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Cdk1 promotes kinetochore bi-orientation and regulates Cdc20 expression during recovery spindle checkpoint arrest

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

1st Editorial Decision 19 May 2011

Thank you for submitting your interesting manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are shown below. I must apologize for the atypically slow refereeing process in this case.

Given the referees' overall positive recommendations, I would like to invite you to submit a substantially revised version of the manuscript, addressing the experimental points raised by referees 1 and 3. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

To be more specific, referee 1 thinks the experiments are well supported as far as they go, but that the relative contribution of both effects (i.e. functions of Cdk1 to maintain Cdc20 levels and to restrain spindle elongation through Ase1 phosphorylation) has to be experimentally tested. Following further discussion with the referee, the following experiments are recommended:

> perform the pGal1-MPS1 expression/release experiment in cells expressing pAdh1-CDC20. Is Cdk1 activity is required for SAC recovery?
> The nocodazole wash out could also be performed with pAdh1-CDC20 cells in the presence of ASE1, ase1-7A or ase1-7E/D.
> evaluate the importance of both mechanisms under different SAC activating conditions.

Referee 3 has suggested a rearrangement of the logical flow of the manuscript - this is not an editorial requirement and we defer to your judgement on this issue. This referee does suggest a number of important experimental issues. Most importantly:

> Effects of Cdk1 inhibition on cell cycle progression have to be monitored more rigorously and at
the suitable concentration of the inhibitor. 500 nM 1NMPP1 might be insufficient for full kinase inhibition; check whether cohesion is retained or lost when they use a higher concentration of 1NMPP1.

> use Cdc20 expression turn-off experiment throughout paper.
> test if cells lacking Cdk1 activity are prone to premature spindle extension in the Met-Cdc20 background.
> Is Cdh1 responsible for the declining levels of Cdc20?

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

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

Yours sincerely,

Editor
The EMBO Journal

REFEE REPORTS

Referee #1

Cdk1 turns off spindle checkpoint and regulates Cdc20 expression during recovery from mitotic arrest.

Liang et al. analyze in this manuscript the role of Cyclin dependent kinase (Cdk1) in the recovery of cells from SAC-induced metaphase arrest. Specifically, they ask whether Cdk1 has only an indirect role in preventing mitotic exit or, in addition, a more direct role in e.g. facilitating kinetochore biorientation. The data presented in this manuscript show convincingly that Cdk1 activity is required to establish kinetochore biorientation in response to nocodazole wash out. This is not because of a role of Cdk1 activity in activating the ability of kinetochores to interact with microtubules. Instead, premature elongation of the metaphase spindle in the absence of Cdk1 activity is probably one reason for the defect in the establishment of kinetochore biorientation. The authors show that the spindle midzone crosslinker Ase1 is under-phosphorylated in nocodazole treated cells. Wash out of nocodazole in the absence of Cdk1 activity therefore leaves Ase1 non-phosphorylated (this normally happens only in anaphase when the phosphatase Cdc14 becomes released from the nucleolus), which then prematurely recruits Cin8, as shown before by Khmelinskii et al. In a final set of experiments, the authors show that expression of CDC20 requires Cdk1 activity. This is on the level of transcription.

This is an interesting manuscript that describes two functions of Cdk1 in the recovery of SAC arrested cells. Cdk1 activity is required to maintain Cdc20 levels and to restrain spindle elongation through Ase1 phosphorylation. Both findings are important and justify publication in EMBO J. However, it could be interesting to evaluate the importance of both mechanisms under different SAC activating conditions. Complete microtubule depolymerization is quite extreme as pointed out by the authors. Are ase1-7A or pADH1-CDC20 synthetically lethal with mutants that activate the SAC (e.g. kinetochore mutants)?

Major point
1. The authors should perform the pGal1-MPS1 expression/release experiment in cells expressing pAdh1-CDC20. I wonder whether in this case Cdk1 activity is required for SAC recovery.

Minor points

1. Why do the authors need a telophase trap - they should explain the importance of the trap on p. 5.

The statement "to prevent mitotic exit" is not convincing because cells can be analyzed before they exit mitosis. In any case, spo12 cdc15-2 cells would have been the better telophase trap because of the role of SLK19 in anaphase spindle formation.

2. On p. 10, bottom, suddenly MPS1 overexpression pops up. What is the rational of this experiment? This is later explained in detail. However, I find it important that the rational behind an experiment is explained when it is described. I would present Figure S4A together with Figure 6.

3. According to a recent publication by the Uhlmann group, Cdc14 is important for the recovery from SAC induced arrest. However, in slk19Δ cdc15-2 cells Cdc14 remains inactive. How does this fit together?

Referee #2

In this study the authors show that Cdk1 activity is required for recovery from a spindle assembly checkpoint arrest because it is required to prevent spindle elongation and to maintain transcription of the Cdc20 gene. They further show that the targets of Cdk1 are likely to be Ase1, which when dephosphorylated recruits the Cin8 kinesin to elongate short spindles, and the Yox1 protein that inhibits the transcription of Cdc20. Overall the experiments are nicely performed and well controlled, but the authors should insert caveats on extending their results to mammalian cells in the discussion, given the differences in biology between yeast and animal cell mitoses. Moreover, the title is somewhat misleading since the authors do not show that Cdk1 turns off the spindle checkpoint, just that it is required to prevent spindle elongation that would otherwise dis-favour kinetochore attachment, and required to maintain Cdc20 levels that are required for mitotic exit. They should also clarify whether all the Cdk1 sites that they mutate in Ase1 have been shown to be phosphorylated, and modify the figure legends to state how many times each experiment was performed and how many cells were analysed in each experiment. The westerns all require molecular weight markers.

Referee #3

In this manuscript, Liang and co-workers establish a requirement for Cdk1 activity in spindle checkpoint recovery. They argue that Cdk1 activity is required to establish chromosome bi-orientation after nocodazole-induced mitotic arrest. They also argue that Cdk1 activity is important for transcriptional regulation of Cdc20.

This is a potentially interesting manuscript, but in its present form it lacks the level of rigor required to have a durable impact. The two effects described here upon interfering with the activity of Cdk1 have very different relevance. The observations on the role of Cdk1 in transcriptional control of the Cdc20 levels in mitosis are very interesting and should be expanded. The other effects (e.g. on bi-orientation and spindle elongation) might be a direct consequence of the interference with Cdc20 expression (e.g. they might result from the stabilization of factors that need to be degraded to get into metaphase and that cannot be degraded in the absence of Cdc20, etc.). In the absence of further experiments, their potential relevance remains unclear.

Specific points

The critique below follows the order of the presentation. As a general point, however, I feel that the paper would gain substantially from inverting the order of the presentation. The paper should begin with a description of the role of Cdk1 and Yox1 in the transcriptional regulation of Cdc20 during mitosis. The declining levels of Cdc20 provide a possible alternative explicable context for the rest of the observations, so that this issue should be sorted out as early as possible in the manuscript. For this, some additional experiments are required, as clarified below.

The other general concern is that the authors might not be using the right level of 1NMPP1 inhibitor, and are therefore achieving partial inhibition of the Cdk1 kinase. This is detailed below.
The authors synchronize cdc28-as1 slk19delta cdc15-2 cells in G1 and release them in nocodazole-containing medium. "After the arrest in metaphase...". Unless I am missing something, there should not be any metaphase at all because nocodazole was used. And indeed, Figure 1A shows that spindles are absent.

In Figure 1B, however, spindles appear to have reformed in the presence of 1NMPP1, which implies that cdc28 activity is not required for spindle formation or that the authors are not inhibiting the kinase to the desired level (i.e. fully, or at least 98%). In fact, 500 nM 1NMPP1 might be insufficient for full kinase inhibition, but it might be consistent with low-level kinase activity responsible for spindle assembly. This would be consistent with recent results from the Nurse laboratory in Nature. In this respect, the concept of "in the absence of Cdk1 activity", which is used at different points in the paper, needs to be rigorously revisited. The authors should use the maximal concentration of 1NMPP1 that does not give a mitotic phenotype in wild type cells. This control will exclude off-target effects of the inhibitor. Then they should repeat their experiments at this high concentration of the inhibitor and re-asses their phenotypes under this condition, i.e. a condition that most likely realizes the highest possible inhibition of Cdc28-as1 without creating dramatic off-target effects. That the authors are achieving sub-optimal inhibition of Cdk1 might be confirmed by the fact that their sisters retain cohesion (although this might be rather a consequence of the declining levels of Cdc20).

This is important: the authors' strategy imposes a block at telophase, which implies that sister chromatid cohesion should have been removed. If this is not the case, it could be 1) because Cdk1 activity is required to get out, i.e. the authors' contention; or 2) because there is sufficient residual Cdk activity so that cells remain checkpoint proficient (I know that Mad1 becomes dephosphorylated, but this is a very weak criterion. For instance, BubR1 undergoes dramatic dephosphorylation when Plk1 becomes inhibited, but the checkpoint is as strong as ever when BubR1 is dephosphorylated). To test this, the authors should check whether cohesion is retained or lost when they use a higher concentration of 1NMPP1 (also, please note that Western blotting might be an unsuitable surrogate marker for cohesion, as demonstrated by a recent Current Biology paper on Cdc14 by F. Uhlmann and co-workers); or 3) because there is insufficient Cdc20 to remove cohesion. This can be tested by assessing the state of cohesion upon deletion of Yox1, as detailed below.

Based on the above, I am not convinced that the authors are correct when they write (Page 6) "These observations suggest that without Cdk1 activity, SAC signaling remains active despite the removal of nocodazole and cells are unable to recover from nocodazole-induced arrest and progress into anaphase". First, the idea that cells "progress into anaphase" is at odds with the statement in the same sentence that "SAC signaling remains active". Second, the observations are consistent with the idea that reduced, but not completely abrogated, Cdk1 activity creates a condition for persistent checkpoint activation (e.g. partly defective spindle re-assembly). In summary, for this part of the paper to be convincing the effects of Cdk1 inhibition on cell cycle progression have to be monitored more rigorously and at the suitable concentration of the inhibitor.

Page 8
The authors introduce the usage of a turn-off experiment to shut down Cdc20 expression. I am unclear why the authors did not use this approach also for the other experiments in the paper. The telophase arrest of slk19delta cdc15-2 cells at non-permissive temperature in the context of this paper is not ideal, because these cells are by definition "checkpoint off" cells, i.e. cells that cannot reactivate the checkpoint because they have transited past anaphase. Although the authors might have not realized this potential problem, they might perceive its importance as soon as they will start using the appropriate concentrations of 1NM-PP1, as cells will start degrading Pds1 and sisters will be parted (after Cdc20 is allowed to re-accumulate via Yox1 deletion, see below).

Page 11
"Collectively, these results indicate that cells lacking Cdk1 activity are prone to premature spindle extension". The authors might elect to repeat these experiments in the Met-Cdc20 background to verify that these cells are indeed blocked in mitosis and are not extending spindles because they are
going out of mitosis.

Page 14
The authors conclude that when kinetochores become bi-oriented, upon over-expression of Mps1, a block to anaphase initiation remains. Chromosomes might indeed bi-orient when Mps1 is overexpressed, as shown in Figure 6A, but what is the evidence that they remain bi-oriented when 1NMPP1 is added?

The authors also demonstrate that the addition of 1NMPP1 to cells arrested in mitosis through the overexpression of Mps1 or the addition of nocodazole causes a dramatic reduction in the levels of Cdc20. This correlates with the inability of the cells to exit mitosis. This very interesting observation, confined to the end of the paper, provides an apparently simple explanation for why cells don't get into anaphase when Cdk1 activity is suppressed.

The authors should reinforce their analysis as follows.

1. An important question is whether Cdh1 at all responsible for the declining levels of Cdc20. My concern is that the authors are monitoring the convolution of two distinct phenotypes, one resulting from the fact that in their experimental scheme cells might become post-anaphase cells (which degrade Cdc20), and one resulting directly from Cdk1 down-regulation. Please note that it is unclear what levels of Cdc20 are sufficient to drive cells out of mitosis, but experiments in several laboratories suggest that at least in mammalian cells very little Cdc20 is sufficient to drive cells out of mitosis.

2. By eliminating Yox1, the authors confirm that it is the crucial repressor of Cdc20 transcription targeted by Cdk1 (Figure 7). This leads to a recovery in the levels of Cdc20 and provides the authors with an opportunity to examine the effects of Cdk1 inhibition in the absence of effects on Cdc20. This is the main reason why I strongly recommend that the authors start the Results with these observations. Else, all the results in the first part of the manuscript can be equally well interpreted as resulting from the loss of Cdc20. Thus, What happens to bi-orientation, spindle elongation, and cell cycle control when yox1 is deleted? In principle, there should be nothing refraining these cells from entering anaphase at this point, unless Cdc28 activity is indeed required for some other function, as claimed by the authors.

Minor points

Page 3
I am aware that the Pines' laboratory proposed a "threshold" model whereby the checkpoint proteins operate on Cdc20 to keep its levels low. The authors are formally entitled to cite this model given the fact that it is published. However, they should consider that in the same paper (Nilsson et al. 2008) the authors show that large overexpression of Cdc20 is insufficient to drive HeLa cells into anaphase, questioning the significance of the threshold model.

Page 4
Correct "D'Angiolellar"

Page 7
"preceded to anaphase". I think the authors mean "proceeded to anaphase"

Page 8
"...but subsequently fail to establish bi-orientation". Where is the evidence that chromosomes do not bi-orient in this particular experiment (in the Cdc20 shut down experiment, I mean) presented?

Page 9
"Cdk1 may not directly regulate bi-orientation via" Ipl1 and Sgo1. A recent paper in Nature by the Watanabe laboratory argues the opposite. If the authors are convinced that their experiments contradict these previous evidence, they should state it.
Reviewer 1 (Major points)

We thank the reviewer for the appreciation of our work and his/her suggestions.

1. The reviewer has suggested that we should perform the pGAL1-MPS1 expression/release experiment in cells expressing pADH1-CDC20. Is Cdk1 activity is required for SAC recovery?

We have attempted several times to replace the native CDC20-promoter by ADH promoter. However, we find that for some reason this manipulation leads to some undesirable rearrangements.

Therefore, we have made use of the strain cdc28-as1 pGAL-MPS1 pMET-CDC20 slk19 cdc15-2 to address the reviewer’s concern. In this strain, native Cdc20 promoter has been replaced by MET3 promoter, so that Cdc20 is continuously expressed as long as cells are cultured in medium lacking methionine (-Met)

In this experiment, α factor synchronized cells were released into –Met +Raff +Gal medium and were arrested in early mitosis by Mps1 expression. Cells were then the released into –Met+Glu medium in the absence or presence of 1NMPP1. As shown in Figure S8A, after the release into -Met+Glu medium, Mps1 was rapidly degraded. Cdc20 expressed from the MET promoter was not affected by Cdk1 inhibition and its level is maintained throughout the experiment. However, Pds1 still remains largely stable in the presence of 1NMPP1.

This shows that even in the presence of constitutive expression of Cdc20, Cdk1 kinase activity is still required for recovery from the arrest. As we have discussed in the manuscript, other functions of Cdk1 such as facilitating the full activation of APC/C by phosphorylation of APC/C subunits may be necessary.

2. “The nocodazole experiment wash-out could also be performed with pADH1-CDC20 cells in the presence of ASE1, ase1-7A or ase1-7E/D.”

As mentioned above, our attempts to replace CDC20 gene’s native promoter with ADH promoter leads to undesirable rearrangements in some of the strains. Hence, we have not been able to perform this particular experiment.

The intended purpose of this experiment is to determine if the expression of Ase1-7A (or Ase1-7D) induces spindle extension in the constant presence of Cdc20. In fact, in one of the experiments included in the manuscript, we have shown that cells expressing ase1-7A allele prematurely extends spindles upon release from nocodazole induced SAC arrest even without the inhibition of Cdk1. Since Cdk1 is not inhibited, Cdc20 expression is maintained at a constant level in these cells. Hence, this experiment already provides the answer to the question posed by the reviewer.

3. The reviewer suggests that it will be useful to evaluate the importance of both mechanisms under different SAC activating conditions.

We have added a paragraph in the Discussion section (Page 20, 1st paragraph) in which we refer to the experimental data presented in the Supplementary Figures (S7A and S7B; new data added to the revised version) and the text-Figures to point out that the two functions (i.e. spindle elongation and Cdc20 expression) are regulated independently by Cdk1 during the recovery from SAC-induced arrest. These conclusions are based on our experiments in which SAC-induced is imposed by two different experimental regimes namely, by nocodazole treatment or by Mps1 overexpression.

(Minor points):

1. “Why do the authors need a telophase trap - they should explain the importance of the trap on p. 5. The statement "to prevent mitotic exit" is not convincing because cells can be analyzed before they exit mitosis. In any case, spo12 cdc15-2 cells would have been the better telophase trap because of the role of SLK19 in anaphase spindle formation.”

As pointed out in the text (now modified to state the reasons more clearly; Page 5, first paragraph), lowering the Cdk1 activity during mitosis causes cells to short-circuit to G1
from metaphase (without going through anaphase), leading to premature mitotic exit and cytokinesis (Supplementary Figure 1C). Premature occurrence of these events adversely affects our ability to make accurate observations. Since cytokinesis in the budding yeast is dependent on Cdc15 function, the use of cdc15-2 and slk19Δ allows us to prevent both mitotic exit and cytokinesis. The reviewer suggests that we could have used the combination of cdc15-2 and spo12Δ mutations (instead of cdc15-2 and slk19Δ). However, we find that the inhibition of premature mitotic exit in cdc15-2 spo12Δ cells is not as robust at 30°C (the temperature we have used in our study).

2. “On p. 10, bottom, suddenly MPS1 overexpression pops up. What is the rational of this experiment? This is later explained in detail. However, I find it important that the rationale behind an experiment is explained when it is described. I would present Figure S4A together with Figure 6.”
   In accordance with the reviewer’s suggestion, we have now modified the text to explain the rationale the first time we use Mps1 and have shifted the Supplementary Figure S4A (old) to Figure 6 (new 6A),

3. “Cdc14 is important for the recovery from SAC induced arrest; however in cdc15-2 slk19Δ cells, Cdc14 remains inactive. How does it fit together?”
   The role of Cdc14, as discussed by Mirchenko and Uhlmann (2010), is not directly implicated in the recovery from SAC. Instead, they argue that Cdc14 is responsible for dephosphorylating Sli15 during anaphase, so that the chromosomal passenger complex can be translocated to the spindle midzone. This ensures that SAC will not be re-engaged after the cells initiate anaphase. In this study, we focus on the function of Cdk1 in the period from nocodazole removal to the time cells finally switch off the SAC signaling and initiate anaphase. As we have shown in Supplementary Figure 1D, cdc15-2 slk19Δ cells recover quite efficiently from SAC induced arrest. This suggests that in our experimental context, recovery from SAC induced arrest is not affected in the absence of Cdc14 function.

Reviewer 2

1. “The title is somewhat misleading since the authors do not show that Cdk1 turns off the spindle checkpoint, just that it is required to prevent spindle elongation that would otherwise disfavor kinetochore attachment, and is required to maintain Cdc20 levels necessary for mitotic exit.”
   In accordance with the reviewer’s suggestions, we have modified the title of the manuscript to be commensurate with our findings.

2. “They should also clarify whether all the Cdk1 sites that they mutate in Ase1 have been shown to be phosphorylated.”
   The seven consensus-Cdk1-phosphorylation sites that have been mutated in this study have not been confirmed for their phosphorylation status in vivo in this manuscript. However as shown by Khmelinskii et al (2007), mutating all seven putative Cdk1 phosphorylation sites to alanine abolishes all detectable phosphorylations on Ase1 (as indicated by the disappearance of band-shifts in Western blotting), implying that these sites account for all in vivo phosphorylation on Ase1.

3. “Modify the figure legends to state how many times each experiment was performed and how many cells were analyzed in each experiment.”
   We have now indicated in the figure legends, the number of times the experiments have been repeated and the number of cells analyzed in each sample.
Reviewer 3

1. “Effects of Cdk1 inhibition on cell cycle progression have to be monitored more rigorously and at the suitable concentration of the inhibitor. 500nM 1NMPP1 might be insufficient for full kinase inhibition”

We have now performed the In vitro kinase assay using Histone H1 as a substrate to show that inhibiting Cdc28-as1 with 250nM or 500nM of 1NMPP1 completely abolishes its mitotic kinase activity (Supplementary Figure S1A). That 500nM is also sufficient to inhibit the mitotic activity in vivo is reflected in the observation that cdc28-as1 cells released from a factor into 500nM 1NMPP1 containing medium failed to enter mitosis and were unable to form bipolar spindle (Supplementary Figure S1B and the reference: Crasta et al, 2006). We chose not to use a concentration higher than 500nM in order to avoid any undesirable off-target effects.

2. “Use Cdc20 expression turn-off experiment throughout paper.”

Since most of our experiments use inhibition of Cdk1 during the release from nocodazole arrest, there is a drastic reduction in Cdc20 level in the cells (Figure 7E). Thus Cdc20 is already in ‘off-state’ in a majority of our experiments. Moreover, there is a technical constraint on using ‘Cdc20-off condition’ in all our experiments. In constructing many of our strains, all available nutrition-markers have already been utilized, leaving no additional markers for introducing cdc20Δ and GAL-CDC20 combination in every strain.

3. “Test if cells lacking Cdk1 activity are prone to premature spindle extension in the MET-CDC20 background.”

We have now conducted such an experiment to examine the issue raised by the reviewer. cdc28-as1 slk19Δ cdc15-2 and cdc28-as1 MET-CDC20 slk19Δ cdc15-2 cells were arrested by nocodazole treatment and subsequently released in the presence of 1NMPP1. As shown in Figure S7A (newly added), 1NM-PP1 treated cdc28-as1 cells exhibit premature spindle extension after the release from nocodazole induced SAC arrest even in the continuous presence of Cdc20 (due to growth in –Met medium). The extent and the kinetics of premature spindle extension in the presence and in the absence of constitutive Cdc20 expression are comparable.

4. “Is Cdh1 responsible for the declining levels of Cdc20?”

We had attempted to investigate whether Cdh1 deletion could prevent the decline in Cdc20 protein level in 1NMPP1 treated cdc28-as1 cells. Therefore, we constructed cdc28-as1 cdh1Δ mutant strain. However, this strain is too sick to permit the desired experiment. Previous reports suggest that Cdh1 is unlikely to be responsible for the Cdc20 degradation in our case. Pan and Chen (Pan and Chen, 2004) reported that Cdc20 is constitutively degraded in SAC arrested S. cerevisiae cells and that this degradation is independent of Cdh1 and the destruction box in Cdc20. However, this degradation was shown to be APC/C dependent.

5. “The authors synchronize cdc28-as1 slk19delta cdc15-2 cells in G1 and release them in nocodazole-containing medium. "After the arrest in metaphase...". Unless I am missing something, there should not be any metaphase at all because nocodazole was used. And indeed, Figure 1A shows that spindles are absent.”

In yeast literature, nocodazole arrest is conventionally referred to as ‘metaphase arrest’. However, we have now replaced the term ‘metaphase arrest’ by ‘arrested in early mitosis’ in the revised version wherever appropriate.

6. “In Figure 1B, however, spindles appear to have reformed in the presence of 1NMPP1, which implies that cdc28 activity is not required for spindle formation or that the authors are not inhibiting the kinase to the desired level (i.e. fully, or at least 98%).”
The statement “in the absence of Cdk1 activity” refers to the fact that we have completely inhibited the mitotic Cdk1 activity. As discussed above, 500nM 1NMPP1 treatment abolished mitotic kinase activity of Cdk1 as shown by in vitro kinase inhibition assay (Fig. S1A). At 500nM, 1NMPP1 treatment in cdc28-as1 cells also prevents bi-polar spindle formation in yeast cells released from a factor arrest.

However, if the inter-SPB bridge is broken (for instance, by Cin8 overexpression), Cdc28-as1 cells assemble bi-polar spindle even in the presence of 1NMPP1 (Crasta et al, 2006). In our experiments utilizing nocodazole arrest, the inter-SPB bridge would be broken in late S phase (as it normally happens in budding yeast). We inhibit the Cdk1 activity only after cells have arrested in early mitosis. Hence, after the release from nocodazole, bipolar spindle can re-form even in the absence of Cdk1 activity.

7. “The authors’ strategy imposes a block at telophase, which implies that sister chromatid cohesion should have been removed. If this is not the case, it could be 1) because Cdk1 activity is required to get out, i.e. the authors' contention; or 2) because there is sufficient residual Cdk activity so that cells remain checkpoint proficient (I know that Mad1 becomes dephosphorylated, but this is a very week criterion. For instance, BubR1 undergoes dramatic dephosphorylation when Plk1 becomes inhibited, but the checkpoint is as strong as ever when BubRI is dephosphorylated). To test this, the authors should check whether cohesion is retained or lost when they use a higher concentration of 1NMPP1 (also, please note that Western blotting might be an unsuitable surrogate marker for cohesion, as demonstrated by a recent Current Biology paper on Cdc14 by F. Uhlmann and co-workers); or 3) because there is insufficient Cdc20 to remove cohesion. This can be tested by assessing the state of cohesion upon deletion of Yox1.”

The reviewer seems to be under the impression that since we are using telophase trap, all our observations are made at telophase arrest and hence in our experiment sister cohesion must have been removed. We wish to clarify that the introduction of a telophase trap into the genetic background of our strains does not mean that our observations are made on cells arrested in telophase. As explained in our response to reviewer 1, the ‘telophase trap’ is meant to prevent premature exit from mitosis (i.e. cyclin degradation) and cytokinesis. It is well known that in yeast, inhibition of Cdk1 activity in ‘metaphase’-arrested cells can lead to cytokinesis and cells can enter G1, without going through anaphase (i.e. without cohesion cleavage). In the absence of Cdk1 activity in metaphase, yeast cells ‘short-circuit’ to G1 and proceed to undergo cytokinesis without cleaving cohesins. For these reasons, it is essential for us to prevent cytokinesis (which can be done by inhibiting the effectors of MEN and FEAR pathway) to observe closely the process of recovery from SAC-induced arrest in the absence of Cdk1 activity. Without a telophase trap, cells (mother and daughter) will split (cytokinesis) during 1NM-PP1 treatment; consequently we will not be able to accurately record the spindle behaviour and other cellular events associated with the recovery process.

1NMPP1 treated cdc28-as1 cells failed to recover from nocodazole induced SAC arrest and in this case, SAC remained active despite the inhibition of mitotic Cdk1 (Mad1 phosphorylation and persistent Pds1). The reason the SAC is not switched off is because cells are unable to efficiently bi-orient their kinetochores due to premature spindle extension in the absence of mitotic Cdk1 activity…. ‘not because cells have residual Cdk1 activity’ as the reviewer suggests. Moreover, it is not entirely clear whether Cdk1 activity is required to maintain checkpoint signaling per se, because when mammalian cells undergo slippage during SAC arrest (following a reduction in Cdk1 activity) and exit from mitosis, Mad1 and Mad2 staining is still observed on kinetochores in these cells (Brito and Rieder, 2006).
8. “Based on the above, I am not convinced that the authors are correct when they write (Page 6) "These observations suggest that without Cdk1 activity, SAC signaling remains active despite the removal of nocodazole and cells are unable to recover from nocodazole-induced arrest and progress into anaphase". First, the idea that cells "progress into anaphase" is at odds with the statement in the same sentence that "SAC signaling remains active". Second, the observations are consistent with the idea that reduced, but not completely abrogated, Cdk1 activity creates a condition for persistent checkpoint activation (e.g. partly defective spindle re-assembly). In summary, for this part of the paper to be convincing the effects of Cdk1 inhibition on cell cycle progression have to be monitored more rigorously and at the suitable concentration of the inhibitor.”

This elaborate comment from the reviewer is due to a typographical error we have made. Our statement should have been: “These observations suggest that without Cdk1 activity, SAC signaling remains active despite the removal of nocodazole and cells are unable to recover from nocodazole-induced arrest and fail to progress into anaphase”.

When cells do not recover from nocodazole induced arrest in these experiments, they do not undergo anaphase. The omission of the phrase “fail to” gave the impression that they undergo anaphase despite the fact that SAC remains on. We apologize for this typographical error. It has been now corrected.

9. Page 8:

“The authors introduce the usage of a turn-off experiment to shut down Cdc20 expression. I am unclear why the authors did not use this approach also for the other experiments in the paper. The telophase arrest of slk19Δ Δ cdc1 Δ 2 cells at non-permissive temperature in the context of this paper is not ideal, because these cells are by definition "checkpoint off" cells, i.e. cells that cannot reactivate the checkpoint because they have transited past anaphase. Although the authors might have not realized this potential problem, they might perceive its importance as soon as they will start using the appropriate concentrations of 1NM-PP1, as cells will start degrading Pds1 and sisters will be parted (after Cdc20 is allowed to re-accumulate via Yox1 deletion, see below).”

We are sorry that the term ‘telophase trap’ has created such a serious misimpression and has generated so many comments from the reviewer. Once again we wish to reiterate that we are not conducting our experiments in telophase-arrested cells; in our experiments, we are not arresting cells in telophase and not reactivating SAC. Cells that have a ‘telophase trap’ built into their genetic background still arrest in early mitosis (or loosely metaphase arrest) when treated with nocodazole (our experimental condition). When Cdk1 activity is inhibited in metaphase, yeast cells tend to short-circuit to G1 (exit M phase) and undergo cytokinesis (without going through anaphase). This phenomenon is quite well known in yeast cell cycle field. Hence, we introduced cdc15-2 slk19Δ mutations to prevent cells from short-circuiting into G1 in the presence of 1NM-PP1.

10. "Collectively, these results indicate that cells lacking Cdk1 activity are prone to premature spindle extension”. The authors might elect to repeat these experiments in the Met-Cdc20 background to verify that these cells are indeed blocked in mitosis and are not extending spindles because they are going out of mitosis.”

As discussed above, cdc28-as1 MET-CDC20 slk19Δ cdc15-2 also exhibit premature spindle extension. We like to emphasize that the spindle extension is not due to cells’ exit from mitosis. Upon Cdk1 inhibition, SAC remain active in cdc28-as1 slk19Δ cdc15-2 cells, Pds1 and Clb2 remain intact (no degradation), and CENV-GFP spots do not segregate with elongating spindles. Moreover, slk19Δ cdc15-2 mutations prevent cells from leaving M phase.

11. Page 14:
“The authors conclude that when kinetochores become bi-oriented, upon over-expression of Mps1, a block to anaphase initiation remains. Chromosomes might indeed be bi-oriented when Mps1 is overexpressed, as shown in Figure 6A, but what is the evidence that they remain bi-oriented when 1NMPP1 is added?”

We now show that in the MPS1 overexpression experiment, cells remain bi-orientated after 1NMPP1 is added (Supplementary Fig. S4C).

12. “The authors also demonstrate that the addition of 1NMPP1 to cells arrested in mitosis through the overexpression of Mps1 or the addition of nocodazole causes a dramatic reduction in the levels of Cdc20. This correlates with the inability of the cells to exit mitosis. This very interesting observation, confined to the end of the paper, provides an apparently simple explanation for why cells don’t get into anaphase when Cdk1 activity is suppressed. The authors should reinforce their analysis as follows:

1. An important question is whether Cdh1 at all responsible for the declining levels of Cdc20. My concern is that the authors are monitoring the convolution of two distinct phenotypes, one resulting from the fact that in their experimental scheme cells might become post-anaphase cells (which degrade Cdc20), and one resulting directly from Cdk1 down-regulation. Please note that it is unclear what levels of Cdc20 are sufficient to drive cells out of mitosis, but experiments in several laboratories suggest that at least in mammalian cells very little Cdc20 is sufficient to drive cells out of mitosis.”

1. As discussed above, previous literature has shown that Cdh1 is unlikely to be responsible for the Cdc20 degradation in our case. Pan and Chen reported that Cdc20 is constitutively degraded in SAC arrested S. cerevisiae cells and this degradation is independent of both the Cdh1 and destruction box in Cdc20. However, it is still APC/C dependent (Pan and Chen, 2004).

2. As we have argued above, 1NMPP1 treated cdc28-as1 slk19A cdc15-2 cells are not post-mitotic because SAC is still active, Pds1 remained uncleaved, CENV-GFP remain unsegregated and Clb2 level remain high (Fig. 1). Moreover, when 1NMPP1 is withdrawn, cells resume anaphase progression (Fig. S2). Also, when cdc28-as1 cells (with telophase trap to prevent cytokinesis) were released in 1NM-PP1 from GAL-MPS1 induced SAC arrest, there was no dramatic spindle extension after Cdk1 inhibition and cells remained arrested in ‘metaphase’. These observations argue against the suggestion that the partial spindle extension and disappearance of Cdc20 is caused by cells’ post-anaphase status when Cdk1 is inhibited.

13. “By eliminating Yox1, the authors confirm that it is the crucial repressor of Cdc20 transcription targeted by Cdk1 (Figure 7). This leads to a recovery in the levels of Cdc20 and provides the authors with an opportunity to examine the effects of Cdk1 inhibition in the absence of effects on Cdc20. This is the main reason why I strongly recommend that the authors start the Results with these observations. Else, all the results in the first part of the manuscript can be equally well interpreted as resulting from the loss of Cdc20. Thus, what happens to bi-orientation, spindle elongation, and cell cycle control when yox1 is deleted? In principle, there should be nothing preventing these cells from entering anaphase at this point, unless Cdc28 activity is indeed required for some other function, as claimed by the authors.”

Our results strongly suggest that the two functions regulated by Cdk1 during the recovery from SAC arrest are independent of each other. As shown in Supplementary Figure S7 (and also discussed above), Yox1 deletion did not prevent the premature spindle extension when Cdk1 is inhibited.

Moreover, even if Cdc20 protein level were maintained by Yox1 deletion (Figure 7E), Cdc20 may not be in its active state to activate APC/C since SAC signaling (released from nocodazole arrest) is still active in these cells in the absence of Cdk1 kinase activity. It is known that Cdc20 is inhibited by MCC if cells fail to attain bi-orientation. Thus APC/C associated functions of Cdc20 are unlikely to be involved.

Minor points:

1. Page 3:
“I am aware that the Pines' laboratory proposed a "threshold" model whereby the checkpoint proteins operate on Cdc20 to keep its levels low. The authors are formally entitled to cite this model given the fact that it is published. However, they should consider that in the same paper (Nilsson et al. 2008) the authors show that large overexpression of Cdc20 is insufficient to drive HeLa cells into anaphase, questioning the significance of the threshold model.”

The paper by Nilsson et al in 2008 was cited on page 3 together with Pan and Chen’s (2004) first proposing the threshold model in budding yeast.
We are aware that in HeLa cells, over-expression of Cdc20 is insufficient to override SAC arrest; however, as presented by Pan & Chen (2004), modest over-expression of Cdc20 in yeast (2 copies of Cdc20) is sufficient to impair spindle checkpoint and cause sensitivity of cells to benomyl.

2. Page 4: Correct "D'Angiolellar"
We have corrected this error.

3. Page 7: "preceded to anaphase". I think the authors mean "proceeded to anaphase"
This has been corrected.

4. Page 8: 
"...but subsequently fail to establish bi-orientation". Where is the evidence that chromosomes do not bi-orient in this particular experiment (in the Cdc20 shut down experiment, I mean) presented?
We did not determine bi-orientation in this experiment as the purpose of this experiment is to show whether kinetochore capturing per se is impaired when Cdk1 is inhibited. This statement refers to the observation in the previous experiment that bi-orientation fails after nocodazole release in the absence of mitotic Cdk1 activity. We have now modified the statement to reflect this fact.

5. Page 9:
"Cdk1 may not directly regulate bi-orientation via Ipl1 and Sgo1. A recent paper in Nature by the Watanabe laboratory argues the opposite. If the authors are convinced that their experiments contradict the previous evidence, they should state it.”
We have discussed Watanabe’s paper in the discussion session [please see page 18 (reference of Tsukahara et al, 2010)].

References:


Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen again by referees 1 and 3 (see below). As you will see, both referees favour of publication, but referee 3 raises two key points based on your response to the referees first report:

1) as suggested in the first round, discuss the potential caveat that Cdc20 removal will affect SAC
early on in the manuscript in a detailed manner. In particular, the referee does not accept your response to point 13 in the original report and this issue has to be discussed, taking the referee's argumentation into account in the revised manuscript.

2) test higher levels of 1NM-PP1 (original points 7 & 9). Since the experiment requires only minor revision and adds value to the dataset, we recommend that it is executed and discussed.

I would like to invite you to submit a revised version of the manuscript, addressing the comments of referee 3 again. Please edit the final revision carefully, as there are a number of typographical errors (for example 'E. Schiebel' is misspelt twice in the reference list.

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

Please resubmit within three months. 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.

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

Yours sincerely

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1

The revised manuscript is now in a state that allows publication in EMBO J. I do not have any further comments.

Referee #3

I am grateful to the authors for their sincere and serious attempts to consider the reviewers' comments. I feel that the overall readability of the manuscript has improved. However, there remain a number of issues that make this manuscript hard to digest and I would like to advise the authors to make a final effort to clear such impediments to the comprehension of this manuscript. To be sure, the comments below are not mandatory. The decision whether they should be taken into account while preparing the final version of the manuscript should emerge from a discussion of the authors with the Editor.

In my first review, I made a clear recommendation that the observation that the levels of Cdc20 decline in the absence of Cdk1 activity should be presented as early as possible in the manuscript. Specifically, I wrote: "The paper should begin with a description of the role of Cdk1 and Yox1 in the transcriptional regulation of Cdc20 during mitosis. The declining levels of Cdc20 provide a possible alternative explicable context for the rest of the observations, so that this issue should be sorted out as early as possible in the manuscript".

There are many good reasons to give proper consideration to this suggestion:

1) First and foremost, control cells retain Cdc20 while cells treated with NMPP1 do not. This is not good, as Cdc20 is crucially implicated in the checkpoint, securin degradation, possibly degradation of spindle factors, etc. (see below). The control is not a good control.
2) The authors incur in this type of narrative problems: "To prevent cells from entering anaphase during the experiment, we replaced endogenous CDC20 by a methionine promoter-driven CDC20 and incubated cells in methionine-medium (+Met) to turn-off Cdc20 expression." (page 8). Here the authors are pretending they do not know that there is no Cdc20 in cells lacking Cdk1 activity, a piece of information they only provide at the end of the manuscript. Wouldn't it be better to spell out problems clearly and at the right time?

3) If Cdc20 levels decline, the context of the spindle checkpoint is completely different in comparison to cells in which Cdc20 is present. This is because Cdc20 is an essential subunit of the checkpoint effector, the MCC. The authors will hopefully agree that in the absence of Cdc20, the concept that the checkpoint cannot be satisfied is meaningless. For instance, the sentence "To investigate whether Cdk1 plays a direct role in bi-orientation outside the SAC context" (page 9) is inexplicable as by definition there cannot be any SAC in the absence of Cdc20. If anything, there might be biochemical surrogates such as Mad1 phosphorylation, but this needs to be spelled out very clearly from the start. Elsewhere (Discussion), the authors write: "Here we have shown that in budding yeast, reduction in Cdk1 activity hinders efficient recovery from SAC, therefore prolonging the time cells spent in a SAC-arrested state." Again, which SAC are the authors referring to if there is no Cdc20?

The authors should do their best to make sure that the levels of Cdc20 are identical in control (non-NMPP1-treated) and treated (NMPP1-treated) cells, so to avoid that the effects of inhibiting Cdk1 become convoluted with possible effects from loosing Cdc20. As I said, this could be done with the Met-Cdc20 system to remove Cdc20 (Cdc20 off), and by depleting Yox1 to stabilize Cdc20 (Cdc20 on).

In their rebuttal, the authors write: "Moreover, even if Cdc20 protein level were maintained by Yox1 deletion (Figure 7E), Cdc20 may not be in its active state to activate APC/C since SAC signaling (released from nocodazole arrest) is still active in these cells in the absence of Cdk1 kinase activity. It is known that Cdc20 is inhibited by MCC if cells fail to attain bi-orientation. Thus APC/C associated functions of Cdc20 are unlikely to be involved." Here the authors neglect substantial previous evidence that Cdc20-APC is active towards many substrates (including Nek2A, Cyclin A, and others) during checkpoint activation, at least in metazoans. So this point is not well taken.

I am also puzzled why the authors opted not to test higher levels of 1NM-PP1 in cells. As there is now very good published evidence (again, the Cudreuse et al. 2010 Nature paper) that 500 nM is not sufficient to completely inhibit the kinase in S. pombe, why not do this very simple experiment instead of arguing about possible unspecific effects? For a number of reasons, I am afraid that the in vitro kinase assay is unconvincing, in particular because the authors do not know a priori what level of inhibition they need to achieve, and their analysis in vitro is intrinsically qualitative.

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Our response to Reviewer #3’s and Editor’s suggestions:

-Discuss the potential caveat that Cdc20 removal will affect SAC early on in the manuscript in a detailed manner.

We have introduced early in the Result section a paragraph mentioning the caveat concerning the low levels of Cdc20 protein when Cdk1 activity is inhibited during recovery from SAC-induced arrest (Pages 6&7). Referring to the results presented in Supplementary Figure S2, we point out that cells deficient in Cdk1 activity fail to recover from SAC-induced arrest even when supplied with Cdc20 (either from MET-CDC20 construct or because of YOX1 deletion, as the reviewer had suggested). This implies that Cdk1 has an additional role in the recovery process, a notion we explore in-depth in the subsequent sections of the manuscript.

In a related context, we have commented further on this issue in the Discussion section (Page 20)

-Test higher levels of 1NM-PP1

We have now used higher concentrations of 1NM-PP1 (1000 nM and 2000 nM) to inhibit Cdk1 activity and monitored the state of the spindle during recovery from SAC-induced arrest under...
conditions where Cdc20 is present during the recovery process. We find, once again (as we did in the experiments using 500 nM 1NM-PP1), that spindles are prematurely extended within 20 min. These experimental results are presented in Supplementary Figure S5. We did not use 1NM-PP1 concentrations higher than 2000 nM, since cell morphology becomes distorted at higher concentrations and cells become too fragile to withstand any processing.