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Controlled and stochastic retention concentrates dynein at microtubule ends to keep endosomes on track

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

1st Editorial Decision 13 July 2010

Thank you for submitting your manuscript for consideration by the EMBO Journal. Please let me apologise once again for the long delay in getting back to you with a decision - I really do understand your frustration with this. In fact, we have still not heard back from referee 3, but at this point it is not clear whether he/she will return his/her report and I do not want to delay things any further. Therefore, I am taking a decision - to invite a revision of your manuscript - based on the two reports I already have to hand. Obviously, if and when the third review does arrive, I will forward it on to you, and may ask you address any concerns raised.

As you will see, both referees express interest in your work, although both - and referee 2 in particular - raise significant concerns that would need to be addressed in a revised version of the manuscript. Most critically, referee 2 questions your quantification of the number of dynein molecules present at the hyphal tips. Demonstrating that your quantification is reliable will be essential for eventual publication. Referee 1's point 1 is also a critical concern that would need to be addressed experimentally. Further, referee 2 comments extensively on the presentation of the manuscript; from an editorial point of view, a major re-write to better highlight the background to and the novelty of the study would be very important.

In the light of the referees' positive recommendations, I would therefore like to invite you to submit a revised version of the manuscript, addressing all the comments of both reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision. Acceptance of your manuscript will thus depend on the completeness of your responses included in the next, final version of the manuscript. 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 initiative, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as a standard revision time, and as a matter of policy, we do not consider any competing manuscripts published during this period as negatively impacting on 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. And once again, I really am sorry for the long delay with this manuscript.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

The manuscript by Schuster et al. uses photobleaching and live cell imaging to investigate the accumulation of dynein at microtubule plus ends in the model organism Ustilago. The authors generate a strain expressing fluorescently labeled dynein and compare this to an internal calibration standard to show that ~55 dynein motors are found in the comet tails at the end of hyphal tips. They use photoactivation/photobleaching approaches to show that there are two pools of dynein, one undergoing rapid turnover and one less dynamic. Pharmacological and inhibitor experiments suggest that the less dynamic dyneins are maintained by a mechanism involving phosphatase activity and EB1. Perturbation of the less dynamic pool leads to an increased "escape" of early endosomes from microtubule plus-ends.

This is a strong and interesting study that uses live imaging, quantitative approaches, and mathematical modeling to address a question of mechanistic interest in an experimentally tractable model organism. As such it makes an important contribution to our understanding of intracellular transport along microtubules. The quality of the data are generally high, and the analysis thoughtful and reasonable. The movies are in general very good, and movie 2 is beautiful.

I do have some specific points that the authors should address in order to improve the work and its impact:

1. The key experiments I was looking for to really support the authors' hypothesis is that when the stationary population is perturbed by either okadaic acid treatment or the dynactin or EB1 peptides, that they see the preferential loss of the stationary populations in the dynamic experiments - the photoactivation and photobleaching experiments.
2. The authors should more clearly state the extent of the region they consider to be a "microtubule end". Is this defined by EB1 comet tail labeling, or some other criteria.
3. The modeling is a strong and well-integrated aspect of the manuscript. However, the authors need to more clearly articulate the assumptions and parameters used for the modeling within the main text. For example, the authors should clarify their assumption that dynein bound at the plus end does not dissociate.
4. At the end of the introduction, the authors summarize their findings by stating that "dynein release occurs without activation". I think this is too strong - they could consider "dynein release can occur" or "usually occurs".
5. It is not clear to me what the authors mean by "traffic jam" of motors in the model they discuss in the Results section.
6. The authors state that "dynein signals left the tip in an apparently random fashion, with a wide range of inter-motor distances" and refer to Figure 4A. This conclusion is not readily apparent to me

from the figure. Similarly, the authors conclude from Figure 4B that the signals turn at various points, but again this is not so clear from the figure. Further analysis is provided in Figure 4C, but the explanation of this analysis was not so clear to me.
7. The authors refer to data not shown to establish that the paGFP3-Dyn2 construct is biologically functional. Unless there is a reason not to, these supporting data should be added to the manuscript.
8. In Figure 5A and movie 5, the intensity of the signal appears to decrease faster than the flux expected from the dynein leaving rate along microtubules. This suggests that the initial rapid loss is not due to dynein actively moving along microtubules, but instead is due to dynein detaching and diffusing away. Can the authors clarify?
9. In Fig. 6, are the authors expressing the wild type Dya1n peptide or the mutant? Please clarify.
10. Some figure panels are arranged in a convoluted manner that doesn't reflect their discussion in the text (ex. E above B and C in Fig. 5). Also, the figure legends are too minimal. More information in the legends would help the reader.

Overall, this is a potentially strong manuscript. Minor revisions would improve the clarity and presentation. Scientifically, the addition of data in regard to point 1 above would strengthen the authors' hypothesis.

Referee #2 (Remarks to the Author):

Schuster et al have investigated endosome transport in hyphal cells. They reason that the dynein-loading of endosomes for retrograde transport uses dynein from two pools at the hyphal tip -- a stochastically retained dynein poplulation and another actively population which is retained by an EB1-dynactin dependent active process.

In general, I think that this is a good paper. However, much of the message is obscured by unclear and lengthy writing. A major revision in the writing and organization is required to make the the message crisp and clearer. I am also not convinced about the basic assumption of ~55 dyneins at the tip (see below).

It would help a general reader to understand the role of motors in the relevant endosome biology of this system. What is the utility of endosomes coming to the tip and going back. Since most of the endosomes turn back rapidly, what is the purpose of this transport of endosomes to the hyphal tip ?? Something on this should be said in the Introduction.

Further specific comments:-

Page 4
"However, numerous recent theoretical studies have emphasized the stochastic behavior of motors"... should be careful about citing ...the study by Ally et al has no theory !!

The authors should cite a recent paper Soppina et al, where a stochastic tug-of-war between motors has been demonstrated both experimentally and through simulations. This is particularly relevant because Soppina et al study endosomes driven by dynein and kinesin-3 (Unc104), which are exactly the motors and cargo of the present manuscript.

"Higher order regulation combines with motor stochastics...". Perhaps they can emphasize more on the implications of this...why should anybody care ??

Page 5
"seen to move towards dynein at MT plus-ends". Remove "towards dynein"...its confusing

what is "most of the organelles rapidly...." in the last line ?? please quantify "most"...seems to be about 97% ?

Page 6 and Fig 2B. The experiment with bleaching steps at high and low laser power is unclear. The authors need to elaborate on this and refer to other work where something similar has been done.

How are the authors sure that the fluorescence response is linear for the entire range of GFP

numbers ?? They calibrate upto ~8 GFP using the nuclear pores. But 55 GFPs is a much larger number and much more fluorescence. If the authors are particular about claiming 55 GFPs, they must show that the florescence response is linear from 1 to 55 (or more) GFPs. Otherwise, the data and the entire foundation of the paper seems weak.

End of Page 8 and beginning of 9...
This part is very poorly written and completely unclear. What is "homogeneity of turning"?? I could not make any sense out of what is written in the the rest of the para.

I am not convinced about a double-exp decay in Fig 5B. Did they try to fit this to a single-exp decay ?? Where is the statistics to show that 2-exp is better than 1-exp fit ?? P values ?

In Fig 5C, the fit to fast population appears fortuitious. How come there are absolutely no points on the blue line between 30 and 150 seconds ?? I am not convinced by the data analysis at all.

How does one understand the handover from kinesin-3 to dynein at a reversal ?? Is the large amount of dynein sitting at the end and occupying MT-sites simply displacing kinesin from the microtubule (like a passive process)? Or, should we understand that the larger force from a large number of dyneins is responsible (a more active process)?? Can the authors comment on this from their data/analysis ??

Additional Correspondence 19 July 2010

I have just received the final referee's report on this manuscript, which I am enclosing below. As you will see, he/she is overall reasonably positive and supports publication after extensive revision. Therefore, this report does not change our overall decision on your manuscript, but I would ask you to address the concerns raised by this referee. In particular, I would draw your attention to points 1 and 2 regarding some of the assumptions of your model and how these relate to the experimental data and the conclusions. Addressing these criticisms to the satisfaction of this reviewer will be essential for eventual publication of your manuscript.

Apologies once again for the delay in getting this to you, and I hope you find this report useful for revising your manuscript.

I look forward to receiving your revision in due course.

REFEREE REPORT

Referee 3 comments:

Controlled and Stochastic retention concentrates dynein at microtubule ends to keep endosomes on track.
Schuster et al.

The authors study the motility or early endosomes in the filamentous fungi U. maydis, which is an easier alternative to neuronal cells. Their conclusions are mostly based on quantitative fluorescence microscopy. Using a triple-GFP construct, a good optical setup, and an internal calibration based on previously characterized nuclear pores, they estimate the number of dynein molecules at the tips of microtubules. In conjunction, they account for the traffic of dynein along the microtubules in a mathematical model, allowing them to relate the accumulation of motors at the microtubule tips, and the measured characteristics of the traffic along the length of the microtubules (density, speed, etc.). The argument is that the model only account for half of the accumulation observed at the microtubule tips, and that therefore 50% of the molecules must be regulated. This argument is invalid because the model was not proven to be 'correct'. This will not be possible, but some of the assumptions can be better justified (see below).

More generally, I expect the amount of dyneins stuck at the end of a microtubules to be very 'model-

dependent'. I am sure that the authors can easily find alternative models where the characteristics of traffic are identical, and yet that lead to different accumulations at the microtubules tips. Thus one should warn the reader that the prediction of the comet size, based on the characteristics of the traffic along microtubules is ill-defined (unreliable) enterprise.

The drug treatment and FRAP data seems to confirm the assumptions, but these are indirect proofs. After considering the data carefully, I was not convinced that half of the motors are 'regulated' by phosphorilation.

The study is nevertheless interesting and in general well executed. The use of mathematical modeling is elegant. I was often disappointed with the logic of the arguments, but these mistakes can be corrected. It will be important to highlight which assumptions are critical in the model, and to leave questions marks /alternative options clearly open to the reader, whenever possible. The current discussion sounds like 'everything fits together' and this harms the work.

The work highlights simple 'stochastic' effects, and downplay the role of 'protein regulation'. This is a welcome clarification of the topic, which is very interesting. In my opinion the article is worth publishing, after extensive revisions.

Specific comments:

1. The model considers a 'two-lane highway' (fig. 4D): dyneins move in one direction on the 'first' lane, and kinesins move in the reverse direction on the 'second' lane. Traffic jams occurs on both lanes independently: motors of the same polarity cannot pass each other, but dyneins and kinesins may cross in reverse direction without obstructing each other, because they are one different lanes.

I would like to see more discussion of these assumptions, because a microtubule potentially has 13 lanes, and each of these lane support traffic in both direction. Things may not be simple, but presumably kinesin and dynein are not able to pass each other if they are on the same lane. Do the authors detect any collisions between oppositely moving particles. Can such events be distinguished from a stochastic reversal?

The assumptions chosen by the authors may not strongly affect the characteristics of the traffic, as long as the density is low, which seems to be the case away from the microtubules tips. They will however be important for the traffic jam at the microtubule tip.

In a 13-lanes models, the ~55 motors will be shared, ~4 motors per lane. The ~3 motors located 'deep' within the jam would blocked by other motors, and not able to reverse directions so readily. Dynein may not be able to bind at the very tip. Only the motors at the edge of the 'jam' still have the opportunity to switch to minus-end directionality. Each layer in the traffic jam would lead to a different time-scale in the FRAP. I am left wondering if such effects would not be enough to account for the measurement.

2. Another critical assumption of the model is that 'dynein motility events' observed along microtubules represent one dynein (and one kinesin). This is made clear by the estimate of the flux: flux (in dynein per unit-time) = flux of motility events. Is the amount of dynein per motile kinesin known? The authors should measure the calibrated intensity and the number of bleach steps for these events, the same way that it was done for the comet at the tip of microtubules. Without a direct demonstration that the motile 'dots' along microtubules involve one kinesin and one dynein, the main conclusions are not justified. For example, if each motile event represented 2 dynein, then the stochastic model would predict the correct number at microtubules tips: no regulation at all?

I would like to see the bleaching curves, and in superposition the results of the step-finding algorithm.

3. In "a large number of dyneins form the comet at MT plus-ends":

"At reduced laser power this curve was shifted toward 8..."
It is shifted toward higher values ... between 3 and 12.
'8' is not even the maximum of likelyhood.

4. In the 'staurosporin treatment":
The accumulation of dynein at the tip is reduced, but this could be a consequence of some change of the traffic along microtubules. Without a quantification of the speed, flux and density of the motile particles in the presence of the drugs, the results are inconclusive.

"The comet serves as a buffer stop for arriving endosomes"

The ability of early endosome to 'pass' the dyneins is also very model dependent...
"The probablity of an EE to turn around is higher if there are more dynein motors on the track" ... is true for almost any scenario.
The model is here very naive: Pesc(N) = (Ppass)^N.
N=55 is really a very crude 1D approximation of the reality.
Considering the 13 protofilaments could give a more reasonable picture (N~4). The idea of 'passing' implies that the kinesins can reach the very tip of microtubules without being hindered by the dyneins located there. Is there some experimental evidence for this?

1st Revision - authors' response 28 September 2010

Reply to referees

Referee #1

1. The key experiments I was looking for to really support the authors' hypothesis is that when the stationary population is perturbed by either okadaic acid treatment or the dynactin or EB1 peptides, that they see the preferential loss of the stationary populations in the dynamic experiments - the photoactivation and photobleaching experiments.
We wish to thank the referee for his/her suggestion; this is an excellent point, and we fully agree. We did the requested experiments and expressed the inhibitory EB1 peptide in a newly generated strain that contained photo-activatable dynein. The outcome of these experiments support our hypothesis, as the show a one phase decay with a short T½ of ~20 seconds. Even though this T½ is slightly higher than that of the fast population of dynein (~10 seconds), these results strongly suggest that interfering with the dynactin-EB1 interaction removes the “less dynamic” dynein population (T½ of ~95 seconds).
These results are now included in Figure 7G and in the text (page 14, middle).

2. The authors should more clearly state the extent of the region they consider to be a "microtubule end". Is this defined by EB1 comet tail labeling, or some other criteria.
When analysing the dimensions of the dynein comet we have used co-localisation with EB1-RFP. Thus the MT end is indeed defined by the EB1 comet. This is now demonstrated in Supplementary Figure 2 and page 5, end of 1st paragraph, and page 6, beginning of 2nd paragraph..

3. However, the authors need to more clearly articulate the assumptions and parameters used for the modeling within the main text. For example, the authors should clarify their assumption that dynein bound at the plus end does not dissociate.
We tried to be clearer in this point and have listed the assumptions of both modelling approach in the text: Results: Page 10/11; Page 12, first half of page; Methods: page 27; Discussion: page 17.

4. *At the end of the introduction, the authors summarize their findings by stating that "dynein release occurs without activation". I think this is too strong - they could consider "dynein release can occur" or "usually occurs".*
We toned the statement down (page 4, last paragraph of introduction)

5. It is not clear to me what the authors mean by "traffic jam" of motors in the model they discuss in the Results section.
We tried to make this clear by avoiding the term “traffic jam” and by better explaining the model conditions (page 12, first paragraph)

6. The authors state that "dynein signals left the tip in an apparently random fashion, with a wide range of inter-motor distances" and refer to Figure 4A. This conclusion is not readily apparent to me from the figure. Similarly, the authors conclude from Figure 4B that the signals turn at various points, but again this is not so clear from the figure. Further analysis is provided in Figure 4C, but the explanation of this analysis was not so clear to me.
We apologise for not being clear in this point. Both figures were just meant to give examples for the behaviour of the dynein signals. We have rephrased the respective passages and have removed Figure 4c . The kymographs are now in the new Figure 4 and it is more clearly said in the text that they illustrate motility behaviour (page 9, middle). We hope the new version is more understandable.

7. The authors refer to data not shown to establish that the paGFP3-Dyn2 construct is biologically functional. Unless there is a reason not to, these supporting data should be added to the manuscript.
We have added the requested data in Supplementary Figure S1, showing that the fusion of triple eGFP and triple paGFP to the endogenous dynein goes not cause in any growth or morphology defect, which is typical for conditional dynein mutants.

8. In Figure 5A and movie 5, the intensity of the signal appears to decrease faster than the flux expected from the dynein leaving rate along microtubules. This suggests that the initial rapid loss is not due to dynein actively moving along microtubules, but instead is due to dynein detaching and diffusing away. Can the authors clarify?
The rapid decay in the kymograph and the movie is due to photo-bleaching. The decay curves are therefore based on image pairs at different time points rather than movies. We added a note to explain this to the figure legend 7 and the supplementary movie legend 6.

9. In Fig. 6, are the authors expressing the wild type Dya1n peptide or the mutant? Please clarify
The former figure 6 is now Figure 7D,E and F. In case of 7E it was the inhibitory Dya1 peptide (Dya1n) and the inhibitory EB1 peptide (Peb1c). In Figure 7F we also present the outcome of expressing the mutant peptide Dya1n*, which carries a point mutation that was predicted to interfere with binding to the EB1 interface. We tried to explain this clearly in the figure legend of Figure 7 (page 40/41)

10. Some figure panels are arranged in a convoluted manner that doesn't reflect their discussion in the text (ex. E above B and C in Fig. 5). Also, the figure legends are too minimal. More information in the legends would help the reader.
We have expanded the figure legends and included key conclusions. We also rearranged several figures to straighten their appearance (e.g. Figure 3).

Referee #2 (Remarks to the Author):

1. *However, much of the message is obscured by unclear and lengthy writing. A major revision in the writing and organization is required to make the message crisp and clearer.*
We have read through the manuscript and agree with the referee. Most of the paper was therefore rewritten and it is now more focussed and redundancies were removed..

2. *It would help a general reader to understand the role of motors in the relevant endosome biology of this system. What is the utility of endosomes coming to the tip and going back. Since most of the endosomes turn back rapidly, what is the purpose of this transport of endosomes to the hyphal tip. Something on this should be said in the Introduction.*
We appreciate this comment. I order to address it we have now included some background on the biology of the endosomes in *Ustilago maydis* in the Introduction (end of page3 and beginning of page 4)

3. *Page 4 "However, numerous recent theoretical studies have emphasized the stochastic behavior of motors"... should be careful about citing ...the study by Ally et al has no theory !!*

We apologise for this mistake; we corrected it and the passage now reads: "..This is in line with numerous reports, showing active regulation of motor proteins and membrane transport…(… Ally…)" (page 3).

4. *The authors should cite a recent paper Soppina et al, where a stochastic tug-of-war bwteen motors has been demonstrated both experimentally and through simulations. This is particularly relevant because Soppina et al study endosomes driven by dynein and kinesin-3 (Unc104), which are exactly the motors and cargo of the present manuscript.*
Again, we aplogise. We should indeed have included this work. It is now cited in the introduction (page 3, bottom) and in the conclusions (page 21, beginning).

5. *"Higher order regulation combines with motor stochastics...". Perhaps they can emphasize more on the implications of this...why should anybody care ??*
We tried to make our point more clear on page 3 (middle) and Discussion (page 21), now emphasizing that stochastics might be underappreciated in cellular processes.

6. *Page 5*
"seen to move towards dynein at MT plus-ends". Remove "towards dynein"...its confusing
We have changed the text to address this comment (page 5, bottom)

7. *what is "most of the organelles rapidly...." in the last line ?? please quantify "most"...seems to be about 97% ?*
We quantified this and found that 88% turn around. This number is now included on page 6, 1st paragraph.

8. *Page 6 and Fig 2B. The experiment with bleaching steps at high and low laser power is unclear. The authors need to elaborate on this and refer to other work where something similar has been done.*
We do appreciate this comment. To address it we have included reference to refer to the work of others in the results part (page 6/7 and page 9, last paragraph). We also removed the high laser power experiments (page 7, beginning), as these were indeed misleading and not necessary (here we do not find 8 steps because too much energy was used).

9. *How are the authors sure that the fluorescence response is linear for the entire range of GFP numbers ?? They calibrate upto ~8 GFP using the nuclear pores. But 55 GFPs is a much larger number and much more fluorescence. If the authors are particular about claiming 55 GFPs, they must show that the florescence response is linear from 1 to 55 (or more) GFPs. Otherwise, the data and the entire foundation of the paper seems weak.*
This is a fair comment that we have not thought of before. In order to address this we have generated a series of additional strains that contained the endogenous genes of the nucloporins Nup107, Nup214 and Nup2 fused to single, double or triple GFP tags. By combining these in the same strains, we were able to visualise nuclear pores that contained 32 and 48 GFP tags. We measured the intensity of these and found an almost perfect linear increase in signal intensity with GFP numbers (Figure 2c).

Furthermore, we tagged the dynein heavy chain with 1xGFP, 2xGFP and 3xGFP and measured the average intensity in the dynein comet. Assuming that the number of dynein motors is ~55, this gives ~110, ~220 and ~330 GFPs in a single comet. We compared the intensities of these comets with each other and with the intensities measured in the strain that contained 48 GFP in a single pore. Again, we found almost perfect linearity (Supplementary Figure S3B). Based on these results we are convinced that the intensities of GFP-tags is additive and increases in a linear fashion with increasing GFP numbers.

All results were added as figures (Figure 2c, Supplementary Figure 3B), and in the text (page 7, 22)

10. *End of Page 8 and beginning of 9...*
This part is very poorly written and completely unclear. What is "homogeneity of turning"?? I could not make any sense out of what is written in the rest of the para.

We do appreciate this comment and apologise for not being clear in this part of the manuscript. We have done our very best to improve this and have completely rewritten (now page 10-12).

11. *I am not convinced about a double-exp decay in Fig 5B. Did they try to fit this to a single-exp decay ?? Where is the statistics to show that 2-exp is better than 1-exp fit ?? P values ?*
In Fig 5C, the fit to fast population appears fortuitious. How come there are absolutely no points on the blue line between 30 and 150 seconds ?? I am not convinced by the data analysis at all.

Again, these comments are much appreciated and we thoroughly revised this point. We have now performed F-tests on the two fittings. In both cases, the recovery after photo-bleaching and the decay after photo-activation, the two-phase recovery or two-phase decay, respectively, is highly favoured over a one phase curve ($P<0.0001$). All details on these tests are now included in the manuscript (page 13, first paragraph; Figure 7B, 7C). We also added a short chapter to the Methods part (page 28/29).

In the previous version of the decay/recovery graphs we used the blue and the green line to demonstrate how the individual populations would behave. This was the reason why no data points were found on the blue line. Reading the comment of the referee we realise that this, again, is misleading. We therefore redid these figures and now present the predicted on-phase and two-phase decay/recovery curves (and all relevant F-test results) in Figure 7B and 7C. Buy this we hope to show graphically that the two-phase curves are fitting the data better than the one-phase curves.

12. *How does one understand the handover from kinesin-3 to dynein at a reversal ?? Is the large amount of dynein sitting at the end and occupying MT-sites simply displacing kinesin from the microtubule (like a passive process)? Or, should we understand that the larger force from a large number of dyneins is responsible (a more active process)?? Can the authors comment on this from their data/analysis ??*
This is a very interesting question. We have addressed this and are upon to submit a manuscript on this. It looks as if dynein is not replacing kinesin but taking over in a transient “tug-of-war”. Release of dynein allows kinesin-3 (which remains bound) to take over again. The current paper is already very long and we therefore did not include any of these data in this manuscript.

Referee 3 comments:

1. *More generally, I expect the amount of dyneins stuck at the end of a microtubules to be very 'model-dependent'. I am sure that the authors can easily find alternative models where the characteristics of traffic are identical, and yet that lead to different accumulations at the microtubules tips. Thus one should warn the reader that the prediction of the comet size, based on the characteristics of the traffic along microtubules is ill-defined (unreliable) enterprise.*
We agree with the referee and apologise for not being accurate in this point. In this revised manuscript we addressed this comment by (1) developing a new and more realistic 13-lane model (for details see comment below) and (2) rephrased the text (Discussion, page 17/18) so that it becomes apparent where the limitations of our modelling approach are.

2. *The drug treatment and FRAP data seems to confirm the assumptions, but these are indirect proofs. After considering the data carefully, I was not convinced that half of the motors are 'regulated' by phosphorilation.*
Indeed, we agree that the aspect of regulation by phosphorylation was the weakest part of our paper. In fact, all we provided were inhibitor experiments (though complementary ones as we used a phosphatase and a kinase inhibitor). Such inhibitor experiments are error-prone and need to be better supported by genetic data. For the sake of focussing the manuscript, but also to cope with the massive amount of additional data (including the new 13-lane model), we removed the data on phosphorylation from this revised manuscript. Instead we have strengthened the data on the role of EB1 and Dynactin in dynein retention (see above, new Figure 7G).

3. *It will be important to highlight which assumptions are critical in the model, and to leave questions marks / alternative options clearly open to the reader, whenever possible. The current discussion sounds like 'everything fits together' and this harms the work.*

We do fully agree with the referee. In this revised version of the paper we have clearly listed the assumptions made in the previous and the new model (page 10, 11, 12, 17) and have tried our very best to provide alternatives where possible throughout the results and discussion (page 11, 18).

4. *The model considers a 'two-lane highway' (fig. 4D): dyneins move in one direction on the 'first' lane, and kinesins move in the reverse direction on the 'second' lane. Traffic jams occurs on both lanes independently: motors of the same polarity cannot pass each other, but dyneins and kinesins may cross in reverse direction without obstructing each other, because they are one different lanes.*

I would like to see more discussion of these assumptions, because a microtubule potentially has 13 lanes, and each of these lane support traffic in both direction. Things may not be simple, but presumably kinesin and dynein are not able to pass each other if they are on the same lane.
Again, this is a very valid point. To address this properly, we decided to develop another, more realistic 13-lane model. This model takes into account that MTs consist of 13 protofilaments, that at least dynein changes lanes quite often (5.1 changes per 1 micrometer, Wang et al. 1995; Biophys J. 69:2011-23). Furthermore, we questioned our initial assumption that no dynein is falling off at the MT plus-end. In fact, we found experimental evidence for an increased cytoplasmic GFP-dynein fluorescence next to the comet (see new Supplementary Figure 4). We were not able to derive a "release rate" from these data, as protein stability and other factors that are unknown will modify the intensity in the cytoplasm and therefore need to be considered. However, we assumed a 2% loss at plus-ends in the new 13-lane model, which reflects the measured loss of endosomes delivered by kinesin-3 (see manuscript). Simulations based on this 13-lane model gave very similar numbers at MT plus-ends. However, we explicitly state in the text, that this realistic model is still an oversimplification that might underestimate the stochastic portion of the dynein in the comet (page 18, top).

The new 13-lane model is introduced in the new Figure 6, a new supplementary movie S5 and in the text (page 17, 27, Supplementary Methods)

5. Do the authors detect any collisions between oppositely moving particles. Can such events be distinguished from a stochastic reversal?
In the experimental data we never saw any colliding motors blocking each others motility. In the 2-lane model we consequently did not consider this possibility. However, after thinking about the referee's comment we realised that collisions indeed might occur, but get rapidly resolved. In our understanding there are two ways to do this, (1) by one motor changing lane or (2) both motors immediately and synchronous changing direction. We consider the second option very unlikely and therefore included the first option in our new 13-lane model.

6. *The assumptions chosen by the authors may not strongly affect the characteristics of the traffic, as long as the density is low, which seems to be the case away from the microtubules tips. They will however be important for the traffic jam at the microtubule tip.*
In a 13-lanes models, the ~55 motors will be shared, ~4 motors per lane. The ~3 motors located 'deep' within the jam would blocked by other motors, and not able to reverse directions so readily. Dynein may not be able to bind at the very tip. Only the motors at the edge of the 'jam' still have the opportunity to switch to minus-end directionality. Each layer in the traffic jam would lead to a different time-scale in the FRAP. I am left wondering if such effects would not be enough to account for the measurement.

As described above, the more realistic 13-lane model led to a very similar conclusion to the 2-lane model. However, we do agree with referee that dynamics within the comet might have an influence on the comet size. Clearly, additional proteins bound to MTs at the tip might affect availability of sites, alternatively local ATP depletion could affect motor activity and cause reduced escape. In the moment we have no means to address these points and get further clarity. We therefore toned our conclusions down and briefly discuss these options in the text (page 18).

7. *Another critical assumption of the model is that 'dynein motility events' observed along microtubules represent one dynein (and one kinesin). This is made clear by the estimate of the flux: flux (in dynein per unit-time) = flux of motility events. Is the amount of dynein per motile kinesin known? The authors should measure the calibrated intensity and the number of bleach steps for these events, the same way that it was done for the comet at the tip of microtubules. Without a direct demonstration that the motile 'dots' along microtubules involve one kinesin and one dynein, the main conclusions are not justified. For example, if each motile event represented 2 dynein, then the stochastic model would predict the correct number at microtubules tips: no regulation at all?*

Again, a very fair point is raised by the referee. Indeed, we did this analysis. Technically, this was very challenging, because travelling signals moved in and out of focus, which made photo-bleaching impossible. We therefore used CCCP (a drug that decouples the respiration chain and reversible reduces ATP levels) which did freeze the signals and allowed bleaching analysis. However, these dynein signals lost their "history" (e.g. is the frozen signal anterograde or retrograde?). We overcame this by simultaneous treatment with CCCP and photo-bleaching under microscopic observation. In these experiments, cells were placed on an agar cushion containing CCCP. The drug gradually decreased ATP and signals stopped moving after a few minutes. At the same time we photo-bleached for example all dynein in subapical or apical regions, respectively. The dynein released from the tip comet (retrograde) or that is delivered to the apical comets (anterograde) moved into the bleached region, where it became immobilised and could be analysed using photo-bleaching techniques and intensity measurements using nucleoporins as internal standards. This procedure did not harm the cells (controls were done), but allowed very accurate measurements. The consistent outcome of both experimental approaches (photo-bleaching and nucleoporin measurements) was that the fast majority of the anterograde and retrograde signals are single dynein motors.

These results are now summarised in a new Figure 4B-4E and in the text (page 9/10, 24),

8. *I would like to see the bleaching curves, and in superposition the results of the step-finding algorithm.*

We have included two example curves showing step-wise photo-bleaching and the respective STEPFINDER algorithm curves in the new Figure 4C.

9. *In "a large number of dyneins form the comet at MT plus-ends":*
"At reduced laser power this curve was shifted toward 8..." It is shifted toward higher values ... between 3 and 12. '8' is not even the maximum of likelyhood.

We appreciate the comment. The text was changed (see also comment 8, referee 2).

7. *In the 'staurosporin treatment": The accumulation of dynein at the tip is reduced, but this could be a consequence of some change of the traffic along microtubules. Without a quantification of the speed, flux and density of the motile particles in the presence of the drugs, the results are inconclusive.*

We agree and did the requested experiments. Indeed, neither speed, flux, nor the density of the motile particles was altered in the presence of Staurosporin. However, in the light of the new data included in this revised version we felt that the phosphorylation/dephosphorylation aspects were too preliminary (because based only on inhibitor studies, see above point 2, same referee). We also needed to shorten the manuscript and felt that these data do not add very much to the core of the story. The therefore removed these results. The "EE falling-off at MT ends" that were previously done using the phosphatise inhibitor Ocadaic Acid are now replaced by experiments done in the presence of high amounts of the inhibitory peptide Peb1c. These new data fully confirm the inhibitors results (see new Figure 8D) and, therefore, the overall conclusion that the comet captures arriving EEs is not challenged.

8. *"The comet serves as a buffer stop for arriving endosomes"*
The ability of early endosome to 'pass' the dyneins is also very model dependent...
"The probablity of an EE to turn around is higher if there are more dynein motors on the track" ... is true for almost any scenario.
The model is here very naive: Pesc(N) = (Ppass)^N.

N=55 is really a very crude 1D approximation of the reality.
Considering the 13 protofilaments could give a more reasonable picture (N~4).
The idea of 'passing' implies that the kinesins can reach the very tip of microtubules without being hindered by the dyneins located there. Is there some experimental evidence for this?
There is indeed experimental evidence for a bypassing of kinesin and dynein. We do find that 1.78% of the EEs that are delivered by Kinesin-3 fall off the end of the MT. This clearly suggests that there is a probability that kinesin can pass dynein without being captured.

We also appreciate that the referee finds the model not sophisticated enough. We set out to improve it, but than realised that this model does not add much to the story. Actually, the experimental fact that EEs fall off at the MT end when dynein numbers are low is important and makes our point. For the sake of focus and having the length of the manuscript in mind, we removed the old model. We hope that the referee finds this acceptable.

2nd Editorial Decision 03 November 2010

Many thanks for submitting the revised version of your manuscript EMBOJ-2010-74833R. Please let me first apologise again for the delay in getting back to you with a decision - this was due to the late return of the final referee's report. However, we do now have comments from all three of the original referees, which are enclosed below. As you will see, referee 1 is fully satisfied with the revision and supports publication without further changes, but referees 2 and 3 - while finding the manuscript to be substantially improved - still raise a number of issues that would need to be addressed in a second round of revision.

Referee 2's comments relate primarily to the presentation of the paper. I agree with the referee that there is quite significant repetition throughout the manuscript, and would recommend that you make every effort to improve conciseness - having the manuscript read by an 'outsider' as suggested would probably be very helpful. In this context, I note that your character count is already significantly over the 55,000 character limit and so I would ask you to try and shorten the text as far as possible. As for his/her comments as to the modelling part of the text, it seems to me that the section on the two-lane model could be cut somewhat, since the 13-lane model is the more representative.

Referee 3 raises a number of important concerns with the 13-lane model, as well as with other aspects of the text, which need to be addressed - his/her report is very explicit and I therefore see no reason to go into detail here. As you will no doubt recognise, this referee is an expert in mathematical modelling, and eventual acceptance of your paper will be contingent on your satisfying these remaining concerns with the validity of the model.

I would therefore like to invite you to revise your manuscript according to the referees' suggestions and as outlined above. Please don't hesitate to get in touch should you have any questions or comments about this.

I'm sorry once again for the delay, and I look forward to receiving your revised manuscript.

REFEREE REPORTS

Referee #1 (Remarks to the Author):

The authors have satisfactorily addressed my concerns. The revised manuscript is more focused and clear, and makes a significant contribution to the literature. I recommend publication in EMBO.

Referee #2 (Remarks to the Author):

The authors have made honest attempts to improve the paper. I think the paper can still do with a lot of editing...

Both models (2-lane and 13-lane) give exactly same result (25 dyneins). What does this mean ?? Why bother to give lengthy descriptions of two models and a figure for each ?? I really think that the modelling part can be reduced significantly and most of it moved to Supplementary information. The two model-figs can be reduced to a single concise figure discussing only one model. Anyway, it doesn't matter ...its all the same !!

The "Discussion" still reads like the "Results". Please be crisp here and dont make the reader go through things twice.

I suggest that the manuscript be shown to somebody not working in the author's Lab for a second opinion, and about how it can be edited. For example, the entire first para in section "A large number of dyneins form the comet at MT plus-ends" can be moved to Exp methods or supplement. Please be kind to the reader and dont take him/her away on digressions in every alternate paragraph.

Referee #3 (Remarks to the Author):

Controlled and Stochastic retention concentrates dynein at microtubule ends to keep endosomes on track.
Schuster et al.

The manuscript has been greatly improved, and the new experiments help to make the story convincing. I have additional comments though, mostly relating to the new 13-lanes model that was added to this version. I also noted that - although the answers to my comments said that the authors agreed with my point of view - the article was not modified accordingly. This was an very important point, and I am very surprised of this discrepancy (see specific below). This must have escaped the author's attention, and should absolutely be corrected before publication.

A. Figure 2:

Figure 2B: the fit in dotted line is certainly not Gaussian, contrary to what is said in the text, because it is skewed (asymmetric around its mean value), and the value at zero is zero.

Discrepancy in 'arbitrary units'.
Figure 2A indicates that 1GFP ~ 2.5 10^3 a.u.
Figure 2D indicates however 1GFP ~ 2.4 10^2 a.u.

B. Figure 7: I would suggest using a log-scale for the Y-axis, on Figure7B and 7G.

C. Comet size:

The dynein comet covers a stretch of ~1 micrometer near the plus-end of the MT (Figure 8B), and this is not accounted for by the model, which predict the number of dynein molecules accumulated at the end, but not the spatial extension of this traffic-jam. This probably indicates that many other proteins, also accumulated at the tip of MTs. It would be useful to indicate this limitation in the text.

D.13-lanes model:

Figure 6:

Please explain the different transition rates P_i: they are not all "probabilities of moving in various direction" as explained in the caption of figure 6. P2 and P4 are transition from the dynein-bound state to the kinesin-bound state, and vice-versa. It took me a while to understand this.

Description of the model in supplementary text:

There seems to be some confusion in the numerical values of the probabilities P_i.
In the 2-lane model, P1=P3 and P2 ~= P4, which is in order.
However in the 13-lane model the P2 ~ 10 P4. I could not make sense of the numerical values, from the time-step and values of v_mean. Since Mr ~ Ma, I would expect P2~P4.

Can you explain P3 = P1 / (1+2*P5)?
I would have rather guessed something like (P3 +2P5) * h_s / h_t = v_mean, or equivalently P3+2P5 = P1.

Can you justify P6 = P1 / 2 ?
P5 is the probability for dynein to change lane, and this was even measured (Wang et al. 1995).
I agree that the rate of sidestepping could be different when the motor is blocked (P6), compared to when the motor is walking (P5). However, setting P6 = P5 would still be the first assumption, I believe, without a measurement relating to the blocked situation. I cannot see the reason to set P6 at P1/2.

Could you provide a plot of the number of motors accumulated at the MT tip, as a function of P6? This is very important!

E. Can the author rationalize their choice of time-step? It seems to me that the results should be independent of the time-step, provided it is small enough, so why choose such an odd value?

F. Discrepancy between the response and the modifications made to the manuscript:

Review 1, my comment 1:

More generally, I expect the amount of dyneins stuck at the end of a microtubules to be very 'model-dependent'. I am sure that the authors can easily find alternative models where the characteristics of traffic are identical, and yet that lead to different accumulations at the microtubules tips. Thus one should warn the reader that the prediction of the comet size, based on the characteristics of the traffic along microtubules is a ill-defined (unreliable) enterprise.

Author's response:

We agree with the referee and apologise for not being accurate in this point. In this revised manuscript we addressed this comment by (1) developing a new and more realistic 13-lane model (for details see comment below) and (2) rephrased the text (Discussion, page 17/18) so that it becomes apparent where the limitations of our modelling approach are.

Manuscript:

Yet, the logical articulation of the manuscript is still as follows:
1. We see 50 dyneins at the ends of MTs.
2. 2 models predict 25 dyneins at the ends of MTs.
3. There must be regulation for at least 25 dyneins.
4. Experiments indeed confirm these predictions.

There is no logical link between 2 and 3. The logical articulation of the article is a fallacy. Point 2 merely shows a lack of imagination.

If the authors really agree with my comment 1, they ought to change the presentation of their work. The authors have added a new model with 13 lanes, and surprisingly it gave almost the same value for the number of dynein accumulated at the end... Even if the authors would present N models that all predict the same value (sic), it is important to explicitly say that it would be possible to find another model predicting 50 dyneins at the MT tips. Maybe it could be as simple as changing P6 in the new 13-lane model. This is why it is important to provide the plot, of the number of motors accumulated at the MT end, as a function of P6...

2nd Revision - authors' response 19 November 2010

Reply to Referees

Referee #2 (Remarks to the Author):

The authors have made honest attempts to improve the paper. I think the paper can still do with a lot of editing...

Both models (2-lane and 13-lane) give exactly same result (25 dyneins). What does this mean ?? Why bother to give lengthy descriptions of two models and a figure for each ?? I really think that the modelling part can be reduced significantly and most of it moved to Supplementary information. The two model-figs can be reduced to a single concise figure discussing only one model. Anyway, it doesn't matter ...its all the same !!

We appreciate this comment and have condensed the manuscript by removing details of the 2-lane model and referring to Ashwin et al. (2010). In the current paper the 2-lane model is mentioned as a step towards the 13-lane model. According to the referee we merged the modelling data and have moved parts of the previous figures to the Supplementary Data (now Supplementary Fig. 4 and 6)

The "Discussion" still reads like the "Results". Please be crisp here and dont make the reader go through things twice.

We have substantially rewritten the discussion to avoid repetition of results. To meet the comments of referee 3, we also have highlighted more strengths and weakness of the model and the biological need for a capture mechanism. The current paper is now 18% shorter than the previous version.

Referee #3 (Remarks to the Author):

Controlled and Stochastic retention concentrates dynein at microtubule ends to keep endosomes on track.
Schuster et al.

The manuscript has been greatly improved, and the new experiments help to make the story convincing. I have additional comments though, mostly relating to the new 13-lanes model that was added to this version. I also noted that - although the answers to my comments said that the authors agreed with my point of view - the article was not modified accordingly. This was an very important point, and I am very surprised of this discrepancy (see specific below). This must have escaped the author's attention, and should absolutely be corrected before publication.

A. Figure 2:

Figure 2B: the fit in dotted line is certainly not Gaussian, contrary to what is said in the text, because it is skewed (asymmetric around its mean value), and the value at zero is zero.

We apologise for this mistake. The text was changed and the red dotted line removed in the figure (now Fig 3B).

Discrepancy in 'arbitrary units'.
Figure 2A indicates that 1GFP ~ 2.5 10^3 a.u.
Figure 2D indicates however 1GFP ~ 2.4 10^2 a.u.

We assume that the Fig 2A referred to actually means Figure 2C. We checked the data again and found that the discrepancy is due to different settings and lasers used. However, the absolute values are not important in these figures and we therefore kept the data as they are, but highlighted the reason for the discrepancy in the figure legend.

B. Figure 7: I would suggest using a log-scale for the Y-axis, on Figure7B and 7G.

We have followed the referee's suggestion and used log scales for B and G in the new Figure 4.

C. Comet size:

The dynein comet covers a stretch of ~1 micrometer near the plus-end of the MT (Figure 8B), and this is not accounted for by the model, which predict the number of dynein molecules accumulated at the end, but not the spatial extension of this traffic-jam. This probably indicates that many other proteins, also accumulated at the tip of MTs. It would be useful to indicate this limitation in the text.

Indeed, this is an important point that needs to be mentioned in the text. At the top of p17, we have added a note to address this.

D.13-lanes model:

Figure 6:

Please explain the different transition rates P_i: they are not all "probabilities of moving in various direction" as explained in the caption of figure 6. P2 and P4 are transition from the dynein-bound state to the kinesin-bound state, and vice-versa. It took me a while to understand this.

We have clarified the meanings of the transition probabilities in the supplementary data more carefully and hope that the readability has been improved.

Description of the model in supplementary text:

There seems to be some confusion in the numerical values of the probabilities P_i.
In the 2-lane model, P1=P3 and P2 ~= P4, which is in order.
However in the 13-lane model the P2 ~ 10 P4. I could not make sense of the numerical values, from the time-step and values of v_mean. Since Mr ~ Ma, I would expect P2~P4.

We apologise again, as there was a typo in the previous version that meant that P2 was indeed a factor of ten too large.

*Can you explain P3 = P1 / (1+2*P5)?*
*I would have rather guessed something like (P3 +2P5) * h_s / h_t = v_mean, or equivalently P3+2P5 = P1.*

We thank the referee for this comment. Indeed, we followed his suggestion and changed our model accordingly.

Can you justify P6 = P1 / 2 ?
P5 is the probability for dynein to change lane, and this was even measured (Wang et al. 1995).
I agree that the rate of sidestepping could be different when the motor is blocked (P6), compared to when the motor is walking (P5). However, setting P6 = P5 would still be the first assumption, I believe, without a measurement relating to the blocked situation. I cannot see the reason to set P6 at P1/2.

Could you provide a plot of the number of motors accumulated at the MT tip, as a function of P6? This is very important!

We have added a lot more discussion about possible values of P6 in the supplement and in the text (end of page 12, beginning page 13; new Fig 6C). We came up with three reasonable values of P6 ranging from P6=P5 assuming that lane-change is not affected by hitting an obstacle, P6=P1/2 assuming that obstacles will cause lane change without change speed of motion along the MT and P6=P7 assuming that blockages can occur but clear on a timescale faster than the time resolution, justified by the fact that we do not see these in our experimental data. These values of P6 range from 0.03 to 0.3542, but as the new Fig 6C shows, they do not have a major influence on comet size.

E. Can the author rationalize their choice of time-step? It seems to me that the results should be independent of the time-step, provided it is small enough, so why choose such an odd value?

We agree with the referee and have changed this to 10ms and re-generated the simulations from the model. This is a balance between resolving the fastest processes in the simulation and the time it takes to compute.

F. Discrepancy between the response and the modifications made to the manuscript:

Review 1, my comment 1:

More generally, I expect the amount of dyneins stuck at the end of a microtubules to be very 'model-dependent'. I am sure that the authors can easily find alternative models where the characteristics of traffic are identical, and yet that lead to different accumulations at the microtubules tips. Thus one should warn the reader that the prediction of the comet size, based on the characteristics of the traffic along microtubules is a ill-defined (unreliable) enterprise.

Author's response:

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If the authors really agree with my comment 1, they ought to change the presentation of their work. The authors have added a new model with 13 lanes, and surprisingly it gave almost the same value for the number of dynein accumulated at the end... Even if the authors would present N models that all predict the same value (sic), it is important to explicitly say that it would be possible to find another model predicting 50 dyneins at the MT tips. Maybe it could be as simple as changing P6 in the new 13-lane model. This is why it is important to provide the plot, of the number of motors accumulated at the MT end, as a function of P6...

We realise the importance of this point now, and we apologise very much for that we did not fully understand this criticism the first time round. We have now made explicit in the results (page 13) and discussion (page 16, bottom) that one of the main modelling assumptions is that motor transport properties are homogenous along the length of the MT. We have included a graph showing the size of the dynein comet under different turning probabilities (P2), thereby showing the limitations of our modelling approach. Indeed a smaller P2 can give 50 or more dynein motors at the tip. This change may be caused by additional proteins or other local condition. In fact we consider the active retention by EB1 being one such factor. This is also discussed in page 17. By this we hope that we have met the referee's request.

The referee also asked us to consider the influence of the value of P6 – the probability of changing lanes at collision – on the comet size. We have found this has surprisingly little influence on the dynein numbers (see our response above and Fig 6C).

With respect to the referee's criticisms of the logical structure of the manuscript, we agree that the model was not placed correctly, and have changed by presenting the experimental data first. After that we concentrate on the 13-lane model and use it to try and present an integrated understanding of the system. In this concept, the size that the model predicts is no longer of such importance- it is more that stochastic transport processes can participate in the formation of the comet.

We finally wish to thank the referee for improving our modelling and the manuscript. We found his/her input very valuable.

2nd Revision - authors' response 21 December 2010

Many thanks for submitting the revised version of your manuscript EMBOJ-2010-74833R1 to the EMBO Journal. Please let me first apologise once again for the delay, but we have now finally heard back from referee 3, whose comments are enclosed below. As you will see, he/she is fully satisfied with the changes made, and whole-heartedly recommends publication. I am therefore pleased to be able to tell you that we can accept your manuscript without further delay. The only thing I need from you before we can accept the study is an Author Contributions statement - we are now implementing this as a required policy. Please can I ask you to send me a modified version of the text file including such a statement, which we can then upload in place of the previous version. Once we have this, we will then be able to formally accept the manuscript.

I am really sorry for the length of time the reviews have taken here, but I hope you agree that - despite the frustration - they have significantly improved the paper, and the result is a really nice story.

Many thanks for choosing the EMBO Journal for publication of this study, and congratulations on a fine piece of work!

REFEREE REPORT

Referee 3 comments:

I read the article again with interest.
The discussion is much better organized, and the results are now presented in a more neutral way, and I believe, in a better scientific style.
Congratulations on the work, and my best wishes for the winter break!

