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ArfGAP1 generates an Arf1 gradient on continuous lipid membranes displaying flat and curved regions

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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.)

1st Editorial Decision 09 September 2009

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees, whose comments are enclosed. As you will see, all three referees appreciate the high technical quality of your work, but express varying opinions as to the degree of conceptual advance provided by the study. Therefore, while referees 1 and 3 are rather positive and recommend publication (pending satisfactory revision), referee 2 finds that the work is primarily confirmatory of previous studies, and does not recommend publication.

Having also discussed your manuscript with an expert editorial advisor, our overall assessment is that your study does provide considerable novel insight into how the curvature-dependent regulation of Arf1-GTP by ArfGAP1 might control vesicle formation. We are therefore willing to over-rule the negative recommendation of referee 2, and to invite you to submit a revised version of your manuscript, addressing all the comments of all three referees. I would also add one comment from our external advisor, who feels that the manuscript would benefit from additional discussion of recent studies showing that Arf1-GTP itself shows curvature-dependent binding, which your analysis seems to contradict. While you do mention this point, it is not fully cited or discussed and I agree with our advisor that this would be valuable.

I should add that it is EMBO Journal policy to allow only a single round of revision. Acceptance of your manuscript will thus depend on the completeness of your responses included in the next, final version of the manuscript.

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

Yours sincerely,
Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

The manuscript by Ambroggio et al. is a very interesting and important study. Using molecular motors or optical tweezers to pull tubes of defined diameter from giant unilamellar vesicles (GUVs), the authors investigate the effect of membrane curvature on the distribution of ArfGAP1 and its substrate Arf1 within this system. These authors have demonstrated previously that ArfGAP1 binds with high specificity to highly curved membranes via its ALPS motifs. Here they visualize directly this process, finding that fluorescently tagged full-length ArfGAP1, as well as its ALPS motifs alone, localize exclusively to tubes in the GUV-tube network. In contrast, Arf1 is distributed quite homogeneously, with only a slight enrichment on the membrane of the tube compared to the GUV. The sensitivity of ArfGAP1 binding to the radius of the tube is striking, with a sharp cut-off at 35 +/- 5 nm. This value corresponds very well to the radius of liposomes required to stimulate ArfGAP1 GTP hydrolysis activity on Arf1. When ArfGAP1 was added to the GUV-tube system with Arf1-GTP, a gradient of Arf1 along the tube was generated. Hence ArfGAP1 activity on the tube is counterbalanced by rapid diffusion of Arf1-GTP into the tube from the GUV. These results suggest the compelling idea that in cells, there is constant diffusion of Arf1-GTP into a forming bud to replace that removed by ArfGAP1 activity localized to the curved tip of the bud. This concept is novel and has important implications for the mechanism of COPI vesicle formation. Not only do these results explain how COPI remains stably bound to the bud membrane during its formation despite the presence of ArfGAP1, they also suggest interesting mechanisms of sorting into COPI vesicles. In addition, this study provides an explanation for the results obtained by several groups showing that the rate of Arf1 cycling on and off membranes in cells is faster than that of COPI, even though COPI depends on Arf1 GTP hydrolysis for its cycling. The experiments presented are carefully designed and carried out, and the conclusions made are convincingly supported by the data. However, some minor additional experiments could help to support the essential conclusions, and changes to the text would clarify the ideas being presented.

1. Page 11. For the k value of their diffusion-reaction model, the authors assume that the rate of GTP hydrolysis catalyzed by ArfGAP1 is identical to the rate of release of Arf1 from membranes. This point should be explicitly mentioned, and the relevant data/references provided.

2. In Figure 4, the authors could test their model more directly and comprehensively by expanding the results shown to tubes of different diameters. To complement the Arf1-GTPgammaS experiment, the authors should verify that the distribution of Arf1-GTP along a thick tube (having a radius greater than that which allows ArfGAP1 binding) is uniform. Theoretically it should be possible to calculate different "characteristic length L" values for tubules of different radii, since the activity of ArfGAP1 varies as a function of curvature. These experimentally determined L values could be compared with the predicted L values from known ArfGAP1 activities on liposomes of different size.

3. Page 13, second paragraph. The authors compare the diffusion coefficient, D, of GTP-bound Arf1-OG in GUV membranes to "...reported values for protein diffusion at the Golgi (including Arf)...". This statement is misleading, because FRAP data for fluorescently labeled Arf1 in cells does not measure diffusion of Arf1-GTP in the Golgi membrane, but rather the exchange of Arf1 between membranes and cytosol. To my knowledge, there is no published data on the diffusion coefficient of Arf1-GTP in Golgi membranes in living cells. The authors should clarify that they are considering the diffusion coefficients of transmembrane proteins for the range of D values that they use in Figure 5, and specify whether these values are only for Golgi proteins or whether they are including TM proteins localized to other cellular compartments.

4. Page 5, end of last paragraph. To quantify the distribution of different proteins on the GUV membrane compared to the tube pulled out from the GUV, the authors calculate what they call a

"sorting ratio". I do not like this term because in these GUV/tube experiments, "sorting" suggests that the proteins are binding indiscriminately to either the GUV or the tube membrane, then undergoing a process that sorts them into one or the other membrane domain. Do the authors consider this a possibility for how the ALPS motifs of ArfGAP1 are binding to membranes? If the ALPS motifs bind ONLY to the highly curved tube membrane, and not to the GUV membrane, it seems to me that this is not a sorting process on the membrane. A term like "distribution ratio" would be more appropriate, especially since the system is being analyzed at equilibrium.

5. Page 7, first sentence. To support the conclusion that Arf1-GTP is diffusing within the membrane of the GUV-tube network, it is important to know the stability of binding of Arf1-OG to membranes. The authors state as data not shown that "the Arf1-OG signal was more stable". It would be best to include this data, or at least to indicate approximately how much longer Arf1-OG remains on the membranes after wash-out with buffer alone.

6. Page 9, first paragraph. The wording of the last two sentences in this paragraph hides the truly striking nature of the results presented. Rather than saying "...this could be done only for Arf1-OG (Fig. S4) but not for ALPS1-ALPS2-Alexa488", the authors should say that this quantitation for Arf1-OG shows a three- to fourfold enrichment in the tube compared to the GUV membrane over the range of tube diameters tested, whereas the corresponding value for ALPS1-ALPS2 would be infinite, since no binding at all to the GUV membrane can be detected. Perhaps it would be better to calculate a "homogeneity value" (the reciprocal of the "sorting value", which would be 1 for a perfectly uniform distribution and zero for exclusive tube localization. Then values could be plotted for ALPS1-ALPS2, at least for the tubes on which this peptide binds (all of which would be 0). The last sentence of this paragraph does not clearly point out the differences in the curves shown in Figure 3. These differences were described in the preceding paragraphs, but the two graphs should be directly compared to emphasize this important point. The most striking difference is that for ArfGAP1, the fitted curve intercepts the x-axis, which is not the case for Arf1, where the initial point is approximately (0,1). In addition, the slope of the curve above this critical point for ArfGAP1 is much steeper for ArfGAP1 than for Arf1. A point for clarification: what exactly do the data points for the Arf1 graph in Fig 3B at a curvature (1/Rtube = 0) correspond to? values on the GUV?

7. Figure 3, right hand panels, and supplementary figures 3 and 4. The authors should use symbols rather than colored circles for the data points, or at least change the colors to something other than red and green. The use of these colors, which correspond to the fluorescence signals shown to the left, might give the impression that the red circles refer to lipids and the green to the Arf1 or ALPS signal. In Figure 3A, center panels, indicate "Tube" and "Guv" as in the Figure 3B, and in the left panels show with dotted white lines where the fluorescence plots are taken from.

8. Page 10, experiments shown in Figure 4. The diameter of the tube should be indicated in the text, ie. "...a membrane tube of 10-15 nm radius was pulled from a GUV...". It should also be mentioned that imaging takes place at a time point after the system has reached equilibrium.

9. The term "vesicle" alone is generally used to refer to small-diameter, highly curved membrane structures. In cells, transport vesicles have the same diameter as the tubes pulled out from GUVs in this study. Therefore it could be confusing to some readers when the authors use the term "vesicle" to refer to the low curvature GUV. Instances I noticed are in the abstract, line 6 and results, page 6, second paragraph. Either "GUV" or "giant vesicle" should be used systematically in this context, throughout the manuscript.

10. Figure 3, legend, line 14. When the authors state "Same as in (A) in the presence of 0.5 uM Arf1-OG...", it implies that ALPS1+ALPS2 is present. Should be "Same as in (A) except that ALPS1+ALPS2 was replaced with 0.5 uM Arf1-OG...".

11. There are several English grammatical errors throughout the manuscript, which may mask the meaning of the ideas being presented (a few instances are listed below).

a. Page 3, second paragraph. "...that allow coupling the GAP activity with the curvature of the underneath membrane" should be "...that allow coupling OF the GAP activity with the curvature of the UNDERLYING membrane". (See also two lines from bottom of page.)
b. Page 6, second paragraph. Instead of "...whereas ArfGAP1 or the ALPS1-ALPS2 peptide

segregated on the tubes (Figure 2)" consider changing to "...whereas ArfGAP1 or the ALPS1-ALPS2 peptide was restricted to the tubes (Figure 2)". The latter is more neutral and does not carry any connotation about the mechanism of the localization to tubes.
c. Page 7, second paragraph. From: "The radius of the tubes pulled from GUVs by molecular motors can hardly be controlled and modified. To circumvent this, we used..." to: "The radius of the tubes pulled from GUVs by molecular motors cannot be readily controlled or modified. To circumvent this limitation, we used...".

Referee #2 (Remarks to the Author):

Antonny and colleagues investigate by biophysical methods the behavior of the small GTPase Arf1, a key component and regulator of COPI vesicles, and ArfGAP1 in liposomal systems. As expected from previous work, in a continuous liposomal system ArfGAP1 distributes to strongly curved areas, whereas ArfGTP does not show such a marked preference. Therefore it is not surprising that in such a system gradients arise of ArfGTP when limiting amounts of ArfGAP1 are present. The system would allow determining the diffusion coefficient of ArfGTP in a liposomal model bilayer, and the authors estimate that a diffusion rate of 1% of the diffusion observed in the liposomal system would suffice to keep a coated COPI bud in a meta-stabile state. The experiments are technically sound and well controlled. The problem is that the data does not really add new aspects to our present concepts of vesicle biogenesis, that in part come from the groups coauthoring this man. Therefore the question is whether the advance in knowledge presented in the manuscript would warrant publication in the EMBO-Journal, and whether the data should be published in a journal more specialized in Biophysics.

Referee #3 (Remarks to the Author):

In the manuscript "ArfGAP1 generates an Arf1 gradient on continuous lipid membranes displaying flat and curved regions" by Ambroggio et. al., the authors elegantly show how the lipid-curvature preference of ArfGAP1 creates a gradient of lipid-bound Arf1-GTP on tubules emanating from a large GUV. This gradient could explain how buds remain stable until after the vesicle has been clipped from the parent membrane. The work is novel and compelling and is sufficient for publication in EMBO journal.

Minor comments:

1. How confident are the authors in their assignment of a curvature threshold of 35 nm for the Alps1-Alps2 motifs of ArfGAP1? In other words, what is the error in this measurement?

2. In general, the manuscript is very well written. However, it could benefit from proofreading as there are minor mistakes in the English language usage. For example, this sentence from the introduction "ArfGAP1 and Gcs1p contain motifs named ALPs that allow coupling the GAP activity with the curvature of the underneath membrane"
would read better as "ArfGAP1 and Gcs1p contain motifs named ALPs that allow coupling of GAP activity to the curvature of the underlying membrane.

1st Revision - Authors' Response 15 October 2009

Response to the expert editorial advisor and to the editor

Having also discussed your manuscript with an expert editorial advisor, our overall assessment is that your study does provide considerable novel insight into how the curvature-dependent regulation of Arf1-GTP by ArfGAP1 might control vesicle formation. We are therefore willing to over-rule the negative recommendation of referee 2, and to invite you to submit a revised version of your manuscript, addressing all the comments of all three referees. I would also add one comment from our external advisor, who feels that the manuscript would benefit from additional discussion of

recent studies showing that Arf1-GTP itself shows curvature-dependent binding, which your analysis seems to contradict. While you do mention this point, it is not fully cited or discussed and I agree with our advisor that this would be valuable.

We agree that the way we placed our new findings in the context of other works was incomplete. In the new discussion, two paragraphs have been added to address the following questions: is Arf1 a sensor of membrane curvature? Does Arf1 induce membrane curvature?
First, we underline that the sensitivity of Arf1 to membrane curvature is weak (at best a 1.3-fold increase in the rate of spontaneous activation) and negligible compared to ArfGAP1 (a 30 to 100-fold increase in activity using the same range of liposome size). So we don't contest the observation of Lundmark et al but we argue that this effect is too small- especially when compared to ArfGAP1- to justify the claim in their title that "Arf family GTP loading is activated by [..] positive membrane curvature."
As for the induction of membrane curvature, our opinion is more balanced. On one hand we do not see membrane deformation upon Arf1-OG recruitment on GUVs with a composition mimicking that of the Golgi and showed that deformation requires the subsequent addition of coatomer (Manneville et al. Proc Natl Acad Sci 2008). On the other hand we donít contest the observation that Arf1-GTP, under some conditions, deforms lipid membranes as observed by Krauss, Beck and Lundmark (all papers are now quoted). We note that these effects were observed at higher Arf1 concentration. We thus conclude that the induction of membrane curvature by Arf-GTP depends on the context: Arf1-GTP alone or in complex with non-coat proteins is unlikely to deform lipid membranes. At higher surface concentration, e.g. when trapped in a coat, Arf1-GTP probably contributes to membrane deformation in synergy with the scaffold coat structure. Last, we quote some observations by Orci on Golgi membrane preparations: 1) Arf1 can be detected both on flat area and on buds by immunoEM, 2) bud formation requires the presence of coatomer.

Response to reviewer 1

1. Page 11. For the k value of their diffusion-reaction model, the authors assume that the rate of GTP hydrolysis catalyzed by ArfGAP1 is identical to the rate of release of Arf1 from membranes. This point should be explicitly mentioned, and the relevant data/references provided.

This is a very important point since no one has shown that GTP hydrolysis in Arf1 causes its simultaneous dissociation from lipid membranes. To address this point we have taken advantage of two fluorescent signals associated with Arf1-OG: tryptophan fluorescence to follow its conformational change upon GTP hydrolysis and FRET between Oregon Green and the lipid Rhodamine PE to follow Arf1-OG dissociation from the liposome surface. As shown in a new figure (Figure 5C) the two events are perfectly correlated in time: thus the rate of GTP hydrolysis catalyzed by ArfGAP1 is identical to the rate of release of Arf1 from membranes.
In addition Figure 5A-B shows FRAP experiments on DOPC GUVs and tubes to determine the diffusion coefficient of Arf1-OG. Figure 5 therefore presents a complete set of new experiments where we directly determined the values of k and D using Arf1-OG on DOPC liposomes instead of quoting previous works, which were not necessarily done under the same conditions. This makes more relevant the good match between the observed value of the characteristic length of the Arf1-OG gradient (8.5 μm) and the calculated value (the square root of D/k = 13 μm).

2. In Figure 4, the authors could test their model more directly and comprehensively by expanding the results shown to tubes of different diameters. To complement the Arf1-GTPgammaS experiment, the authors should verify that the distribution of Arf1-GTP along a thick tube (having a radius greater than that which allows ArfGAP1 binding) is uniform. Theoretically it should be possible to calculate different "characteristic length L" values for tubules of different radii, since the activity of ArfGAP1 varies as a function of curvature. These experimentally determined L values could be compared with the predicted L values from known ArfGAP1 activities on liposomes of different size.

We agree, however this is technically very challenging. The Arf1-OG gradient can be observed onlyon very long tubes as already shown in Figure 4 (panel B vs panel A). If making a long thin

tube is not very difficult, the problem arises when we lower membrane tension to increase the tube diameter. At low tension and in the presence of Arf1-GTP, the tube fluctuates and buckles out of the confocal plane, preventing quantification of the fluorescence.

3. Page 13, second paragraph. The authors compare the diffusion coefficient, D, of GTP-bound Arf1-OG in GUV membranes to "...reported values for protein diffusion at the Golgi (including Arf)...". This statement is misleading, because FRAP data for fluorescently labeled Arf1 in cells does not measure diffusion of Arf1-GTP in the Golgi membrane, but rather the exchange of Arf1 between membranes and cytosol. To my knowledge, there is no published data on the diffusion coefficient of Arf1-GTP in Golgi membranes in living cells. The authors should clarify that they are considering the diffusion coefficients of transmembrane proteins for the range of D values that they use in Figure 5, and specify whether these values are only for Golgi proteins or whether they are including TM proteins localized to other cellular compartments.

Sorry for the confusion, we do agree. In the revised text we clearly state that there is no measurement of the lateral diffusion coefficient of Arf on Golgi membranes and discuss the approaches used in vivo. We now write (p17/18): "The lateral mobility of Arf1-GTP on Golgi cisternae is not precisely known since values of D given by FRAP experiments probably integrate several steps including the cycling of Arf between the cytosol and membranes (Vasudevan et al, 1998; Presley et al, 2002)". Note however that the exact value of D is not very important. Rather the key point here is the order of magnitude: a sharp Arf gradient within a small distance requires Arf1-GTP to diffuse much more slowly than most transmembrane or peripheral proteins. Thus the blue area used now in Figure 6C shows the range of values of D from 1 to 100% of unrestricted diffusion assuming that the fastest diffusion coefficient of Arf1-GTP as measured in vitro is 3-5 μm2/s.

4. Page 5, end of last paragraph. To quantify the distribution of different proteins on the GUV membrane compared to the tube pulled out from the GUV, the authors calculate what they call a "sorting ratio". I do not like this term because in these GUV/tube experiments, "sorting" suggests that the proteins are binding indiscriminately to either the GUV or the tube membrane, then undergoing a process that sorts them into one or the other membrane domain. Do the authors consider this a possibility for how the ALPS motifs of ArfGAP1 are binding to membranes? If the ALPS motifs bind ONLY to the highly curved tube membrane, and not to the GUV membrane, it seems to me that this is not a sorting process on the membrane. A term like "distribution ratio" would be more appropriate, especially since the system is being analyzed at equilibrium.

We agree and have replaced the term sorting ratio by distribution ratio as suggested. Of course we cannot rule out that ALPS1-ALPS2 binds faintly to the GUVs, but in that case binding is so weak that it cannot be distinguished from the noise. Please note that in the new version of the manuscript, we have considered the possibility that ALPS1-ALPS2 binding to the GUV is within the same range as the Alexa488 noise. The advantage of this hypothesis is that it permits us to determine a lower limit for the distribution ratio of ALPS1-ALPS2 in the tubes and to compare this lower limit to the distribution ratio of Arf1-OG. This comparison further emphasizes the contrasting sensitivity of the two proteins to membrane curvature (new Figure 3C).

5. Page 7, first sentence. To support the conclusion that Arf1-GTP is diffusing within the membrane of the GUV-tube network, it is important to know the stability of binding of Arf1-OG to membranes. The authors state as data not shown that "the Arf1-OG signal was more stable". It would be best to include this data, or at least to indicate approximately how much longer Arf1-OG remains on the membranes after wash-out with buffer alone.

We agree. The supplementary figure S1 now includes a panel (B) showing the stability of binding of Arf1-OG to membranes after wash-out with buffer.

6. Page 9, first paragraph. The wording of the last two sentences in this paragraph hides the truly striking nature of the results presented. Rather than saying "...this could be done only for Arf1-OG (Fig. S4) but not for ALPS1-ALPS2-Alexa488", the authors should say that this quantitation for Arf1-OG shows a three- to fourfold enrichment in the tube compared to the GUV

membrane over the range of tube diameters tested, whereas the corresponding value for ALPS1-ALPS2 would be infinite, since no binding at all to the GUV membrane can be detected. Perhaps it would be better to calculate a "homogeneity value" (the reciprocal of the "sorting value", which would be 1 for a perfectly uniform distribution and zero for exclusive tube localization. Then values could be plotted for ALPS1-ALPS2, at least for the tubes on which this peptide binds (all of which would be 0). The last sentence of this paragraph does not clearly point out the differences in the curves shown in Figure 3. These differences were described in the preceding paragraphs, but the two graphs should be directly compared to emphasize this important point. The most striking difference is that for ArfGAP1, the fitted curve intercepts the x-axis, which is not the case for Arf1, where the initial point is approximately (0,1). In addition, the slope of the curve above this critical point for ArfGAP1 is much steeper for ArfGAP1 than for Arf1. A point for clarification: what exactly do the data points for the Arf1 graph in Fig 3B at a curvature (1/Rtube = 0) correspond to? values on the GUV?

OK. We have rewritten this part and included a new panel in figure 3 (C) where we directly compare the binding curves of ALPS1-ALPS2 and Arf1-OG on tubes of decreasing radius. For this and as detailed in point 4, we assume that ALPS1-ALPS2- Alexa488 binds faintly to the GUV surface such as to give a binding signal within the same range as the Alexa488 noise. We could then compare in a quantitative manner Arf-OG and ALPS1-ALPS2 for their sensitivity to membrane curvature (Figure 3C). This comparison better illustrates the exquisite sensitivity of ALPS1-ALPS2 to membrane curvature, which, despite being underestimated, contrasts with the permissive behavior of Arf1-OG. The point at zero curvature indeed corresponds to values on the GUV. This is indicated in the legend.

7. Figure 3, right hand panels, and supplementary figures 3 and 4. The authors should use symbols rather than colored circles for the data points, or at least change the colors to something other than red and green. The use of these colors, which correspond to the fluorescence signals shown to the left, might give the impression that the red circles refer to lipids and the green to the Arf1 or ALPS signal. In Figure 3A, center panels, indicate "Tube" and "Guv" as in the Figure 3B, and in the left panel show with dotted white lines where the fluorescence plots are taken from.

Yes it was not appropriate to use colors corresponding to the lipid and protein probes. Different colors have been used in the new figures. We have also indicated the size of the tube in the center panels of Figure 3A. For the quantification of the fluorescence signals in the ALPS1-ALPS2 experiment, we did not consider a line but a rectangle area (illustrated in the figure): this is better explained in the method section: “Igreen and Ired were measured from a rectangular region of interest that included the horizontal tube. These 2-D data sets were transformed into a 1-D array by averaging along vertical lines, leading to a strong increase in the signal-to-noise ratio.”

8. Page 10, experiments shown in Figure 4. The diameter of the tube should be indicated in the text, ie. "...a membrane tube of 10-15 nm radius was pulled from a GUV...". It should also be mentioned that imaging takes place at a time point after the system has reached equilibrium.

We agree. It is now written: “At each step and after equilibration, a confocal image was taken along the tube to visualize the fluorescent lipid and the bound protein” (p7).
“…were transferred to a micromanipulation chamber containing 10 nM ArfGAP1 and a membrane tube (radius 10 – 15 nm) was pulled from a GUV by optical tweezers (p10)”.

9. The term "vesicle" alone is generally used to refer to small-diameter, highly curved membrane structures. In cells, transport vesicles have the same diameter as the tubes pulled out from GUVs in this study. Therefore it could be confusing to some readers when the authors use the term "vesicle" to refer to the low curvature GUV. Instances I noticed are in the abstract, line 6 and results, page 6, second paragraph. Either "GUV" or "giant vesicle" should be used systematically in this context, throughout the manuscript.

We agree. The terms giant vesicle or GUV are now systematically used.

10. Figure 3, legend, line 14. When the authors state "Same as in (A) in the presence of 0.5 uM

Arf1-OG...", it implies that ALPS1+ALPS2 is present. Should be "Same as in (A) except that ALPS1+ALPS2 was replaced with 0.5 uM Arf1-OG...".

OK

11. There are several English grammatical errors throughout the manuscript, which may mask the meaning of the ideas being presented (a few instances are listed below).
a. Page 3, second paragraph. "...that allow coupling the GAP activity with the curvature of the underneath membrane" should be "...that allow coupling OF the GAP activity with the curvature of the UNDERLYING membrane". (See also two lines from bottom of page.)
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c. Page 7, second paragraph. From: "The radius of the tubes pulled from GUVs by molecular motors can hardly be controlled and modified. To circumvent this, we used..." to: "The radius of the tubes pulled from GUVs by molecular motors cannot be readily controlled or modified. To circumvent this limitation, we used...".

Thank you for pointing out these grammatical errors.

Response to reviewer 2

Antonny and colleagues investigate by biophysical methods the behavior of the small GTPase Arf1, a key component and regulator of COPI vesicles, and ArfGAP1 in liposomal systems. As expected from previous work, in a continuous liposomal system ArfGAP1 distributes to strongly curved areas, whereas ArfGTP does not show such a marked preference. Therefore it is not surprising that in such a system gradients arise of ArfGTP when limiting amounts of ArfGAP1 are present. The system would allow determining the diffusion coefficient of ArfGTP in a liposomal model bilayer, and the authors estimate that a diffusion rate of 1% of the diffusion observed in the liposomal system would suffice to keep a coated COPI bud in a meta-stabile state. The experiments are technically sound and well controlled. The problem is that the data does not really add new aspects to our present concepts of vesicle biogenesis, that in part come from the groups coauthoring this man. Therefore the question is whether the advance in knowledge presented in the manuscript would warrant publication in the EMBO-Journal, and whether the data should be published in a journal more specialized in Biophysics.

To our knowledge this is the first reconstitution of a biochemical reaction between two proteins on a continuous membrane with flat and curved regions. Our study highlights the importance of Arf1 diffusion, something that was not really appreciated in the past when we and other groups proposed models for COPI vesicle formation. Our study also demonstrates the contrasting behavior of Arf and ArfGAP1 as regards to membrane curvature. This clarification is important after the confusing report by Lundmark. We think that these 3 points are the main merits of this study.
On the other hand, we agree that we are far from understanding protein dynamics during vesicle formation. However this is a general issue in the field because of the lack of satisfactory experimental strategy exists to study with good time (sub second) and size (≈ 10 nm) resolutions biochemical reactions on membranes.

Response to reviewer 3

In the manuscript "ArfGAP1 generates an Arf1 gradient on continuous lipid membranes displaying flat and curved regions" by Ambroggio et. al., the authors elegantly show how the lipid-curvature preference of ArfGAP1 creates a gradient of lipid-bound Arf1-GTP on tubules emanating from a large GUV. This gradient could explain how buds remain stable until after the vesicle has been clipped from the parent membrane. The work is novel and compelling and is sufficient for publication in EMBO journal.

Minor comments:

1. How confident are the authors in their assignment of a curvature threshold of 35 nm for the Alps1-Alps2 motifs of ArfGAP1? In other words, what is the error in this measurement?

We did not vary the radius of the tube in a continuous manner but in a stepwise manner and therefore we did not achieve a resolution better than a few nm within a single experiment. However the experiment was repeated 8 times using different GUVs. All binding curves show a similar binary shape, with almost no binding of ALPS1-ALPS2 below R = 30 - 40 nm and then linear increase in binding. From these 8 independent experiments we obtained a mean value of 36 nm for the threshold and the corresponding Standard Deviation was ± 5 nm. Three representative experiments are shown in Fig 3A.

2. In general, the manuscript is very well written. However, it could benefit from proofreading as there are minor mistakes in the English language usage. For example, this sentence from the introduction "ArfGAP1 and Gcs1p contain motifs named ALPs that allow coupling the GAP activity with the curvature of the underneath membrane" would read better as "ArfGAP1 and Gcs1p contain motifs named ALPs that allow coupling of GAP activity to the curvature of the underlying membrane.

Thank you for pointing these grammatical errors.

2nd Editorial Decision 22 October 2009

Many thanks for submitting the revised version of your manuscript EMBOJ-2009-72198. Having carefully read your response letter and your revised manuscript, I find that you have satisfactorily addressed the relatively minor concerns of referees 1 and 3.

I therefore do not deem it necessary to send your manuscript back out for re-review, and I am pleased to be able to tell you that we should now be able to accept it without further modification.

You should receive the formal acceptance email shortly.

Yours sincerely,
Editor
The EMBO Journal

