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Recruitment of arfaptins to the trans-Golgi network by PI(4)P and their involvement in cargo export

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(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.)

Editor: Isabel Arnold/Karin Dumstrei

1st Editorial Decision 11 September 2012

Thank you for submitting your manuscript for consideration by The EMBO Journal. Three referees have now evaluated it, and their comments are shown below. You will be pleased to see that all three referees are positive in principle and would support publication after appropriate revision. Still, it also becomes clear that referees 1 and 2 put forward a number of major issues that will need to be addressed during revision. In particular, both referees think that the PI4P interaction data need to be strengthened, co-depletion experiments for arfaptin 1 and 2 will be required, and the conclusion that arfaptin 1 acts as a negative regulator of TGN trafficking needs to be substantiated. We should be happy to consider a revised version of the manuscript that addresses the reviewers' concerns in an adequate manner and to their satisfaction. I should add that it is EMBO Journal policy to allow only a single round of revision, and that acceptance or rejection of your manuscript will therefore depend on the completeness of your responses in this revised version as well as on the final assessment by the referees.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Peer Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may

be able to grant an extension.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS

Referee #1

In this manuscript, Cruz-Garcia and colleagues propose that the BAR domain-containing proteins arfaptin 1 and 2 localize to the TGN by binding PtdIns(4)P. They show that a highly conserved region upstream of the BAR domain modulates this phosphoinositide binding, and furthermore contains a conserved Ser100 which serves as a phospho-regulatory site for arfaptin 1 (but not arfaptin 2). Protein kinase D (PKD) can phosphorylate this residue, and the phospho-mimetic S100E displays reduced membrane binding and TGN localization. Finally, the authors propose that arfaptin 1 and 2 play differential roles in TGN trafficking. Depletion of arfaptin 2, but not 1, by siRNA reduces ssHRP trafficking and cathepsin D maturation. Conversely, over-expression of arfaptin 1 negatively affects ssHRP trafficking out of the TGN. Taken together, the authors propose that arfaptins 1 and 2 play distinct roles in TGN trafficking.

Overall, this is a straightforward analysis of the biochemistry and cell biology of the arfaptins. The authors propose a PKD-mediated phospho-regulatory mechanism that governs this arfaptin 1 lipid binding. However, their model for "distinct" functions of arfaptins 1 and 2 in TGN trafficking requires additional experiments to fully flesh out. Specific comments regarding this are outlined below. Addressing concerns 1, 2, and 3 will be necessary before the manuscript is suitable for publication. Addressing concerns 4 and 5 may further add to the manuscript.

Major concerns:

1) Specificity of arfaptins for binding PtdIns(4)P: as the authors describe, BAR domain-containing proteins interact with lipid membranes via electrostatic contacts. In Figure 1D, they show that the arfaptins can bind to liposomes containing negatively charged PS, and bind even better when PtdIns(4)P is added. They correlate this binding to a region upstream of the BAR domain which may confer "lipid specificity" to the arfaptins. Can this region be better defined? Is it structured? Many BAR domains feature an upstream membrane inserting "wedge" that contains positively-charged and hydrophobic residues (so called N-BAR domains). The regions of the arfaptins immediately preceding their BAR domains may fit the criteria to be such "wedges". Indeed, this would explain several of the experiments presented here.
Also, additional liposome binding experiments are required to substantiate whether arfaptins bind specifically to PtdIns(4)P over other phosphoinositides. At the minimum, a "dot blot" using different phosphoinositides would help to support this model.

2) Arfaptin 1 as a negative regulator of TGN trafficking: the authors propose that arfaptin 1 functions as a negative regulator in TGN trafficking. The main evidence for this is that, when either wildtype or S100A mutant arfaptin 1 are over-expressed, they reduce ssHRP trafficking out of the Golgi (Figure 5D-F). This experiment is very artificial. Protein over-expression can often perturb a trafficking pathway in non-physiological ways. Further experiments are needed to confirm if arfaptin 1 is truly a negative regulator of TGN trafficking. For instance, does the over-expression of arfaptin 2 also reduce ssHRP trafficking? Can the over-expression of arfaptin 1 merely titrate out other proteins that are required for proper TGN trafficking, such as Arf1? Does co-overexpression of Arf1 rescue this over-expression phenotype? The authors claim that over-expression of S100E arfaptin 1 does not affect ssHRP trafficking, but this is not surprising considering the mutant does not localize to the TGN. More direct evidence for negative regulation is needed to substantiate this model.

3) What affect does arfaptin 1/2 knockdown have on TGN trafficking machinery?: the authors conduct several siRNA experiments knocking down either arfaptin 1 or 2 separately. What happens when both are knocked down together? Many BAR proteins have paralogues that function redundantly on the same pathway (amphiphysin1/2, FCHo1/2, sorting nexins, etc.) Knocking down

both together may reveal previously unseen phenotypes.
Furthermore, the authors propose a multi-stage pathway in which DAG and Arf1 recruit PKD, which in turn modulates PtdIns(4)P levels and thus promotes arfaptin recruitment. From this model, Arf1/Arl do not physically interact with the arfaptins, yet other studies suggest a direct interaction. Can this be reconciled with the present study? In other words, can the authors better show that it is the phosphoinositide and not the Arf1/Arl that is mediating TGN localization of the arfaptins?

4) Are there Golgi morphological changes associated with loss or perturbed arfaptin1/2 function?: The authors propose trafficking defects when the arfaptins are lost or (for arfaptin 1) over-expressed. First, is this trafficking defect general for all TGN cargoes? Are the arfaptins thoughts to mediate specific cargo movements, or are they players in all TGN trafficking pathways (eg. clathrin, COPI, etc.)? Is M6P receptor trafficking affected? Second, are there gross morphological changes within the TGN when the arfaptins are compromised? Close examination by light microscopy or, even better, electron microscopy may indicate defects and provide mechanistic insights to arfaptin function.

5) Are the constructs used in Figure 2, which define the "minimal" localizing regions of arfaptin, stable?: The observations in Figure 2B set the basis for subsequent investigations on the region "upstream" of the BAR domain. Are these protein truncations stable? A loss of stability may give a false negative. Western blots would help substantiate this finding.

Minor comments:

In general, the language requires some editing and syntactical changes throughout the manuscript.

Referee #2

Cruz-Garcia et al in their Ms identify a role for the BAR domain proteins arfaptin 1 and 2 via association with PI4P in cargo export from the TGN. It is shown that arfaptins bind to PI4P containing liposomes in sedimentation assays and this association is perturbed by mutation of conserved aromatic acids within the region preceding the BAR domain proper. Furthermore, arfaptin 1 but not arfaptin 2 is shown to be directly phosporylated by PKD within the PI4P binding stretch, a modification that releases the protein from membranes and the TGN. Overexpression of WT-arfaptin 1 but not its depletion by siRNA interferes with secretion of a signal sequence containing HRP reporter. Furthermore, arfaptin 2 depleted cells show defects in secretion of ssHRP, VSVG, and missort lysosomal hydrolases to the medium. Based on these data a model is proposed according to which arfaptins differentially regulate cargo exit at the TGN.

The observation that arfaptins associate with PI4P and differentially regulate cargo exit from the TGN is potentially interesting and may warrant publication in The EMBO J. However, the present stage Ms appears premature with a number of open questions remaining that would need to be addressed prior to publication. In addition to technical concerns, there are also some conceptual issues.

1. My most important point pertains to the concept of arfaptin 1 vs. arfaptin 2 function. The authors claim an inhibitory role of arfaptin 1 in cargo exit from the TGN. However, this claim is not supported by the data. If this was the case one would expect that knockdown of arfaptin 1 accelerates secretion of ssHRP and VSVG. The data in figs. 5 and 6 show that this is not the case. A careful kinetic analysis would be required to address this point. Moreover, as many BAR domain proteins dimerize another option is that the potential regulatory role of arfaptin 1 may only become overt under certain conditions, i.e. a wave of cargo export or signal-induced activation or inactivation of PKD.

Does one observe arfaptin 1/2 heterodimers and what is the effect of phosphorylation on dimerization?

In any case the current data set is inconsistent with a model where arfaptin 1 is a negative regulator of cargo flux through the TGN en route to the cell surface.

2. Surprisingly, the authors do not make an effort to carry out knockdown/ rescue experiments in any one of their assays. This would be required to ascertain specificity of the observed phenotypes and to analyze the physiological relevance of the PKD-mediated phosporylation of arfaptin 1. As it stands the only evidence in favor of a physiological role of this modification are the overexpression experiments shown in fig. 5; yet, this pathway appears to operate in an arfaptin 1-independent manner.

3. What is the effect of co-depletion of arfaptin 1 and 2 on TGN export? One might expect profound ultrastructural changes in TGN morphology if indeed cargo export was blocked.

4. The data regarding PI4P association of arfaptins appear rather preliminary. First, sedimentation assays are error-prone due to effects of protein aggregation (which may sometimes even be lipid-induced). Second, what should have been conducted is a careful analysis of the lipid binding profile of arfaptins. This is mandatory as many BAR domain proteins associate with charged membrane surfaces in a rather non-selective manner. Third, in order to substantiate the proposal that PI4P at the TGN is required for membrane association of arfaptins in living cells better tools are needed than PAO (a horrible poison!). Recently, several techniques have been developed that allow for the acute or sustained depletion of PIPs including PI4P, i.e. by rapamycin or rapalog-induced translocation of a PI4P-selective phosphatase.

5. The paper entirely misses statistical analyses, which are state-of-the-art in the field nowadays.

Referee #3

This paper shows clearly that arfaptin 1 and arfaptin 2 bind PI4P and this interaction requires a particular tryptophan residue for binding in vitro and Golgi localization in cells. The authors identify a targeting domain in both proteins and PKD phosphorylation of S100 of arfaptin 1 (in this domain) but not arfaptin 2 blocks PI4P binding and Golgi association. Exogenous expression but not depletion of arfaptin 1 block secretion of HRP; depletion of arfaptin 2 slows G protein delivery to the cell surface and blocks HRP secretion. In general, the work is well done and the data are very clean; upon revision, the findings will be of broad interest to the readers of EMBO J.

Main comment. The authors show that depletion of either arfaptin 1 or 2 leads to an increase in lysosomal enzume secretion from cells. They infer that this means arfaptin 1 or 2 participate actively in this process (see abstract and conclusion). Unfortunately, many perturbations lead to enhanced hydrolase secretion. An important control would be to try a GRASP siRNA or another protein that acts earlier in the Golgi to be sure that they are not just seeing Golgi disruption rather than a specific hydrolase secretion defect. In addition, the block should lead to accumulation of mannose 6-phosphate receptors in the TGN. This should be tested to verify their conclusion. In many cell types, these receptors are predominantly in late endosomes at steady state by light microscopy and that should shift in depleted cells. Otherwise, well done.

Other specific comments:
1. Fig. 1A. CERT is still on the Golgi upon PAO treatment in contrast to what the authors write. OR? Yes the Golgi has a different appearance.

2. Fig. 4B why is the GST-arfaptin 1 wild type on the Golgi in cells expressing constitutively active PKD but not endogenous arfaptin1 (4C)? This goes against what authors state. Please clarify.

3. Fig. 6 D underestimates inhibition as the blot in C is saturated for the controls at 45 minutes. Please state this or scan a lower exposure blot.

4. Results page 2--staining was primarily cytoplasmic (and nuclear).

5. Results page 3--you could not identify a shorter mutant? Do you mean localize?
Trp and tyr are known to bind other proteins--do you mean in these specific cases or in general?
Bottom of same paragraph--you couldn't make the mutant proteins or plasmid?

6. Results page 4--These results show the importance of those residues in PI4P binding in vitro and

IN GOLGI LOCALIZATION in cells (you haven't shown this was due to PI4P here).

7. Abstract. what do the authors mean by "a similar mechanism"? It can't be similar if it uses a different domain. Please modify. Also, the authors state that arfaptin 1 is a negative regulator. That is not really accurate. Overexpression is inhibitory but that may also be true for arfaptin 2 and may be due to artificial titration of some key component. Also, the lack of phenotype in arfaptin 1 depleted cells may be because arfaptin 2 is still there and more important. Please soften text. If they saw increased secretion upon arfaptin 1 depletion, it would be correct to call the protein a negative regulator.

1st Revision - authors' response 27 November 2012

Our point-by-point answer to the comments of the reviewers follow.

Referee #1

In this manuscript, Cruz-Garcia and colleagues propose that the BAR domain-containing proteins arfaptin 1 and 2 localize to the TGN by binding PtdIns(4)P. They show that a highly conserved region upstream of the BAR domain modulates this phosphoinositide binding, and furthermore contains a conserved Ser100 which serves as a phospho-regulatory site for arfaptin 1 (but not arfaptin 2). Protein kinase D (PKD) can phosphorylate this residue, and the phospho-mimetic S100E displays reduced membrane binding and TGN localization. Finally, the authors propose that arfaptin 1 and 2 play differential roles in TGN trafficking. Depletion of arfaptin 2, but not 1, by siRNA reduces ssHRP trafficking and cathepsin D maturation. Conversely, over-expression of arfaptin 1 negatively affects ssHRP trafficking out of the TGN. Taken together, the authors propose that arfaptins 1 and 2 play distinct roles in TGN trafficking.

Overall, this is a straightforward analysis of the biochemistry and cell biology of the arfaptins. The authors propose a PKD-mediated phospho-regulatory mechanism that governs this arfaptin 1 lipid binding. However, their model for "distinct" functions of arfaptins 1 and 2 in TGN trafficking requires additional experiments to fully flesh out. Specific comments regarding this are outlined below. Addressing concerns 1, 2, and 3 will be necessary before the manuscript is suitable for publication. Addressing concerns 4 and 5 may further add to the manuscript.

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We thank the reviewer for this suggestion. A predicted amphipathic helix (residues 93 to 112) is identified in the region preceding the BAR domain of arfaptin1 and 2 (Figure 2D). This region, in principle, has the capacity to insert into the membranes. It is also interesting that only arfaptin1 contains a PKD phosphorylation site within the amphipathic helix. PKD-dependent phosphorylation could thus alter the structure of the amphipathic helix and therefore its binding to the membrane. This item is now discussed extensively in the paper.

Also, additional liposome binding experiments are required to substantiate whether arfaptins bind specifically to PtdIns(4)P over other phosphoinositides. At the minimum, a "dot blot" using different phosphoinositides would help to support this model.

The protein-lipid overlay assay is a useful approach but many colleagues have discouraged us from relying on the data from this kind of experiment. Regardless, we now include data in the

supplemental section (Figure S2). Our data shows that that recombinant non-tagged arfaptin1 binds to PI(3)P and PI(5)P and to less extent to PI(4)P, phosphatidylserine, and PI(3,5)P_2. Recombinant non-tagged arfaptin2 binds mainly to PI(3)P, PI(4)P, PI(5)P, and phosphatidylserine. However, there is no PI(3,5)P_2, PI(3)P, or PI(5)P at the Golgi membranes so the significance of the binding data, on its own, is not clear. This is explained in the text.

2) Arfaptin 1 as a negative regulator of TGN trafficking: the authors propose that arfaptin 1 functions as a negative regulator in TGN trafficking. The main evidence for this is that, when either wildtype or S100A mutant arfaptin 1 are over-expressed, they reduce ssHRP trafficking out of the Golgi (Figure 5D-F). This experiment is very artificial. Protein over-expression can often perturb a trafficking pathway in non-physiological ways. Further experiments are needed to confirm if arfaptin 1 is truly a negative regulator of TGN trafficking. For instance, does the over-expression of arfaptin 2 also reduce ssHRP trafficking?

We did overexpress arfaptin2, as suggested by the reviewer, and found it not to affect the secretion of ssHRP to the extent observed with the overexpression of wild type arfaptin1. However, this could be due to any number of reasons and we have therefore removed the discussion on these proteins as negative regulators of constitutive protein secretion.
To further ascertain the role of arfaptins in constitutive secretion, in addition to test ssHRP secretion, we monitored the effect of arfaptin knockdown (both single and double) on the secretion of another protein called PAUF and general protein secretion. Our results reveal no obvious role of arfaptins in PAUF and constitutive protein secretion (Figure 5C and S5). Knockdown of the single arfaptin in Drosophila S2 cells was also without an effect on the secretion of ssHRP (Figure 5F). Taken together these results indicate that arfaptins are not required for general protein secretion. We now claim these are not required for general protein secretion.
Our new data indicates the requirement of arfaptins in the regulated secretion of chromogranin A (Cg A) and the data is included in the revised manuscript (Figure 6).

Can the over-expression of arfaptin 1 merely titrate out other proteins that are required for proper TGN trafficking, such as Arf1? Does co-overexpression of Arf1 rescue this over-expression phenotype? Over expression of Arf1 affects the structure of Golgi membranes and the experiment is therefore not particularly insightful.
The authors claim that over-expression of S100E arfaptin 1 does not affect ssHRP trafficking, but this is not surprising considering the mutant does not localize
to the TGN. More direct evidence for negative regulation is needed to substantiate this model.

We agree with the reviewer and we have changed the text and state that arfaptins have no role in constitutive protein secretion. There is no mention of their role as negative regulators in constitutive protein secretion.

3) What affect does arfaptin 1/2 knockdown have on TGN trafficking machinery?: the authors conduct several siRNA experiments knocking down either arfaptin 1 or 2 separately. What happens when both are knocked down together? Many BAR proteins have paralogues that function redundantly on the same pathway (amphiphysin1/2, FCHo1/2, sorting nexins, etc.) Knocking down both together may reveal previously unseen phenotypes.

The double knockdown of arfaptin1 and 2 did not alter ssHRP secretion or the pattern or levels of total secreted proteins in HeLa cells (^{35}S-Met labeling experiment). The data are now included in the text (Figure 5B and C).

Furthermore, the authors propose a multi-stage pathway in which DAG and Arf1 recruit PKD, which in turn modulates PtdIns(4)P levels and thus promotes arfaptin recruitment. From this model, Arf1/Arl do not physically interact with the arfaptins, yet other studies suggest a direct interaction. Can this be reconciled with the present study? In other words, can the authors better show that it is the phosphoinositide and not the Arf1/Arl that is mediating TGN localization of the arfaptins?

In the revised version, we discuss the view that both PI(4)P and Arf1/Arl1 are required for the recruitment of arfaptins to the TGN.

4) Are there Golgi morphological changes associated with loss or perturbed arfaptin1/2 function?:

The authors propose trafficking defects when the arfaptins are lost or (for arfaptin 1) over-expressed. First, is this trafficking defect general for all TGN cargoes? Are the arfaptins thoughts to mediate specific cargo movements, or are they players in all TGN trafficking pathways (eg. clathrin, COPI, etc.)?

We now show that arfaptins do not have a role in constitutive protein secretion. We now include new data on their involvement in the secretion of chromogranin A by the regulated secretory pathway (Figure 6).

Is M6P receptor trafficking affected?

We did not test the trafficking of the M6Pr. Instead, we examined the trafficking of lysosomal hydrolases in Drosophila S2 cells and found no obvious change upon the knockdown of the single D. melanogaster arfaptin. In addition, we did not observe effect on the localization of CI-M6Pr in HeLa upon arfaptin knockdown, which further suggests that the defect we had reported was unlikely to be direct. We have now removed the discussion on the potential involvement of arfaptins in the trafficking of lysosomal hydrolases.

Second, are there gross morphological changes within the TGN when the arfaptins are compromised?
Close examination by light microscopy or, even better, electron microscopy may indicate defects and provide mechanistic insights to arfaptin function.

There is no obvious effect upon arfaptin1 and/or 2 knockdown on the organization of the Golgi complex in HeLa cells at the level of light microscopy when it is examined by immunofluorescence with anti-CI-M6Pr and GRASP55 antibodies.

5) Are the constructs used in Figure 2, which define the "minimal" localizing regions of arfaptin, stable?: The observations in Figure 2B set the basis for subsequent investigations on the region "upstream" of the BAR domain. Are these protein truncations stable? A loss of stability may give a false negative. Western blots would help substantiate this finding.

Yes, we can detect them by western blotting. The data is included in the paper (Figure S3).

Minor comments:

In general, the language requires some editing and syntactical changes throughout the manuscript.

The paper is modified extensively and we thank the reviewer for highlighting the sloppy writing.

Referee #2

Cruz-Garcia et al in their Ms identify a role for the BAR domain proteins arfaptin 1 and 2 via association with PI4P in cargo export from the TGN. It is shown that arfaptins bind to PI4P containing liposomes in sedimentation assays and this association is perturbed by mutation of conserved aromatic acids within the region preceding the BAR domain proper. Furthermore, arfaptin 1 but not arfaptin 2 is shown to be directly phosporylated by PKD within the PI4P binding stretch, a modification that releases the protein from membranes and the TGN. Overexpression of WT-arfaptin 1 but not its depletion by siRNA interferes with secretion of a signal sequence containing HRP reporter. Furthermore, arfaptin 2 depleted cells show defects in secretion of ssHRP, VSVG, and missort lysosomal hydrolases to the medium. Based on these data a model is proposed according to which arfaptins differentially regulate cargo exit at the TGN.

The observation that arfaptins associate with PI4P and differentially regulate cargo exit from the TGN is potentially interesting and may warrant publication in The EMBO J. However, the present stage Ms appears premature with a number of open questions remaining that would need to be addressed prior to publication. In addition to technical concerns, there are also some conceptual issues.

1. My most important point pertains to the concept of arfaptin 1 vs. arfaptin 2 function. The authors claim an inhibitory role of arfaptin 1 in cargo exit from the TGN. However, this claim is not supported by the data. If this was the case one would expect that knockdown of arfaptin 1 accelerates secretion of ssHRP and VSVG. The data in figs. 5 and 6 show that this is not the case. A careful kinetic analysis would be required to address this point. Moreover, as many BAR domain proteins dimerize another option is that the potential regulatory role of arfaptin 1 may only become overt under certain conditions, i.e. a wave of cargo export or signal-induced activation or inactivation of PKD.

To further ascertain the role of arfaptins in constitutive secretion, in addition to test ssHRP secretion, we monitored the effect of arfaptin knockdown (both single and double) on the secretion of another protein called PAUF and general protein secretion. Our results reveal no obvious role of arfaptins in PAUF and general protein secretion (Figure 5C and S5). Knockdown of the single arfaptin in Drosophila S2 cells was also without an effect on the secretion of ssHRP (Figure 5F). Taken together these results indicate that arfaptins are not required for general protein secretion. We now claim these are not required for general protein secretion.
Indeed, we examined the trafficking of lysosomal hydrolases in Drosophila S2 cells and found no obvious change upon the knockdown of the single arfaptin of D. melanogaster. We have now removed the discussion on the potential involvement of arfaptins in the trafficking of lysosomal hydrolases.
Our new data indicates the requirement of arfaptins in the regulated secretion of chromogranin A (Cg A) and the data is included in the revised manuscript (Figure 6).

Does one observe arfaptin 1/2 heterodimers and what is the effect of phosphorylation on dimerization?

Ricci and colleagues have recently reported that PKD phosphorylates arfaptin1 in Ser100 and that the phosphomimetic mutant of arfaptin1 can still interact with the wild type form. This is based on a GST pull-down assay with cell extracts (Gehart et al, Dev Cell, 2012). We have noticed that arfaptins can form homodimers and heterodimers in solution. This event is independent of the PKD phosphorylation. However, because PKD is active on the TGN and not in the cytoplasm, we would like to test this with arfaptins on liposomes or isolated Golgi membranes. We plan to address this issue more rigorously in the future and apologize for not being able to include this additional data in the present manuscript.

In any case the current data set is inconsistent with a model where arfaptin 1 is a negative regulator of cargo flux through the TGN en route to the cell surface.

We have removed our description on the potential role of arfaptin1 as negative regulators of constitutive secretion.

2. Surprisingly, the authors do not make an effort to carry out knockdown/ rescue experiments in any one of their assays. This would be required to ascertain specificity of the observed phenotypes and to analyze the physiological relevance of the PKD-mediated phosphorylation of arfaptin 1. As it stands the only evidence in favor of a physiological role of this modification are the overexpression experiments shown in fig. 5; yet, this pathway appears to operate in an arfaptin 1-independent manner.

We cannot perform rescue experiments with the reagents that we have now in the lab. We have found the levels of the exogenously expressed, siRNA-resistant, arfaptins were too high (around 10-fold higher than the endogenous levels of the arfaptins) and, thefore, we could not discriminate between a potential rescue phenotype or non-specific effects due to the high level of overexpression.

3. What is the effect of co-depletion of arfaptin 1 and 2 on TGN export? One might expect profound ultrastructural changes in TGN morphology if indeed cargo export was blocked.

The double knockdown of arfaptin1 and 2 did not alter ssHRP secretion or the pattern or levels of total secreted proteins in HeLa cells (^{35}S-Met labeling experiment). The data are now included in the text (Figure 5B and C).

There is no obvious effect upon arfaptin1 and/or 2 knockdown on the organization of the Golgi complex in HeLa cells at the level of light microscopy when it is examined by immunofluorescence with anti-CI-M6Pr and GRASP55 antibodies.

4. The data regarding PI4P association of arfaptins appear rather preliminary. First, sedimentation assays are error-prone due to effects of protein aggregation (which may sometimes even be lipid-induced). Second, what should have been conducted is a careful analysis of the lipid binding profile of arfaptins. This is mandatory as many BAR domain proteins associate with charged membrane surfaces in a rather non-selective manner. Third, in order to substantiate the proposal that PI4P at the TGN is required for membrane association of arfaptins in living cells better tools are needed than PAO (a horrible poison!). Recently, several techniques have been developed that allow for the acute or sustained depletion of PIPs including PI4P, i.e. by rapamycin or rapalog-induced translocation of a PI4P-selective phosphatase.

We started the analysis of the binding of arfaptins to phospholipids by using a liposome flotation assay. We observed the same binding to PI(4)P and phosphatidylserine as in the sedimentation assay. However, with the flotation, the quantitation became difficult because of the inability to collect the floated fractions from the gradient. We therefore decided to work only with the sedimentation assay.
We now include in the revised version the results of a protein-lipid overlay assay showing the preferential binding of arfaptins to phosphatidylinositol monophosphates and phosphatidylserine (Figure S2).
Although the PAO treatment can induce secondary effects, such as the inhibition of tyrosine phosphatases, it has been reported to reduced the PI(4)P pool of the Golgi complex by immunofluorescence (Hammond et al, Biochem J, 2009). Indeed, PAO treatment has been used to show that proteins like FAPPs or GBF1 are recruited to the Golgi complex in a PI(4)P-dependent manner (Godi et al, Nat Cell Biol, 2004; Dumaresq-Doiron et al, JCS, 2010). We could not perform the analysis of the effect of the acute depletion of PI(4)P on arfaptin localization, as suggested by the reviewer, because this approach is extremely tedious and requires extensive quantitative imaging. We discussed with Dr. Tamas Balla (it is his approach, afterall) and he agreed that although this is useful, it is technically challenging to quantitate. Instead, we show and explain in the revised manuscript that PAO treatment does not affect the ability of Arf1 and Arl1, the factors known to mediate the binding of arfaptins to the Golgi, to recruit other effectors on the Golgi membranes such as β-COP and p230, respectively (Figure 1B and S1).

5. The paper entirely misses statistical analyses, which are state-of-the-art in the field nowadays.

Statistical analyses are included in the revised manuscript.

Referee #3

This paper shows clearly that arfaptin 1 and arfaptin 2 bind PI4P and this interaction requires a particular tryptophan residue for binding in vitro and Golgi localization in cells. The authors identify a targeting domain in both proteins and PKD phosphorylation of S100 of arfaptin 1 (in this domain) but not arfaptin 2 blocks PI4P binding and Golgi association. Exogenous expression but not depletion of arfaptin 1 block secretion of HRP; depletion of arfaptin 2 slows G protein delivery to the cell surface and blocks HRP secretion. In general, the work is well done and the data are very clean; upon revision, the findings will be of broad interest to the readers of EMBO J.

Main comment. The authors show that depletion of either arfaptin 1 or 2 leads to an increase in lysosomal enzume secretion from cells. They infer that this means arfaptin 1 or 2 participate actively in this process (see abstract and conclusion). Unfortunately, many perturbations lead to enhanced hydrolase secretion. An important control would be to try a GRASP siRNA or another protein that acts earlier in the Golgi to be sure that they are not just seeing Golgi disruption rather than a specific hydrolase secretion defect.

We agree with the reviewer but instead of knocking down GRASP proteins, we tested the effect of the knockdown of the single arfaptin protein in Drosophila S2 cells on both protein secretion and the export of lysosomal hydrolases. Knockdown of arfaptin did not affect ssHRP secretion or the

trafficking of lysosomal hydrolase in S2 cells. We have therefore removed these analysis and description from the paper.

In addition, the block should lead to accumulation of mannose 6-phosphate receptors in the TGN. This should be tested to verify their conclusion. In many cell types, these receptors are predominantly in late endosomes at steady state by light microscopy and that should shift in depleted cells. Otherwise, well done.

We did not see any obvious effect on the localization of CI-M6Pr. The description on the potential involvement of arfaptins in the export of lysosomal hydrolases is removed from the paper.

Other specific comments:
1. Fig. 1A. CERT is still on the Golgi upon PAO treatment in contrast to what the authors write. OR? Yes the Golgi has a different appearance.

A different image showing the effect of PAO treatment on GFP-CERT localization and a better explanation of the finding based on this experiment is now included in the revised manuscript.

2. Fig. 4B why is the GST-arfaptin 1 wild type on the Golgi in cells expressing constitutively active PKD but not endogenous arfaptin1 (4C)? This goes against what authors state. Please clarify.

Figure 4B is now explained more clearly in the revised manuscript.

3. Fig. 6 D underestimates inhibition as the blot in C is saturated for the controls at 45 minutes. Please state this or scan a lower exposure blot.

New experiments (analysis of the arfaptin knockdown on PAUF secretion, the secretion of newly synthesized proteins by metabolic labeling, and ssHRP secretion in Drosophila S2 cells) indicate that arfaptins have no obvious role in constitutive protein secretion. We have removed the former Figure 6 and the new findings are explained in the text (Figure 5 and S5).

4. Results page 2--staining was primarily cytoplasmic (and nuclear).

This is corrected in the revised manuscript.

5. Results page 3--you could not identify a shorter mutant? Do you mean localize?
Trp and tyr are known to bind other proteins--do you mean in these specific cases or in general?
Bottom of same paragraph--you couldn't make the mutant proteins or plasmid?

In the revised manuscript, the Tyr104 is not discussed and the focus is on Trp99. The sentence regarding the identification/localization of shorter mutant has been removed to clarity our discussion.

6. Results page 4--These results show the importance of those residues in PI4P binding in vitro and IN GOLGI LOCALIZATION in cells (you haven't shown this was due to PI4P here).

Changed as suggested.

7. Abstract. what do the authors mean by "a similar mechanism"? It can't be similar if it uses a different domain. Please modify. Also, the authors state that arfaptin 1 is a negative regulator. That is not really accurate. Overexpression is inhibitory but that may also be true for arfaptin 2 and may be due to artificial titration of some key component. Also, the lack of phenotype in arfaptin 1 depleted cells may be because arfaptin 2 is still there and more important. Please soften text. If they saw increased secretion upon arfaptin 1 depletion, it would be correct to call the protein a negative regulator.

We have removed the discussion on arfaptin 1 as a negative regulator of protein secretion based on the new data.

2nd Editorial Decision 18 December 2012

Thank you for submitting your revised manuscript to the EMBO Journal. Your manuscript has now been re-reviewed by the original three referees.

As you can see below, we received mixed feedback on your revision. Referee #3 is basically satisfied with the revised version, while referee #2 indicates that several of the initial raised issues have not been adequately addressed. Referee #1, on the other hand, finds that in light of the recent Dev Cell paper (Gerhart et al.) that the advance and insight provided is not sufficient for publication in the EMBO Journal. Regarding this last issue, our policy is that competing papers published during the revision period do not compromise the novelty or advance of a study under revision with us. In other words, we will not take the Dev Cell paper into consideration.

Regarding the remaining issues raised by referee #2. These were issues that were also brought up during initial review. I have looked carefully into each of them and I am in agreement with the referee that significant revisions are still needed for publication here. Specifically the following points needs to be addressed

1) Ref#2 point #2 and Ref#1: Rescue experiments must be carried out

2) Ref #2 point #3: PI4P binding assays using flotation assays and PI4P depletion assays are also needed.

3) Ref #2 general comments: To look at CgA secretion in arfaptin1 and 2 double knockdowns.

4) Ref #2 general comments: Confirmation of the findings using a second SG marker

We don't need further insight into if arfaptin dimerization is regulated by phosphorylation - referee #2 point #1.

I recognize that some of the issues raised might be technically challenging to carry out, but this is what is needed for publication here. We normally allow only one major revision, but in this case I can offer that if you are able to experimentally address the points raised above that we would be willing to consider a revised manuscript. I will involve referee #2 in the re-review of the revision.

REFEREE REPORTS

Referee #1

In their revised manuscript, Cruz-Garcia and colleagues demonstrate that Arfaptins contain a conserved amphipathic helix (AH) that promotes binding to PtdIns(4)P liposomes and to Golgi membranes. They show that this AH is phosphorylated by PKD, and propose that this phospho-regulation modulates Arfaptin localization by the AH. Finally, they demonstrate that both Arfaptins are involved in regulated secretion from the Golgi, including the secretion of chromogranin A (Cg A).

Initially, I had several major concerns about this manuscript, and acknowledge that the authors have conducted many of the suggested experiments to address these concerns. One of these concerns involved dissecting why the phosphorylation of Ser100 negatively-regulated Arfaptin localization to the Golgi. The authors have now uncovered an important AH that is essential to Golgi localization. The regulation of AH membrane binding by PKD is thus a clearly defined regulatory mechanism, and a significant discovery. The authors have also conducted dual siRNA experiments, and changed many of their conclusions based on the results.

The authors note that while this manuscript has been in review, another similar manuscript was published in Developmental Cell (Gerhart et al, Oct 2012). Gerhart and colleagues show that PKD phosphorylates Ser132 of Arfaptin-1. Furthermore, they demonstrate that Arfaptins negatively regulate Arf1 in the production of Golgi secretory vesicles.

Unfortunately, the Dev Cell paper establishes several of the key features regarding the function of the Arfaptins. However, it is noteworthy that Cruz-Garcia and colleagues have added new experiments that moderately change the scope and conclusions of this manuscript. They now provide evidence for the AH as a membrane targeting module. This provides a mechanistic basis for the phospho-regulation by PKD. While the authors do distinguish an AH, which they incorporate into their model, it still seems hard to justify publication in EMBO J given the previously published work of Gerhart et al.

Specific comments:

1) The authors report that it is difficult to conduct "rescue" experiments, since siRNA-resistent constructs massively over-express Arfaptins. These experiments are important controls, and should be reported for the Cg A secretions, despite the expression problems.

Referee #2

Cruz-Garcia et al in their revised Ms now conclude that arfaptins are involved the biogenesis of secretory granules. This a substantial turnaround from the original version in which they claimed opposing roles for arfaptins 1 and 2 in regulating constitutive secretion and lysosomal sorting. Neither of these proposals seems to have withstood the new experiments conducted in the time frame for the revision of this paper.
Instead it appears that KD of either arfaptin 1 or 2 reduces but does not abolish secretion of chromogranin A, with more severe effects of arfaptin 1. Neither double KDs nor rescue experiments have neen conducted. Moreover, the underlying mechanism is unclear (i.e. with respect to PI4P binding and phosphorylation of arfaptins- this remains after all the main message of the paper) and confirmation by a second SG marker is missing from the analysis. I am also surprised to see that the authors have chosen not to conduct a number of experiments suggested in the original round of review.

1. The role of arfaptin dimerization and its potential regulation by phosphorylation has not been addressed. In agree that these data may seem less critical as both arfaptins now appear to do the same. However, if double KD of both isoforms turns out not have additive effects then this issue would become important.
2. I cannot side with the authors' argument that rescues cannot be carried out due to DN effects of OE. In this case commercially available cell lines that allow for regulated inducible expression of proteins could be used. While this is time-consuming I regard such data as essential for publication in a high impact journal such as The EMBO J.
3. The same holds true for the analysis of PI4P binding using flotation assays and acute PI4P depletion. There are many labs in the field who have successfully used flotation assays. Again, I agree these are technically more challenging but certainly doable and provide much more reliable data. The key point here is also specificity (that cannot be reflected in lipid dot blot overlay assays as the lipid head groups are randonly oriented on the spot!) for PI4P vs. other PIPs, such as PI3P, PI5P, PI3,4P2, P4,5P2, PI3,5P2, or PI3,4,5,P3, as well as other acidic phospholipids. Again, the point is crucial as most BAR domains display little specificity and can interact with a number of charged headgroups.

All-in-all, I remain of the opinion that the Ms though potentially interesting is premature for publication in The EMBO J.

Referee #3

The authors have tried to do a careful job of replying to all of the referee comments and the revised paper is much improved. The authors should add the distinction between regulated and constitutive secretion to the title and abstract to be sure this aspect is clear. In addition, there are many language errors throughout the text (example: constitute secretion?) that must be fixed before acceptance of the paper for publication.

2nd Revision - authors' response 05 April 2013

1) Ref#2 point #2 and Ref#1: Rescue experiments must be carried out

For the rescue of the CgA secretion phenotype in BON cells after arfaptin1 or 2 knock down we have tried the following approaches. The BON cells are difficult to transfect with plasmids (transfection index around 10-15%), we therefore decided to co-transfect a plasmid coding for CgA fused to GFP and the corresponding vectors coding for the siRNA-resistant versions of arfaptins. We took this approach to increase the chance of obtaining higher proportion of cells co-expressing the siRNA-resistant form of arfaptin, and secretory cargo (CgA-GFP) for detection by western blotting. Under these conditions, arfaptin1 or 2 were knocking down, CgA-GFP was expressed and secreted, but we could not detect by western blotting in a reproducible manner the expression of the siRNA-resistant forms of arfaptins, which were tagged with a Myc-tag at the C-terminus. After extensive trials, we failed to obtain sufficiently high levels of expression of the siRNA-resistant forms of arfaptins in BON cells. This has been technically challenging and we have not been able to improve the transfection efficiency by using various transfection reagents, conditions and the constructs. However, the data from the double knockdown of both arfaptins gives us the confidence to state that only arfaptin1 is required for Cg A secretion.

2) Ref #2 point #3: PI4P binding assays using flotation assays and PI4P depletion assays are also needed.

We have performed the binding assays by floating rather than sedimenting the liposomes (Figures 1, 3, and 5). We have also used the PI(4)P depletion procedure by recruiting Sac1 phosphatase at the TGN. This was done in collaboration with Dr. Tamas Balla and the data is now included in the paper (Figure 2).

3) Ref #2 general comments: To look at CgA secretion in arfaptin1 and 2 double knockdowns.

The experimental data is now included in the paper (Figure 7). Our results reveal that knockdown of arfaptin1 causes a 65% reduction in the secretion of Cg A. Arfaptin 2 knockdown had a moderate effect (25% inhibition) and the double knockdown caused 65% reduction in Cg A secretion.

4) Ref #2 general comments: Confirmation of the findings using a second SG marker.

We haven't done this and we sincerely hope that this is not a reason to further delay the publication of our paper. There is already data on the requirement of arfaptin1 in insulin secretion (Ricci and colleagues, Dev. Cell. 2012). Our findings that Cg A secretion in BON cells is also affected by arfaptin1 knockdown provides the evidence of another cargo in an another cell line that relies on arfaptin1 for its secretion by the regulated secretory pathway.

Acceptance 25 April 2013

Thank you for submitting your revised manuscript to The EMBO Journal. Your revision has now been re-reviewed by the original referee #2. As you can see below, the referee raises issues regarding the lack of rescue experiment for the Arfaptin kd experiments. I have also asked for further advice from referee #3 on this issue. Referee #3 appreciates the efforts that you have undertaken to try to resolve this and finds that the paper should be published here despite the lack of the rescue experiments.

I have also discussed the paper and the remaining concerns with the team and while we very much would have wished to see the inclusion of the rescue experiments, we also appreciate the efforts undertaken to address this and the findings reported. We have therefore decided to accept the manuscript as is.

REFEREE REPORT

Referee #2

In their second revision the authors have added liposome flotation as well as acute PI4P depletion experiments to strengthen their conclusion that arfaptins specifically bind to PI4P, which facilitates their recruitment to the Golgi. Moreover, double KD experiments indicate that arfaptin 1 is required for CgA secretion; whether this involves PI4P binding to arfaptin 1 remains unclear as no rescue experimemts are provided.

I appreciate the efforts and the new data and the explanation as to why no rescue data can be provided at this time. The latter is unfortunate and in my opinion compromises the key conclusion of the paper. The problem remains that the biochemical data indicate that arfaptins can bind to PI4P. However, the only other lipid this is compared to is PS, an much more abundant component of the cytoplasmic leaflet of many intracellular membranes. While there are certainly good chances that PI4P is also the physiologically relevant PI species the actual data do not address this point. As stated before further specificity controls using alternate PI species should have been included- this is not an unusual thing to ask for and rather standard in the field! With this caveats in mind and in the absence of rescue experiments that would allow to test the role of PI4P binding with respect to arfaptin 1-mediated CgA secretion the paper remains a borderline case for The EMBO J.

