PP1 promotes cyclin B destruction and the metaphase-anaphase transition by dephosphorylating CDC20

James Bancroft, James Holder, Zoe Geraghty, Tatiana Alfonso-Perez, Daniel Murphy, Francis Barr, and Ulrike Gruneberg

Corresponding author(s): Francis Barr, University of Oxford

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RE: Manuscript #E20-04-0252
TITLE: PP1 promotes cyclin B destruction and the metaphase-anaphase transition by dephosphorylating CDC20

Dr. Barr,

As you will see below, all three reviewers are excited about your findings. However, all three also raise a number of points that will need to be addressed. While most of the reviewers' concerns should be straightforward to address, a few will require significant effort and new experiments. As Reviewer #1 indicates, it's important to present rescue results for the knockdown experiments. Both Reviewers #2 and #3 raise the possibility that the effects of knocking down PP1 might be mediated through the SAC. Both Reviewers suggest some nice experiments to probe this possibility. Although I focus my comments on these two sets of experiments, because they may required the most effort on your part, I find all of the reviewer comments insightful and in need of responses in a revised manuscript.

I hope these comments are helpful as you complete this work for a revised manuscript.

Sincerely,

Mark Solomon
Associate Editor
Molecular Biology of the Cell

Dear Prof. Barr,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made, as described in the Monitoring Editor's decision letter above and the reviewer comments below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers' comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter.") Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you haveopted out of publishing the review history.

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In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

To submit the rebuttal letter, revised manuscript, and figures, use this link: Link Not Available

Please contact us with any questions at mboc@ascb.org.

Thank you for submitting your manuscript to Molecular Biology of the Cell. We look forward to receiving your revised paper.

Sincerely,

Eric Baker
Journal Production Manager

MBoC Editorial Office
mbc@ascb.org

Reviewer #1 (Remarks to the Author):

Review of MBC E20-04-0252 (Gruneberg)

In this study, the authors provide evidence that PP1 promotes the metaphase-anaphase transition in human cells by dephosphorylating Cdc20. This point seems like a significant one in mitotic regulation. Overall, this work seems well-executed and the conclusions seem reasonably sound. I have the following criticisms.

1. For all of the blots in the paper with the anti-PPP1CA-pT320 antibodies, there is no corresponding blot for either the PPP1CA or PPP1CC protein. There needs to be at least one figure where the authors show that changes in the abundance of the protein(s) are not a factor in the functional observations.

2. My understanding is that tautomycetin can inhibit PP2A at high concentrations, and 5 micromolar does not seem like a low concentration. The authors should specifically validate that the drug does not inhibit PP2A at the concentrations used.

3. For siRNA experiments, it is usually best to perform rescue experiments. The authors mention that one set of siRNAs was used for Figures 2, 3, and 6 and another set for Figures 5, 7, and S2. Were these sets tested side-by-side to see if they give the same results? Also, did the authors try knocking down PP1-alpha and PP1-gamma separately? Perhaps one of these could be more specific for the particular processes examined in this paper.

4. Figures 3A and 3B. It seems that a statistical analysis was done, but I did not see any information about it. Elsewhere in the paper, there are plots with lots of data points but no information on significance. Some figures have error bars with no explanation . I did not see reference to how many times experiments were done.

5. It is a little confusing that the authors switch back and forth by referring to the enzymes as PPP1CA, B, C in some places and then using Greek letter names in others.

6. Figure 6B. The PhosTag experiment could be explained better, and the labeling of the bands improved.

7. I does not seem to quite fit that the Cdc20-6A mutant is not defective for the spindle checkpoint when, as the authors mention in the Introduction, phosphorylation of Cdc20 by Cdk1 promotes incorporation into the MCC. Some discussion would be helpful.

Minor Point.

All of the micrographs in the main paper are black-and-white, whereas Figure S2 is in color. Color figures might look better in the main text.

Reviewer #2 (Remarks to the Author):

See Attached.

Reviewer #3 (Remarks to the Author):

This manuscript by Bancroft et al. describes the role of PP1 in dephosphorylating CDC20, thus promoting its association with the APC/C and allowing degradation of Cyclin B and metaphase-anaphase transition in human cells, bringing it in line with the observations made in C. elegans (Kim et al., 2017). By using siRNA knockdown experiments in conjunction with CDK inhibition in metaphase-released HeLa cells, the authors establish the importance of the α and γ isoforms of PP1 in timely Cyclin B degradation. Using live cell microscopy, the authors then show that Cyclin B degradation delay persists after Mad2 eviction from kinetochores in PP1-inhibited or -depleted cells. Furthermore, using CDK1 phosphorylation-deficient CDC20-6A, they describe the role for PP1 particularly in dephosphorylation of CDC20 at the metaphase-anaphase transition. The mechanism underlying timely activation of APC/C-CDC20 during mitotic progression is of general interest. Yet, the conclusion that PP1's role in CDC20 dephosphorylation at the metaphase-anaphase transition is separate from its upstream roles in stabilizing kinetochore-microtube interactions and spindle assembly checkpoint silencing, or indeed its downstream requirement for reactivating PP2A-B55 is less convincing and should be examined carefully as some data could be interpreted differently. The manuscript would be improved if the following points were addressed:

Major points:
1. One of the major concerns is about evidence of the temporal order in action of three mitotic phosphatases highlighted in Figure 8. The authors present no evidence that PP2A-B56 activity is restricted to early mitosis, and there is a temporal window of PP2A-B56 and PP1 activity. As CDK1-dependent PP1 inhibition is not complete, some PP1 is active in early mitosis. Unless the authors have evidence that PP2A-B56 acts first and then PP1 to dephosphorylates CDC20, Figure 8 would be rather misleading as it indicates IN and OUT of PP1 and PP2A-B56, respectively. Can the authors alter their figure to accurately represent their data?

2. Figure 6B shows that CDC20 is slowly dephosphorylated in the presence of PP1 inhibitor although the dephosphorylation of PP1 is completely inhibited at every time point. Is it possible that the delayed dephosphorylation of CDC20 could be an indirect effect because of delayed degradation of cyclin B (Inactivation of CDK1)?

3. Figure 6C and 6G are crucial experiments for the authors' model: PP1 dephosphorylates CDC20 (pT70) at M-A transition. The experiments should be repeated and presented with error bars. More importantly, PP1 inhibition causes PP2A-B55 inhibition indirectly through preventing Gwl (MASTL) inactivation and thus it is not clear whether PP2A-B55 as well as PP1 are involved in CDC20 dephosphorylation under these conditions. Can the authors clarify this?

4. A number of observations made by the authors such as Cyclin B degradation and M-A transition delay observed upon PP1 inhibition or depletion can be explained by persistence of inhibitory phosphorylation events in SAC signaling that are removed by PP1. To deconvolute PP1 requirement in these processes, can the authors inhibit SAC signaling (by Mps1 inhibition for instance) in conjunction with PP1 depletion in Figure 3 or Figure 4, for instance?

5. Figure 7E: It seems that the scatter plot of CDC20-6A under siPP1 is more similar to that of WT under siPP1 than to that of CDC20-WT or 6A without depletion of PP1. As PP1 is implicated in release from SAC rather than mounting it, it is also important to examine the impact on PP1-mediated Cdc20 dephosphorylation at M-A transition by Mps1 inhibition. Can the authors show that cells depleted for endogenous PP1 and CDC20 and expressing CDC20-6A more rapidly degrade cyclin B and progress to anaphase than those expressing CDC20-WT upon SAC silencing?

Minor points:
1. Throughout the manuscript, there are several depletion experiments under different conditions. It would be more convincing if efficient depletion results (SDS-PAGE and immunoblot) are presented. Otherwise, the lack any observed effect upon depletion of PP2A-B55 could be explained by poor depletion efficiency, for instance.
2. Figure S1A and B: the text refers to destruction profiles of both Cyclin B and securin; yet the figure only shows Cyclin B degradation. Could the authors clarify the text?
3. Figure 1C, D and Figure 2E: could the authors clarify the number of repeats performed for these experiments/show error bars within the quantification?
4. Figure 3 and subsequent figures: could the authors uniformly indicate how many cells were counted for each condition, the details of the statistical analyses performed and the associated p-values?
5. Figure 4A-C: Please indicate the time points of "last MAD2 loss" and "50 mins after last MAD2 loss" as shown in Figure 5. Similarly, please mark the time points of "LCC", "LMC" and "ANA" in Figure S2.

Reviewer #1 (Remarks to the Author):

Review of MBC E20-04-0252 (Gruneberg)

In this study, the authors provide evidence that PP1 promotes the metaphase-anaphase transition in human cells by dephosphorylating Cdc20. This point seems like a significant one in mitotic regulation. Overall, this work seems well-executed and the conclusions seem reasonably sound. I have the following criticisms.

1. For all of the blots in the paper with the anti-PPP1CA-pT320 antibodies, there is no corresponding blot for either the PPP1CA or PPP1CC protein. There needs to be at least one figure where the authors show that changes in the abundance of the protein(s) are not a factor in the functional observations.
This omission has been corrected. Blots showing the total pool of PPP1CA (PP1α) are now included in Figure 1 and Figure S1. Additional Western blot controls are also shown in Figure S4A and S4B.

2. My understanding is that tautomycetin can inhibit PP2A at high concentrations, and 5 micromolar does not seem like a low concentration. The authors should specifically validate that the drug does not inhibit PP2A at the concentrations used.
Inhibitor selectivity is always a concern. For this reason, we use both the PP1 inhibitor tautomycetin in Figure 1, and selective depletion of PP1 catalytic subunits using siRNA in Figure 2. This approach provides independent support for our conclusions that the effects are due to PP1 activity.

Regarding tautomycetin specificity, published biochemical analysis and structural biology show that tautomycetin is a highly specific and irreversible inhibitor of PP1 due to the formation of a covalent adduct to the PP1-specific cysteine residue Cys127 (Choy et al (2017) J Am Chem Soc 139(49):17703-17706 (PMID: 29156132). By binding into the hydrophobic groove of the catalytic subunit and forming this covalent adduct, tautomycetin occludes the catalytic site and shows selectivity of at least 10^2-10^3 for PP1 compared the other PPPs.

In terms of relevant cell biology and application to studies of mitosis, we have previously characterised the use of tautomycetin and calyculin to inhibit either PP1 specifically, or both PP1 and PP2A. This is shown in Figures 1 and S1 of Hayward et al (2019) Journal of Cell Biology 218(10):3188-3199 (PMID: 31511308). In that case 5 μM tautomycetin, the concentration used here, does not inhibit the PP2A-dependent turnover of phosphorylation sites on the checkpoint protein MPS1 in mitosis. We therefore conclude that it is sufficiently selective for PP1 under the mitotic conditions we use in the current work.

3. For siRNA experiments, it is usually best to perform rescue experiments. The authors mention that one set of siRNAs was used for Figures 2, 3, and 6 and another set for Figures 5, 7, and S2. Were these sets tested side-by-side to see if they give the same results? Also, did the authors try knocking down PP1-alpha and PP1-gamma separately? Perhaps one of these could be more specific for the particular processes examined in this paper.
We have used two different sets of siRNA duplexes directed to the different PP1 catalytic subunits, targeted against either the ORF or the 3'-UTR as explained in the Methods section of the manuscript. One reason for this choice was to reduce the possibility of a common off-target effect. Western blots to demonstrate efficient PP1 depletion with the first set of siRNA duplexes were already included in the manuscript (Figure 2). We have now added Western blots and immunofluorescence panels to demonstrate efficient depletion with the second set of oligos in Figure S2.

We have tested siRNA single knock down of PP1α or PP1γ in the course of the work presented in this manuscript and a series of previous studies of mitosis (Zeng et al. (2010) Journal of Cell Biology 191(7):1315-1332 (PMID:21187329); Cundell et al. (2013) Mol Cell 52(3): 393-405 (PMID:24120663); Espert et al. (2014) Journal of Cell Biology 206(7):833-842 (PMID:25246613)). Single depletions resulted in less pronounced mitotic defects than co-depletion of both PP1α and PP1γ, in agreement with the published idea that these two proteins act partially redundantly (Liu et al. (2010) Journal of Cell Biology 188(6): 809-820 (PMID:20231380); Trinkle-Mulcahy et al. (2006) Journal of Cell Biology 172(5):679-92 (PMID:16492807)). To look at the bulk role of PP1 in mitotic exit we therefore co-deplete both PP1α and PP1γ. The triple co-depletion with PP1β was found to result in high levels of cell death and was not used for that reason.

Rescue experiments are difficult to perform for the PP1 catalytic subunit co-depletion, since both transgenes have to be expressed simultaneously to the correct level, at the right time, and crucially overexpression has to be avoided. This has proven technically challenging. For our future studies of PP1, we are currently working on strategies to overcome these limitations using a very different approach to the one presented here. This is a project in its own right which goes beyond the scope of our current manuscript. As explained we used a variety of methods to study the role of PP1, not only siRNA knockdown.

In summary, we are confident in our findings for two reasons: (i) the tautomycetin PP1-inhibitor experiments already in the manuscript serve as an independent control for PP1 depletion; (ii) we use different siRNA duplexes to confirm the findings.

4. Figures 3A and 3B. It seems that a statistical analysis was done, but I did not see any information about it. Elsewhere in the paper, there are plots with lots of data points but no information on significance. Some figures have error bars with no explanation. I did not see reference to how many times experiments were done.
We have included a paragraph detailing how measurements and the relevant statistical analysis were performed in the revised Methods section.

5. It is a little confusing that the authors switch back and forth by referring to the enzymes as PPP1CA, B, C in some places and then using Greek letter names in others.
Naming for PP1 is now consistent throughout the manuscript text.

6. Figure 6B (now Figure 7B). The PhosTag experiment could be explained better, and the labeling of the bands improved.
The description of the PhosTag experiment has been expanded on page 14 of the revised version of the manuscript:

"To investigate the kinetics of CDC20 dephosphorylation at the metaphase-to-anaphase transition in the presence and absence of PP1 activity, we first used PhosTag SDS-PAGE to visualise the phosphorylation status of CDC20. The PhosTag reagent binds to phosphate groups on proteins and thus results in reduced mobility and enhanced separation of the phosphorylated species away from non-phosphorylated forms on SDS-PAGE (Kinoshita et al., 2006)."

7. It does not seem to quite fit that the Cdc20-6A mutant is not defective for the spindle checkpoint when, as the authors mention in the Introduction, phosphorylation of Cdc20 by Cdk1 promotes incorporation into the MCC. Some discussion would be helpful.
We have revised the introduction to more clearly explain how CDC20 phosphorylation might bias incorporation into the MCC without expression of $CDC20^{6A}$ resulting in a checkpoint deficiency. Published work shows that CDC20 phosphorylation reduces the affinity for the APC/C and thus increases the free pool of CDC20. This free pool of CDC20 is presumably what feeds into MCC formation. An alternative is that phosphorylated CDC20 is a better substrate for the MCC formation pathway. These aren't mutually exclusive possibilities and further work is needed to explain the precise mechanism by which CDC20 phosphorylation promotes MCC formation. Our observation that cells expressing only $CDC20^{6A}$ do not show obvious spindle checkpoint defects is possibly more consistent with the first model, since the mutation would not directly alter MCC formation.

In summary, the most parsimonious explanation is that phosphorylation of CDC20 does not actively promote incorporation into MCC but does impair association with APC/C. We have tried to capture this in the revised introduction.

Minor Point.

All of the micrographs in the main paper are black-and-white, whereas Figure S2 is in color. Color figures might look better in the main text.
We considered this option but found that the contrast is better with monotone greyscale images and have therefore left the main figures as they were. Common visual impairments make interpretation of image data difficult for many individuals, so we generally try to use greyscale where comparison of intensity is important and always provide single channel data alongside merged images for localisation data.

Reviewer #2 (Remarks to the Author):
In the manuscript entitled "PP1 promotes cyclin B destruction and metaphase-anaphase transition by dephosphorylating CDC20" the authors analyze the role of protein phosphatase 1 (PP1) in mitotic progression. They propose that PP1 is required for activation of the APC/C, the ubiquitin ligase required for mitotic exit, by removing inhibitory phosphates on Cdc20, the key APC/C co-activator in mitosis. This is an interesting concept, especially in light of previous studies suggesting mechanisms for Cdc20 dephosphorylation, such as PP2A-B56 docked on Apc1 in *Xenopus laevis* (Fujimitsu et al, EMBO 2020) and kinetochore-localized PP1 in *C. elegans* (Kim et al, Genes and Development 2017). However, there is an important concern about the conclusion that needs to be addressed before publication, in addition to clarification of other points.
Major Comment:
The authors need to address more carefully the mechanism by which PP1 promotes APC/C activation. Throughout the manuscript, they show that PP1 inhibition delays cyclin B1 degradation and mitotic exit, consistent with a delay in APC/C activation. However, PP1 is also known to be important for inactivation of the spindle assembly checkpoint (e.g. Vanoosthuyse and Hardwick, Current Biology 2009) and a defect in checkpoint inactivation would also result in delayed cyclin B1 degradation. To address this point, the authors look at cyclin B1 degradation kinetics following loss of Mad2 from kinetochores, which they use as a marker for checkpoint inactivation, and found that cyclin B1 degradation remained slower in cells inhibited of PP1. The authors then conclude that PP1 must be directly required for APC/C activation. However, an alternative possibility is that PP1 is required for checkpoint silencing downstream of Mad2 kinetochore removal by, for instance, promoting disassembly of mitotic checkpoint complexes. The only way to distinguish between these possibilities is to eliminate checkpoint signaling altogether. The authors should conduct the cyclin B1 degradation

analysis in cells in which checkpoint signaling was abolished (for instance, by inhibition of the checkpoint kinase Mps1 or depletion of Mad2). If, under these conditions, PP1 inhibition still caused a delay in cyclin B1 degradation, their claim for PP1 playing a direct role in APC/C activation will be well-supported. Similarly, the experiment in which they suppress the PP1 phenotype using a non-phosphorylatable Cdc20 mutant should also be conducted in the absence of checkpoint signaling.

To help address this question, we have added live cell imaging data in which we treat control or PP1-depleted cells undergoing an unperturbed mitosis with an MPS1 inhibitor to artificially silence the spindle checkpoint as suggested by the reviewer (see new Figure 6). In both control and PP1-depleted cell populations, MAD2 was lost from kinetochores within 4 minutes of the addition of the MPS1 inhibitor, confirming that spindle checkpoint signalling had stopped. Under these conditions, cyclin B destruction was substantially delayed in the PP1 depleted cells compared to the control cells. This supports the conclusion that PP1 has a role in regulating cyclin B destruction independently of ongoing spindle checkpoint signalling. Other experiments shown in Figure 7 and 8 show that the APC/C co-activator CDC20 is likely to be a crucial target of PP1 in this context. Some precise mechanistic details remain for future studies to address as we discuss in the manuscript. Published work shows that CDC20 phosphorylation reduces the affinity for the APC/C. Whether or not CDC20 phosphorylation also has an impact on MCC turnover as proposed by the referee is not known. Future studies will certainly be needed to understand the precise mechanism by which CDC20 phosphorylation acts, and we agree with the reviewer that a detailed analysis of MCC turnover would be informative. However, that is beyond the scope of this current work.

Since the publication of the review article on yeast spindle checkpoint signalling from 2009 cited in this comment, evidence has emerged for key differences between the spindle checkpoint silencing and mitotic exit pathways in yeast and mammalian cells. Although budding and fission yeast cells have proven to be excellent models to study the cell cycle, some differences, especially relating to the control of mitotic exit, are relevant when considering for our work. Phosphatase function and regulation shows some major differences, for example the central role of CDC14 in the yeast mitotic exit network (MEN) or septum initiation network (SIN) pathways is fulfilled by PP1 and PP2A-B55 in animal cells. In yeast, as the reviewer notes, PP1 has been implicated as a key checkpoint silencing activity, whereas it has become clear since that review was written that in mammalian cells this place is taken by a combination of PP2A-B56 (Espert et al. (2014) Journal of Cell Biology, 206(7):833-42 (PMID:25246613); Hayward et al. (2019) Journal of Cell Biology, 218(10):3188-3199 (PMID: 31511308); Qian et al. (2017) Mol. Cell, 68(4):715-730 (PMID: 29129638) and PP1 (Nijenhuis et al. (2014) Nat. Cell. Biol., 16(12):1257-64 (PMID: 25402682)). This literature, and the data presented in our manuscript support the view that PP1 plays earlier and later roles during mitosis. Our focus here was creating conditions that allowed us to test the role of PP1 in regulation of the APC/C at the metaphase-anaphase transition. That doesn't speak against earlier roles in cell rounding, spindle formation and checkpoint signalling, or later roles in MCC turnover, mitotic exit and regulation of chromatin architecture.

1) In figure 6, the authors characterize the effect of PP1 inhibition on Cdc20 dephosphorylation upon mitotic exit. The analysis of this data is complicated by the fact that PP1 inhibition also delays exit from mitosis, as evidenced by a delay in cyclin B1. In other words, is the kinetic effect on Cdc20 dephosphorylation observed upon PP1 inhibition simply reflecting delayed exit from mitosis? To address this, the authors need to compare dephosphorylation at time points where cyclin B1 levels are equivalent and also analyze later time points.

CDK1-cyclin B and PP1 activities are coupled by a feedback loop controlled by the stability of cyclin B. CDK inhibits PP1, which autocatalytically dephosphorylates itself then becomes re-phosphorylated while CDK activity remains sufficiently high. As we show in this manuscript, PP1 activity towards CDC20 provides a further feedback element regulating cyclin B stability. As the reviewer notes, picking apart this network of regulation is challenging. Figures 7 and 8 address this issue. In Figure 7 we perform a careful time course analysis of CDC20 dephosphorylation in mitotic exit. In Figure 8, we use the CDC20^{6A} mutant to examine the consequences of removing this regulation for cyclin B stability, chromosome segregation and mitotic exit. The data presented in revised Figure 8 show that the CDC20^{6A} mutant can rescue the delay in cyclin B destruction observed in the absence of PP1. This observation is most consistent with the idea that CDC20 dephosphorylation feeds back to increase the rate of cyclin B destruction.

2) The authors find that inhibition of PP1, either through the small-molecule tautomycetin or siRNA-mediated depletion of the PP1 isoforms a and g resulted in a mitotic delay. On the other hand, depletion of PP1b did not have a significant effect, suggesting that PP1a and PP1g are the main phosphatases that promote mitotic exit. However, it appears that PP1a/g double siRNA does not fully recapitulate the effect. of tautomycetin (for instance, compare Fig. 4D and 5D). Did the authors analyze triple depleted cells? Even though PP1b does not have an effect on its own, it may contribute to mitotic exit in the absence of PP1 a/g . This may also explain why a sub-population of PP1 a/g-depleted cells displayed wild-type kinetics of cyclin B1 destruction (shown in Fig. 3E)

We have previously compared PP1α, β and γ single depletions, as well as PP1α/γ double and PP1$\alpha/\beta/\gamma$ co-depletions. PP1$\alpha/\beta/\gamma$ co-depletions showed the same phenotype as PP1α/γ co-depletions but with strongly decreased cell viability. For these reasons, in all future experiments we used PP1α/γ co-depletion. With siRNA-mediated depletion it is difficult to achieve knockout (complete loss), and the simplest explanation for the slight differences between siRNA depletion and tautomycetin-mediated inhibition experiments is that PP1α and PP1γ were not completely depleted by siRNA in all cells. A chemical inhibitor typically gives a more uniform response across the cell population.

Minor issues:
-The authors showed that CDK1 inhibition results in accelerated dephosphorylation of the inhibitory T320 residue in PP1 with kinetics parallel to that of cyclin B destruction. However, it is important to confirm that total PP1 levels are unchanged by CDK1 inhibition.
Blots showing that total PP1 remains unchanged have now been included in Figure 1 and Figure S1.

-Information on "n=sample size" is missing from the legend of Fig. 3.
We have included a paragraph detailing how measurements and the relevant statistical analysis were performed in the revised Methods section. Sample sizes are described in the figure legend.

-The statement "depletion of PP1beta had "no obvious" effect on cyclin B destruction compared to the control condition" should be toned down, as PP1beta depletion does appear to mildly delay cyclin B1 degradation.
Single cell imaging revealed that PP1β-depleted cells showed more heterogeneous mitotic exit timing than controls, but the kinetics of cyclin B destruction once initiated were similar to the control (Figure 2). This effect was seen in the ensemble biochemical analysis (Figure 1). Taken together, we concluded that the rate of cyclin B destruction is not affected by depletion of PP1β. This is more accurately described in the manuscript text.

-In page 6, Hein et al 2017 did not suggest that B55 was the Cdc20 phosphatase.
This paper from the Nilsson group does suggest that PP2A-B55 is the major CDC20 phosphatase. The published abstract and relevant portion of the text (italic, blue) is copied below:
"Here we demonstrate that the phosphothreonine preference of PP2A–B55 provides an essential regulatory element of mitotic exit. To allow rapid activation of the anaphase-promoting complex/cyclosome (APC/C) co-activator Cdc20, inhibitory phosphorylation sites are conserved as threonines while serine substitutions delay dephosphorylation and Cdc20 activation."

A more direct statement comes from the main text of that same article:
"This indicates that PP2AB55 is a mitotic exit phosphatase for Cdc20 and APC1, but we cannot exclude that other protein phosphatases can dephosphorylate these sites."

Our text does not misrepresent these statements or the published work of the authors. Therefore, the text has not been changed.

-Information on statistics is missing from Fig. 4E, 5E and 7E.
We have included a paragraph detailing how measurements and the relevant statistical analysis were performed in the revised Methods section. Sample sizes are described in the relevant figure legends (former Figure 7E is now Figure 8E.)

-(+) signs in Fig. 2F and Fig. 8 (now Figure 9) might not be relevant.
Figure labelling has been updated to remove + signs.

Reviewer #3 (Remarks to the Author):

This manuscript by Bancroft et al. describes the role of PP1 in dephosphorylating CDC20, thus promoting its association with the APC/C and allowing degradation of Cyclin B and metaphase-anaphase transition in human cells, bringing it in line with the observations made in C. elegans (Kim et al., 2017). By using siRNA knockdown experiments in conjunction with CDK inhibition in metaphase-released HeLa cells, the authors establish the importance of the α and γ isoforms of PP1 in timely Cyclin B degradation. Using live cell microscopy, the authors then show that Cyclin B degradation delay persists after Mad2 eviction from kinetochores in PP1-inhibited or -depleted cells. Furthermore, using CDK1 phosphorylation-deficient CDC20-6A, they describe the role for PP1 particularly in dephosphorylation of CDC20 at the metaphase-anaphase transition. The mechanism underlying timely activation of APC/C-CDC20 during mitotic progression is of general interest. Yet, the conclusion that PP1's role in CDC20 dephosphorylation at the metaphase-anaphase transition is separate from its upstream roles in stabilizing kinetochore-microtube interactions and spindle assembly checkpoint silencing, or indeed its downstream requirement for reactivating PP2A-B55 is less convincing and should be examined carefully as some data could be interpreted differently. The manuscript would be improved if the following points were addressed:

Major points:
1. One of the major concerns is about evidence of the temporal order in action of three mitotic phosphatases highlighted in Figure 8. The authors present no evidence that PP2A-B56 activity is restricted to early mitosis, and there is a temporal window of PP2A-B56 and PP1 activity. As CDK1-dependent PP1 inhibition is not complete, some PP1 is active in early mitosis. Unless the authors have evidence that PP2A-B56 acts first and then PP1 to dephosphorylates CDC20, Figure 8 would be rather misleading as it indicates IN and OUT of PP1 and PP2A-B56, respectively. Can the authors alter their figure to accurately represent their data?
We are not suggesting that bulk PP2A-B56 activity is restricted to earlier mitosis. We propose that PP2A-B56 activity towards some important mitotic substrates is restricted to early mitosis due to high CDK activity, and that

PP2A-B56 is then redirected to other substrates as the landscape of phosphorylation changes. This is consistent with the reported role for phosphorylation in directing PP2A-B56 binding to BubR1 (Kruse et al. (2013) J Cell Sci 126(Pt 5):1086-92 (PMID: 23345399); Suijkerbuijk et al. (2012) Dev Cell 23(4):745-55 (PMID: 23079597). In this case it is therefore probably better to refer to the pool of PP2A-B56 recruited by BUBR1. A modified version of the schematic depicting this has been included in the revised manuscript (Figure 9).

2. Figure 6B (now Figure 7B) shows that CDC20 is slowly dephosphorylated in the presence of PP1 inhibitor although the dephosphorylation of PP1 is completely inhibited at every time point. Is it possible that the delayed dephosphorylation of CDC20 could be an indirect effect because of delayed degradation of cyclin B (Inactivation of CDK1)?
CDK1-cyclin B and PP1 activities are coupled by a feedback loop controlled by the stability of cyclin B. CDK inhibits PP1, which autocatalytically dephosphorylates itself then becomes re-phosphorylated while CDK activity remains sufficiently high. As we show in this manuscript, PP1 activity towards CDC20 provides a further feedback element regulating cyclin B stability. This isn't an indirect effect, it is an integral component of the feedback loop. Figures 7 and 8 address this issue in detail. In Figure 7 we perform a careful time course analysis of CDC20 dephosphorylation in mitotic exit. In Figure 8, we use the CDC20^{6A} mutant to examine the consequences of removing this regulation for cyclin B stability, chromosome segregation and mitotic exit. The data presented in revised Figure 8 show that the CDC20^{6A} mutant can rescue the delay in cyclin B destruction observed in the absence of PP1. It is unlikely that PP1 is completely inhibited, Figure 1C suggests that under these conditions the half-life of the inhibitory pT320 phosphorylation is increased from ~2 min to >60min, and inhibition is >80%. These observations are most consistent with the idea that CDC20 dephosphorylation feeds back to increase the rate of cyclin B destruction.

3. Figure 6C and 6G are crucial experiments for the authors' model: PP1 dephosphorylates CDC20 (pT70) at M-A transition. The experiments should be repeated and presented with error bars.
These figure panels (now Figure 7) have been extended with additional data as requested.

More importantly, PP1 inhibition causes PP2A-B55 inhibition indirectly through preventing Gwl (MASTL) inactivation and thus it is not clear whether PP2A-B55 as well as PP1 are involved in CDC20 dephosphorylation under these conditions. Can the authors clarify this?
In live cell imaging we do not see any delays at the metaphase to anaphase transition when PP2A-B55 is depleted in the absence of "errors" (this manuscript Figure 5 and Hayward, Alfonso-Perez et al. (2019) Journal of Cell Biology 218(4):1108-1117 (PMID: 30674583)), whereas depletion or inhibition of PP1 does lead to a delay at the metaphase-to-anaphase transition. The same is true in biochemical time courses, and we have now included an additional time course experiment where the cyclin B destruction kinetics of siPP1 or siB55 depleted cells are compared to the control (Figure S4A-S4D). This shows that PP1-depletion leads to a notable delay in CDC20 dephosphorylation and cyclin B destruction, while B55-depletion does not. Together, this makes it far more likely that PP1, and not PP2A-B55, directly initiates CDC20 dephosphorylation. We agree, that at the same time PP1 will inactivate Greatwall leading to PP2A-B55 activation so that PP2A-B55 will contribute to CDC20 dephosphorylation later in anaphase. This is explained in the text.

4. A number of observations made by the authors such as Cyclin B degradation and M-A transition delay observed upon PP1 inhibition or depletion can be explained by persistence of inhibitory phosphorylation events in SAC signaling that are removed by PP1. To deconvolute PP1 requirement in these processes, can the authors inhibit SAC signaling (by Mps1 inhibition for instance) in conjunction with PP1 depletion in Figure 3 or Figure 4, for instance?
Using GFP-MAD2 as a read-out, our experiments show that spindle checkpoint proteins are lost from kinetochores in the absence of PP1 activity and cyclin B destruction is not initiated with normal kinetics. From these data we can be reasonably sure that the events leading to spindle checkpoint activation are reversed in PP1 depleted cells. To formally address this point, we have now added additional live cell imaging data in which we treat control or PP1-depleted cells undergoing unperturbed mitosis with an MPS1 inhibitor to artificially silence the spindle checkpoint (new Figure 6). In both cell populations, MAD2 is lost from kinetochores within a few minutes of the addition of the inhibitor. This confirms that spindle checkpoint signalling has been stopped. However, cyclin B destruction was substantially delayed in the PP1 depleted cells compared to the control cells. This supports the conclusion that PP1 has a role in regulating cyclin B destruction independently of ongoing spindle checkpoint signalling.
We cannot completely exclude that MCC disassembly is affected by the absence of PP1, and that is an interesting possibility for future investigations.

5. Figure 7E (now Figure 8E): It seems that the scatter plot of CDC20-6A under siPP1 is more similar to that of WT under siPP1 than to that of CDC20-WT or 6A without depletion of PP1. As PP1 is implicated in release from SAC rather than mounting it, it is also important to examine the impact on PP1-mediated Cdc20 dephosphorylation at M-A transition by Mps1 inhibition. Can the authors show that cells depleted for endogenous PP1 and CDC20 and expressing CDC20-6A more rapidly degrade cyclin B and progress to anaphase than those expressing CDC20-WT upon SAC silencing?

The new Figure 6 in our revised manuscript demonstrates that PP1 depletion impairs cyclin B destruction independently of any role of PP1 in the silencing of the spindle assembly checkpoint. Our manuscript demonstrates that the delay in cyclin B destruction is largely due to the role of PP1 in initiating CDC20 dephosphorylation but as explained above we cannot completely exclude that PP1 has additional roles in regulating MCC disassembly.

Minor points:

1. Throughout the manuscript, there are several depletion experiments under different conditions. It would be more convincing if efficient depletion results (SDS-PAGE and immunoblot) are presented. Otherwise, the lack any observed effect upon depletion of PP2A-B55 could be explained by poor depletion efficiency, for instance.
Blots demonstrating efficient depletion of the different factors have now been added to Figure S2C and Figure S4A.

2. Figure S1A and B: the text refers to destruction profiles of both Cyclin B and securin; yet the figure only shows Cyclin B degradation. Could the authors clarify the text?
This has been corrected in the revised text.

3. Figure 1C, D and Figure 2E: could the authors clarify the number of repeats performed for these experiments/show error bars within the quantification?
This information has been added to relevant figure legends in the revised manuscript.

4. Figure 3 and subsequent figures: could the authors uniformly indicate how many cells were counted for each condition, the details of the statistical analyses performed and the associated p-values?
We have included a paragraph detailing how measurements and the relevant statistical analysis were performed in the revised Methods section. Sample sizes are described in the relevant figure legends.

5. Figure 4A-C: Please indicate the time points of "last MAD2 loss" and "50 mins after last MAD2 loss" as shown in Figure 5. Similarly, please mark the time points of "LCC", "LMC" and "ANA" in Figure S2 (now figure S3).
Labels have been added to the figures as requested. Note the original Figure S2 is now S3.

RE: Manuscript #E20-04-0252R
TITLE: "PP1 promotes cyclin B destruction and the metaphase-anaphase transition by dephosphorylating CDC20"

Dr. Barr,

Thank you for the extensively revised manuscript, which is now acceptable for publication in Molecular Biology of the Cell. I appreciate the effort required to address the reviewers' concerns.

Sincerely,

Mark Solomon
Associate Editor
Molecular Biology of the Cell

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