JCB

Fam20C regulates protein secretion by Cab45 phosphorylation

Tobias Hecht, Birgit Blank, Martin Steger, Victor Lopez, Gisela Beck, Bulat Ramazanov, Matthias Mann, Vincent Tagliabracci, and Julia von Blume

Corresponding Author(s): Julia von Blume, Yale School of Medicine

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November 7, 2019

Re: JCB manuscript #201910089

Dr. Julia von Blume
Yale School of Medicine
333 Cedar Street
New Haven 06511

Dear Dr. von Blume,

Thank you for submitting your manuscript entitled "Fam20C regulates protein secretion by Cab45 phosphorylation". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

You will see that overall the reviewers find your report that Fam20C and phosphorylation of Cab45 are important for protein secretion a potentially interesting observation. However, the reviewers have concerns that further insight into the relevance of this regulation during transport vesicle formation is required for the readership of JCB. In particular, experiments addressing if phosphorylation specific to Cab45 regulates secretion are essential to include (Reviewer #1 point 1, Reviewer #2 point 1, Reviewer #3 point 3). The suggestions of reviewers 1 and 3 to use phosphomimetic mutants of Cab45 seems particularly helpful. Experiments to determine a detailed mechanistic model of how phosphorylation of Cab45 enhances secretion are not required for the current study. However, slightly further insight into the effect of phosphorylation of Cab45, for example testing oligomerization as suggested by Reviewers #1 and #3, as well as the effect of Cab45 phosphorylation on LyzC binding should be attempted, as these will strengthen your conclusions and help provide the basis for future studies examining the mechanism in detail. Regarding the concerns of reviewer #3 about your budding assay (point 5), we agree that the control reaction is not correct as showing cytosol and ATP dependence is essential.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:

Text limits: Character count for an Article is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

Figures: Articles may have up to 10 main text figures. Figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, http://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

IMPORTANT: It is JCB policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.

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When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised in this letter.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

Elizabeth Miller, PhD
Monitoring Editor

Andrea L. Marat, PhD
Scientific Editor

Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

Von Blume and coworkers study the calcium and Cab45 regulated secretion of specific cargoes from the Golgi. Calcium influx into the Golgi triggers Cab45 oligomerization and cargo binding; the complexes are then packaged into sphingomyelin enriched vesicles and transported to the plasma membrane for release. This manuscript describes the discovery of Fam20C as a Golgi localized kinase that phosphorylates Cab45 to enhance transport vesicle formation from the Golgi lumen. They use the RUSH system to show that knock out of Fam20C slows secretion; Fam20C co-localizes with Cab45 in the TGN; they show that Cab45 is phosphorylated and a phosphomimetic version seems to enhance vesicle release from the Golgi. The story will surely be of broad interest to readers of JCB and is done at a high level of quality. But the reader is still left wondering how this post-translational modification influences transport vesicle formation.

1. Although we learn that phosphorylation speeds the process there is no clue how. It seems that cargoes are broadly phosphorylated which would in principle, decrease their tendency to aggregate

with phospho-Cab45. One outstanding question that the authors should be able to easily address is whether cargoes need phosphorylation as much as Cab45 does. What happens to Cab45 cargo secretion rate in Fam20C knock out cells rescued with phosphomimetic Cab45-is Cab45's pseudo-phosphorylation sufficient for enhanced vesicle trafficking? (Not clear if LyzC is being phosphorylated or not--the proposed experiment would have only phosphomimetic Cab45 present in the Golgi). (Please clarify legends as to which cells are used in each case). Also, what about a phosphorylated non-Cab45 bulk flow cargo-is it's rate changed? These are important questions that will help the authors be sure that it is specifically Cab45 phosphorylation that enhances one specific trafficking pathway.
2. The authors provide lovely CD spectra for alpha helix content but have great assays for Cab45 oligomerization. They should really try to examine calcium triggered oligomerization of their phospoho-mimetic form. Gel filtration with a western blot perhaps?
3. Minor-the authors use chemiluminescence for their blots which is non-linear so they need to use great care in quantitation of such blots (such as Fig. 1C that is surely saturated signal.

Reviewer #2 (Comments to the Authors (Required)):

Previous work by von Blume et al. identified the calcium-binding luminal Golgi protein Cab45 as a key player in sorting at the TGN. Here they show that the secretory pathway-localized protein kinase Fam20C phosphorylates Cab45, thereby promoting secretion of the Cab45-binding secretory protein LyzC and accelerating the budding of sphingomyelin-rich vesicles from the TGN. Fam20C has been shown previously to phosphorylate secreted proteins, and this is the first report that it acts on a component of the secretory apparatus.

This manuscript is well structured and presents high-quality experiments. The conclusions are generally convincing, although some of the effects are mild (on the order of 1.5X). Overall, there is good evidence that Cab45 is a substrate of Fam20C and that phosphorylation of Cab45 influences its function. But the broader significance is less clear. There is no hint that Fam20C-mediated phosphorylation of Cab45 is a regulated process. Rather, Cab45 may be phosphorylated as part of its normal maturation to a functional protein. That finding is interesting, but I'm not sure it advances our understanding of TGN sorting enough to qualify for presentation to the JCB audience.

Additional points:

1. Is LyzC itself phosphorylated by Fam20C? That point seems important for the argument that the Fam20C-dependent enhancement of LyzC secretion is due to activation of Cab45.

2. Fig. S1 seems to show only about a 5x reduction in Fam20C in the knockout cells. Why isn't the reduction complete? Perhaps the authors can explain a bit further.

3. On line 236, Figure 4F should be Figure 4E.

4. I'm puzzled by the results of Figure 4E, which imply that the phosphomimetic mutant of Cab45 is secreted even at 20{degree sign}C. Is the idea that a significant fraction of the mutant Cab45 has already left the TGN when the 20{degree sign}C block is initiated?

5. In Figure 5A, are all of the images taken at the same exposure? It's surprising that the tagged LyzC seems to persist in the Golgi for more than 40 minutes after accumulating there quantitatively

in just 20 minutes. Was cycloheximide added? If not, then at 60 minutes we may be looking mainly at newly synthesized protein. This detail is relevant because the question is whether the phosphomimetic mutant accelerates secretion or whether it simply generates more vesicles at steady-state.

Reviewer #3 (Comments to the Authors (Required)):

In this manuscript, the authors demonstrate that a Golgi-localized protein kinase, Fam20C, is important for the secretion of a client of Cab45, Lysosome C (LyzC). The punctate structures of Fam20C, presumably vesicles derived from the TGN, were partially colocalized with a sphingomyelin marker. Further analysis indicates that Cab45 was phosphorylated by Fam20C. Moreover, the authors provide evidence suggesting that Fam20C-dependnet Cab45 phosphorylation drives sorting and secretion of Cab45 and its clients. These evidences include: 1) a phosphorylation-mimetic mutant of Cab45 (Cab45-5pXE) showed a significant stronger accumulations at the punctate structures in close proximity to the TGN; 2) phosphorylation of Cab45 affected the number of punctate structures of LyzC but not CatD during post-Golgi trafficking, influenced the efficiency of LyzC secretion, influenced the efficiency of releasing of Cab45 into vesicles and affected the number of sphingomyelin-containing punctate structures; 3) knockdown of isoforms of sphingomyelin synthase significantly decreased in the number of Cab45-5pXE punctate structures in cells; 4) the kinase activity of Fam20C regulated the number of Cab45 punctate structures during post-Golgi trafficking and regulated the release of Cab45 into transport vesicles.

Overall, the manuscript describes interesting observations demonstrating that Fam20C and phosphorylation of Cab45 are important for protein secretion. However, the evidence in the manuscript is not sustained to support the major conclusions and the molecular mechanisms underlying the observations remain largely unclear. The manuscript could be significantly strengthened if the authors could provide evidence showing mechanistic insight into how phosphorylation of Cab45 enhances the secretion process. In addition, analyses need to be performed to investigate the nature of the punctate structures observed in this manuscript. Moreover, additional experiments need to be performed to show that Fam20C regulates proteins secretion through phosphorylation of Cab45 and to strengthen their analyses using a semi-intact budding assay.

The major issues that need to be addressed before it is suitable for publication in JCB and the suggested experiments to address these issues are outlined below:

1). The authors provide evidence suggesting that phosphorylation of Cab45 promotes secretion of Cab45 and its client, LyzC, but their study did not provide mechanistic insight into this observation. Cab45 has been shown to binds LyzC in a Ca2+ dependent manner through GST pull-down experiments [1]. Does phosphorylation of Cab45 regulate the interaction between Cab45 to its client? The authors need to perform GST-pull down experiments to test whether the phosphorylation-mimetic mutant Cab45-5pXE and the phosphorylation-deficient mutant Cab45-5pXA show different binding affinities for LyzC. In addition, Ca2+ dependent oligomerization of Cab45 has been observed [2]. Similar experiments need to be performed to test whether Cab45 phosphorylation affects oligomerization of Cab45.

2) The authors claimed that the punctate structures observed in the RUSH system after addition of biotin are vesicles. However, it is also possible that some of these structures are endosomes. In addition, it is unclear whether these punctate structures are delivered to the plasma membrane for

secretion or lysosomes for degradation. Thus the nature of the punctate structures need to be further investigated. Colocalization analyses need be performed to test whether these punctate structures of LyzC and Cab45 detected in the RUSH system are colocalized with early endosomal markers, late endosomal markers, recycling endosomal markers or lysosomal markers.

3) The function of Fam20C in cargo sorting and secretion need to be further analyzed. As Fam20C has a lot of substrates, additional experiments need to be performed to test whether Fam20C acts through Cab45 to regulate the secretion process. The following are the suggested experiments to address these issues:

a) The authors demonstrate that there were defects in the secretion of LyzC in Fam20C KO ells (Figure 1B). The authors need to analyze whether there are defects of secretion of CatD in Fam20C KO cells. The authors also need to test whether expressing wild type Fam20C or kinase dead mutant Fam20C (Fam20C-D478A) recuse the defects of secretion of LyzC in Fam20C KO cells.

b) Experiments need to be performed to test whether expressing Cab45-5pXE or Cab45-5pXA in Fam20C KO cells changes the efficiency of LyzC secretion.

5) The author analyzed Cab45 vesicular budding using a semi-intact budding assay (Figure 6C, E, F) but the results are not sustained to support the major conclusions. The following control experiments need to be performed to strengthen this part:

a) Experiments need to be performed to test whether release of Cab45 into vesicles is dependent on cytosol and dependent on ATP regeneration system.

b) Another cargo protein, TGN46, is shown to be packaged into vesicles in this in vitro assay [3]. Thus TGN46 needs be utilized as another cargo for the experiments performed in Figure 6C and 6E.

c) A significant greater amount of Cab45 was detected in the vesicle fraction produced from the Fam20C KO cells transfected with Fam20C-wt compared to the cells transfected with Fam20C-D478A (Figure 6E-F). Similar experiments can be performed to test whether the amount of Cab45-5pXE and Cab45-5pXA in vesicle fractions are changed in Fam20C KO cells transfected with Fam20C-wt and in cells transfected with Fam20C-D478A.

6) The authors demonstrate that Cab45-5pXE increased the efficiency of secretion of LyzC (Figure 5C-D, page 15, line 304-306). Although quantification showed an enhancement (Figure 5D), we cannot detect a clear enhancement of LyzC signal in the medium of cells expressing Cab45-5Pxe (Figure 5C, compare lanes 4 and 6). Moreover, secretion of CatD in cells expressing Cab45-wt, Cab45-5pXA or Cab45-5pXE needs to be analyzed.

Additional issues:
1)"Figure 4F" in line 236 needs to be replaced with "Figure 4E".
2)The authors need to provide methods describing how the number of punctate structures and the percentage of colocalizated punctate structures were counted and quantified.

References:

1.von Blume, J., et al., Cab45 is required for Ca(2+)-dependent secretory cargo sorting at the trans-Golgi network. J Cell Biol, 2012. 199(7): p. 1057-66.
2.Crevenna, A.H., et al., Secretory cargo sorting by Ca2+-dependent Cab45 oligomerization at the trans-Golgi network. J Cell Biol, 2016. 213(3): p. 305-14.
3.Wakana, Y., et al., A new class of carriers that transport selective cargo from the trans Golgi network to the cell surface. EMBO J, 2012. 31(20): p. 3976-90.

Yale SCHOOL OF MEDICINE
Department of Cell Biology

JULIA von BLUME
Associate Professor of Cell Biology

PO Box 208002
New Haven CT 06520-8002
T 203 737-2746
julia.vonblume@yale.edu
medicine.yale.edu/cellbio

office
Sterling Hall of Medicine (SHM)
Room C425C, Lab Room C434
333 Cedar Street
New Haven CT 06510

To the
Journal of Cell Biology
Andrea Marat
Elizabeth Miller
The Rockefeller University Press
1114 First Avenue, 3rd floor
New York, NY 10065-8325

March 6th, 2020

Resubmission of #201910089

Dear Andrea, dear Liz,

Thank you very much for the useful and supportive suggestions to improve the quality of our manuscript. On behalf of my coauthors, I am submitting the revised version of our paper entitled “Fam20C regulates protein secretion by Cab45 phosphorylation”. We have addressed the comments of the reviewers, rearranged the paper, and added new Figures. We have summarized the major concerns of the reviewers and can now present the following new data:

1. **Phosphorylated Cab45 not phosphorylation of LyzC is necessary for its secretion**

We can now show that Fam20C does not phosphorylate the Cab45 client LyzC. Furthermore, we can demonstrate that the phosphorylation mimicking Cab45 mutant can rescue the secretion defect caused by the loss of Fam20C, which further strengthens the significance of Cab45 phosphorylation in secretion.

2. Trafficking of Osteopontin a Fam20C substrate and "bulk flow" cargo is not affected by Cab45 phosphorylation mutants

Osteopontin (OPN) is a secreted "bulk flow" cargo protein that is phosphorylated by Fam20C on more than 26 residues. We generated an OPN RUSH system, and we were able to follow the transport of OPN from the ER (without biotin) to the Golgi (t = 20 min after Biotin addition) and into secretory vesicles (t = 40, t = 50 min after Biotin addition**) (Figure 5G, Figure S4C)**. Overall, we could not determine any significant differences in the amount of OPN vesicles in HeLa control versus Cab45-KO cells, demonstrating that OPN is not a Cab45 client **(Figure 5G, Figure S4C)**. Similarly, we can show that phosphomimetic Cab45 exclusively changes the secretion rate of a Cab45 client (LyzC) but not of OPN, since ectopic expression of Cab45-5pXE or Cab45-5pXA in cells with Cab45-KO background does not change OPN vesicle numbers. We further confirmed that CatD secretion and trafficking to lysosomes are not affected by the phosphorylation of Cab45 **(Figure S4A, B, D and E)**.

3. Cab45 oligomerization but not client binding is affected by phosphorylation

These experiments provided a significant new understanding of the mechanism of phosphorylation and led to a change in our primary hypothesis. To provide a more mechanistic inside into the significance of Cab45 phosphorylation on cargo sorting, we have generated recombinant GFP-tagged Cab45 wild type, Cab45-5pxA, and Cab45-5pxE. We performed oligomerization assays using confocal microscopy and characterized the nature of the different oligomers **(new Figure 7)**. Overall our data suggest that upon incubation with Ca^{2+}, the Cab45-5pXA mutant forms mainly larger oligomeric structures with higher intensities compared to oligomers formed by Cab45-wt and Cab45-5pXE. These findings indicate an oligomerization-prone behavior of the phosphorylation-deficient mutant, assuming that the formation of Cab45 oligomers might be regulated via phosphorylation by Fam20C **(new Figure 7)**. Importantly, client binding to the different mutants is not changed **(Figure S2C and S2D)**. We have reconsidered our original hypothesis and show a new cartoon for how we, based on our new data, imagine the role of Cab45 phosphorylation in client sorting **(Figure 8)**.

4. Conditions of Cab45 vesicular budding assays

We have further validated the requirements for the semi-intact vesicle budding assay. We observed while ATP is required for the reaction, rat liver cytosol is not necessary for budding of Cab45 vesicles **(Figure S5A)**. We have seen the same result in a previous study (Deng et al., 2018, Figure 1). Accordingly, we have repeated the entire set of budding assays in the paper **(Figure 6C and D, Figure 6F, and G)**.

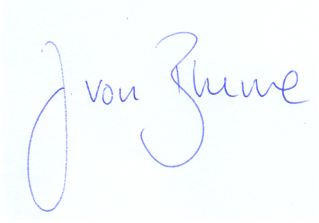
5. RUSH-LyzC and RUSH-Cab45 are not trapped in endosomal or lysosomal compartments

We intensively tested the identity of our secretory vesicles, and we could clearly show that they do not colocalize with early endosomes, recycling endosomes or lysosomes **(Figure S1C, Figure S5C)**. We also show that all Cab45 mutants are correctly sorted into sphingomyelin-rich vesicles at the TGN. We have characterized this specific type of vesicle intensively in recent studies (Deng et al., 2018).

We have included all new results to the manuscript and addressed major and minor. The comments of the reviewers (listed below). The reviewer´s questions are written in bold.

If you have further questions, please do not hesitate to contact me.

Sincerely,



Julia von Blume, PhD
Associate Professor of Cell Biology
Department of Cell Biology
Yale School of Medicine

Reviewer #1 (Comments to the Authors (Required)):

Von Blume and coworkers study the calcium and Cab45 regulated secretion of specific cargoes from the Golgi. Calcium influx into the Golgi triggers Cab45 oligomerization and cargo binding; the complexes are then packaged into sphingomyelin enriched vesicles and transported to the plasma membrane for release. This manuscript describes the discovery of Fam20C as a Golgi localized kinase that phosphorylates Cab45 to enhance transport vesicle formation from the Golgi lumen. They use the RUSH system to show that knock out of Fam20C slows secretion; Fam20C co-localizes with Cab45 in the TGN; they show that Cab45 is phosphorylated and a phosphomimetic version seems to enhance vesicle release from the Golgi. The story will surely be of broad interest to readers of JCB and is done at a high level of quality. But the reader is still left wondering how this post-translational modification influences transport vesicle formation.

1a. Although we learn that phosphorylation speeds the process there is no clue how. It seems that cargoes are broadly phosphorylated which would in principle, decrease their tendency to aggregate with phospho-Cab45. One outstanding question that the authors should be able to easily address is whether cargoes need phosphorylation as much as Cab45 does. What happens to Cab45 cargo secretion rate in Fam20C knock out cells rescued with phosphomimetic Cab45-is Cab45's pseudo-phosphorylation sufficient for enhanced vesicle trafficking? (Not clear if LyzC is being phosphorylated or not--the proposed experiment would have only phosphomimetic Cab45 present in the Golgi). (Please clarify legends as to which cells are used in each case).

Do cargoes need phosphorylation as much as Cab45 does?

- To experimentally address this important question, we performed an *in-vitro* kinase assay, similar to the one that was performed with Cab45. To this end, recombinant LyzC was incubated with Fam20C-wt or Fam20C-D478A (kinase-dead) in the presence of [γ-32P] ATP. 32P incorporation was measured by autoradiography. Osteopontin (positive ctrl.) but not LyzC was phosphorylated by Fam20C-wt but not Fam20C-D478A. This assay clearly showed that LyzC is not phosphorylated by Fam20C **(Figure 3B)**.

- In addition, we checked cargo (LyzC) secretion in Fam20C-KO cells rescued with the different Cab45 variants, as suggested by the reviewer. Fam20C-KO cells were transfected with LyzC-Flag and either Cab45-wt, Cab45-5pXA, or Cab45-5pXE. Secretion assays were performed as described in the paper (20 degree block followed by a release for 1 h). In line with our submitted results, we could show that significantly more LyzC is secreted in cells expressing Cab45-5pXE but not in control cells or cells expressing Cab45-wt and Cab45-5pXA **(Figure 5D and 5E)**.

b. Also, what about a phosphorylated non-Cab45 bulk flow cargo-is it's rate changed? These are important questions that will help the authors be sure that it is specifically Cab45 phosphorylation that enhances one specific trafficking pathway.

- Osteopontin (OPN) is a secreted “bulk flow” cargo protein that is phosphorylated by Fam20C on more than 26 residues. We generated an OPN RUSH system, and we were able to follow the transport of OPN from the ER (without biotin) to the Golgi (t = 20 min after Biotin addition) and into secretory vesicles (t = 40, t = 50 min after Biotin addition) **(Figure 5G, Figure S4C)**. Overall, we could not

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Altogether our results indicate that LyzC is not phosphorylated by Fam20C and that enhanced cargo secretion is caused by phosphorylated Cab45.

2. The authors provide lovely CD spectra for alpha helix content but have great assays for Cab45 oligomerization. They should really try to examine calcium triggered oligomerization of their phospoho-mimetic form. Gel filtration with a western blot perhaps?

- We thank the reviewer for the great suggestion. To address this issue, we have generated recombinant GFP-tagged Cab45 wild type, Cab45-5pxA and Cab45-5pxE. We performed oligomerization assays using confocal microscopy and characterized the nature of the different oligomers **(new Figure 7)**. Overall our data suggest that upon incubation with Ca^{2+}, the Cab45-5pXA mutant forms mainly larger oligomeric structures with higher intensities compared to oligomers formed by Cab45-wt and Cab45-5pXE. These findings indicate a potential oligomerization-prone behavior of the phosphorylation-deficient mutant, assuming that the formation of Cab45 oligomers might be regulated via phosphorylation by Fam20C **(new Figure 7)**.

3. Minor-the authors use chemiluminescence for their blots which is non-linear so they need to use great care in quantitation of such blots (such as Fig. 1C that is

surely saturated signal.

We have inserted a new blot in Figure 1B and we checked all our images for oversaturation. We have adapted our quantifications accordingly.

Reviewer #2 (Comments to the Authors (Required)):

Previous work by von Blume et al. identified the calcium-binding luminal Golgi protein Cab45 as a key player in sorting at the TGN. Here they show that the secretory pathway-localized protein kinase Fam20C phosphorylates Cab45, thereby promoting secretion of the Cab45-binding secretory protein LyzC and accelerating the budding of sphingomyelin-rich vesicles from the TGN. Fam20C has been shown previously to phosphorylate secreted proteins, and this is the first report that it acts on a component of the secretory apparatus.

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Additional points:

1. Is LyzC itself phosphorylated by Fam20C? That point seems important for the argument that the Fam20C-dependent enhancement of LyzC secretion is due to activation of Cab45.

- To experimentally address this critical question, we performed an *in-vitro* kinase assay, similar to the one that was performed with Cab45. To this end, recombinant LyzC was incubated with Fam20C-wt or Fam20C-D478A (kinase-

dead) in the presence of [γ-32P] ATP. 32P incorporation was analyzed by autoradiography. Osteopontin (positive ctrl.) was phosphorylated by Fam20C-wt but not Fam20C-D478A. LyzC was not phosphorylated by Fam20C, suggesting a specific role of phosphorylated Cab45 in the trafficking of LyzC **(Figure 3B)**.

- Also, we analyzed cargo (LyzC) secretion in Fam20C-KO cells rescued with the different Cab45 variants, as suggested by the reviewer. Fam20C-KO cells were transfected with LyzC-Flag and either Cab45-wt, Cab45-5pXA or Cab45-5pXE, respectively. Secretion assays were performed as described in the paper (20 degree block followed by a release for 1 h). In line with our submitted results, we could show that significant more LyzC is secreted in cells expressing Cab45-5pXE but not in control cells or cells expressing Cab45-wt and Cab45-5pXA **(Figure 5D and 5E)**.

2. Fig. S1 seems to show only about a 5x reduction in Fam20C in the knockout cells. Why isn't the reduction complete? Perhaps the authors can explain a bit further.

- We used the CRISPR/Cas9 technology to knockout Fam20C in HeLa cells. Forward and reverse sequencing revealed the deletion of 22 bp leading to premature termination of protein translation. Additionally, we investigated the knockout at the protein level by mass spectrometry analysis (Figures S1A and S1B). Table 1 shows an extract of the mass spectrometry results. Both cell lines were measured in triplicates. Whereas in HeLa-wt cells, Fam20C was detectable in all three replicates, in “Fam20C-KO” cells, only the second replicate could detect Fam20C. This detection was identified in HeLa-wt cells via MS/MS, whereas identification in Fam20C-KO cells was only by matching. Since intensities were approx. 10 times lower, we therefore assume that detection in Fam20C-KO was due to contamination (data analysis with the MPI MS core

facility leader). We did not exclude this value from analysis, that's why Volcano Blot only showed a reduction, not a complete knockout.

Table 1	Fam20C-KO			HeLa-wt		
Replicate	1	2	3	1	2	3
Intensity	0	9939400	0	82556000	110110000	162670000
Identification type	-	By matching	-	By MS/MS	By MS/MS	By MS/MS
MS/MS count	0	0	0	2	1	3

3. On line 236, Figure 4F should be Figure 4E.

- We thank the reviewer for pointing this out. We have restructured the paper and we were careful about the numbering.

4. I'm puzzled by the results of Figure 4E, which imply that the phosphomimetic mutant of Cab45 is secreted even at 20{degree sign}C. Is the idea that a significant fraction of the mutant Cab45 has already left the TGN when the 20{degree sign}C block is initiated?

- Exactly, Cab45-5pXE localizes in a vesicular form around the Golgi (post- Golgi vesicles, **Figure 4C, and 4D**), even at steady state. Considering our budding assays, we see increased budding of Cab45-5pXE and therefore hypothesize that phosphorylation of Cab45 by Fam20C triggers Golgi export **(Figure 6C)**. The fraction of Cab45-5pXE that has already left the TGN (vesicles at steady state) is

not affected by blocking Golgi export (by 20 °C block) and could be detected via western blot **(Figure 4D and 4E)**.

5. In Figure 5A, are all of the images taken at the same exposure? It's surprising that the tagged LyzC seems to persist in the Golgi for more than 40 minutes after accumulating there quantitatively in just 20 minutes. Was cycloheximide added? If not, then at 60 minutes we may be looking mainly at newly synthesized protein. This detail is relevant because the question is whether the phosphomimetic mutant accelerates secretion or whether it simply generates more vesicles at steady-state.

- (Former Figure 5A, now **Figure S3A**). Yes, all the images were taken at the same exposure, however, to better visualize the budded cytosolic vesicles, the contrast was increased equally for images at time t = 20, 40, 60 min. We realized that the contrast might have been changed more in images of this LyzC-RUSH Figure, compared to LyzC-RUSH Figure 1E. This resulted in exaggerated Golgi staining in 5A; we have changed this.

- The fact that we see LyzC even 40 minutes after biotin addition is similar to other cargoes that we have tested using the RUSH assay. Since cycloheximide was not added to the RUSH assay, we agree that newly synthesized protein might be in the Golgi after 60 minutes. Nevertheless, we assume that this proportion is insignificant since the “original” protein was synthesized for several hours, trapped in the ER and released all at the same time by adding biotin to the cell culture medium.

- Concerning your question, if Cab45-5pXE accelerates secretion or generates more vesicles at steady state, we think that this goes hand in hand with each other. We have to emphasize that the accelerated vesicles seen in the RUSH

assay **(Figure 3SA)**, contain LyzC. This means that the budding of cargo is accelerated. We show in **Figure 5B, 5C, 5D and 5E** increased secretion of LyzC in cells expressing Cab45-5pXE. In line with this, in-*vivo* vesicle budding **(Figure 5F)**, showed co-localization between LyzC-mCh and EQ-SM-oxGFP in Cab45-wt and phosphomutant cell lines, as well as co-localization between LyzC-mCh and different GFP-Cab45 variants **(Figure S3B)**. Overall this suggests proper sorting into sphingomyelin-rich secretory vesicles. These data demonstrate that we do not just see an acceleration of vesicular budding.

Reviewer #3 (Comments to the Authors (Required)):

In this manuscript, the authors demonstrate that a Golg-localized protein kinase, Fam20C, is important for the secretion of a client of Cab45, Lysosome C (LyzC). The punctate structures of Fam20C, presumably vesicles derived from the TGN, were partially colocalized with a sphingomyelin marker. Further analysis indicates that Cab45 was phosphorylated by Fam20C. Moreover, the authors provide evidence suggesting that Fam20C-dependnet Cab45 phosphorylation drives sorting and secretion of Cab45 and its clients. These evidences include: 1) a phosphorylation-mimetic mutant of Cab45 (Cab45-5pXE) showed a significant stronger accumulations at the punctate structures in close proximity to the TGN; 2) phosphorylation of Cab45 affected the number of punctate structures of LyzC but not CatD during post-Golgi trafficking, influenced the efficiency of LyzC secretion, influenced the efficiency of releasing of Cab45 into vesicles and affected the number of sphingomyelin-containing punctate structures; 3) knockdown of isoforms of sphingomyelin synthase significantly decreased in the number of Cab45-5pXE punctate structures in cells; 4) the kinase activity of Fam20C regulated the number of Cab45 punctate structures during post-Golgi trafficking and regulated the release of Cab45 into transport vesicles.

Overall, the manuscript describes interesting observations demonstrating that Fam20C and phosphorylation of Cab45 are important for protein secretion. However, the evidence in the manuscript is not sustained to support the major conclusions and the molecular mechanisms underlying the observations remain largely unclear. The manuscript could be significantly strengthened if the authors could provide evidence showing mechanistic insight into how phosphorylation of Cab45 enhances the secretion process. In addition, analyses need to be performed to investigate the nature of the punctate structures observed in this manuscript. Moreover, additional experiments need to be performed to show that

Fam20C regulates proteins secretion through phosphorylation of Cab45 and to strengthen their analyses using a semi-intact budding assay.

The major issues that need to be addressed before it is suitable for publication in JCB and the suggested experiments to address these issues are outlined below:

1). The authors provide evidence suggesting that phosphorylation of Cab45 promotes secretion of Cab45 and its client, LyzC, but their study did not provide mechanistic insight into this observation. Cab45 has been shown to binds LyzC in a Ca2+ dependent manner through GST pull-down experiments [1]. Does phosphorylation of Cab45 regulate the interaction between Cab45 to its client? The authors need to perform GST-pull down experiments to test whether the phosphorylation-mimetic mutant Cab45-5pXE and the phosphorylation-deficient mutant Cab45-5pXA show different binding affinities for LyzC.

- We thank the reviewer for this comment. We have performed pulldown experiments using recombinant His-tagged wild type Cab45, Cab45-5pxE and Cab45-5pxA mutants, respectively. Interestingly, Cab45 wild type Cab45-5pxE, and Cab45-5pxA bind to the same extent to recombinant LyzC **(Figure S2C and D)**. These data showed that the phosphorylation mutations have no impact on client binding.

In addition, Ca2+ dependent oligomerization of Cab45 has been observed [2]. Similar experiments need to be performed to test whether Cab45 phosphorylation affects oligomerization of Cab45.

- This is a crucial point that we have now investigated. To address this issue, we have generated recombinant GFP-tagged Cab45 wild type, Cab45-5pXA, and Cab45-5pXE. We performed oligomerization assays using confocal microscopy

and characterized the nature of the different oligomers **(new Figure 7)**. Overall our data suggest that upon incubation with Ca^{2+}, the Cab45-5pXA mutant forms mainly larger oligomeric structures with higher intensities compared to oligomers formed by Cab45-wt and Cab45-5pXE. These findings indicate a potential oligomerization-prone behavior of the phosphorylation-deficient mutant, assuming that the formation of Cab45 oligomers might be regulated via phosphorylation by Fam20C **(new Figure 7)**.

2) The authors claimed that the punctate structures observed in the RUSH system after addition of biotin are vesicles. However, it is also possible that some of these structures are endosomes. In addition, it is unclear whether these punctate structures are delivered to the plasma membrane for secretion or lysosomes for degradation. Thus the nature of the punctate structures need to be further investigated. Colocalization analyses need be performed to test whether these punctate structures of LyzC and Cab45 detected in the RUSH system are colocalized with early endosomal markers, late endosomal markers, recycling endosomal markers or lysosomal markers.

- This is a valid point, and we have performed colocalization experiments of RUSH-LyzC **(Figure S1C)** and RUSH-Cab45 **(Figure S5C)**. These data show that the observed vesicles do not colocalize with early endosomes (EEA), recycling endomes (Rab11) or lysosomes (LAMP1). We have intensively characterized RUSH LyzC in a previous paper (Deng et al., 2018). Here we followed the vesicles by life cell imaging and TIRF to show the fusion of these vesicular structures with the cell surface. In addition, we want to refer to LyzC secretion assays **(Figure 5B and 5C)** that were performed, confirming the results of the LyzC RUSH approach.

3) The function of Fam20C in cargo sorting and secretion need to be further

analyzed. As Fam20C has a lot of substrates, additional experiments need to be performed to test whether Fam20C acts through Cab45 to regulate the secretion process. The following are the suggested experiments to address these issues:

a) The authors demonstrate that there were defects in the secretion of LyzC in Fam20C KO cells (Figure 1B). The authors need to analyze whether there are defects of secretion of CatD in Fam20C KO cells.

- We want to highlight that CatD is a lysosomal hydrolase that is transported to the endosomal/lysosomal compartments. In this regard, we used RUSH-CatD as a cargo that is not sorted via Cab45 (Crevenna et al., 2016; Deng et al., 2018). This showed, that phosphomutants of Cab45 influence sorting of Cab45-cargo LyzC **(Figures S3A, 5A, 5B and 5C)** but not of CatD **(Figures S4D and S4E)**.

- According to the reviewer question, we first tested secretion of CatD in HeLa control and Fam20C-KO cells **(Figure S4A)**, revealing that there was no secretion of endogenous CatD since we did not detect CatD in cell culture supernatants after performing a 20 °C block followed by a release at 37 °C. As a result, we were not able to perform this experiment.

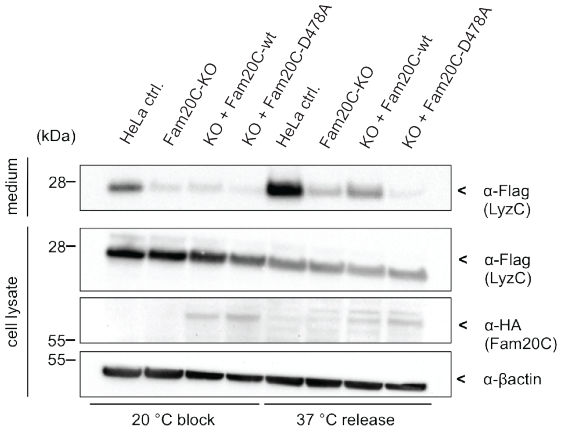
- We, in addition, tested the correct transport of RUSH-CatD to lysosomes by immunofluorescence imaging **(Figure S4B)**. These data clearly show that there is no change in CatD trafficking to lysosomes. This in addition supports the results of **Figure S4A**, which showed no CatD secretion.

- Furthermore, we have analyzed another non-Cab45 but secretory cargo: Osteopontin (OPN) is a secreted “bulk flow” cargo protein that is phosphorylated by Fam20C on more than 26 residues. We generated an OPN RUSH system, and we were able to follow the transport of OPN from the ER (without biotin) to

the Golgi (t = 20 min after Biotin addition) and into secretory vesicles (t = 40, t = 50 min after Biotin addition**) (Figure 5G, Figure S4C)**. Overall, we could not determine any significant differences in the amount of OPN vesicles in HeLa control versus Cab45-KO cells, demonstrating that OPN is not a Cab45 client **(Figure 5G, Figure S4C)**. Similarly, we can show that phosphomimetic Cab45 exclusively changes the secretion rate of a Cab45 client (LyzC) but not of OPN since ectopic expression of Cab45-5pXE or Cab45-5pXA in cells with Cab45-KO background does not change OPN vesicle numbers.

The authors also need to test whether expressing wild type Fam20C or kinase dead mutant Fam20C (Fam20C-D478A) recuse the defects of secretion of LyzC in Fam20C KO cells.

- Due to the lack of time, we have only performed one experiment to prove that wild type Fam20C rescues LyzC secretion in Fam20C knock out cells. Therefore, we will not include the result into the paper, but the blot is shown below and confirms the results of the RUSH assay (**Figure 1D**) where we performed the same rescue experiment. These data show that Fam20C rescues secretion of LyzC while the catalytic inactive mutant does not.



b) Experiments need to be performed to test whether expressing Cab45-5pXE or Cab45-5pXA in Fam20C KO cells changes the efficiency of LyzC secretion.

- We analyzed cargo (LyzC) secretion in Fam20C-KO cells rescued with the different Cab45 variants, as suggested by the reviewer. Fam20C-KO cells were transfected with LyzC-Flag and either Cab45-wt, Cab45-5pXA or Cab45-5pXE. Secretion assays were performed as described in the paper (20 degree block followed by a release for 1 h). In line with our submitted results, we could show that significantly more LyzC is secreted in cells expressing Cab45-5pXE but not in control cells or cells expressing Cab45-wt and Cab45-5pXA. We included the results in the paper **(Figure 5D and 5E)**.

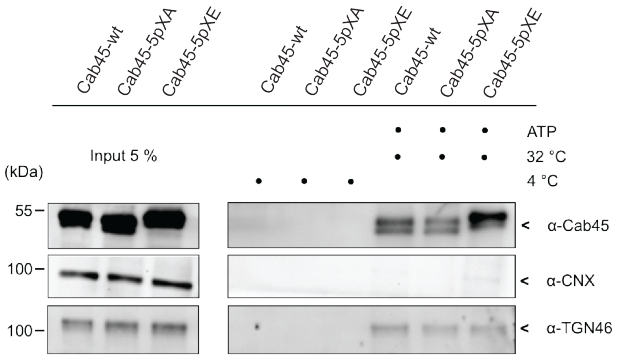
5) The author analyzed Cab45 vesicular budding using a semi-intact budding assay (Figure 6C, E, F) but the results are not sustained to support the major conclusions. The following control experiments need to be performed to strengthen this part:

a) Experiments need to be performed to test whether release of Cab45 into vesicles is dependent on cytosol and dependent on ATP regeneration system.

We agree that our control experiments did not totally support our conclusion. We, therefore, tested budding of Cab45 (endogenous) and Cab45-wt (ectopically expressed) in dependency of ATP and cytosol (**Figure 5SA**). Cab45 vesicles bud also in the absence of rat liver cytosol – as already shown in Deng et al., 2018, Figure 1. We repeated all experiments without rat liver cytosol, and budding still works (**Figures 6C, 6D, 6F and 6G**).

b) Another cargo protein, TGN46, is shown to be packaged into vesicles in this in vitro assay [3]. Thus TGN46 needs be utilized as another cargo for the experiments performed in Figure 6C and 6E.

- We performed the experiment as described in **6C**, checking the vesicular fraction for TGN46. By doing so, we couldn't detect significant differences in TGN46 budding. Since there is no known connection between TGN46 and Cab45, we think that unchanged TGN46 levels as seen here are because not all Cab45-vesicles contain TGN46.



c) A significant greater amount of Cab45 was detected in the vesicle fraction produced from the Fam20C KO cells transfected with Fam20C-wt compared to the cells transfected with Fam20C-D478A (Figure 6E-F). Similar experiments can be performed to test whether the amount of Cab45-5pXE and Cab45-5pXA in vesicle fractions are changed in Fam20C KO cells transfected with Fam20C-wt and in cells transfected with Fam20C-D478A.

- We were not able to address this question since expression levels of the two transfected constructs Cab45-5pXA/E and Fam20C-wt/D478 in Fam20C-KO cell lines were not comparable.

- Here we want to highlight that we have performed both experiments separately as included in the paper. As mentioned by the reviewer, Cab45 was detected in a higher amount in vesicular fractions when Fam20C-KO cells were transfected with Fam20C-wt (**Figure 6F and 6G).** The Cab45 RUSH approach shows a similar result, depicting more formed cytosolic vesicles, 40 min after biotin addition (**Figure 6E**). In addition, Cab45-5pXE showed vesicular localization (**Figures 4C, 4D, and 4E**) and a greater amount in the vesicular fraction performing semi-intact budding assays (**Figures 6C and 6D**).

6) The authors demonstrate that Cab45-5pXE increased the efficiency of secretion of LyzC (Figure 5C-D, page 15, line 304-306). Although quantification showed an enhancement (Figure 5D), we cannot detect a clear enhancement of LyzC signal in the medium of cells expressing Cab45-5Pxe (Figure 5C, compare lanes 4 and 6). Moreover, secretion of CatD in cells expressing Cab45-wt, Cab45-5pXA or Cab45-5pXE needs to be analyzed.

- We apologize for this statement and changed it in the manuscript. We determined a significant difference of LyzC secretion between cells expressing Cab45-5pXE (phosphorylation mimicking) and Cab45-5pXA (phosphorylation deficient). We agree that differences in the secretion of LyzC in Cab45-5pXE and Cab45-wt are not significant. Concerning this, we want to emphasize that Cab45-wt might be phosphorylated in the cell by Fam20C and so conform like Cab45-5pXE.

- For a detailed explanation see comment 3a. CatD was not secreted into the cell culture supernatant of HeLa control or Fam20C knock out cells, respectively (Figure S4A). Therefore, no secretion assays were performed for phosphomutant cell lines.

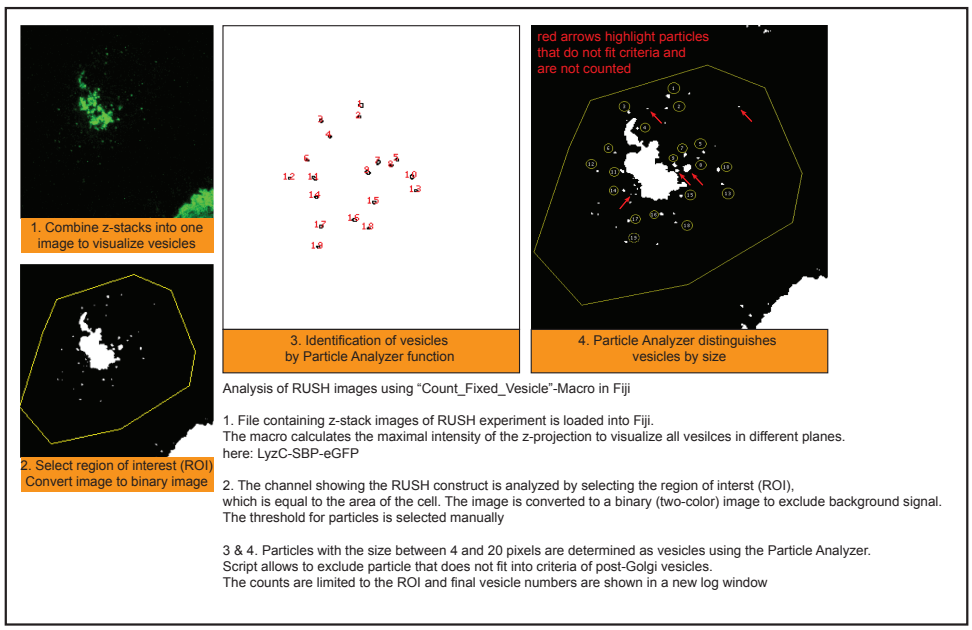
Additional issues:

1)"Figure 4F" in line 236 needs to be replaced with "Figure 4E".

We thank the reviewer for pointing this out. We have restructured the paper and we were careful about the numbering.

2) The authors need to provide methods describing how the number of punctate structures and the percentage of colocalizated punctate structures were counted and quantified.

We have inserted a more detailed description of our data analysis in Materials and Methods. We have also made a diagram to show how we quantified our RUSH assays (see below).



March 23, 2020

RE: JCB Manuscript #201910089R

Dr. Julia von Blume
Yale School of Medicine
333 Cedar Street
New Haven 06510

Dear Dr. von Blume:

Thank you for submitting your revised manuscript entitled "Fam20C regulates protein secretion by Cab45 phosphorylation". We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below), as well as text edits to thoroughly address the final comments of reviewer #1.

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

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4) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (either in the figure legend itself or in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, if you used parametric tests, please indicate if the data distribution was tested for normality (and if so, how). If not, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

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7) Please be sure to provide the sequences for all of your primers/oligos and RNAi constructs in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers (where appropriate) for all of your antibodies. Please also indicate the acquisition and quantification methods for immunoblotting/western blots.

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b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
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e. Fluorochromes
f. Camera make and model
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14) A separate author contribution section following the Acknowledgments. All authors should be mentioned and designated by their full names. We encourage use of the CRediT nomenclature.

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Sincerely,

Elizabeth Miller, PhD
Monitoring Editor
Journal of Cell Biology

Andrea L. Marat, PhD
Scientific Editor
Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

The authors have taken to heart the comments of all the reviewers and tried to address all points

raised. A few problems remain. In Fig. 7 they are comparing oligomers of their various mutants. The X-axis should be size class, not WAE, so that the data are easier to comprehend by the reader. What is not clear is why the 5pXE mutant is so like the wild type protein. Oligomerization of Cab45 appears to be less phosphorylation regulated than calcium dependent and this experiment doesn't seem to reveal new information related to cargo sorting (conclusion line 37, 38, 95 is not strong?). In addition, since the Cab45 doesn't change it's cargo affinity with phosphorylation, it suggests more likely that phospho Cab45 is binding something else to enhance secretion (which is clearly shown). Thus lines 583-587 don't make sense--and the authors need to try to think (and discuss) more clearly the possible mechanism. In this light, Fig. 8 does not clarify the findings of this study. Yes, the authors have shown that the cargo need not be phosphorylated and the phosphomimetic is sufficient to activate the pathway but we still have not learned really why, or? Please verify the validity of all summary statements in abstract, intro and discussion.

Reviewer #3 (Comments to the Authors (Required)):

In this manuscript, the authors clearly demonstrate that a Golg-localized protein kinase, Fam20C, is important for the secretion of Cab45 client by regulating Cab45 phosphorylation. The authors have addressed all the major issues and the revised manuscript has been significantly enhanced. The findings are novel and the results support the conclusions. In particular, the new results demonstrate that Cab45 oligomerization is affected by phosphorylation, providing interesting mechanistic insight. Thus, the revised manuscript is suitable for publication in JCB. One minor issue is that "Figure S1C" in page 12, line 238 should be replaced with "Figure 1F".