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Dynactin helps target Polo-like kinase 1 to kinetochores via its left-handed beta-helical p27 subunit

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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.)

Editor: Bernd Pulverer

1st Editorial Decision 16 March 2012

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by two referees whose comments are shown below.
I am afraid that we will not be able to publish the manuscript, at least in its current form.

While both referees affirm some potential interest of the dataset, they both raise multiple largely consistent issues that would require major experimental revision. We appreciate that this may well be beyond the scope of the project and note that such large scale revision is at odds with our policy to undertake only a single, finite round of experimental revision.

In brief, referee 1 finds the phenotypic analysis superficial and suggests that the structural analysis lacks context. The experimental suggestions that would have to be addressed in depth are:
> extend localization analysis in the presence of nocodozole (partially done already).
> test PBIP1.
> show Cdk1 activity-dependent binding of p27 to Plk1 in vitro.
> remove kinetochore dynein through spindly depletion and look at Plk1, Mad1 and p27 kinetochore localization.
> mitotic index is not enough - extend to live imaging.
> controls for rescue experiment: Westerns and ideally use single copy expression system.
> based on the structure, mutate hydrophobic face.

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> does purified p27 form dimers/oligomers/heterodimers with p25 in vitro?
The referee notes in general that more primary data must be included, including data that may be contained in the cited 'Imai et al., in preparation' study.
The referee also suggest an elegant and potentially rather definitive experiment that we would like to encourage: tether p27 in an ectopic location away from rest of dynactin complex and assess if Plk1 is recruited there.

Referee 2 notes that the novelty is somewhat reduced by the Lowery et al. (2007) study.
The referee goes on to note a lack direct evidence for Plk1 binding to p27/p25.
Like referee 1, s/he state that the structure is not publishable without the Plk1 binding site and further downstream analysis.
> need evidence for p27/p25 heterodimer - do both bind Plk1? - be more clear about Plk1 interaction site.
> need actual data for mitotic defect in the p27 RNAi experiment. Show Plk1 at spindle poles and cytokinetic bridges. Show life imaging.
Again, the referee notes that data from Imai et al. would need to be included in a revision.
The referee also raises a caveat: why does p27 not have a major role in Plk1 recuitment, and yet have a strong overall phenotype?

Should you be able to address these criticisms in full, ideally with the tether experiment which would raise level of the dataset significantly, we could consider a revised manuscript. As noted, it is EMBO Journal policy to allow a single round of revision only and acceptance or rejection of the manuscript will depend on the completeness of your responses in this revised version. I do realize that addressing all the referees' criticisms will require a lot of additional time and effort and be technically challenging. I would therefore understand if you wish to publish the manuscript rapidly and without any significant changes elsewhere, in which case please let us know so we can withdraw it from our system.

If you decide to revise the manuscript for the EMBO Journal, please include a detailed point-by-point response to the referees' comments. Please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website:
http://www.nature.com/emboj/about/process.html

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

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

REFEREE REPORTS

Referee #1

This manuscript reports the phenotype of depleting the dynactin p27 subunit and also described the atomic structure of this subunit. The major conclusion from the manuscript is that p27 may provide one of several binding sites for the kinase Plk1 at kinetochores. The data reported here have potential to be published in EMBO J. but the current version of the manuscript needs significant reworking, including new experiments, prior to any further consideration. The two major problems are that the phenotypic analysis is superficial and the structure is appended on at the end and lacks context. Comments below may help address these concerns.

1) The authors conclude from the p27 RNAi experiments that:
i) levels of Plk1 and Mad1 are reduced at kinetochores
ii) mitosis is accelerated

iii) there are potentially attachment defects causing chromosome alignment problems

All of these conclusions need significant strengthening.

First, the quantitative localization analysis needs to be performed in nocodazole - this helps discriminate between loss of a binding site versus other more complex effects that are observed for kinetochore proteins. The degree of arrest must first be verified by measuring the mitotic index. This should be straightforward to do for Plk1 and Mad1 (I know they show Plk1 in nocodazole in the supplement but this needs to be a primary figure with images included). The authors also do not test PBIP1, which has been described as a self-primed Plk1 anchor at kinetochores (Kang et al. 2006, cited by the authors). They need to quantify kinetochore localization of PBIP1 in the p27 depletion. Also, as p27 is well-expressed in bacteria, Cdk1-actiivty dependent binding to the Polo box should be assessed in vitro - such experiments would directly test whether p27 phosphorylated by Cdk1 provides a Plk1- binding site. A strong test for p27 acting as a direct anchor for Plk1 in vivo would be to artificially tether it to a different location independently of the rest of the dynactin complex and test if Plk1 gets mislocalized - such experiments are feasible (e.g. using Lac repressor fusion proteins in cell lines with a single integrated Lac operator array) but may be beyond the scope of a revision.

On a related note, the reduction in the levels of Mad1 or Plk1 at the kinetochore are not specific to p27 depletion. The data in Fig.3 suggest Mad1, Plk1 and the 3F3/2 epitope are all reduced if any of the dynactin subunits are knocked down. The reduction is strongest for the p150 depletion which also prevents dynein recruitment. This result is a bit surprising for Mad1 given prior published work. Two potential interpretations are that i) other dynactin subunit inhibitions remove p27 from the kinetochore - is this correct? ii) there may be an effect of altering dynein/dynactin levels at the kinetochore independently of p27. To distinguish these possibilities, the authors should remove kinetochore dynein by another means (e.g. Spindly depletion) and test if they also observe a similar reduction in Plk1 and Mad1. They also need to clearly describe what happens to p27 kinetochore localization in the various perturbations.

Second, the phenotype of the p27 depletion must be described by live imaging. From the presented data it is difficult to assess the effects of p27 depletion on cell division. A reduced mitotic index is not sufficient to conclude that mitosis is accelerated (the depletion may instead affect the rate of mitotic entry due to a problem in interphase). The only way to conclude that mitosis is accelerated is to quantify the nuclear envelope breakdown to anaphase onset timing in ~100 cells from live imaging analysis of cell expressing fluorescent histone H2b - this is standard in the field.

Third, the attachment and alignment defect data must be accompanied by galleries of images - at the resolution of light microscopy it is not straightforward to confidently assign end-on versus lateral attachments and precisely how these were scored needs to be described. Without primary data it is not possible to evaluate the conclusions.

Finally, the rescue experiments with WT vs T186A mutant protein must include evidence that both proteins are expressed equivalently and target normally - ideally the rescue should be performed using single-copy transgenes (e.g. using the Flp-In system from Invitrogen); however, proper blotting and immunostaining controls with transient transfection would be acceptable. These controls are essential to establish the key result of the paper.

2) The crystal structure appears as an afterthought and no functional tests in vivo or in vitro are performed. Given the authors' model (that the highly hydrophobic face is used to form a dimer with p25) and the available rescue system for p27, the authors should mutate some of the hydrophobic residues, test if the mutant proteins are expressed / localized normally, and compare p27 and p25 localization. With respect to in vitro followup, what is the oligomeric state of the purified p27? Is it a homodimer using light-scattering or analytical ultracentrifugation? Has co-expression of p25 & p27 been attempted? Co-expression would directly test if heterodimers are formed and enable mutational analysis in vitro. If p25 fails to be expressed in bacteria an alternative strategy would be to express p25 using in vitro translation and use the purified p27 to analyze binding (this would only be feasible if p27 does not form a tight heterodimer). Some efforts to develop insight from the crystal structure are needed to integrate this interesting piece of data into the paper.

Other comments:

1) The title is a too broad & uninformative. Something along the lines below would be better:
"The p27 subunit of dynactin forms a left-handed beta helix and provides a binding site for Plk1 at kinetochores"

2) Experimental details are minimal and makes the functional analysis difficult to decipher. As noted above, primary data must be included.

Referee #2

This paper describes a role for the dynactin subunits p27 and p25 in cdk1 phosphorylation and PLK1 recruitment. p27/p25 RNAi has a very limited effect on dynactin function, but results in reduced PLK1 levels at kinetochores and premature anaphase onset. Structural analysis of p27 reveals a monomer with a rare LBH fold.

This is a very interesting paper, and the novel aspects of the reported p27 RNAi effect are intriguing. However, the paper has a number of serious problems:

1. The identification of the Plk1-binding site in p27 was apparently predicted by prior sequence analysis (Lowery et al., 2007), somewhat reducing the novelty of the current finding.

2. More important, a direct demonstration of Plk1 binding to p27/p25 binding is lacking, and, therefore, verification of the predicted site is indirect and incomplete.

3. The crystallographic analysis does little to advance our understanding of the main thesis of this paper, that p2 serves as a novel kinetochore anchoring site for PLK1. To be included in this paper, the structural analysis would need to include the Plk1-binding region. The structural work in its current state is out of place in the study.

4. An additional paper "in preparation" is referenced on several issues: the lack of effect of p27/p25 removal from the dynactin complex on its structural integrity, the loss of the entire dynactin molecule through Arp11 RNAi, and the coordinate knockdown of p27 and p25 peptides. These issues are important to the current study and should be included.

5. The severity of the mitotic phenotype would seem to suggest a major role for p27/p25 in recruitment of PLK1 to kinetochores relative to other recruitment factors. Is this the case? If so this point should be addressed in the manuscript.

6. The relationship between p27 and p25 sequences needs to be described or shown. Among other questions, is the PLK1 binding site conserved between the two genes? How is it known that p27 and p25 form an obligate heterodimer rather than a mixture of hetero- and homodimers? They are referred to as part of the pointed end complex - is this based on cofractionation analysis? Is the Plk1-binding sequence in p27 conserved in p25? Presumably this sequence is related to the Polo box? What is the consensus sequence for Plk1? Which part o f dynactin has been shown to be "part of the polo-binding box interactome - is it p27? If so, this information needs to be presented up front in the paper, so that it is evident that the authors are verifying a predicted site, rather than discovering a new one.

7. How might the free pool of p27/p25 contribute to the mitotic effects reported in this manuscript? Is this a sizeable fraction of total p27/p25?

8. The TBH fold can't be unique if it has been found in bacteria

9. The spots in Fig. 1A should be better labeled to indicate which are thought to be phosphorylated vs. unmodified.

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10. Images of spindle defects, chromosome misalignment, end-on vs. lateral or no attachment, are crucial to the interpretation of the RNAi effects, and should be provided in substantial detail for Fig. 2 and 3.

11. The previous reports that Bub1, BuBR1 etc. don't require dynein or dynactin for kinetochore localization should be cited.

12. Plk1 at spindle poles and cytokinetic bridges is never shown and needs to be, particularly as the only real control for the specificity of p27 RNAi for kinetochore Plk1 binding.

13. In Fig 3K, why is Mad1 reduced by 80% in Arp11 and p150Glued siRNA-treated cells? These results imply only a small reduction in kinetochore dynein activity, despite the severe reduction in dynein IC staining in Fig. 2.

14. p12 - Plk1 doesn't target directly to dynein.

15. The authors' model for Plk1 function doesn't make sense. Plk1 is said to keep dynein in an "idling" state during prometaphase, but this contradicts functional evidence for dynein activity during chromosome alignment.

16. In order to know that reduced mitotic index is due to premature anaphase onset, live imaging should be performed on p27 siRNA-treated cells.

1st Revision - authors' response 26 July 2012

Author response to critiques:

First of all, we would like to thank the Editor for his support of this manuscript and the two reviewers for their extremely fair, constructive and useful critiques. Much of the experimental data requested was already in hand and much more primary data (i.e., micrographs) has been added. In response to the critiques we conducted two major new experiments that we agree added a great deal: (1) live-cell imaging to directly observe mitotic progression and (2) *in vitro* binding between Plk1 (specifically, its Polo-binding domain, or PBD) and purified, *in vitro* phosphorylated p27/p25 heterodimers.

It was also clear from the critiques that the original manuscript was overly brief and not organized in a way that told the story as clearly as it might have. We have completely reworked the treatment by adding much more primary data, the two new experiments mentioned above, and reorganizing the order of presentation. We think the revised manuscript is significantly improved by these changes and are very pleased to present it to you.

Please note: Our response to the Editor's summary of the required changes is included at the very end of this document.

Referee 1:

We would like to thank the referee for his/her overall support of this manuscript, the thorough evaluation and very useful suggestions.

Overview: The two major problems are that the phenotypic analysis is superficial and the structure is appended on at the end and lacks context.

The original submission was excessively streamlined and most of the primary immunolocalization data was distilled into bar graphs that quantified the phenomena we were describing. This gave an erroneous impression that our analysis was superficial, which could not have been farther from the truth. We have reconstructed all the figures to include original data images and quantitation, and have added new figures to the supplement to document additional controls. The paper now contains 7 figures (up from 5) and 6 supplemental figures (up from 1).

We completely reorganized the paper in the interest of telling a more compelling story. The structural work is now fully integrated into the body of the paper to make the context more clear.

The quantitative localization analysis (of Plk1 and Mad1) needs to be performed in nocodazole - this helps discriminate between loss of a binding site versus other more complex effects that are observed for kinetochore proteins. The degree of arrest must first be verified by measuring the mitotic index. This should be straightforward to do for Plk1 and Mad1 (I know they show Plk1 in nocodazole in the supplement but this needs to be a primary figure with images included).

We have examined the localization of both proteins in nocodazole-treated cells and see no significant difference in kinetochore intensity compared with untreated controls. These data (images and quantitation) have been added to Figures 6 and 7.

The authors also do not test PBIP1, which has been described as a self-primed Plk1 anchor at kinetochores (Kang et al. 2006, cited by the authors). They need to quantify kinetochore localization of PBIP1 in the p27 depletion.

We thank the reviewer for this suggestion. We see no change in PBIP1 localization (Supplemental Figure 3B), reinforcing the idea that Plk1 can bind p27 directly.

Also, as p27 is well-expressed in bacteria, Cdk1-actiivty dependent binding to the Polo box should be assessed in vitro - such experiments would directly test whether p27 phosphorylated by Cdk1 provides a Plk1- binding site.

This important experiment is now included as Figure 3F.

A strong test for p27 acting as a direct anchor for Plk1 in vivo would be to artificially tether it to a different location independently of the rest of the dynactin complex and test if Plk1 gets mislocalized - such experiments are feasible (e.g. using Lac repressor fusion proteins in cell lines with a single integrated Lac operator array) but may be beyond the scope of a revision.

Given the strength of the new in vitro evidence that phosphorylated p27 can bind the PBD directly and specifically (new Figure 3F), we elected not to perform this experiment.

On a related note, the reduction in the levels of Mad1 or Plk1 at the kinetochore are not specific to p27 depletion. The data in Fig.3 suggest Mad1, Plk1 and the 3F3/2 epitope are all reduced if any of the dynactin subunits are knocked down. The reduction is strongest for the p150 depletion which also prevents dynein recruitment. This result is a bit surprising for Mad1 given prior published work. Two potential interpretations are that i) other dynactin subunit inhibitions remove p27 from the kinetochore - is this correct?

We apologize for the confusion. Yes, all of the siRNA treatments used here remove p27 from kinetochores. Arp11 siRNA depletes cells of dynactin (including p27), so these proteins are no longer available to bind kinetochores. Depletion of $p150^{Glued}$ causes dynactin to be lost from kinetochores, so once again p27 is lost. In all cases, loss of p27/dynactin from kinetochores results in loss of Plk1.

To put it another way, Arp11 and $p150^{Glued}$ depletion have "broad stroke" inhibitory effects, whereas p27 depletion is an incisive, selective tool for looking at just the function of p27 and p25. The strength of this study is the fact that we are able to remove p27 and Plk1 from kinetochores while leaving dynein and dynactin in place and not interfering with spindle pole focusing or initial kinetochore engagement with MTs. This makes our results easy to interpret because there are no concerns about possible the downstream effects of loss of dynein and dynactin from the spindle.

The effects we see on Mad1 localization are a bit more complicated. Metaphase kinetochores in cells depleted of Arp11 or $p150^{Glued}$ have higher levels of Mad1 than controls because the cells have reduced levels of dynein at their kinetochores, so there is less "stripping". What is most interesting is the fact that Mad1 behaves differently when p27 is depleted as compared to depletion of Arp11 or $p150^{Glued}$. In this case, metaphase kinetochore levels of Mad1 are normal, but prometaphase

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kinetochore levels are reduced relative to controls. The fact that we only obtain this finding under conditions where kinetochores have normal steady state levels of dynein and dynactin, but reduced levels of Plk1 is the most likely explanation of the spindle assembly checkpoint bypass we observe. Plk1 phosphorylation of some kinetochore component (e.g., dynein itself or Mad1 itself) is necessary for normal Mad1 recruitment and/or retention on unattached kinetochores. If this does not occur, the spindle assembly checkpoint is bypassed. This is now clearly explained the text.

ii) there may be an effect of altering dynein/dynactin levels at the kinetochore independently of p27. To distinguish these possibilities, the authors should remove kinetochore dynein by another means (e.g. Spindly depletion) and test if they also observe a similar reduction in Plk1 and Mad1.

We thank the reviewer for this suggestion. Spindly depletion correlates with loss of dynactin (we evaluated multiple subunits, including p27), dynein, Plk1 and Mad1. This is as expected given the loss of dynactin (Supplemental Figure 4).

They also need to clearly describe what happens to p27 kinetochore localization in the various perturbations.

This is now shown in Figure 2A (micrographs) and B (quantitation).

Second, the phenotype of the p27 depletion must be described by live imaging. From the presented data it is difficult to assess the effects of p27 depletion on cell division. A reduced mitotic index is not sufficient to conclude that mitosis is accelerated (the depletion may instead affect the rate of mitotic entry due to a problem in interphase). The only way to conclude that mitosis is accelerated is to quantify the nuclear envelope breakdown to anaphase onset timing in ~100 cells from live imaging analysis of cell expressing fluorescent histone H2b - this is standard in the field.

We fully recognize that the only way to draw conclusions about mitotic progression is to observe cells going through mitosis, so we thank the reviewer for encouraging us to do this important experiment. We imaged mitotic cells using phase contrast microscopy (which we and others have found to be less perturbing than fluorescence) and found the difference between control and p27/p25 depleted cells to be quite striking – the latter have a minimal metaphase, which shortens the time from NEBD to anaphase by about 10 minutes. The data are included in Figure 1E and F and Movies.

Third, the attachment and alignment defect data must be accompanied by galleries of images - at the resolution of light microscopy it is not straightforward to confidently assign end-on versus lateral attachments and precisely how these were scored needs to be described. Without primary data it is not possible to evaluate the conclusions.

All the figures illustrating mitosis-specific cellular phenomena (Figure 1: mitotic progression, Figure 2: kinetochore composition, Figure 6: Plk1 binding, Figure 7: functional effects on kinetochores; the experiments to which the referee refers) now contain images as well as quantitation. In addition, we have included 3 new supplemental data figures that are rich in micrographs.

Finally, the rescue experiments with WT vs T186A mutant protein must include evidence that both proteins are expressed equivalently and target normally - ideally the rescue should be performed using single-copy transgenes (e.g. using the Flp-In system from Invitrogen); however, proper blotting and immunostaining controls with transient transfection would be acceptable. These controls are essential to establish the key result of the paper.

We are well aware of the caveats surrounding protein overexpression (particularly of dynactin subunits), so all rescue experiments used a plasmid that yields expression of the target protein at near endogenous levels via the actin promoter. We have verified that expression levels are equivalent on immunoblots. We have verified that both wild type and the T186A variant are incorporated into dynactin as shown (Supplemental Figure 1), and they don't associate with kinetochores in the absence of dynactin (Supplemental Figure 2), indicating that they target normally. Supplemental Figure 1 also includes data showing coordinate depletion of p27 and p25

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and the fact that p27 expression rescues p25 protein levels, addressing one of the concerns of Referee 2.

The crystal structure appears as an afterthought and no functional tests in vivo or in vitro are performed. Given the authors' model (that the highly hydrophobic face is used to form a dimer with p25) and the available rescue system for p27, the authors should mutate some of the hydrophobic residues, test if the mutant proteins are expressed / localized normally, and compare p27 and p25 localization.

The crystal structure is not an afterthought, but a complementary effort to address structure-function relationships in the complex. We did not anticipate that the phosphorylation site will be within a disordered fragment, but that should not divert attention from the fact that we now have structural data for the main domain of p27.

We elected not to do an extensive mutagenesis study as we feel it goes too far beyond the scope of the present paper.

With respect to in vitro followup, what is the oligomeric state of the purified p27? Is it a homodimer using light-scattering or analytical ultracentrifugation? Has co-expression of p25 & p27 been attempted? Co-expression would directly test if heterodimers are formed and enable mutational analysis in vitro. If p25 fails to be expressed in bacteria an alternative strategy would be to express p25 using in vitro translation and use the purified p27 to analyze binding (this would only be feasible if p27 does not form a tight heterodimer). Some efforts to develop insight from the crystal structure are needed to integrate this interesting piece of data into the paper.

We have carefully analyzed p27 in solution and we determined that it is a monomer. We discussed co-expression of p25/p27 in the original submission and we refer to this effort explicitly in the structural section of the revised manuscript. Co-expression of p27 and p25 was used to generate heterodimers for in vitro phosphorylation and PBD-binding experiments (Figure 3). The problem is not that p25 fails to be expressed, but that it is somewhat unstable and is highly dependent on p27. The assay that is proposed is not likely to yield unequivocal data because isolated p25 cannot be used for this purpose. We simplified and qualified the description of the model in the revised version.

Other comments:

1) The title is a too broad & uninformative. Something along the lines below would be better: "The p27 subunit of dynactin forms a left-handed beta helix and provides a binding site for Plk1 at kinetochores"

Thank you for the suggestion. We have changed the title to make it more descriptive.

2) Experimental details are minimal and makes the functional analysis difficult to decipher. As noted above, primary data must be included.

Considerably more primary data is included now, and more experimental details have been added to the Figure legends and the Methods.

Referee #2:

We appreciate the general interest voiced by the referee and regret that the abbreviated form of the original submission had so many problems. We feel we have rectified all of them and hope the referee agrees.

1. The identification of the Plk1-binding site in p27 was apparently predicted by prior sequence analysis (Lowery et al., 2007), somewhat reducing the novelty of the current finding.

This is not correct and we apologize for the confusion. Our findings are completely novel. The Plk1 interactome published by Lowery and coworkers contained two dynactin subunits, p150^{Glued} and dynamitin. It did not contain p27 or any other dynactin subunits. The interactome results suggested

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that dynactin is somehow associated with Plk1, but did not demonstrate that the interaction was direct, nor did it identify p27 as the actual binding partner.

2. More important, a direct demonstration of Plk1 binding to p27/p25 binding is lacking, and, therefore, verification of the predicted site is indirect and incomplete.

As detailed above, we have demonstrated that phospho-p27 can bind the polo binding domain directly and specifically and have included this analysis as Figure 3F.

3. The crystallographic analysis does little to advance our understanding of the main thesis of this paper, that p2 serves as a novel kinetochore anchoring site for PLK1. To be included in this paper, the structural analysis would need to include the Plk1-binding region. The structural work in its current state is out of place in the study.

Elucidation of the atomic structure of any new protein is always informative. It so happens that the phosphorylation site is within a disordered fragment, what shows that it is easily accessible to the kinase. The elucidation of the structure opens new avenues of investigation not only in this paper, but also for others in the field. The structure and functional studies are complementary and we have made an effort to revise the manuscript to make this come across more effectively.

4. An additional paper "in preparation" is referenced on several issues: the lack of effect of p27/p25 removal from the dynactin complex on its structural integrity, the loss of the entire dynactin molecule through Arp11 RNAi, and the coordinate knockdown of p27 and p25 peptides. These issues are important to the current study and should be included.

Our paper describing the biochemical effects of dynactin subunit depletion was a large study by itself. It has been accepted with minor revisions by Molecular Biology of the Cell and will be published shortly. We have provided the manuscript in the supplement.

5. The severity of the mitotic phenotype would seem to suggest a major role for p27/p25 in recruitment of PLK1 to kinetochores relative to other recruitment factors. Is this the case? If so this point should be addressed in the manuscript.

Our results clearly indicate that Plk1 recruitment mediated by p27 is essential for proper checkpoint function. Plk1 binding to p27 on dynactin provides a mechanism for bringing Plk1 close to dynein and other proteins in the immediate vicinity. The fact that loss of this pool of Plk1 from kinetochores has such a profound effect on mitotic progression highlights the importance of this particular Plk1 recruitment mechanism in checkpoint control. This is now discussed clearly in the text.

6. The relationship between p27 and p25 sequences needs to be described or shown. Among other questions, is the PLK1 binding site conserved between the two genes? How is it known that p27 and p25 form an obligate heterodimer rather than a mixture of hetero- and homodimers? They are referred to as part of the pointed end complex - is this based on cofractionation analysis? Is the Plk1-binding sequence in p27 conserved in p25? Presumably this sequence is related to the Polo box? What is the consensus sequence for Plk1? Which part o f dynactin has been shown to be "part of the polo-binding box interactome - is it p27? If so, this information needs to be presented up front in the paper, so that it is evident that the authors are verifying a predicted site, rather than discovering a new one.

We sincerely apologize for the lack of clarity. The p27 and p25 sequences are provided in Figures 4 and 5, respectively. They are vaguely similar, and owing to the LbH fold, the structure of the central part of p25 is predicted to be quite similar to p27. Despite this general similarity, p25 does not contain sites for Cdk1 phosphorylation and Plk1 binding (please note that the Plk1 binding site is usually requires the presence of a residue that has been phosphorylated by Cdk1). Our in vitro analysis verifies that p25 is not phosphorylated by Cdk1 in vitro, which means it cannot be "primed" for Plk1 binding. This is now clearly spelled out in the text.

p27 and p25 were shown to be part of a subcomplex of dynactin in our biochemical analysis of dynactin in Eckley et al, 1999. They copurify by gel filtration and ion exchange and other work from our lab indicates that they are always present in equal stoichiometries. Bacterially expressed

p27 is monomeric, whereas p25 is unstable on its own and can only be isolated in a complex with p27. All this evidence indicates that p27 and p25 form obligate heterodimers.

As mentioned above, p27 is NOT in the polo-binding interactome. The Plk1 binding site we describe here is completely new. This is now clearly explained.

7. How might the free pool of p27/p25 contribute to the mitotic effects reported in this manuscript? Is this a sizeable fraction of total p27/p25?

The free pool is somewhat variable, but always small (≤ 10%). We did two different types of experiment to show that the free pool does not underlie the mitotic effects: (1) express p27 in cells depleted of dynactin (via Arp11 depletion) or p150^{Glued} (these data are the p27 "rescue" bars in Figure 6F and G) and (2) boost free p27 levels in control cells by expressing extra p27 (Figure 6F and G, royal blue bars, and Supplemental Figure 6). Neither treatment yields a mitotic effect and expression of just p27 in the absence of dynactin does not "rescue" anything, indidating that p27 is active only when it is incorporated into dynactin.

8. The TBH fold can't be unique if it has been found in bacteria

The LbH fold has indeed been seen in bacterial proteins, and we removed the adjective from the sentence in which it was used. In Fig 4 we refer to 'unique features' of the fold, not the fold itself.

9. The spots in Fig. 1A should be better labeled to indicate which are thought to be phosphorylated vs. unmodified.

We have done this. Thank you for the suggestion.

10. Images of spindle defects, chromosome misalignment, end-on vs. lateral or no attachment, are crucial to the interpretation of the RNAi effects, and should be provided in substantial detail for Fig. 2 and 3.

Thank you for the suggestion. We have done this (Figure 1 and 7). The spindle morphology defects are shown in our Molecular Biology of the Cell paper which will be published shortly.

11. The previous reports that Bub1, BuBR1 etc. don't require dynein or dynactin for kinetochore localization should be cited.

The citations are added.

12. Plk1 at spindle poles and cytokinetic bridges is never shown and needs to be, particularly as the only real control for the specificity of p27 RNAi for kinetochore Plk1 binding.

We appreciate the importance of this and have added images to accompany the quantitation (Figure 6A, B and C).

13. In Fig 3K, why is Mad1 reduced by 80% in Arp11 and p150Glued siRNA-treated cells? These results imply only a small reduction in kinetochore dynein activity, despite the severe reduction in dynein IC staining in Fig. 2.

Our data show that metaphase kinetochores that have normal levels of dynein (control and p27 depletion) have nearly undetectable Mad1 (Figure 7E). Kinetochores depleted of dynactin and dynein (Arp11 or p150^{Glued} depletion) have considerably more Mad 1 than this. To put it another way, if we normalized Mad1 staining to control metaphase kinetochores, cells depleted of Arp11 or p150Glued would have several fold higher Mad1. The large increase *relative to controls is the best indicator of the loss of dynein from kinetochores.*

14. p12 - Plk1 doesn't target directly to dynein.

The manuscript was largely rewritten and this phrasing was removed.

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15. The authors' model for Plk1 function doesn't make sense. Plk1 is said to keep dynein in an "idling" state during prometaphase, but this contradicts functional evidence for dynein activity during chromosome alignment.

We apologize for the lack of clarity of the model. A number of possible mechanisms are now proposed that involve known Plk1 targets that are thought to play important roles in the spindle assembly checkpoint. How Plk1 phosphorylation is proposed to alter dynein activity at kinetochores is now explained more clearly.

16. In order to know that reduced mitotic index is due to premature anaphase onset, live imaging should be performed on p27 siRNA-treated cells.

As stated above, we completely agree that this is an important addition and have included it (Figure 1E and F, Movies).

Editor overview:

extend localization analysis in the presence of nocodozole (partially done already).

Done (Figures 6H and I, 7G)

test PBIP1

Done (Supplemental Figure 3B)

show Cdk1 activity-dependent binding of p27 to Plk1 in vitro.

Done (Figure 3F)

remove kinetochore dynein through spindly depletion and look at Plk1, Mad1 and p27 kinetochore localization.

Done (Supplemental Figure 4)

mitotic index is not enough - extend to live imaging.

Done (Figure 1E and F, Movies)

controls for rescue experiment: Westerns and ideally use single copy expression system.

Rescue constructs are expressed at very close to endogenous levels, as verified by blotting (explained in text, p27 incorporates into dynactin (Supplemental Figure 1) and does not mistarget in the absence of dynactin (Supplemental Figure 2).

based on the structure, mutate hydrophobic face.

We elected not to do an extensive mutagenesis study as we feel it goes too far beyond the scope of the present paper.

does purified p27 form dimers/oligomers/heterodimers with p25 in vitro?

Yes, p27 and p25 form heterodimers. These were used in the in vitro phosphorylation and PBD binding experiments (Figure 3). This is now clearly explained in the text.

The referee also notes in general that more primary data must be included, including data that may be contained in the cited 'Imai et al., in preparation' study.

The revision contains much more primary data, both in the body of the paper and in the supplement. Our paper analyzing the biochemical effects of dynactin subunit depletion (formerly Yeh et al., in preparation) will be published soon in Molecular Biology of the Cell so the data will be readily

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available to readers; we have included the manuscript in the supplement. Direct demonstration that wild type p27 and the T186A variant both rescue p25 and are incorporated into dynactin is included (Supplemental Figure 1).

The referee also suggest an elegant and potentially rather definitive experiment that we would like to encourage: tether p27 in an ectopic location away from rest of dynactin complex and assess if Plk1 is recruited there.

The experiment we did to demonstrate that p27 can bind Plk1 directly in vitro (Figure 3F) is very convincing, so we elected not to perform this additional experiment.

Referee 2 notes that the novelty is somewhat reduced by the Lowery et al. (2007) study.

We believe this is an oversimplification, Our findings are completely novel. The Lowery study may have reported the presence of two dynactin subunits in the (very large) Plk1 interactome, but the authors did not demonstrate that Plk1 binds dynactin directly, nor did they identify p27 as a binding partner.

The referee goes on to note a lack direct evidence for Plk1 binding to p27/p25.

This is now addressed experimentally (Figure 3F).

Like referee 1, s/he state that the structure is not publishable without the Plk1 binding site and further downstream analysis.

Unfortunately, the Plk1-binding site is unstructured. The paper has been reorganized to weave the structure in better. We are of the view that the structure complements the functional part of the story well and will be useful and of interest to the community.

need evidence for p27/p25 heterodimer - do both bind Plk1? - be more clear about Plk1 interaction site.

Only p27 binds Plk1. This has been clarified.

need actual data for mitotic defect in the p27 RNAi experiment. Show Plk1 at spindle poles and cytokinetic bridges. Show life imaging.

We have done all this and the data are included in the body of the paper (Figure 1E and F, Figure 6A, B and C) with one exception: the effects of dynactin subunit knockdown on spindle morpohology will be published in our soon-to-be-published Molecular Biology of the Cell paper (see supplement).

Again, the referee notes that data from Imai et al. would need to be included in a revision.

Our other paper (Yeh et al., see supplement) will be published shortly.

The referee also raises a caveat: why does p27 not have a major role in Plk1 recuitment, and yet have a strong overall phenotype?

About half of Plk1 is lost, and this pool seems to be of particular importance for the spindle assembly checkpoint, because the checkpoint bypass phenotype is incredibly strong. We believe this is because dynactin p27 recruits Plk1 to exactly the site where it needs to work to assemble the checkpoint machinery, keep it stable and/or maintain its localization.

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2nd Editorial Decision 20 September 2012

Thank you for re-submitting your revised manuscript for consideration by the EMBO Journal. It has now been seen again by the two referees (their comments are shown below).

You will be pleased to see that the referees are in principle in favour of further considering this study, but a number of important issues have been raised:

1) Referee 1 and 2 both question if the checkpoint defect has been definitively shown in the absence of better live imaging. Indeed, referee 1 suggest and additional experiment with tagged histone H2b, which we would strongly encourage you to add in revision. Referee 2 notes problems with the data presentation in figure 1.
2) Referee 1 notes an important discrepancy of the spindly depletion
defect on Mad1 localization with a number of previous papers which would have to be addressed in detail within the manuscript.
3) Referee 2 requests a new experiment is added to show that p27
overexpression restores Mad1 levels at kinetochores. We appreciate that this experiment was not suggested explicitly in the last round and will therefore not enforce it addition beyond this encouragement.

Referee 1 added the following additional remarks informally after review, which help to clarify his/her concerns. Please note that referee 2 has commented to us that s/he strongly encourage addition of the H2b live imaging experiment suggested by referee 1.
.....
If the spindle checkpoint is indeed compromised, then one would expect live imaging to show that p27-depleted cells initiate anaphase in the presence of uncongressed chromosomes, which would lead to the lagging chromosomes in anaphase such as depicted in Figure 1C, bottom right. Whether this is the case is difficult to ascertain from the phase contrast movies, and the authors' own description of the movies is not very clear: "Instead of pausing in metaphase for a few minutes, the cells initiated anaphase as soon as the chromosomes had converged into a single mass" (page 5, line 8). Are the authors implying that all chromosomes managed to congress before anaphase onset? This would not be expected given the congression defect observed in the fixed cell data. What is needed is a more precise description of chromosome behavior during the accelerated mitosis of p27-depleted cells. If anaphase onset is observed in the presence of uncongressed chromosomes, the live imaging data would perfectly match the fixed cell data. Furthermore, since anaphase onset in the presence of uncongressed chromosomes is the hallmark of a defective spindle checkpoint, this would make their case very compelling. Although this type of analysis is best done by imaging fluorescent H2b, the phase contrast movies may be of sufficient resolution to judge the state of chromosome congression at anaphase onset. I agree with referee 2 that the green lines encircling the chromosomes in Figure 1 are not very helpful.
.....

Given the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of both reviewers.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

We would suggest that two months should be sufficient for this experimental revision. 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 deadline, please let us know in advance and we may be able to grant an extension.

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

REFEREE COMMENTS

Referee #1:

The authors have included more experiments that address the concerns I raised, and the additional data supports the main claims of the paper. Furthermore, the authors have reorganized the manuscript to improve the presentation of the data. Their efforts have strengthened the manuscript significantly, and I am confident that it would be received with considerable interest by the readers of EMBO J.

My main remaining issue rests with newly added live-imaging analysis. The authors filmed cells by phase contrast and observe that mitosis is accelerated in p27-depleted cells, as predicted by their fixed-cell data. However, it would be greatly preferable if analysis of the p27 depletion and one other dynactin subunit was conducted using a fluorescent H2b-expressing cell line. This would allow the observation of chromosome alignment in more detail and facilitate the interpretation of the defects documented by fixed cell analysis in Figures 1 and 7. Also, if anaphase onset in the presence of unaligned chromosomes was observed, it would provide compelling evidence for a checkpoint defect. Can this be ascertained from the phase-contrast images?

Minor points:

- The p27 tethering experiment I suggested was not done. Although I will not insist on it given that this is a substantial revision, I still think that this type of experiment could provide compelling in vivo evidence for a direct role of p27 in Plk1 recruitment, which would perfectly complement the beautiful new in vitro data of Figure 3F.

- The authors see reduced Mad1 localization at unaligned kinetochores after depletion of Spindly, which is known to delocalize dynein and dynactin from kinetochores. This is in agreement with their dynactin depletion data but contrasts with previous studies in human cells (Chan et al. JCB 2009, Gassmann et al. Genes Dev 2010, Barisic et al. MBC 2010), in which Spindly depletions were reported to have no effect on targeting of Mad1 or Mad2, which depends on Mad1 for kinetochore localization. The authors need to explain why their results contradict those of three independent studies.

- For the title, I would recommend something like below, as Plk1 is only partially reduced (~50%) and other Polo-targeting mechanisms are known.

"Dynactin uses its left-handed beta-helical p27 subunit to target A POOL of Polo-like kinase 1 to kinetochores"

Referee #2:

This paper is a resubmission of an earlier manuscript, which has been dramatically improved. The data are very convincing and should make for a very useful addition to the literature. There are a few points that still need attention.

1. Fig. 1. The authors attempt to distinguish between delayed chromosome alignment and premature anaphase onset. In panels C, bottom left, we see a clump of chromosomes supposedly representing the former, and at bottom right, lagging chromosomes. The first result implies that p27 RNAi does, in fact, interfere with congression, though the manuscript argues against this possibility, an issue that might be resolved by looking at disjunction of sister chromatids. Also, panels E are difficult to understand. The green line circumscribing chromosomes makes the raw image impossible to

interpret. Better and clearer time lapse images showing premature anaphase onset are important in documenting a particularly novel aspect of this work, and would improve the paper.

2. Have the authors tried to restore Mad1 levels at prometaphase kinetochores by p27 overexpression?

3. Hasn't p62 been partially characterized?

4. p4: "the first such tertiary fold.." presumably the authors mean "vertebrate" or "eukaryotic" rather than human.

5. 4, 6-7 lines from bottom, reference to Figure 1A and B is switched.

6. p4 and later: "~endogenous levels" seems incorrect. The authors probably mean that they see an effect only in high expressing cells.

7. p9: The Plk consensus binding site in p27 is at the C-terminal end of a predicted a-helix. Is this the case for Plk-binding proteins for which more detailed structural information is available?

8. p14 bottom: "dynein and dynactin are recruited to kinetochores via..." NudE and NudEL should be included in the list here and elsewhere.

9. p16: Top sentence is very unclear.

2nd Revision - authors' response 11 December 2012

Author response to critiques:

We would like to thank the Editor and the reviewers for their continuing support of this manuscript. The first revision was viewed very favorably and concerns were minimal, save one that was expressed by both reviewers: imaging of mitotic chromosome behavior in cells expressing FP-tagged histone 2B to see how well they congress prior to anaphase. We were of the shared view that this experiment was worthwhile, but had not yet performed it owing to concerns re. the notorious sensitivity to fluorescence illumination of cells in mitosis. We have now performed this experiment and obtained results that are in complete agreement with our previous conclusions based on fixed cell and phase contrast live cell imaging. We see uncongressed chromosomes in cells depleted of p27 and p25 which demonstrates unambiguously that congression does not go to completion prior to anaphase entry, consistent with some sort of checkpoint aberration. We also attended to all the minor issues raised by the two reviewers. We hope that all will agree that our study is now ready for publication.

Editor summary:
1) Referee 1 and 2 both question if the checkpoint defect has been definitively shown in the absence of better live imaging. Indeed, referee 1 suggest and additional experiment with tagged histone H2b, which we would strongly encourage you to add in revision. Referee 2 notes problems with the data presentation in figure 1.

H2B imaging has been performed and Figure 1E (previously phase contrast) was replaced with fluorescent images (H2B-mCherry).

2) Referee 1 notes an important discrepancy of the spindly depletion
defect on Mad1 localization with a number of previous papers which would have to be addressed in detail within the manuscript.

This has been addressed.

3) Referee 2 requests a new experiment is added to show that p27

overexpression restores Mad1 levels at kinetochores. We appreciate that this experiment was not suggested explicitly in the last round and will therefore not enforce it addition beyond this encouragement.

We appreciate the leeway and have elected not to perform this additional experiment.

Referee 1:
The authors have included more experiments that address the concerns I raised, and the additional data supports the main claims of the paper. Furthermore, the authors have reorganized the manuscript to improve the presentation of the data. Their efforts have strengthened the manuscript significantly, and I am confident that it would be received with considerable interest by the readers of EMBO J.

We very much appreciate the enthusiasm of the referee for our work.

My main remaining issue rests with newly added live-imaging analysis. The authors filmed cells by phase contrast and observe that mitosis is accelerated in p27-depleted cells, as predicted by their fixed-cell data. However, it would be greatly preferable if analysis of the p27 depletion and one other dynactin subunit was conducted using a fluorescent H2b-expressing cell line. This would allow the observation of chromosome alignment in more detail and facilitate the interpretation of the defects documented by fixed cell analysis in Figures 1 and 7. Also, if anaphase onset in the presence of unaligned chromosomes was observed, it would provide compelling evidence for a checkpoint defect. Can this be ascertained from the phase-contrast images?

[From informal comments to the editor] If the spindle checkpoint is indeed compromised, then one would expect live imaging to show that p27-depleted cells initiate anaphase in the presence of uncongressed chromosomes, which would lead to the lagging chromosomes in anaphase such as depicted in Figure 1C, bottom right. Whether this is the case is difficult to ascertain from the phase contrast movies, and the authors' own description of the movies is not very clear: "Instead of pausing in metaphase for a few minutes, the cells initiated anaphase as soon as the chromosomes had converged into a single mass" (page 5, line 8). Are the authors implying that all chromosomes managed to congress before anaphase onset? This would not be expected given the congression defect observed in the fixed cell data. What is needed is a more precise description of chromosome behavior during the accelerated mitosis of p27-depleted cells. If anaphase onset is observed in the presence of uncongressed chromosomes, the live imaging data wouldperfectly match the fixed cell data. Furthermore, since anaphase onset in the presence of uncongressed chromosomes is the hallmark of a defective spindle checkpoint, this would make their case very compelling. Although this type of analysis is best done by imaging fluorescent H2b, the phase contrast movies may be of sufficient resolution to judge the state of chromosome congression at anaphase onset. I agree with referee 2 that the green lines encircling the chromosomes in Figure 1 are not very helpful.

We analyzed mitotic progression in cells expressing histone 2B-mCherry and find that p27-depleted cells do indeed initiate anaphase in the presence of uncongressed chromosomes. After some discussion with kinetochore experts, we realize that a good explanation of our findings is that the cells have unresolved merotelic attachments, a condition that does not activate the checkpoint. Rather than causing the checkpoint to be bypassed, unresolved merotelic attachments allow the checkpoint to be effectively ignored. Formation of a large number of stable merotelic attachments in lieu of proper monotelic attachments (which take longer to form) might at least partly explain the accelerated mitotic progression we observe. We have also edited the text to more clearly explain our findings and discuss this additional scenario.

Minor points:

The p27 tethering experiment I suggested was not done. Although I will not insist on it given that this is a substantial revision, I still think that this type of experiment could provide compelling in vivo evidence for a direct role of p27 in Plk1 recruitment, which would perfectly complement the beautiful new in vitro data of Figure 3F.

We appreciate the reviewer's suggestion, but feel that we have already provided strong and compelling evidence for a direct role of p27 in Plk1 recruitment to kinetochores so we elected not to

perform this additional experiment.

The authors see reduced Mad1 localization at unaligned kinetochores after depletion of Spindly, which is known to delocalize dynein and dynactin from kinetochores. This is in agreement with their dynactin depletion data but contrasts with previous studies in human cells (Chan et al. JCB 2009, Gassmann et al. Genes Dev 2010, Barisic et al. MBC 2010), in which Spindly depletions were reported to have no effect on targeting of Mad1 or Mad2, which depends on Mad1 for kinetochore localization. The authors need to explain why their results contradict those of three independent studies.

Thank you for pointing out this apparent discrepancy. All our work involved cells treated with siRNAs for 72 h. We found that the experimental conditions used in the studies cited (48 h in siRNA) were not sufficient to fully deplete Spindly. The more extensive Spindly depletion we observe at 72 h may explain the discrepancy, a point we now discuss in the text. We would also like to point out that Spindly depletion did, in fact, result in loss of Mad2 in Chan et al., 2009 (Figure 5), a fact that is not emphasized by the authors.

In any case, it is clear that the mechanisms by which Mad1 and Mad2 are recruited to kinetochores are complicated and not yet fully unexplained. Given this, we feel any apparent discrepancy does not detract from our overall conclusions or the impact of our work.

- For the title, I would recommend something like below, as Plk1 is only partially reduced (~50%) and other Polo-targeting mechanisms are known.

"Dynactin uses its left-handed beta-helical p27 subunit to target A POOL of Polo-like kinase 1 to kinetochores"

We appreciate this suggestion and have retitled the manuscript, "Dynactin helps target Polo-like kinase 1 to kinetochores via its left-handed beta-helical p27 subunit"

Referee #2:

This paper is a resubmission of an earlier manuscript, which has been dramatically improved. The data are very convincing and should make for a very useful addition to the literature. There are a few points that still need attention.

Thank you for your appreciation of our efforts and out work.

1. Fig. 1. The authors attempt to distinguish between delayed chromosome alignment and premature anaphase onset. In panels C, bottom left, we see a clump of chromosomes supposedly representing the former, and at bottom right, lagging chromosomes. The first result implies that p27 RNAi does, in fact, interfere with congression, though the manuscript argues against this possibility, an issue that might be resolved by looking at disjunction of sister chromatids. Also, panels E are difficult to understand. The green line circumscribing chromosomes makes the raw image impossible to interpret. Better and clearer time lapse images showing premature anaphase onset are important in documenting a particularly novel aspect of this work, and would improve the paper.

We have always thought that p27/p25 depletion interferes with complete chromosome congression and proper spindle assembly, and we regret if previous versions of the manuscript were not clear about this. We agree that the phase contrast images provided in the first revision did not allow ready observation of individual chromosomes and were difficult to interpret. As suggested by R1, we have imaged cells expressing H2B-mCherry and find that anaphase initiates in cells that contain uncongressed chromosomes.

2. Have the authors tried to restore Mad1 levels at prometaphase kinetochores by p27 overexpression?

We have not attempted this experiment.

3. Hasn't p62 been partially characterized?

Yes, it has; apologies. Our earlier overly simple statement has been appropriately embellished.

4. p4: "the first such tertiary fold.." presumably the authors mean "vertebrate" or "eukaryotic" rather than human.

This has been corrected to read "vertebrate". (Most LBH structures are from prokaryotic proteins. This is the first animal Type I LBH structure to be solved. One insect Type II LBH (distinct, but similar) has been solved and one plant Type I LBH has been solved. Because we did not emphasize the difference between Type I and Type II, "vertebrate" is the most precise modifier.

5. 4, 6-7 lines from bottom, reference to Figure 1A and B is switched.

This has been corrected.

6. p4 and later: "~endogenous levels" seems incorrect. The authors probably mean that they see an effect only in high expressing cells.

siRNA-resistant p27 (WT or mutant) was exogenously expressed from the pCAGIG vector, which contains a relatively weak promoter. Quantitative blotting reveals that protein levels are similar to endogenous levels. We have attempted to clarify this in the text, and emphasize that exogenously expressed p27 is not present in excess and is fully incorporated into dynactin.

7. p9: The Plk consensus binding site in p27 is at the C-terminal end of a predicted a-helix. Is this the case for Plk-binding proteins for which more detailed structural information is available?

This does not appear to be a common theme. Plk1 binding sites are found at a variety of positions in proteins. Co-crystals with Plk1 are usually made using short peptides whose structures are extended in the crystals; their structural contexts are unknown.

8. p14 bottom: "dynein and dynactin are recruited to kinetochores via..." NudE and NudEL should be included in the list here and elsewhere.

This has been corrected.

9. p16: Top sentence is very unclear.

We agree and apologize. The description of how Plk1 phosphorylation is thought to affect kinetochore dynein behavior has been rewritten for clarity.

3rd Editorial Decision — 07 January 2013

Thank you for re-submitting your revised manuscript for consideration by the EMBO Journal. It has now been seen by referee 1, whose comments are enclosed.
We are pleased to accept this manuscript in principle for publication, after suitable revision.

As you will see, while the referee raises a number of substantial issues which can be addressed by textural revision, s/he remains in principle in favour of publication, pending satisfactory revision.

We also note that the scale bars are not always presented/defined (e.g. fig 1, 2). Please present statistical information as accurately as possible (e.g. note independent experiments where approaprite). We generally discourage 'data not shown' where data can be shown as supplementary information (e.g. p. 5).
We encourage the display of 'source data' underlying graphs and blots where appropriate and encourage you to submit this information upon revision for inclusion as 'Source Data' files within the figures.

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The structural data should be deposited at PDB and the accession number noted within the paper (see our guide to authors: http://www.nature.com/emboj/about/authors.html#a3.4).

When preparing your letter of response to the referee's comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community.

As a matter of policy, competing manuscripts published during the revisiom 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.

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

REFEREE COMMENTS

Referee #3 (Remarks to the Author):

The authors have addressed the issues I raised to my satisfaction, and I can recommend publication after the following points in the manuscript text have been addressed:

- In the results section, the authors should let the reader know up front what cell line they are using (right now this information is restricted to the methods section).

- For the new Figure 1E, statistics should be added in the figure legend, i.e. how many cells were filmed, and how many of those exhibited uncongressed chromosomes at anaphase onset?

- I assume that the numbers in the graph of Figure 1F are derived from the prior phase contrast imaging, not the new fluorescence imaging shown in Figure 1E. This should be clearly stated in the figure legend.

- Page 5, line 5:
"The combination of chromosome alignment defects and a reduced number of cells in mitosis suggests that cells are proceeding through mitosis more rapidly than normal, due either to lack of activation of the spindle assembly checkpoint in the face of defects or checkpoint bypass".

It is unclear what the authors mean by "checkpoint bypass" (also used on page 5, line 18; page 16, line 13) and, consequently, how that is different from a "lack of activation of the spindle checkpoint".

- Page 5, line 18:
"Treatment of cells with nocodazole was found to result in mitotic arrest (data not shown), indicating that the checkpoint was operational, however, it was not activated or bypassed in cells depleted of p27 and p25."

Were the p27-depleted cells also treated with nocodazole to check for mitotic arrest, or was this only done to control cells? Please clarify in the text.

- Page 11, lines 21-24:
"The reduction in Plk1 and 3F3/2 staining seen in cells depleted of dynactin subunits was not a downstream consequence of a loss of microtubule-dependent tension because similar results were observed in nocodazole-treated cells".

Since loss of tension actually generates the 3F3/2 epitope, reduced 3F3/2 staining cannot be a "downstream consequence".

- Page 13, lines 15-19:
"Depletion of p27/p25, by contrast, does not result in a loss of tension, as expected given that kinetochores in these cells still have normal steady state levels of dynein. This suggests that inhibition of Plk1 binding to dynactin at kinetochores does not cause accelerated mitotic progression

by altering the tension generating mechanism, but instead by interfering with the generation of and/or prematurely terminating the "wait-anaphase" signal."

The authors seem to imply that decreased inter-kinetochore tension would be an explanation for accelerated mitosis, but this is not so, because low tension actually engages the checkpoint.

- Page 14, lines 14-17:
"Although it has been reported that Spindly depletion has no effect on Mad1 (or Mad2) localization (Chan et al, 2009; Barisic et al, 2010; Gassmann et al, 2010), all previous studies relied on shorter depletion intervals (48h vs. 72h) which may explain the discrepancy."

Given that the experiments were performed in cell lines from different species (monkey COS7 cells in this study, human cells in the other studies), I don't think simply comparing the duration of the RNAi treatments is meaningful. Rather, RNAi treatment per se may be more efficient in COS7 cells in this instance, or there may be cell line and/or species-specific differences in how Spindly affects Mad1 recruitment.

- Page 17, lines 12-18:
"Attenuation of a Plk1-dependent switch might yield constitutively or prematurely motile dynein, which has the potential to impact the checkpoint in multiple ways. Dynein-based removal of Mad1 would prevent conversion of Mad2 from its autoinhibited form to the form that is incorporated into the MCC (Fava et al, 2011). Alternatively, constitutively active dynein might prematurely remove the fully assembled MCC, triggering anaphase. Finally, enhanced dynein activity would be expected to generate more tension at the kinetochore and increase intrakinetochore stretch which has been correlated with checkpoint satisfaction (Maresca & Salmon, 2009)."

It should be noted that the authors observe decreased Mad1 staining at kinetochores even when cells are treated with nocodazole, where there are no microtubules that dynein could use to strip Mad1 from kinetochores, so the explanations above seem a bit speculative.

3rd Revision - authors' response 10 January 2013

Author response to critiques:

Once again, thanks to the Editor and Referee for their careful attention to our manuscript and support of our work. We are happy to provide the additional information requested and have also modified the text to address the Referee concerns.

Editor comments:
We also note that the scale bars are not always presented/defined (e.g. fig 1, 2).

A scale bar was added to Figure 1. All the panels in Figure 2 are the same magnification so the scale bar in the lower right panel applies to all.

Please present statistical information as accurately as possible (e.g. note independent experiments where approaprite).

We have clarified this in the Figure Legends and Methods.

We generally discourage 'data not shown' where data can be shown as supplementary information (e.g. p. 5).

We now summarize the findings in question (mitotic indices under various conditions) in the text of the results and have added a new Supplemental Figure as well.

We encourage the display of 'source data' underlying graphs and blots where appropriate and encourage you to submit this information upon revision for inclusion as 'Source Data' files within the figures.

We have provided excel spreadsheets that include all the data summarized in the bar graphs as well as scans of the original blots.

The structural data should be deposited at PDB and the accession number noted within the paper.

The data were deposited in the PDB at the time of first submission and the accession number was provided in the supplement. We have now added the accession number to the legend of Figure 4 as well.

Referee (Remarks to the Author):

The authors have addressed the issues I raised to my satisfaction, and I can recommend publication after the following points in the manuscript text have been addressed:

In the results section, the authors should let the reader know up front what cell line they are using (right now this information is restricted to the methods section).

This is now clearly stated at the beginning of the results.

For the new Figure 1E, statistics should be added in the figure legend, i.e. how many cells were filmed, and how many of those exhibited uncongressed chromosomes at anaphase onset?

We added this information to the Figure Legend.

I assume that the numbers in the graph of Figure 1F are derived from the prior phase contrast imaging, not the new fluorescence imaging shown in Figure 1E. This should be clearly stated in the figure legend.

Apologies for the confusion: The numbers are from the new fluorescence imaging. The precise times are slightly different than those obtained by phase contrast imaging, but the trends are identical, adding confidence to our observations.

- Page 5, line 5:
"The combination of chromosome alignment defects and a reduced number of cells in mitosis suggests that cells are proceeding through mitosis more rapidly than normal, due either to lack of activation of the spindle assembly checkpoint in the face of defects or checkpoint bypass".

It is unclear what the authors mean by "checkpoint bypass" (also used on page 5, line 18; page 16, line 13) and, consequently, how that is different from a "lack of activation of the spindle checkpoint".

We realize our original phrasing was sloppy and appreciate having this brought to our attention. The sentence now reads, "due to lack of activation of the spindle assembly checkpoint in the face of defects (i.e., checkpoint bypass)".

We have deleted "or bypassed" from p. 5, line 19 so the sentence now reads, "…however, it was not activated in cells depleted of p27 and p25."

We have rephrased the sentence on p16, line 13 to read, "Instead, the checkpoint, if it is activated at all, appears to be prematurely satisfied……."

Page 5, line 18:
"Treatment of cells with nocodazole was found to result in mitotic arrest (data not shown), indicating that the checkpoint was operational, however, it was not activated or bypassed in cells depleted of p27 and p25."

Were the p27-depleted cells also treated with nocodazole to check for mitotic arrest, or was this only done to control cells? Please clarify in the text.

We determined the effects of nocodazole treatment under all four conditions (control, p27 knockdown, Arp11 and p150 knockdown). We now explicitly state in the Results the mitotic indices in control and p27 knockdown, and have added a graph showing all the data to the supplement.

Page 11, lines 21-24:
"The reduction in Plk1 and 3F3/2 staining seen in cells depleted of dynactin subunits was not a downstream consequence of a loss of microtubule-dependent tension because similar results were observed in nocodazole-treated cells".

Since loss of tension actually generates the 3F3/2 epitope, reduced 3F3/2 staining cannot be a "downstream consequence".

Apologies for the lack of precision. We have modified the text to read,
"The reduction in Plk1 and 3F3/2 staining seen in cells depleted of dynactin subunits reflected the intrinsic composition of the kinetochore because similar results were observed in nocodazole-treated cells in which microtubule-dependent tension has been eliminated".

Page 13, lines 15-19:
"Depletion of p27/p25, by contrast, does not result in a loss of tension, as expected given that kinetochores in these cells still have normal steady state levels of dynein. This suggests that inhibition of Plk1 binding to dynactin at kinetochores does not cause accelerated mitotic progression by altering the tension generating mechanism, but instead by interfering with the generation of and/or prematurely terminating the "wait-anaphase" signal."

The authors seem to imply that decreased inter-kinetochore tension would be an explanation for accelerated mitosis, but this is not so, because low tension actually engages the checkpoint.

Sorry for the ambiguity. This has been edited to read: "Depletion of p27/p25, by contrast, does not result in a loss of tension, as expected given that kinetochores in these cells still have normal steady state levels of dynein. This suggests that inhibition of Plk1 binding to dynactin at kinetochores does not alter the tension generating mechanism, but instead interferes with the generation of and/or prematurely terminates the "wait-anaphase" signal."

Page 14, lines 14-17:
"Although it has been reported that Spindly depletion has no effect on Mad1 (or Mad2) localization (Chan et al, 2009; Barisic et al, 2010; Gassmann et al, 2010), all previous studies relied on shorter depletion intervals (48h vs. 72h) which may explain the discrepancy."

Given that the experiments were performed in cell lines from different species (monkey COS7 cells in this study, human cells in the other studies), I don't think simply comparing the duration of the RNAi treatments is meaningful. Rather, RNAi treatment per se may be more efficient in COS7 cells in this instance, or there may be cell line and/or species-specific differences in how Spindly affects Mad1 recruitment.

Agreed. We changed this to read, "Although it has been reported that Spindly depletion has no effect on Mad1 (or Mad2) localization (Chan et al, 2009; Barisic et al, 2010; Gassmann et al, 2010), efficiency of knockdown or sensitivity of Mad1 recruitment to Spindly levels in the different cell lines used in these studies may explain the discrepancy."

Page 17, lines 12-18:
"Attenuation of a Plk1-dependent switch might yield constitutively or prematurely motile dynein, which has the potential to impact the checkpoint in multiple ways. Dynein-based removal of Mad1 would prevent conversion of Mad2 from its autoinhibited form to the form that is incorporated into the MCC (Fava et al, 2011). Alternatively, constitutively active dynein might prematurely remove the fully assembled MCC, triggering anaphase. Finally, enhanced dynein activity would be expected to generate more tension at the kinetochore and increase intrakinetochore stretch which has been correlated with checkpoint satisfaction (Maresca & Salmon, 2009)."

It should be noted that the authors observe decreased Mad1 staining at kinetochores even when cells are treated with nocodazole, where there are no microtubules that dynein could use to strip Mad1

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from kinetochores, so the explanations above seem a bit speculative.

We appreciate the Referee's concern, but have elected not to edit this further. Because dynein sits in close proximity to dynactin which makes it a likely substrate for dynactin-associated Plk1, and because we did not study Mad1 dynamics at kinetochores in any detail, we still feel it is reasonable to share with the reader our thoughts as to how altered dynein activity might contribute to the phenotypes we observe. We are of the view that the closing paragraph of the discussion is an acceptable place for a bit of speculation.

We would like to close by thanking, once again, the Editor and Referee for their thoughtful suggestions and attention to our work.

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