THE
EMBO
JOURNAL

Manuscript EMBO-2010-76857

Nek9 is a Plk1-activated kinase that controls early centrosome separation through Nek6/7 and Eg5

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Review timeline:

Submission date:	22 December 2010
Editorial Decision:	26 January 2011
Revision received:	11 April 2011
Editorial Decision:	02 May 2011
Revision received:	05 May 2011
Accepted:	09 May 2011

Transaction Report:

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

1st Editorial Decision 26 January 2011

Thank you very much for submitting your research manuscript for consideration to The EMBO Journal editorial office.

I did receive a full set of comments (enclosed below) on your paper that reports on Nek-kinases in centrosome separation downstream of Plk1. Despite appreciating the potential general interest in this report, these scientists still request further mechanistic clarifications (particularly related to Plk1's role in Nek-activation) and request experimental support for its operation as potential general mechanism.

As specifically outlined in the comments from ref#3, further putative PBD-binding sites should be tested and epistasis between the kinases better defined. Similar to concerns raised by ref#1, potential contribution(s) of alternative Plk1-targets should in light of the presented results at least be discussed.

With definitive molecular insight being a critical measure for further consideration here, I urge you to take these remarks serious and invest the necessary time and experimental efforts to convince our referees from the significance of your findings.

Formerly, I do have to remind you that it is EMBO_J policy to allow a single round of major revisions only and that the final decision on your manuscript entirely depends on content and strength of its final version.

Thank you for the opportunity to consider your work for publication.

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Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1:

In this manuscript the authors explore the contribution of Nek kinases to centrosome separation in mammalian cells. They provide in vitro biochemical data demonstrating that Plk1 interacts with Nek9 through the PBD and that Plk1 phosphorylates and activates Nek9, and that both CDK1 and Plk1 are necessary for activation of Nek9 at mitosis. The in vitro experiments were generally convincing. My concerns are with the subsequent experiments that attempt to link the activation of Nek with Eg5 localization and function in mitotic cells.

In Figure 5 the authors examine centrosome separation in interphase cells, and conclude that activation of Nek6/9 can promote centrosome separation, and that this depends on Eg5. However, Eg5 is not found at centrosomes in interphase cells and is not microtubule associated because it is not phosphorylated at Thr926 in interphase cells. Thus it is important to determine how Eg5 is required for centrosome separation in interphase cells, ie is it phosphorylated at S1033? In Figure 7, the WB shows that in an exponentially growing population of cells, Eg5 is not phosphorylated at S1033.

It is likely that Plk1 has other targets in addition to Nek at centrosomes because active Nek is not as effective as Plk1 in centrosome separation. This should be discussed.

In Figure 7 the authors explore the localization of Eg5 and show that wild type localizes to centrosomes, but that the localization of Eg5S1033A is reduced. In the image showing localization to centrosomes, the motor is also decorating all the microtubules strongly; I suspect that it is overexpressed, or perhaps it is binding more strongly to microtubules than to centrosomes.

The rescue of centrosome localization of Eg5 (and centrosome separation) shown in Figure 8B was not convincing. The control cell shows strong co-localization of Eg5 and gamma tubulin signals, but the cells expressing Nek6/9 (after depletion of Plk1) did not. In both cases the gamma tubulin "dots" were surrounded by some Eg5 staining that appears to be on microtubules, not precisely at the centrosome; the overlay is not yellow (this is also seen in the interphase cells shown in Fig S6). This result is consistent with data in Figure 7 showing that there is centrosome separation in cells expressing Eg5S1033A (panel B). I think that the data show that Eg5 phosphorylation down stream of Nek is important, but that something else is also needed for localization of the motor and subsequent separation of centrosomes. Perhaps the localization is to microtubules, not centrosomes.

Referee #2:

The manuscript by Bertarn et al reports the mechanism of Nek9 activation and its downstream event that is relevant to centrosome separation in prophase. They provide evidence that Plk1 phosphorylates Nek9 at T210, which results in activation of Nek9. They also showed that Nek9 phosphorylation by CDK1 at S869 allows interaction of Plk1 with Nek9, which is consistent with the notion that CDK could act as a priming kinase for Plk1. They also dissected the roles of Nek9 and Nek6/7 in separation of duplicated centrosomes by RNAi-mediated depletion of Nek9 and Nek6/7. They found that these kinases contribute to centrosome separation, but less efficiently compared with Plk1 and EG5. Nevertheless, they showed that suppression of centrosome separation by Plk1-depletion, but not by EG5-depletion, can be rescued quite well by Nek9 active form. Probably the discrepancy needs to be explained. Further, their data show that centrosome separation by EG5 requires Nek9/6-mediated S1033 phosphorylation as well as Cdk1-mediated T926 phosphorylation on EG5, although effect of S1033 phosphorylation is less effective. Their data suggest that EG5 S1033 phosphorylation controls centrosomal localization of EG5. In this way, the manuscript reports some novel aspects of EG5 regulation that are mediated by CDK1-Plk1-Nek pathway, and thus interesting. However, I have a couple of comments to be addressed (see below). In addition, I would like to point out that the manuscript was not written straightforwardly and therefore it was not easy to go through the manuscript. I found many unnecessary, decorative and

ambiguous descriptions. Especially, discussion needs to be improved as the current form is just lengthy. 1. By mass spectrometry analysis phosphorylation of Nek5 at T210 was not found. Why? 2. Does Fig. 2A suggest that CDK1 is not functioning as a priming kinase in vitro? Is this consistent with in vivo data? 3. In page 7, line 2 from the bottom, do the authors want to say "Nek phosphorylation at T210 in mitosis"? 4. >2 micro meter distance between the sister centrosomes is determined to qualify their separation. But why 2 micro meter? 5. In Figure 8B bottom panels, separation of EG5 signals or centrosomes is so obvious. If the Nek9 RCC1 or Nek6 could efficiently rescue the phenotypes induced by Plk1 siRNA as shown in Fig. 6, more significant (for example more than 3-5 micro meter) separation of centrosomes could be easily visualized with EG5 staining. 6. The title could be more more friendly to non-specialists by avoiding the repeated use of Nek.

Referee #3:

In this manuscript the authors investigate the activation of Nek9 through exploring Cdk phosphorylation sites and Plk1 binding and phosphorylation. Using a series of biochemical measurments, RNAi and overexpression studies, and cell biological assays, the authors make a reasonable case for Cdk1-induced Plk1 PBD binding to Nek9, with subsequent Plk1-dependent Nek9 activation. They show that defects in centrosome separation in Plk1 knock-downs can be rescued, at least in part, using active forms of Nek6 or Nek9. Finally, they show that at Nek6/7 phosphorylation of Eg5, along with Cdk1 phosphorylation, is important for centrosome separation, providing a potential mechanistic link from Plk1 to Eg5 through Nek9 activation and Nek9-mediated activation of Nek6/7.

In general, I think the data are reasonable, and the overall conclusions are consistent with the mechanism, with the exception that I do not believe the data support a direct Plk1-mediated phosphorylation of Nek9 on its activation loop. Instead, Plk1 may facilitate Nek9 auto-activation, and the data as they are do not exclude this possibility. This should be investigated in detail, and some additional experiments performed to show direct interactions between full-length Plk1 and Nek9-wt and S869A mutants. Finally, similar experiments in murine cells looking at the other putative PBD-binding sites are important to demonstrate the universality of the proposed mechanism.

Major points:

1- Figure 1 - the authors should show that the S869A mutant of Nek9 does not bind to full-length Plk1 in mitotic cell extracts.

2- I am concerned in Figure 2B that Plk1 may not be directly phosphorylating T210 on Nek9. The 210 site is a poor match for the Plk1 phosphorylation motif, and is a much better match for typical Nek kinases. The K81M mutant likely has diminished activity but is probably not be truly a dead kinase. In this case Plk1 phosphorylation at other sites (S76 is a great Plk1 site, and the authors claim 6 total Plk1 sites) may trigger Nek9 auto-phosphorylation and activation. The authors could use a mutation in the catalytic Asp of Nek9, which generally makes a much more inactive kinase to demonstrate this using both their antibody and mass-spectrometry.

3- Similar experiments should be conducted in murine cells that lack the PBD site at S869 to evaluate the other putative PBD sites, and show the generality of this model for Plk1-mediated centrosome separation.

4- In Figure 4A, the authors should also examne Nek9/Nek6/7 combined knock-outs to insure that the phenotypes are epistatic, and therefore in agreement with the model that Nek9 is upstream of Nek6/7 rather than in separate pathways.

5- Figure 8B - Eg5 recruitment appears aberrant in the Plk1 knock-down cells trabsfected with active Nek9 or Nek6, despite some restoration of centrosome separation. Doesn't this indicate additional Plk1-dependent roles in centrosome separation that are Nek9:Nek6/7 independent?

6- Figure 8A needs a statistical analysis from a moderate numbers of cells, not a single example of the phenotype.

Minor points

7- In figure 1C, the GST-Plk1 blot region in GST pull-downs looks odd, as though the region of the blot is damaged. The authors should show a clearer blot to demonstrate whether there is or is not protein in this part of the blot.

8- The blot in Fig 2B lacks MW markers. It would be helpful to see the markers to know that we are really looking at the right Nek9 band.

9- Figure 9 should contain representative micrographs.

1st Revision - Authors' Response 11 April 2011

Referee #1

1. (...) In Figure 5 the authors examine centrosome separation in interphase cells, and conclude that activation of Nek6/9 can promote centrosome separation, and that this depends on Eg5. However, Eg5 is not found at centrosomes in interphase cells and is not microtubule associated because it is not phosphorylated at Thr926 in interphase cells. Thus it is important to determine how Eg5 is required for centrosome separation in interphase cells, ie is it phosphorylated at S1033? In Figure 7, the WB shows that in an exponentially growing population of cells, Eg5 is not phosphorylated at S1033.

In untreated interphase cells Eg5 is not phosphorylated at Ser1033 and is not found at centrosomes. This is in agreement with our proposal that Eg5[Ser1033] phosphorylation and centrosomal recruitment of the kinesin are necessary for centrosome separation, as these organelles if duplicated remain predominantly together in interphase cells. In our manuscript (Figure 5 and S8) we tried to convey the fact that when artificially activated in interphase cells, Nek9 and Nek6 are capable of inducing Eg5 recruitment to the vicinity of the centrosome (Figure S8A) and Eg5-dependent centrosome separation (Figure 5). We have now added new data as Figure S8B showing that, in addition, expression of either active Nek9[Δ346-732] or Nek6 results in increased phosphorylation of Eg5[Ser1033] (without inducing any significant effect on the cell cycle distribution of the cells, see Figure S5). Altogether our results lead us to conclude that active Nek9 and Nek6 are able to ectopically induce centrosome separation in interphase cells as a result of Eg5[Ser1033] phosphorylation and untimely Eg5 recruitment.

Regarding the relationship between Eg5[Thr926] and Eg5[Ser1033] phosphorylation, our results with non-phosphorylable mutants of Eg5 (Figure 7) clearly show that both are necessary for efficient centrosomal accumulation of Eg5 in cells with normal levels of Nek9 or Nek6 activity. We think that Eg5 recruitment may become independent of Thr926 phosphorylation in conditions in which Nek9 or Nek6 activities are experimentally increased above physiological levels. This would result in an increase of the pool of Eg5[Ser1033-P] above a threshold that could allow for Eg5 accumulation at centrosomes and the induction of ectopic centrosome separation. Our anti-Eg5[Ser1033-P] antibody is not suitable for immunocytochemistry and thus we are not able to determine the phosphorylation state of Eg5 in individual cells, nevertheless and supporting this hypothesis, figure S8B suggest that cells overexpressing Nek9[Δ346-732] or specially Nek6 may have high levels of Eg5[Ser1033-P] when compared to mitotic cells.

We have added a comment on this in the discussion.

2. It is likely that Plk1 has other targets in addition to Nek at centrosomes because active Nek is not as effective as Plk1 in centrosome separation. This should be discussed.

We now comment on this point in the Discussion relating it to Eg5 localization (point 4).

3. In Figure 7 the authors explore the localization of Eg5 and show that wild type localizes to centrosomes, but that the localization of Eg5S1033A is reduced. In the image showing localization to centrosomes, the motor is also decorating all the microtubules strongly; I suspect that it is overexpressed, or perhaps it is binding more strongly to microtubules than to centrosomes.

During prophase, Eg5 is indeed localized to centrosomes and surrounding microtubules. This can be observed both with recombinant (Figure 7) and endogenous (Figure 8) Eg5. A similar kinesin localization is observed in response to ectopic Nek9 or Nek6 activation resulting in interphase

centrosome separation (Figure S8A). In response to this and other comments of the referees, in our manuscript we now describe and discuss more extensively Eg5 localization at centrosomes and surrounding microtubules.

4. The rescue of centrosome localization of Eg5 (and centrosome separation) shown in Figure 8B was not convincing. The control cell shows strong co-localization of Eg5 and gamma tubulin signals, but the cells expressing Nek6/9 (after depletion of Plk1) did not. In both cases the gamma tubulin "dots" were surrounded by some Eg5 staining that appears to be on microtubules, not precisely at the centrosome; the overlay is not yellow (this is also seen in the interphase cells shown in Fig S6). This result is consistent with data in Figure 7 showing that there is centrosome separation in cells expressing Eg5S1033A (panel B). I think that the data show that Eg5 phosphorylation down stream of Nek is important, but that something else is also needed for localization of the motor and subsequent separation of centrosomes. Perhaps the localization is to microtubules, not centrosomes.

As we have noted above, we think that our results show that during prophase Eg5 is recruited to both centrosomes and surrounding microtubules. We agree with this and other referees that in our rescue experiments (Figure 8B), while pericentrosomal localization of Eg5 is consistently observed, localization of the kinesin specifically to the centrosome is not always so evident. We now show in Figure 8 what we think are more representative examples of the observed phenotypes and we have added as supplementary material (Figure S7) additional examples of cells in which Plk1 downregulation is rescued by Nek9 or Nek6 expression. We have also added a comment about Eg5 localization in our manuscript, where we also discuss the suggested possibility that Eg5 centrosomal recruitment may depend, in addition to Nek9/Nek6/7 signaling, on other yet-to be described inputs.

Referee #2

(...) I have a couple of comments to be addressed (see below). In addition, I would like to point out that the manuscript was not written straightforwardly and therefore it was not easy to go through the manuscript. I found many unnecessary, decorative and ambiguous descriptions. Especially, discussion needs to be improved as the current form is just lengthy.

We have tried to make the manuscript more straightforward and unambiguous. In addition we have shorten the discussion and attempted to simplify it.

1. By mass spectrometry analysis phosphorylation of Nek9 at T210 was not found. Why?

Previous results (Roig et al (2005) Mol Biol Cell 16: 4827-4840) show that only a small percentage of Nek9 (<5%) is activated and thus phosphorylated at Thr210 during mitosis, and that Nek9[Thr210-P] has a greatly retarded electrophoretical mobility. Both factors may be responsible for the lack of detection of peptides containing Thr210-P in our analysis. In contrast to Thr210, other non-activating sites are phosphorylated at high stoichiometry in mitotic Nek9 (resulting in a slight change in electrophoretical mobility that affects ~100% of the kinase) and have been more easily detected.

We thus note in our manuscript that the sample analyzed by MS possibly contained mostly inactive Nek9.

2. Does Fig. 2A suggest that CDK1 is not functioning as a priming kinase in vitro? Is this consistent with in vivo data?

While our results (see Figure 3) indicate that in vivo CDK1 activity is necessary for Nek9 activation and that the putative CDK1 site Ser869 is necessary for Plk1 binding to Nek9, as the referee points out CDK1 phosphorylation is not necessary for Nek9 phosphorylation and activation by Pk1 in vitro (Figure 3). In vitro independence of priming phosphorylation has been described for different Plk1 substrates (Santamaria et al. (2011) Mol Cell Proteomics 10: M110.004457) and at least in our case this may reflect high Plk1 abundance and activity levels as well as the lack of any competing phosphatases or substrates in the assays as compared to in vivo conditions.

3. In page 7, line 2 from the bottom, do the authors want to say "Nek phosphorylation at T210 in mitosis"?

We want to convey that while CDK1 is responsible for a significative fraction of Nek9 phosphorylation in mitosis resulting in an observable mobility shift, Nek9 activation (and thus Thr210 phosphorylation) is ultimately dependent on both CDK1 and Plk1.

We have changed the wording of the sentence to make this more clear ("Thus while CDK1 activity is necessary for Nek9 phosphorylation in mitosis and the resulting change in electrophoretical mobility, Nek9 Thr210 phosphorylation and mitotic activation requires both CDK1 and Plk1").

4. >2 micro meter distance between the sister centrosomes is determined to qualify their separation. But why 2 micro meter?

2 μm has been used before as the distance to separate paired or unsplit centrosomes form split centrosomes (Meraldi and Nigg (2001) J Cell Sci 114: 3749-3757). In our work we have used this arbitrary distance to categorize pairs of centrosomes in three groups: together (i.e. with overlapping centrosomal markers such as γ-tubulin), physically separated (i.e. not overlapping) but close together as to be unsplit (< 2 μm apart), and fully separated (> 2 μm apart).

We acknowledge that an alternative and sometimes more effective manner to describe centrosome separation is the use of box plots of intercentrosomal distances and thus now use this system in both Figure 4 and 6.

5. In Figure 8B bottom panels, separation of EG5 signals or centrosomes is so obvious. If the Nek9 RCC1 or Nek6 could efficiently rescue the phenotypes induced by Plk1 siRNA as shown in Fig. 6, more significant (for example more than 3-5 micro meter) separation of centrosomes could be easily visualized with EG5 staining.

In Figure 8B we now show what we thing are more representative examples of cells in which Plk1 downregulation is rescued by active Nek9 or Nek6. We also show additional examples of cells as supplementary material (Figure S7) . We have in addition added to Figure 6 a box plot of intercentrosomal distances. We think that in this manner we now convey better the fact that Nek9 RCC1 or Nek6 expression are indeed able to support significant Eg5 recruitment and centrosome separation in cells that lack normal levels of Plk1.

6. The title could be more more friendly to non-specialists by avoiding the repeated use of Nek.

Although we would like to refer to Nek9 as "the NIMA-family protein kinase Nek9" thus introducing it for non-specialists, the title length constrains (less than 100 characters including spaces) makes this difficult without removing the description of other important aspects of our work (i.e. that Nek9 controls early or prophase centrosome separation through Nek6/7 and Eg5). We would be happy to accept suggestions about alternative ways to do this.

Referee #3

 # (...) In general, I think the data are reasonable, and the overall conclusions are consistent with the mechanism, with the exception that I do not believe the data support a direct Plk1-mediated phosphorylation of Nek9 on its activation loop. Instead, Plk1 may facilitate Nek9 auto-activation, and the data as they are do not exclude this possibility. This should be investigated in detail, and some additional experiments performed to show direct interactions between full-length Plk1 and Nek9-wt and S869A mutants. Finally, similar experiments in murine cells looking at the other putative PBD-binding sites are important to demonstrate the universality of the proposed mechanism.

Major points:

1. Figure 1 - the authors should show that the S869A mutant of Nek9 does not bind to full-length Plk1 in mitotic cell extracts.

We have added a supplementary figure (S2C) showing specifically that in contrast to wild type Nek9, Nek9[S869)] does not interact with endogenous Plk1 in mitosis.

2. I am concerned in Figure 2B that Plk1 may not be directly phosphorylating T210 on Nek9. The 210 site is a poor match for the Plk1 phosphorylation motif, and is a much better match for typical Nek kinases. The K81M mutant likely has diminished activity but is probably not be truly a dead

kinase. In this case Plk1 phosphorylation at other sites (S76 is a great Plk1 site, and the authors claim 6 total Plk1 sites) may trigger Nek9 auto-phosphorylation and activation. The authors could use a mutation in the catalytic Asp of Nek9, which generally makes a much more inactive kinase to demonstrate this using both their antibody and mass-spectrometry.

We have performed the corresponding experiment with Nek9[D176A] (new Figure S3) and observed that this mutant of the catalytic Asp of Nek9 is phosphorylated by Plk1 at Thr210 similarly to Nek9[K81M]. We thus think that taken together our data shows that Plk1 can phosphorylate Nek9 Thr210 directly leading to its activation. Nevertheless we agree with the reviewer that Plk1 phosphorylation of other Nek9 sites outside the activation loop may additionally contribute to Nek9 activation by triggering autophosphorylation of Thr210 (possibly by interfering with the autoinhibition of Nek9 by its own RCC1 domain). We now discuss this in our manuscript. We have started a detailed study of the biochemical basis of Nek9 activation by Plk1 that we hope in the future can clarify the specific contribution of each of the Nek9 residues phosphorylated by this Polo-family kinase during Nek9 activation.

3. Similar experiments should be conducted in murine cells that lack the PBD site at S869 to evaluate the other putative PBD sites, and show the generality of this model for Plk1-mediated centrosome separation.

We have repeated the coimmunoprecipitation of Nek9 and Plk1 (Figure 1A) in mouse embryo fibroblast cells (new Figure S2A) and thus show that the mitotic interaction of both kinases is conserved in mice. This suggests that our data can be generalized to even these organism that lack a suitable S[S/T]P Plk1 binding site in a similar position to that of human Nek9[Ser869], but conserve an S[S/T]P motif suitable to interact with Plk1, such as mouse 749SSP751 -or putatively Xenopus laevis 908SSP910.

4. In Figure 4A, the authors should also examne Nek9/Nek6/7 combined knockouts to insure that the phenotypes are epistatic, and therefore in agreement with the model that Nek9 is upstream of Nek6/7 rather than in separate pathways.

We have added the requested condition to figure 4A. Our new results show that the downregulation of Nek6, Nek7 and Nek9 together is not significantly different to the downregulation of the kinases individually or of Nek6 plus Nek7 ($p>0.05$). We think that our results support a model in which Nek9 is upstream of Nek6/7.

5. Figure 8B - Eg5 recruitment appears aberrant in the Plk1 knock-down cells trabsfected with active Nek9 or Nek6, despite some restoration of centrosome separation. Doesn't this indicate additional Plk1-dependent roles in centrosome separation that are Nek9:Nek6/7 independent?

Eg5 recruitment in Plk1 knock-down cells transfected with active Nek9 or Nek6 shows indeed some differences with that of control cells (i.e. it is less focussed at or around centrosomes). We agree that this may indicate that Plk1-dependent roles exist during centrosome separation that are Nek9/Nek6/7-independent (alternatively it could be the result of differences in the levels of Nek activity obtained after expression of the recombinant kinases). We now note this in our manuscript, and in response to this and other referee comments we show what we think are micrographs that better convey this fact. We have also added additional examples of the observed phenotypes as supplementary material (Figure S7).

6. Figure 8A needs a statistical analysis from a moderate numbers of cells, not a single example of the phenotype.

We now show in Figure 8A the quantification of the signal corresponding to pericentrosomal Eg5 for the different siRNAs used.

Minor points:

7. In figure 1C, the GST-Plk1 blot region in GST pull-downs looks odd, as though the region of the blot is damaged. The authors should show a clearer blot to demonstrate whether there is or is not protein in this part of the blot.

This was most probably a scanning artifact. We have redigitalized the corresponding Coomassie-stained membrane to better show that the mentioned region is not damaged.

8. The blot in Fig 2B lacks MW markers. It would be helpful to see the markers to know that we are really looking at the right Nek9 band.

We have added MW markers to figure 2B.

9. Figure 9 should contain representative micrographs.

We have added representative examples of prometaphase cells to Figure 9 showing DNA, γ-tubulin (as a centrosomal marker) and myc-Eg5 (GFP in control cells) staining.

2nd Editorial Decision 02 May 2011

Your revised manuscript has now been re-assessed by two of the original referees.

As you will see, both still have some concerns related to the paper. Ref#1 is not sure about accurate labeling of the pro-metaphase graph in figure 9, penetrance of the rescue results as well as some suggestions for text changes.

Ref#2 is not fully satisfied with the MS-analysis of mostly inactive Nek. If you might already have data at hand that would address this point (you mentioned detailed mechanistic studies on Nek-activation in the rebuttal), it would be good to include this here. I would however at this point not insist on this.

Overall, I kindly ask you to consider the referee remarks before providing the ultimate version of your study for eventual acceptance.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1:

The manuscript is greatly improved and provides new information regarding the regulation of centrosome separation downstream of Plk1. I have a few comments on the revised manuscript.

In figure 6, the constitutively active form of Nek9 is abbreviated differently than in the text; this should be changed.

In Figure 9, and the text that discusses this figure, I was confused regarding the proportion of cells expressing Eg5S1033A that were in prometaphase. The authors state that cells entered prometaphase at a rate similar to controls (63% at 60 min) and that at 60 mins more than 57% were in prometaphase. The graph looks like the value is about 57% for the prometaphase panel. Please clarify. This experiment also shows that many cells that enter prometaphase have unseparated centrosomes; together this data shows that timely progression through mitosis requires phosphorylation of Eg5 at serine 1033, and likewise, centrosome separation is impacted by this phosphorlation event.

However, I am still not convinced that the accumulation of Eg5 at centrosomes is fully rescued by the expression of active Nek6/9. In figure 8, the distribution of Eg5 is not the same in the control (where very bright centrosome staining is seen with little Eg5 on microtubules) and in the cells expressing Nek6 or Nek9(const. activ). In the latter experiments, there is Eg5 all along the microtubules, and the bright, distinct centrosome staining is not present. However, the authors do not overstate their findings, so a precise understanding of how Nek phosphorylation of Eg5 regulates accumulation at centrosomes may await further experimentation.

In the abstract, I would change the phrase "is necessary for the accumulation of Eg5 around centrosomes......" to "contributes to the accumulation of Eg5 at centrosomes and is necessary for subsequent centrosome separation and timely mitosis".

Minor Page 3, O'regan should be O'Regan. Page 11, second paragraph, "these..." should be "those present in exponentially growing...".

Referee #2:

I appreciate very much the authors' responses to my comments and believe that the manuscript is improved. I just am frustrated by their comment that the samples they analyzed by MS possibly contained mostly inactive Nek9. I don't see the importance on the description of the phosphorylation sites on the inactive Nek9. Phosphorylation sites on active Nek9 needs to be analyzed. Other responses are fine. I also would like to add there are still some redundant descriptions that could be polished up (i. e. page 5, 9 lines at the bottom) and mistyping (i.e. Nek9[S869)]).

2nd Revision - Authors' Response 05 May 2011

Referee #1

1. In figure 6, the constitutively active form of Nek9 is abbreviated differently than in the text; this should be changed.

In the text of our manuscript we refer to the constitutively form of Nek9 as "Nek9 [Δ346-732]". In our figures to simplify the labeling we have used "Nek9 RCC1" to refer to the same Nek9 form. We noted this in the legend to Figure 5 ("Nek9 RCC1, Nek9[Δ346-732]"); to avoid any confusion, we now have also added a similar text to the legends of Figure 6 and Figure 8B. Additionally we have changed the initial description of Nek9[Δ346-732] in the text to "a constitutively active form of the kinase that lacks the autoinhibitory RCC1 domain" (page 9).

2. In Figure 9, and the text that discusses this figure, I was confused regarding the proportion of cells expressing Eg5S1033A that were in prometaphase. The authors state that cells entered prometaphase at a rate similar to controls (63% at 60 min) and that at 60 mins more than 57% were in prometaphase. The graph looks like the value is about 57% for the prometaphase panel. Please clarify.

This was our error and we have corrected it in the text. 63% of cells expressing Eg5[S1033A] were in prometaphase at 30 min while more than 57% remained at this phase at 60 min. Thus "Cells transfected with Eg5 siRNAs plus Eg5[Ser1033Ala] entered prometaphase at a similar rate than control cells (63% at 60 min)" should be "...than control cells (63% at 30 min)".

3. In the abstract, I would change the phrase "is necessary for the accumulation of Eg5 around centrosomes......" to "contributes to the accumulation of Eg5 at centrosomes and is necessary for subsequent centrosome separation and timely mitosis".

We have changed the sentence.

4. Page 3, O'regan should be O'Regan.

We have corrected this.

5. Page 11, second paragraph, "these..." should be "those present in exponentially growing...".

We have corrected this.

Referee #2

1. I just am frustrated by their comment that the samples they analyzed by MS possibly contained mostly inactive Nek9. I don't see the importance on the description of the phosphorylation sites on

the inactive Nek9. Phosphorylation sites on active Nek9 needs to be analyzed. Other responses are fine.

We are also somehow frustrated by the lack of phosphopeptides corresponding to fully active Nek9 in our analysis. We attribute this (at least partially) to the low cellular abundance of active Nek9, and we are presently trying to devise ways to overcome it. Nevertheless, we are convinced of the importance of the MS data described in our manuscript, as it establishes the phosphorylation state of the predominant form of Nek9 in mitotic cells (as opposed to exponentially growing cells), together with our in vitro data suggests that Nek9 is a bona fide CDK1 substrate, and provides a basis to understand the activation mechanism of the kinase.

2. I also would like to add there are still some redundant descriptions that could be polished up (i. e. page 5, 9 lines at the bottom) and mistyping (i.e. Nek9[S869)]).

We have corrected the mistyping and modified the sentence in page 5. We have also tried to identify and modify redundant, long or unclear descriptions in the manuscript:

Page 7: we have changed "a phosphospecific antibody that specifically recognizes Nek9[Thr210-P]" to "a phosphospecific antibody that recognizes Nek9[Thr210-P]"

Page 16: we have split the long initial sentence in the last paragraph: "...in these and other organisms Ser750 is conserved. We thus suggest..."

Page 17: we have split the long sentence ending the first paragraph: "...the absence of activation of the Nek9/Nek6/7 module. This results in lack of Eg5 phosphorylation at Ser1033...".

Page 17: we have changed the order of the last sentence: "Attesting to the redundancy of the two centrosome separation pathways and the robustness of the mechanisms that results in spindle bipolarity, cells that fail to phosphorylate Eg5 [Ser1033] remain longer in prometaphase, but for the most part reach metaphase (although with a marked delay) and progress to later mitotic phases

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