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Nuclear envelope-associated dynein drives prophase centrosome separation and enables Eg5-independent bipolar spindle formation

Jonne Raaijmakers, Roy G.H.P. van Heesbeen, Johnathan L. Meaders, Erica F. Geers, Belen Fernandez-Garcia, Rene H. Medema, Marvin Tanenbaum

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Transaction Report:

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

1st Editorial Decision 23 May 2012

Thank you for submitting your manuscript on the generation and characterization of Eg5-independent cell lines for consideration by The EMBO Journal. We have now received the input of three experts (as copied below), who consider these results potentially interesting and in principle suitable for reporting in our journal. Nevertheless, especially referees 2 and 3 also raise a number of substantive criticism that we feel would need to be decisively addressed before publication may be warranted. These concerns pertain to various aspects of presentation (background, interpretation, data), but also to important technical concerns. Should you be able to fully address these points, then we could consider a revised manuscript further for publication. In this respect, it will be essential that you experimentally address referee 3's major points 2 and 3; furthermore, it will be important to satisfactorily respond to the various well-taken concerns of referee 2. Finally, I notice that referee 3' request for deeper analysis of the drug resistance analyses would likely require significant further work in order to allow sufficiently definitive conclusions - I would therefore not insist on inclusion of such data in the revised version. However, the well-taken caveats pointed out by referee 3 will necessitate rewriting and refocussing of major parts of the study, in order to tone down the drug resistance and therapeutic claims and to instead keep the focus on the biological questions and findings; I should stress that this too will be a pre-requisite for eventual acceptance of the study.

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. Therefore, please do not hesitate to contact me should you have any further questions regarding the reports or this decision.

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We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

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

Yours sincerely,
Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

This manuscript describes a dynein-dependent mechanism that drives centrosome separation in prophase and thereby contributes to robust bipolar spindle assembly.
Remarkably, by incubating HeLa cells in low doses of STLC for several weeks, the authors manage to obtain clones of cells that divide and proliferate in the absence of Eg5 activity. This is surprising in the light of the large amount of data that support an essential function for Eg5 in bipolar spindle assembly.
However, the authors perform a set of well thought and well performed control experiments that convincingly show that indeed these cells grow in the absence of Eg5 activity, although mitotic time is slightly increased and these cells proliferate slightly slower than the parental cells.
This suggests that alternative pathways in these cells can take over all the function of Eg5 to drive centrosome separation and bipolar spindle formation and the authors aim at identifying these potential alternatives.
Confirming earlier results they find that in these cells, kinesin-12 becomes essential for bipolar spindle formation. However, their data suggest that another mechanism must drive centrosome separation in prophase.
They find that nuclear envelope associated dynein perform this function and this also occurs in cells having normal Eg5 activity.
Interestingly they show that the contribution of the different pathways may be different in different cell types.
In their final model, the authors propose that Eg5 drives centrosome separation by sliding the microtubules between the two centrosomes whereas dynein attached to the nuclear envelope promotes individual uncoordinated movements of the centrosomes. Although they provide some data to support this idea, it is difficult to envisage how the uncoordinated movement of the centrosomes (in the EIC cells) results in centrosome separation that seems as efficient as in control parental cells. The authors discuss a mechanism that could explain how the dynein dependent uncoordinated movements could function on p.12, but this is not convincing and I would suggest to revise or eliminate this part.

Minor comment:
The same authors used a different nomenclature for kinesin-12 (KIF15) in a previous paper. It is quite confusing for the reader and it would be helpful to at least mention the they refer to KIF15 somewhere in the text

Overall, this work is well performed and interesting. It provides novel data and an integrated view of

redundant mechanisms at play to ensure the assembly of a bipolar spindle. It is also interesting in the context of cancer therapy as discussed by the authors.

Referee #2 (Remarks to the Author):

In the present paper the authors carry out a nice set of experiments to derive Eg5-independent cell lines in which they can dissect the contributions of other proteins to establishing spindle bipolarity. Through these studies they show that the nuclear envelope pool of dynein is most critical for this process. While many of the experiments are cleanly performed and controlled for nicely, there are some issues that must be dealt with before publication. Most significantly the authors need to address several discrepancies between their own work and the published literature to clarify major points of novelty and mechanism.

1. The authors repeatedly state that they have uncovered a novel pathway. I disagree. Vaisberg and McIntosh showed nearly 20 years ago that inhibition of dynein by good old-fashioned microinjection of inhibitory antibodies blocked centrosome separation during prophase. While they did not speculate about the mechanism involving dynein at the nuclear envelope they clearly established a role for dynein early in mitosis. Furthermore, the authors have completely ignored work from Reinsch and Karsenti, who generated a reconstituted assay to describe the movement of nuclei on microtubules in a dynein-dependent process. Both of these studies need to be stated up front in the paper, and the terms novel pathway need to be removed.
2. There is some contradiction between the present studies and those of others regarding the extent of centrosome separation in prophase. In the current study the authors show data of centrosomal separation with small standard deviations. However previous work from Toso et al show that in HeLa cells ~50% of the cells show prophase centrosome separation whereas another 50% show prometaphase centrosome separation. How can the authors reconcile such data?
3. In Figure 1D, I don't understand why the authors scored the mitotic index when the percentage of monopolar spindles is the typical assay for Eg5.
4. The images in Figure 3D do not agree with the quantified data. The detached centrosomes are not clearly visible in the images.
5. The fact that inhibition of dynein does not result in a block in centrosome separation in prophase does not mean it is not involved in this normally in the presence of Eg5. There appears to be something different about the heLa line used here relative to the Toso paper because the current cell line has a different percentage of monopoles/bipoles. In addition it is possible that the dynein inhibition by siRNA is incomplete in the present studies.
6. Figure 4 is poorly organized. Many of the siRNA treatment conditions of repeated but shown in multiple graphs. In addition, the layout of having to find panel E in the middle is making it tough on others.
7. The exposure times of the cell proliferation assays need to be reduced. The first time I looked at the figure I honestly thought they were cartoon schematics of the experiments and completely missed that there were small colonies in places in the grey or blue circles.
8. The discussion could be shorted by 25% with no loss in clarity or expression of new ideas.

Referee #3 (Remarks to the Author):

In this manuscript Medema and co-workers described a series of experiments using Eg5-independent cells (EIC) to study mechanisms of centrosome separation at prophase. The authors provide data suggesting that that nuclear envelope (NE)-associated dynein drives centrosome separation at prophase. While aspects of the work are intriguing, I have several major concerns about the significance and strength of their conclusions.

My main concern is about significance. The interplay between dynein and Eg5 in spindle assembly has been extensively analyzed over the last several years, and also in several recent studies (Tanenbaum et al. EMBO J 2008, Ferenz et al. Current Biology 2009). Studies from Hoyt and Scholey labs have led to models that account for the authors observation of how dynein and Eg5 contribute to centrosome separation. Even if the authors addressed few technical concerns (see below), I believe that the advance from the studies reported in this manuscript is relatively modest.

The authors motivate the study by suggesting that their findings will shed light on resistance to inhibitors of the mitotic kinesin, Eg5/kinesin-5. However, the data presented in the manuscript are not directly addressing this point. Currently, it is also unclear if resistance to Eg5 inhibitors is a clinical problem at all. It seems that all the Eg5 inhibitors are not successful in the clinic due to limited efficacy (i.e. not better than standard of care, dose-limiting toxicity, etc). Moreover, resistance in the clinical context can be multi-factorial and linked to a particular drug. As far as I know, STLC is not in clinical trials. Therefore, it seems that the manuscript should be re-written so that it is better linked to the data. Using a drug-resistant cell lines to examine mechanism can be a good strategy, particularly when the genetic backgrounds of the sensitive and resistant cells are matched. This can be quite difficult in human cells and is certainly not the case here, greatly limiting the strength of the conclusions.

Other concerns:
1. The analysis of 'drug resistance' seems largely incomplete. A more systematic analysis is appropriate- e.g. which genes are over-expressed in the clones vs wildtype cells? Can an unbiased analysis be carried out using microarrays, or equiv. In addition, the results should be described more clearly: e.g. during EIC clone generation, are the three clones reported the only ones that they found to be STLC resistant? What is the total number of clones and percentage of clones that are truly Eg5-independent? What are other mechanisms of resistance? These questions need to be addressed, if the authors wish to focus on drug-resistance.

2. To probe the function of the NE-associated dynein, the authors deplete dynein components (Lis1, DIC) or dynein interaction partners (BICD2, CENPF) by RNAi. As it is known that RNAi can have off-targets (for example, Nigg et. al. Chromosoma Volume 119, Number 2, 149-165), the authors need to do 'rescue' experiments with RNAi-resistant lines to support the key results (e.g. for BICD2 or CENPF).

3. The authors conclude that NE-associated dynein drives prophase centrosome separation based on BICD2 and CENPF RNAi experiments. To support this key conclusion, it would be necessary to show that dynein localization at NE (and not spindle pole, kinetochore etc) is specifically altered upon RNAi of these proteins. The specificity in depletion is central to their conclusions.

4. The authors state that the NE-dynein pathway "functions in the presence of full Eg5 activity". However, the experiments used to support this claim were done with partial inhibition of Eg5. Though there is no apparent centrosome separation defects at low doses of STLC, this doesn't mean cells have full Eg5 activity. The authors need to clarify.

1st Revision - authors' response 15 August 2012

Referee #1 (Remarks to the Author):

This manuscript describes a dynein-dependent mechanism that drives centrosome separation in prophase and thereby contributes to robust bipolar spindle assembly.
Remarkably, by incubating HeLa cells in low doses of STLC for several weeks, the authors manage to obtain clones of cells that divide and proliferate in the absence of Eg5 activity. This is surprising in the light of the large amount of data that support an essential function for Eg5 in bipolar spindle assembly.
However, the authors perform a set of well thought and well performed control experiments that convincingly show that indeed these cells grow in the absence of Eg5 activity, although mitotic time is slightly increased and these cells proliferate slightly slower than the parental cells.
This suggests that alternative pathways in these cells can take over all the function of Eg5 to drive centrosome separation and bipolar spindle formation and the authors aim at identifying these potential alternatives.
Confirming earlier results they find that in these cells, kinesin-12 becomes essential for bipolar spindle formation. However, their data suggest that another mechanism must drive centrosome separation in prophase.
They find that nuclear envelope associated dynein perform this function and this also occurs in cells

having normal Eg5 activity.
Interestingly they show that the contribution of the different pathways may be different in different cell types.

In their final model, the authors propose that Eg5 drives centrosome separation by sliding the microtubules between the two centrosomes whereas dynein attached to the nuclear envelope promotes individual uncoordinated movements of the centrosomes. Although they provide some data to support this idea, it is difficult to envisage how the uncoordinated movement of the centrosomes (in the EIC cells) results in centrosome separation that seems as efficient as in control parental cells. The authors discuss a mechanism that could explain how the dynein dependent uncoordinated movements could function on p.12, but this is not convincing and I would suggest to revise or eliminate this part.

We have now revised this part in the discussion.
We acknowledge that the model we discuss is speculative and we have now clearly indicated this in the text. However, we feel we should discuss potential mechanisms for this dynein-dependent separation, since this is central to our manuscript. If the feeling remains that this section is not relevant, we can of course eliminate it.

Minor comment:
The same authors used a different nomenclature for kinesin-12 (KIF15) in a previous paper. It is quite confusing for the reader and it would be helpful to at least mention the they refer to KIF15 somewhere in the text

We apologize for the confusion. In our initial manuscript we named the protein by its human name, Kif15, but for simplicity we have since then referred to the protein by its family name kinesin-12. We now mention both names in both the discussion and the results section.

Overall, this work is well performed and interesting. It provides novel data and an integrated view of redundant mechanisms at play to ensure the assembly of a bipolar spindle. It is also interesting in the context of cancer therapy as discussed by the authors.

Referee #2 (Remarks to the Author):

In the present paper the authors carry out a nice set of experiments to derive Eg5-independent cell lines in which they can dissect the contributions of other proteins to establishing spindle bipolarity. Through these studies they show that the nuclear envelope pool of dynein is most critical for this process. While many of the experiments are cleanly performed and controlled for nicely, there are some issues that must be dealt with before publication. Most significantly the authors need to address several discrepancies between their own work and the published literature to clarify major points of novelty and mechanism.

1. The authors repeatedly state that they have uncovered a novel pathway. I disagree. Vaisberg and McIntosh showed nearly 20 years ago that inhibition of dynein by good old-fashioned microinjection of inhibitory antibodies blocked centrosome separation during prophase. While they did not speculate about the mechanism involving dynein at the nuclear envelope they clearly established a role for dynein early in mitosis. Furthermore, the authors have completely ignored work from Reinsch and Karsenti, who generated a reconstituted assay to describe the movement of nuclei on microtubules in a dynein-dependent process. Both of these studies need to be stated up front in the paper, and the terms novel pathway need to be removed.

We regret to have left out discussion of these papers in our original submission. We now cite the Vaisberg and McIntosh paper at first mention of NE-dynein, as well as in the discussion. We also removed our claim that we have uncovered a "novel" pathway. We would like to note though, that while Vaisberg and McIntosh indeed found a role for dynein in prophase centrosome separation using antibody injection, subsequent studies using RNAi were unable to confirm this. Consistent with this we find that depletion of dynein in parental Hela cells does not perturb prophase centrosome separation. Thus, for the field it was unclear if dynein was involved in this process or not. Here, we solve this paradox by showing that two redundant pathways control centrosome

separation and that the relative contribution of each pathway differs per cell type, likely explaining the contradictory results. We therefore feel that our results make a critical contribution to the field.

2. There is some contradiction between the present studies and those of others regarding the extent of centrosome separation in prophase. In the current study the authors show data of centrosomal separation with small standard deviations. However previous work from Toso et al show that in HeLa cells ~50% of the cells show prophase centrosome separation whereas another 50% show prometaphase centrosome separation. How can the authors reconcile such data?

We do observe some variability in the extent of prophase centrosome separation between cells. However, we find that nearly 100% of our HeLa cells do undergo separation of the centrosomes in prophase, at least to some extent. In agreement, we find that two different HeLa clones, as well as U2OS cells and RPE cells, show nearly 100% centrosome separation in prophase. We agree that these data are not completely consistent with the data of Toso et al, but we cannot explain the underlying reason for this difference. All we can say is that in every cell line we have tested, we find that the vast majority of cells separate their centrosomes in prophase. This makes it unlikely that the high number of cells that separate their centrosomes in prophase is a unique characteristic of our used clone, since we find that this is a general feature of different cell types and HeLa clones. Consistent with our data, another recent report from the Khodjakov lab found that in an untransformed cell line all cells separate their centrosomes in prophase (Magidson et al., 2011). We have now highlighted this difference in the manuscript.

3. In Figure 1D, I don't understand why the authors scored the mitotic index when the percentage of monopolar spindles is the typical assay for Eg5.

We have chosen to score the mitotic index in Fig. 1D, because it allows a direct comparison between Eg5 depletion and Hec1 depletion. We feel that presenting the data in this way most clearly shows that the EICs are still efficiently transfected by siRNA and are not impaired in the ability to maintain a mitotic arrest. While parental cells clearly arrest in mitosis after depletion of Eg5 and Hec1, the EICs only arrest after depletion of Hec1 and do not arrest after Eg5 depletion.

4. The images in Figure 3D do not agree with the quantified data. The detached centrosomes are not clearly visible in the images.

Although we do not see centrosome detachment in all EICs depleted for DHC, we agree that the picture of the EICs treated with DHC siRNA is not representative for the data shown in Supplementary figure 3A in terms of centrosome detachment. We have replaced this image with a more representative image.

5. The fact that inhibition of dynein does not result in a block in centrosome separation in prophase does not mean it is not involved in this normally in the presence of Eg5.

We agree with this point and we did not mean to suggest in the manuscript that there is no involvement for dynein in the presence of Eg5. Actually, we see in one Hela clone a small, but significant decrease in inter-centrosomal distance after dynein depletion in the presence of Eg5 (Figure S1B).

There appears to be something different about the heLa line used here relative to the Toso paper because the current cell line has a different percentage of monopoles/bipoles.

We agree that there might be differences between the HeLa clones used in this study and the HeLa clones used in the study of Toso et al., 2009. As mentioned above, we have tested multiple cell lines and different HeLa clones and consistently find that nearly 100% of the cells separate their centrosomes in prophase. We have now highlighted this difference in our manuscript.

In addition it is possible that the dynein inhibition by siRNA is incomplete in the present studies.

The siRNA used in this study to deplete DHC has been extensively validated in previous studies (see methods section) and we show on western blot a strong loss of DIC that coincides with depletion of DHC. Although we cannot exclude that there is a small amount of dynein left after

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treatment with DHC siRNA, the differential contribution of dynein between parental cells and the EICs indicated that the dynein-dependent pathway is more dominant in the EICs (or cells treated with a low dose of STLC), but does not argue that dynein is not involved normally, when Eg5 is present. However, it indicates that the dynein-dependent pathway only becomes *essential* when Eg5-activity is compromised.

6. Figure 4 is poorly organized. Many of the siRNA treatment conditions of repeated but shown in multiple graphs. In addition, the layout of having to find panel E in the middle is making it tough on others.

We apologize for this. We have now split the figure up into two parts (Figure 4 and Figure 5) and moved part of the data to the supplemental results.

7. The exposure times of the cell proliferation assays need to be reduced. The first time I looked at the figure I honestly thought they were cartoon schematics of the experiments and completely missed that there were small colonies in places in the grey or blue circles.

We repeated the proliferation assays and included better images.

8. The discussion could be shorted by 25% with no loss in clarity or expression of new ideas.

We have substantially shortened our discussion section.

Referee #3 (Remarks to the Author):

In this manuscript Medema and co-workers described a series of experiments using Eg5-independent cells (EIC) to study mechanisms of centrosome separation at prophase. The authors provide data suggesting that that nuclear envelope (NE)-associated dynein drives centrosome separation at prophase. While aspects of the work are intriguing, I have several major concerns about the significance and strength of their conclusions.

My main concern is about significance. The interplay between dynein and Eg5 in spindle assembly has been extensively analyzed over the last several years, and also in several recent studies (Tanenbaum et al. EMBO J 2008, Ferenz et al. Current Biology 2009).

Indeed, many studies have addressed the function of both dynein and Eg5 in cell division. However, Eg5 and dynein are complex proteins with many functions in different phases of mitosis and the findings described in this manuscript regarding the cooperativity of Eg5 and dynein in prophase have not been described before anywhere.

Studies from Hoyt and Scholey labs have led to models that account for the authors observation of how dynein and Eg5 contribute to centrosome separation. Even if the authors addressed few technical concerns (see below), I believe that the advance from the studies reported in this manuscript is relatively modest.

We completely agree that the Scholey and Hoyt labs have published an enormous amount of excellent and important work on microtubule motors and spindle assembly. However, neither lab has published findings indicating that nuclear envelope-associated dynein co-operates with Eg5 to promote prophase centrosome separation. In fact, the Hoyt lab uses *S. cerevisiae* as a model organism for their studies. In *S. cerevisae*, dynein does not localize to the nuclear envelope, nor do yeast cells have a prophase centrosome separation pathway similar to mammalian cells. Thus, their previous work does not take away from the novelty and importance of our current study.

The authors motivate the study by suggesting that their findings will shed light on resistance to inhibitors of the mitotic kinesin, Eg5/kinesin-5. However, the data presented in the manuscript are not directly addressing this point. Currently, it is also unclear if resistance to Eg5 inhibitors is a clinical problem at all. It seems that all the Eg5 inhibitors are not successful in the clinic due to limited efficacy (i.e. not better than standard of care, dose-limiting toxicity, etc). Moreover, resistance in the clinical context can be multi-factorial and linked to a particular drug. As far as I

know, STLC is not in clinical trials. Therefore, it seems that the manuscript should be re-written so that it is better linked to the data. Using a drug-resistant cell lines to examine mechanism can be a good strategy, particularly when the genetic backgrounds of the sensitive and resistant cells are matched. This can be quite difficult in human cells and is certainly not the case here, greatly limiting the strength of the conclusions.

As suggested by the reviewer, we have refocused our manuscript on the biological part. We have completely removed the following sections from the manuscript:

Introduction:

"Indeed, several drugs targeting Eg5 have shown potent anti-tumor activity in animal models and are currently being evaluated in clinical trials (Hayashi et al, 2008; Knight & Parrish, 2008; Liu et al, 2008; Rath & Kozielski, 2012; Sakowicz et al, 2004)."

"Redundant pathways may be able to take over the essential functions of Eg5, thereby facilitating resistance to Eg5 inhibitors. However, it is currently unclear if Eg5-independent pathways can promote resistance to Eg5 inhibitors."

Discussion:

"and it is unclear if cancer cells can become resistant to Eg5 inhibitors by bypassing Eg5 function in bipolar spindle assembly"

"Eg5 has been considered an attractive drug target for cancer therapy, since Eg5 was originally thought to provide an irreplaceable function in bipolar spindle assembly. However, this study shows that tumor cells can proliferate in the complete absence of Eg5 activity and surprisingly, full resistance to Eg5 inhibitors occurred in a time scale of only several weeks. These results suggest that tumors could become resistant to Eg5 inhibitors, not only through point mutations in the inhibitor binding pocket (Brier et al, 2006; Maliga & Mitchison, 2006; Tcherniuk et al, 2010), but also through upregulation of Eg5-independent pathways that allow bipolar spindle assembly in the absence of Eg5 activity. Importantly, we show that targeting either kinesin-12 or NE-associated dynein can eliminate this resistance, providing novel therapeutic targets to kill Eg5-resistant cells. An important question that will need to be addressed in the future is what the relative contribution of each of these mechanisms of resistance is to the clinical resistance observed in patients treated with Eg5 inhibitors."

Other concerns:
1. The analysis of 'drug resistance' seems largely incomplete. A more systematic analysis is appropriate- e.g. which genes are over-expressed in the clones vs wildtype cells? Can an unbiased analysis be carried out using microarrays, or equiv. In addition, the results should be described more clearly: e.g. during EIC clone generation, are the three clones reported the only ones that they found to be STLC resistant? What is the total number of clones and percentage of clones that are truly Eg5-independent? What are other mechanisms of resistance? These questions need to be addressed, if the authors wish to focus on drug-resistance.

We have removed the focus on drug resistance, as noted above. An unbiased analysis of gene expression is a great idea for future research, but we have not performed such experiments for the current manuscript, in line with the editorial decision.

2. To probe the function of the NE-associated dynein, the authors deplete dynein components (Lis1, DIC) or dynein interaction partners (BICD2, CENPF) by RNAi. As it is known that RNAi can have off-targets (for example, Nigg et. al. Chromosoma Volume 119, Number 2, 149-165), the authors need to do 'rescue' experiments with RNAi-resistant lines to support the key results (e.g. for BICD2 or CENPF).

We agree that this is an important point. In order to show that the phenotypes described here are not caused by RNAi off-target effects, we have now performed the suggested rescue experiments. We

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show in figure 4D and E that expression of mouse HA-BICD2 can fully rescue prophase centrosome separation after BICD2 depletion. We have been unable to generate a RNAi resistant cell line for full length CENPF due to the large size of the CENPF gene (>10kb). However, we have been able to independently confirm the specificity of CENPF RNAi, as we now show that four different siRNA's (all "ON-TARGET PLUS" siRNA's from Dharmacon, which are chemically modified to reduce off-target effects) targeting CENPF result in a strong reduction in prophase centrosome separation. Together, these results show that the phenotypes presented in this paper are specific.

3. The authors conclude that NE-associated dynein drives prophase centrosome separation based on BICD2 and CENPF RNAi experiments. To support this key conclusion, it would be necessary to show that dynein localization at NE (and not spindle pole, kinetochore etc) is specifically altered upon RNAi of these proteins. The specificity in depletion is central to their conclusions.

In figure S2, we now show that after depletion of BICD2, p150glued and DIC are specifically depleted from the NE, consistent with our previous findings (Splinter et al., 2010). We also show that CENPF depletion results in loss of dynein activators Nde1/NdeL1 from the NE, consistent with findings from the Doye lab (Bolhy et al., 2011). Interestingly, CENPF depletion does not detectably decrease the steady state levels of the dynein intermediate chain at the NE, suggesting that the major role of CENPF is to activate the dynein motor at the NE through recruitment of Nde1/NdeL1. Consistent with this, we confirmed findings from Bolhy et al that loss of CENPF results in inhibition of NE-dynein, as centrosomes detach from the NE in CENPF RNAi (Supplemental figure 3B and Bolhy et al. 2011).
Also, we have analyzed dynactin localization to the centrosome in prophase, and find it is not decreased upon depletion of BICD2 and CENPF (Supplemental figure 2C). We found that Nde1/L1 does not localize to the centrosomes during prophase (supplemental figure 2C), which makes it very unlikely that the effects that we observe after CENPF depletion are due to loss of dynein activity at the centrosome. Furthermore, we do not observe dynein localization to the kinetochore in prophase (only after NEB), so therefore we conclude that KT-dynein is not relevant for centrosome separation during prophase.

4. The authors state that the NE-dynein pathway "functions in the presence of full Eg5 activity". However, the experiments used to support this claim were done with partial inhibition of Eg5. Though there is no apparent centrosome separation defects at low doses of STLC, this doesn't mean cells have full Eg5 activity. The authors need to clarify.

We based this claim on the experiments performed in figure 6, in which centrosome movement is analyzed after Plk4 depletion. In the presence of full Eg5 activity movement of single centrosomes is completely dependent on NE-dynein.

Acceptance letter 04 September 2012

Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by one of the original referees (see comments below), and I am happy to inform you that there are no further objections towards publication in The EMBO Journal.
You shall receive a formal letter of acceptance shortly!

Yours sincerely,
Editor
The EMBO Journal

Referee #2

(Remarks to the Author)
The authors have done a very good job of addressing my concerns by the inclusion of new data, by the presentation of revised data that is superior to the original data, and by rewriting of the manuscript. The manuscript is now acceptable for publication.