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Sec24p and Sec16p cooperate to regulate the GTP cycle of the COPII coat.

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1st Editorial Decision 13 July 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. Three referees have now evaluated it, and their comments are shown below. As you will see, while all three referees consider the study as interesting, it becomes clear that more work will be needed before they support publication of the study here. I will not repeat all individual points here, but apart from certain issues with the conclusiveness of the data there are essentially two further issues that will need to be addressed. First, the mechanistic link between Sec16 and Sec24 needs to be strengthened at the molecular level, including an analysis of the effect of bona fide cargo, along the lines pointed out by all three referees. Second, as also mentioned by referee 1, the manuscript is extremely hard to follow for a non-specialist reader. I therefore need to urge you to make a major effort and re-write the manuscript, maybe with the help of a colleague who does not work directly in the field. Taking together all these points, we should therefore be able to consider a revised version of the manuscript, in which these points are addressed in an adequate manner and to the full satisfaction of the referees. Please do not hesitate to get back to me in case you would like to discuss any aspect of the revision further.

I should add that it is EMBO Journal policy to allow only a single round of revision, and 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.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1

This article describes two mutations in genes encoding two COPII subunits that affect the COPII coat assembly/disassembly. I was very much looking forward to reading the manuscript, but I am disappointed mostly by its lack of clarity of the text that somehow masks the biology.
Furthermore, even though I am sure that something mechanistically interesting is present in the description of these two mutations and their potential link, as far as the latter is concern, I have not found it in the present manuscript. Last, some of the experiments presented are not convincing enough to draw some of the conclusions of the paper.

1) The first mutation is the m11 mutation in Sec24 that leads to an overproliferation of ER presumably because the size of the COPII vesicles produced is smaller with m11-sec24 than in presence of WT Sec24. In the presence of this mutation, budding is not inhibited when the in vitro reaction is driven by GMP-PNP (one round of budding) but about 50% inhibited when driven by GTP. It is unclear that this 50% is solely accounted for by the smaller size of the vesicles (this is a lovely result) or whether there is also less vesicle budded altogether. This could be tested.

As a minor comment, on page 5, I would put the part starting " We note...budding" before the description that it is not substrate specific.

2) A genetic interaction between Sec24 and sed4, a mysterious protein with no assigned function, is then detected but not explained. With only Sec24-m11 as a source of Sec24, Sed4 is required. This is potentially interesting but in the absence of a role for Sed4, it remains a genetic interaction.
I think the interaction with Sed4 is worth examining further since it has such a clear and specific to m11 mutation, phenotype in vivo.
Furthermore, how the GTPase activity is modified when Sed4 is included in the assay should be tested. It could be that sed4 directly or indirectly hides or occupies the Sec16 N-terminus (see below 6.3).

3) In a third part of the manuscript, the authors embark on unraveling the relationship between the Sec24-

m11 mutant and Sec16. Sec16 is a candidate mostly because the in vitro budding criteria (no inhibition by GMP-PNP but by GTP) corresponds to a Sec16 free budding reaction and a genetic interaction between Sec16-2 and Sec24-m11 genetically interact (see their page 9).
This part is very hard to understand and I wonder whether there would not be a clearer way to bring the message about.
-Sec23/24 stimulates the GTPase activity of Sar1 in vitro as reported before.
-When Sec16DN is added to the reaction, this stimulation is decreased. And this inhibition needs the presence of Sec13/31.

If the conclusion were that Sec24 binding to Sec16 (on the central domain) somehow slows the GTPase activity of Sar1 and in turn regulates the COPII coat binding and size of vesicle, it would be lovely.
It would then follow that when Sec24-m11 is introduced in the reaction, it does not bind anymore to Sec16 to the same extent and the inhibitory effect would not be enacted, so that GTP hydrolysis by Sar1 would not prevent COPII disassembly. This would explain the small size of the COPII coated vesicles.

However, this is not the case. Full length Sec16 does not inhibit the Sec23/24 stimulated GTPase activity of Sar1 (as shown by Supek). Only Sec16DN has this inhibitory effect and frankly, I do not see in this paper how this would work.
My tentative explanation that full length Sec16 also exerts this slowing effect, is to postulate a mechanism that hides Sec16 N-terminus, thus mimicking a Sec16DN situation. In vivo, this mechanism would be of course be modulated. "Hiding" the N-terminus would slow down the GTP hydrolysis by Sar1 and allow proper size vesicles to bud.

4) What could do this hiding?
Since Sec16DN binding to liposomes recruits Sec23/24-m11 but less of Sec31 (fig6B), Sec31 could perhaps be the mechanism that modulates Sec16 N-terminus accessibility? So, Sec31 binding to Sec16-Nterminus would leads to an inhibition of the Sec24/23 stimulation of the Sar1 GTPase activity.

5) Now what happens with Sec24-m11?
When Sec24-m11 is present, it would somehow prevent Sec31 binding to Sec16Nter. In turn, it would be exposed and would not lead to the inhibition of the Sec23/24 stimulation of the GTPase activity.
I guess part of it is what it shown in Figure 6C and D, but In C and D the gels looks different. With WT Sec24, the Sec31 band is tiny and it is not clear whether the intensity really changes in the different conditions (at least it is now quantified).
With Sec24m11 the Sec31 band is stronger but it does not seems to go down when Sec16DN is introduced in the reaction (except at the highest concentration).
Why are the gels so different and what is the conclusion of this experiment?

This is all speculation, I realize, but I do not find in the manuscript experiments that help me understand the link between Sec24-m11 and Sec16DN.

6) Perhaps some of the key could be in extending Figure 5A.

6.1. Is Sec24-m11/Sec23 able to stimulate Sar1 GTPase activity as the WT complex using the same technique as in Fig5A.
Here we need a 4 panels-figure presenting results of experiments with WT Sec23/24 and WT Sec16, WT Sec23/24 and Sec16 DN (as shown), Sec24-m11/Sec23 and WT Sec16 and Sec24-m11/Sec23 and Sec16-DN.

6.2. Is Fig5C derived from measurements similar to 5A.
In 5C, what does COPII means? Is it purified COPII with WT Sec16?

7) Can the authors check what the Sec16 N terminus binds to, at least Sec31 and Sec13? How is the binding modulate by Sec24 and Sec24-m11. Could Sed4 be binding to N terminus?

8) Further comments

8.1 Can some of this be shown in vivo? The liposome assay is powerful to a certain extent but what is the effect of Sec24-m11 combined to Sec16DN in vivo?

8.2 The model as drawn is very confusing and does not seem to recapitulate what the text says.
For instance, Sec13 has been show to interact with Sec16 and that is not taken into account in the model. I did not really get what competes with Sec16 for binding to Sec31 Also the title of this section is cryptic. Sec16p competes for Sec13/31 binding but compete with what?

8.3 What is Sec16-2 mutant should be explained.

8.4 Was Sec16 picked up in the initial synthetic dosage lethality screen?

8.5 For the experiments in fig.4. Could they use a mutant Sec23 that does not bind to sec16? In that case the recruitment of Sec23/24 to the PC/PE liposomes would be only a result of Sec24 interaction with Sec16 in the presence of Sar1. Have the authors tried not including Sec23 at all in the assay?

8.6 I think that showing Sec24 interactions with small domains of Sec16 by Y2H is a bit weak if not shown otherwise..

8.7 Fig 4C. There is a clear difference between lanes 5 and 6, suggesting that Sec24-m11/sec23 might be more recruited that Sec24/23. Havinf said this, the labes are so faint that it is difficult to really be sure.

8.8 I could hardly see the changes that are described in the western blots (Fig.6A). How was the quantification done?
In Fig.6D there is no loading to compare the lanes. What about Sar1?

9) In conclusion, the paper needs more experimental data to test a possible mechanism that is drawn from the present results. I am not sure that it is feasible but a firmer link between Sec16DN and Sec24-m11 needs to be established. Also some of the experiments should be strengthened and further quantified.

The paper would also benefit of less dryness and more words to make the story more palatable for the broad readership of EMBO J. Not every reader is an expert in yeast genetics and COPII biology. The assay needs to be better described in fitting predictions or hypothesis that are presented and the jargon should be removed. This would definitely add to the clarity.

Referee #2

The findings reported in the paper are potentially interesting and could help reveal the significance of Sec16 in protein secretion. It is known that the binding of Sec31 to Sec24 controls the rate of rate of Sar1-GTP hydrolysis. In this paper the author report that Sec24-M11 does not bind Sec16 and has faster kinetics of Sar1-GTP hydrolysis. The coats dissociate prematurely, and the vesicles generated as a result are smaller in size. In principle, this scheme could help reveal the mechanism of Sec16 dependent size regulation of COPII vesicle. However, the authors should address the following concerns to validate the significance of their proposal in intact cells.

1. Does the expression of aa 565-1235 of Sec16 affect the size of COPII vesicle in intact cells. Does it affect the kinetics of protein secretion? By the way, is Sec24 M-11 localized to the ER exit site?

2. Figure 4C. The addition of Sec24M-11 affects the level of Sar1p recruitment to the liposomes (compare lane 5 vs 6 ; 7 vs 8). Is this important? Could this perturb the overall GTP levels being monitored?

3. Figure 5/ supplementary S3A. Why does the wild type Sec16 not affect the Sar1-GTP hydrolysis? This is troubling and should be resolved.

4. Figure 6C does not make sense. The authors show that addition of Sec16DN inhibits the recruitment of Sec31 in a concentration dependent manner. However, there is no recruitment of Sec31 to sec24 even at the lowest concentration of Sec16DN (compare 11 with 33 and 55).

5. Figure 6D. Why does Sec16DN inhibit the recruitment of Sec31 to Sec24-M11? I don't want to nit-pick but why does Sec31 appear as a doublet in 6C and not in 6D?

Minor issue.

Figure 1C and D. Please explain the growth conditions of the temperature sensitive cells. The cells are dead at 38{degree sign}C, the pulse chase is performed at 37{degree sign}C but is the ER morphology perturbed only at 37{degree sign}C? In the budding assays (Figure 2) the authors should also describe the incubation temperature.

Referee #3

This article contains considerable fascinating information relating to the mechanism of the GTPase cycle of the COPII coat. The paper is beautifully described within the abstract itself which highlights nicely the important findings of the paper in relation to the inter-relationship of Sec24 and Sec16 during COPII assembly.

The data are convincing and well controlled as well as being nicely presented. The data in figures 1 & 2 are very convincing indeed and provide clear data building on previous assays largely developed with the Schekman lab. The data in figure 3 provide a good distinction between cargo-binding functions of Sec24 and general COPII function.

There is only one area where I would have expected to see further experimental development. The model of cargo priming described on page 13 (& Fig 7) suggests that Sec16 might interact more tightly with Sec24 when cargo is bound. Since this is clearly central to the model I would have expected this to be tested experimentally using bona fide secretory cargo proteins (not just SNAREs). In addition, the lack of effect of Bet1p and Sec4p reported as unpublished perhaps even argues against this model. Consequently I would expect explanations to be given equal weight.

Figure 5B seems to be from a single experiment. The importance of these data requires at least 3 repeats and statistical validation (essential since these data could be used by others for mathematical modelling).

The data relating to Sed4p are interesting but some clarification is required over the presence (or otherwise) of Sed4p in other species, notably metazoans. To my knowledge Sed4p is not found in many organisms and so the relevance of these data (while clearly of high significance to the COPII pathway in S. cerevisiae).

The numbering of residues is useful but should be extended to the schematic figure 7. Also - how does the region 565-1235 relate to the ACE described in terms of structure?

One point of confusion that perhaps merits further discussion by the authors relates to Figure 5A. If Sec16 is functionally coupled to COPII through the M11 site then what is the functional relevance of the other interaction sites with other COPII subunits? While this is not something I expect to be tested experimentally, further discussion would be welcome here.

A sentence on Page 11 states that "....Sec16p competes for interaction with Sec23p, effectively displacing

Sec31p...". I am somewhat confused by the context of the sentence which goes to the heart of the paper. I don;t entirely follow how this relates to 7A and suggest that this section might be written in clearer, shorter sentences (notably for the benefit of the more general reader).

I have nothing other than minor comments in relation to this work and recommend publication.

If at all possible, the supplemental data should be included in the main figures.

Page 12 - the authors refer to TFG-1 as an additional regulator of COPII function yet my reading fo the recent Witte et al paper suggests that it is required for correct assembly of COPII i.e. it is more than a regulator, perhaps a key component. This should be clarified more clearly.

Typographical errors:
Intro line 3; resident what?
line 8: year missing in ref

Additional correspondence (author) 18 July 2011

Many thanks for your email and the handling of our manuscript. The reviews clearly demand additional experimentation, some of which is feasible and some of which is beyond the current scope of the manuscript. Before we embark on a significant investment of time and effort, I would definitely appreciate some input from you as to the level of your enthusiasm for our work and the degree of additional data that you imagine would be acceptable to at least some of the reviewers.

Clearly, the manuscript needs significant rewriting, and this will probably also entail a refocusing of the findings to the role of the GTPase cycle of the coat in governing vesicle scission from the ER membrane, which is an important finding and the most well-supported by our data. In addition to the EM already included in the first submission, we also have fluorescence loss in photobleaching experiments that show a significant difference in the lifetime of ER exit sites in wt vs. sec24-m11 mutants (the lifetime is lengthened in the mutants, consistent with inefficient release of small vesicles leading to a somewhat more stable exit site).

We agree that the Sed4 part of the story is underdeveloped and are happy to remove it since it seems to muddy the waters.

Unfortunately, two of the major criticisms of the reviewers are currently beyond our ability to characterize to molecular detail. We have been unable to detect a defect in direct interaction between Sec24 and Sec16 in the presence of the m11 mutant, but this is consistent with published data that suggest Sec16 as a multi-valent binder of the COPII coat. We are currently exploring the astute observation of reviewer 2 that the mutant form of Sec24 binds liposomes and/or Sar1 more efficiently than wt to dissect the potential for this to be functionally relevant. At this stage, further dissection of Sec16 and its interactions is beyond our capability.

Next is the issue of cargo in this process. Clearly we believe this is fundamental and was a major focus of the original submission largely because of the potential for this level of regulation more than concrete evidence. We have searched for a role for the "usual suspects" but saw no effect - of course, absence of evidence isn't evidence of absence (or something like that), so it could be that we just don't have the conditions right. I imagine that with a significant rewriting and a de-emphasis on the cargo aspect, this will be less of an issue.

Of course, I understand completely that you are not in a position to know the minds of the reviewers as to what would be acceptable or not, but given your experience I would appreciate hearing your advice. I still firmly believe that our findings are highly significant and shed important light on the poorly understood

role of Sec16 in vesicle formation, the molecular function of GTP hydrolysis by the coat and the surprising role for Sec24 in that process.

Additional correspondence (editor) 05 August 2011

Thank you for your message asking for advice on your revision. Let me first of all apologise for the long delay in getting back to you with a reply. I was on vacation and returned to the office only earlier this week. In the meantime I have now had a chance to look into the matter.

First, I would agree with you that refocusing the findings to the GTPase cycle and the role of Sec24 and Sec16 is a good idea and that it is thus OK to remove the Sed4 part from the paper.

Now, a key issue, in particular after refocusing the paper as you suggest, is that the mechanistic link between Sec16 and Sec24 as well as the effect of cargo needs to be understood better. Both points are not necessarily "nice to have" issues, but are required to make a stronger and more direct case for your model that when Sec24p engages cargo, its interaction with Sec16p attenuates GTPase activity, prolonging coat association with the membrane for vesicle budding, but that coat complexes disassemble rapidly (because of the high GTPase activity) when cargo isn't bound. The main significance and impact of the study depends on more direct evidence for this model. Now, I would not necessarily insist on all the specific experiments suggested by the referees, in particular if you see alternative, technically feasible ways to strengthen your point. Still, toning down these issues and keeping the study at the present level of mechanistic analysis will not be sufficient.

Obviously, the referees will need to see the study again, and I will specifically discuss the required depth of deeper mechanistic understanding with them at that point. Along these lines, I should point out that the study was rather difficult to follow in its original form and that some of the specific points raised by the referees may also be due to this caveat.

Taking together all these thoughts, I would suggest looking into how the core conclusions of the study and the model you are putting forward can be strengthened by more direct mechanistic evidence in addition to addressing the feasible points raised by the referees. Furthermore, major effort should go into re-writing the study in a more straightforward and digestible way. I will then look at the study again in depth, consult with the referees and take it from there.

Do these thoughts help?

Additional correspondence (author) 08 August 2011

Many thanks for your detailed comments - they help a lot. I think it's clear from your response that our current efforts to address the mechanistic holes in our study will likely not be sufficient to satisfy your requirements. To be blunt, we have tried from many different angles to substantiate the interactions (and disruption by mutation) between Sec16 and Sec24 but simply are unable to provide strong evidence of a clear effect. I firmly believe that this is because Sec16 is an incredibly complex protein that interacts with every other component of the COPII coat and that dissecting the details of how all of these interactions

interplay with each other functionally is beyond our current ability. This is, of course, disappointing to me and others in the field, since the function of this fundamentally important protein is so obscure. I had hoped that our finding of a GTPase regulation and an unexpected role for Sec24 in this regulation was a significant enough finding to warrant publication even without the complete dissection of the pathway.

Similarly, our work on the influence of cargo is clearly of fundamental importance and where we are focusing a lot of effort, but having tested our "best guesses" as to the likely cargo regulators (what cargo could be more important than the SNAREs required for fusion?), we are left looking for a needle in a haystack. We have taken a genetic approach to try to further understand the Sec24 mutation that we describe, and find in fact that additional mutations around the Sed5 binding pocket rescue the lethality of our mutant, suggesting that perhaps Sed5 is indeed involved. If this is true then our biochemical assays are clearly not reporting on this function, so further investigation is needed in order to fully understand how Sed5 might be acting. Again, I think this is a more long-term study to follow our current findings.

The one area where we have made nice progress is in understanding the dynamics of the coat on ER exit sites in the presence of our Sec24 mutation. Using FLIP to look at the lifetime of coat on exit sites we see a significant difference when our mutant form of Sec24 is the sole copy. We think this is reporting on the effect of increased GTPase activity, which we are currently trying to validate using other mutants that may also impact GTPase activity, and with Sar1 mutants that are defective in vesicle release (which we expect to reverse this change since heightened GTPase activity seems to cause premature scission). These findings complement well our new focus on the effect of GTPase cycle on vesicle formation using our knowledge of Sec16 and Sec24 to examine in vivo dynamics.

Since this line of experiments is outside of what you recommend we achieve (which practically is beyond us at the moment in terms of timely resubmission) I would love to hear whether you would still be interested in a revised manuscript along these lines. Of course, I completely understand if your interest only extends to the cargo link, which I agree is largely speculative at this stage.

I appreciate your input and look forward to hearing your thoughts.

Additional correspondence (editor) 11 August 2011

Thank you for your response explaining the situation in a bit more detail. Obviously, at the end of the day all will depend on how insightful the final version of the manuscript will be and how big a step forward the study will therefore provide. My suggestion therefore is to do the revision as well as you can. I will then evaluate the study again in depth at the editorial level and, if appropriate, involve our referees again. We will then take it from there. Given the caveats you point out, it is hard to predict the outcome of this new evaluation without actually seeing the final version. This procedure will allow us to evaluate the final version of the study in a fairer and more informed way.

I am looking forward to receiving your revision in due course.

Yours sincerely,

Editor
The EMBO Journal

Response to reviewers.

We thank the reviewers for their thoughtful and constructive comments. We have carefully considered these criticisms and have significantly amended the manuscript, revising for improved clarity and including additional experiments. We have refocused the manuscript to emphasize the importance of our findings to our understanding of the functional relevance of the GTPase cycle of the coat: previously GTPase activity on Sar1 was thought to simply govern coat assembly and disassembly, but this model is not fully supported by newer findings that implicate GTPase activity with vesicle scission and find increased coat stability even following GTP hydrolysis when cargo proteins are present. Our findings are highly significant in several ways: (1) we finally assign a molecular function to the enigmatic protein, Sec16 in inhibiting GTPase activity of the full COPII coat (albeit in a manner that is itself subject to regulation that remains to be dissected); (2) we demonstrate that Sec24 participates in this catalytic activity through mutation of a site that surprisingly impacts the ability of Sec16 to inhibit GTPase activity; (3) the effect of this altered GTPase modulation is the generation of smaller vesicles and more stable ER exit sites suggesting that a major function of GTPase activity of Sar1 is to govern vesicle release. In addition to these broad revisions, we have also addressed the specific comments:

Reviewer 1.

1) ... the m11 mutation in Sec24 that leads to an overproliferation of ER presumably because the size of the COPII vesicles produced is smaller with m11-sec24 than in presence of WT Sec24. In the presence of this mutation, budding is not inhibited when the in vitro reaction is driven by GMP-PNP (one round of budding) but about 50% inhibited when driven by GTP. It is unclear that this 50% is solely accounted for by the smaller size of the vesicles (this is a lovely result) or whether there is also less vesicle budded altogether. This could be tested.

Our interpretation is indeed that the reduction in vesicle size accounts for the diminished budding, although we cannot rule out a reduction in the number of vesicles released. This is actually not trivial to test: the best "inert" marker for release of vesicles is the lipid contained within the membrane, but this is generally not quantitative and may yield the same difficulties in interpretation as a reduction in cargo load. Simply counting the number of vesicles released is not possible since these are enriched in a high speed pellet prior to electron microscopy and only a small sample of the pellet processed for EM.

As a minor comment, on page 5, I would put the part starting " We note...budding" before the description that it is not substrate specific.

The ordering of this section has been rearranged to improve clarity as suggested by the reviewer.

2) A genetic interaction between Sec24 and Sed4, a mysterious protein with no assigned function, is then detected but not explained. With only Sec24-m11 as a source of Sec24, Sed4 is required. This is potentially interesting but in the absence of a role for Sed4, it remains a genetic interaction. I think the interaction with Sed4 is worth examining further since it has such a clear and specific to m11 mutation, phenotype in vivo. Furthermore, how the GTPase activity is modified when Sed4 is included in the assay should be tested. It could be that sed4 directly or indirectly hides or occupies the Sec16 N-terminus (see below 6.3).

We agree that the role of Sed4 in this process is interesting and warrants further investigation. However, we focused on pursuing the role of Sec16 as a top priority for a number of reasons. Firstly, Sec16 is conserved in higher eukaryotes yet remains enigmatic in terms of function despite its clear importance in trafficking and organization of ER export. Conversely, Sed4 is found only in budding yeast and thus is not likely to be

universal in its molecular action. Furthermore, the molecular function of Sed4 is complicated since different assays seem to yield different effects for Sed4 on the GTPase activity of Sar1. These contradictory results obtained for Sed4 function confuse the issue: initially, the cytoplasmic domain of Sed4 was shown to negatively regulate the GAP activity of Sec23 (Saito-Nakano et al., Genes Cells 2000) whereas a more recent study uncovered a GAP stimulating effect for the same domain (Kodera et al., Traffic 2011). We postulate that these conflicting results stem from the use of assays that don't include the "active" form of Sec16, and this is something that we are actively pursuing but that remains beyond the scope of this paper. Our rationale for not pursuing Sed4 and instead focusing on Sec16 is explained in some more detail in the revised manuscript (p. 8).

3) … the authors embark on unraveling the relationship between the Sec24-m11 mutant and Sec16. Sec16 is a candidate mostly because the in vitro budding criteria (no inhibition by GMP-PNP but by GTP) corresponds to a Sec16 free budding reaction and a genetic interaction between Sec16-2 and Sec24-m11 genetically interact (see their page 9). This part is very hard to understand and I wonder whether there would not be a clearer way to bring the message about.

This section of the manuscript has been reorganized to improve the flow of logic, which hopefully clarifies these issues. The genetic screening approach that identified a synthetic interaction between Sec24-m11 and Sed4 led us to test for a similar genetic interaction with Sec16 (which we found), which in turn led us to probe the potential catalytic role of Sec16 using in vitro assays. We first recapitulated published experiments that suggest full-length Sec16 has no impact on GTPase activity on Sar1, but then went on to dissect Sec16 into different functional domains, one of which (Sec16-ΔN) showed an inhibitory effect on the GTPase activity of the full coat (ie. Sar1/Sec23/Sec31). We then probed the mechanism for this activity (preventing recruitment of Sec31) and show that this effect is indeed diminished in the context of Sec24-m11.

If the conclusion were that Sec24 binding to Sec16 (on the central domain) somehow slows the GTPase activity of Sar1 and in turn regulates the COPII coat binding and size of vesicle, it would be lovely. It would then follow that when Sec24-m11 is introduced in the reaction, it does not bind anymore to Sec16 to the same extent and the inhibitory effect would not be enacted, so that GTP hydrolysis by Sar1 would not prevent COPII disassembly. This would explain the small size of the COPII coated vesicles. However, this is not the case. Full length Sec16 does not inhibit the Sec23/24 stimulated GTPase activity of Sar1 (as shown by Supek). Only Sec16DN has this inhibitory effect and frankly, I do not see in this paper how this would work. My tentative explanation that full length Sec16 also exerts this slowing effect, is to postulate a mechanism that hides Sec16 N-terminus, thus mimicking a Sec16DN situation. In vivo, this mechanism would be of course be modulated. "Hiding" the N-terminus would slow down the GTP hydrolysis by Sar1 and allow proper size vesicles to bud.

Indeed, this is exactly our model: we postulate that the N-terminal domain is autoinhibitory, preventing the GTPase inhibitory action of the central domain of Sec16. In vivo, this autoinhibition would be relieved by additional factors that may also be regulated, perhaps by phosphorylation of Sec16, as recently published by the Rabouille lab. This model is more clearly spelled out in the revised manuscript (pp. 9, 13).

4) What could do this hiding? Since Sec16DN binding to liposomes recruits Sec23/24-m11 but less of Sec31 (fig6B), Sec31 could perhaps be the mechanism that modulates Sec16 N-terminus accessibility? So, Sec31 binding to Sec16-Nterminus would leads to an inhibition of the Sec24/23 stimulation of the Sar1 GTPase activity.

We consider it unlikely that Sec31 itself releases the N-terminal domain since incubations that contain full-length Sec16 supplemented with Sec13/31 do not show the GTPase inhibition. One important point is that Sec31 does not rely on Sec16 to be recruited to the COPII coat: it is sufficient on its own to bind to Sar1/Sec23/Sec24. The effect of adding Sec16 (either full-length or the truncated form) is to recruit additional Sec23/24 (by virtue of the scaffolding function of Sec16) but in the case of Sec16-ΔN this increase in Sec23/24 fails to result in similar stimulation of Sec31 recruitment. Our tentative explanation is that Sec16-ΔN (ie. in the permissive conformation with the N-terminal autoinhibition relieved) binds to the same site on Sar1/Sec23 that Sec31 is recruited to and that this blocks binding of Sec31. Clearly, structural

information is required to gain full insight into the nature of these interactions, which are complex and difficult to dissect.

The question of what regulates the postulated autoinhibition of Sec16 is an important one, but is beyond the scope of this current study. We speculate that Sed4 itself may play this role, by virtue of its genetic and physical interactions (now spelled out more clearly on p. 8), but given the non-ubiquity of Sed4 and the recent identification of other potential Sec16 modulators (including TFG-1 and ERK-mediated phosphorylation), the field of candidate regulators is wide open and akin to looking for a needle in a haystack at this point.

5) Now what happens with Sec24-m11? When Sec24-m11 is present, it would somehow prevent Sec31 binding to Sec16Nter. In turn, it would be exposed and would not lead to the inhibition of the Sec23/24 stimulation of the GTPase activity. I guess part of it is what it shown in Figure 6C and D, but In C and D the gels looks different. With WT Sec24, the Sec31 band is tiny and it is not clear whether the intensity really changes in the different conditions (at least it is now quantified). With Sec24m11 the Sec31 band is stronger but it does not seems to go down when Sec16DN is introduced in the reaction (except at the highest concentration). Why are the gels so different and what is the conclusion of this experiment?

Figure 6 has now been simplified and reorganized to improve clarity and the flow of logic. We show a diminished effect of the Sec16-ΔN inhibition when Sec24-m11 is present, then go on to demonstrate that interaction of Sec24-m11 with full-length (autoinhibited) Sec16 is not impaired but that when smaller domains are dissected using yeast 2-hybrid analysis we see a dramatic reduction in interaction signal. This interaction has been incredibly difficult to dissect biochemically, despite enormous effort on our part. Sec16 is a very large and proteolytically sensitive protein that interacts with all components of the COPII coat via its various (and sometimes overlapping) domains. The gels previously included that examined this interaction on the surface of liposomes looked different because the experiments were done on separate occasions using different percentages of acrylamide in the SDS-PAGE. These gels have been removed to simplify the figure, however our conclusion stands that the Sec24-m11 mutant still retains some Sec16-ΔN interaction since we still see the diminished recruitment of Sec31 to liposomes in incubations containing both Sec16-ΔN and Sec24-m11 (former Fig. 6D, now stated as unpublished observations). Since Sec16 binds to all components of the coat, the significance of this recruitment is difficult to dissect, leading us to turn to the yeast 2-hybrid approach, which affords detection of interactions between smaller domains of the given proteins. We have repeated the yeast 2-hybrid experiments using 2 different independent systems and find the same result: diminished interaction between $Sec16_{565\text{-}1235}$ and Sec24-m11 as compared with wild-type Sec24. The important point here is that by 2 independent methods – GTPase activity and yeast 2-hybrid analysis – we see a clear effect of the Sec24-m11 mutation with respect to Sec16 function/interaction.

6) Perhaps some of the key could be in extending Figure 5A.
6.1. Is Sec24-m11/Sec23 able to stimulate Sar1 GTPase activity as the WT complex using the same technique as in Fig5A. Here we need a 4 panels-figure presenting results of experiments with WT Sec23/24 and WT Sec16, WT Sec23/24 and Sec16 DN (as shown), Sec24-m11/Sec23 and WT Sec16 and Sec24-m11/Sec23 and Sec16-DN.

This figure has been combined with the previous Figure 6 and simplified to improve clarity and the flow of logic. We have included some of the tryptophan fluorescence experiments that were previously in the supplemental figure and simplified some of the additional experiments that further explore the Sec16-ΔN-mediated effect on GTPase activity. We have not included the tryptophan fluorescence experiments for Sec24-m11 since these measurements are not quantitative, and instead we show radioactive GTPase assays that are quantitative and recapitulate the tryptophan fluorescence experiments, quantifying the GTPase inhibition by Sec16-ΔN and showing a clear effect of the Sec24-m11 mutation in diminishing the impact of Sec16-ΔN (but no effect on GTPase activity in the absence of Sec16-ΔN).

6.2. Is Fig5C derived from measurements similar to 5A. In 5C, what does COPII means? Is it purified COPII with WT Sec16?

Fig. 5C (Fig. 5E) is derived from a quantitative ^{33}P-GTPase assay; COPII refers to the full coat (Sar1/Sec23/Sec24/Sec13/Sec31). These points are spelled out more clearly in the figure legend.

7) Can the authors check what the Sec16 N terminus binds to, at least Sec31 and Sec13? How is the binding modulate by Sec24 and Sec24-m11. Could Sed4 be binding to N terminus?

We would love to be able to dissect these interactions and are working to develop tools and reagents to do these experiments. We are currently limited to yeast 2-hybrid analysis since the different Sec16 domains are difficult to express, are proteolytically sensitive and share overlapping binding specificities making discrete domains difficult to dissect. Our model is that the N-terminal domain folds back over the central domain to obscure the site of interaction with Sar1/Sec23. We are currently mutagenizing all the relevant components to further dissect these interactions, but at this stage these experiments are beyond the scope of this study.

8) Further comments
8.1 Can some of this be shown in vivo? The liposome assay is powerful to a certain extent but what is the effect of Sec24-m11 combined to Sec16DN in vivo?

We have addressed the in vivo consequences of the Sec24-m11 mutation in several ways. We used in vivo imaging to monitor the kinetics of turnover of GFP-Sec13 in wild-type and *sec24-m11* mutant cells. We show a significantly longer lifetime of Sec13 at ER exit sites in the mutant, consistent with less coat being released with each round of budding presumably as the result of the smaller vesicles being released. These data are included in Figure 6. We also tested the genetic consequences of overexpressing Sec16-ΔN in the *sec24-m11* mutant and found that the m11 mutation potentiates the toxicity of this active fragment. Since the active Sec16 fragment is somewhat toxic on its own, interpreting the synthetic lethality of this particular combination is difficult, other than to suggest that messing too much with the GTPase cycle of the coat is toxic to cells.

8.2 The model as drawn is very confusing and does not seem to recapitulate what the text says. For instance, Sec13 has been show to interact with Sec16 and that is not taken into account in the model. I did not really get what competes with Sec16 for binding to Sec31 Also the title of this section is cryptic. Sec16p competes for Sec13/31 binding but compete with what?

The model has been removed and the text clarified in terms of what we meant by "competing" interactions between Sec16, Sec23 and Sec31: we simply mean that the presence of Sec16 prevents binding of Sec31 to Sec23. This language should have been clearer and is hopefully remedied in the revised submission.

8.3 What is Sec16-2 mutant should be explained.

The *sec16-2* mutation is Leu1088Pro, and is now mentioned in the text (p. 8).

8.4 Was Sec16 picked up in the initial synthetic dosage lethality screen?

Sec16 was not identified in the original synthetic dosage lethality screen since it is an essential gene and thus not represented in the haploid deletion library that is the basis for the genome-wide screen (explained on p. 8).

8.5 For the experiments in fig.4. Could they use a mutant Sec23 that does not bind to sec16? In that case the recruitment of Sec23/24 to the PC/PE liposomes would be only a result of Sec24 interaction with Sec16 in the presence of Sar1. Have the authors tried not including Sec23 at all in the assay?

Such a mutant form of Sec23 is not known, although we are actively searching for mutations that perturb the Sec23-Sec16 interaction. We have tried a number of experiments (liposome binding and GTPase assays) using monomeric Sec23, but the quantification of these experiments is difficult since removing Sec24 from the coat diminishes the global recruitment/stability of the coat on the liposome and thus quantifying changes is difficult. Similarly, omitting Sec23 also causes a reduction in global coat stability

leaving dissection of individual binding events problematic.

8.6 I think that showing Sec24 interactions with small domains of Sec16 by Y2H is a bit weak if not shown otherwise..

We completely agree and have tried extensively to recapitulate these findings using purified proteins. As described above, Sec16 is notoriously difficult to work with due to its inherent proteolytic susceptibility and its propensity to interact with every relevant COPII coat protein, often via overlapping domains. For this reason we have been unable to fully explore the intricacies of the Sec24/Sec16 interaction in a simplified system and are instead pursuing additional genetic means to further dissect this interaction. That said, our data clearly support our contention that the Sec24-m11 mutant is defective in a Sec16-mediated GTPase event.

8.7 Fig 4C. There is a clear difference between lanes 5 and 6, suggesting that Sec24-m11/sec23 might be more recruited that Sec24/23. Havinf said this, the labes are so faint that it is difficult to really be sure.

This is an astute observation and one that we have also noticed. The significance of this increased recruitment to liposomes is not clear (and not entirely reproducible), but we have checked for an increased interaction with Sar1 (which was not observed) and an increase in GTPase activity (also not detected, see Fig. 5E). The fact that this phenomenon is not consistently observed leads us to question its importance/relevance.

8.8 I could hardly see the changes that are described in the western blots (Fig.6A). How was the quantification done? In Fig.6D there is no loading to compare the lanes. What about Sar1?

This figure has been modified to improve clarity. The gels shown are SYPRO-stained SDS-PAGE gels (not westerns) and quantification was done using a TYPHOON imager. These details have been added to the methods section.

9) In conclusion, the paper needs more experimental data to test a possible mechanism that is drawn from the present results. I am not sure that it is feasible but a firmer link between Sec16DN and Sec24-m11 needs to be established.

In the revised manuscript we have included additional data that support our model that the Sec24-m11 mutant causes an increase in the rate of GTP hydrolysis on Sar1, resulting in the release of smaller vesicles and increased residence at ER exit sites. We believe that these new additions, combined with our extensive re-writing of the manuscript support our model and clarify our findings.

Reviewer 2.

1. Does the expression of aa 565-1235 of Sec16 affect the size of COPII vesicle in intact cells. Does it affect the kinetics of protein secretion? By the way, is Sec24 M-11 localized to the ER exit site?

In living cells, COPII vesicles are consumed very rapidly so are not generally detected by electron microscopy. Imposing a fusion block allows vesicles to accumulate, but also rapidly induces secondary effects, making direct relationships difficult to discern. However, overexpression of $Sec16_{565-1295}$ has previously been shown to impair growth slightly (Espenshade et al., J. Cell Biol. 1995). We can recapitulate these results and show that overexpression of $Sec16_{565-1235}$ is mildly toxic in wild-type cells and more toxic in a *sec24-m11* strain. Overexpression of Sec16-ΔN is lethal in a sec24-m11 strain and moderately toxic in a wild-type strain, consistent with our domain dissection that suggests the 565-1235 fragment contains the bulk of the GAP-inhibitory activity whereas Sec16-ΔN contains additional additive or synergistic domains. Our conclusion from these genetic experiments is that messing too much with the GTPase cycle is toxic and that mutant analysis is too blunt a tool to fully dissect what is a complex in vivo balance. These findings could be included as supplementary data, but we hesitate since synthetic sick/lethal interactions can be difficult to dissect mechanistically and we are currently examining these in vivo effects more extensively.

2. Figure 4C. The addition of Sec24M-11 affects the level of Sar1p recruitment to the liposomes (compare lane 5 vs 6 ; 7 vs 8). Is this important? Could this perturb the overall GTP levels being monitored?

This is an astute observation and one that we have noticed on several occasions, although not always consistently. We have looked very carefully for evidence of an increased interaction between Sec24-m11 and Sar1 and have not observed one. Although it's true that we don't normalize our GTPase assay to account for Sar1 recruitment we don't believe the marginal enhancement of Sar1 binding to liposomes is a cause for concern.

3. Figure 5/ supplementary S3A. Why does the wild type Sec16 not affect the Sar1-GTP hydrolysis? This is troubling and should be resolved.

Our hypothesis is that full-length Sec16 is auto-inhibited, which is why previous attempts to assign a catalytic function for this protein were not successful. By identifying a smaller active fragment, we have discovered a molecular function for Sec16 and propose that additional factors that are present in the complex environment of the cell provide further layers of regulation in terms of relieving this autoinhibition. This model is spelled out more explicitly on p. 13.

4. Figure 6C does not make sense. The authors show that addition of Sec16DN inhibits the recruitment of Sec31 in a concentration dependent manner. However, there is no recruitment of Sec31 to sec24 even at the lowest concentration of Sec16DN (compare 11 with 33 and 55).

We agree that this figure was difficult to understand and we have removed it to improve clarity since it only recapitulated the findings shown in Figure 6B (now Figure 5C) using a different liposome mixture.

5. Figure 6D. Why does Sec16DN inhibit the recruitment of Sec31 to Sec24-M11? I don't want to nit-pick but why does Sec31 appear as a doublet in 6C and not in 6D?

We were also surprised by this result, but since Sec16-ΔN retains some GTPase inhibitory activity in the context of Sec24-m11 (new Fig. 5E) we postulate that this residual inhibition (now data not shown) perhaps explains the partial inhibition. Clearly, the mechanism of COPII regulation by Sec16 is somewhat complex and awaits additional dissection, most likely at the structural level. On a practical note, Sec31 is rather sensitive to proteolytic cleavage and we routinely see a slightly smaller cleavage product that varies in abundance from prep to prep. This cleaved product does not seem to correlate with any changes in observable function.

Minor issue: Figure 1C and D. Please explain the growth conditions of the temperature sensitive cells. The cells are dead at 38ºC, the pulse chase is performed at 37ºC but is the ER morphology perturbed only at 37ºC? In the budding assays (Figure 2) the authors should also describe the incubation temperature.

These details are now included in the relevant methods and figure legends. The difference in restrictive temperature is a quirk of subtly different strain backgrounds.

Reviewer 3.

The model of cargo priming described on page 13 (& Fig 7) suggests that Sec16 might interact more tightly with Sec24 when cargo is bound. Since this is clearly central to the model I would have expected this to be tested experimentally using bona fide secretory cargo proteins (not just SNAREs). In addition, the lack of effect of Bet1p and Sec4p reported as unpublished perhaps even argues against this model. Consequently I would expect explanations to be given equal weight.

Our reasoning in focusing initially on the SNAREs was based on the assumption that these proteins are in some ways the most essential primary cargoes, since each vesicle needs to bear these proteins (especially Bet1) in order to fuse with the Golgi. Indeed this was the initial premise of the "priming" hypothesis that implied the coat should build around the proteins that are needed for delivery rather than relying on

stochastic selection to ensure capture. On a more practical note, these proteins are also simple to express, purify and reconstitute into synthetic liposomes, making them readily assessable in our assays. However, a negative result with these proteins may simply mean that we haven't found the correct conditions that recapitulate their effect. Alternatively, "bona fide" cargo that transiently pass through the ER may correspond to the regulatory step. Searching for such effects is akin to searching for a needle in a haystack as a candidate-based approach and is beyond the scope of the current study. In our revised manuscript, we have de-emphasized the cargo to more accurately focus on the GTPase cycle of the coat and its relationship to vesicle size and coat stability and revised the discussion of cargo packaging to also take into account secretory cargoes, especially large cargoes that might want to regulate vesicle release (p. 14).

Figure 5B seems to be from a single experiment. The importance of these data requires at least 3 repeats and statistical validation (essential since these data could be used by others for mathematical modelling).

This figure has been moved to supplemental data since it largely recapitulates the tryptophan fluorescence findings. The more important data are shown in Figure 5E and are the result of at least 3 independent experiments with appropriate statistical validation.

The data relating to Sed4p are interesting but some clarification is required over the presence (or otherwise) of Sed4p in other species, notably metazoans. To my knowledge Sed4p is not found in many organisms and so the relevance of these data (while clearly of high significance to the COPII pathway in S. cerevisiae).

We have now included a brief discussion of the limited orthology of Sed4 and spell out that the non-ubiquity of this component was our underlying rationale for further pursuing Sec16 at a functional/mechanistic level (p. 8).

The numbering of residues is useful but should be extended to the schematic figure 7. Also - how does the region 565-1235 relate to the ACE described in terms of structure?

The model shown in Figure 7 has been removed since it seemed to complicate our study rather than illuminate it. We now include a more detailed description of the domain structure (p. 9). The functional significance of the Sec13/Sec16 interaction remains unclear since abrogation of the Sec16 blade insertion region has only marginal in vivo phenotypic costs.

One point of confusion that perhaps merits further discussion by the authors relates to Figure 5A. If Sec16 is functionally coupled to COPII through the M11 site then what is the functional relevance of the other interaction sites with other COPII subunits? While this is not something I expect to be tested experimentally, further discussion would be welcome here.

We propose that Sec16 has (at least) two functions: its "scaffold" function as a binding platform for recruitment of the coat to defined regions of the ER, and its role as a regulator of the GTPase cycle of the coat. This is made more explicit (p. 13). Sec16 interacts with Sec23, Sec13 and Sec31 in addition to Sec24. The central domain, which binds Sec24, Sec13 and Sec31, has most of the GTPase inhibitory activity whereas the C-terminal domain (that binds Sec23) has some residual activity. The mechanistic importance of the Sec13 interaction is not clear and is a complex issue given that the binding of Sec13 to Sec16 seems mutually exclusive with Sec13 binding to Sec31. Dissecting this complexity and deciphering the functional relevance of all these interactions is a challenging task beyond the scope of this paper but is addressed in the discussion (p. 13).

A sentence on Page 11 states that "....Sec16p competes for interaction with Sec23p, effectively displacing Sec31p...". I am somewhat confused by the context of the sentence which goes to the heart of the paper. I don;t entirely follow how this relates to 7A and suggest that this section might be written in clearer, shorter sentences (notably for the benefit of the more general reader).

We have amended and clarified this section, including removing the model. We have removed the confusing and imprecise "competition" language and now spell out that the presence of Sec16-ΔN, while

increasing recruitment of Sec23, precludes recruitment of Sec31 and that this failure to bind Sec31 is likely the cause of the observed decrease in GTPase activity (p. 10, 13)

Page 12 - the authors refer to TFG-1 as an additional regulator of COPII function yet my reading fo the recent Witte et al paper suggests that it is required for correct assembly of COPII i.e. it is more than a regulator, perhaps a key component. This should be clarified more clearly.

We have changed the wording of our description of TFG-1 and included reference to recent findings from the Rabouille lab on phosphorylation of Sec16 as a potential means of regulation (p. 13).

Typographical errors:
Intro line 3; resident what?
line 8: year missing in ref

These typographical errors have been corrected.

2nd Editorial Decision 10 November 2011

Thank you for sending us your revised manuscript. Our original referees have now seen it again, and you will be pleased to learn that in their view you have addressed all criticisms in a satisfactory manner and that the paper will now be publishable in The EMBO Journal.

Prior to formal acceptance, there are a number of editorial issues that need further attention:

* Please include an author contribution section and a conflict of interest statement into the main body of the manuscript text after the acknowledgement section.

* Please include the whole supplementary information (including the legends for the supplementary figures) into one merged Pdf file.

* Please include the number of independent repeats into the legend of figure 5D and a scale bar (and description) into supplementary figure S1D.

* We now encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. Would you be willing to provide files comprising the original, uncropped and unprocessed scans of all gels used in the figures? We would need 1 file per figure (which can be a composite of source data from several panels) in jpg, gif or PDF format, uploaded as "Source data files". The gels should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. These files will be published online with the article as a supplementary "Source Data". Providing such data is voluntary. Please let me know if you have any questions about this policy.

Thank you very much again for considering our journal for publication of your work.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

The authors have performed a large amount of work to clarify the manuscript both in term of presentation and experiments. Although there are still unclear issues, the message is now well presented in an understandable manner to non yeast geneticists.
I therefore support publication of this article in the EMBO Journal

Referee #2 (Remarks to the Author):

I am satisfied with the revisions. The paper is suitable for publication.

Referee #3 (Remarks to the Author):

The manuscript has been extensively revised and is in my opinion now suitable for publication in EMBO Journal. The improvements with regard to the structure and ordering are particularly welcome.

2nd Revision - authors' response 10 November 2011

This is great news. Thank you so much for handling our submission with such care. I'm thrilled that our work will be published in The EMBO Journal.

I've uploaded new files that include the changes and additions that you requested. I have opted to not upload source data.

3rd Editorial Decision 11 November 2011

Thank you for sending us your amended manuscript. After looking through everything, I realised that there are three minor points that need to be corrected before I can formally accept the manuscript:

* There is a discrepancy between the supplementary figure S1 and its legend: the figures has panels A-D, the legend describes A-C only.

* Please remove the supplementary figure legends from the main manuscript text.

* Figure 5D: two independent repeats (n=2) are not sufficient for statistical analysis such as the calculation of averages and error bars. One solution to this would be to choose a plot representation that shows individual dots for the two data points per condition. Another one would be to show one representative experiment and to state this in the figure legend. Another (the preferred) option would be to include one additional independent experiment (n=3).

I am sorry that I have to be insistent at this point. However, at this last step and prior to transfer of the paper to our production team, we need to make sure that everything is well in order.

Thank you for your kind cooperation.

Yours sincerely,

Editor
The EMBO Journal

3rd Revision - authors' response 11 November 2011

My apologies for not catching these things myself. I've amended the files as you requested and resubmitted them online. For Figure 5D I included an additional measure of band intensity from the experiment shown in 5C to allow the reader to see the raw data, before ratios were calculated.

