Endosomal cargo recycling mediated by Gpa1 and Phosphatidylinositol-3-Kinase is inhibited by glucose starvation

Kamilla Laidlaw, Katherine Paine, Daniel Bisinski, Grant Calder, Karen Hogg, Sophia Ahmed, Sally James, Peter O'Toole, and Chris MacDonald

Corresponding author(s): Chris MacDonald, University of York

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)
1st Editorial Decision

RE: Manuscript #E21-04-0163

TITLE: "Endosomal recycling to the surface mediated by Gpa1 and PI3-Kinase is inhibited by glucose starvation"

Dear Chris,

Thank you for submitting your manuscript, "Endosomal recycling to the surface mediated by Gpa1 and PI3-Kinase is inhibited by glucose starvation", to be considered for publication in Molecular Biology of the Cell. I asked two colleagues who are experts in the field to review the manuscript, and their comments are attached verbatim. As you will see, while both reviewers find your observations to be of potential interest to the readers of MBoC, they both agree that the conclusions are not supported by the data shown and that the paper is generally too preliminary. In particular, both reviewers identify aspects of your model of starvation-dependent signaling via G-alpha protein and PI3-Kinase to be overly speculative, lacking in detail, and not well integrated into a cohesive story. The reviewers also point out a number of experiments that lack critical controls and a number of points in the manuscript that lack clarity. Both reviewers provide a rather substantial list of experiments that would be required to clarify your model and support the conclusions drawn. The reviewers are both very clear and meticulous in their critiques, and so I refer you to their comments for details.

Given the nature of these reviews, I am afraid that I must reject this manuscript. In my view, the extensive number of new experiments that would be required to address the reviewer concerns would take substantial and undue effort and a longer time to complete than is reasonable for a revision. I am sorry that I do not have better news for you at this time, but hope that the reviewer comments provide some guidance as your project progresses.

Thank you for the opportunity to examine this work. We hope that as your studies progress you will consider submitting future manuscripts to Molecular Biology of the Cell (MBoC).

If you have any questions regarding the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

Sincerely,

Michael S. (Mickey) Marks

Monitoring Editor
Molecular Biology of the Cell

Reviewer #1 (Remarks to the Author):

The presented study focuses on the regulation of endosomal recycling by the presence or absence of glucose. Previous studies have documented that glucose starvation results in the rapid degradation of numerous cell surface proteins, in particular nutrient transporters. This study finds that this downregulation is mediated in part by an impaired endosome-to-PM recycling pathway. The authors implicate the G-alpha proteins Gpa1 and Gpa2 as well as the PI-3 kinase Vps34 in this regulation. Specifically, the expression levels of Gpa2, which are regulated by Mig1, are key for the glucose-dependent change in endosomal recycling. Although the authors present an intriguing model, many aspects of this model remain too speculative. Furthermore, the writing in several sections of the manuscript is confusing and not very precise.

Major issues:
- Both the Gpr1/Gpa2 sensing system and Ras2, shown to affect recycling, feed into cAMP regulation. Why is cAMP not considered as a potential regulator of endosomal recycling? Based on published models, this would be the obvious regulatory route. Also based on published data, the Ras system is activated by glucose independently of Gpr1 (e.g. S. Colombo, JBC 2004), which contradicts statements in the manuscript. This would support the idea that the common output, cAMP, is key for the regulation. I am not an expert in this field and so if these published models of Ras and Gpr1 are not correct the author should comment. Other discrepancy in the manuscript is the statement that Gpr1 is activated by Gpa2 (the other way around?).
- The key regulatory steps in the proposed model are the sequestration of Gpa1 by high levels of Gpa2, which then leads to reduced production of PI-3P by Vps34. These regulatory steps need to be better analyzed. Is during glucose starvation Gpa1 recruited away from endosomes to the PM and is this relocalization dependent on binding to Gpa2? Are co-IPs of Gpa1 and Gpa2 possible and if yes is there more Gpa1 interacting with Gpa2 during glucose starvation? Is the localization of Gpa1 affecting PI-3P levels for recycling: is the localization of PI-3P dependent sorting nexins affected? The fact that vps34 and vps15 mutants have reduced recycling is not support for the model. The loss of PI-3P causes massive disruption of all endosomal/lysosomal functions and therefore it is expected that recycling together with all the other functions would be affected.
- The deletion of GPA1 is problematic because it causes constitutive activation of the mating signaling pathway which should
cause cell cycle arrest in G1. The fact that gpa1 mutants are growing indicates either the presence of suppressor mutations or my ignorance in this signaling pathway. This issue should be addressed (in text or experiment).

- The timing of the proposed regulation is not clear. It is known that an acute loss of glucose results in a very efficient endocytic response with rapid loss of nutrient transporters. However, when cells have adapted to the alternative carbon source (e.g. raffinose or galactose) nutrient transporters are more or less stable at the PM. The proposed model suggest that the regulation requires a transcriptional/translational response which is expected to be too slow for the acute regulation (a few minutes), but might be important for long term adaptation. Maybe the turnover rates of cell surface proteins are faster in raffinose-adapted cells. In any case, the proposed regulation has to be put into context of acute versus adapted response. How fast are Gpa2 levels rising after glucose starvation? Does this increase in protein mirror the accumulation of Ste3-DUB in endosomes? There might be 2 distinct regulatory pathways active: a fast/acute one via cAMP and a slow/adaptive one via Gpa2 expression.

- The endosomal localization of Ste3-GFP-DUB in the various experiments has to be quantified. It could be a simple as counting number of intracellular dots per cell.

Minor points:

- Why is the mRNA increase of the mig1mig2 mutant only 2 fold but 6 fold in raffinose growth?
- What are the conclusions from Figure 6H?
- Have all the tagged proteins been tested for functionality (already published or test).
- The explanation to Figure 4D is very confusing. What is the scoring (what method)? Reg1 is mentioned in the text as top scoring but is at the bottom in the table.
- Why is the localization in Figure 2C of Ste3-GFP-DUB so messy (compared to all the other localizations shown of this protein)?

Reviewer #2 (Remarks to the Author):

This manuscript by Laidlaw, Paine et al reports many interesting results on the regulation of endosomal recycling in yeast, notably by - glucose starvation and glucose-sensing/signaling components 
- the class III PI3K Vps34/Vps15 complex 
- the heterotrimeric G proteins Gpa1 and Gpa2

Overall, there are a lot of interesting and convincing observations but I find it hard to link them together. Several panels lack controls. A few key experiments are missing to reach the authors’ conclusions as explained below. A few panels do not bring much to the story and/or existing knowledge in the field. Overall, I feel the manuscript is too preliminary.

First, the authors confirm that glucose starvation inhibits recycling. For this, they use their previously published reporter, Ste3-GFP-DUB, and show that it recycles despite YAP1802 overexpression that increases endocytic rates. The authors then nicely demonstrate that glucose starvation leads to a increase in intracellular Ste3-GFP-DUB, suggesting deficient recycling, and confirm this with FM4-64 recycling assay. Overall, these data confirm those obtained by Lang et al. 2004 that recycling is compromised in glucose-starved yeast.

Second, the authors exploit a previously published screen (MacDonald & Piper, 2017) highlighting that several factors whose deletion abrogates recycling are linked to glucose signaling. They confirm the previously published recycling defect for Ste3-GFP-DUB in these mutants and focus on one candidate, gpa1∆, whose corresponding gene encodes a Galpha subunit of heterotrimeric G protein involved in the mating response signaling pathway but also which interacts with class III PI3K. They nicely demonstrate that GPA1 deletion leads to increased vacuolar trafficking (presumably decreased recycling) of several cargoes (2F) and decreased FM recycling (2E), which are both very strong results.

The authors then go on to study whether the recycling defects observed in the gpa1∆ can be attributed to an interference with PI3K function. They show that deletion of VPS15 or VPS34 leads to FM recycling defects but this is only a correlation. Additionally, data obtained with overexpression of Vps34 (WT or activated allele) suggests that increased PI3K activity could also be detrimental for recycling, but to a lower extent. At this stage and in the absence of more insights, it's not clear how these results should be interpreted.

The authors then go on to show that over-expression of Gpa1 or expression of a constitutively active allele also leads to recycling defects, possibly through the regulation of PtdIns levels, although this is not experimentally addressed. Overall, I found this part too weak and preliminary at this stage. Whereas the recycling defects displayed by the gpa1∆ mutants are clear, I think we lack evidence that this is linked to PI3K regulation.

From the published observation that deletion of the gene encoding the PP1 accessory protein Reg1 prevents constitutive Gpa1-QL signaling, and the known functions of Reg1 in the glucose-mediated repression of gene expression, the authors attempt to link their observations on Gpa1 function in recycling to those observed in the context of glucose starvation. It appears clearly that the deletion of REG1 leads to decreased recycling, both Ste3-GFP-DUB and FM. From this, the authors suggest that there is an "inhibitor" that is expressed in reg1∆ cells (4E, 4F) and this is further supported by similar data obtained in the mig1∆
mig2Δ mutant (6C). Transcript expression and genetic analyses reveal that Gpa2, another Galpha protein involved in glucose signaling through Gpr1, and potential interactor of Gpa1, is likely to be this inhibitor. This is confirmed by an artificial overexpression (Fig 7). I am convinced by this aspect of the work but I think seeing whether (and how much) this translates in protein levels would also be informative (Gpa2 expression levels - especially to compare with experiments in which Gpa2 is overexpressed -Fig 7A). Yet it remains difficult to interpret these results. Gpa2 is a Galpha for Gpr1, which is actually one of the hit genes involved in Ste2-GFP-DUB recycling (see 2B and 2C) so I feel this should probably be looked in a deeper way.

Second, it seems that there is no further confirmation of the Gpa1 / Gpa2 interaction other than large-scale data (Ho et al. 2002) and the co-localization. I think this is very limited to put together a model (Fig 5) whereby Gpa2 titrates Gpa1 at the PM. Overall, although I agree that Gpa2 is likely the factor that inhibits recycling in glucose-repression mutants, the mechanism is not clear. The last part on the localization of Gpa2 using Apotome SIM indicates that Gpa2 has a reticulate distribution at the membrane, like many other PM proteins, but is not detected in internal structures suggesting a function at the plasma membrane - but it does not inform much on the questions addressed here.

Overall, the authors suggest a model which is plausible and may very well be valid but in my opinion was not robustly tested. The dots need to be connected with more experiments. Would the overexpression of Gpa1 rescue the overexpression of Gpa2? Would the ectopic localization of Gpa2 change the localization of Gpa1? etc

1/ Could the authors show Ste3-GFP alone in this condition (without DUB, or with catalytically inactive DUB)? I understand that Ste3-GFP-DUB is merely used as a recycling reporter but following the observations on Mup1 in panel 1A, it would be nice to know what happens to Ste3-GFP.

Also, could the authors remind the readers here why Ste3-GFP-DUB localization at the PM is due to an increased recycling rather than lack of internalization (which could be caused by deubiquitination at the PM).

2/ 2F. The differences are minor. How many times was this experiment repeated? In any case they are not comparable to the effects of the deletion (3C).

3/ Again, what is the new information in panel 3A? As the authors mention, it is well known that vps15Δ or vps34Δ mutants have vacuolar morphology defect and although Airyscan does bring better resolution to these structures, this is not used in the paper (the authors could have looked at Ste3-GFP-DUB for example). Same for 3B, this is confirmation of previous phenotypes.

4/ I think that proof of a functional interaction would be required to state that PI3K is responsible for recycling "in collaboration with Gpa1" (l.1 page 4). For example, Heenan et al. Biochemistry 2009 (PMID 19445518) show that mutation of Arg1261 in Vps15 reduces the interaction with Gpa1, this could be used to make this point. Other strategies may also be considered.

5/ Same thing when the authors attempt to link the reg1Δ defects with those of gpa1Δ based on their similar phenotypes - I disagree with the statement later in page 4, "In support of the idea that Reg1 functions in the same pathway as Gpa1". This would require action on one component or another to go past the correlation.

6/ I am not sure that the deletion of SNF3 alone (4E) can confer phenotypes due to the presence of the ohnologous gene RGT2. In any case, these pathways (Reg1 and Snf3) govern very different aspects of glucose signalling so I don't think this is the most informative.

7/ Fig 6A. It seems to me that the "nucleus" panels are exactly the same between both conditions. Are these the same cells between both conditions, if so how is the glucose/raffinose exchange operated? Similarly, I don't think this panel (and panel 6B) is very informative - it is well known that Mig1-GFP is displaced from the nucleus to the cytosol upon glucose starvation, and the authors don't need to show this data to involve Mig1 in the regulation of Gpa2 expression.

8/ The authors need to show proof of interaction between Gpa1/2 to be able to use this in their model.

9/ Many panels lack controls. 2E lacks an empty-vector control. 4E lacks a WT control. 6F lacks controls / WT and gpa1Δ. etc.

Minor points.
-Please label figures on the figure page.
-Could the authors show more cells in their micrographs when possible? Several experiments are based on expression from plasmids and the expression level can be different, that can cause different localizations.
-one would expect that mutants with defective recycling like gpa1Δ have increased resistance to drugs transported by the corresponding permeases (as in Shi et al. MBoC 2011 for Can1/canavanine). This would help consolidate the findings. Are there evidence in the literature that this is the case?
RESPONSE TO REVIEWERS

We would like to sincerely thank the reviewers for all the comments we received, it highlighted several key weaknesses of the original paper that we fully agree needed to be addressed. We also appreciate comments on how to better present and describe the model. We have tried to take all these comments on board, reconcile any differences of opinion, and consolidate old and new data into a coherent story regarding the regulation of endosomal trafficking in yeast. The proposed experiments have essentially all supported the original model and we hope this resubmitted version might be appropriate for consideration at MBoC. We have also tapered our conclusions to allow alternative views to be considered and present the model in a less cavalier fashion. In addition to the specific responses detailed below, we have better improved the study by:

- Providing a genetic explanation via genome sequencing for the viability of gpa1Δ yeast cells (Figure 3A - 3F).
- Showing that Gpa1 functionally connects with PI3K to regulate recycling, and a point mutation that disrupts interaction results in defective recycling (Figure 4F - 4H).
- Providing evidence that glucose starvation triggers an increase in overall protein levels of Gpa2 by immunoblot, in addition to a distribution shift of Gpa2 from the cytoplasm to the plasma membrane (Figure 8A - 8F).
- Demonstrating that Gpa1 and Gpa2 interact at the surface using a FRET based approach (Figure 10A, 10B, S8).
- Confirming the model that glucose starvation perturbs downstream PI3K function via Gpa2 (Figure 11A - 11B).
- Validated new reagents we have generated for this study that have not previously tested for function. Including the ability of fluorescently tagged versions of Gpa1 to rescue mating defects of gpa1Δ defects (Figure S1) and Gpa2 to rescue the small growth phenotype of gpa2Δ cells (Figure S4).

For simplicity, the figure subpanels referenced in our response match the new figure layout, which is quite different from the original submission due to the volume of extra data. But we also highlight the original figures where it is helpful.
The presented study focuses on the regulation of endosomal recycling by the presence or absence of glucose. Previous studies have documented that glucose starvation results in the rapid degradation of numerous cell surface proteins, in particular nutrient transporters. This study finds that this downregulation is mediated in part by an impaired endosome-to-PM recycling pathway. The authors implicate the G-alpha proteins Gpa1 and Gpa2 as well as the PI-3 kinase Vps34 in this regulation. Specifically, the expression levels of Gpa2, which are regulated by Mig1, are key for the glucose-dependent change in endosomal recycling. Although the authors present an intriguing model, many aspects of this model remain too speculative. Furthermore, the writing in several sections of the manuscript is confusing and not very precise.

We have included many new experiments to better validate the intriguing model succinctly summarised here, and both reviewers played a critical part in this process; so, thank you. In addition to better supporting the model, we are much clearer about any speculative aspect of the study in the discussion.

Major issues:
1 - Both the Gpr1/Gpa2 sensing system and Ras2, shown to affect recycling, feed into cAMP regulation. Why is cAMP not considered as a potential regulator of endosomal recycling? Based on published models, this would be the obvious regulatory route. Also based on published data, the Ras system is activated by glucose independently of Gpr1 (e.g. S. Colombo, JBC 2004), which contradicts statements in the manuscript. This would support the idea that the common output, cAMP, is key for the regulation. I am not an expert in this field and so if these published models of Ras and Gpr1 are not correct the author should comment. Other discrepancy in the manuscript is the statement that Gpr1 is activated by Gpa2 (the other way around?).

We agree there could be a different regulatory mechanism via Gpr1/Gpa2/Ras2/cAMP that controls endosomal recycling, but we also note that gpa2Δ cells are not defective in recycling (Figures 7F). However, this paper focuses on the mechanisms related to the discovery that gpa1Δ cells exhibit a severe recycling defect (MacDonald & Piper JCB 2017). As Gpa1 is an effector of PI3K, we pursued this line of inquiry instead of cAMP. Although Gpa2 has better defined cellular roles, we find Gpa2 responds to glucose and inhibits recycling of many different protein/lipid cargoes (Figures 7, 8 & 9) governed by Gpa1-PI3K. We don’t find strong evidence for this to be connected to cAMP levels, which might not be expected (see below), but we cannot exclude this possibility. It might be future work on Gpr1/Ras2 might help establish if there are mechanisms related to PI3K regulation documented in our study.

Importantly, although it has been shown that Gpa2 is required for cAMP production when cells are shifted from low to high glucose (Colombo et al JBC 1998), this same study and others demonstrate neither deletion or overexpression of GPA2 influences cAMP levels in sustained glucose conditions (such as those used in our study that reveal recycling defects). In pheromone signalling, Gpa1 associates with the Beta-Gamma subunits Ste4 and Ste18. The Beta-Gamma dimer for Gpa2 has not been identified, but has been sought. Such searches reasonably tested for an interaction with Ste4-Ste18 but yielded no evidence of Gpa2 binding, and related studies have revealed modulating the Gpa1-Ste4-Ste18 module has no effect on cAMP production (reviewed in Versele et al 2001 EMBO Reports). This suggests signalling via Gpa1, which is required for recycling, is not regulated via cAMP.

The errors in language have been corrected, thanks for flagging this.

2 - The key regulatory steps in the proposed model are the sequestration of Gpa1 by high levels of Gpa2, which then leads to reduced production of PI-3P by Vps34. These regulatory steps need to be better analyzed. Is during glucose starvation Gpa1 recruited away from endosomes to the PM and is this relocalization dependent on binding to Gpa2? Are co-IPs of Gpa1 and Gpa2 possible and if yes is there more Gpa1 interacting with Gpa2 during glucose starvation? Is the relocalization of Gpa1 affecting PI-3P levels for recycling: is the localization of PI-3P dependent sorting nexins affected? The fact that vps34 and vps15 mutants have reduced recycling is not support for the model. The loss
of PI-3P causes massive disruption of all endosomal/lysosomal functions and therefore it is expected that recycling together with all the other functions would be affected.

Thank you for these various suggestions. In support of the model, we created an endogenously expressed version of Gpa2-GFP and used it to demonstrate that raffinose treatment increases Gpa2 protein levels (Figures 8A - 8B). Unfortunately, biochemical analysis was not possible, despite many attempts and optimisations of disrupting cells (including spheroplasting, high pressure French press, glass beads) and lysate making conditions (optimising detergents, inhibitors, buffer conditions) we were unable to generate detergent soluble lysates that retained either Gpa1 or Gpa2 at full length (Rebuttal Figure 1a). We even tried to purify from large 1-2L volumes of yeast cultures following lysis followed by concentration to generate lysates for IP. However, attempts to capture full length protein using high affinity GFP nanobodies conjugated to beads was unsuccessful, and we were only able to immunoisolate GFP alone (Rebuttal Figure 1b). Optimisations of mCherry tagged Gpa1 was very similar, with only degraded fragments detectable after lysis via various optimised protocols (Rebuttal Figure 1c).

Instead, we were able to show that Gpa1 and Gpa2 interact at the plasma membrane using FRET (Figure 10A - 10B). More sophisticated experiments could not be performed as suggested (e.g. pull down or FRET experiments during raffinose induced GPA2 levels) because endogenously tagged Gpa2-GFP was barely detectable by conventional confocal and required super resolution microscopy to get accurate localisations (Airyscan, Figure 8; Apotome SIM, Figure 10). To get protein at sufficient levels for FRET experiments we over-expressed Gpa1 and Gpa2 and included relevant negative controls and quantifications (Figure S8). However, we were able to use 3D super resolution microscopy to show that raffinose treatment redistributes endogenously expressed cytosolic Gpa2 to the plasma membrane (Figure 8C - 8D).
Following this wonderful suggestion, we provide Figure 11, which shows the localisation of the PI3P binding proteins (the PX-domain containing sorting nexin Snx41 and the FYVE domain containing ligase Pib1) is perturbed in raffinose media and this mis-localisation is phenocopied in cells grown in glucose media but over-expressing Gpa2 from a plasmid.

Finally, we agree PI3K null cells are somewhat of a sledgehammer approach that severely disrupts endosomal processes. Therefore, the more subtle experiments in the paper that reveal recycling defects (such as expressing a hyperactive allele of Vps34 (Figure 4), deletion of the PI3K effector Gpa1 (Figure 2), over expression of the Gpa2 inhibitor (Figure 8, 9) are all consistent with PI3K regulation of surface protein recycling in response to available glucose (Figure 11).

3 - The deletion of GPA1 is problematic because it causes constitutive activation of the mating signaling pathway which should cause cell cycle arrest in G1. The fact that gpa1 mutants are growing indicates either the presence of suppressor mutations or my ignorance in this signaling pathway. This issue should be addressed (in text or experiment).

GPA1 was originally proposed as a haploid-specific essential gene, involved in mating in both cell types (Mijajima et al Cell 1987). This study showed suppressor mutations from gpa1Δ mutants, that are defective for mating phenotype, are very common. This means it is possible to grow yeast strains lacking GPA1, which we and others have taken advantage of to understand the role of Gpa1 (Klein et al 2000 PNAS, Gillen et al 1998 JCS, Medici et al, EMBO 1997, etc). However, to obtain a genetic explanation for this we took a genome-sequencing approach. Although we did identify 27 mutations that were shared in both gpa1Δ null mutants but not the wild type control, none of these were in regions likely to impact genes or expression (not in ORFs or 1000bp up/downstream of ORFs). However, we identified several promising mutations from this analyses that explain suppression of lethality (see below).

Whilst it is true that under normal physiological conditions constitutive signalling in gpa1Δ cells will result in cell arrest, it has been previously showing that a temperature sensitive allele of the MAPKKK Ste11 will bypass this defect when cells are grown at, or near, restrictive temperature of 34°C (Gillen et al 1998 JCS). We discovered the BY4741 gpa1Δ mutant strain we have used lacks Ste11 activity by virtue of a premature stop codon in STE11 after residue 416 (the Ste11 kinase domain encompasses residues 410-476).

The explanation for the viability of BY4742 mutants lacking GPA1 is less obvious. GO analysis of the mutated genes our sequencing identified only revealed one mutation likely to impact biological processes related to Gpa1, and this was a point mutation in the MAPKK gene MKK1. As Ste11 and Mkk1 directly interact and have functional cross talk (Leng & Song 2016 FEBS letters) it is possible this mutation also renders gpa1Δ mutants viable.

Given the two described discoveries above, it is important to note that the actual parental background BY4742 (different from that used in the original Mijajima et al Cell 1987 study) also harbours two mutations: point mutations both SST1 and SAC7 that were identified from GO terms associated with biological processes related to Gpa1. These mutations, alone or on combination in the gpa1Δ strains might account for viability. We have distilled down this extensive analysis to a single new Figure 3.

4 - The timing of the proposed regulation is not clear. It is known that an acute loss of glucose results in a very efficient endocytic response with rapid loss of nutrient transporters. However, when cells have adapted to the alternative carbon source (e.g. raffinose or galactose) nutrient transporters are more or less stable at the PM. The proposed model suggest that the regulation requires a transcriptional/translational response which is expected to be too slow for the acute regulation (a few minutes), but might be important for long term adaptation. Maybe the turnover rates of cell surface proteins are faster in raffinose-adapted cells. In any case, the proposed regulation has to be put into context of acute versus adapted response. How fast are Gpa2 levels rising after glucose starvation? Does this increase in protein mirror the accumulation of Ste3-DUB in endosomes? There might be 2 distinct regulatory pathways active: a fast/acute one via cAMP and a slow/adaptive one via Gpa2 expression.

This is a misunderstanding and we have altered the text at the end of the first results section (Glucose starvation inhibits surface recycling) to explain better. For glucose starvation experiments we avoid exchanging the cells with media lacking
any sugar, as a more dramatic change in osmotic potential would create a much more dramatic physiological change in addition to loss of glucose related signal transduction pathways (that we want to investigate). This is especially pertinent as changes in osmotic potential are known to affect surface membrane proteins via eisosomes (Riggi et al., 2018 Nature Cell Biology) and endocytosis (Riggi et al., 2019 JCB). Instead, we acutely exchange with the trisaccharide raffinose, which cannot be directly converted to energy (de la Fuente & Sols 1961 Biochem. Acta). Our raffinose treated cells behave as though they are starved for glucose during the experimental periods we assess (up to the first 2 hours). Furthermore, although surface proteins are internalised in response to raffinose, there are significant levels of varied proteins, like Ste3, Fur4, and Mup1, at the plasma membrane following 2 hours treatment (see time lapse microscopy in Laidlaw et al 2021 JCS). These surface populations are therefore available for regulation through the mechanism we describe in this study.

There is no adaption period to raffinose, the phenotypes we document are assessed in the period less than 2 hours from starvation, where we find increased transcript levels of GPA2, increased protein levels of Gpa2, redistribution of cytosolic Gpa2, recycling defects, etc.

We now also acknowledge timing more clearly at the start of the final discussion paragraph, including some references from relevant mechanisms that might integrate with our discoveries to regulate surface protein trafficking at both faster and slower time scales.

5 - The endosomal localization of Ste3-GFP-DUB in the various experiments has to be quantified. It could be a simple as counting number of intracellular dots per cell.

We have included more quantifications across the manuscript and specifically those requested by the reviewer (see Figures 2D, 5F and 7G).

Minor points:
6 - Why is the mRNA increase of the mig1mig2 mutant only 2 fold but 6 fold in raffinose growth?

We have previously noted that expression of other genes in response to raffinose versus deletion of MIG1 and MIG2 is similarly affected. The acute starvation (that results in Mig1 translocation and derepression) is almost immediate and for example results in a 7.1±1.4 increase in expression of YAP1801 (Laidlaw et al 2021 JCS). The creation of mig1Δ mig2Δ null cells is far from acute, and minimally involves many, many generations to isolate a clonal population of double mutants. These mutants still show a significant 2.1±0.3 increase in expression of YAP1801. We assume growth during identification and isolation of mig1Δ mig2Δ mutants allow for adaption to attenuate the severity of the response compared to glucose starvation.

We assume the same with expression of GPA2, which is similar, as mentioned, to YAP1801. Taken together with significant increase under both conditions, we propose GPA2 is regulated in response to glucose via Mig1.

7 - What are the conclusions from Figure 6H?

Figure 6H is now Figure S3. Recycling efficiency is connected to the ratio of Gpa1 in endosomes and surface, but the function of Rcy1 is not known and we only speculate now as to why Gpa1 levels at endosomes is reduced in conditions with less recycling. We agree this does not lend itself to a necessarily obvious conclusion, so we have moved these micrographs to the supplemental material. Happy to remove entirely if this is deemed better.

8 - Have all the tagged proteins been tested for functionality (already published or test).

Most have previously been shown to complement and are referenced. Ste3-GFP-Dub is an artificial cargo used to assess recycling, so technically would not be expected to complement a specific phenotype. We now include complementation analysis of the important constructs that had not been previously characterised, such as fluorescently tagged: Gpa1 rescuing mating defects of gpa1Δ (Figure S1); and Gpa2 rescuing cellular size defect in gpa2Δ cells (Figure S3).
9 - The explanation to Figure 4D is very confusing. What is the scoring (what method)? Reg1 is mentioned in the text as top scoring but is at the bottom in the table.

Figure 4D is now Figure 5D. I do apologise about this; it is a very complicated facet to the story that I was keen to included but never did a sufficiently good job at explaining. In short, there was a paper documenting a screen for proteins that suppress the effects of constitutively active Gpa1 (Slessera 2006 Cell). This paper made no mention of several of the top scoring mutants. We point out that cells lacking REG1, which was the most effective mutant from the Gpa1 screen, would phenocopy the mig1Δ mig2Δ cells in our study. i.e. these cells have high levels of Gpa2, which is why they were identified as a Gpa1 regulator.

10 - Why is the localization in Figure 2C of Ste3-GFP-DUB so messy (compared to all the other localizations shown of this protein)?

A different microscope was used - which we now make more obvious reference to in the figure legend. This result is also supported by the flow cytometry (Figure 2D) from a requested experiment during a previous round of review. This basic characterisation was performed to show localisation with standard confocal, and we do not pursue this line of inquiry so did not feel it was worth performing super resolution microscopy for strains not discussed further. The gpa1Δ result is of validated using a series of distinct experimental avenues across various figures.

Reviewer #2 (Remarks to the Author):

This manuscript by Laidlaw, Paine et al reports many interesting results on the regulation of endosomal recycling in yeast, notably by
- glucose starvation and glucose-sensing/signaling components
- the class III PI3K Vps34/Vps15 complex
- the heterotrimeric G proteins Gpa1 and Gpa2

Overall, there are a lot of interesting and convincing observations but I find it hard to link them together. Several panels lack controls. A few key experiments are missing to reach the authors' conclusions as explained below. A few panels do not bring much to the story and/or existing knowledge in the field. Overall, I feel the manuscript is too preliminary.

We thank reviewer 2 for the appraisal and hope the additional experiments help link the initial work and provide more robust support for the model, in addition to the inclusion of more controls and quantifications.

First, the authors confirm that glucose starvation inhibits recycling. For this, they use their previously published reporter, Ste3-GFP-DUB, and show that it recycles despite YAP1802 overexpression that increases endocytic rates. The authors then nicely demonstrate that glucose starvation leads to a increase in intracellular Ste3-GFP-DUB, suggesting deficient recycling, and confirm this with FM4-64 recycling assay. Overall, these data confirm those obtained by Lang et al. 2004 that recycling is compromised in glucose-starved yeast.

Second, the authors exploit a previously published screen (MacDonald & Piper, 2017) highlighting that several factors whose deletion abrogates recycling are linked to glucose signaling. They confirm the previously published recycling defect for Ste3-GFP-DUB in these mutants and focus on one candidate, gpa1Δ, whose corresponding gene encodes a Galpha subunit of heterotrimeric G protein involved in the mating response signaling pathway but also which interacts with class III PI3K. They nicely demonstrate that GPA1 deletion leads to increased vacuolar trafficking (presumably decreased recycling) of several cargoes (2F) and decreased FM recycling (2E), which are both very strong results.
The observations are consistent with previous reports from the labs of Mara Duncan and Howard Riezman, both referenced, hopefully validating the rationale for our effort to identify molecular regulators. We also presume increased vacuolar trafficking is caused by reduced recycling, due to an increase in endosomal retention and elevated signal for degradation through the endosomal ubiquitination machinery.

The authors then go on to study whether the recycling defects observed in the gpa1Δ can be attributed to an interference with PI3K function. They show that deletion of VPS15 or VPS34 leads to FM recycling defects but this is only a correlation. Additionally, data obtained with overexpression of Vps34 (WT or activated allele) suggests that increased PI3K activity could also be detrimental for recycling, but to a lower extent. At this stage and in the absence of more insights, it's not clear how these results should be interpreted.

The authors then go on to show that over-expression of Gpa1 or expression of a constitutively active allele also leads to recycling defects, possibly through the regulation of PtdIns levels, although this is not experimentally addressed. Overall, I found this part too weak and preliminary at this stage. Whereas the recycling defects displayed by the gpa1Δ mutants are clear, I think we lack evidence that this is linked to PI3K regulation.

Agreed, and also raised by Reviewer 1. We have now added data to show that the functional connection between Vps15 and Gpa1 is required for efficient control of recycling (Figure 4D-4H), in addition to PI3K (which as Reviewer 1 mentioned might be expected), its effector Gpa1 (Figure 2) and this activity must be finely tuned for correct sorting (Figure 4D-4E). Furthermore, we have confirmed the model by revealing novel effects of raffinose and GPA2 overexpression on endosomal proteins that rely on PI3K production of PtdIns3P (Figure 11).

From the published observation that deletion of the gene encoding the PP1 accessory protein Reg1 prevents constitutive Gpa1-QL signaling, and the known functions of Reg1 in the glucose-mediated repression of gene expression, the authors attempt to link their observations on Gpa1 function in recycling to those observed in the context of glucose starvation. It appears clearly that the deletion of REG1 leads to decreased recycling, both Ste3-GFP-DUB and FM. From this, the authors suggest that there is an "inhibitor" that is expressed in reg1Δ cells (4E, 4F) and this is further supported by similar data obtained in the mig1Δ mig2Δ mutant (6C). Transcript expression and genetic analyses reveal that Gpa2, another Galpha protein involved in glucose signaling through Gpr1, and potential interactor of Gpa1, is likely to be this inhibitor. This is confirmed by an artificial overexpression (Fig 7). I am convinced by this aspect of the work but I think seeing whether (and how much) this translates in protein levels would also be informative (Gpa2 expression levels - especially to compare with experiments in which Gpa2 is overexpressed -Fig 7A). Yet it remains difficult to interpret these results. Gpa2 is a Galpha for Gpr1, which is actually one of the hit genes involved in Ste2-GFP-DUB recycling (see 2B and 2C) so I feel this should probably be looked in a deeper way. Second, it seems that there is no further confirmation of the Gpa1 / Gpa2 interaction other than large-scale data (Ho et al. 2002) and the co-localization. I think this is very limited to put together a model (Fig 5) whereby Gpa2 titrates Gpa1 at the PM. Overall, although I agree that Gpa2 is likely the factor that inhibits recycling in glucose-repression mutants, the mechanism is not clear.

The last part on the localization of Gpa2 using Apotome SIM indicates that Gpa2 has a reticulate distribution at the membrane, like many other PM proteins, but is not detected in internal structures suggesting a function at the plasma membrane - but it does not inform much on the questions addressed here.

Overall, the authors suggest a model which is plausible and may very well be valid but in my opinion was not robustly tested. The dots need to be connected with more experiments. Would the overexpression of Gpa1 rescue the overexpression of Gpa2? Would the ectopic localization of Gpa2 change the localization of Gpa1? etc
Again, a completely valid point. We now demonstrate that the protein levels reflect the changes in mRNA, with an increase of Gpa2 in glucose starved cells (Figure 8A - 8B). There is also a redistribution of Gpa2 to the surface in these conditions (Figure 8C - 8D). These observations do a much better job to support the model and explain the various cargo recycling defects documented in our first submission (Figure 9). We have added text to explain the results and the implication that cAMP/Gpr1/Ras2 might integrate with these functions at some other level (also mentioned by other reviewer). We have toned down the conclusions and model regarding this point, and in general, we tried really hard to validate the large-scale interaction documented by Ho et al, but this was unsuccessful biochemically (see Rebuttal Figure above). However, using over-expression constructs were able to validate a Gpa1-Gpa2 interaction using FRET (Figure 10A - 10B).

We hope you agree these various new experiments combined provide a more robust test of the original model, and we really appreciate all the comments to achieve this, detailed below.

1/ Could the authors show Ste3-GFP alone in this condition (without DUB, or with catalytically inactive DUB)? I understand that Ste3-GFP-DUB is merely used as a recycling reporter but following the observations on Mup1 in panel 1A, it would be nice to know what happens to Ste3-GFP. Also, could the authors remind the readers here why Ste3-GFP-DUB localization at the PM is due to an increased recycling rather than lack of internalization (which could be caused by deubiquitination at the PM).

We find that GFP tagged cargoes like Ste3, Fur4, Gap1 that predominantly localise to the vacuole make any subtle trafficking differences at the PM difficult to assess. Instead, we have previously used two cargoes that mainly localise to the PM, Mup1 and Can1, to show AP180 overexpression induces endocytosis (Laidlaw et al 2021 JCS). We now explain this to nicely set up the Ste3-GFP-DUb analysis. The important feature of this fusion protein is that it chiefly reports on endosomal organisation and recycling, which the Ste3-GFP-DUb and FM4-64 cargoes allow.

The reason we have concentrated on an engineered protein reporter is that Ste3, and all other endogenous cargoes, are also susceptible to increases in endocytic rates. The crucial point of Figure 1 is to dissect internalization and recycling, with the Ste3-GFP-DUb and FM4-64 cargoes allow.

2/ 2F. The differences are minor. How many times was this experiment repeated? In any case they are not comparable to the effects of the deletion (3C).

This is an example profile from an experiment that has been performed multiple times. It shows over a million data points recorded over 10-minutes. Efflux measurements include an ‘on the day’ WT control as we presume FM4-64 uptake, labelling and recycling are all susceptible to subtle changes (e.g. room temperature). The data is presented in similar format to the original documentation of the assay (Wiederkehr et al 2000 JCB). Furthermore, our protocol gives efflux profiles for rcy1Δ and vps4Δ mutants that are near identical to the Riezman lab, so we assume the assay is properly calibrated and the recycling defects in gpa1Δ cells bona fide, a notion substantiated by the additional defects of protein cargoes Mup1, Fur4 and Ste3.

Furthermore, the FM4-64 recycling defect of gpa1Δ is actually very substantial, with efflux deficiencies similar to deletion of established recycling regulators like the Rag GTPases (MacDonald & Piper 2017 JCB) and hos2Δ mutants (Amoiradaki et al, 2021 JMS). Our evidence suggests that modifying regulation of PI3K does not mimic deletion of PI3K activity, which might be predicted as mentioned (see reviewer 1, point #2). The profiles for the PI3K nulls are indeed very extreme and much more defective than any mutants I am previously aware of being measured using this assay, including rcy1Δ, tlg1Δ, tlg2Δ, and sec18-1 at restrictive temperature (Wiederkehr et al 2000 JCB). We know these cells uptake the dye (Figure 4A) but the endosomal organisation of these mutants is very disrupted, which also have severe growth defects (Figure 4B). It is possible that the wash steps, which must be performed in cold media, abrogate the endosomal system and reduce FM4-64 recycling further.
3/ Again, what is the new information in panel 3A? As the authors mention, it is well known that vps15Δ or vps34Δ mutants have vacuolar morphology defect and although Airyscan does bring better resolution to these structures, this is not used in the paper (the authors could have looked at Ste3-GFP-DUB for example). Same for 3B, this is confirmation of previous phenotypes.

We considered it better to demonstrate the endosomal morphology beside the novel demonstration of FM4-64 efflux measurements in PI3K mutants (as discussed in previous point). Therefore, although this initial figure panel A does not demonstrate ‘new’ information (although the spatial resolution is a vast improvement of anything I could find in the literature) it does set up the other subpanels in this Figure. We do show, for the first time, more subtle manipulations of PI3K also impact recycling:

1. Increasing PI3K activity, which has no impact on growth (we only included Figure 4B as we didn’t think growth had been compared in PI3K nulls with hyperactive Vps34 alleles) but does perturbs the recycling pathway (Figure 4D - 4E). This adds to recent discoveries that hyperactive Vps34 perturbs some, but not all, post Golgi trafficking routes (Steinfeld et al 2021 MBoC).

2. The PI3K effector Gpa1 is required for recycling (Figure 2).

3. The functional connection between Gpa1 and PI3K in recycling is suggested through the new data showing Vps15 mutants that cannot being Gpa1 are also defective in recycling (Figure 4F - 4G).

We are happy to remove these micrographs due to being insufficiently novel, but ideally only if it does not reduce the reader’s ability to connect all the distinct discoveries.

We were unable to obtain Mat alpha strains lacking either VPS15 or VPS34 (from the deletion collection we have purchased or by making the mutants using homologous recombination). We are very grateful to Lois Weismann for providing us with PI3K mutants, but these are both in a Mat A strain that unfortunately should not express Ste3 or Ste3-GFP-DUb. As we have failed in our renewed efforts over the last 8 months to generate Mat Alpha PI3K mutants we stably integrated Ste3-GFP-DUb at the HIS3 locus in MatA cells. Although some protein appears to be synthesised, and it does not localise to the surface, instead resembling other recycling mutants and accumulating in puncta (Rebuttal Figure 2), we did not feel that conceptually this experiment makes sense, so we have not included it in the resubmission. As before, happy for editor/reviewer guidance on this.

4/ I think that proof of a functional interaction would be required to state that PI3K is responsible for recycling "in collaboration with Gpa1" (l.1 page 4). For example, Heenan et al. Biochemistry 2009 (PMID 19445518) show that mutation of Arg1261 in Vps15 reduces the interaction with Gpa1, this could be used to make this point. Other strategies may also be considered.

We entirely agree and performed the suggested experiment (thank you!) to reveal that disruption of this interaction causes a defect in surface recycling (Figure 4F, 4G, 4H). The defect is in a similar range to gpa1Δ (Figure 2).
5/ Same thing when the authors attempt to link the reg1Δ defects with those of gpa1Δ based on their similar phenotypes - I disagree with the statement later in page 4, "In support of the idea that Reg1 functions in the same pathway as Gpa1". This would require action on one component or another to go past the correlation.

We have modified the text to overly imply these effects are directly related and do not use "pathway" to describe this relationship. The additional data showing an interaction does better connect Gpa1 and Gpa2 as functional regulators (Figure 10), in addition to the protein levels and localisation of Gpa2 being controlled by glucose (Figure 8), with the ability to ultimately modulate PI3K activity (Figure 11). With these additions, I now believe the model presented as a Reg1 > Mig1 > Gpa2 impinges on PI3K-Gpa1 is much better supported.

6/ I am not sure that the deletion of SNF3 alone (4E) can confer phenotypes due to the presence of the ohnologous gene RGT2. In any case, these pathways (Reg1 and Snf3) govern very different aspects of glucose signalling so I don't think this is the most informative.

We created all these reagents and performed this experiment at the request of a reviewer from a previous round. We are glad our results supported expectation that although Reg1 is involved in recycling (via Mig1 > Gpa2) that Snf3 is not. For full transparency I have included previously suggested experiments and responses (in red and grey) at the end of this rebuttal. In light of this, and as some readers might share the views of the original reviewer, I have opted to keep this result in the paper. However, if this is an issue, I think it can be removed without losing the overall message and we would be happy to comply for the best story.

7/ Fig 6A. It seems to me that the "nucleus" panels are exactly the same between both conditions. Are these the same cells between both conditions, if so how is the glucose/raffinose exchange operated? Similarly, I don't think this panel (and panel 6B) is very informative - it is well known that Mig1-GFP is displaced from the nucleus to the cytosol upon glucose starvation, and the authors don't need to show this data to involve Mig1 in the regulation of Gpa2 expression.

These micrographs represent the same cells imaged over different time periods, with the glucose media exchanged using a microfluidic system. We have updated the legend to better describe this. To avoid this concern, we re-made this figure with different cells, including a larger field of view and the DIC and segmentation mask (Figure 7A).

Related to the previous reviewer comment (Reviewer 2, point #6). Instead of simply referencing previous work showing Mig1 is glucose responsive (which we also do), we considered it important to demonstrate that the raffinose conditions and protocols used to perform transcriptomic, biochemical and cell biological assays across the paper trigger the expected phenotypes (in addition to our discoveries), in expected timeframes.

8/ The authors need to show proof of interaction between Gpa1/2 to be able to use this in their model.

Agreed, see Reviewer 1, point #2 and rebuttal figure, and Figures 10 and S7

9/ Many panels lack controls. 2E lacks an empty-vector control. 4E lacks a WT control. 6F lacks controls / WT and gpa1Δ. etc.

Figure 2E is now Figure 2F. In this experiment we have not transformed the cells so did not include an empty vector condition. Apologies for the confusion, we have now amended the FM4-64 recycling assay methods section to better highlight the appropriate controls used for these efflux assays. Any comparisons between cells are performed 1) on the same day and 2) in the same media. If a plasmid transformation is being compared against wild-type, the wild-type cells will be transformed with an empty vector control. If plasmid over-expression is induced in the presence of copper, the empty vector control will be exposed to the same copper conditions for the same incubation period.
Figure 4E is now Figure 5E / Figure 6F & 6G are now combined in Figure 7F. These figures have been broken down into subpanels to make dissection of different experimental aims easier to explain. For example, controls for 5E are shown in only the top scoring mutants and many other places in this paper (1B, 1C, 2C, 2D, 7C, 9A) and other manuscripts. Additionally, Reviewer 1, point #6 requested that we quantify these phenotypes. Not only does this increase robustness of results, including statistical analyses from large numbers, but it also unites the data much better (for example data represented in 5B, 5C and 5E are collated and quantified in a single graph in 5F, which allows easy comparison across conditions.

We have added many more experiments and controls to support the model. Furthermore, these Ste3-GFP-DUb experiments are complemented by additional and distinct experimental approaches (e.g. FM4-64 efflux assays), hopefully bolstering the robustness of our observations.

Minor points.
-Please label figures on the figure page
Done

-Could the authors show more cells in their micrographs when possible? Several experiments are based on expression from plasmids and the expression level can be different, that can cause different localizations

Many of these experiments were performed at a) very low cell density to allay concerns about nutrition and b) with imaging scan speeds maximised with small fields of view (described in Laidlaw et al JCS 2021). We take the point on board and in addition to incorporating more cells to micrographs (See Figures 7, 11, and S4) but we also include quantifications from large numbers of cells throughout the manuscript (for example those requested by reviewer 1 in Figures 2, 5 and 7 and the new data quantified in in Figures 8, 10 and 11.

-one would expect that mutants with defective recycling like gpa1Δ have increased resistance to drugs transported by the corresponding permeases (as in Shi et al. MBoC 2011 for Can1/canavanine). This would help consolidate the findings. Are there evidence in the litterature that this is the case?

The supplemental data of Shi et al MBoC 2011 does not include the canavanine resistance quantifications for every strain tested. So it might be that gpa1Δ have a phenotype that was not considered sufficient to be included alongside the top scoring mutants. Whilst we can be confident about hits identified from genetic screens, as they can be validated, we can’t be confident that mutants in a survey of 5000-6000 candidates were thoroughly tested. Furthermore, yeast growth in the presence of canavanine is an indirect measure of Can1 recycling, and other pleiotropic effects from this toxic compound might alter Can1 trafficking in unexpected ways (e.g. we were surprised about Ste3-GFP.

In support of our model, the Saccharomyces cerevisiae genome database does state that gpa1Δ mutants have ‘abnormal chemical compound accumulation’ suggesting the surface proteome is affected by deletion of GPA1. The SGD also shows ‘decreased starvation resistance’ which conceptually ties into our glucose mediated control of recycling via Gpa1 and helps allay concerns that our large series of results are not consistent with the literature.
REBUTTAL TO ORIGINAL REVIEWS - THIS INCLUDES DESCRIBING ALL NEW DATA ORIGINALLY SUBMITTED TO MBoC IN APRIL 2021

We thank the reviewers for comments related to the recycling pathway in response to glucose starvation. We have used these comments to improve the text and explanations, alongside new data that has been used to:

a) Address the reviewer concerns about experimentation and interpretations in paper (individual points discussed below)
b) Perform critical experiments to demonstrate observed effects are specific to reduced trafficking through the recycling pathway (Figs. 1A, 1B)
c) Show that gpa1Δ defects in recycling are broad, in addition to the engineered reporter, lipid dye recycling and various endogenous cargoes are also shown to be defective in recycling (Figs. 2A - 2F)
d) Include more mechanistic insight about the functional role of Gpa1, in particular showing PI3-kinase has a role in cargo recycling (Figs. 3A - 3F)
e) More data and better explanation about the glucose-mediated transcriptional control of recycling (Figs. 4C - 4F, 5A - 5B, and 6D) including upstream data with Reg1 and constitutively active Gpa1
f) Include more experiments demonstrating the effects of Gpa2 over-expression on different cargoes and how Gpa2 localisation implies this inhibitory role on endosomal recycling is indirect (Figs 7D and 8A - 8E)

Reviewer #1

For Fig. 5, it would be beneficial to include a flowchart to summarize the relationships between Mig1, Gpa1, Gpa2, Gpr1, Ras, etc. framed in the context of membrane recycling and endocytosis.

We agree this relationship is confusing, and not fully understood. However, we now include a full schematic (Figure 5A, 5B) that depicts much of what is understood about these factors, and also incorporates out discoveries and working model for how all these factors function in a pathway to control cell surface recycling.

For Fig. 5B and 5H, it is not intuitive what the authors mean by FM4-64 efflux. Please explain how this was measured (i.e., what was the readout?).

Apologies, we have relied too heavily on references to describe this approach, an issue also mentioned by Reviewer 2. Each experiment typically represents about ~1 million yeast cells measured over 10 minutes, and a single population plotted over time. All comparisons were made from cells loaded with dye and efflux measurements made at the same time. This detail has been added to the methods section and also elaborated in both the main results section and the figure legends.

For Fig. 5F, please include data on gpa1Δgpa2Δ to show which gene would have a more dominant effect.

Great experiment suggestion. We generated a gpa1Δ gpa2Δ double null strain and stably integrated the recycling reporter to find that recycling is also defective in this background (Fig. 6F). We conclude that PI3P-kinase-Gpa1 is crucial for efficient recycling and that Gpa2 is a non-essential regulator of the pathway.

We also extended this idea and created a gpa2Δ mutant in the mig1Δ mig2Δ strain. This demonstrates that the Ste3-GFP-DUb recycling defects of mig1Δ mig2Δ cells (Fig. 6C) are suppressed in a gpa2Δ mig1Δ mig2Δ strain (Fig. 6G). This nicely complements data that shows over-expressing Gpa2 causes recycling defects (Fig. 7A - 7D), as it suggests the elevated expression of GPA2 in mig1Δ mig2Δ cells (Fig. 6E) are solely responsible for the various recycling defects observed.

Reviewer #2

Figure 5: Several of the Ste3-GFP-Dub experiments are qualitative in nature and hard to interpret. For example, Figure 5D: Ste3-GFP-Dub is claimed to be more internalized in Mig1/2 double KO cells. However, based on the data presented the PM pools of Ste3-GFP-Dub are the same.
Importantly, we now use extra experiments in Figure 1A and 1B to point out that Ste3-GFP-DUb, which has been characterised extensively (MacDonald & Piper 2017 JCB), is exclusively localised at the plasma membrane at steady state in wild-type cells. Therefore, any intracellular signal in defective recycling conditions / mutants can be used as an indication of increased endocytosis / decreased recycling.

The abundance of PM pools cannot be accurately assessed from visualisation. To avoid such an interpretation, we have performed flow cytometry measurements of various cells expressing Ste3-GFP-DUb to demonstrate that Ste3-GFP-DUb levels do not increase in recycling defective (gpa1Δ, ras2Δ, and gpr1Δ) cells. Therefore, any observable intracellular signal cannot be explained by elevated expression and we believe represent molecules that have not recycled efficiently and are now observable at steady state. We also added quantification of recycling efficiency (e.g. Fig. 7B) to complement micrographs. Additionally, throughout the paper, recycling defects are often shown through the completely independent FM4-64 recycling assay.

In addition to the controls described above, we have added additional arrows indicating key plasma membrane (PM) and endosome (E) markers for the Ste3-GFP-DUb localisation micrographs, to avoid confusion.

From the data presented, it is unclear if this is due to just a block in recycling or an increased endocytic uptake, as your previous data suggest.

This is a good point and to address it, we have added an in depth explanation at the start of the results section, including new data (Figs. 1A, 1B). We mention that the Ste3-GFP-DUb fusion protein seems to preferentially report on recycling, by virtue of it having no discernible localisation differences in 3 separate manipulations to increase endocytosis levels: 1) ligand induced internalization, 2) endocytosis triggered by elevated temperature, 3) increased endocytosis by over-expression of AP180 adaptors. Therefore, as mentioned above intracellular signal relates to recycling defects. And again, we bolster these localisation phenotypes with FM4-64 efflux assays, as it measures efflux of dye that has already been loaded to endosomes. Therefore, efflux is calculated as a percentage of uptaken dye, so rates are focussed on recycling (not internalization) kinetics.

That said, it is clearly very important to note and discuss (in introduction and discussion) that glucose starvation results in molecular mechanisms that modulate two distinct and opposing membrane trafficking pathways that work together to downregulate surface proteins. We also highlight the importance of our discovery that both of these trafficking responses to glucose starvation are centred around the transcriptional regulator Mig1 control (Figs. 5A, 5B). Following glucose starvation and Mig1 translocation (or de-repression) endocytosis rates are elevated following increased expression of AP180s whilst recycling is reduced by increased expression of the recycling inhibitor Gpa2.

This is similar in Fig 5G. It is stated here that over-expression of Gpa2-mCherry inhibits surface recycling of Ste3-GFP-Dub (page 8). This is hard to conclude based only on the data presented. If this were true, some Ste3-GFP alone may be completely sorted into the vacuole lumen in Gpa2-mCherry over-expression. Is this the case?

We now quantify the effects on Gpa2 over-expression of Ste3-GFP-DUb trafficking from >200 cells (Fig. 7B). We are very grateful for the suggestion of testing Gpa2 over-expression in other cargoes like Ste3-GFP to complement the Ste3-GFP-DUb data, as also requested by Reviewer 3. Our expectations were the same, the model would predict that Ste3-GFP would be more (possibly entirely) in the vacuole. Unexpectedly, although less Ste3-GFP is localised to the plasma membrane upon Gpa2 over-expression, vacuolar sorting is also inhibited with cargo accumulating in bright prevacuolar structures (Fig. 7D, lower). We assume overexpression of the Galpha subunit Gpa2 perturbs trafficking of the Ste3 specifically as it is a GPCR, in a manner distinct from the Gpa1-PI3P-kinase trafficking mechanism we describe.

Does this data represent FM4-64 readings from one cell, a population of cells, or multiple populations of cells averaged? Be more specific in the Figure Legends.

Please refer to response to Reviewer 1’s second point, above.

Reviewer #3

The conclusions regarding the role of Gpa1/2 in cargo recycling during carbon starvation are mainly based on
study of Ste3-GFP-DUB, an artificial cargo. Analysis of additional cargoes known to recycle would be useful to confirm the roles the Gpa1/2 in recycling.

This was a and also mentioned by Reviewer 2 above specifically for Ste3-GFP. Unlike the data with Ste3-GFP, which is trickier to disentangle (see above), we now show over-expression of Gpa2-mCherry induces endocytosis of Mup1 and Can1 (Fig. 7D, upper two rows). Not only does Ste3-GFP have the complication of being a GPCR, but at steady state much of the GFP tagged signal is accumulated in the vacuole. This is not true of Mup1-GFP or Can1-GFP (in media lacking methionine and arginine, respectively), so these results very clearly support the hypothesis, and we thank the reviewer for the suggestion.

Minor point:
"in response to depleted glucose levels sensed by Mig1" : it is not established that Mig1 is a glucose sensor. Glucose sensing is mediated by the Snf3 and Rgt2 plasma membrane sensors that control Mig1. The authors could extend their work by studying the roles of Snf3/Rgt2 in YAP1801/2 and Gpa1/2 control.

Apologies, this was poorly worded, what we meant was that Mig1 is a glucose responsive factor; we have removed the mistake. Thanks for the suggestion to test the role of surface glucose sensors to these downstream effects. We do not find elevated endocytosis (Laidlaw et al. 2020) or decreased recycling (Fig. 4E) in cells lacking the Snf3 glucose sensor. We assume that although the Snf3 > Rgt2 > Tpk1/2/3 > Rgt1 pathway regulates expression of Mig2, as Mig2 only accounts for approximately 6% of Mig1/Mig2 repression (Lutfiyya and Johnston 1996 MCB) then the glucose > Mig1 pathway we focus on in this study is responsible for the effects we document.

This experimental suggestion and result led us to if the Snf1/ AMPK pathway was involved. We found reg1Δ cells have decreased protein and lipid recycling (Fig. 4E, 4F). So, we further conclude Reg1/Glc7 > Snf1 > Mig1 >Gpa2 pathways we have uncovered are functionally distinct from the Snf3.

These new observations also help rationalise the involvement of Gpa1-PI3Kinase in recycling traffic, as reg1Δ mutants were the very top scoring hit of a genome-wide screen for deletion mutants that attenuate the negative effects of constitutively active Gpa1 (Slessareva et al., 2006 Cell). Although this result was not discussed in the paper, our model would explain these results as reg1Δ mutant, (just like mig1Δ mig2Δ cells - a double mutant not tested in the screen) would have elevated levels of Gpa2 to inhibit the negative effects of constitutively active Gpa1. This rationale and the model/Figure 5 are now built in to the middle of the paper, to best explain this series of observations.

Another cargo such as Snc1, actively recycling to the cell surface, would have probably been a better choice for testing reduced recycling in the analyzed mutants.

We believe Snc1 recycling follows a different pathway (Xu et al, eLife 2017) to the cargoes studied in this manuscript and we are trying to mechanistically dissect these pathways in a different project. Unfortunately, the endogenous cargoes we use mainly enter the MVB pathway, making their recycling much more difficult to study in yeast.

The authors conclude from Fig. 5N that tagged Gpa1 co-localizes to some extent with Sec7, but none quantification is shown, and this co-localization does not seem convincing.

We agree the colocalization experiments do not convincingly demonstrate a clear Golgi or MVB localisation of either Gpa1 or Gpa2, so we show the localisation data to simple propose: 1) that Gpa1 localises to the surface and intracellular endosomal structures, as others have documented and 2) we include additional 4D lattice structured illumination microscopy to suggest that colocalization of Gpa2-GFP with any intracellular structures is very rare.
Dear Chris,

Thank you for submitting your revised manuscript "Endosomal cargo recycling mediated by Gpa1 and Phosphatidylinositol-3-Kinase is inhibited by glucose starvation" to be considered for publication in Molecular Biology of the Cell. I very much appreciate the effort that you put into the revision. I asked the original reviewers to review the revised paper, and I am pleased to say that they, too, appreciated your efforts and find the revised manuscript to be vastly improved compared to the original. I agree with both reviewers, and am pleased to provisionally accept the manuscript on behalf of MBoC.

Both reviewers have very minor comments that need to be addressed before full acceptance. Reviewer #1 cites a number of typographical errors that should be amended, and Reviewer #2 points out a missing figure legend and a conclusion that needs to be toned down. With these corrections I will be pleased to fully accept your manuscript for publication. I trust that these corrections can be done quickly.

Congratulations on a job well done!

Sincerely,

Mickey Marks
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. MacDonald,

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).

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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.

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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.
Reviewer #1 (Remarks to the Author):

The authors addressed all major issues I raised in my review and therefor I support the publication of this manuscript. I encourage the authors to carefully spell check the manuscript. I noticed several mistakes, including the label of the Y-axis in Figure 2D.

Reviewer #2 (Remarks to the Author):

The authors have answered favorably to many of my points. At this stage, I would recommend publication because they have gone through lengthy rounds of review, added lots of new data and made their best to address the comments so in my opinion this should be published. I trust that this lab or others will clarify the points that are not fully answered at this stage.

One last point. Thank you for doing the suggested experiment using Vps15-R1261A. I agree that there is an effect of the mutation on FM recycling and that it is comparable to that observed in gpa1Δ. But when compared to vps15Δ, the conclusion in the text is too strong ("was sufficient to inhibit efficient FM4-64 recycling"). Same in the discussion ("was sufficient to disrupt recycling to a similar degree as GPA1 deletion" - disrupt is too strong). If the Vps15/Gpa1 interaction is indeed abolished by this mutation, then an additional, Gpa1-independent function of Vps15 is missing in the model. This is beyond the scope now, but shouldn't it be acknowledged and discussed?

Minor point. Legend / 4H is missing
Rebuttal for final revision

Reviewer #1 (Remarks to the Author):

The authors addressed all major issues I raised in my review and therefore I support the publication of this manuscript. I encourage the authors to carefully spell check the manuscript. I noticed several mistakes, including the label of the Y-axis in Figure 2D.

Thank you for the many great suggestions that helped us improve this manuscript. We have corrected the improper spelling of ‘puncta’ in the y-axis of Figure 2D and corrected various typos from the methods and figure legends.

Reviewer #2 (Remarks to the Author):

The authors have answered favorably to many of my points. At this stage, I would recommend publication because they have gone through lengthy rounds of review, added lots of new data and made their best to address the comments so in my opinion this should be published. I trust that this lab or others will clarify the points that are not fully answered at this stage.

Thanks for the great ideas to improve our study. We agree there are more questions, some of which we have raised in the discussion, that will be important for us and others to tackle in the future.

One last point. Thank you for doing the suggested experiment using Vps15-R1261A. I agree that there is an effect of the mutation on FM recycling and that it is comparable to that observed in gpa1Δ. But when compared to vps15Δ, the conclusion in the text is too strong (“was sufficient to inhibit efficient FM4-64 recycling”). Same in the discussion (“was sufficient to disrupt recycling to a similar degree as GPA1 deletion” - disrupt is too strong). If the Vps15/Gpa1 interaction is indeed abolished by this mutation, then an additional, Gpa1-independent function of Vps15 is missing in the model. This is beyond the scope now, but shouldn’t it be acknowledged and discussed?

This was a cool experiment that really helped us tie the regulation of PI3K in recycling to its effector Gpa1. It was only when writing the resubmission that I noticed the levels of defect in these two mutants was similar, so mentioned in the discussion. We agree this implies cells lacking GPA1 or lacking an interaction between Gpa1 and Vps15 are similar to vps15Δ or vps34Δ mutants, which is not true (as elaborated in our last response to reviewers). To remedy this issue we have modified the text (from disrupt to attenuate) and then gone on to call out the difference between the extreme defects (seen in glucose starvation and PI3K deletion) and more subtle defects found in gpa1Δ or Vps15-RA mutants. Finally we point out the level of defects in these latter mutations is similar to other recycling factors, we then include some citations that were all already in the text). Changes:

The finding that a single point mutation in Vps15, which disrupts the interaction of the Gpa1 effector with PI3K (Heenan et al., 2009) was sufficient to attenuate recycling to a similar degree as GPA1 deletion (Figure 4F-H) suggests that the recycling defects of gpa1Δ cells could be explained by improper PtdIns3P production. We note disruption of these regulators do not exhibit the extreme FM4-64 recycling defects found in glucose starved (Figure 1D) or PI3K-null cells (Figure 5), but they are similar to deletion of other factors previously shown to perturb recycling, such as Rcy1, the Rag GTPases, and the Rpd3 complex (Wiederkehr et al., 2000; MacDonald and Piper, 2017; Amoiradaki et al., 2021).

Minor point. Legend / 4H is missing

I tried to include this earlier in the legend (with C & D) but now appreciate that it will easily not get noticed there. Therefore, I have amended the legend to show separate descriptions for C, D and H.
Dear Chris,

Thank you for making the final requested changes to your manuscript, "Endosomal cargo recycling mediated by Gpa1 and Phosphatidylinositol-3-Kinase is inhibited by glucose starvation" in response to the reviewer comments. I am pleased to officially accept your paper for publication in MBoC. Congratulations on a job well done, and thank you so much for choosing to publish your nice story in MBoC!

Sincerely,

Mickey

Michael Marks
Monitoring Editor
Molecular Biology of the Cell

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Dear Dr. MacDonald:

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.

Within approximately four weeks you will receive a PDF page proof of your article.

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