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Directionality of individual kinesin-5 Cin8 motors is modulated by loop 8, ionic strength and microtubule geometry

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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 29 July 2011

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are enclosed. As you will see, all three reviewers express interest in your work, and the majority are clearly in favour of publication here. We would therefore like to invite you to submit a revised version of your manuscript. There are, however, a few important concerns that would need to be addressed first:

- Referee 1 suggests some analysis of loop 8 phosphorylation site mutants.
- Referee 2 asks about the oligomeric state of Cin8 and its ability to cross-link microtubules.
I find both these suggestions valuable and would strongly encourage you to undertake these analyses.

- Referee 3 raises serious concerns as to whether you have conclusively demonstrated bidirectional transport in vivo. This is a valid criticism, and clearly an important one. Any further data you may have to address this point would be very useful. Referee 2, however, points out that the different kinetics do suggest genuine bidirectional transport. At a minimum, a discussion of the arguments and caveats here would be essential.

Given the recent paper from the Surrey lab, I think it would be in everyone's best interests to move swiftly here. I do, however, find the above experiments important. Perhaps you could get back to me to let me know how much time you anticipate needing to conduct the necessary revisions, and we can discuss further if necessary.

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

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:

This paper presents a surprising and important finding that the yeast kinesin motor Cin8 can switch between plus and minus end directions of movement in a salt dependent manner. This is indeed big news in the motor field, as this is first well documented bidirectional motor. This study was somewhat scooped by a similar report by Surrey and co-workers in Science this year. However, I consider this work to be more in-depth. I think that the importance of the finding and the additional information provided in this study (very importantly the role of loop 8 in this process) merits its publication in EMBO J. The combination of in vivo studies in yeast along with single molecule measurements is very nice. = I think that it would benefit the authors and EMBO J to publish the study rapidly although there might be some consideration of the experiments to address the role of cdk phosphorylation as it could be quick and is speculated upon in the paper.

- The last line of the abstract is somewhat trite and could be rephrased into something more specific about the findings.

- The title also might benefit from revision to stating the result more directly. Control of directionality is still mysterious, since the authors manipulate with salt. It is likely controlled by different mechanisms in a cell, so I do not think that authors yet understand this.

- Loop 8 clearly is not the answer, since its deletion does not eliminate bidirectionality. This is somewhat surprising to me, since I might have thought that it would be given that it is the unique structural feature for Cin8. This might be better emphasized in the paper, since the search is still on for the key structural element that confers the switch. However, it is a good start and the in vivo results are interesting.

- The one slightly disappointing element is that the roles of the two CDK phosphorylation sites in the loop 8 insert are not explored. This seems relatively easy to do to see if the S to A mutant generates a motility defect in the spindle.

- The length of the paper is not bad, but could be made somewhat more concise in a revision. Figs. 2-4 might be creatively rearranged into 2 figures and a supplement of some of the detailed data.

Referee #2:

This follows closely on Roostalu, the first report of bidirectional motion from Cin8, published in Science in May of this year. Roostalu et al found that the balance between minus ended biased

diffusion and plus ended microtubule sliding motility was ionic strength dependent, but that the key point was not the ionic strength per se, but how many motors were engaged with a particular microtubule. A specific molecular mechanism was not determined and it remains deeply unclear how an individual motor finds out whether it is in a team or not, in order to decide which way to move. Like this first report, the present work uses a combination of in vivo observations and in vitro reconstitution to try to get at the problem of bidirectionality.

There are some experiments here that are similar to those done by Roostalu, and some that take a somewhat different approach, do test the assertions made by Roostalu et al., and do throw extra light. The present authors do not report a mechanism, but they do report a set of further clues to the mechanism, in particular showing that the loop 8 deletion can shift the ionic strength dependence of the directionality. This work is edging towards being too preliminary, but on balance I think these extra clues do contribute enough to warrant publication at this point, given the immediacy, excitement and puzzlement of the problem.

The new aspects in this work, relative to the Roostalu et al report are:

1. A viable loop 8 mutant, in which the very long loop 8 of Cin8 is swapped for the short loop 8 of Kar1, is investigated for its ability to move plus and minus endwards.

2. Individual molecules of Cin8-3GFP are shown to be able to switch direction depending on ionic strength, independent of their forming into teams. This finding contradicts the main conclusion of Roostalu et al.

3. In vivo tracking shows that the motor moves both plus endwards and (albeit rarely) minus endwards in vivo. The finding of minus endwards movement in vivo is novel. This finding relies on the polewards movements in S. cerevisae corresponding to motion towards the minus ends of pole-attached microtubules.

Criticisms:

Relating to point 3, there is a worry that these might be movements towards the plus ends of long microtubule attached to the opposite pole, especially as the minus end motility events are relatively rare. This potential caveat needs acknowledging and discussing. The fact that the minus end motility and plus end motility have different velocity distributions can be used as an argument that the minus-endwards events are genuine. The in vitro data showing minus end motility are clear.

Please given velocities in nanometers per sec.

The most significant weakness of the current work is the lack of data on the assembly state of the constructs & on their ability to crosslink microtubules. Are the expressed 3GFP-Cin8 proteins tetrameric(two antiparallel homodimers) as argued in the Roostalu work, or dimeric (as might occur when so many GFPs are attached the C-terminus of the molecules)? It is important to address this point before publication. A closely related and equally important question is, can these constructs crosslink / bundle (and slide) microtubules in solution? This should be rather easy to test, but there is no mention of it in the ms. If crosslinking does not occur, that is potentially a useful experimental twist compared to the experiments of Roostalu et al.

This is particularly important since the discussion of this paper refers to the possibility that the ability of Cin8 to make active crosslinks might be a key part of its regulation. It is suggested that Cin8s bidirectional motility does not play a significant role in spindle assembly, with its crosslinking activity being more important than its motility. Please reword this confusing part of the discussion, instead referring to Cin8's role or lack of role in specific processes with specific microtubule-motor geometries. For example, does Cin8's role in focusing kinetochore clusters not imply that it needs to crosslink and actively draw together the minus ends of microtubules? Or is passive crosslinking envisaged?

Can the plots in figure 4 be remade to show the shift in the ionic strength dependence of the transition zone between plus end directed and minus end directed motility? The Pd99 mutation shifts this zone. At the moment the velocities of plus and minus end directed episodes are plotted but there

is no indication of their relative frequency. Also, please show the minus-endwards velocities in 4B as negative velocities, as in 4A. It would be useful to plot the ATP concentration dependence of both plus and minus-endwards motility in figure 4, rather than having the plus-endwards data in supplementary.

Referee #3:

In this interesting study by Gerson-Gurwitz et al, the authors study the directionality of Cin8 motors, expanding upon a previous study to analyze possible mechanisms for the observed bi-directionality of Cin8 motors. The in vitro work in this study is interesting, as these studies provide additional insight on the molecular mechanism for the observed in vitro motor birectionality. In contrast, the in vivo results and implications are not convincing. Following are comments:
1. In Figure 1C,D, the authors note both "plus-end directed" and "minus-end directed" Cin8 motility in the anaphase spindle. While it is interesting that Cin8 motility can be observed in living anaphase spindles, the conclusion that minus-end directed and plus-end directed motility are observed is not convincing, as follows:

a. Electron-microscope reconstructions of anaphase spindles at similar lengths show that the midzone is substantial, and a percentage of non-kinetochore microtubules run the entire length of the spindle (see Fig. 6,Winey et al, 1995; Fig. 5A, Gardner et al, JCB, 2008). For this reason Cin8 motors could be traveling in the plus-end or the minus-end direction in the zones noted in Fig. 5D, but, because of the mix of microtubule polarities in this area, it is not possible to definitively classify the motion as plus-end or minus-end directed. Thus, it is not convincing to state that the in vivo data provides evidence that Cin8 moves in both a plus- and minus- end direction on the spindle during anaphase based on this data.

b. The spindle itself appears to be translocating in the top image of Fig. 1D, and so motor motility is difficult to distinguish separately from the spindle translocation.

2. The in vitro and in vivo results in which the behavior of Cin8 was studied in the absence of Kip1 and Ase1 motors is very interesting. I would suggest that these results be moved to the main text.

3. Similarly, the result that Cin8 changes directionality by varying the salt concentration is nice. The discussion of this result centers around Fig. 3A-C, but also included in these figures are results from the Cin8delta99 mutant. It would improve clarity if the mutant results were moved to another figure. In addition, it would be helpful to add a summary graph showing the mean velocity (with error bars) as a function of ionic strength to clearly demonstrate the gradual switch toward plus-end motility with decreasing ionic strength.

4. The Cin8delta99 result in which, at 30 nM NaCl, the presence of loop 8 in the WT cin8 results in plus-end directed motion, while cin8delta99 was minus-end directed, is an interesting result. As above, I suggest moving Fig. 3A(c) and B(b) into a new figure that highlights these graphs specifically.

5. The in vivo examination of the Cin8delta99 mutant shown in Fig. 5 is problematic, as follows:
a. The authors conclude that Cin8delta99-3GFP is asymmetrically distributed on the anaphase spindle. There is no quantification to support this conclusion, and although the authors state that the asymmetric distribution is in contrast to WTCin8-3GFP, the WT image in Fig. 1A also appears to be asymmetric, similar to the mutant in Fig. 5A. In addition, there is no mechanistic model or reasoning for why the mutant would result in an asymmetric distribution of motors on the spindle.

b. The authors state that Cin8delta99 "fails to reach the midzone region and support spindle elongation", and yet Cin8 in the midzone region appears more intense in Fig. 5A as compared to Fig. 1A. In addition, the authors state that the Cindelta99 mutants do not exhibit spindle-collapse phenotypes, suggesting that these motors are able to support spindle elongation, albeit at a slower rate (Table II).

c. Similar to the argument for the WT spindles, it is not appropriate to classify motor movement in the spindle as "plus-end directed" or "minus-end directed" without further information about the

microtubule polarity to which the motors are attached.

6. While the authors provide new, interesting in vitro data on the molecular mechanism for the bidirectional switch in the Cin8 motor, convincing data for the physiological presence and relevance of this switch remains unclear. The argument that bi-directionality is important since Cin8 motors need to be actively shuttled between spindle poles and the midzone seems unnecessary, as diffusion of unattached Cin8 motors would be rapid owing to the small size of the yeast spindle.

7. Minor: Introduction p. 3; and on p. 11; "...and focus the kinetochore clusters". The following two references are missing: Wargacki et al, Cell Cycle (2010) and Gardner et al, Cell (2008).

1st Revision - Authors' Response 28 September 2011

Please find enclosed the revised version of our manuscript EMBOJ-2011-78530

"Directionality of individual kinesin-5 Cin8 motors is modulated by loop 8, ionic strength and microtubule geometry". We performed a number of additional experiments to address the referees' comments and have achieved a clearer understanding of the surprising directionality switch of Cin8. We also rewrote and rearranged the manuscript according to their suggestions. We thank the referees for their positive judgment and helpful comments and we feel that the manuscript has been much improved.

Specifically, we performed the following additional experiments:

1. To better characterize the bi-directional movement of Cin8 *in vivo*, on the mitotic spindle, we imaged a larger number of spindles on which we could observe the motility of Cin8. We achieved better resolution and more statistics on midzone- and spindle-pole body (SPB)-directed single motor movements. The new data is summarized in Fig. 7 and Table 1, and shows that the velocity of Cin8 movements towards the midzone is significantly slower compared to Cin8 movements towards the SPBs. This clearly indicates that these two types of movements are different, further supporting the notion that Cin8 moves in both plus- and minus-end directions on the spindle MTs in living cells.

2. We show that Cin8-3GFP can bundle MTs in solution and support viability of cin8Δkip1Δ cells (supplementary figure S4), which is based on our previous work (Hildebrandt, et al., J Biol Chem, 2006; Gheber, et al., J Biol Chem, 1999). This experiment provides the evidence that the expressed Cin8-GFP constructs are functional and perform in their native tetrameric form (page 6 line 20 - page 7 line 7).

3. We examined motility on anaphase mitotic spindles of a phosphorylation-deficient Cin8 mutant that carries mutations to alanine at the two Cdk1 sites in the loop8 of Cin8 (Cin8-2A). The results are summarized in Fig. 7 and Table 1 and indicate that similarly to the "loopless" cin8Δ99 mutant, Cin8-2A is diminished in its ability to move towards the plus-end of spindle MTs.

4. We followed the motility of single (purified) Cin8-GFP motors between overlapping MTs *in vitro*. These experiments show that at constant and close to physiological buffer conditions, the interaction of Cin8 with one MT is dramatically different from the interaction with two antiparallel MTs. While the motors rapidly run into the minus-end direction on single MTs, they push in the plus direction between antiparallel MTs and move slowly and erratically in the fluorescence assays. Binding between parallel MTs in high salt also occasionally induced a change in Cin8 motility, but the motors appear to mostly not bind to both MTs. These new results are presented in Fig. 8.

In the following we provide point-by-point responses to the referees' comments.

Referee #1:

1. R: *The last line of the abstract is somewhat trite and could be rephrased into something more specific about the findings.*

A: The abstract was rewritten and the previous last sentence was deleted in favor of more specific statements.

2. R: *The title also might benefit from revision to stating the result more directly. Control of directionality is still mysterious, since the authors manipulate with salt. It is likely controlled by different mechanisms in a cell, so I do not think that authors yet understand this.*

A: The title was changed to more directly describe our results

3. R: *Loop 8 clearly is not the answer, since its deletion does not eliminate bidirectionality. This is somewhat surprising to me, since I might have thought that it would be given that it is the unique structural feature for Cin8. This might be better emphasized in the paper, since the search is still on for the key structural element that confers the switch. However, it is a good start and the in vivo results are interesting.*

A: We have now emphasized this point in the discussion and expanded on other factors that may induce the switch to the plus-end motility on the spindle, in particular phosphorylation and MT overlap geometry (page 13 line 22 – page 14 line 5).

4. R: *The one slightly disappointing element is that the roles of the two CDK phosphorylation sites in the loop 8 insert are not explored. This seems relatively easy to do to see if the S to A mutant generates a motility defect in the spindle.*
A: To address this comment, we produced a new mutant (Cin8-2A) and analyzed spindle movements of this Cin8 mutant, deficient in phosphorylation of the two Cdk1 sites in loop 8 (Table 1 and Fig. 7). Our data is consistent with the notion that phosphorylation of Cdk1 sites modifies the switch to plus-end motility of Cin8 in cells. Both, the "loopless" mutant (which lacks two of the three Cdk1 sites in the catalytic domain of Cin8) and Cin8-2A show reduced motility towards the midzone. The reduction in plus-end motility of Cin8-2A is smaller, though, than that of Cin8Δ99, indicating that the effect of the loop 8 insert on Cin8 directionality cannot be solely attributed to the lack of phosphorylation in this region. (page 13 line 22 – page 14 line 5)

5. R: *The length of the paper is not bad, but could be made somewhat more concise in a revision. Figs. 2-4 might be creatively rearranged into 2 figures and a supplement of some of the detailed data.*

A: To clarify the presented data (and to address comments of referees # 2 and 3), we rearranged the figures and placed the original Figure 4A in the supplementary material section (Figure S5). We also eliminated some redundancy and tightened the prose.

Referee #2:

1. R: …*Relating to point 3, there is a worry that these might be movements towards the plus ends of long microtubule attached to the opposite pole, especially as the minus end motility events are relatively rare. This potential caveat needs acknowledging and discussing. The fact that the minus end motility and plus end motility have different velocity distributions can be used as an argument that the minus-endwards events are genuine. The in vitro data showing minus end motility are clear.*

A: We now more extensively discuss this point in the text Page 5 line 6-19. In addition, to address the referee's concern (and a similar point by referee #3), we increased the number of spindles in which Cin8 motility was examined (Table 1 and Fig.7). The numbers now are adequate for statistical analysis and clearly show that velocities, as well as run lengths of Cin8 movements in the two directions are different. We now also more extensively discuss published evidence that overlapping MTs in the late phase of anaphase only overlap very closely to the spindle midzone.

2. R: *Please give velocities in nanometers per sec.*

A: Velocity units were changed to nm/s throughout the manuscript except for the descriptions of spindle dynamics, where it is much more customary to state velocities in μm/min.

3. R: *The most significant weakness of the current work is the lack of data on the assembly state of the constructs & on their ability to crosslink microtubules. Are the expressed 3GFP-Cin8 proteins tetrameric (two antiparallel homodimers) as argued in the Roostalu work, or dimeric (as might occur when so many GFPs are attached the C-terminus of the molecules)? It is important to address this point before publication. A closely related and equally important question is, can these constructs crosslink / bundle (and slide) microtubules in solution? This should be rather easy to test, but there is no mention of it in the ms. If crosslinking does not occur, that is potentially a useful experimental twist compared to the experiments of Roostalu et al.*

A: We performed more experiments and now discuss the assembly state of the expressed GFPp-tagged motors more extensively. Several lines of evidence presented in the manuscript indicate that Cin8, tagged with three GFP at the C-terminus, is likely to be a tetramer.

a - Fluorescence intensity traces of individual Cin8-3GFP and Eg5Kin (a dimer with two GFPs in a complex, (Lakamper, et al., J Mol Biol, 2010)) motors presented in Supplementary figure S3A show that the initial fluorescence intensity of Cin8-3GFP is ~5.2 times higher than the intensity of Eg5Kin (Supplementary Figure S3A). This is consistent with Cin8-3GFP being a tetramer (containing twelve GFP molecules in a complex) and not a dimer, whose initial intensity should have been ~3 times higher than that of Eg5Kin.

b – In the *in vitro* experiments, we examined several forms of Cin8, two of them tagged with one GFP and one tagged with three GFP (page 5 line 22 – 26): All forms of Cin8 were minus-end directed and fast, regardless of the number of GFP molecules they were tagged with. Moreover, both Cin8-1GFP, purified from yeast cells and Cin8-3GFP, examined in whole cell extracts, were switched by lowering ionic strength from fast, minus-end directed to slow plus-end directed motility (Fig. 4A and B). The similarity in activities of the GFP and 3GFP-tagged forms indicates that the oligomeric state of the proteins is the same.

c - New data added in the revised version shows that purified Cin8-GFP crosslinks and slides antiparallel MTs (Figure 8), ((Roostalu, et al., Science, 2011) also found that Cin8, tagged with one GFP is a tetramer). Thus, based on (b) and (c), we conclude that Cin8-3GFP is mostly a tetramer as well.

d - To further address this concern, we examined directly whether Cin8-3GFP can bundle MTs (supplementary figure S4A). We show that only extracts that overexpress Cin8-3GFP bundle MTs, while extracts of cin8-deleted cells or cells with endogenous expression of Cin8-3GFP do not. In our previous studies with biotinated Cin8 (Cin8-BCP), we showed that when overexpressed, tetrameric but not dimeric forms of Cin8 can bundle MTs in whole cell extracts (Hildebrandt, et al., J Biol Chem, 2006; Gheber, et al., J Biol Chem, 1999).

e - We found that Cin8-3GFP and Cin8-GFP, expressed from CEN plasmids, support viability of cells carrying chromosomal deletions of CIN8 and KIP1 (Supplementary figure 4B). Since we previously reported that only tetrameric Cin8 can support viability in *cin8*Δ*kip1*Δ cells (Hildebrandt, et al., J Biol Chem, 2006), we conclude that Cin8-GFP and Cin8-3GFP are tetramers.

4. R: *...This is particularly important since the discussion of this paper refers to the possibility that the ability of Cin8 to make active crosslinks might be a key part of its regulation. It is suggested that Cin8s bidirectional motility does not play a significant role in spindle assembly, with its crosslinking activity being more important than its motility. Please reword this confusing part of the discussion, instead referring to Cin8's role or lack of role in specific processes with specific microtubule-motor geometries. For example, does Cin8's role in focusing kinetochore clusters not imply that it needs to crosslink and actively draw together the minus ends of microtubules? Or is passive crosslinking envisaged?*

A: This part of the discussion was clarified and rewritten, with specific emphasis on the roles of Cin8 during anaphase spindle elongation and kinetochore clustering, page 14 line 20 – page 15 line 21. One of the mechanism by which Cin8 can place the kinetochores near the SPBs is active transport of the kinetochores towards the minus-end of kMTs. In anaphase there clearly is a role for Cin8's motility as it has to push the overlapping MTs in the spindle midzone apart.

5 R: *Can the plots in figure 4 be remade to show the shift in the ionic strength dependence of the transition zone between plus end directed and minus end directed motility? The Pd99 mutation shifts this zone. At the moment the velocities of plus and minus end directed episodes are plotted but there is no indication of their relative frequency. Also, please show the minus-endwards velocities in 4B as negative velocities, as in 4A. It would be useful to plot the ATP concentration dependence of both plus and minus-endwards motility in figure 4, rather than having the plus-endwards data in supplementary.*

A: We have rearranged and supplemented the figures following the very useful suggestions and have addressed all these comments. The new figures are numbered 4-6.

Referee #3:

1. R: *In Figure 1C,D, the authors note both "plus-end directed" and "minus-end directed" Cin8 motility in the anaphase spindle. While it is interesting that Cin8 motility can be observed in living anaphase spindles, the conclusion that minus-end directed and plus-end directed motility are observed is not convincing, as follows:*

a. R: Electron-microscope reconstructions of anaphase spindles at similar lengths show that the midzone is substantial, and a percentage of non-kinetochore microtubules run the entire length of the spindle (see Fig. 6,Winey et al, 1995; Fig. 5A, Gardner et al, JCB, 2008). For this reason Cin8 motors could be travelling in the plus-end or the minus-end direction in the zones noted in Fig. 5D, but, because of the mix of microtubule polarities in this area, it is not possible to definitively classify the motion as plus-end or minus-end directed. Thus, it is not convincing to state that the in vivo data provides evidence that Cin8 moves in both a plus- and minus- end direction on the spindle during anaphase based on this data.

A: This comment is related to the first comment of referee # 2. Indeed, in short anaphase spindles (< 5 µm), it is difficult to be sure of MT polarity because of substantial overlap. For this reason, in the revised manuscript, we describe Cin8 movements more conservatively, as midzone-directed and SPB-directed and then outline our rationale for classifying them as plus- or minus-end directed.

Several lines of evidence have convinced us that indeed we see plus- and minus-end directed movements of Cin8 on the anaphase spindles.

a – The midzone-directed movements of Cin8 were observed in short, 3-5 µm, as well as long > 5µm anaphase spindles. However, the majority of the SPB-directed movements that we observe are in the long (> 5 µm) spindles (Fig. 7). Previous reports show that mixed polarity of MT is restricted to short-intermediate anaphase spindles, less than 5 µm long (Winey et al, 1995, Figures 5 and 6 and Table I; Gardner et al, 2008, figure 5A). In the long anaphase spindles, there are only four interpolar MTs. Two emanate from each pole and overlap only in the middle of the spindle (Winey et al, 1995, Figure 6 and Table I). Thus, the SPB-directed movements that we observe in the long anaphase spindles are most likely to be on MTs emanating from the same pole towards which Cin8 motility is directed (minus-end directed). Similarly, midzone-directed movements observed in the long spindles are highly likely to be plus-end directed.

b - The midzone-organizing proteins Ase1, which binds to antiparallel MT array of the midzone, was shown to occupy only the middle 10-20% of the long anaphase spindles (Schuyler, et al., J Cell Biol, 2003; Fridman, et al., EMBO Rep, 2009). This indicates that in these spindles, the overlapping region of interpolar MTs is confined to a small middle portion of the spindle. We observe SPB- and midzone-directed Cin8 motility events outside of this middle region (Fig. 7), indicating that they are likely to occur on parallel arrays of interpolar MTs.

c - Most importantly, in order to address the point of Cin8 directionality on the spindle, we characterized Cin8 movements on a considerably larger ensemble of cells than in the previous version of the paper. We performed these analyses in cells expressing WT Cin8 as well as Cin8Δ99

and phosphorylation-deficient Cin8-2A (Table I and Fig. 7). In all Cin8 variants, the SPB-directed movements were significantly faster and shorter than midzone-directed movements. These results strongly indicate that we are observing two types of Cin8 motility events: midzone-directed in the plus-end direction and pole-directed, in the minus-end direction of the spindle MTs.

These points are now elaborated in the first paragraph of the results section.

b. R: *The spindle itself appears to be translocating in the top image of Fig. 1D, and so motor motility is difficult to distinguish separately from the spindle translocation.*

A: The top image in Fig. 1D was replaced and additional images are now shown in Fig. 7. Statistical analysis of Cin8 movements is summarized in Table I.

2. R*: The in vitro and in vivo results in which the behavior of Cin8 was studied in the absence of Kip1 and Ase1 motors is very interesting. I would suggest that these results be moved to the main text.*

A: Results with *kip1*Δ and *ase1*Δ cells and extracts were moved to the main text, Figure 3.

3+4. R: *Similarly, the result that Cin8 changes directionality by varying the salt concentration is nice. The discussion of this result centers around Fig. 3A-C, but also included in these figures are results from the Cin8delta99 mutant. It would improve clarity if the mutant results were moved to another figure. In addition, it would be helpful to add a summary graph showing the mean velocity (with error bars) as a function of ionic strength to clearly demonstrate the gradual switch toward plus-end motility with decreasing ionic strength.*

4. R: *The Cin8delta99 result in which, at 30 nM NaCl, the presence of loop 8 in the WT cin8 results in plus-end directed motion, while cin8delta99 was minus-end directed, is an interesting result. As above, I suggest moving Fig. 3A(c) and B(b) into a new figure that highlights these graphs specifically.*

A: To address these and related comments by referees # 1 and 2, we rearranged the figures (new Figs 4-6) and added a new panel showing average Cin8 velocity (+/- SEM) as a function of ionic strength (Fig. 6C).

5. R: *The in vivo examination of the Cin8delta99 mutant shown in Fig. 5 is problematic, as follows:*
a. The authors conclude that Cin8delta99-3GFP is asymmetrically distributed on the anaphase spindle. There is no quantification to support this conclusion, and although the authors state that the asymmetric distribution is in contrast to WTCin8-3GFP, the WT image in Fig. 1A also appears to be asymmetric, similar to the mutant in Fig. 5A. In addition, there is no mechanistic model or reasoning for why the mutant would result in an asymmetric distribution of motors on the spindle.

A: We clarified the discussion of this point and added more data. While WT Cin8 detached from the spindle in mid-late anaphase, Cin8Δ99 remains attached to the spindle (Supp figure S6). To better demonstrate this point, we added representative images of Cin8, Cin8Δ99 and Cin8-2A localization on the anaphase spindles (SI Fig. S6C). The distribution of the Cin8Δ99 motor appeared to be more asymmetrically distributed on the spindle but since this was difficult to quantify, we toned down this point in the manuscript page 10 line 5-6.

b. R: *The authors state that Cin8delta99 "fails to reach the midzone region and support spindle elongation", and yet Cin8 in the midzone region appears more intense in Fig. 5A as compared to Fig. 1A. In addition, the authors state that the Cindelta99 mutants do not exhibit spindle-collapse phenotypes, suggesting that these motors are able to support spindle elongation, albeit at a slower rate (Table II).*

A: We have added more data on Cin8Δ99 movements in the anaphase spindles (Table I). The new quantitative analysis of these movements clearly shows that the midzone-directed movements of Cin8Δ99 are significantly reduced compared to the WT Cin8 (Table 1). Nevertheless there is an increased Cin8 signal in the midzone. Lack of Cin8Δ99 detachment from the spindle is likely to be one of the reasons for the higher midzone signal of Cin8Δ99-3GFP. In addition, as we now mention

in the revised discussion (page 13 line 24 – page 14 line 4), one of the roles of Cin8 phosphorylation in its catalytic domain may be to induce its dissociation from a complex with Ase1. Since Cin8 was shown to interact with Ase1 and since Ase1 recruits Cin8 to the midzone (Khmelinskii, et al., Dev Cell, 2009) and Fig. 3E), lack of phosphorylation is likely to cause prolonged binding of Cin8 to the midzone. Since in the Cin8Δ99 sequence two of the three Cdk1 sites in Cin8's catalytic domain are missing, this mutant can be regarded as "phosphorylation deficient", which could explain its persistent attachment to the spindle and midzone. Nonetheless, although Cin8Δ99 exhibits increased binding to the midzone, the rate of anaphase spindle elongation is reduced in cells expressing Cin8Δ99, indicating that this mutant is diminished in the ability to provide efficient plus-end directed MT sliding.

c. *Similar to the argument for the WT spindles, it is not appropriate to classify motor movement in the spindle as "plus-end directed" or "minus-end directed" without further information about the microtubule polarity to which the motors are attached.*

A: Our new analysis of the spindle movements of Cin8Δ99 is summarized in Table I, which shows that this mutant (as well as WT Cin8 and Cin8-2A) moves faster towards the SPBs than towards the midzone. Also, please see our detailed response to the related point regarding the WT Cin8 (point 1a).

6. R: *While the authors provide new, interesting in vitro data on the molecular mechanism for the bidirectional switch in the Cin8 motor, convincing data for the physiological presence and relevance of this switch remains unclear. The argument that bi-directionality is important since Cin8 motors need to be actively shuttled between spindle poles and the midzone seems unnecessary, as diffusion of unattached Cin8 motors would be rapid owing to the small size of the yeast spindle.*

A: Thus far, the *in vivo* function of Cin8 in spindle elongation is consistent with its plus-end directionality between antiparallel MTs (Roostalu, et al., Science, 2011) and Fig. 8). The minus-end directionality of single Cin8 molecules was only recently discovered and its physiological significance remains unclear. Therefore we can not, in this manuscript, provide a conclusive result on the physiological role of the phenomenon. Nevertheless, we think it is useful to propose several possible roles for the minus-end directionality of Cin8 in the discussion (page 14 line 22 – page 15 line 23). During anaphase B, the *S. cerevisiae* spindle elongates from 2 to 8-10 μm and translocates through the mother-bud neck. Although the nuclear envelope remains intact, detachment from the spindle and diffusion would be inefficient mechanism to shuttle from the midzone to the kinetochores. An additional possible function for the minus-end directed motility was added in this context (kinetochores clustering). These are suggestions which we believe are adequate in the discussion. However, the comment regarding Cin8 shuttling between spindle poles and the midzone was removed from the end of the introduction.

7. R: Minor: Introduction p. 3; and on p. 11; "...and focus the kinetochore clusters". The following two references are missing: Wargacki et al, Cell Cycle (2010) and Gardner et al, Cell (2008).

A: The missing references were added.

2nd Editorial Decision 11 October 2011

Many thanks for submitting the revised version of your manuscript EMBOJ-2011-78530R. It has now been seen again by referees 1 and 3, whose comments are enclosed below. As you will see, both find the manuscript to be substantially improved and are fully supportive of publication. Referee 3 has a couple of minor comments - I tend to agree with him/her that clarity might be improved by increasing the bin size in figure 3B, but I leave it up to you whether or not to make this change. I do just have a couple of editorial issues to be dealt with before we can accept the study:

- Please can you include "Author Contributions" and "Conflict of Interest" statements, below the Acknowledgments section?

- In the legends to figures 4 and 5, can you clarify the terminology 'MB175' etc? While you do state

that buffer conditions are indicated, it's not totally clear (without checking back to the materials and methods) what the numbers refer to!

- In tables 1 and 2, can you clarify the statistical test used to demonstrate significance?

If you can just make these final changes and then resubmit the manuscript (you should find that all the files are carried forwards with your revision so you don't need to upload everything again), we will then be able to accept it without further delay. If I could ask you to deal with these things promptly, that would be a huge help: obviously we want to make sure that your paper gets a 2011 publication date, so we need the final version back asap!

Many thanks and best wishes,

Yours sincerely,

Editor

The EMBO Journal

REFEREE REPORTS:

Referee #1:

The authors have addressed the comments of my original review, including performing an important additional experiment. I now think that this work is suitable for publication in the EMBO J.

Referee #3:

The authors have done a nice job with the revision, and I support publication as-is. I have the following minor suggestions:

1) The characterization of SPB-directed movements vs midzone-directed movements is carefully done and I am satisfied with the wording. I do think the authors should be cautious about interpreting this result (as they are), since the difference in velocities could also be due to movements tracking polymerization or depolymerization of microtubule ends.

2) Figure 3B is a bit busy, one suggestion would be to reduce the bin size.

2nd Revision - Authors' Response 16 October 2011

Thank you for your mail and for the great news of close acceptance of our manuscript.

We have made all the corrections as were suggested by you and by referee #3:

1 - changed 3B with increased binning and changed the color of one category, for clarity.
2 - added information regarding statistical analysis for significance determinations in tables 1 and 2
3 - added clarification of buffer and salt conditions in figures 4 and 5.
4 - included author contribution and conflict of interest.

We also made two additional very minor changes:
1 - added an affiliation of one of the authors, which was forgotten in the previous version
2 - corrected panel 4D which was missing an error bar of one of the points

I have uploaded the new manuscript file and files of the corrected Figures. 3 and 4. I also added

caption of movies, exactly as they appear in the movie list of the supplementary file.

We sincerely thank you for handling this manuscript.