nature portfolio

Peer Review File

Unveiling an Arpp-19 phosphorylation switch that grants chromosome stability

Corresponding Author: Professor Domenico Grieco

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Cell cycle progression is strictly regulated by the opposing actions of kinases and phosphatases. The PP2A-B55 family of phosphatases are particularly critical for regulated entry into and exit out of mitosis. During mitosis, these phosphatases are inhibited by two small phosphorylated proteins, ENSA and Arpp19. Phosphorylation of ENSA and Arpp-19 by the Greatwall kinase has been shown to be essential for the inhibitory properties of these proteins. Serpico and colleagues here demonstrate that Arpp-19 is also phosphorylated by Cdk1, and that this phosphorylation seems to be critical for the action of Arpp-19 as cells expressing phospho-null or phospho-mimetic versions of Arpp-19 display significant defects during cell cycle progression.
The paper is on the whole very nicely conducted and the results seem convincing, and the topic is definitely of interest to a wide readership, so I am very enthusiastic about this manuscript. There are however, some issues that should be addressed:
1. I have a slight concern with the fact that all of the experiments where transgenic forms of Arpp-19 were used seem to have been conducted with transiently transfected cells rather than stable cell lines. In this setting, transfection efficiency and non-homogeneous transfection of cell populations can be problematic for the interpretation of the results. Such problems are not apparent in the figures in the paper, in contrast, e.g. in Figure 2C, the results for at least the cells that are shown look very homogeneous. Since the transfection efficiency is unlikely to have been 100%, this is a bit surprising. What was the transfection efficiency in these experiments, and could the authors stain for the transgene to demonstrate that the observed effects only happen in the transfected cells?
2. The interplay between the Greatwall and the Cdk1 phosphorylation sites is very interesting but Figure 3d, which analyses this, is confusing. The Arpp-19 S23A and S23D mutants are compared to the WT at different time points (30 or 50 mins). This means the conditions for the A and the D mutants are not comparable. The experiment should be conducted as a time course, showing for each condition (WT, S23A and S23D) 0, 30 and 50 mins after nocodazole release. There should also be further discussion of the relationship between the two phosphorylation sites and their phosphatases in the Discussion part of the paper. The authors suggest that CDk1-phosphorylated Arpp-19 may inhibit Fcp1 in a similar manner to Greatwall-phosphorylated Arpp-19 inhibiting PP2A-B55, but this would only really make sense if Fcp1 was then the phosphatase dephosphorylating the Greatwall site on Arpp-19.This has indeed been suggested (Hégarat et al., 2014), but generally (and by the authors), PP2A-B55 is thought to be the main phosphatase for the DpSG-site on Arpp-19. So what exactly is then the relationship between PP2A-B55 and Fcp1, and why does the depletion of Fcp1 also lead to stabilisation of the DpSG site (Figure 4b)? This should really be discussed more; it's a bit difficult to fully understand what the authors think their data mean. A model at the end would be very helpful!
3. Minor point: The sequence alignment should be included in the main figure. It's important to know where the two phospho-sites are.

Reviewer #2

(Remarks to the Author)
Title: Cdk1 phosphorylates Arpp-19 to grant chromosome stability

The authors claim to have identified a new phosphorylation switch involved in regulating mitotic exit and chromosome stability. Indeed, it is known that Cyclin B1 / CDK1 complex is tightly regulated during mitosis to allow mitotic progression,

correct chromosome segregation and mitotic exit. CDK1 is dephosphorylated and inactivated by the phosphatase PP2A-B55 which must be inhibited during mitosis to allow CDK1 activity. This happens via Arpp-19/ENSA phosphorylated by Gwl, itself phosphorylated by CDK1. The authors work identified a new phosphorylation site S23 on Arpp-19 that would be phosphorylated by CDK1 itself and dephosphorylated by Fcp1, that contributes to the inactivation of PP2A-B55 and is crucial to maintain chromosome stability and mitosis exit timing. The authors successfully generated an antibody specific of this site. This S23-Arpp-19 site seems more important than the Gwl phosphorylation site to maintain mitosis integrity. This work could improve our understanding of the regulation of the Cyclin B1 / CDK1 complex during mitosis progression.

Although there are some interesting and novel findings presented, some of the conclusions are currently not adequately supported by the presented evidence. In particular, the title is “Cdk1 phosphorylated Arpp-19 to grant chromosome stability” while there is presently no evidence of direct targeting of Arpp-19 S23 by CDK1. There are several points that need to be addressed (see details below), with quantification, statistical analysis often missing, and additional experiments are needed to strengthen and support the manuscript before publication.

1. There is no statistical analysis in the paper. The authors have performed sufficient experiments (three biological replicates in most cases) to perform some basic statistical testing, as without this it is difficult to interpret the significance of the data presented.

2. The Figure 1 should have more than panels a and b as each panel has different figures and methods. Figure one should be reformatted with IF and WB separated from the same panel.

3. In Figure 1, the immunofluorescence shows lagging chromosomes in anaphase cells treated with siRNA. The authors also stained for CREST but do not appear to use this to additionally quantify the nature of the lagging chromatin material. The authors could use these images to quantify the proportion of lagging chromosomes with CREST signal, as a useful confirmation that the origin of the lagging chromosomes is indeed the consequence of mitotic defects (as opposed to defects during chromosome replication in the preceding S-phase for example). Most lagging chromosomes should be CREST-positive, indicating whole lagging chromosomes.

4. In Figure 2, the authors show that a nocodazole treatment and release induces a high proportion of anaphase cells in the S23A condition (20min – 50min), suggesting that the cells were quicker to progress through mitosis. Here, in lines 107-108 the authors commented “S23A-Arpp-19-complemented cells appeared to exit mitosis faster than control as about 25 to 30% were already in anaphase by 20 min post nocodazole wash out” but cells in S23A condition might be stuck in anaphase or take longer to go through anaphase/telophase and could exit mitosis with the same timing as the WT S23 cells, as there is no quantification of the telophase proportion nor mitosis rate nor timing. To be sure of that, the authors should measure the mitotic rate of every condition from their fixed immunofluorescence. Indeed, as the proportion of anaphase cells is even higher after the nocodazole release of 50min and if cells truly exit mitosis quicker, then the overall rate of mitotic cells vs interphase cells will be lower in the S23A conditions compared to the others. This would be more convincing to show that not only S23A mutant cells progress quicker through mitosis until anaphase but also do exit mitosis quicker with segregation errors. The authors could also perform a complementary live imaging experiment to track every stage of mitosis and measure the timing length of mitosis and compare between conditions to show that in the S23A mutant cells the mitosis timing length is shorter and that cells exit mitosis quicker than in the other conditions. That would be the ideal experiment but more complicated to perform.

5. In the same lines 107-109, the author also mentioned “in anaphase by 20 min post nocodazole wash out with abnormal spindles and substantial segregation defects”. There is no data that show the normality or abnormality of spindles in Figure 2. If this is only based on the images presented Figure 2, a quantification of the abnormality of spindles must be performed accompanied with an explanation about how this is measured from the images, if not this should be removed from the text.

6. Same lines 107-109, the author mentioned the proportion of lagging chromosomes after 20 min of nocodazole release which is not showed in any plot. The authors should either add the quantification of lagging chromosomes after 20min and/or 50min of nocodazole release or remove this from the text.

7. In Figure 2b in the Western Blot the authors wrote “S423D” instead of “S23D”, please correct.

8. The title of the third result section is “S23-Arpp-19 is phosphorylated by CDK1 in human cells”. Here the authors show that the S23 phosphorylation is lost in response to the CDK1 inhibitor RO3306, the result here is very clear. However, this is not a sufficient proof that S23-Arpp-19 is a direct substrate of CDK1. It could be true, but it could also be that with CDK1 being inactive, there is no need for the S23-Arpp-19 to be phosphorylated anymore or that S23-Arpp-19 is phosphorylated by an intermediate kinase like Gwl that is itself phosphorylated by CDK1 and so if CDK1 is inactive, the intermediate kinase is not activated. In Figure 4b, RO3306 alone inhibits both the phosphorylation of S23 and DpSG even if DpSG is not a direct target of CDK1 as phosphorylated by Gwl. This emphasises that the inhibition of S23 by RO3306 is not sufficient to say that S23 is a direct target of CDK1. The manuscript title should also be changed to reflect this, or, to prove that S23-Arp-19 is a direct target of CDK1 the authors should perform a kinase assay to identify the specific substrates of CDK1 in cells enriched in mitosis.

9. All Figures 3 and 4 are representative Western blot with no quantification. These blots should be quantified and represented as plots with the standard deviation representing the variability between the three biological replicates and with a corresponding statistical analysis.

10. In Figure 3c the authors show a “weak transient interaction of Arpp-19 with Fcp1” during mitosis leading to the dephosphorylation of S23 (lines 206-207). The Fcp1 signal here is very weak, is this the best blot out of the three replicates? This is not convincing and should be repeated with an increased level of proteins for a better signal and use an siRNA against Fcp1 to confirm the specificity of the Fcp1 weak band. To confirm the interaction between Fcp1 and Arpp-19 the authors could also perform an immunofluorescence colocalization assay as well as a proximity ligation assay if the antibodies are suitable for imaging. In the Discussion, at lines 250-251 the authors wrote that “S23-Arpp-19 dephosphorylation at mitosis exit required the phosphatase Fcp1 that transiently interacted with Arpp-19 in mitosis, dissociating from it along with S23-Arpp-19 dephosphorylation during mitosis exit”. There is insufficient evidence to support this conclusion.

11. In Figure 3D, the authors are comparing DpSG phosphorylation between the overexpressed Arpp-19 mutants S23A and S23D. Why is the nocodazole release timepoint different between the two mutants with 30min for the mutant S23A and 50min for the mutant S23D? If there is a reason please explain, if it is a mistake please correct.

12. Lines 256-261 is long and complicated; we advise to re-write this paragraph.

Reviewer #3

(Remarks to the Author)
This study by Serpico et al. investigates the critical role of Cdk1 phosphorylation of Arpp-19, specifically at serine 23 (S23), in ensuring chromosome stability during mitosis in human cells. The research highlights how this phosphorylation event helps regulate the progression of mitosis and guarantees the proper segregation of chromosomes. The phosphorylation of Arpp-19 by Cdk1 differs from a related protein, mammalian Ensa, which lacks the S23 site, underscoring the unique function of Arpp-19 in mitotic processes.
The study conducted several experiments to explore the role of S23 phosphorylation in Arpp-19. When this phosphorylation was blocked (using a non-phosphorylatable mutant, S23A), cells exhibited increased chromosome segregation errors, including a higher occurrence of lagging chromosomes during anaphase. Conversely, when a phospho-mimicking mutant (S23D) was expressed, cells displayed delayed mitotic exit, indicating that the timing of mitosis exit is finely tuned by the phosphorylation status of Arpp-19 at S23. The research also showed that dephosphorylation of Arpp-19 at S23 is necessary for mitotic exit and that this dephosphorylation is carried out by the phosphatase Fcp1, rather than PP2A-B55, suggesting distinct roles for different phosphatases in regulating Arpp-19 during mitosis.

This research adds valuable insight into the molecular intricacies of mitosis, emphasizing the critical role of protein phosphorylation and dephosphorylation in maintaining chromosomal integrity.
I find this work very interesting, and even if it is mainly descriptive, it provides an important insight into the regulation of mitosis. But before accepting this work for publication, I would like the authors to clarify two points that seem important to the scientific community
1. The authors should investigate whether WT and mutant forms of Arpp19 localize differently at the cellular level. Indeed, inhibition of the PP2A-B55 phosphatase could be modified according to the localization of the Arpp19 inhibitor. This could partly explain the phenotypes observed by the authors.
2. I agree with the authors that only Arpp19 has a serine followed by a proline at position 23. However, as an important part of the discoveries on the role of Greatwall and PP2A-B55 was made with the xenopus oocyte model, I checked the sequences of the two PP2A-B55 inhibitors: Arpp19 and Ensa. To be confirmed by the authors, but it seems to me that the xenopus serine 23 equivalent is conserved for Ensa and Arpp19.
It would be interesting for the authors to test whether the Ensa form of the xenopus can rescue the depletion of the Arpp19 form of the cells used by the authors.
This would show that :
a. that only serine 23 is responsible for the observed phenotypes
b. that the domain containing serine 23 is required for the function of the Arpp19 or Ensa inhibitors.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have addressed all my concerns. This is a very interesting and very nicely conducted manuscript; I am very happy to support publication.

Reviewer #2

(Remarks to the Author)
The authors identified a novel phosphorylation switch that regulates mitotic exit and maintains chromosome stability. In this revised version, the authors have undertaken substantial additional work to address the reviewers’ comments. Specifically, they have performed and incorporated statistical analyses to demonstrate the significance of their findings. Figures and their accompanying text have been updated and clarified, and new experiments have been carried out to enhance both the

quality and consistency of the manuscript. The revisions have therefore significantly strengthened the study that is now in my opinion appropriate for publication in the journal.

Reviewer #3

(Remarks to the Author)
The authors have done excellent work. The revisions requested by myself and the two other reviewers have improved this important work for the scientific community. I see nothing that would prevent this work from being published in Nature Communications.

Dear Dr. Georgieva, we wish to thank you and the Reviewers for the high standards of excellence by which our manuscript (COMMSBIO-24-5600) was handled and commented.

Point-by-point reply to the Reviewers' comments to manuscript COMMSBIO-24-5600:

Reviewer #1 (Remarks to the Author):
Cell cycle progression is strictly regulated by the opposing actions of kinases and phosphatases. The PP2A-B55 family of phosphatases are particularly critical for regulated entry into and exit out of mitosis. During mitosis, these phosphatases are inhibited by two small phosphorylated proteins, ENSA and Arpp19. Phosphorylation of ENSA and Arpp-19 by the Greatwall kinase has been shown to be essential for the inhibitory properties of these proteins. Serpico and colleagues here demonstrate that Arpp-19 is also phosphorylated by Cdk1, and that this phosphorylation seems to be critical for the action of Arpp-19 as cells expressing phospho-null or phospho-mimetic versions of Arpp-19 display significant defects during cell cycle progression.
The paper is on the whole very nicely conducted and the results seem convincing, and the topic is definitely of interest to a wide readership, so I am very enthusiastic about this manuscript.

Our reply: We thank this Reviewer for the words of appreciation about our manuscript.

There are however, some issues that should be addressed:
1. I have a slight concern with the fact that all of the experiments where transgenic forms of Arpp-19 were used seem to have been conducted with transiently transfected cells rather than stable cell lines. In this setting, transfection efficiency and non-homogeneous transfection of cell populations can be problematic for the interpretation of the results. Such problems are not apparent in the figures in the paper, in contrast, e.g. in Figure 2C, the results for at least the cells that are shown look very homogeneous. Since the transfection efficiency is unlikely to have been 100%, this is a bit surprising. What was the transfection efficiency in these experiments, and could the authors stain for the transgene to demonstrate that the observed effects only happen in the transfected cells?

Our reply: Accordingly, data concerning transfection efficiency in both HeLa and hTERT-RPE1 cells are now shown in Supplementary Figure 1 of this revised version. For all Arpp-19 vectors, transfection efficiency was around 73% and 63% for HeLa and hTERT-RPE1, respectively. Because of time limitations, we could not repeat the experiments to score mitotic defects in transgene-stained cells. However, we would like to point out that the transfection efficiency, which is similar for all constructs, is in approximate agreement with the complementation efficacy of WT-Arpp-19 expression in endogenous Arpp19-deficient background.

2. The interplay between the Greatwall and the Cdk1 phosphorylation sites is very interesting but Figure 3d, which analyses this, is confusing. The Arpp-19 S23A and S23D mutants are compared to the WT at different time points (30 or 50 mins). This means the conditions for the A and the D mutants are not comparable. The experiment should be conducted as a time course, showing for each condition (WT, S23A and S23D) 0, 30 and 50 mins after nocodazole release. There should also be further discussion of the relationship between the two phosphorylation sites and their phosphatases in the Discussion part of the paper. The authors suggest that CDk1-phosphorylated Arpp-19 may inhibit Fcp1 in a similar manner to Greatwall-phosphorylated Arpp-19 inhibiting PP2A-B55, but this would only really make sense if Fcp1 was then the phosphatase dephosphorylating the Greatwall site on Arpp-19. This has indeed been suggested (Hégarat et al., 2014), but generally (and by the authors), PP2A-B55 is thought to be the main phosphatase for the DpSG-site on Arpp-19. So what exactly is then the relationship between PP2A-B55 and Fcp1, and why does the depletion of Fcp1 also lead to stabilisation

of the DpSG site (Figure 4b)? This should really be discussed more; it's a bit difficult to fully understand what the authors think their data mean. A model at the end would be very helpful!

Our reply: Our reply: Accordingly, we now show dephosphorylation kinetics at 0, 30 and 50 min for Arpp-19 WT, S23A and S23D in Figure 3d of this revised version. In addition, in the Discussion section of this revised version, we expanded the discussion on the relationship between PP2A-B55 and Fcp1 and provided a plausible explanation of why the depletion of Fcp1 also leads to stabilization of the DpSG site upon brief treatment with a Cdk1 inhibitor in prometaphase-arrested cells. We also added a scheme illustrating our model as recommended (Figure 4d of this revised version).

3. Minor point: The sequence alignment should be included in the main figure. It's important to know where the two phospho-sites are.

Our reply: Accordingly, the sequence alignment, that now includes also Xenopus ENSA following Reviewer 3 recommendations, has been moved to main Figure 1a.

We sincerely thank this Reviewer as we believe that her/his criticisms have substantially helped to improve the quality of our manuscript.

Reviewer #2 (Remarks to the Author):
Title: Cdk1 phosphorylates Arpp-19 to grant chromosome stability
The authors claim to have identified a new phosphorylation switch involved in regulating mitotic exit and chromosome stability. Indeed, it is known that Cyclin B1 / CDK1 complex is tightly regulated during mitosis to allow mitotic progression, correct chromosome segregation and mitotic exit. CDK1 is dephosphorylated and inactivated by the phosphatase PP2A-B55 which must be inhibited during mitosis to allow CDK1 activity. This happens via Arpp-19/ENSA phosphorylated by Gwl, itself phosphorylated by CDK1. The authors work identified a new phosphorylation site S23 on Arpp-19 that would be phosphorylated by CDK1 itself and dephosphorylated by Fcp1, that contributes to the inactivation of PP2A-B55 and is crucial to maintain chromosome stability and mitosis exit timing. The authors successfully generated an antibody specific of this site. This S23-Arpp-19 site seems more important than the Gwl phosphorylation site to maintain mitosis integrity. This work could improve our understanding of the regulation of the Cyclin B1 / CDK1 complex during mitosis progression.

Our reply: We thank this Reviewer for appreciating the relevance of our work.

Although there are some interesting and novel findings presented, some of the conclusions are currently not adequately supported by the presented evidence. In particular, the title is "Cdk1 phosphorylated Arpp-19 to grant chromosome stability" while there is presently no evidence of direct targeting of Arpp-19 S23 by CDK1.

Our reply: Accordingly, the title has been changed into "A novel Arpp-19 phosphorylation switch grants chromosome stability", in agreement with the Reviewer opinion that our data show that S23-Arpp-19 phosphorylation is dependent on Cdk1 activity but do not prove that phosphorylation is direct and that it could also be dependent on an intermediary kinase. In addition the text has been amended taking into account the fact that we do not have data showing direct S23-Arpp-19 phosphorylation by Cdk1, although indicating that that site is in a Cdk1 phosphorylation consensus context as also suggested by the Reviewer and described in https://www.pnas.org/doi/10.1073/pnas.0708966105.

There are several points that need to be addressed (see details below), with quantification, statistical analysis often missing, and additional experiments are needed to strengthen and support the manuscript before publication.

1. There is no statistical analysis in the paper. The authors have performed sufficient experiments (three biological replicates in most cases) to perform some basic statistical testing, as without this it is difficult to interpret the significance of the data presented.

Our reply: Accordingly, statistical analysis of the replicates and significance (p-values) have now been provided for all experiments and indicated in the graphs and/or in the Figure legends.

2. The Figure 1 should have more than panels a and b as each panel has different figures and methods. Figure one should be reformatted with IF and WB separated from the same panel.

Our reply: Accordingly, the revised Figure 1 has been reformatted as suggested and also includes sequence alignment and data concerning complementation experiments with Xenopus Ensa, as recommended by Reviewers 1and 3.

3. In Figure 1, the immunofluorescence shows lagging chromosomes in anaphase cells treated with siRNA. The authors also stained for CREST but do not appear to use this to additionally quantify the nature of the lagging chromatin material. The authors could use these images to quantify the proportion of lagging chromosomes with CREST signal, as a useful confirmation that the origin of the lagging chromosomes is indeed the consequence of mitotic defects (as opposed to defects during chromosome replication in the preceding S-phase for example). Most lagging chromosomes should be CREST-positive, indicating whole lagging chromosomes.

Our reply: We are sorry for not having clearly indicated in the previous version, but our assesments of lagging chromosomes was indeed done counting CREST-positive lagging chromosomes. We have now clearly stated that in this revised version.

4. In Figure 2, the authors show that a nocodazole treatment and release induces a high proportion of anaphase cells in the S23A condition (20min – 50min), suggesting that the cells were quicker to progress through mitosis. Here, in lines 107-108 the authors commented “S23A-Arpp-19-complemented cells appeared to exit mitosis faster than control as about 25 to 30% were already in anaphase by 20 min post nocodazole wash out” but cells in S23A condition might be stuck in anaphase or take longer to go through anaphase/telophase and could exit mitosis with the same timing as the WT S23 cells, as there is no quantification of the telophase proportion nor mitosis rate nor timing. To be sure of that, the authors should measure the mitotic rate of every condition from their fixed immunofluorescence. Indeed, as the proportion of anaphase cells is even higher after the nocodazole release of 50 min and if cells truly exit mitosis quicker, then the overall rate of mitotic cells vs interphase cells will be lower in the S23A conditions compared to the others. This would be more convincing to show that not only S23A mutant cells progress quicker through mitosis until anaphase but also do exit mitosis quicker with segregation errors. The authors could also perform a complementary live imaging experiment to track every stage of mitosis and measure the timing length of mitosis and compare between conditions to show that in the S23A mutant cells the mitosis timing length is shorter and that cells exit mitosis quicker than in the other conditions. That would be the ideal experiment but more complicated to perform.

Our reply: Accordingly, we have now differentially scored anaphase from telophase cells, at the 50 min time point, in the experiments shown in Figure 2 and changed the graph accordingly. The data show that telophase is reached earlier in the S23A-Arpp-19-expressing cells and later in S23D-Arpp19-expressing cells relatively to WT-Arpp-19 expressing cells. Standard deviations, within 3 independent experiments, are shown in the graph, statistical significance of the different mitotic stage distribution, in the various Arpp-19 variants expressing cells during mitosis exit, is indicated (p-values) in the Figure legend. The time lapse experiment suggested would be ideal but, unfortunately, we do not have the adequate setting to perform it.

5. In the same lines 107-109, the author also mentioned "in anaphase by 20 min post nocodazole wash out with abnormal spindles and substantial segregation defects". There is no data that show the normality or abnormality of spindles in Figure 2. If this is only based on the images presented Figure 2, a quantification of the abnormality of spindles must be performed accompanied with an explanation about how this is measured from the images, if not this should be removed from the text.

Our reply: Accordingly, we have removed the sentence regarding spindle abnormalities from the text.

6. Same lines 107-109, the author mentioned the proportion of lagging chromosomes after 20 min of nocodazole release which is not showed in any plot. The authors should either add the quantification of lagging chromosomes after 20min and/or 50min of nocodazole release or remove this from the text.

Our reply: Accordingly, we have maintained the sentence concerning the increased frequency of lagging chromosomes in S23A-Arpp-19- relatively to WT-Arpp-19-expressing cells upon release from prometaphase arrest and shown the quantitation of this from three new experiments (Supplementary Figure 2 of this revised version) performed under similar condition to those indicated for Figure 2.

7. In Figure 2b in the Western Blot the authors wrote "S423D" instead of "S23D", please correct.

Our reply: The error has been corrected.

8. The title of the third result section is "S23-Arpp-19 is phosphorylated by CDK1 in human cells". Here the authors show that the S23 phosphorylation is lost in response to the CDK1 inhibitor RO3306, the result here is very clear. However, this is not a sufficient proof that S23-Arpp-19 is a direct substrate of CDK1. It could be true, but it could also be that with CDK1 being inactive, there is no need for the S23-Arpp-19 to be phosphorylated anymore or that S23-Arpp-19 is phosphorylated by an intermediate kinase like Gwl that is itself phosphorylated by CDK1 and so if CDK1 is inactive, the intermediate kinase is not activated. In Figure 4b, RO3306 alone inhibits both the phosphorylation of S23 and DpSG even if DpSG is not a direct target of CDK1 as phosphorylated by Gwl. This emphasises that the inhibition of S23 by RO3306 is not sufficient to say that S23 is a direct target of CDK1. The manuscript title should also be changed to reflect this, or, to prove that S23-Arp-19 is a direct target of CDK1 the authors should perform a kinase assay to identify the specific substrates of CDK1 in cells enriched in mitosis.

Our reply: Accordingly, the title has been changed into "A novel Arpp-19 phosphorylation switch grants chromosome stability" and the text changed to indicate that S23-Arpp-19 phosphorylation may depend directly or indirectly on Cdk1 activity. We agree with the Reviewer that our data do show that S23-Arpp-19 phosphorylation is dependent on Cdk1 activity but do not prove direct phosphorylation and that this could also be dependent on an intermediary kinase, as we also

proposed in the final scheme of this revised version (Figure 4d). However, we indicated that S23-Arpp-19 is in the context of Cdk1 phosphorylation consensus, as documented by the PNAS paper https://www.pnas.org/doi/10.1073/pnas.070896610 that the Reviewer suggested to us. We tried the experiments suggested by the Reviewer to determine whether Cdk1 would directly phosphorylate Arpp-19. However, our results were inconclusive, we could not attempt the proximity ligation experiment as our anti-pS23-Arpp-19 antibody did not perform well for immunofluorescence or immunoprecipitation.

9. All Figures 3 and 4 are representative Western blot with no quantification. These blots should be quantified and represented as plots with the standard deviation representing the variability between the three biological replicates and with a corresponding statistical analysis.

Our reply: Accordingly, plots of quantification, including standard deviation and statistical significance, have been provided for the blots in Figures 3 and 4 in the Figures and relative Figure legends.

10. In Figure 3c the authors show a "weak transient interaction of Arpp-19 with Fcp1" during mitosis leading to the dephosphorylation of S23 (lines 206-207). The Fcp1 signal here is very weak, is this the best blot out of the three replicates? This is not convincing and should be repeated with an increased level of proteins for a better signal and use an siRNA against Fcp1 to confirm the specificity of the Fcp1 weak band. To confirm the interaction between Fcp1 and Arpp-19 the authors could also perform an immunofluorescence colocalization assay as well as a proximity ligation assay if the antibodies are suitable for imaging. In the Discussion, at lines 250-251 the authors wrote that "S23-Arpp-19 dephosphorylation at mitosis exit required the phosphatase Fcp1 that transiently interacted with Arpp-19 in mitosis, dissociating from it along with S23-Arpp-19 dephosphorylation during mitosis exit". There is insufficient evidence to support this conclusion.

Our reply: Accordingly, the coimmunoprecipitation conditions between Arpp-19 and Fcp1 have been improved and shown in new Figure 4c and Fcp1 antibody specificity in coimmunoprecipitations by using siRNA to knockdown Fcp1 is now shown in Supplementary Figure 5. Technical reasons precluded us from performing the experiments suggested, immunofluorescence colocalization assay as well as a proximity ligation assay (our antibody to detect endogenous Arpp-19 performed very poorly in indirect immunofluorescence). The sentence in the Discussion has been rephrased to make it more descriptive and reduce emphasis on a possible cause/effect relationship between dephosphorylation and dissociation.

11. In Figure 3D, the authors are comparing DpSG phosphorylation between the overexpressed Arpp-19 mutants S23A and S23D. Why is the nocodazole release timepoint different between the two mutants with 30min for the mutant S23A and 50min for the mutant S23D? If there is a reason please explain, if it is a mistake please correct.

Our reply: Accordingly, we now show dephosphorylation kinetics at 0, 30 and 50 min for Arpp-19 WT, S23A and S23D in Figure 3d of this revised version.

12. Lines 256-261 is long and complicated; we advise to re-write this paragraph.

Our reply: Accordingly, the discussion has been thourougly revised and rewritten shortening paragraphs length.

We sincerely thank this Reviewer as we believe that her/his criticisms have substantially helped to improve the quality of our manuscript.

Reviewer #3 (Remarks to the Author):

This study by Serpico et al. investigates the critical role of Cdk1 phosphorylation of Arpp-19, specifically at serine 23 (S23), in ensuring chromosome stability during mitosis in human cells. The research highlights how this phosphorylation event helps regulate the progression of mitosis and guarantees the proper segregation of chromosomes. The phosphorylation of Arpp-19 by Cdk1 differs from a related protein, mammalian Ensa, which lacks the S23 site, underscoring the unique function of Arpp-19 in mitotic processes.

The study conducted several experiments to explore the role of S23 phosphorylation in Arpp-19. When this phosphorylation was blocked (using a non-phosphorylatable mutant, S23A), cells exhibited increased chromosome segregation errors, including a higher occurrence of lagging chromosomes during anaphase. Conversely, when a phospho-mimicking mutant (S23D) was expressed, cells displayed delayed mitotic exit, indicating that the timing of mitosis exit is finely tuned by the phosphorylation status of Arpp-19 at S23. The research also showed that dephosphorylation of Arpp-19 at S23 is necessary for mitotic exit and that this dephosphorylation is carried out by the phosphatase Fcp1, rather than PP2A-B55, suggesting distinct roles for different phosphatases in regulating Arpp-19 during mitosis.

This research adds valuable insight into the molecular intricacies of mitosis, emphasizing the critical role of protein phosphorylation and dephosphorylation in maintaining chromosomal integrity.

I find this work very interesting, and even if it is mainly descriptive, it provides an important insight into the regulation of mitosis.

Our reply: We thank this reviewer for the words of appreciation about our manuscript.

But before accepting this work for publication, I would like the authors to clarify two points that seem important to the scientific community

1. The authors should investigate whether WT and mutant forms of Arpp19 localize differently at the cellular level. Indeed, inhibition of the PP2A-B55 phosphatase could be modified according to the localization of the Arpp19 inhibitor. This could partly explain the phenotypes observed by the authors.

Our reply: Accordingly, experiments to detect localization of WT and mutant forms of Arpp-19, by immunofluorescence analysis, have now been performed and shown in Supplementary Figures 1 and 3. By our immunofluorescence procedures no significant differences in localization were scored.

2. I agree with the authors that only Arpp19 has a serine followed by a proline at position 23. However, as an important part of the discoveries on the role of Greatwall and PP2A-B55 was made with the xenopus oocyte model, I checked the sequences of the two PP2A-B55 inhibitors: Arpp19 and Ensa. To be confirmed by the authors, but it seems to me that the xenopus serine 23 equivalent is conserved for Ensa and Arpp19.

It would be interesting for the authors to test whether the Ensa form of the xenopus can rescue the depletion of the Arpp19 form of the cells used by the authors.

This would show that :

a. that only serine 23 is responsible for the observed phenotypes

b. that the domain containing serine 23 is required for the function of the Arpp19 or Ensa inhibitors.

Our reply: Accordingly, we have now performed this interesting experiment suggested by the Reviewer and found that Xenopus Ensa can, indeed, substantially rescue mitotic defects in human cells caused by Arpp-19 depletion. Data are now shown in revised Figure 1f.

We sincerely thank this Reviewer as we believe that her/his criticisms have substantially helped to improve the quality of our manuscript.