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Stable MCC binding to the APC/C is required for a functional Spindle Assembly Checkpoint

Jamin B. Hein and Jakob Nilsson

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Transaction Report:

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

Editor: Nonia Pariente

1st Editorial Decision 04 June 2013

Thank you for your submission to EMBO reports. We have now received the three enclosed reports on it. As you will see, all referees raise a substantial number of serious concerns about the conclusiveness of the data and inconsistencies in the data interpretation, as well as pinpoint several technical issues that would need to be addressed.

The main problems with the study are the questions regarding the competition of the IR and C-box mutants with endogenous Cdc20 (also voiced by an additional editorial advisor who initially saw the study) and the speculative nature of the "active" APC/C, as well as the discrepancy between your current results and previously published data. From the analysis of these comments it is clear that publication of your study cannot be considered at this stage and that a significant amount of experimentation of uncertain outcome would be required. However, given that all referees provide a constructive critique, if you can satisfy the cited concerns in full by obtaining further, decisive data in support of your current claims within the next three months, we would be willing to consider a revised manuscript. If you need more time to perform the experiments, please let me know.

Please note that that it is EMBO reports policy to allow a single round of revision and, therefore, acceptance or rejection of the manuscript will depend on the next, final round of peer-review. Thus, to save you from any frustrations in the end, I would advise against returning an incomplete revision. Given the nature of the comments and the uncertainty of the results of the experiments required, I would also understand if you rather chose to seek rapid publication elsewhere at this stage.

Should you decide to embark on a revision, revised manuscripts should be submitted within three months; they will otherwise be treated as new submissions, except if a short extension has been discussed with the editor. Revised manuscripts may not exceed 29,000 characters (including spaces and figures legends). When submitting your revised manuscript, please include:
an RTF-formatted version of the manuscript text
JPEG, TIFF, PowerPoint or EPS-formatted figure files
a separate single PDF file of all the Supplementary information (in its final format)
a letter detailing your responses to the referee comments.

We also welcome the submission of cover suggestions or motifs that might be used by our Graphics Illustrator in designing a cover.

I look forward to seeing a revised form of your manuscript when it is ready. Should you choose to submit your study elsewhere, please let us know.

REFEREE REPORTS:

Referee #1:

The question of how the MCC inhibits APC/C function is of great interest. In the submitted manuscript, Hein and Nilsson report that the IR dipeptide motif of Cdc20 is important for binding of the MCC to the APC/C. This is an important finding. However, the mitotic checkpoint field suffers from the fact that individual findings are published without adequately placing them into the context of other published results. I am well aware of the fact that this challenge goes beyond the scope of the submitted manuscript. However, since the finding by Hein and Nilsson conflicts with previous published results by the Pines laboratory (Izawa & Pines, JCB 2012 and NCB 2011) more data should be provided to explain the discrepancy. In particular I ask the authors to address the following points:
major points:
p.6.: "....the ionic strength of the buffer used in that study is lower than ours likely explaining the difference". Apart from the stylistic problem of this sentence, I do not understand its statement because:
According to the quantification shown in S2B, Cdc20ΔIR/ΔC-Box binds under both buffer conditions less efficiently to the APC/C compared to the WT protein. Therefore, differences in the ionic strength do not explain the different findings. In the publication of Izawa, physiological buffer conditions were used.
This is a very important point because it relates to the key finding of the manuscript: How does the MCC bind to the APC/C? Previous studies revealed that Cdc20 binding to the APC/C under SAC conditions occurs via the KILR motif whereas the IR motif is required for binding when Cdc20 acts as an APC/C co-activator. More data are required to convincingly show that the IR motif indeed is important for MCC binding to the APC/C and to conclusively explain the discrepancy to previously published results.
p. 6. "...Cdc20ΔIR/ΔC-Box displayed elevated levels of all MCC components..." The quantification in 2B suggests that Cdc20ΔIR/ΔC-Box binds 1.5-1.6 times more BuBR1 than the WT protein. I cannot see this in the WB shown in 2A.
Fig. 1D: Why are the error bars in the Cdc20 RNAi cases so huge and the delay in non-rescued Cdc20-RNAi cells so mild?
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The authors present good evidence that MCC formation is not sufficient for robust spindle assembly checkpoint (SAC) arrest, rather MCC needs to stably interact with the APC/C. They analyse the role of the C box and the IR motif of Cdc20, and demonstrate that the latter is particularly important for stable MCC-APC/C binding and SAC arrest. This is not a complete surprise but I think that it is a significant point in understanding the biochemical mechanism of SAC arrest. Several mechanisms have been postulated, and Cdc20 sequestration is quite a popular model, but this study shows that this is unlikely to be sufficient for a good SAC arrest, at least in vertebrate cells.

The work is interesting for the specialist, and I think is of significance for the general reader as the SAC impacts on many fields of cell cycle regulation, mitosis and centromere/kinetochore function. However, a number of issues need to be addressed before publication:

1) Relating to page 5 (para 2). The authors need to quantitate the efficiency of Cdc20 depletion in these experiments. Competition between endogenous Cdc20 and the different mutant alleles is a key issue here, and so the relative protein levels need to be shown.

2) Fig 1D and page 5. Why are the mutants causing a longer mitosis than the Cdc20 RNAi treatment on its own? The authors argue that the mutants are competing with the remaining endogenous Cdc20 (this is a case where we need to see the relative levels of both proteins), but they later conclude that the IR mutants are very poor at such competition. The IR mutation reduces the effect of the C box, however, this does not account for the whole phenotype: even the poorly competing IR mutant leads to a significantly longer delay than the depletion. Further explanation is required for this delay.

3) Is the IR mutant Cdc20 protein more stable than wild-type Cdc20 (as shown in SFig3) because it is less prone to APC/C dependent ubiquitination? Cycloheximide block and chase experiments should also be formed to look at Cdc20 turnover. Alternatively Cdc20-ubiquitin ladders could be looked for (in the presence of MG132). If the authors have access to an APC/C in vitro ubiquitination assay, they should monitor auto-ubiquitination of their Cdc20 mutants in vitro.

4) The authors need to discuss the high levels of p31 binding in more detail, in both Fig 2A. Recent studies (eg. Ref 34, Westhorpe) have argued that p31 acts by binding to free MCC and disrupting it, by extracting Mad2. It is less effective in targeting MCC bound to the APC/C. Here the p31 seems to stay associated with Cdc20 in the form of MCC (and Mad2 is high also) - why would that be affected by these mutations?

5) Fig 3D - here high levels of Cdc20 are found associated with the APC/C in the Cdc20 mutants, and this is particularly noticeable for the endogenous Cdc20 protein (with C box and double mutant). Does this suggest that two molecules of Cdc20 might be bound by a single APC/C particle? To test this the authors could perform a sequential IP: first pull down APC4, then release with peptide, then pull down with anti-YFP - do both forms of Cdc20 IP? If so this wouldprovide support for models such as in Primorac and Muisacchio (JCB 201,177).

6) Why is BubR1 a doublet in Fig 3D? Is this affected by PPase treatment? This is interesting as the delta-C box Cdc20 mutant seems to enhance the upper band.

Referee #3:

The work by Hein et al. deals with the mechanism by which the spindle assembly checkpoint (SAC) blocks the activation of APC/C by its mitotic activator Cdc20. In response to unattached kinetochores, SAC proteins Mad2 and BubR1 bind to Cdc20, forming the mitotic checkpoint complex (MCC). MCC binds to APC/C with higher affinity than free Cdc20 does, but it is unclear whether this affinity difference between MCC and Cdc20 is required for the checkpoint in human cells. This current study provides insight into this issue.

The authors report an interesting observation that mutating the two APC/C-binding motifs of Cdc20, the C-box and the IR motif, has different effects on the status of the checkpoint. It was known that mutating either motif could weaken the interaction between Cdc20 and APC/C, resulting in an inactive APC/C activator. Unexpectedly, it is described here that, in the presence of wild type, endogenous Cdc20, overexpression of the Cdc20 ΔIR mutant in human cells compromises the SAC while overexpression of the ΔC-box mutant does not. The authors suggest that the defect of the ΔIR mutant is due to a more severe weakening of the MCC-APC/C interaction comparing with ΔC-box mutant. An important implication of their finding is that the SAC requires the stable interaction between APC/C and MCC. Another conclusion made in the paper is that MCC specifically targets the active pool of APC/C. The argument is based on their observation that after depletion of exogenous Cdc20, both apo-APC/C and free Cdc20 exist in checkpoint-active cell extracts. Presumably, this pool of APC/C is incapable of being activated by Cdc20.

Overall, the paper contains some interesting findings. Although consistent with their conclusions, these results fall far short of proving them. The notion that MCC specifically targets active APC/C is even more speculative. The authors should provide direct biochemical evidence supporting their key conclusions. For example, they can isolate MCC-APC/C from wild-type, ΔC, or ΔIR Cdc20-expressing cells with the endogenous Cdc20 depleted, and test whether addition of purified recombinant Cdc20 can displace ΔIR Cdc20 (but not WT or ΔC) from the complex. To support their second conclusion, they should test whether recombinant purified Cdc20 can activate the apo-APC/C that is not depleted from the extracts. These biochemical experiments are essential, as cellular experiments can be complicated by unforeseen factors.

1st Revision - authors' response 20 September 2013

Referee #1:

The question of how the MCC inhibits APC/C function is of great interest. In the submitted manuscript, Hein and Nilsson report that the IR dipeptide motif of Cdc20 is important for binding of the MCC to the APC/C. This is an important finding. However, the mitotic checkpoint field suffers from the fact that individual findings are published without adequately placing them into the context of other published results. I am well aware of the fact that this challenge goes beyond the scope of the submitted manuscript. However, since the finding by Hein and Nilsson conflicts with previous published results by the Pines laboratory (Izawa & Pines, JCB 2012 and NCB 2011) more data should be provided to explain the discrepancy. In particular I ask the authors to address the following points:

We thank the reviewer for the positive comments and suggestions. We acknowledge that we could not cite and cover all of the literature associated with the SAC and APC/C but this is a short report and the body of literature is large making it impossible to cover all aspects. We have in the revised version discussed in more detail the difference with the Pines lab results. In particular we have emphasized the fact that we use Cdc20R499E while the Pines lab use a mutant were the IR motif is deleted which is at least one major difference. We have therefore also renamed in all the text Cdc20ΔIR to Cdc20 R499E to make this point clearer.

major points:

p.6.: "....the ionic strength of the buffer used in that study is lower than ours likely explaining the difference". Apart from the stylistic problem of this sentence, I do not understand its statement because:
According to the quantification shown in S2B, Cdc20ΔIR/ΔC-Box binds under both buffer conditions less efficiently to the APC/C compared to the WT protein. Therefore, differences in the ionic strength do not explain the different findings. In the publication of Izawa, physiological buffer conditions were used.

I think our point did not come clearly through. What we were trying to say is that for Cdc20 wt

APC/C binding is not affected by ionic strength but in Cdc20C-box/IR the interaction with the APC/C is. We have now done an even more careful comparison on the effect of the NaCl concentration on the interaction between the different Cdc20 proteins and the APC/C (New Supplemental Figure S2A-B). As we predicted the interaction between APC/C and Cdc20 R499E is more sensitive to increases in NaCl concentration than Cdc20 and Cdc20ΔC-box.

This is a very important point because it relates to the key finding of the manuscript: How does the MCC bind to the APC/C? Previous studies revealed that Cdc20 binding to the APC/C under SAC conditions occurs via the KILR motif whereas the IR motif is required for binding when Cdc20 acts as an APC/C co-activator. More data are required to convincingly show that the IR motif indeed is important for MCC binding to the APC/C and to conclusively explain the discrepancy to previously published results.

In addition to the clear results in Figure 2 on the effect of mutating the IR motif on the interaction with the APC/C we have performed a new competition experiment with recombinant Cdc20 and the different Cdc20 mutants (New Figure 4C-E). This again supports that mutating the IR motif of Cdc20 weakens the interaction with the APC/C while the effect of mutating the C-box is less strong. This combined with the in vivo effects we describe in Figure 3 hopefully convinces the reviewer that the IR motif is important for MCC-APC/C interaction.

p. 6. "...Cdc20ΔIR/ΔC-Box displayed elevated levels of all MCC components..." The quantification in 2B suggests that Cdc20ΔIR/ΔC-Box binds 1.5-1.6 times more BuBR1 than the WT protein. I cannot see this in the WB shown in 2A.

We use Licor technology to quantify all our blots. The actual numbers measured are:

Experiment 1 Licor values:

Cdc20 WT: 90.8, BubR1 levels: 22.4
Cdc20 ΔC-Box/R499E: 68.7, BubR1 levels: 25.4
In this experiment 1,5 times more BubR1 in Cdc20ΔC-box/R499E

Experiment 2 Licor values:

Cdc20 WT: 55,6 BubR1 levels: 9.0
Cdc20 C-Box/R499E: 47.5, BubR1 levels: 13.9
In this experiment 1,8 times more BubR1 in Cdc20ΔC-box/R499E

Fig. 1D: Why are the error bars in the Cdc20 RNAi cases so huge and the delay in non-rescued Cdc20-RNAi cells so mild?

We have now explained this in more detail in the revised manuscript. The reason for the mild delay is that very little Cdc20 is required for driving cells through mitosis as for instance described in (Wolthuis et al 2008) so even though our depletion is efficient there remains enough to drive mitosis although at a reduced speed. The reason for the large error bars reflects that the level of Cdc20 depletion varies from cell to cell such that some cells will arrest for very long while others will not.

p. 5. Why should Cdc20 with mutated IR or C-box compete with the remaining endogenous Cdc20 for APC/C binding? These mutants should be unable to bind the APC/C and, therefore, should not be able to compete with residual Cdc20.

The fact that the different Cdc20 mutants induce a longer delay that the depleted cells has puzzled us but is a very consistent result. It must somehow reflect that they are interfering with the activity of the remaining endogenous Cdc20. At least for the single mutants and in particularly the C-box mutant they might have maintained some ability to bind the APC/C, which could contribute in interfering with endogenous Cdc20. However we agree with the reviewer that for the double mutant this can hardly be the explanation for the increased metaphase delay. We have in the revised manuscript proposed that the expression of the inactive Cdc20 mutants might interfere with activating mechanisms of endogenous Cdc20 be they of biochemical or spatial nature. There is

likely still a lot to be learned on Cdc20 regulation making it difficult to address this precisely at the moment.

Referee #2:

The authors present good evidence that MCC formation is not sufficient for robust spindle assembly checkpoint (SAC) arrest, rather MCC needs to stably interact with the APC/C. They analyse the role of the C box and the IR motif of Cdc20, and demonstrate that the latter is particularly important for stable MCC-APC/C binding and SAC arrest. This is not a complete surprise but I think that it is a significant point in understanding the biochemical mechanism of SAC arrest. Several mechanisms have been postulated, and Cdc20 sequestration is quite a popular model, but this study shows that this is unlikely to be sufficient for a good SAC arrest, at least in vertebrate cells.

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1) Relating to page 5 (para 2). The authors need to quantitate the efficiency of Cdc20 depletion in these experiments. Competition between endogenous Cdc20 and the different mutant alleles is a key issue here, and so the relative protein levels need to be shown.

We have in the revised manuscript added a blot of the depletion efficiency (New Figure 1B) and in the text written the amount of endogenous Cdc20 remaining (approximately 15% remains).

2) Fig 1D and page 5. Why are the mutants causing a longer mitosis than the Cdc20 RNAi treatment on its own? The authors argue that the mutants are competing with the remaining endogenous Cdc20 (this is a case where we need to see the relative levels of both proteins), but they later conclude that the IR mutants are very poor at such competition. The IR mutation reduces the effect of the C box, however, this does not account for the whole phenotype: even the poorly competing IR mutant leads to a significantly longer delay than the depletion. Further explanation is required for this delay.

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We have tried extensively using different approaches to see if we could detect and quantify in vivo ubiquitination of the different Cdc20 mutants but this has proved very difficult. We have repeated the time-lapse filmings and degradation curves now in the presence of cycloheximide and this again shows that mutating the IR motif stabilizes Cdc20 strongly while the C-box have less of an effect (New supplemental Figure 2C). The observed degradation depends on the presence of nocodazole (for the C-box mutant) and is blocked by proteasome inhibition supporting that the degradation we are observing requires MCC-APC/C interaction. The most straightforward explanation for why mutating the IR motif inhibits Cdc20 degradation is that the MCC is no longer stably associated with the APC/C. Although we do not have evidence at the level of Cdc20 ubiquitination we suspect

that this is the step affected and this is also supported by the work from the Morgan lab (Foster and Morgan, 2012) were Cdc20ΔIR is not getting efficiently ubiquitinated in vitro upon Mad3 addition (Figure 2B in that paper).

4) The authors need to discuss the high levels of p31 binding in more detail, in both Fig 2A. Recent studies (eg. Ref 34, Westhorpe) have argued that p31 acts by binding to free MCC and disrupting it, by extracting Mad2. It is less effective in targeting MCC bound to the APC/C. Here the p31 seems to stay associated with Cdc20 in the form of MCC (and Mad2 is high also) - why would that be affected by these mutations?

We have now added an additional experiment to support the observations from the Taylor lab that p31 can only target free MCC. In our p31 purifications we are also unable to detect APC/C components (New supplemental Figure 1B). At least this explains the increase in p31 levels for Cdc20ΔC-box/R499E. However as the reviewer points out this is at odds with the increase in MCC components as p31 should remove these from Cdc20. At the moment we do not have a good explanation for the increase in MCC components on Cdc20ΔC-box/IR but we discuss in the revised manuscript that the complete lack of APC/C binding might make MCC formation on the mutant more efficient. This might simply be due to the fact that MCC formation on free Cdc20 compared to APC/C-Cdc20 is more efficient (experiment in Figure 4 of Kulukian et al 2009 also supports that free Cdc20 is more efficiently inhibited than APC/C-Cdc20)

5) Fig 3D - here high levels of Cdc20 are found associated with the APC/C in the Cdc20 mutants, and this is particularly noticeable for the endogenous Cdc20 protein (with C box and double mutant). Does this suggest that two molecules of Cdc20 might be bound by a single APC/C particle? To test this the authors could perform a sequential IP: first pull down APC4, then release with peptide, then pull down with anti-YFP - do both forms of Cdc20 IP? If so this wouldprovide support for models such as in Primorac and Muisacchio (JCB 201,177).

Although we have not done exactly the suggested experiment the new experiment in Figure 4 C-E addresses this point to some extent as well. In this experiment we added increasing concentrations of recombinant Cdc20 to extracts from the YFP-Cdc20 expressing cells and following incubation isolated the YFP-Cdc20. As can be seen in Figure 4E the recombinant Cdc20 can bind even to YFP-Cdc20 beads suggesting that an additional molecule of Cdc20 associates with the co-purifying APC/C. This effect is even stronger when C-box or IR is mutated. Although this result could support the idea that 2 molecules of Cdc20 can interact with the APC/C we cannot ignore the observations of APC/C dimers from yeast (Passmore et al 2005), which off course could explain why additional Cdc20 molecules can bind.

6) Why is BubR1 a doublet in Fig 3D? Is this affected by PPase treatment? This is interesting as the delta-C box Cdc20 mutant seems to enhance the upper band.

BubR1 is strongly phosphorylated in mitosis and its phosphorylation by Plk1 causes the observed upper band of the doublet (see for instance Elowe et al 2007). Depending on how the gel ran we sometimes see a clear separation of the different BubR1 species like in Figure 3D in the original submission but sometimes they are not as clearly separated. The reason why this upper band is enhanced in Cdc20ΔC-box is likely that these cells delay in mitosis as we show in Figure 3A even when endogenous Cdc20 remains. When we collect mitotic cells by shake-off there will always be a fraction of cells that are not in mitosis due to the shake-off procedure but this is likely less in Cdc20ΔC-box due to the mitotic arrest as also evident by slightly more phosphorylated APC3 on the same blot.

Referee #3:

The work by Hein et al. deals with the mechanism by which the spindle assembly checkpoint (SAC) blocks the activation of APC/C by its mitotic activator Cdc20. In response to unattached kinetochores, SAC proteins Mad2 and BubR1 bind to Cdc20, forming the mitotic checkpoint complex (MCC). MCC binds to APC/C with higher affinity than free Cdc20 does, but it is unclear whether this affinity difference between MCC and Cdc20 is required for the checkpoint in human cells. This current study provides insight into this issue.

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Overall, the paper contains some interesting findings. Although consistent with their conclusions, these results fall far short of proving them. The notion that MCC specifically targets active APC/C is even more speculative. The authors should provide direct biochemical evidence supporting their key conclusions. For example, they can isolate MCC-APC/C from wild-type, ΔC, or ΔIR Cdc20-expressing cells with the endogenous Cdc20 depleted, and test whether addition of purified recombinant Cdc20 can displace ΔIR Cdc20 (but not WT or ΔC) from the complex. To support their second conclusion, they should test whether recombinant purified Cdc20 can activate the apo-APC/C that is not depleted from the extracts. These biochemical experiments are essential, as cellular experiments can be complicated by unforeseen factors.

We thank the reviewer for the comments and constructive ideas. We have performed the suggested competition experiment which shows that recombinant Cdc20 can more easily displace APC/C from Cdc20R499E than from Cdc20 or Cdc20ΔC-box (New Figure 4C-E) as we proposed.
The suggested in vitro ubiquitination experiment will probably show that you can activate apo-APC/C with recombinant Cdc20 (as also shown in Westhorpe et al 2011 Figure 5F) and one would instead need to compare the activity of apo-APC/C, APC/C-BCC (Bub3-BubR1-Cdc20) and APC/C-MCC. The problem is that to compare these APC/C complexes (which will also be challenging to purify) we would need to get rid of Cdc20 from APC/C-BCC and APC/C-MCC first which is not trivial. Although one can wash BCC and MCC off with high salt this also affects the activity of the APC/C in our hands as APC11 is also washed reduced. Thus a meaningful comparison at a biochemical level will be very difficult to do.

We agree that the proposal that the MCC targets an active pool of APC/C is too speculative at the moment and we have in the revised manuscript more focused on the fact that stable MCC binding to the APC/C is important for the SAC and end with two lines on why this might be.

2nd Editorial Decision 08 October 2013

I have now received the enclosed reports on your revised study from the same referees that assessed the initial version. As you will see, although they find the study much improved, they all have some outstanding concerns. As most of these are minor, we have decided to open an exceptional, second round of revision in this case to allow you to address them.

Particularly important is the experiment requested by referee 1 with a Cdc20 mutant lacking the IR motif. Upon further discussion with the referees, both 2 and 3 considered this is a valid point and a desirable experiment which would contribute to make the story cleaner and easier to reconcile with the literature. Please address also the issues raised by referees 2 and 3.

Browsing through the manuscript myself, I have realize that in figure 1A and 3A, information regarding the statistical test applied and the P values considered significant is missing from the figure legends. Likewise, the legend of SF2C does not specify the number of independent

experiments performed and the identity of the error bars. I also note that figure 2B represents the median of two experiments, but errors are shown, which is incorrect (for guidance, please refer to: Cumming et al. JCB 2007). You would ideally have to increase the number of independent experiments to three to perform statistical analysis, or show the individual data points in the graph (without errors).

Once all the remaining issue have been addressed, we would be happy to consider publication of your study in EMBO reports.

We now encourage the publication of original source data -particularly for electrophoretic gels and blots, but also for graphs- with the aim of making primary data more accessible and transparent to the reader. If you agree, you would need to provide one PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figures and an Excel sheet or similar with the data behind the graphs. The files should be labeled with the appropriate figure/panel number, and the gels should have molecular weight markers; further annotation could be useful but is not essential. In your case, as you have so many blots and have cropped them extensively for presentation purposes, it would be particularly informative to include the full blots as source data. The source files will be published online with the article as supplementary "Source Data" files and should be uploaded when you submit your final version. If you have any questions regarding this please contact me.

I look forward to seeing a new revised version of your manuscript as soon as possible, and always on or before Dec 4th, as we cannot consider revisions that are submitted more than 6 months after an initial decision was made (you could submit it later, but it would then be considered a resubmission rather than a revision and its novelty would be assessed afresh). In addition, please send a letter detailing your responses to the outstanding referee concerns.

REFEREE REPORTS:

Referee #1 (Report):

The key message of the work by Hein and colleagues is that binding of the MCC to the APC/C requires the IR tail of Cdc20 and this binding is critical for SAC function. This is an interesting finding. Yet, I am still puzzled by the approach used by Hein et al. To me, the most straightforward way to show that the IR motif is important for MCC function would be to remove it. Instead, the authors mutate it to IE and, thereby, convert a positive charge into a negative charge. This makes it difficult to place the submitted manuscript in the context of already published data by the pines lab showing that the IR tail is dispensable for Cdc20 binding under checkpoint arrest conditions. Therefore, I am still puzzled by the fact that the authors observed different phenotypes than the Pines lab.

Referee #2 (Report):

I am happy that the authors have improved the manuscript, and addressed the major issues we raised. A few minor points could still be improved:

1) with respect to the observation that the Cdc20 mutants "interfere with the activity of the remaining endogenous Cdc20" and lead to significant mitotic delays. It is good to see quantitation of the knockdown (new Fig. 1B), but ideally we would have seen this data from cells expressing the mutants too.

It is possible that the mutant Cdc20 proteins interfere (to different extents) with remaining wild-type Cdc20 through some form of dimerization (this could be on or off the APC/C), or that the remaining wild-type Cdc20 protein is "destabilised" to different extents by the mutants.

Such possibilities should be dicussed, in addition to the proposed interference "with activating

mechanisms" at top p6.

2) R499E is still labelled delta-IR in Fig 4B .

3) Why is Mad1 probed for in Supp Fig 1B. It is not referred to here. Do the authors think that it is relevant to the mode of p31 action?
Is Mad1 detectable in the Cdc20 pull-down (Fig 2A) where abnormally hi levels of p31 are found associated with the double mutant (delta Cbox/R499E)?

Referee #3 (Report):

The authors have performed some of the requested competition experiments. The results are generally consistent with their original conclusions. These data strengthen the manuscript. Publication is recommended.

The authors might want to clarify why more recombinant Cdc20 is incorporated into APC/C-Cdc20deltaC as compared to the wild-type APC/C-Cdc20, and yet APC/C remains constant (Figure 4C-E). Does this imply that recombinant Cdc20 and Cdc20deltaC (as a part of MCC) can co-exist on APC/C?

Correspondence - authors 12 October 2013

Thank you very much for your email and the continued interest in our work. It is off course with some frustration to us that reviewer 1 did not bring on this concern during the first round of review as it was clearly stated that we used Cdc20 R499E (the mutant used in Nilsson et al 2008). However I can also appreciate that the requested experiments will help settle discrepancies between our study and that of Izawa and Pines and we are therefore happy to do it. I should mention that I off course have discussed our results with Daisuke and Jon as I know them quite well and Daisuke acknowledge that he did notice an effect of deleting the IR motif in certain experiments which is also clear if one looks at figure 3D in his paper.
Given that we do not have the constructs and stable lines of YFP-Cdc20 del IR (which will take 6 weeks to make) and the short time left before 4/12 I suggest the following strategy which I hope you agree on:

We will obtain the stable lines used in Daisuke and Pines for Cdc20 WT and Cdc20 del IR and in APC/C purifications test the effect of increasing salt and the effect of having endogenous Cdc20 present. This I believe is the key experiment to test if deletion of the IR also affects MCC binding to the APC/C similar to what we see with Cdc20 R499E. It is not unusual in biology to mutate a basic residue to an acidic residue to determine if that residue has a role in binding so I do suspect that the Cdc20R499E has a stronger effect than deleting the IR but this does not change the conclusions of our work.

Let me know if you agree on the outlined strategy.

Correspondence - editor 14 October 2013

Thanks you for your email. I disagree that the issue brought up by referee 1 is new. S/he already asked you to address the discrepancy with Jon's data before, and just isn't satisfied by the ways you have addressed it in revision. As the other two referees agreed this would be a fair and useful experiment to ask for, we decided to request it.

That said, I do think your plan sounds very reasonable and would address this question

appropriately. Using the Daisuke and Pines lines is most fitting, actually!

I look forward to receiving the re-revised version of your study.

2nd Revision - authors' response 27 November 2013

Referee #1 (Report):

The key message of the work by Hein and colleagues is that binding of the MCC to the APC/C requires the IR tail of Cdc20 and this binding is critical for SAC function. This is an interesting finding. Yet, I am still puzzled by the approach used by Hein et al. To me, the most straightforward way to show that the IR motif is important for MCC function would be to remove it. Instead, the authors mutate it to IE and, thereby, convert a positive charge into a negative charge. This makes it difficult to place the submitted manuscript in the context of already published data by the pines lab showing that the IR tail is dispensable for Cdc20 binding under checkpoint arrest conditions. Therefore, I am still puzzled by the fact that the authors observed different phenotypes than the Pines lab.

We have now performed similar experiments with Cdc20 where the IR motif is deleted and obtain similar results as for Cdc20 R499E (New supplemental Figure 3). Using the cell lines published by the Pines lab we see a clear defect in MCC binding when the IR motif is deleted. Furthermore we see the same SAC defect as for Cdc20 R499E (New Supplemental Figure 3D).
We suspect that the reason why the Pines lab did not observe this is due to their mild buffer conditions and we have commented on this in relation to our results with their cell lines.

Referee #2 (Report):

I am happy that the authors have improved the manuscript, and addressed the major issues we raised. A few minor points could still be improved:

1) with respect to the observation that the Cdc20 mutants "interfere with the activity of the remaining endogenous Cdc20" and lead to significant mitotic delays. It is good to see quantitation of the knockdown (new Fig. 1B), but ideally we would have seen this data from cells expressing the mutants too.

We have provided this data as new Figure S1B.

It is possible that the mutant Cdc20 proteins interfere (to different extents) with remaining wild-type Cdc20 through some form of dimerization (this could be on or off the APC/C), or that the remaining wild-type Cdc20 protein is "destabilised" to different extents by the mutants.

It does not appear that expression of the mutants affect the stability of the remaining endogenous Cdc20 (New Figure S1B) although small differences would be impossible to tell as we are working with cells were we have depleted Cdc20 already by RNAi.

Such possibilities should be dicussed, in addition to the proposed interference "with activating mechanisms" at top p6.

We have discussed these possibilities in the revised manuscript.

2) R499E is still labelled delta-IR in Fig 4B .

Corrected.

3) Why is Mad1 probed for in Supp Fig 1B. It is not referred to here. Do the authors think that it is relevant to the mode of p31 action?
Is Mad1 detectable in the Cdc20 pull-down (Fig 2A) where abnormally hi levels of p31 are found associated with the double mutant (delta Cbox/R499E)?

Mad1 is a known ligand of p31 (via its interaction with Mad2) and we merely probed for Mad1 as a positive control. We do not see Mad1 in Cdc20 IPs not even using sensitive mass spectrometry approaches.

Referee #3 (Report):

The authors have performed some of the requested competition experiments. The results are generally consistent with their original conclusions. These data strengthen the manuscript. Publication is recommended.

The authors might want to clarify why more recombinant Cdc20 is incorporated into APC/C-Cdc20deltaC as compared to the wild-type APC/C-Cdc20, and yet APC/C remains constant (Figure 4C-E). Does this imply that recombinant Cdc20 and Cdc20deltaC (as a part of MCC) can co-exist on APC/C?

We believe this is the case. When we mutate the C-box this allows recombinant Cdc20 to bind but does not displace the Cdc20 molecule with a mutated C-box. We have commented on this in the revised manuscript.

3rd Editorial Decision 09 December 2013

Thank you for your patience while referee 1 assessed your last minor revision. S/he now fully supports publication and has no further comments. I have also had time to go through your file in detail and I am twriting with an 'accept in principle' decision, which means that I will be happy to accept your manuscript for publication once a few minor issues/corrections have been addressed, as follows.

- There is still some information missing regarding the quantitative and statistical analyses of the data. In the legend to figures 1E, 3A, 3B, and supp 3D the identity of the error bars (and even what the boxes mean for several of them) is missing, as are the p-values that are considered significant (**, ***, etc) and in some cases the statistical test applied. In Supp fig 2C the legend doesn't state what is represented by the bars (median, mean,...) or the identity of the errors depicted (SD, SE). Please ensure that all figure legends include all relevant information.

- We now encourage the publication of original source data for the key experiments in an article - particularly for electrophoretic gels and blots, but also for graphs- with the aim of making primary data more accessible and transparent to the reader. If you agree, you would need to provide one PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figures and an Excel sheet or similar with the data behind the graphs. The files should be labeled with the appropriate figure/panel number, and the gels should have molecular weight markers; further annotation could be useful but is not essential. The source files will be published online with the article as supplementary "Source Data" files and should be uploaded when you submit your final version. If you have any questions regarding this please contact me.

After all remaining corrections have been attended to, you will receive an official decision letter from the journal accepting your manuscript for publication in the next available issue of EMBO reports. This letter will also include details of the further steps you need to take for the prompt inclusion of your manuscript in our next available issue.

Thank you for your contribution to EMBO reports.

3rd Revision - authors' response 11 December 2013

final revision received

4th Editorial Decision 11 December 2013

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. As you are aware, this File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

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