control, we did not perform a statistical test. This fact is highlighted in the figure legend.

**Reviewer #2 (Significance (Required)):**
The final significance of this paper is not clear because it is mostly descriptive without really defining specific changes in cell physiology in any detail.

**Author response:** We thank the reviewer for pointing this out, and apologize for including too many messages without clearly identifying the important and mechanistic findings of the manuscript. These aspects have been clarified and better integrated into the revised manuscript.

**Reviewer #3:**

**Reviewer #3 (Evidence, reproducibility and clarity (Required)):**
Overall, this is a high-quality fly genetic study of Rab21, and the authors presented evidence to suggest that in fly midgut enterocytes, Rab21 regulates upd3, EGFR signaling, proliferation, and autophagy.

**Major comment**

**Reviewer comment 1:** The conceptual significance should be emphasized by the authors further. What was previously found in mouse and human for Rab21 should be detailed in introduction.

**Author response:** We thank the reviewer for their helpful comments, which have resulted in an improved manuscript. As suggested, we have emphasized the conceptual significance, using new data incorporated in the revised manuscript (please read Reviewer 1- Comment #6). Although there is not much known about Rab21 in mammalian intestines, we have provided these details in the introduction (manuscript lines 120-126).

**Some moderate comments**

**Reviewer comment 2:** In Introduction, "Mutations in membrane trafficking genes are associated with a large array of human diseases [2]. Importantly, some of these mutations affect several tissues, while others are restricted to one organ system, and can be used to shed light on the cell type-specific functions of vesicular trafficking genes." These statements seem too general, vague, and lack details.

**Author response:** We apologize for the lack of clarity and have modified this text (manuscript lines 56-61).

**Reviewer comment 3:** The author should also elaborate on "Rab21 is modulated by stress in both flies and in mouse models of IBD [25,41]." What were found by these studies? Should comment on human Rab21, whether mutation or overexpression have been found.

**Author response:** We have included additional details about these aspects in the introduction (manuscript lines 126-129, 183-184, 710-713).
Reviewer comment 4: "microvillus inclusion and chylomicron retention disease, are associated with mutations in the membrane trafficking related-genes myosin 5B (MYO5B) and secretion associated Ras related GTPase 1B (SAR1B), respectively [2,5,6]." Here, the author should cite work for STX3. Also missing references reporting the contribution of Rab11a and Cdc42 to microvillus development in next section that discussed enterocyte morphogenesis (PMID: 22354172; PMID: 28596241).

Author response: We apologize for this oversight and have added the missing references (manuscript lines 73, 74, 88, 92).

Reviewer comment 5: In Results: "with no restricted basal or apical localization (Suppl. Fig. 1A)." Was this referring to GFP or endogenous Rab21? If it was GFP, then polarized distribution may not be expected.

Author response: This refers to the expression of a GFP:Rab21 transgene using the Rab21 promoter. We have clarified this in the text (line 187).

Reviewer comment 6: "As previously observed in human and mouse intestines, these data show that Rab21 is expressed throughout the fly gut, with high expression in enterocytes, suggesting potential important functions in these cells." What was previously observed in human and mouse intestines for Rab21?

Author response: We now changed the sentence to make this clearer and describe the expression patterns observed in previous investigations of Rab21 in mammals in the introduction (manuscript line 187).

Reviewer comment 7: Fig. 1G was described after 1H.

Author response: We have corrected this.
Reviewer comment 8: "Rab21 loss-of-function through either RNAi or Rab21DN expression negatively affected lifespan (Suppl. Fig. 2E)." Do they mean Rab21 loss-of-function increased the lifespan?

Author response: This sentence has been changed (manuscript line 234). It meant that loss of Rab21 in enterocytes reduces survival.

Reviewer comment 9: "Autophagy and EGFR signaling are independent in enterocytes." Instead of making this sweeping claim, consider change this subtitle to relate to Rab21.

Author response: We have made this modification (manuscript line 385).

Reviewer comment 10: Fig. 4E, can the authors look at apoptosis in yki/Rab21 double RNAi, to establish the link? This is very interesting.

Author response: We thank the reviewer for this interesting suggestion. As suggested, we performed the experiment and observed that co-depletion of Rab21 and Yki rescues apoptosis. We have included these data in the revised manuscript (Fig. 4E).

Reviewer comment 11: Fig. 5 was impressive. Are these vesicular genes function in the same pathway of Rab21?

Author response: We thank the reviewer for their positive assessment of Figure 5. All the genes shown affect specific steps of endosomal and autophagy trafficking and some are related to previously described functions for Rab21.

Reviewer comment 12: Fig. 6D: "inhibition of EGFR signaling was unable to rescue the increased mitotic activity observed in guts depleted of autophagic genes (Figure 6D)." Can inhibition of upd3 rescue the increased mitotic activity in these flies?
**Author response:** This is an interesting question. While our manuscript was under review at *Review Commons*, a manuscript studying the role of autophagy in enterocytes showed that it indeed does (Nagai et al., 2021). Therefore, we did not assess this for our revised manuscript, but have mentioned this new finding in the manuscript.

**Reviewer comment 13:** Elaborate about TMT-based quantitative proteomic analysis, whether it only assesses differential protein abundance rather than assess Rab21 interactome?

**Author response:** The TMT approach we used gives an overview of differential protein abundances and does not assess the Rab21 interactome.

**Reviewer comment 14:** Have the authors considered transcriptome analysis of Rab21RNAi midgut?

**Author response:** We opted for proteomic instead of transcriptomic analyses because we already had an idea of the signaling pathways impacted by Rab21 depletion. We reasoned that the proteomic study could give us a different overview of the physiological aspects impaired by enterocyte Rab21 depletion.

**Reviewer comment 15:** Fig. 7 seemed detached from the major theme and early parts of the paper.

**Author response:** We agree with this remark. To overcome this issue, we acquired new tools and performed more experiments (please read Reviewer 1- Comment #6), which improves the cohesion between Fig.7 and the earlier results.

**Reviewer #3 (Significance (Required)):**

The data are strong and potentially significant in terms of enterocyte-intrinsic regulation of inflammatory and growth signaling. The conceptual significance should be emphasized further by the authors in revision.

**Author response:** We have now provided new data and modified the text to emphasize the conceptual significance of our study.
2nd Editorial Decision

RE: Manuscript #E21-03-0139R

TITLE: Rab21 in enterocytes participates to intestinal epithelium maintenance

Dear Prof. Jean:

Thank you for submitting your revised manuscript (previously reviewed through Review Commons) to Molecular Biology of the Cell. I have now gone through your revisions and compared these to the comments of the reviewers. I feel you have addressed nearly all of their comments and that the manuscript is quite close to being ready for publication.

Below is a list of additional comments on the manuscript and figures that I feel must be addressed. I am also attaching copies of pdf files of the manuscript and the supplementary figure legends (now combined with the supplementary table) to which I have added comments in Adobe Acrobat. Feel free to contact me through the journal if you find that my comments are unclear.

I look forward to receiving a re-revised version of your manuscript. Please ensure that the line numbers in the point-by-point response match those in the revised manuscript, as this will greatly facilitate my review of the next revision.

Sincerely,

Julie Brill
Monitoring Editor
Molecular Biology of the Cell

OVERALL COMMENTS ON MANUSCRIPT

1. Using the Rab21-GAL4 driver to drive expression of UAS-GFP:Rab21 could result in overexpression because GAL4 itself can amplify gene expression by binding multiple UAS sites (this is really not equivalent to putting a transgene under direct control of the Rab21 promoter). Thus, I feel your response to Reviewer #3, comment 5 is not quite adequate. If the GFP:Rab21 fusion is overexpressed, this could potentially mask any apicobasal polarization of the protein. For this reason, a caveat about the interpretation of the localization data should be included in the manuscript.

2. In several places in the manuscript, manipulations of Rab21 are described as "loss-of-function experiments", but RNAi and dominant-negative constructs are not the same as null mutations, which are the standard for "loss-of-function experiments". I suggest finding a different way of describing these experiments or just spelling out what was actually done.

3. The data included in response to Reviewer #1, comment 3 (absolute quantification of pH#+ cells in the distal part of the posterior midgut) should be included as a supplemental figure.

4. In general, it would be clearer if Drosophila protein names were capitalized throughout the manuscript (both in the text and in the figures), as this will make it clear to the reader that proteins are being described.

5. The word "guts" is a colloquial term and is a bit overused for my taste. I suggest using "intestines" instead in a number of places in the text (these are indicated in the uploaded pdfs).

6. In quite a few of the figures, the layout is awkward, and a number of the panels have text that is too small to easily read when printed.

7. The figure legends are quite long and could be trimmed without losing essential information.

8. The degenerate Rab21 sequence listed as Table 1 should be included as a supplementary figure rather than a table, and it would help to show an alignment with the starting sequence, as well as the locations of the hairpin RNAs in RNAi-1 and 2.

SPECIFIC COMMENTS ON THE FIGURES

Figure 1D: The stem cells marked with arm staining that are pointed at by the arrows are quite small. An inset would help the reader see these cells more clearly.

Figure 1E: The font used at the top of this panel is quite small and hard to read (except for "Myo1A").

Figure 1F: Why does the image not extend to the edges in the lower panel? Also, there is something in the box below the "Rab21^RNAi" label.

Figure 1H and 1I: The labels on the graphs are much too small to read; the graphs could be larger and still fit nicely in the figure.

Figure 1J: Why are there error bars for the Rab21 DN genotype if N=2?

Figure 2A: The diagram is quite pixilated and seems low-resolution. The labels for the cell types are incredibly small and nearly impossible to read.

Figure 2B: The blue label for DAPI is invisible on the black background. It should be a brighter/ lighter colour so it can be seen.

Figure 4E: Stats are not appropriate for experiments where N=2.

Figure 5A: The diagram is quite fuzzy and pixilated and seems low-resolution. The yellow-orange colour of the label for the CORVET complex is nearly impossible to see on a white background. A few genes are missing from the list on the right: Atg1, Vps8, Rab4. Also, the colours for endocytosis and the Wash complex are very close and difficult to distinguish.

Figure 6E: As mentioned above, "loss of function" suggests that null mutants were examined, but this is not the case here. Use of some other term or abbreviating knockdown (KD) and dominant-negative (DN) (while defining these abbreviations in the figure legend) would better convey what was actually done.

Figure 8A: This diagram is fuzzy and pixilated similar to the others. The blue text should say "lipid transport", not "lipids".
mobilization", as apolpp transports lipids in the hemolymph ("lipid mobilization" suggests release of lipids from lipid droplets, etc.).

Figure S1B: Rab21 label should be italicized to indicate that the transcript is being examined. Also, it is very hard to see the blue and green on black in the lower right panel, and the nuclei the arrowheads are pointing at are not visible in the grayscale DAPI panel at the top because the scale bar is on top of one of the cells. Perhaps the scale bar could be moved to the Rab21 panel (middle panel on the right) and the arrowheads could be added to the DAPI panel as well as the merged panel.

Figure S2C: Why are there so many cells with high levels of Arm staining in the panel on the right? Is there an effect on Wnt signaling?

Figure S2F: SDS does not to be in italics in the upper right panel.

Figure S3D: The right-most arrowhead is missing from the panel on the left.

Figure S3E: The beta-Gal signal in the middle panel looks overexposed in many of the cells, which appear as solid blocks of white. An image with a lower exposure in this channel should be substituted or the reason for using an overexposed image should be explained in the figure legend. Also, the white beta-Gal label is hard to read because of the bright white cells; it would help to put the label over a black box, and the green label (if there is one) in the panel on the right is entirely invisible.

Figure S4A-D: Replace the word "dots" with "puncta".

Figure S4E: It is really hard to see any details of the green GFP-LAMP signal on a black background. It would help to show the green channel as separate grayscale images.

Figure S7E: Stats are not appropriate for experiments where N=2.

Dear Prof. Jean,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made, as described in the Monitoring Editor's decision letter above and the reviewer comments below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers' comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter.") Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you have opted out of publishing the review history.

Authors are allowed 180 days to submit a revision. If this time period is inadequate, please contact us at mboc@ascb.org.

Revised manuscripts are assigned to the original Monitoring Editor whenever possible. However, special circumstances may preclude this. Also, revised manuscripts are often sent out for re-review, usually to the original reviewers when possible. The Monitoring Editor may solicit additional reviews if it is deemed necessary to render a completely informed decision.

In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

To submit the rebuttal letter, revised manuscript, and figures, use this link: Link Not Available

Please contact us with any questions at mboc@ascb.org.

Thank you for submitting your manuscript to Molecular Biology of the Cell. We look forward to receiving your revised paper.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org
RESPONSES TO MONITORING EDITOR COMMENTS

I. OVERALL COMMENTS ON MANUSCRIPT

Monitoring editor comment 1: Using the Rab21-GAL4 driver to drive expression of UAS-GFP:Rab21 could result in overexpression because GAL4 itself can amplify gene expression by binding multiple UAS sites (this is really not equivalent to putting a transgene under direct control of the Rab21 promoter). Thus, I feel your response to Reviewer #3, comment 5 is not quite adequate. If the GFP:Rab21 fusion is overexpressed, this could potentially mask any apicobasal polarization of the protein. For this reason, a caveat about the interpretation of the localization data should be included in the manuscript.

Author response: We thank the editor for pointing out this important aspect. We agree that this could be the case, thus we have included a sentence regarding limits of the GAL4-UAS system to characterize apicobasal polarization (manuscript lines 192-193).

Monitoring editor comment 2: In several places in the manuscript, manipulations of Rab21 are described as "loss-of-function experiments", but RNAi and dominant-negative constructs are not the same as null mutations, which are the standard for "loss-of-function experiments". I suggest finding a different way of describing these experiments or just spelling out what was actually done.

Author response: We apologize for this overstatement and have corrected it in the text.

Monitoring editor comment 3: The data included in response to Reviewer #1, comment 3 (absolute quantification of pH3+ cells in the distal part of the posterior midgut) should be included as a supplemental figure.

Author response: The figure has been included in Supplementary figure 5.

Monitoring editor comment 4: In general, it would be clearer if Drosophila protein names
were capitalized throughout the manuscript (both in the text and in the figures), as this will make it clear to the reader that proteins are being described.

**Author response:** We capitalized *Drosophila* protein names throughout the manuscript.

**Monitoring editor comment 5:** The word "guts" is a colloquial term and is a bit overused for my taste. I suggest using "intestines" instead in a number of places in the text (these are indicated in the uploaded pdfs).

**Author response:** We thank the editor for highlighting our overuse of the word “guts”. We have replaced it with the suggested word “intestines” and have additionally changed it with different alternatives throughout the text to minimize the use of ‘guts’.

**Monitoring editor comment 6:** In quite a few of the figures, the layout is awkward, and a number of the panels have text that **is too small to easily read when printed**.

**Author response:** We have increased the size of the text throughout all the figures and made sure everything was readable when printed.

**Monitoring editor comment 7:** The figure legends are quite long and could be trimmed without losing essential information.

**Author response:** We have trimmed the figure legends as suggested.

**Monitoring editor comment 8:** The degenerate Rab21 sequence listed as Table 1 should be included as a supplementary figure rather than a table, and it would help to show an alignment with the starting sequence, as well as the locations of the hairpin RNAs in RNAi-1 and 2.
Author response: The degenerate Rab21 sequence is now included in the additional Supplementary Figure 8 where we show an alignment with the wildtype sequence and highlight locations of Rab21 RNAi-1 and 2 used in our study.

II. SPECIFIC COMMENTS ON THE FIGURES

Monitoring editor comment 9: Figure 1D: The stem cells marked with arm staining that are pointed at by the arrows are quite small. An inset would help the reader see these cells more clearly.

Author response: We added zoom views of stem cells highlighted by the arrows.

Monitoring editor comment 10: Figure 1E: The font used at the top of this panel is quite small and hard to read (except for "Myo1A").

Author response: We have increased font size.

Monitoring editor comment 11: Figure 1F: Why does the image not extend to the edges in the lower panel? Also, there is something in the box below the "Rab21^RNAi" label.

Author response: The images previously used showed intestines that were differentially oriented. We now have changed them with new images showing similarly positioned intestines. Also, we have increased the box label to cover the bottom part of the image completely.

Monitoring editor comment 12: Figure 1H and 1I: The labels on the graphs are much too small to read; the graphs could be larger and still fit nicely in the figure.

Author response: We increased the size of the graphs and labels.
**Monitoring editor comment 13:** Figure 1J: Why are there error bars for the Rab21 DN genotype if N=2?

**Author response:** We apologize for the error. We removed error bars for the Rab21 DN condition.

**Monitoring editor comment 14:** Figure 2A: The diagram is quite pixilated and seems low-resolution. The labels for the cell types are incredibly small and nearly impossible to read.

**Author response:** Looking at the source files, we uploaded during the submission, we noticed that the diagram was adequate. However, when we compared the source files to the compiled manuscript or to the compressed files generated by the MBoC system after reading your comment, we noticed, as you stated, that most diagrams appeared pixelated (to highlight these differences, please find below screenshots of (1) compressed file and (2) original file). As mentioned by the editor, labels for cell types were too small, we have now increased their sizes. We also confirmed that source files are used for publication and thus should not appear pixelated if the manuscript is accepted.

![Diagram showing cell types and labels](image1)

**Monitoring editor comment 15:** Figure 2B: The blue label for DAPI is invisible on the black background. It should be a brighter/lighter colour so it can be seen.

**Author response:** We thank the editor for pointing this out. We now used a brighter color and put a black box under the DAPI label to make it more visible.
Monitoring editor comment 16: Figure 4E: Stats are not appropriate for experiments where $N=2$.

Author response: We apologize for the error. We have now corrected it.

Monitoring editor comment 17: Figure 5A: The diagram is quite fuzzy and pixilated and seems low-resolution. The yellow-orange colour of the label for the CORVET complex is nearly impossible to see on a white background. A few genes are missing from the list on the right: Atg1, Vps8, Rab4. Also, the colours for endocytosis and the Wash complex are very close and difficult to distinguish.

Author response: Concerning diagram resolution, please refer to our response to comment 14 (to highlight these differences, please find below screenshots of (1) compressed file and (2) original file). We thank the editor for pointing out color issues with the diagram. We have now changed the colors of the mentioned groups.

Monitoring editor comment 18: Figure 6E: As mentioned above, "loss of function" suggests that null mutants were examined, but this is not the case here. Use of some other term or abbreviating knockdown (KD) and dominant-negative (DN) (while defining these abbreviations in the figure legend) would better convey what was actually done.

Author response: We apologize for the misuse of ‘loss of function’. We have now corrected it.
Monitoring editor comment 19: Figure 8A: This diagram is fuzzy and pixilated similar to the others. The blue text should say "lipid transport", not "lipids mobilization", as apolpp transports lipids in the hemolymph ("lipid mobilization" suggests release of lipids from lipid droplets, etc.).

Author response: Concerning diagram resolution, please refer to our response to comment 14 (to highlight resolution differences, please find below screenshots of (1) compressed file and (2) original file). We corrected the blue text for “lipid transport”.

![Diagram](image1.png)

Monitoring editor comment 20: Figure S1B: Rab21 label should be italicized to indicate that the transcript is being examined. Also, it is very hard to see the blue and green on black in the lower right panel, and the nuclei the arrowheads are pointing at are also not visible in the grayscale DAPI panel at the top because the scale bar is on top of one of the cells. Perhaps the scale bar could be moved to the Rab21 panel (middle panel on the right) and the arrowheads could be added to the DAPI panel as well as the merged panel.

Author response: As suggested by the editor, we moved the scale bar to the Rab21 panel. We have also increased the blue and green signals to make them more visible on the black background.
Author response: Armadillo staining highlights the membrane of enterocytes, intestinal stem cells (ISC) and enteroblasts (EB), with ISC and EB harboring higher Arm staining. Therefore, we believe that the many cells with high Arm staining upon Rab21 depletion might represent the increased ISC population we observed with Dl staining (Fig. 2E), or an increased number of EB (which we have not quantified due to the lack of reporter). Coherent with this idea, most of the cells with high Arm are small cells. From the literature, it is known that activation of Wingless/Wnt pathway in enterocytes is required to inhibit JAK/STAT signaling in neighboring cells, by blocking expression of Upd2 and Upd3 in enterocytes. Unfortunately, we have not assessed Wnt signaling effects in Rab21 RNAi condition, but given that Upd3 is increased upon Rab21 depletion, we speculate that Wnt signaling in enterocytes is not increased.

Monitoring editor comment 22: Figure S2F: SDS does not to be in italics in the upper right panel.

Author response: We thank the editor for noticing the error. We have now corrected it.

Monitoring editor comment 23: Figure S3D: The right-most arrowhead is missing from the panel on the left.

Author response: We thank the editor for noticing the missing arrow. We have now added it.

Monitoring editor comment 24: Figure S3E: The beta-Gal signal in the middle panel looks overexposed in many of the cells, which appear as solid blocks of white. An image with a lower exposure in this channel should be substituted or the reason for using an overexposed image should be explained in the figure legend. Also, the white beta-Gal label
is hard to read because of the bright white cells; it would help to put the label over a black box, and the green label (if there is one) in the panel on the right is entirely invisible.

**Author response:** We thank the editor for pointing out the difficulties in interpreting the data from figure S3E. We have now provided images with a lower exposure for Upd3 staining.

**Monitoring editor comment 25:** Figure S4A-D: Replace the word "dots" with "puncta".

**Author response:** We replaced the word “dots” with “puncta”.

**Monitoring editor comment 26:** Figure S4E: It is really hard to see any details of the green GFP-LAMP signal on a black background. It would help to show the green channel as separate grayscale images.

**Author response:** We added separate grayscale images of the GFP:Lamp channel.

**Monitoring editor comment 27:** Figure S7E: Stats are not appropriate for experiments where N=2.

**Author response:** We apologize for the error. We have now removed the stats.

III. COMMENTS ON THE PDF MANUSCRIPT

**Monitoring editor comment 28:** was there no sense probe or non-specific probe used as a control?

**Author response:** Unfortunately, we did not use a non-specific probe or a sense probe in the FISH experiments, and we do agree that it would have been preferable. However, we did test multiple anti-sense probes for different genes and observed different expression patterns for the individual genes tested. Significantly, these expression patterns reflected
published databases. Given this, and the fact the Rab21 FISH pattern was similar to published datasets and that it overlapped extensively with the Rab21-Gal4>GFP:Rab21 approach, we concluded that the FISH protocol reflected Rab21 localization.

_Monitoring editor comment 29: missing Nagai et al., 2021 reference on upd3 and autophagy genes (cited in the text)._  

**Author response:** We thank the editor for the comment, we have now added the citation.
RESPONSES TO REFEREE COMMENTS

Reviewer #1:

Reviewer comment 1: While the authors assert that the RNAi constructs targeting Rab21 have been previously validated, it would be helpful to demonstrate knockdown efficiency (by qRT-PCR) for the two Rab21 RNAi constructs (in enterocytes if possible). Complementation needs to be shown for both RNAi constructs for key assays (PH3 and CC3 for example).

Author response: We thank the reviewer for their helpful comments, which have resulted in an improved manuscript. As suggested, qRT-PCR and western blot analyses validating the Rab21 RNAi efficiencies in enterocytes have been added to the manuscript (Supp. Fig. 2A, B). Concerning the complementation requirements mentioned by the reviewer, we would like to emphasize that we provide rescue evidence for intestinal organization (Fig. 1G) as well as pH3 positivity for both RNAi reagents used (Fig. 3D). Moreover, we used a dominant negative construct (Rab21-DN) to further assess their specificity in various settings. Thus, we believe that the two rescue experiments and the use of two independent tools (RNAi and the DN construct) adequately demonstrates the specificity of our reagents and have not performed additional complementation experiments, electing to focus on other requested experiments that we believed are more beneficial to the manuscript. Nonetheless, we have added a sentence regarding the limitations of the RNAi approach (Lines 272 to 277).

Reviewer comment 2: The CC3 data in 3A mostly looks like an outlier effect. Not sure this result is even correct. Conversely the data in 3E and F looks quite compelling and the mechanisms connecting Rab21 to this change is not pursued. This is the strongest phenotype in the paper.

Author response: Upon close examination, although there were few individual intestines with high cCASP3 staining, we were able to support increased cell death in Rab21-depleted guts by SYTOX assays (Fig. 3B) and qRT-PCR of the caspase activator hid (Supp. Fig. 3A). Furthermore, the functional significance of apoptosis to the observed Rab21 phenotype was demonstrated by rescue experiments with the caspase inhibitor p35 (Fig. 4C, D). Importantly, additional rescue experiments with yki RNAi
also reinforced the experiment by again demonstrating that depletion of Rab21, with two independent, RNAi reagents leads to increased cCASP3 staining (Fig. 4E).

We thank the reviewer for acknowledging the importance of the Upd3 and JAK/STAT phenotypes. As mentioned, we did not follow up on the direct involvement of Rab21 in these cell responses, and we apologize for that. The reason why we did not pursue it further is because we believe that JAK/STAT pathway activation is a downstream effect of Rab21 depletion, due to increased Upd3 secretion, which is enhanced via Yki activity and cell death (Fig. 4F). We showed that apoptosis inhibition rescues JAK/STAT activation in Rab21-depleted enterocytes (Fig. 4C), supporting our hypothesis. Following the reviewer’s suggestion, we have added epistatic experiments showing that co-depletion of Rab21 and Yki is sufficient to reduce JAK/STAT activation (Fig 4C), which is consistent with this hypothesis and further strengthens the link between Rab21, Yki, and JAK/STAT.

Reviewer comment 3: The data in Fig 5 shows week effects for many genes and does not advance the mechanism. The proportion of pH3+ cells is very small in all experiments. While some manipulations result in statistically significant changes in this measure, is this a biologically meaningful or relevant effect? Can this be normalized to the number of stem cells instead of the total number of cells?

Author response: The screen targeted genes involved in specific membrane trafficking steps and genes related to previously described Rab21 functions, to get insights about the precise trafficking pathways phenocopying Rab21 depletion in enterocytes. It is important to note that pH3+ cell percentages were assessed as the total number of pH3+ cells in a region of interest (in the distal part of the posterior midgut) divided by the total number of cells (revealed using DAPI staining) in the same region. Hence, the small percentages result from the low number of pH3+ cells compared to total cells. We used this approach to determine if the increase in pH3+ cells was dependent or independent of increased cell density. The quantification method used has been better detailed in the materials and methods section (lines 903 to 906). To keep data representation consistent throughout the manuscript, we decided to keep the proportion of pH3+ cells. Nonetheless, we have graphed the absolute number of pH3+ cells below (also included in Supp. Fig. 5), as suggested by the referee. We believe that the large differences observed using this type of representation supports meaningful and relevant differences in pH3+ cells.
Proliferation is increased upon knockdown of specific membrane trafficking genes Absolute quantification of pH3+ cells in the distal part of the posterior midgut.

Reviewer comment 4: The statistical tests used for determining significant differences between groups are often incorrect: for example a one-way ANOVA should be used for all experiments with >2 conditions.

Author response: In the revised manuscript, we have used \( t \)-tests and Mann Whitney U tests for pairwise comparisons as done in the literature (Chakrabarti et al., 2014; Chen et al., 2020; Du et al., 2020; Hao et al., 2020; Houtz et al., 2017; Jin et al., 2020; Li et al., 2018; Liang et al., 2017; Mundorf et al., 2019; Nagai et al., 2021; Nie et al., 2019; Xiang et al., 2017; Xu et al., 2017; You et al., 2014; Zhang et al., 2019; Zhou and Boutros, 2020). After discussing the matter with our institute statistician, we have used one-way ANOVA followed by Dunnett’s tests when multiple conditions were tested (Fig.1 F, Fig. 2C, D, E, Fig. 3A, B, C, E, Fig. 5C, D, Fig. 6A, C, Fig. 8D, Fig. 9E, Supp. Fig. 3C, Supp. Fig. 4A, B, C, D, Supp. Fig. 5A, B, Supp. Fig. 6B).
Reviewer comment 5: The survival data are compelling and would be better suited in figure 1 rather than in the supplemental material (an analysis of developmental delay and/or size/weight would further support the effects of Rab21 loss on viability).

Author response: As suggested, we incorporated the survival data into Figure 1 (Fig. 1J). Since Rab21 knockdown is only performed in adult flies, we do not expect developmental delays. Size/weight analyses would be of great interest and represent nice follow up experiments for future studies.

Reviewer comment 6: The proteomics experiment included at the end (figure 7) seems out of place and leaves the reader wondering about the role of the differentially abundant proteins in rab21 deficient flies - strongly suggest removing these data from the manuscript unless detailed follow-up experiments are performed. In their current state, these data do not support the rest of the narrative.

Author response: We agree that without follow-up, the proteomic data leaves the reader with a lot of questions. Since these data provide exciting fodder for future studies, and are therefore a potential resource for the community, we decided to include the data in the manuscript. The proteomic experiment was initially designed to get insights into the physiological defect(s) caused by Rab21 depletion, hoping that it would uncover specific roles of Rab21 in enterocytes causing the various phenotypes. The results have been promising, as they identified defects in unexpected physiological processes (sugar transport, lipid metabolism, as well as nutrient digestion). We think these data are of interest and point to Rab21’s functions in enterocytes. To expand on these results as suggested by the referee, and solidify the manuscript, we decided to perform follow up experiments assessing the main physiological aspects highlighted by the proteomic data.

Although, it was difficult to find tools, we managed to obtain transgenic flies or antibodies for three important hits: Tret1-1 (a trehalose transporter), Apolpp (an apolipoprotein) and Hermes (a monocarboxylate transporter), all of which were more abundant in Rab21-depleted flies. Using transgenic flies expressing HA-tagged Tret1-1 and an antibody against Apolpp, we confirmed that these proteins accumulate in the intestinal epithelium (Fig. 8B, 9B) upon Rab21 loss. Unfortunately, we were unable to generate recombinant lines (Myo1Ats, GFP:hermes or Rab21RNAi, GFP:hermes) allowing us
to analyze hermes abundance upon Rab21 depletion in enterocytes. Apolpp and Tret1-1 are involved in lipid and carbohydrate transport, respectively, therefore we investigated both aspects using different approaches:

For **Apolpp**, based on its function (Figure 8A; manuscript lines 470-473), we evaluated lipid transport and lipid droplet morphology upon Rab21 depletion from enterocytes. Interestingly, we found decreased lipid droplet sizes upon *Rab21* knockdown (Fig. 8C). To further refine these observations, we also assessed lipid droplet morphology in the main TAG storage organ: the fat body. Nile Red staining highlighted an increase in lipid droplet sizes when Rab21 was depleted from enterocytes compared to controls (Fig 8E). We also quantified circulating lipids (mono-, di-, and triacylglycerol) in the hemolymph of adult flies, and did not detect any differences (Fig. 8D). Because Apolpp is mainly produced by the fat body, we also investigated the impact of *apolpp* knockdown in the fat body on the intestinal epithelium. Surprisingly, fat body *apolpp* knockdown tended to decrease proliferation (although not significantly) while tissue morphology was not affected (Suppl. Fig. 7A-B). Although *apolpp* is barely expressed in the gut (Palm et al. 2012), we nevertheless attempted *Apolpp* depletion in enterocytes. Interestingly, we observed that lipid droplets sizes were decreased in apolpp-depleted enterocytes compared to controls (Suppl. Fig. 7C), while their density was increased (Suppl. Fig. 7C). Tissue morphology was also affected, while proliferation was unchanged (Suppl. Fig. 7D-E). Finally, we co-knockdown *Rab21* and *apolpp* in enterocytes to assess if Apolpp accumulation was responsible for some of the epithelial defects observed upon *Rab21* knockdown. Apolpp co-depletion with Rab21 did not affect the increased proliferation or lipid droplet size modulation caused by *Rab21* knockdown (Fig. 8F-G). Nonetheless, these findings are exciting and indicate a role for Rab21 in lipid metabolism in gut enterocytes, something previously unknown. These results also emphasize potential cross-talk and compensation between the fat body and the gut upon defective lipid absorption or mobilization in the gut. Finally, these results have potential clinical significance, as two recent manuscripts linked SNPs in human *RAB21* to improper lipid levels.

**Tret1-1** is a trehalose transporter. Confirming the mass spectrometry data, we observed higher levels of transgenic HA-tagged Tret1-1 upon Rab21 knockdown (Fig. 9B). Given the increased Tret1-1, we assessed circulating concentrations of trehalose in the hemolymph of adult flies depleted of enterocyte Rab21 (Fig. 9E). Because trehalose is composed of two glucose molecules, and previous studies
highlighted a role for it in circulating glucose transport regulation, we also quantified the glucose level in the hemolymph. This experiment highlighted a decreasing tendency of both trehalose and glucose levels upon Rab21 depletion (Fig. 9E). To assess Tret1-1 involvement in gut maintenance, we depleted Tret1-1 in enterocytes and analyzed intestinal stem cell proliferation and tissue architecture. No changes in proliferation were observed, but the tissue architecture was modified by Tret1-1 knockdown (Fig. 9C-D). Finally, to test if increased levels of Tret1-1 were linked to Rab21 phenotypes, we co-depleted Rab21 and Tret1-1 in enterocytes. These experiments did not show a rescue of Rab21 loss of function-related phenotypes (Fig. 9F).

In these experiments, we confirmed two hits from the mass spectrometry analysis. Furthermore, we highlighted a lipid uptake (and/or) mobilization defect in Rab21-depleted guts and found a role of Apolpp in enterocytes. Furthermore, we confirmed that Rab21 also affects Tret1-1 levels, with slight decreases in circulating glucose and trehalose levels. Epistasis experiments demonstrated that the intestinal phenotypes observed upon Rab21 knockdown are not caused by either increased ApoLpp or Tret1-1. These novel experiments did not allow us to pinpoint the cellular mechanism by which enterocyte Rab21 loss causes compensatory proliferation and tissue disorganization. Nonetheless, we identified two new important functions for Rab21 linked to lipid and trehalose transport or metabolism in enterocytes. How Rab21 is involved in these specific functions in enterocytes remains to be elucidated. These findings will serve as foundation for future studies aimed at better define trafficking events in enterocytes and how they affect their absorptive functions.

**Reviewer comment 7:** How is it possible that early endosome formation is perturbed in Rab21-deficient IECs yet late endosome and lysosome formation are normal? The authors should address this more carefully and thoroughly in the discussion.

**Author response:** This is now addressed in the revised manuscript (manuscript lines 354-358).

**Reviewer comment 8:** The authors should more explicitly indicate what time point(s) were analyzed. Were all experiments performed at 10 days post-eclosion? If so, why? Analysis of the effects of cell death and proliferation on physiology is difficult to interpret from a single time point, and kinetic experiments would greatly benefit this study.
Author response: All experiments from the manuscript were performed at 10 days post-eclosion, except the proteomic experiment, which was done at 5 days to get an accurate idea of Rab21-specific contributions rather than secondary effects. We did perform a time-course analysis of proliferation and cell junction marker disruption upon Rab21 depletion (Rab21 RNAi-1 and -2, and with Rab21DN) at earlier time points (3 and 5 days). While these phenotypes were already strong for Rab21 RNAi-1 at 3 and 5 days, Rab21 RNAi-2 and Rab21DN did not show strong phenotypes at these time points (Suppl. Fig. 2D, 3C). In a gut autophagic-deficient context, it was recently shown (Zhang et al., 2019) that all pathways known to enhance intestinal stem cell proliferation can rescue overproliferation at an early time point (5d) but at a later time point (10d). Therefore, we opted for the 10 day time point for epistasis studies to (1) easily discriminate between a rescue or lack of one and (2) characterize specific downstream effectors. Experiments performed at earlier time points have been added to the manuscript (Suppl. Fig. 2D, 3C) as well as a discussion of the stronger impact of Rab21 RNAi-1 at earlier time points 1 (manuscript lines 268-275).

Reviewer comment 9: The Myo1A driver used in this study has expression restricted to enterocytes. However, in many of the experiments reported in this manuscript (eg Fig3, Fig4, Fig5, and Fig6), the assays lack sufficient cellular specificity and resolution to delineate autocrine vs paracrine effects. Moreover, the mosaicism of the knockdown is not clear. Many experiments would be more convincing if performed with dual transgenes or labeling (e.g., pH3 staining with prospero or DL:GFP; SYTOX / upd3:LacZ / 10xSTATE92E-GFP + Myo1A reporter).

Author response: As described at the beginning of the manuscript (Fig. 1A-C), Rab21 is not homogeneously expressed in enterocytes. Its expression is higher in distal enterocytes of the posterior midgut, while it is weakly expressed in the proximal part. Importantly, even in the distal region, Rab21 is not present in all enterocytes (Fig. 1B-D). Therefore, what reviewer refers to as “knockdown mosaicism” is probably a consequence of Rab21’s particular expression pattern. We would expect that Rab21 depletion would affect only enterocytes expressing it. To gain a broad overview of Rab21’s effects and pinpoint its specific functions, we initially decided not to look at the cellular level, particularly because we are unable to discriminate between enterocytes that have been depleted of Rab21 versus those that do not express it. Thus, it is difficult to get any information about autocrine vs
paracrine effects by looking at cell types. Nevertheless, we agree with the referee that dual transgenes/labeling for pH3 with Delta and Prospero is informative in this context; therefore, we performed these experiments and incorporated them into the revised manuscript (Suppl. Fig. 3B). Given the scope of the manuscript and the fact that we already showed dual-labeling for dying cells with an enterocyte reporter (Fig. 3A), we believe that although interesting, performing new dual transgenes labelling for upd3:LacZ and stat92E will not advance our mechanistic findings further. Nonetheless, we provided ‘zoomed-in’ views of upd3:LacZ stain to allow better assessment of the affected cells (Suppl. Fig. 3D). In addition, we included upd3:LacZ/cCASP3 dual staining to highlight their lack of strong overlap (Suppl. Fig. 3E).

**Reviewer comment 10:** Many signal transduction pathways converge on ERK phosphorylation and the authors over-interpret their observations: in the absence of EGFR knockdown or pharmacological blockade, it is impossible to determine whether differences in dpERK staining are in fact due to changes in EGFR signaling.

**Author response:** ERK phosphorylation is indeed the consequence of various transduction pathways. Because we could rescue the increased proliferation induced upon Rab21 depletion via overexpression of EgfrDN, which often used in the field to conclude on the requirement of EGFR signaling (Buchon et al. 2010, Gervais et al., 2019), we concluded that increased dpERK was linked to increased Egfr signaling. To support this conclusion, we have added qRT-PCR for the Egfr pathway target genes Mkp3 and argos upon knockdown of Rab21, showing increased transcript levels for these genes (Suppl. Fig. 6A).

**Reviewer comment 11:** The author's use of the term "homeostasis" is an overstatement of their findings: the data reported in this manuscript do not test enterocyte functions, and the inference of lost intestinal homeostasis is largely based on the proportions of apoptotic and proliferation cells, which is an oversimplification.

**Author response:** By “homeostasis”, we meant tissue equilibrium. Because this equilibrium is lost (cell type proportions impaired, proliferation increased, cell junctions affected, etc.) upon depletion of Rab21 we believed that it was correct to say that Rab21 was required for proper tissue homeostasis. We have modified the term “homeostasis” throughout the revised manuscript.
**Reviewer comment 12:** The authors should attempt to reconcile the apparent increase in apoptosis, the modest increase in pH3 staining, and the striking increase in total cellularity in the discussion.

**Author response:** Apart from an increase in apoptosis and pH3 staining, we also showed increases in prospero⁺ and delta⁺ cells (Fig.2D, E) which mostly account for the increase in total cellularity. This point is addressed in the revised discussion (manuscript lines 549-550).

**Reviewer comment 13:** In figure 3A, the cCASP3 staining in the Rab21 RNAi image seems much more than ~1.5 fold greater than the control (which is the effect size portrayed in the quantification). Are these images truly representative?

**Author response:** While the image is representative, we have exchanged it with one showing a weaker effect (Fig.3A). We have also provided more details regarding the image analysis pipeline in the materials and methods section.

**Reviewer comment 14:** The vast majority of the findings presented in this manuscript are derived from epistasis experiments, and while a summary model would help the reader synthesize the convergent and independent pathways that result in the observed phenotypes, the authors should take care when linking genes in the Rab21 pathway; moreover, cross-talk between cell types should be more carefully assessed.

**Author response:** We totally agree with the reviewer. We have modified the initial summary model to make it easier for the reader to understand and added a more general one including all epistatic experiments performed (Fig. 4F). Assessing crosstalk between cells is an interesting aspect; unfortunately, we believe that it represents the next step in our experiments and will be studied in more detail in follow-up studies. Nonetheless, we have mentioned this in the revised discussion (manuscript lines 601-607).
Reviewer comment 14: The title is misleading: with the exception of the barrier function assay (the results of which have been relegated to supplemental material), none of the data presented in the manuscript are from experiments which test the "functions" of enterocytes.

Author response: We agree with this comment and have modified the title accordingly.

Reviewer comment 15: key details regarding experimental design and materials and methods are generally lacking.

Author response: We have made our best efforts to incorporate all necessary information in the revised materials and methods section (manuscript lines 761-762, 807-810, 832-839, 848-850, 859-862, 903-908, 916-917, 920-922).

Reviewer comment 16: Please clarify sample sizes in figure legends and indicate what the points of scatter dot plots represent (individual flies, fields, etc).

Author response: Although this information was provided in the methods, we now have incorporated it in the figure legends as well. Dots from scatter dot plots correspond to individual flies. If an experiment has no graph, the sample size is indicated in the figure legend. The number of independent experiments corresponds to \( N \) and is incorporated in all figure legends. All quantifications/analyses were performed in the distal part of the posterior midgut. This information has been incorporated in the materials and methods section (manuscript lines 922-924).

Reviewer comment 17: Please clarify in the materials & methods how image analysis was performed for quantification of "normalized signal intensity" (Figs 3A, 3B, 3E, 3F, 5D, 6A, 6C).

Author response: We apologize for not making this point clearer in our original submission. This point has been clarified in the revised manuscript (manuscript lines 907-908).

Reviewer comment 18: In figure 1G, why is quantification of lumen area expressed as a normalization instead of absolute area? Also, please clarify what region(s) were analyzed for this quantification.
Author response: We have included the absolute area measurement in the revised manuscript (Fig. 1I). Distal parts of posterior midguts were analyzed. This information has also been added to the manuscript (manuscript lines 903-904).

Reviewer comment 19: The quantification shown in figure 1H is rather coarse and could be improved: cell density, intestinal thickness, or intestinal cellularity would be more satisfying metrics.

Author response: We hope that we are not misinterpreting the comment, but cell density analyses were incorporated in the original manuscript. As suggested by the reviewer, in order to support quantification shown in Fig. 1H, we now have incorporated cell density analysis in figure 1 (Fig. 1F) from the revised manuscript.

Reviewer comment 20: In the materials and methods, please provide sequences and/or a schematic (suitable for supplemental information) for the Rab21Deg allele.

Author response: We have added this sequence information into the manuscript (Supp. Fig. 8).

Reviewer comment 21: The composition of the loading buffer used for protein extraction is confusing: Tris is listed twice (at two different concentrations and pH)

Author response: We thank the reviewer for noticing this error, which has been corrected (manuscript lines 857-862).

Reviewer comment 22: Regarding TEM in the materials and methods: reference 58 is likely inappropriate as this paper did not perform TEM on drosophila. Also, please include the following details: what region(s) of the intestine were analyzed, what embedding medium was used, what fixatives and stains were used after sectioning, what was the operating keV during image acquisition.

Author response: This information has been incorporated (manuscript lines 831-840).
Reviewer comment 23: The authors should add additional detail in the materials and methods regarding in situ hybridization assays: what kit(s) were used for probe synthesis and labeling, was a sense probe used for a control, and provide catalog numbers for reagents used.

Author response: This information has been added in the materials and methods section (manuscript lines 807-810).

Reviewer #1 (Significance (Required)):
Nature and significance of advance: The study advances of the body of knowledge regarding the roles of Rab family small GTPases in intestinal epithelial cell biology using an in vivo model (i.e. Drosophila). While this protein has been studied in epithelial cell culture systems, its role(s) in intestinal epithelial biology in vivo remain poorly characterized. Moreover, the data presented suggest the possibility of evolutionarily conserved roles for this protein in the intestine.

Context: A quick google search indicates there isn't much literature investigating the role of Rab21 in vivo

Audience: This work would appeal to scientists interested in: novel in vivo models to investigate the functions of Rab GTPases, regulators of endocytic recycling and trafficking pathways

Expertise: Intestinal epithelial cell biology.

Author response: We thank the reviewer for highlighting the novelty of this study and how it will be of interest to a broad public.

Reviewer #2:

Reviewer comment 1: First, the major effect of Rab21 loss in the intestine seems to be an effect on the progenitor cells and lineage differentiation. This is interesting because previous studies in mammalian
systems suggested that levels of Rab21 were low in the proliferative crypts in the small intestine. This difference should be discussed.

**Author response:** We thank the reviewer for their helpful comments, which have resulted in an improved manuscript. In our study, we are assessing Rab21 function in differentiated cells—enterocytes. Although knockdown of enterocyte Rab21 affects intestinal stem cells, this effect is likely not direct, as shown by our epistasis experiments (Fig. 3 and 4). In mammals, Rab21 has low and high expression levels in intestinal stem cells and villi, respectively (Zhang et al., 2016), which is akin to what we observed in flies. This point is now discussed in the revised manuscript (manuscript lines 552-555).

**Reviewer comment 2:** The screens in Figure 5 examine autophagy and find some subtle effects and suggest an effect of Rab21 on EGFR signaling, a finding that has previously been noted in mammalian cells. It is unclear to this reader why effects of Rab21 in relation to the recycling system (Rab8 and Rab11) are not examined.

**Author response:** A few studies have shown that knockdown of Rab11 in fly enterocytes leads to inflammation and increased proliferation (D’Agostino et al., 2019; Nie et al., 2019; Yu et al., 2014). Rab11 acts synergistically with the RasV12 oncogene to induce inflammation (Nie et al., 2019). To assess whether Rab21 phenotypes are associated with defects in endosomal recycling, we overexpressed Rab11 in Rab21-depleted enterocytes and monitored if increased recycling could rescue depletion of Rab21, using proliferation as a readout (Suppl. Fig. 5C). Rab11 activation in the context of Rab21 knockdown did not rescue the increased proliferation, suggesting that the effects of Rab21 and Rab11 are independent, or that Rab21 acts upstream of Rab11 and its loss is dominant and non-rescuable by increased Rab11 activity.

**Reviewer comment 3:** The studies show changes in the expression of some SLC proteins but there is little investigation of the importance of these changes and even their relevance to endocytosis on the apical versus basolateral membranes. Indeed this is one of the most confusing aspects of this work is there is no clarification of what the significance is for Rab21 in apical versus basolateral membrane endocytosis and how specific cargoes are altered.
Author response: We completely agree with this comment. The problem we had at the time of submission was that most SLC proteins we identified had no described localization patterns in enterocytes. Unfortunately, during our revision, we only managed to obtain constructs for Tret1-1 and Hermes (SLC16A12), and Hermes knock-in flies proved difficult to work with. Given these limitations, we were unable to test the impact of Rab21 depletion on an SLC protein and study the apico-basal localization of that SLC. Since Rab21 does not show an apico-basal polarity, we would hypothesize that both types of cargos could be affected. We have addressed the point raised by the referee in greater details in the discussion. Nonetheless, we have acquired some tools that have allowed us to expand on the importance of Rab21 on Tret1-1 levels (please read the reply to Reviewer 1’s Comment #6”).

Reviewer comment 4: It is unclear to me what the authors mean by "intestinal homeostasis". Is this normal proliferation? Normal differentiation? Normal expression of transporters? In the end I am left with a number of interesting findings that are relatively correlative but do not lead to clear insights into cell physiology.

Author response: By using “homeostasis” we meant “tissue equilibrium”. Therefore, changes in tissue composition, due to modifications in intestinal cell numbers or ratios would modify this equilibrium. For easier understanding, we have replaced the term “homeostasis” throughout the manuscript. To expand on the physiological effects of Rab21, we now performed new experiments to validate two proteins modulated by its depletion in enterocytes (please see Reviewer 1- Comment #6). We have also modified the discussion for clarity and to discuss the limitations of our findings.

**Other concerns:**

Reviewer comment 5: The introduction refers to a number of papers dealing with vesicle trafficking associated with Rab11 and Rab8, these should really be referred to as Rab11a and Rab8a.

Author response: We have made this correction (manuscript lines 79, 81 and 85).
Reviewer comment 6: The exact mutations for dominant negative and dominant active Rab21 forms should be noted at their first introduction.

Author response: We added the information in the text for dominant negative Rab21 (manuscript line 703). Because we do not show data with activated Rab21 in the revised manuscript, we did not include this information.

Reviewer comment 7: Many of the references appear incomplete.

Author response: The references have been corrected.

Reviewer comment 8: There is no statistical analysis performed in figure 1G.

Author response: Because we only have two individual samples (arising from two independent experiments) from the LacZ control, we did not perform a statistical test. This fact is highlighted in the figure legend.

Reviewer #2 (Significance (Required)):
The final significance of this paper is not clear because it is mostly descriptive without really defining specific changes in cell physiology in any detail.

Author response: We thank the reviewer for pointing this out, and apologize for including too many messages without clearly identifying the important and mechanistic findings of the manuscript. These aspects have been clarified and better integrated into the revised manuscript.

Reviewer #3:

Reviewer #3 (Evidence, reproducibility and clarity (Required)):
Overall, this is a high-quality fly genetic study of Rab21, and the authors presented evidence to suggest that in fly midgut enterocytes, Rab21 regulates upd3, EGFR signaling, proliferation, and autophagy.

**Major comment**

Reviewer comment 1: The conceptual significance should be emphasized by the authors further. What was previously found in mouse and human for Rab21 should be detailed in introduction.

Author response: We thank the reviewer for their helpful comments, which have resulted in an improved manuscript. As suggested, we have emphasized the conceptual significance, using new data incorporated in the revised manuscript (please read Reviewer 1- Comment #6). Although there is not much known about Rab21 in mammalian intestines, we have provided these details in the introduction (manuscript lines 114-119).

**Some moderate comments**

Reviewer comment 2: In Introduction, "Mutations in membrane trafficking genes are associated with a large array of human diseases [2]. Importantly, some of these mutations affect several tissues, while others are restricted to one organ system, and can be used to shed light on the cell type-specific functions of vesicular trafficking genes." These statements seem too general, vague, and lack details.

Author response: We apologize for the lack of clarity and have modified this text (manuscript lines 55-58).

Reviewer comment 3: The author should also elaborate on "Rab21 is modulated by stress in both flies and in mouse models of IBD [25,41]." What were found by these studies? Should comment on human Rab21, whether mutation or overexpression have been found.

Author response: We have included additional details about these aspects in the introduction (manuscript lines 120-123, 176-177, 674-677).
Reviewer comment 4: "microvillus inclusion and chylomicron retention disease, are associated with mutations in the membrane trafficking related genes myosin 5B (MYO5B) and secretion associated Ras related GTPase 1B (SAR1B), respectively [2,5,6]." Here, the author should cite work for STX3. Also missing references reporting the contribution of Rab11a and Cdc42 to microvillus development in next section that discussed enterocyte morphogenesis (PMID: 22354172; PMID: 28596241).

Author response: We apologize for this oversight and have added the missing references (manuscript lines 70, 74, 85, 89).

Reviewer comment 5: In Results: "with no restricted basal or apical localization (Suppl. Fig. 1A)." Was this referring to GFP or endogenous Rab21? If it was GFP, then polarized distribution may not be expected.

Author response: This refers to the expression of a GFP:Rab21 transgene using the Rab21 promoter. We have clarified this in the text (line 181-182).

Reviewer comment 6: "As previously observed in human and mouse intestines, these data show that Rab21 is expressed throughout the fly gut, with high expression in enterocytes, suggesting potential important functions in these cells." What was previously observed in human and mouse intestines for Rab21?

Author response: We now changed the sentence to make this clearer and describe the expression patterns observed in previous investigations of Rab21 in mammals in the introduction (manuscript line 113-120).

Reviewer comment 7: Fig. 1G was described after 1H.

Author response: We have corrected this.
Reviewer comment 8: "Rab21 loss-of-function through either RNAi or Rab21DN expression negatively affected lifespan (Suppl. Fig. 2E)." Do they mean Rab21 loss-of-function increased the lifespan?

Author response: This sentence has been changed (manuscript line 232). It meant that loss of Rab21 in enterocytes reduces survival.

Reviewer comment 9: "Autophagy and EGFR signaling are independent in enterocytes." Instead of making this sweeping claim, consider change this subtitle to relate to Rab21.

Author response: We have made this modification (manuscript line 406).

Reviewer comment 10: Fig. 4E, can the authors look at apoptosis in yki/Rab21 double RNAi, to establish the link? This is very interesting.

Author response: We thank the reviewer for this interesting suggestion. As suggested, we performed the experiment and observed that co-depletion of Rab21 and Yki rescues apoptosis. We have included these data in the revised manuscript (Fig. 4E).

Reviewer comment 11: Fig. 5 was impressive. Are these vesicular genes function in the same pathway of Rab21?

Author response: We thank the reviewer for their positive assessment of Figure 5. All the genes shown affect specific steps of endosomal and autophagy trafficking and some are related to previously described functions for Rab21.

Reviewer comment 12: Fig. 6D: "inhibition of EGFR signaling was unable to rescue the increased mitotic activity observed in guts depleted of autophagic genes (Figure 6D)." Can inhibition of upd3 rescue the increased mitotic activity in these flies?
Author response: This is an interesting question. While our manuscript was under review at Review Commons, a manuscript studying the role of autophagy in enterocytes showed that it indeed does (Nagai et al., 2021). Therefore, we did not assess this for our revised manuscript, but have mentioned this new finding in the manuscript.

Reviewer comment 13: Elaborate about TMT-based quantitative proteomic analysis, whether it only assesses differential protein abundance rather than assess Rab21 interactome?

Author response: The TMT approach we used gives an overview of differential protein abundances and does not assess the Rab21 interactome.

Reviewer comment 14: Have the authors considered transcriptome analysis of Rab21RNAi midgut?

Author response: We opted for proteomic instead of transcriptomic analyses because we already had an idea of the signaling pathways impacted by Rab21 depletion. We reasoned that the proteomic study could give us a different overview of the physiological aspects impaired by enterocyte Rab21 depletion.

Reviewer comment 15: Fig. 7 seemed detached from the major theme and early parts of the paper.

Author response: We agree with this remark. To overcome this issue, we acquired new tools and performed more experiments (please read Reviewer 1- Comment #6), which improves the cohesion between Fig.7 and the earlier results.

Reviewer #3 (Significance (Required)):
The data are strong and potentially significant in terms of enterocyte-intrinsic regulation of inflammatory and growth signaling. The conceptual significance should be emphasized further by the authors in revision.

Author response: We have now provided new data and modified the text to emphasize the conceptual significance of our study.
Dear Dr. Jean:

I have now read your re-revised manuscript, as well as your response to my previous comments and feel that the manuscript is in excellent shape and is nearly ready for publication. Below is a list of the few small changes I feel still need to be made. After these changes are made, I will be delighted to accept the manuscript for publication.

Best wishes,

Julie Brill
Monitoring Editor
Molecular Biology of the Cell

MINOR CORRECTIONS TO MANUSCRIPT
lines 216-217: this sentence is a bit awkward; I suggest removing the commas
line 292: replace “lead” with “led”
line 352: insert “a” before “slight increase”
line 406: replace “5D” with “5E” to correspond to the revised figure
line 438: insert comma after “autophagy”
line 616: capitalize “Hippo”
lines 710-766: edit the fly stock genotypes so the genes and alleles are italicized, for example, w[1118], y[1], sc[*], v[1], etc.
line 1362: insert hyphen between Myo1A[ts] and GAL4

MINOR CORRECTIONS TO SUPPLEMENTARY FIGURE LEGENDS
Supp. Fig. 3 legend:
p. 2, line 4: insert space before “(C)”
Supp Fig. 5 legend:
p. 3, line 1: replace “proteins depleted” with “genes as”
p. 3, line: replace “(C)” with “(D)”
p. 3, line 4: replace “(A, B)” with “(B, C)” and “(C)” with “(D)” if appropriate (make sure the legend is in agreement with changes to the figure)
Supp. Fig. 6 legend:
p. 3, line 12: add space before “Dunnett’s test”
Sup. Fig. 7 legend:
p. 4, line 3: replace “corresponds” with “correspond”

Sincerely,
Julie Brill
Monitoring Editor
Molecular Biology of the Cell

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Dear Prof. Jean,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript requires minor revisions before it can be published in Molecular Biology of the Cell, as described in the Monitoring Editor’s decision letter above and the reviewer comments (if any) below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor’s and reviewers’ comments have been addressed. (The file type for this letter must be “rebuttal letter”; do not include your response to the Monitoring Editor and reviewers in a “cover letter.”) Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you have opted out of publishing the review history.
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In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

To submit the rebuttal letter, revised version, and figures, please use this link (please enable cookies, or cut and paste URL):

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Thank you for submitting your manuscript to Molecular Biology of the Cell. Please do not hesitate to contact this office if you have any questions.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

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Dear Dr. Jean:

I am delighted to accept your manuscript for publication in Molecular Biology of the Cell. Thank you for sending the journal your very interesting work!

I think the fly community might be interested in the methods involved in the TMT proteomics you performed on fly guts, so I encourage you to develop a publicly available protocol if you are interested in doing this.

Sincerely,

Julie Brill
Monitoring Editor
Molecular Biology of the Cell

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Dear Prof. Jean:

Congratulations on the acceptance of your manuscript.

A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at www.molbiolcell.org/toc/mboc/0/0 is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

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