THE
EMBO
JOURNAL

Manuscript EMBO-2011-77747

Myosin-5, kinesin-1 and myosin-17 cooperate in secretion of fungal chitin synthase

Martin Schuster, Steffi Treitschke, Sreedhar Kilaru, Justin Molloy, Nicholas J Harmer and Gero Steinberg

Corresponding author: Gero Steinberg, University of Exeter

Review timeline:

Submission date:	29 March 2010
Editorial Decision:	31 March 2010
Resubmission:	30 March 2011
Editorial Decision:	18 May 2011
Revision received:	05 August 2011
Editorial Decision:	23 August 2011
Revision received:	02 September 2011
Accepted:	06 September 2011

Transaction Report:

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

1st Editorial Decision 31 March 2010

Thank you for submitting your manuscript for consideration by the EMBO Journal. I have now had a chance to read it carefully and to discuss it with my colleagues and I am sorry to say that we cannot offer to publish it.

We appreciate that you have characterised the myosin motor domain of the ustilago maydis Msc1 protein, a virulence associated factor that consists of an N terminal myosin head domain linked to a C terminal chitin synthase domain. Your data demonstrate that Mcs1-MMD associates with actin filaments, consistent with previous findings on related family members, but that it can not walk along filaments. Instead, Mcs1 seems to be important for tethering vesicles at the plasma membrane, analogous to what has been suggested for the unconventional myosin Vs. While we do recognise that this function has not been proposed for the myosin 17 family, and that very little is known about the molecular roles of these proteins, the downstream consequences of this activity - in terms of regulating fungal growth and/or pathogenesis - are not investigated in depth. Moreover, we find that the previous work demonstrating a similar function for myosin Vs somewhat limits the conceptual novelty here. Given these parallel concerns, I am sorry to say that we do not feel that your study is well suited to publication in the EMBO Journal, and we can not offer to consider it further here.

Please note that we publish only a small percentage of the many manuscripts that we receive at the EMBO Journal, and that the editors have been instructed to subject only those manuscripts to external review which are likely to receive enthusiastic responses from our reviewers and readers. I

© European Molecular Biology Organization

am sorry to have to disappoint you on this occasion, but I hope that this negative decision will not prevent you from considering the EMBO Journal for publication of future studies.

Yours sincerely,

Editor
The EMBO Journal

Additional Correspondence 19 July 2010

I recently submitted a manuscript on a myosin-chitin synthase, a fungal virulence factor in plant pathogens.
You expressed a principle interest, but had concerns about novelty of the tethering mechanism of Myosin-17 and asked for analysis of plant pathology of the mutants.
I was not able to include the latter, as it was already in revision at another journal, and consequently you reject the paper (EMBOJ-2010-74328).

We now have fully revised the story and have elucidated the mechanism by which secretory vesicles that carry myosin-chitin synthase are delivered and how in molecular detail the myosin-chitin synthase cooperate with the delivery mechanism.

By showing that the majority of the secretory vesicles (against all expectation) move bidirectional (involving myosin-5, kinesin-1 and dynein). This bi-directional motility of vesicles that are actually supposed to undergo unidirectional secretion at the cell tip provides a reason for the myosin-chitin synthase tethering activity:
The virulence factor plays a key role in exocytosis by imbalancing the bi-directional motility towards the growth region, thereby allowing polarised secretion.

You have rejected a previous version, a descision that I fully respect. However, this new story gives (A) the first details on a mechanism of secretion in filamtous (and in particular pathogenic) fungi and (B) the reason why fungi have invented the myosin-chitin synthase. We also found the same myosin-chitin synthase in Septoria tritici, where it also located at the hyphal tip in a microtubule dependent way, suggesting that the work is of general importance for plant pathogens. We thus have worked out the first fungal specific secretion pathway that is essential for fungal virulence. I am convinced that this story is suited for the Embo J, but before I format it for Embo J, I would like to know whether you are in principle willing to consider it. Of course I would respect any descision on this, either now or at later stages.

Additional Correspondence 20 July 2010

Many thanks for your correspondence regarding a possible resubmission of this manuscript. I have now had the chance to read your message and the new abstract. I have to say that it is not entirely clear to me what is new in your revised version - am I right in thinking that the main thing you have added is an in-depth characterisation of the movement of the secretory vesicles, showing that these vesicles are in fact moving bidirectionally?

In any case, I do recognise the interest at the moment in understanding how the balance between different motor activities regulates polarised transport, and can see that your manuscript may give additional insight into this question, as well as providing a detailed characterisation of the U. maydis Mcs1 protein. I would therefore suggest that you submit your full manuscript to us. Without being able to look at the study in full, I am a little reluctant to make a firm commitment to sending it out for in-depth review, but I would minimally seek advice from an expert editorial advisor in the field: this is usually a very quick process so shouldn't delay things too much. I would also ask you to upload the related manuscript file (Treitschke et al) when you submit - I'm sure you understand that potential referees will need to see this paper in order to assess yours.

© European Molecular Biology Organization

I hope this quick assessment is useful for you, and I look forward to receiving your revised manuscript.

Resubmission 30 March 2011

Here we investigate the delivery and secretion of a class V chitin synthase in the corn pathogen *Ustilago maydis*, which carries an N-terminal myosin 17 motor domain. In previous work we have shown that this motor domain is important for secretion but not for polar delivery of chitin synthase vesicles. However, the role of class V chitin synthase and the delivery mechanisms remained elusive.

In this paper we show that F-actin and microtubules form in dependent routes for secretion and that kinesin-1 and myosin-5 are motors for transport of class V chitin synthase. Surprisingly, there activity is counteracted by dynein, which results in most secretory vesicles not being exocytosed but rather moving back to the cell center. The activity of the myosin-17 motor domain is required for breaking the symmetry of bi-directional transport, thereby allowing polarized secretion, which is required for successful plant infection.

Class V chitin-synthases/myosin 17 motors are found in most filamentous fungi and are virulence factors in numerous human and plant pathogens. This work explains their crucial importance and describes a novel and fungal-specific secretion pathway. This promises new ways of controlling fungal infections.

This article was submitted following conversation with the Editor regarding a former version of the manuscript. Many thanks for considering our work.

2nd Editorial Decision 18 May 2011

Thank you for submitting your manuscript for consideration by the EMBO Journal. I'm really sorry for the delay in getting back to you with a decision: it took quite some time until we were able to find three appropriate referees, and there was also a delay until the third reviewer was able to return his/her reports. However, I do now have the comments from all three reviewers, which are enclosed below.

As you will see, the reviewers express interest in your work, but all also raise a number of major concerns that would need to be addressed before we can consider publication here. Their reports are explicit, so I see little need to go into detail here, but if you have any questions or comments about the revision, please don't hesitate to get in touch.

I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the 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 REPORTS

Referee #1 (Remarks to the Author):

The manuscript by Schuster et al uses a genetically amenable model (Ustilago) to investigate the cooperation between actin and microtubule cytoskeletons in delivering chitin synthase-Myosin17 to the fungal cell wall. This work follows several recent papers by the same group. The model system allows fluorescent labelling of specific motors combined with a simple linear geometry to address transport of vesicles. In general, it is a nice paper and a worthy contribution to the EMBO J. However, I have some concerns/comments which would need to be addressed before I can recommend publication.

1) In their earlier work (Schuster et al PNAS 2011), it was concluded that kin3 and dynein are the only motors responsible for motility of EEs towards hyphal tip, and therefore all the motion is on MTs. There was no mention of actin/myosin in EE motion. However, this paper talks about transport of CSVs, where no role for kin3 is found. This paper looks at the process of polarized secretion. The distinction between the two systems is not very clear. How are the authors sure that the cargo population in the these two papers have no overlap. This has to be made clear to the reader at the beginning of the manuscript

2) A major finding of the paper is that in addition to MTs, a peripheral actin route close to the cell wall is required for the CSVs. However, the authors report this just as an observation and do not go into the possible biological implications of this. Is it that the actin route also "leaks" chitin synthase to the cell walls as CSVs move along actin, with the majority of the chitin being delivered to the apical tip. To me, this makes sense because it would also be neccessary to replenish chitin all along the cell wall even though the secretion is polarized mainly to the tip. Infact, it may be possible that the MT pathway does the majority of long-range CSV delivery to the tip and the actin pathway delivers some to the tip, and a fair amount along the cell wall along the way.

Have the authors tried to look at such "leaky" delivery in their experiments ?? I strongly feel that this should be investigated and it presence/absence should be reported in this paper.

3) Why was benomyl used to depolymerize MTs ?? Usually nocadozole is the drug of choice.

Referee #2 (Remarks to the Author):

The manuscript from Schuster et al. addresses the mechanism by which the cell wall forming enzyme Mcs1 is delivered to the polar growth region of the pathogenic fungus Ustilago Maydis. It is a follow-up of an earlier paper of the Steinberg lab, which reported that the Myosin motor domain of Mcs1 is not needed for vesicle motility but required for virulence (Treitschke et al 2010). In that work, the authors proposed a model in which long-range motility was microtubule-dependent, whereas Mcs1 mediates short-range motility of vesicles within the hyphel apex. The current manuscript present evidence for a modified model, in which the actin-dependent motor Myo5 contributes to long-range transport independently of microtubule-based transport and in which the Myosin Motor domain of Msc1 anchors vesicles at the apex, rather than mediating short-range motility.

Overall, this is an interesting paper, which addresses some outstanding issues of the previous paper. For example, a role for MyoV in transport of (a subset of) CSVs is now convincingly demonstrated. Nevertheless, the key conclusion that the Myosin Motor domain of Mcs1 acts in vivo as a tether without any motor activity currently lacks sufficient support. More specifically, the following points need to be addressed.

Major comments:

1. Secretion
The data shown for stable membrane insertion of Mcs1 are not so strong. What happens with Mcs1 upon membrane insertion? Does it stay together in one patch or does it diffuse away? Figure 2D should show more frames after overlapping with Sso1 to illustrate Mcs1 fate. Similarly, Video 3 should run longer. Most kymographs also don't show stable insertion events. Or is secretion identified by sudden disappearances, such as the one near the kymograph bottom in Figure 2F?

2. Motility quantification
Currently, Figure 3 does not offer much insight that goes beyond Figure 7 of Treitschke et al. Therefore, the authors should add a quantification that discriminates between anterograde and retrograde runs. If the motor domain of Mcs1 indeed anchors CSVs to facilitate secretion, movement frequencies and/or run lengths in both directions should differ in control cells, but less so in deltaMM cells. Furthermore, a quantification of CSV fate similar to Figure 2E is needed for deltaMM cells (in Figure 3 or 7, see below).

3. Myo5-dependent CSV motility
Figure S5 shows an interesting result and should be moved to the main figures, because it presents evidence for different subsets of CSVs, decorated with different motors. Furthermore, I would encourage the authors to develop the Myo5 part a bit more, for example by adding dual-color time lapse data for Myo5 and CSVs to confirm that only a subset has Myo5. In addition, what is known about the actin polarity in the bundles? Is all motility unidirectional upon Benomyl treatment? Is the motility imaged independent of MT (Video S7) always unidirectional?

4. Mcs1 motor domain activity
A major conclusion of the manuscript is that the Myosin Motor domain of Msc1 is unlikely to be a walking motor, but rather contributes to CSV localization by tethering these particles at the apex. However, the DeltaMM has only a small effect on the apical pausing time (from 4 to 3 seconds), which argues against the central model of the manuscript. Instead, the effect of a rigor mutant on Msc1 membrane proximity seems much more striking. However, the model of the manuscript does not provide an explanation for Figure 7D. In my opinion, the best explanation for decreased membrane proximity of the rigor mutant would be a loss of motor activity, suggesting that in vivo the Myosin Motor domain of Mcs1 powers short range motility (in fact, as suggested by the yellow arrow in Figure 8).
To better understand how the motor domain activity contributes to secretion, the authors should quantify the fraction of CSV that get secreted for DeltaMM cells (similar to Figure 2E).

Minor comments:

1. The title is somewhat confusing, as Myosin-17 and chitin synthase both refer to the same cell-wall forming enzyme Mcs1.

2. What are the bright spots that appear in the actin labeling? Aggregates or some bona fide structure?

3. Figure 1D should also contain data for Benomyl treatment in control cells.

4. What happens near the red arrow in Figure 2F? Does the particle split in two?

5. p.7 - "Anterograde transport and subsequent fusion of these vesicles..." Replace "these" with "CSV"

Referee #3 (Remarks to the Author):

Schuster et al.,: " Myosin-5, kinesin-1 and myosin -17 cooperate in secretion of fungal chitin synthase"

This manuscript by Schuster et al., highlights the cooperation between microtubule and actin-based motor proteins during polarized delivery of chitin-synthase containing exocytic vesicles into the

apical growth zone of the fungus U. maydis. This enzyme is a multispanning transmembrane protein, which contains at its N-terminus the myosin-17 motor domain. In previous studies the authors have shown (Treitschke et al., 2010) that although myosin-17 motor activity is required for apical localization of the chitin-synthase and for correct cell wall formation, myosin-17 motor activity is not required for microtubule-dependent movement of the secretory vesicles (Treitschke et al., 2010). The current manuscript by Schuster et al. is a continuation of this previous work and now demonstrates convincingly that kinesin-1 and myosin-5 are required for anterograde transport, whereas dynein is important for retrograde movement.

This manuscript advances our understanding of the complex interplay between the different cytoskeletal motor proteins and is therefore of wide interest to readers working in vesicle transport. . The authors suggest a novel function for myosin-17 in the cell periphery in capturing exocytic vesicles and thereby interrupting bidirectional motility of these vesicles.
Overall the manuscript is very well written and a pleasure to read. The results are adequately documented and the experiments overall are well performed and controlled.

More specific points:

1. The authors only measure the rate of exocytosis in cells expressing the kinesin-1 mutant. However, invertase secretion for example should also be measured for the myosin-5, myosin-17 and the dynein mutants. In cells expressing the temperature-sensitive dynein mutant secretory vesicles accumulate in the apical growth region, a disruption of bi-directional transport has occurred. Is there an increase in secretion (> 15%) in cells expressing the mutant dynein, which is no longer able to remove the excess apically delivered exocytic vesicles? These experiments could support the author's conclusion that myosin-17 breaks the symmetry of bi-directional transport. Is there an increase in secretion in cells expressing the myosin-17 rigor mutant? Expression of the myosin-17 rigor mutant leads to a significantly extended residence time at the apical domain.

2. The authors perform in vitro characterization of the motor properties of myosin-17 using an in vitro motility assay. In this assay actin filaments are immobilized on the surface of coverslips and myosin-17 bound to vesicles added. Under these conditions the authors observed ATP-dependent binding of myosin-17, indicating that the motor domain is active, however, no translocation along actin filaments was observed. These are important experiments, however they are very difficult to do, especially with immobilized actin filaments and a membrane bound myosin. Therefore the authors should be very cautious with their interpretation. It might be premature to conclude that myosin-17 is indeed not walking along actin filaments, but only reversibly binds and therefore "docks" the secretory vesicles in the cell periphery. To support this conclusion more thorough experiments are needed. A positive control should be included that demonstrates that under the conditions they use coverslip-immobilised actin filaments can support translocation of a myosin with an expected step size similar to myosin-17.

3. Deletion of myosin-5 indicates that this myosin plays a major role in anterograde transport and the authors suggest that myosin-5 captures the secretory vesicles in the cell periphery (see discussion). How the role of myosin-5 different to myosin-17? Does myosin-5 work in parallel or subsequently to kinesin-1? Discuss what further experiments would answer these questions.

1st Revision - authors' response 05 August 2011

Reply to Referees

Referee #1

1) *In their earlier work (Schuster et al PNAS 2011), it was concluded that kin3 and dynein are the only motors responsible for motility of EEs towards hyphal tip, and therefore all the motion is on MTs. There was no mention of actin/myosin in EE motion. However, this paper talks about transport of CSVs, where no role for kin3 is found. This paper looks at the process of polarized secretion. The distinction between the two systems is not very clear. How are the authors sure that the cargo population in the these two papers have no overlap. This has to be made clear to the reader at the beginning of the manuscript.*

We apologise for not being clear in this point. Indeed, Mcs1 is endocytosed and we expect that the chitin synthase passes through the early endosomes. However, the point in this paper is the polar exocytosis of Mcs1, which we think is not based on early endosome motility. To make this clearer we have generated 2 new kinesin-3 motor mutant strains (kin3 null mutant and a Kin3rigor mutant, both described in Wedlich-Söldner et al., 2002). In both mutants the manipulation of the kinesin-3 motor (deletion or tight binding to the microtubule) abolishes transport of the endosomes. However, the formation of an apical G_3Mcs1 cap was not impaired. Furthermore, we used our newly developed quantitative chitin synthase secretion assay (see below) and show that fluorescent recovery of G_3Mcs1 after photo-bleaching is not impaired in both mutants. Thus, early endosomes (and kinesin-3) are not taking a significant part in delivery of the chitin synthase to the growth region. This is consistent with the finding that kinesin-1 is involved in G_3Mcs1 motility. These data are now included in a new Supplementary Figure S4 and early in the text (page 8).

2) A major finding of the paper is that in addition to MTs, a peripheral actin route close to the cell wall is required for the CSVs. However, the authors report this just as an observation and do not go into the possible biological implications of this. Is it that the actin route also "leaks" chitin synthase to the cell walls as CSVs move along actin, with the majority of the chitin being delivered to the apical tip. To me, this makes sense because it would also be necessary to replenish chitin all along the cell wall even though the secretion is polarized mainly to the tip. In fact, it may be possible that the MT pathway does the majority of long-range CSV delivery to the tip and the actin pathway delivers some to the tip, and a fair amount along the cell wall along the way.

Have the authors tried to look at such "leaky" delivery in their experiments ?? I strongly feel that this should be investigated and it presence/absence should be reported in this paper.

We wish to thank the referee for this brilliant idea! Indeed, we were so much engaged with the mechanistic details that we overlooked this important question. We now analysed the potential "leaking" (lateral insertion) of Mcs1 in a quantitative way. Using FRAP experiments, we indeed find a gradient of secretion, with ~55% occurring at the bud apical tip, ~30% at the side of the bud and ~16% at the mother cell. Thus, we think that the referee is correct in assuming that F-actin/myosin-5 distributes the chitin synthase along the lateral parts of the cell. The combination of the F-actin and the microtubule MT-dependent transport generates a flow to the tip that can not be balanced by dynein, thus leading to a polarization. The myosin-17 seems to help in this polarization mechanism. These data are included in this revised manuscript now (Figure 3G, 3H, page 8). The conclusion drawn is summarized on page 21 and in Figure 9.

3) Why was benomyl used to depolymerize MTs ?? Usually nocadozole is the drug of choice.

In fungal cells the fungicide benomyl is used to destroy the MTs. In contrast to nocodazole, which works but has irreversible effects on MTs, benomyl is well characterized. Therefore, we used benomyl in our studies. We previously analyzed the effect of benomyl on MTs in *Ustilago* (Fuchs et al. 2005, MBoC). This reference is now cited in the text (page 5).

Referee #2:

1. Secretion
The data shown for stable membrane insertion of Mcs1 are not so strong. What happens with Mcs1 upon membrane insertion? Does it stay together in one patch or does it diffuse away? Figure 2D should show more frames after overlapping with Sso1 to illustrate Mcs1 fate. Similarly, Video 3 should run longer. Most kymographs also don't show stable insertion events. Or is secretion identified by sudden disappearances, such as the one near the kymograph bottom in Figure 2F?

We apologise for not being clear in this point. Upon secretion, the G3Mcs1 signal remained largely immobile and only rarely lateral diffusion was seen. This suggested that the cortical F-actin cytoskeleton (and maybe the myosin-17 motor domain) participate in anchorage of secreted chitin synthase complexes. However, secreted signals remained stationary even in the absence of F-actin (after Latrunculin A treatment) or when the motor domain of Mcs1 was deleted (not shown). Thus, we consider it most likely that the secreted chitin synthase complex anchors to the extracellular cell wall. We have included these data in a new Supplementary Figure S3 and in the text (page 8).

© European Molecular Biology Organization

2. Motility quantification
Currently, Figure 3 does not offer much insight that goes beyond Figure 7 of Treitschke et al. Therefore, the authors should add a quantification that discriminates between anterograde and retrograde runs. If the motor domain of Mcs1 indeed anchors CSVs to facilitate secretion, movement frequencies and/or run lengths in both directions should differ in control cells, but less so in deltaMM cells. Furthermore, a quantification of CSV fate similar to Figure 2E is needed for deltaMM cells (in Figure 3 or 7, see below).

We thought about discriminating between anterograde and retrograde motility. However, due to bleaching events our experiments always favour anterograde motility (as retrograde can only be measured after a phase of anterograde and therefore less retrograde will be measured). Our main point, however, was that deltaMM cells do not differ from control cells. We agree that this is not much of advancement over previous data in Treitschke et al. 2010 (just that we measured in yeast-like cells now) and have, therefore, shifted the former Figure 3 to the Supplementary Figures (now Figure S6, mentioned on page 13).

To meet the second point of the referee, we quantified the CVS fate in deltaMM cells. Furthermore we applied our FRAP-based secretion assay. Consistent with the data on reduced pausing in deltaMM cells (but not complete absence), we found that secretion is not completely abolished, but significantly impaired. Thus, b Both experiments show that the MMD of Mcs1 is important for secretion, which is confirming our main point. These data are now included (Figure 8F, 8G; page 16)

3. Myo5-dependent CSV motility
Figure S5 shows an interesting result and should be moved to the main figures, because it presents evidence for different subsets of CSVs, decorated with different motors. Furthermore, I would encourage the authors to develop the Myo5 part a bit more, for example by adding dual-color time lapse data for Myo5 and CSVs to confirm that only a subset has Myo5. In addition, what is known about the actin polarity in the bundles? Is all motility unidirectional upon Benomyl treatment? Is the motility imaged independent of MT (Video S7) always unidirectional?

To meet the referees' request, we moved Figure S5 to the main text (now Figure 6).

To further strengthen the finding that Myo5 moves CSVs we co-imaged G3Myo5 and mCherry$_3$Mcs1. As said in the previous version this proved to be very difficult, as the mCherry$_3$Mcs1 signal was rapidly bleaching. However, we have added a new Movie S10 that shows co-migration of Mcs1 and Myo5 and therefore supports our case.

Nothing is known about the orientation of F-actin in *Ustilago*. To assess this question we analysed the motility of G$_3$Myo5 and found that ~98.3% of all these myosin signals very moving towards the growth region, suggesting that the plus-ends of the F-actin cables is located in the bud. We have included this finding in the text (page 11).

4. Mcs1 motor domain activity
A major conclusion of the manuscript is that the Myosin Motor domain of Msc1 is unlikely to be a walking motor, but rather contributes to CSV localization by tethering these particles at the apex. However, the DeltaMM has only a small effect on the apical pausing time (from 4 to 3 seconds), which argues against the central model of the manuscript. Instead, the effect of a rigor mutant on Msc1 membrane proximity seems much more striking. However, the model of the manuscript does not provide an explanation for Figure 7D. In my opinion, the best explanation for decreased membrane proximity of the rigor mutant would be a loss of motor activity, suggesting that in vivo the Myosin Motor domain of Mcs1 powers short range motility (in fact, as suggested by the yellow arrow in Figure 8).
To better understand how the motor domain activity contributes to secretion, the authors should quantify the fraction of CSV that get secreted for DeltaMM cells (similar to Figure 2E).

We appreciate the view of the referee that the motor domain of Mcs1 might support short range motility. While we have no positive data for such a function, we certainly cannot exclude such role. However, we consider an active transport unlikely because (1) we do not find in vitro motility (which, however, is a negative result that could have many reasons); (2) the sequence conservation of myosin-17 is very weak (it is almost an outgroup amongst the myosins); (3) introducing a rigor point mutation tightly binds Mcs1 to F-actin and therefore should reduce secretion. However, using the FRAP-based secretion assay we find

© European Molecular Biology Organization

a significant increase in secretion (*P*: 0.0051). We have included these data in a new Figure S4 and have mentioned the observations in the text (page 16 and 20).

Minor comments:

1. The title is somewhat confusing, as Myosin-17 and chitin synthase both refer to the same cell-wall forming enzyme Mcs1.

We appreciate this concern. However, we clarify the dual nature of Mcs1 as a class V chitin synthase AND a myosin-17 myosin in the abstract. Doing this in the titled seemed to be even more confusing and therefore not appropriate. We hope the referee accepts that we, therefore, preferred to stay with the previous title.

2. What are the bright spots that appear in the actin labeling? Aggregates or some bona fide structure?

These are fungal-specific sites of endocytosis. This is now mentioned in the text and a reference supporting this is included (page 5).

3. Figure 1D should also contain data for Benomyl treatment in control cells.

Benomyl data were added.

4. What happens near the red arrow in Figure 2F? Does the particle split in two?
Yes, we do think it does. This was a rare event, its physiological implication is not clear. We have not preceded this, but have added a note to the figure legend.

5. p.7 - "Anterograde transport and subsequent fusion of these vesicles..." Replace "these" with "CSV"

We have slightly rephrased the sentence.

Referee #3

1. The authors only measure the rate of exocytosis in cells expressing the kinesin-1 mutant. However, invertase secretion for example should also be measured for the myosin-5, myosin-17 and the dynein mutants.

We agree with the referee that the data provided are incomplete. In fact, when we thought about this secretion assay we came to the conclusion that providing invertase secretion data is actually missing the point. The paper starts with a morphogenic defects in cells that were defective in the cytoskeleton. Morphogenesis depends on cell wall strength which, in turn, is based on the activity of chitin synthases. Indeed, chitin synthase delivery and secretion is the main point of the paper. Therefore, it seemed most logical to address secretion of chitin synthases, rather than providing data on secretion of a metabolic enzyme (invertase).

To address this point we developed a FRAP (fluorescent recovery after photo-bleaching)-based chitin synthase secretion assay that measures the rate of insertion of chitin synthases into the plasma membrane after photo-bleaching of specific areas of the cell. Using this assay, we tested secretion of the polar chitin synthases Chs5, Chs6 and Mcs1 and show now that secretion of all three chitin synthases depends on microtubules and on F-actin. These secretion data are more coherent with the rest of the paper and therefore replace the invertase secretion results. These data are now included in a new Figure 2 and in the text (end of page 6, beginning page 7).

In cells expressing the temperature-sensitive dynein mutant secretory vesicles accumulate in the apical growth region, a disruption of bi-directional transport has occurred. Is there an increase in secretion (> 15%) in cells expressing the mutant dynein, which is no longer able to remove the excess apically delivered exocytic vesicles? These experiments could support the author's conclusion that myosin-17 breaks the symmetry of bi-directional transport. Is there an increase in secretion in cells expressing the

myosin-17 rigor mutant? Expression of the myosin-17 rigor mutant leads to a significantly extended residence time at the apical domain.

We wish to thank the referee for this brilliant suggestion. Using the FRAP-based secretion assay, we measured the secretion rate of G_3Mcs1^{rigor} and G_3Mcs1 in temperature-sensitive dynein mutants as requested. We found that in both cases secretion increased, which was found to be of high statistical significance in case of G_3Mcs1^{rigor}, (*P*: 0.0051). This is an interesting result, as the introduction of the rigor mutation is expected to immobilize Mcs1, thereby increasing the residence time. If myosin-17 acts as a short range motor, this mutation should decrease secretion, whereas if it acts as a tether one would expect an increase in secretion. We find such an increase, which supports one of our main points (myosin-17 is a tether). The new data have been included these data in a new Figure S4 and have mentioned the observations in the text (page 16 and 20).

2. The authors perform in vitro characterization of the motor properties of myosin-17 using an in vitro motility assay. In this assay actin filaments are immobilized on the surface of coverslips and myosin-17 bound to vesicles added. Under these conditions the authors observed ATP-dependent binding of myosin-17, indicating that the motor domain is active, however, no translocation along actin filaments was observed. These are important experiments, however they are very difficult to do, especially with immobilized actin filaments and a membrane bound myosin. Therefore the authors should be very cautious with their interpretation. It might be premature to conclude that myosin-17 is indeed not walking along actin filaments, but only reversibly binds and therefore "docks" the secretory vesicles in the cell periphery. To support this conclusion more thorough experiments are needed. A positive control should be included that demonstrates that under the conditions they use coverslip-immobilised actin filaments can support translocation of a myosin with an expected step size similar to myosin-17.

We extensively discussed this point amongst us. Indeed, myosin-5 was shown to move in these assays (Sakamoto et al. 2003, J. Biol. Chem.). However, the architecture of this motor is different (e.g. a much longer lever arm). In addition, our data suggest that myosin-17 activity is locally regulated and does not interfere with the myosin-5 delivery mechanism. Thus the absence of motility in our assays might reflect sub-optimal conditions under which the motor is very slow or not active at all. Considering all these uncertainties we thought it is most appropriate to "tone down" our conclusions. We have done this in the text (pages 15, 19, 20, 21). However, when taking all data together (the low sequence similarity, the increased secretion rate of $Mcs1^{rigor}$, the in vitro motility assays), we still feel that a tethering function of Mcs1 is the most likely scenario.

3. Deletion of myosin-5 indicates that this myosin plays a major role in anterograde transport and the authors suggest that myosin-5 captures the secretory vesicles in the cell periphery (see discussion). How the role of myosin-5 different to myosin-17? Does myosin-5 work in parallel or subsequently to kinesin-1? Discuss what further experiments would answer these questions.

We apologize for this confusion. In fact, in the previous manuscript we wrote the misleading sentence "this raises the question of how myosin-5 captures the CSVs at the cell periphery. Further studies are needed to provide insight into this loading process."
This was deleted from the revised manuscript.

Myosin-5 transports CSVs along peripheral actin cables to the cell tip, whereas kinesin-1 takes vesicles along MTs to the bud. Both therefore operate in parallel. Dynein takes vesicles backwards, and the question that we wanted to raise is "how does myosin-5 receive the vesicles from dynein" or better "does myosin-5 receive vesicles from the MT-based transport system". In this revised version this is stated more clearly (at the end of page 18 and 21).

3rd Editorial Decision 23 August 2011

Many thanks for submitting the revised version of your manuscript EMBOJ-2011-77747R. It has now been seen again by referees 1 and 2, whose comments are enclosed below. As you will see, referee 1 is now fully satisfied with the revision (I leave it up to you whether or not to include the acknowledgment to the 'anonymous referee'!), but referee 2 does have one remaining concern that I would ask you to address in a final revision of the manuscript: I hope this should not be too difficult.

From my side, I also need to ask you to include an Author Contributions statement (below the abstract) and to state clearly in the figure legends which statistical test was used to demonstrate statistical significance in the relevant panels.

Please let me know if you have any questions or comments about this revision; otherwise I look forward to receiving the final version.

REFEREE REPORTS

Referee #1 (Remarks to the Author):

I think the authors have improved the paper significantly in response to comments of all the referees. It should be ready for publication

In the interest of scientific ethics they should acknowledge an "anonymous referee" for suggesting the experiments to check whether chitin synthase is being leaked to the cell wall by an actin route as well. This is of course if EMBO J allows this.

Referee #2 (Remarks to the Author):

The authors have addressed most of my concerns in their revised version of the manuscript. Nevertheless, one important issue still remains and should be addressed appropriately.

I agree that observation of increased secretion with the rigor mutant would be evidence for an anchoring, rather than a motoring, role. Nevertheless, Figure S4C does not directly show evidence for such an increase.
In the original manuscript, and in the current Figure 8G, secretion rate is reported as number of insertions per um per 5 minutes. However, at various locations in the new manuscript, including Figure S4C, secretion rate is reported as the % recovery in 15 or 20 minutes, which does not directly reflect a secretion rate. First of all, the "15 or 20 minutes" is confusing, because in order to judge the value of Figure S4C, it is crucial that the time after bleaching is the same. More importantly, particles can just be stuck near the cell wall, without being secreted. In fact, Figure 8D shows that this is exactly what happens in the case of the rigor construct. Therefore, recovery of fluorescence does not solely arise from secretion and cannot be used to estimate or compare secretion rates. To test increased secretion for the rigor mutant, secretion needs to be analyzed as insertions per um per 5 minutes and added to Figure 8F.

Minor comment:
It would be helpful to add the control data (Figure 3F) as dotted lines to Figure 8F, just as was done in Figure 8G.

2nd Revision - authors' response 02 September 2011

We have addressed all reviewers' and your comments and have improved the manuscript according to their suggestion. The changes include:

(1) We have included an Author contribution statement and in the figure legends named the statistical tests used to determine significant difference between data sets.

(2) We have added a note to the Acknowledgement to express our appreciation for the referee's comments.

(3) We have tested for increased secretion for the rigor mutant by analyzing insertions per um per 5 minutes. The data fully confirm our previous statement and were added to Figure 8G.

(4) Control values were indicated by red dotted lines in Figure 8F.

© European Molecular Biology Organization