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Periodic changes of cyclin D1 mRNA stability are regulated by PC4 modifications in the cell cycle

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September 27, 2023

Re: JCB manuscript #202308066

Prof. Yin Dong
Sun Yat-Sen Memorial Hospital
107 Yan Jiang West Road, YueXiu District
Guangzhou 510235
China

Dear Prof. Dong,

Thank you for submitting your manuscript entitled "Periodic changes of cyclin D1 mRNA stability are regulated by PC4 modifications in the cell cycle." The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

You will see that the reviewers are enthusiastic about your study but request a few additional experiments which we agree are needed in order to strengthen the conclusions. Reviewer #1 notes a discrepancy in the mRNA levels of CCND1 in wildtype cells between different figures and asks to resolve this by repeating the experiment with all relevant cell types. Reviewer #2 suggests testing if the TRIM28 interaction with PC4 is altered by phoshpho mutants. Reviewers #2&3 also ask whether the effects seen in PC4 depleted cells are mainly due to loss of cyclin D1 and not other cell cycle regulators. Reviewer #2 suggests testing this by expressing CCND1 in PC4 depleted cells. Additionally, quantifications of protein level changes in PC4 knockdown and overexpression cells, rather than single blot images, would be important to add. The reviewers have also provided other comments aimed at clarifying and better explaining the data and conclusions which you will need to address by text and figure revisions. How PC4 functions in cells that mainly express cyclins D2 and D3 is an interesting question that could be a discussion point but we do not think it needs to be addressed experimentally in this paper.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:

Text limits: Character count for an Article is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, and acknowledgments. Count does not include materials and methods, figure legends, references, tables, or supplemental legends.

Figures: Articles may have up to 10 main text figures. Figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, https://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

IMPORTANT: It is JCB policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.

Supplemental information: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

Please note that JCB now requires authors to submit Source Data used to generate figures containing gels and Western blots with all revised manuscripts. This Source Data consists of fully uncropped and unprocessed images for each gel/blot displayed in the main and supplemental figures. Since your paper includes cropped gel and/or blot images, please be sure to provide one Source Data file for each figure that contains gels and/or blots along with your revised manuscript files. File names for Source Data figures should be alphanumeric without any spaces or special characters (i.e., SourceDataF#, where F# refers to the associated main figure number or SourceDataFS# for those associated with Supplementary figures). The lanes of the gels/blots should be labeled as they are in the associated figure, the place where cropping was applied should be marked (with a box), and molecular weight/size standards should be labeled wherever possible. Source Data files will be made available to reviewers during evaluation of revised manuscripts and, if your paper is eventually published in JCB, the files will be directly linked to specific figures in the published article.

Source Data Figures should be provided as individual PDF files (one file per figure). Authors should endeavor to retain a minimum resolution of 300 dpi or pixels per inch. Please review our instructions for export from Photoshop, Illustrator, and

PowerPoint here: https://rupress.org/jcb/pages/submission-guidelines#revised

The typical timeframe for revisions is three to four months. While most universities and institutes have reopened labs and allowed researchers to begin working at nearly pre-pandemic levels, we at JCB realize that the lingering effects of the COVID-19 pandemic may still be impacting some aspects of your work, including the acquisition of equipment and reagents. Therefore, if you anticipate any difficulties in meeting this aforementioned revision time limit, please contact us and we can work with you to find an appropriate time frame for resubmission. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised in this letter.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu.

Sincerely,

Arshad Desai, PhD
Monitoring Editor
Journal of Cell Biology

Dan Simon, PhD
Scientific Editor
Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

In this paper, Pan et al sought to identify RNA binding proteins (RBPs) that maintain hepatocellular carcinoma (HCC). The authors identified PC4 as strongly correlating with patient survival. Through a fairly significant amount of work, the authors found that PC4 stabilizes CCND1 in a cell-cycle dependent manner and its ability to stabilize CCND1 is regulated by two opposing post-translational modifications. The authors characterize TRIM28-mediated K68 ubiquitination as an activation switch and CK2-mediated S17 phosphorylation as an inactivation switch. The authors also show that inhibiting PC4 can reduce HCC tumor growth and sensitive tumors to the CDK4/6 inhibitor palbociclib, consistent with its ability to stabilize CCND1.
Overall, the manuscript presents a fairly comprehensive characterization of this new mechanism of regulating CCND1 mRNA stability. The paper is well written, easy to follow, and the data is overall convincing. Given the importance of cyclin D1 in cancer, and the success of CDK4/6 inhibitors in the clinic, the results reported here would be of general interest to several fields. There are a few minor points that should be addressed before publication.

1. Figure 1D requires labeling. What are the numbers at the top? Do they refer to time? If so, is it hours or minutes? What does the color-bar represent? The figure legend does not explain these values either.

2. CCND1 stability appears quite variable between experiments, even in control conditions. For example, CCND1 in Figure S1C appears much less stable than in Figure 1D. In fact, in Figure S1C, the stability of CCND1 in control cells is similar to the stability of CCND1 in shPC4 cells. Could the authors comment on this discrepancy? Ideally the authors would conduct the four conditions from Figure S1C and Figure 1D at the same time so as to better compare the results more directly.

3. The authors should note in the figure legends how many repeats there were for each experiment. This is particularly important for figures with western blots.

4. Why doesn't PC4-S17A mutant lead to more CCND1 mRNA in M phase (see Figure 4K)? If S17 phopshorylation turns PC4 "off", then CCND1 mRNA should be more stable in M phase in PC4-S17A cells compared to PC4-WT cells. Could the authors comment on this discrepancy?

5. Figure 7K: when printed, one cannot see any fluorescence in any of these panels. The contrast or brightness needs to be adjusted.

6. A comment about all the figures: I found the labels and legends in general to be too small to read. Increasing the font size would help the reader immensely. In addition, increasing the thickness of lines in line graphs would also be helpful. For example, in Figure 2F, it's hard to read the legend and know which line is which.

Reviewer #2 (Comments to the Authors (Required)):

Summary- This impressive manuscript from Pan and colleagues describes the regulation of mRNA stability as an important regulator of cell cycle progression. The cell cycle is driven by oscillations in protein abundance and activities. The most well established drivers of these oscillations are dynamics in the transcription of genes, protein phosphorylation which controls the activity of many important cell cycle regulators, and the destruction of cell cycle proteins by the ubiquitin system. Despite this knowledge, remarkably little is known about changes in the stability of mRNAs that encode critical cell cycle proteins by binding to RNA binding proteins, or RBPs. The current study focuses on a small RBP PC4, which emerged from a computational screen for regulators of HCC proliferation. The authors show here that PC4 control the stability of several cell cycle transcripts, and most notably, one encoding Cyclin D1, a co-activator subunit for the CDK4/6 kinases that play critical roles in cell cycle progression. They show that PC4 is modified both by phosphorylation and ubiquitin in a cell cycle dependent fashion, and that this controls regulation of the CCND1 transcript. Further, their data suggest that regulation of the CCND1 transcript is important for cell cycle progression and tumorigenesis. Altogether, this is a very comprehensive study, and worthy of publication. I only have a few small suggestions that could improve the overall manuscript.

Points-
1- A potential caveat to the interpretation of some of these experiments is that the authors show that PC4 is also bound to, and regulating the stability of, the cell cycle mRNAs Skp2 and E2F2. Both of these also represent key regulators of G1/S. It is therefore often hard to know if the effects that are observed phenotypically are indeed due to changes in CCND1, or to all of these collectively. This difference does not undermine the overall findings. It would be interesting to know if the cell cycle phenotypes observed in Fig 5, for example, could be rescued by expression of CCND1. If not, the authors could speculate that this is caused by more broad impacts on cell cycle genes, which might include E2F2 and Skp2.
2- The authors casually refer to screening data from "Dang's analysis" in the first section of the results. It is unclear what that analysis is, what the pipeline used to identify important RBPs was, how data was analyzed, etc.. This should be described in better detail. As it reads, it is unclear if the analysis was done on their work or are they summarizing and analyzing only the work from the Dang et al paper? Related to that same section, the gene/protein labels in Fig 1A are too small to read and need to be fixed.
3- I am confused by Figs S2H and S2I. In S2H, the data shows that most of the ubiquitination is happening on K68 in PC. Then, in Fig S2I, they show that this is largely through K63 linked ubiquitination. This is because mutation of PC4-S68 virtually eliminates K63-linked ubiquitination. Altogether, that suggests that K63-linked ubiquitination on PC4, at K68, represents nearly all of the ubiquitination of PC4. However, they also show that PC4 is K48 ubiquitinated, and that this persists in the S68 mutant. I am not sure how to reconcile those differences. I think this could be explained through written changes in the text.
4- It is unclear if there is any difference in CCND1 mRNA stability in Figure 3L. I think this has to do with the fact that a heat map is being used to display these data.
5- I am confused about the data and interpretation of Fig 4E. Since CK2 depletion increases the ubiquitination of the PC4-S17E mutant protein, I believe that the correct interpretation would be that the kinase is doing something independent of that phosphorylation site to regulate ubiquitination. However, the authors interpretation is that one happens after the other. I am not following that logic. I think they need to explain this better or consider reinterpreting that result. Also related to Figure 4E, the phos-tag gel shows a huge decrease in electrophoretic mobility in the PC4 S-to-E mutant. However, phos-tag gels specifically interact with the presence of a phosphate groups on the modified protein, and swapping an amino acid, to the very best of my understanding, should have no impact on its electrophoretic mobility?
6- Related to the previous two points, there does not appear to be any change in TRIM28 binding to PC4 in CK2 inhibited cells in Fig 4G. Yet the ubiquitination of PC4 is remarkably increased? To help address this confusion, I would suggest performing a PC4 IP with the phoshpho mutants that have already been generated, and blot for TRIM28. Repeating this in the presence of either CK2 overexpression or inhibition would be very helpful.
7- I am skeptical that the data in Fig 5B has anything to do with CCND1, since a double thymidine block and release is likely to trap cells past the point where they would need Cyclin D. This could be the result of SKP2 reduction? See above.
8- It appears as though the APC/C ubiquitin ligase is not turning on to degrade geminin in the PC4 overexpressing cells in Fig 5F. This could play an important role in driving cell cycle, independent of cyclin D. Are the authors certain that they have marked cells at mitotic exit for these experiments? If that is true, they might speculate as to what is going on.
9- Data in Figure 7C, examining overall Pc4 ubiquitination in cancers, is hard to interpret because there is much more trim28 in the tumor cells.
10- In the table in Fig 1C PTTG1IP is listed twice.
11- What is meant by "common PC4-binding sites" in describing RIPseq?
12- State more clearly in the many text of the results that Fig 3B is an in vitro assay using purified components.

Reviewer #3 (Comments to the Authors (Required)):

In this study, Pan et al. report that an RNA-binding protein PC4 regulates cyclin D1 mRNA stability. Cyclin D1 plays an important function in driving proliferation of cancer cells. Hence, understanding how this protein is regulated is of great biological and clinical importance. Previous studies documented that cyclin D1 is regulated transcriptionally and at the level of protein degradation. Here, Pan et al. propose a new level of cyclin D1 regulation, namely though cyclin D1 mRNA stability.

I have the following questions/suggestions for the authors.

1. If PC4 plays a biologically significant role in regulating cyclin D1 levels, I would expect that depletion of PC4 will reduce the levels of cyclin D1 mRNA and protein, while overexpression will have an opposite effect. I did not find this information in the manuscript.
2. The authors mention that stability of cyclin D1 mRNA fluctuates across the cell cycle, and postulate that this is driven by PC4. Is then the fluctuation lost in PC4-depleted cells?
3. The authors state that "PC4 deficiency induced G1 phase arrest, linking PC4 to the cell-cycle transition (Figure 5A)". However, in Figure 5A the fraction of S-phase cells is decreased from 25% to 13%, which does not constitute "an arrest".
4. What is the evidence that this effect is mediated by cyclin D1 depletion? Does it occur in RB-negative cells (which do not arrest after cyclin D1 depletion)?
5. Likewise, what is the evidence that the effects seen in other cell lines in vitro and in vivo are mediated through D1, rather than targeting of several cell cycle proteins? The authors mentioned that transcripts bound to PC are enriched in the cell cycle category. Is it really that cyclin D1 downregulation is entirely responsible for the effect? Was there a significant decrease of cyclin D1 protein levels? Was the effect abrogated in RB1-negative cells?
6. The authors show that PC4 loss sensitized cells to palbociclib. This effect is not necessarily mediated by D1 depletion, and it could be mediated by depletion of CDK2. What is the evidence that the effect depends on cyclin D1?
7. The authors found that PC4 regulates cyclin D1, but not D2 or D2. Would PC4 depletion have any impact on proliferation of cells which express mainly cyclins D2 and D3?

Dear Arshad Desai and Dan Simon,

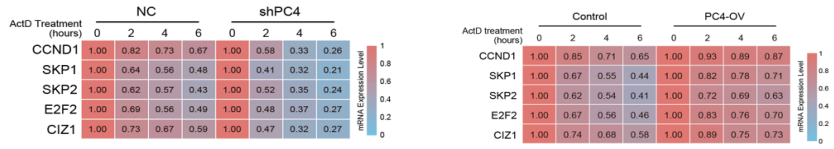
We would like to express our gratitude for the opportunity to revise our manuscript titled "Periodic changes of cyclin D1 mRNA stability are regulated by PC4 modifications in the cell cycle." We sincerely appreciate the constructive comments provided by the reviewers, as they have been immensely valuable in improving our article. In response to the reviewers' comments, we have diligently made modifications throughout the manuscript and hope that the revised version meets the necessary criteria for publication in the Journal of Cell Biology. All modifications within the document have been highlighted using red text. Below, we provide point-by-point responses to the reviewers' comments:

Reviewer #1 (Comments to the Authors (Required)):
In this paper, Pan et al sought to identify RNA binding proteins (RBPs) that maintain hepatocellular carcinoma (HCC). The authors identified PC4 as strongly correlating with patient survival. Through a fairly significant amount of work, the authors found that PC4 stabilizes CCND1 in a cell-cycle dependent manner and its ability to stabilize CCND1 is regulated by two opposing post-translational modifications. The authors characterize TRIM28-mediated K68 ubiquitination as an activation switch and CK2-mediated S17 phosphorylation as an inactivation switch. The authors also show that inhibiting PC4 can reduce HCC tumor growth and sensitive tumors to the CDK4/6 inhibitor palbociclib, consistent with its ability to stabilize CCND1.
Overall, the manuscript presents a fairly comprehensive characterization of this new mechanism of regulating CCND1 mRNA stability. The paper is well written, easy to follow, and the data is overall convincing. Given the importance of cyclin D1 in cancer, and the success of CDK4/6 inhibitors in the clinic, the results reported here would be of general interest to several fields. There are a few minor points that should be addressed before publication.
We appreciate the time and effort that Reviewer #1 dedicated to assessing our manuscript.

1. Figure 1D requires labeling. What are the numbers at the top? Do they refer to time? If so, is it hours or minutes? What does the color-bar represent? The figure legend does not explain these values either.
We thank the reviewer for the helpful comments. We have included the unit (hours) for the numbers presented at the top of Figure 1D. Furthermore, we have included a description of the color bar and integrated it into the figure legend.

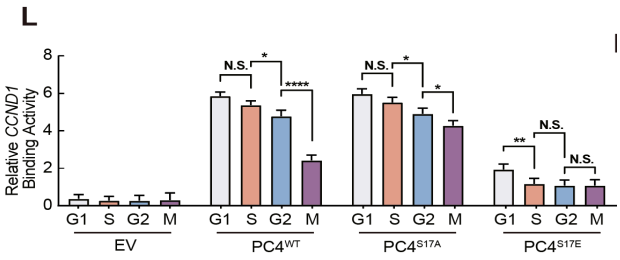
2. CCND1 stability appears quite variable between experiments, even in control conditions. For example, CCND1 in Figure S1C appears much less stable than in Figure 1D. In fact, in Figure S1C, the stability of CCND1 in control cells is similar to the stability of CCND1 in shPC4 cells. Could the authors comment on this discrepancy? Ideally the authors would conduct the four conditions from Figure S1C and Figure 1D at the same time so as to better compare the results more directly.

We acknowledge the reviewer's observation regarding the discrepancy in mRNA levels of CCND1 between Figure S1C and Figure 1D in control cells. To address this concern, we conducted a repeat experiment simultaneously under four different conditions using Huh7 cells. Our findings indicate that the stability of CCND1 was comparable in the NC and control groups. However, it notably decreased in the PC4-knockdown group and increased in the PC4-overexpression group (see below). Therefore, we have incorporated these updated data into the corresponding figures (Figures S1C and 1D).



3. The authors should note in the figure legends how many repeats there were for each experiment. This is particularly important for figures with western blots.
We appreciate the reviewer for bringing this to our attention, and we have now included the number of repeats for each experiment in the corresponding figure legend.

4. Why doesn't PC4-S17A mutant lead to more CCND1 mRNA in M phase (see Figure 4K)? If S17 phosphorylation turns PC4 "off", then CCND1 mRNA should be more stable in M phase in PC4-S17A cells compared to PC4-WT cells. Could the authors comment on this discrepancy?
We acknowledged the reviewer's comment and conducted a meticulous repetition of the experiment to compare the binding capacity of PC4 to *CCND1* mRNA during M phase in both $PC4^{S17A}$ and $PC4^{WT}$ cells. The subsequent results demonstrated that $PC4^{S17A}$ exhibited a slightly stronger binding affinity to *CCND1* mRNA compared to wild-type cells during mitosis. This difference can primarily be attributed to the fact that $PC4^{S17A}$ remains active without being switched off by CK2. Therefore, we have integrated this revised data into Figure 4L.



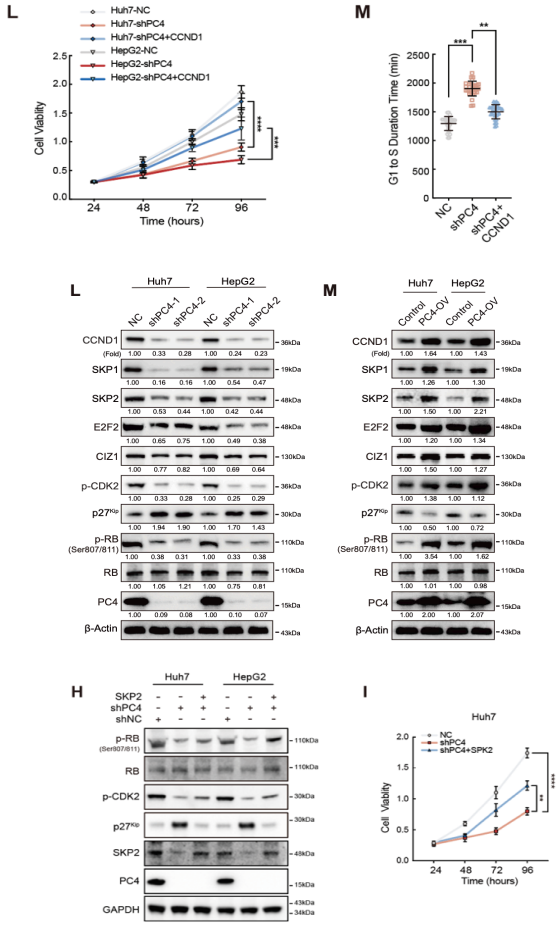
5. Figure 7K: when printed, one cannot see any fluorescence in any of these panels. The contrast or brightness needs to be adjusted.
We thank the reviewer for pointing this out and have replaced the images in Figure 7K with higher brightness levels.

6. A comment about all the figures: I found the labels and legends in general to be too small to read. Increasing the font size would help the reader immensely. In addition, increasing the thickness of lines in line graphs would also be helpful. For example, in Figure 2F, it's hard to read the legend and know which line is which.
We thank the reviewer for pointing this out and have increased the font size of the labels and legends in each figure and also enhanced the thickness of the lines in all the line graphs.

Reviewer #2 (Comments to the Authors (Required)):
Summary-
This impressive manuscript from Pan and colleagues describes the regulation of mRNA stability as an important regulator of cell cycle progression. The cell cycle is driven by oscillations in protein abundance and activities. The most well-established drivers of these oscillations are dynamics in the transcription of genes, protein phosphorylation which controls the activity of many important cell cycle regulators, and the destruction of cell cycle proteins by the ubiquitin system. Despite this knowledge, remarkably little is known about changes in the stability of mRNAs that encode critical cell cycle proteins by binding to RNA binding proteins, or RBPs. The current study focuses on a small RBP PC4, which emerged from a computational screen for regulators of HCC proliferation. The authors show here that PC4 control the stability of several cell cycle transcripts, and most notably, one encoding Cyclin D1, a co-activator subunit for the CDK4/6 kinases that play critical roles in cell cycle progression. They show that PC4 is modified both by phosphorylation and ubiquitin in a cell cycle dependent fashion, and that this controls regulation of the CCND1 transcript. Further, their data suggest that regulation of the CCND1 transcript is important for cell cycle progression and tumorigenesis. Altogether, this is a very comprehensive study, and worthy of publication. I only have a few small suggestions that could improve the overall manuscript.
We thank the reviewer for their supportive comments.

Points-
1. A potential caveat to the interpretation of some of these experiments is that the authors show that PC4 is also bound to, and regulating the stability of, the cell cycle mRNAs Skp2 and E2F2. Both of these also represent key regulators of G1/S. It is therefore often hard to know if the effects that are observed phenotypically are indeed due to changes in CCND1, or to all of these collectively. This difference does not undermine the overall findings. It would be interesting to know if the cell cycle phenotypes observed in Fig 5, for example, could be rescued by expression of CCND1. If not, the authors could speculate that this is caused by more broad impacts on cell cycle genes, which might include E2F2 and Skp2.
We are acknowledged the reviewer for raising this important issue. We agree that PC4 plays a role in promoting G1/S phase transition and cell proliferation through its impact on various cell cycle genes, rather than solely on CCND1 itself. Depletion of PC4 led

to extended G1 phase, impaired cell growth, and re-introduction of CCND1 only partially restored these effects in PC4-depleted cells (Figures 5L and 5M). Moreover, the mRNA and protein levels of four other critical G1/S regulators (SKP1, SKP2, E2F2, and CIZ1) were reduced upon PC4 knockdown in both Huh7 and HepG2 cells （Figures 1L）. Conversely, the opposite was observed in PC4-overexpressing cells, suggesting that PC4 influences the mRNA stability and expression of multiple genes involved in G1/S transition (Figure 1M). Additionally, reintroducing SKP2 into cells depleted of endogenous PC4 partially increased the expression of p-CDK2 and p-RB (Figure S6H) and restored the impaired cell growth (Figures S6I), indicating that PC4 may promote cell cycle progression by regulating the gene expression of several key regulators involved in G1/S transition. We have incorporated the new data and addressed this issue in the relevant sections of the manuscript.



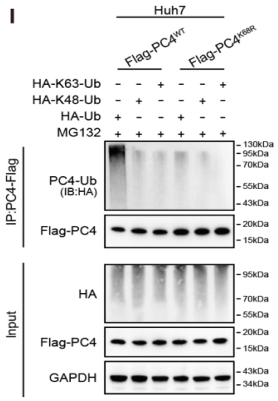
2. The authors casually refer to screening data from "Dang's analysis" in the first section of the results. It is unclear what that analysis is, what the pipeline used to identify important RBPs was, how data was analyzed, etc. This should be described in

better detail. As it reads, it is unclear if the analysis was done on their work or are they summarizing and analyzing only the work from the Dang et al paper? Related to that same section, the gene/protein labels in Fig 1A are too small to read and need to be fixed.

We thank the reviewer for the critical comments. To identify functional RBPs that promote and maintain HCC, we developed a two-step screening system. In the first step, we utilized the dataset from Dang's research, which consisted of 672 RBP expressions and associated patient outcomes in 1,225 clinical samples of HCC, to investigate whether the expression of any RBPs correlated with HCC prognosis. Through this analysis, we validated that the differential expression of 148 RBPs was significantly associated with overall patient survival. Among the top 20 RBPs that were highly expressed in tumours with poor prognosis, four candidates had undefined roles in HCC and were selected based on their essentiality in liver cancer cell proliferation experiments. After the second cell proliferation screening, PC4 knockdown had the most significant inhibitory effect on HCC cell growth. We have made modifications to the manuscript's description and increased the font size of the gene/protein labels in Figure 1A.

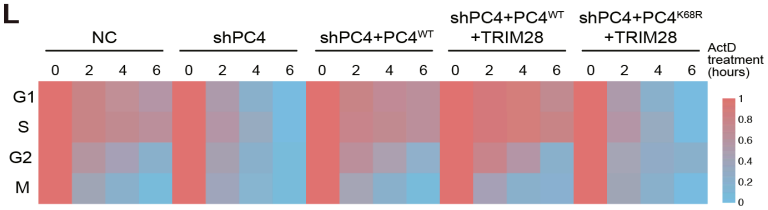
3. I am confused by Figs S2H and S2I. In S2H, the data shows that most of the ubiquitination is happening on K68 in PC4. Then, in Fig S2I, they show that this is largely through K63 linked ubiquitination. This is because mutation of PC4-K68 virtually eliminates K63-linked ubiquitination. Altogether, that suggests that K63-linked ubiquitination on PC4, at K68, represents nearly all of the ubiquitination of PC4. However, they also show that PC4 is K48 ubiquitinated, and that this persists in the K68 mutant. I am not sure how to reconcile those differences. I think this could be explained through written changes in the text.

We appreciate the reviewer's valuable comments. To investigate K63-linked ubiquitination on PC4 at K68 more comprehensively, we optimized the PC4 immunoprecipitation protocols and compared the levels of different ubiquitin chains between PC4 K68-mutant and wild-type samples on the same blot. Our new experiment revealed the following findings: (1) PC4 is conjugated to both K63- and K48-linked polyubiquitin chains (Figure S2I, Lane 2 and Lane 3). (2) The K68R mutation significantly reduced PC4 ubiquitination (Figure S2I, Lane 1 and Lane 4). (3) The K68R mutation almost completely abolished K63-linked ubiquitination of PC4 (Figure S2I, Lane 6). (4) K48 ubiquitin supported PC4 modification in both $PC4^{K68R}$ and $PC4^{WT}$ cells, indicating that K63 ubiquitin, but not K48 ubiquitin, specifically supports PC4 modification at the K68 site. Based on these new findings, we now conclude that K63-linked polyubiquitination predominantly occurs on the K68 residues of PC4. We have updated the data in Figure S2I, and we believe these additional experiments provide stronger evidence for our conclusion. We thank the reviewer for prompting us to explore further.



4. It is unclear if there is any difference in CCND1 mRNA stability in Figure 3L. I think this has to do with the fact that a heat map is being used to display these data.

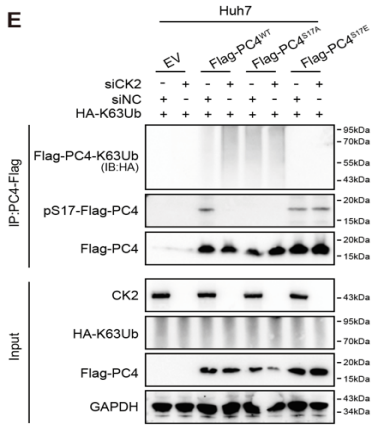
We appreciate the reviewer for bringing this to our attention. In order to more accurately compare the difference in CCND1 mRNA stability, we conducted additional experiments using PC4-depleted cells. These experiments allowed us to observe the changes in CCND1 mRNA levels in various rescue groups throughout the cell cycle. As expected, we found that during interphase, TRIM28 overexpression further enhanced the stability of CCND1 mRNA in PC4^{WT}cells, but not in PC4^{K68R} mutant cells. The disparity between the distinct groups is more pronounced in the new experiments, thereby strengthening our conclusions. We have updated Figure 3L with the new data. We sincerely thank the reviewer once again for the valuable comments.



5. I am confused about the data and interpretation of Fig 4E. Since CK2 depletion increases the ubiquitination of the PC4-S17E mutant protein, I believe that the correct interpretation would be that the kinase is doing something independent of that phosphorylation site to regulate ubiquitination. However, the authors interpretation is that one happens after the other. I am not following that logic. I think they need to explain this better or consider reinterpreting that result. Also related to Figure 4E, the phos-tag gel shows a huge decrease in electrophoretic mobility in the PC4 S-to-E mutant. However, phos-tag gels specifically interact with the presence of a phosphate groups on the modified protein, and swapping an amino acid, to the very best of my understanding, should have no impact on its electrophoretic mobility?

Thanks for your helpful comments, and we agree with the reviewer. To further

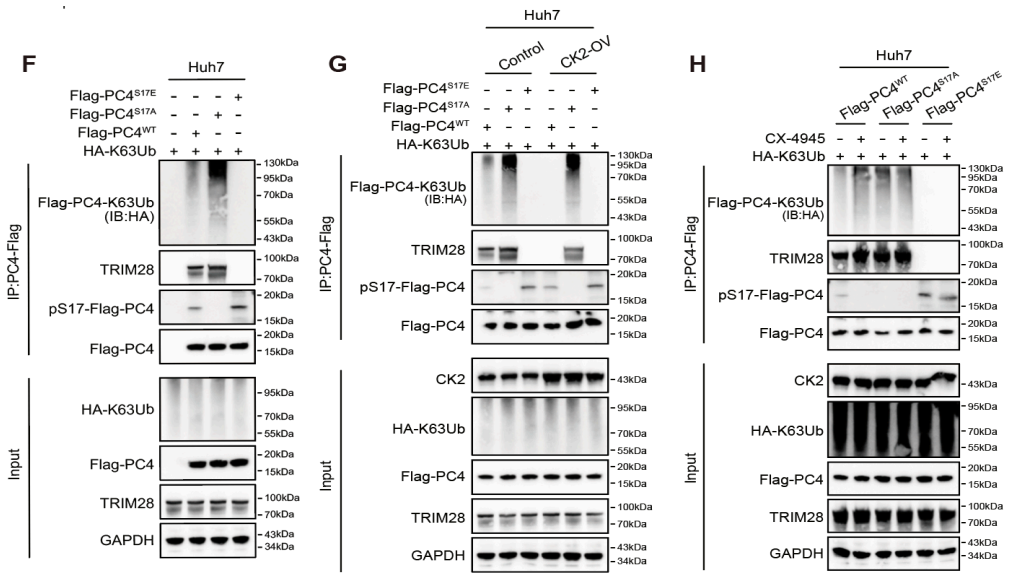
investigate whether CK2 regulates PC4 ubiquitination dependent on the phosphorylation site, we conducted new PC4-IP experiments using different PC4-mutant cells. We optimized the protocol to better detect the phosphorylation signal of PC4 at the S17 site by using a specific PC4-S17 phosphorylation antibody in SDS-page gels instead of a general PC4 antibody in the phos-tag gels. In three repeated experiments, we observed the following results: (1) Compared to $PC4^{WT}$, the $PC4^{S17A}$ mutation moderately increased the level of K63-linked ubiquitination of PC4, while the $PC4^{S17E}$ mutation significantly decreased this level. (2) Silencing CK2 enhanced K63-linked ubiquitination in $PC4^{WT}$ cells but did not affect the abundance of ubiquitination in $PC4^{S17A}$ cells. (3) Silencing CK2 failed to reverse the reduction of PC4 ubiquitination induced by the S17E mutation. Based on these findings, we concluded that PC4 K63-linked ubiquitination occurs after S17 dephosphorylation. We apologize for the confusion caused by our previous results and have updated Figure 4E accordingly. We have also modified the text to reflect these new findings.



6. Related to the previous two points, there does not appear to be any change in TRIM28 binding to PC4 in CK2 inhibited cells in Fig 4G. Yet the ubiquitination of PC4 is remarkably increased? To help address this confusion, I would suggest performing a PC4 IP with the phoshpho mutants that have already been generated, and blot for TRIM28. Repeating this in the presence of either CK2 overexpression or inhibition would be very helpful.

We greatly appreciate the valuable comments from the reviewer. Following to the reviewer's suggestion, we conducted new PC4 IP experiments to compare the K63-linked ubiquitination level of PC4 and the interaction between PC4 and TRIM28 in $PC4^{WT}$, $PC4^{S17A}$, and $PC4^{S17E}$ cells, respectively. Consistent with our previous findings, phosphorylation of S17 ($PC4^{S17E}$) prevented the interaction between TRIM28 and PC4, leading to a decrease in downstream ubiquitination. In $PC4^{WT}$ cells, inhibition of CK2 enhanced this interaction and TRIM28-mediated PC4-K68 linked polyubiquitination, while CK2 overexpression blocked these events. However, in $PC4^{S17A}$ cells, CK2 had no effect on the interaction of TRIM28 and $PC4^{S17A}$, nor on the K63-linked

polyubiquitination of PC4^{S17A}. Therefore, we conclude that CK2-mediated phosphorylation of PC4 at S17 disrupts the binding between TRIM28 and PC4, thereby inhibiting subsequent K68 ubiquitination. We believe that these new results provide clearer evidence and strengthen our conclusion. We have updated Figures 4F-4H accordingly. We have also modified the text to reflect these new findings.



7. I am skeptical that the data in Fig 5B has anything to do with CCND1, since a double thymidine block and release is likely to trap cells past the point where they would need Cyclin D. This could be the result of SKP2 reduction? See above.

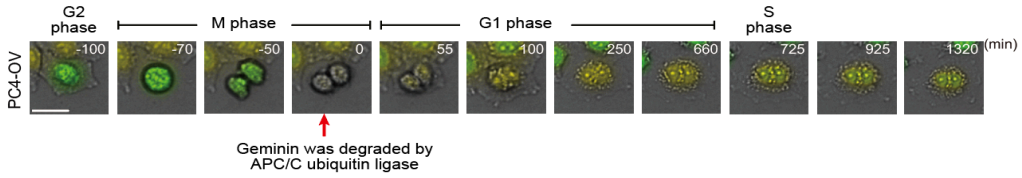
We agree with the reviewer's comments. According to the results shown in Figure 1, PC4 appears to promote the G1/S phase transition by regulating the stability of several key factors involved in G1/S phase transition. We speculate that PC4 plays a crucial role in stabilizing the mRNAs of CCND1, E2F2, SKP1, SKP2, and CIZ1, which are involved in activating the E2F:RB pathway. This pathway, in turn, promotes the G1/S transition. On one hand, PC4 enhances the stability of CCND1 mRNA, leading to increased protein abundance. Consequently, high levels of CCND1 facilitate the effects of the cyclin D-Cdk4/6 complex on RB phosphorylation. On the other hand, PC4 upregulates the expression of SKP1, SKP2 and CIZ1 mRNA. The elevated levels of the SCF:SKP2 complex result in the degradation of p21 and p27, while increased CIZ1 expression efficiently represses p21 activity. These processes collectively lead to the activation of cyclin E-Cdk2, hyperphosphorylation of RB, and dissociation of E2F. Additionally, increased expression of E2F2 caused by PC4 further promotes E2F-driven gene expression during the G1/S transition, resulting in a shorter G1 phase duration and faster entry into the S phase.

Therefore, we concur with the reviewer's suggestion that the impaired G1/S transition observed in Figure 5B, after a double thymidine block and release, is

probably caused by reduced SKP2 levels resulting from PC4 depletion. It is important to note that our study primarily focused on investigating the mechanism by which PC4 regulates CCND1 mRNA stability, given its significance in cell cycle progression and tumorigenesis. We genuinely appreciate the valuable suggestions provided by the reviewer, and we have revised our conclusion that PC4 promotes G1-S transition and cell proliferation partially through a CCND1-dependent manner.

8. It appears as though the APC/C ubiquitin ligase is not turning on to degrade geminin in the PC4 overexpressing cells in Fig 5F. This could play an important role in driving cell cycle, independent of cyclin D. Are the authors certain that they have marked cells at mitotic exit for these experiments? If that is true, they might speculate as to what is going on.

We appreciate the reviewer's careful attention to our experiment. In this study, we utilized the FUCCI-system to focus on the G1 phase, which is marked by the cell entering this phase (indicated by a yellow color) and continuing until the transition to the S phase (when the cell color changes to green). Our comparison of the G1 phase across different groups revealed that PC4 knockdown led to significant cell-cycle arrest in the G1 phase. Conversely, PC4 overexpression resulted in a shorter G1 phase. We thought that we initially observed that geminin was not being degraded by APC/C ubiquitin ligase in the selected PC4-overexpression cell. However, it is possible that with further live imaging at the next time point, we may observe geminin degradation. To address this matter, we have selected a different cell that offers clearer visualization of the entire process. This enables us to observe the progression from the G2 phase, entry into the M phase, geminin degradation during mitosis, transition into the subsequent G1 phase, and eventually entry into the S phase. We apologize for any confusion caused by the previous images, and the new set of images in Figure 5F will provide stronger evidence to support our conclusions.



9. Data in Figure 7C, examining overall PC4 ubiquitination in cancers, is hard to interpret because there is much more TRIM28 in the tumor cells.

We concur with the reviewer's perspective. Our speculation is that the overall level of PC4 ubiquitination is elevated in HCC samples due to two main factors: 1) the increased protein expression of TRIM28 in tumor cells, and 2) the enhanced interaction between TRIM28 and PC4 in HCC tissues. In summary, the higher ubiquitination of PC4 mediated by TRIM28 in HCC indicates a heightened RBP function of PC4 in tumor cells, which holds potential clinical relevance for prognostic analysis of HCC.

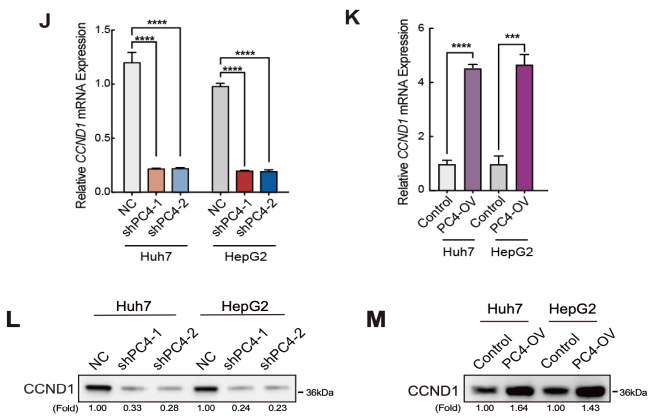
10. In the table in Fig 1C PTTG1IP is listed twice.
We appreciate the reviewer's comments, and we have removed the duplicated "PTTG1IP" in Figure 1C.

11. What is meant by "common PC4-binding sites" in describing RIPseq?
The pie chart in Figure S1A depicts the distribution of PC4-RIPseq peaks, indicating a notable enrichment towards protein-coding transcripts. We have revised the manuscript description to highlight that PC4 prominently bound to protein-coding transcripts.

12. State more clearly in the many texts of the results that Fig 3C is an in vitro assay using purified components.
We are grateful for the reviewer's insightful comments, and we have made the corrections to the text describing the results in Figure 3C by saying "The *in vitro* ubiquitylation assay using recombinant proteins also showed the polyubiquitylation of PC4 occurred exclusively when TRIM28 was present."

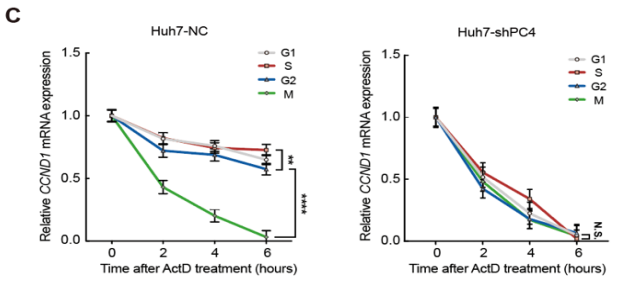
Reviewer #3 (Comments to the Authors (Required)):
In this study, Pan et al. report that an RNA-binding protein PC4 regulates cyclin D1 mRNA stability. Cyclin D1 plays an important function in driving proliferation of cancer cells. Hence, understanding how this protein is regulated is of great biological and clinical importance. Previous studies documented that cyclin D1 is regulated transcriptionally and at the level of protein degradation. Here, Pan et al. propose a new level of cyclin D1 regulation, namely though cyclin D1 mRNA stability. I have the following questions/suggestions for the authors.
We appreciate the reviewer's thorough evaluation.

1. If PC4 plays a biologically significant role in regulating cyclin D1 levels, I would expect that depletion of PC4 will reduce the levels of cyclin D1 mRNA and protein, while overexpression will have an opposite effect. I did not find this information in the manuscript.
We appreciate the reviewer's mention of this. As anticipated by the reviewer, we conducted experiments in both Huh7 and HepG2 cells. Our findings demonstrate that depletion of PC4 significantly reduced the expression of CCND1 mRNA and protein (Figures 1J and 1L). Conversely, we observed contrasting effects in PC4-overexpressing cells (Figures 1K and 1M).



2. The authors mention that stability of cyclin D1 mRNA fluctuates across the cell cycle, and postulate that this is driven by PC4. Is then the fluctuation lost in PC4-depleted cells?

We thank the reviewer for pointing this out. In Figure 2K, we noticed that the fluctuation in CCND1 mRNA stability during cell cycle was no longer present where PC4 was knocked down. We also conducted additional RNA half-life experiments and verified that the changes in CCND1 mRNA stability during cell cycle were eliminated in PC4-depleted cells. We have now updated the data in Figure S2C and included a corresponding description in the manuscript.



3. The authors state that "PC4 deficiency induced G1 phase arrest, linking PC4 to the cell-cycle transition (Figure 5A)". However, in Figure 5A the fraction of S-phase cells is decreased from 25% to 13%, which does not constitute "an arrest".

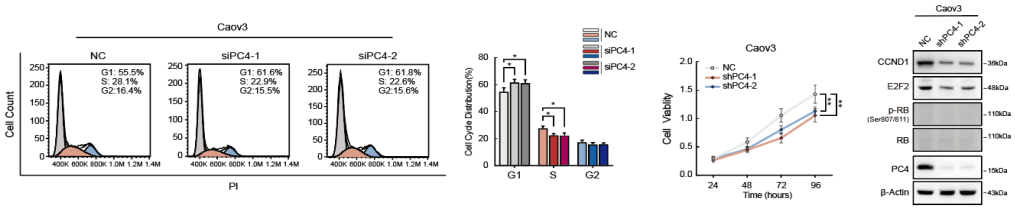
We thank for the reviewer's helpful comments. We have modified the statement in the manuscript as follows: "As expected, PC4 deficiency induced an elevation of cells in the G1 phase, accompanied by a reduction in the non-G1 phases (G2/M/S), linking PC4 to the cell-cycle transition."

4. What is the evidence that this effect is mediated by cyclin D1 depletion? Does it occur in RB-negative cells (which do not arrest after cyclin D1 depletion)?

We appreciate the reviewer for the useful comment. Indeed, we conducted flow

cytometry and cell proliferation assays after depleting CCND1 in liver cancer cells. Our findings revealed that CCND1 knockdown led to an increased number of cells in the G1 phase and defects in cell growth (Figure 5J-5K and S6A-S6C), though the phenotypic effects were not as pronounced as the significant impact observed upon PC4 knockdown. Furthermore, when we complemented CCND1 in PC4-knockdown cells, we observed partially alleviated the extension of the G1 phase (Figures 5M) and the cell growth defects observed in PC4-knockdown cells (Figures 5L and S6D-S6G). This suggests that PC4 promotes cell cycle progression by regulating the expression of several cell-cycle genes, not solely CCND1. As mentioned earlier (Figures 1L and 1M), our results demonstrate that PC4 facilitates the G1/S phase transition by controlling the stability of key factors involved in this process. We speculate that PC4 plays a crucial role in stabilizing the mRNAs of CCND1, E2F2, SKP1, SKP2, and CIZ1, which are involved in activating the E2F:RB pathway. This pathway, in turn, promotes the G1/S transition.

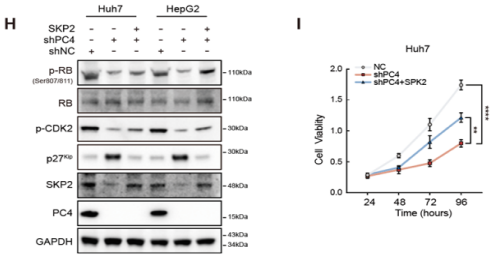
Additionally, we investigated the effects of PC4 depletion on cell growth and cell cycle progression in RB-negative CAOV3 cells. Our results showed a slightly slower G1/S transition and a minor inhibition of cell proliferation upon PC4 depletion (see below). We hypothesize that PC4 may mainly influence E2F2 expression to regulate the cell cycle in RB-negative cells. Further experiments will be performed to fully interpret these findings. Therefore, we cannot conclude at this stage that the effect of PC4 on the cell cycle is solely mediated by CCND1 control. We have modified the descriptions in the manuscript accordingly. We are grateful for the reviewer's valuable suggestions.



5. Likewise, what is the evidence that the effects seen in other cell lines in vitro and in vivo are mediated through D1, rather than targeting of several cell cycle proteins? The authors mentioned that transcripts bound to PC4 are enriched in the cell cycle category. Is it really that cyclin D1 downregulation is entirely responsible for the effect? Was there a significant decrease of cyclin D1 protein levels? Was the effect abrogated in RB1-negative cells?

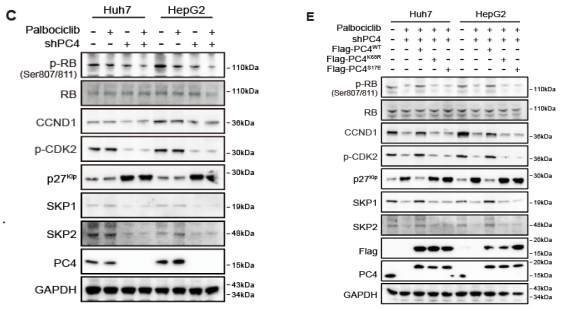
We acknowledge the reviewer's point. Currently, we cannot definitively conclude that CCND1 downregulation, resulting from PC4 depletion, is solely responsible for blocking cell-cycle progression and inhibiting cancer cell growth. There are several reasons for this. Firstly, PC4 plays a role in binding to and stabilizing a group of mRNAs involved in the G1/S transition. Its depletion extends the G1 phase, impairs cell growth, and reintroducing CCND1 only partially restores these effects in PC4-depleted cells.

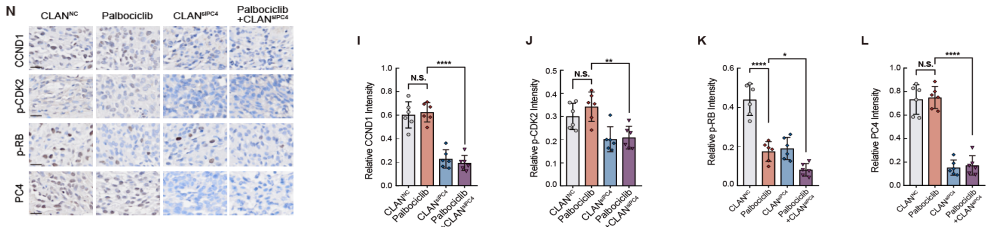
Secondly, PC4 depletion affects other cell-cycle genes such as SKP2, which it also binds to. Introducing SKP2 partially rescues the phenotype in PC4-depleted cells (Figure S6H and S6I), suggesting that PC4-induced SKP2 downregulation contributes to impaired cell cycle progression. While PC4 regulates multiple gene expressions to control cell cycle progression, our focus in this paper is on elucidating the mechanism by which PC4 modulates the fluctuation of CCND1 mRNA stability during the cell cycle, as it is crucial for cancer cell proliferation.



6. The authors show that PC4 loss sensitized cells to palbociclib. This effect is not necessarily mediated by D1 depletion, and it could be mediated by depletion of CDK2. What is the evidence that the effect depends on cyclin D1?

We agree with the reviewer's point that the effect of PC4 loss on increasing the sensitivity of HCC cells to Palbociclib is not necessarily mediated by CCND1 depletion. We have conducted new experiments and observed that both PC4 depletion and inhibition of PC4-RNA binding capacity reduced CCND1 and SKP2 protein expressions *in vitro* and *in vivo*. Therefore, PC4 loss sensitizes HCC cells to Palbociclib mainly through two mechanisms. Firstly, PC4 depletion decreases CCND1 protein expression, which may hinder the cyclin D-Cdk4/6 complex from phosphorylating RB. Secondly, PC4 depletion reduces SKP2 expression, leading to the inhibition of p27 degradation, suppression of CDKdk2 activation, and prevention of RB hyperphosphorylation. We have updated the Figures 7C, 7E, 7N and S7I-7L with the new data and modified the descriptions in the manuscript accordingly. We appreciate the valuable suggestions provided by the reviewer.





7. The authors found that PC4 regulates cyclin D1, but not D2 or D2. Would PC4 depletion have any impact on proliferation of cells which express mainly cyclins D2 and D3?

We appreciate the reviewer for raising this point. As PC4 is both an RNA binding protein and a transcription factor, it is possible that it may regulate cellular growth by influencing the expression of *CCND2* and *CCND3* mRNA in other cells. This intriguing question merits further investigation.

Once again, thank you very much for your interest in our work and for helping us improve the quality of our paper. We hope that our manuscript will be considered for publication in your journal. If there are any further suggestions or corrections, please feel free to contact us.

Best regards,

Chunmeng Shi, PhD
Professor
Third Military Medical University (Army Medical University), Chongqing, China

Dong Yin, PhD
Professor
Sun Yat-Sen University, Guangzhou, China

December 11, 2023

RE: JCB Manuscript #202308066R

Prof. Chunmeng Shi
Army Medical University
Gaotanyan Street, Shapinba District
Chongqing 400000
China

Dear Prof. Shi,

Thank you for submitting your revised manuscript entitled "Periodic changes of cyclin D1 mRNA stability are regulated by PC4 modifications in the cell cycle." The manuscript has been re-assessed by Reviewers 1&2. Reviewer #3 was not available to re-review. We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

Full guidelines are available on our Instructions for Authors page, https://jcb.rupress.org/submission-guidelines#revised. **Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.**

1) Text limits: Character count for Articles is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, and acknowledgments. Count does not include materials and methods, figure legends, references, tables, or supplemental legends.

2) Figure formatting: Articles may have up to 10 main text figures. Scale bars must be present on all microscopy images, including inset magnifications. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis. Please add scale bars to Figures 7K & S1F (magnifications) and MW markers to Figures 1F/G, 3J, & 4K.

Also, please avoid pairing red and green for images and graphs to ensure legibility for color-blind readers. If red and green are paired for images, please ensure that the particular red and green hues used in micrographs are distinctive with any of the colorblind types. If not, please modify colors accordingly or provide separate images of the individual channels.

3) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Please, indicate whether 'n' refers to technical or biological replicates (i.e. number of analyzed cells, samples or animals, number of independent experiments). If independent experiments with multiple biological replicates have been performed, we recommend using distribution-reproducibility SuperPlots (please see Lord et al., JCB 2020) to better display the distribution of the entire dataset, and report statistics (such as means, error bars, and P values) that address the reproducibility of the findings.

Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (both in the figure legend itself and in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, if you used parametric tests, please indicate if the data distribution was tested for normality (and if so, how). If not, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

4) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions (at least in brief) in the text for readers who may not have access to referenced manuscripts. The text should not refer to methods "...as previously described." Please also indicate the acquisition and quantification methods for immunoblotting/western blots.

5) For all cell lines, vectors, constructs/cDNAs, etc. - all genetic material: please include database / vendor ID (e.g., Addgene, ATCC, etc.) or if unavailable, please briefly describe their basic genetic features, even if described in other published work or gifted to you by other investigators (and provide references where appropriate). Please be sure to provide the sequences for all of your oligos: primers, si/shRNA, RNAi, gRNAs, etc. in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers/vendor identifiers (where appropriate) for all of your antibodies, including secondary. If antibodies are not commercial, please add a reference citation if possible.

6) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
d. Imaging medium
e. Fluorochromes
f. Camera make and model
g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

7) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

8) Supplemental materials: Articles are generally allowed up to 5 supplemental figures and 10 videos. You currently exceed this limit but, in this case, we will be able to give you the extra space. Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section. Please include one brief sentence per item.

9) eTOC summary: A ~40-50 word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person. It should begin with "First author name(s) et al..." to match our preferred style.

10) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

11) A separate author contribution section is required following the Acknowledgments in all research manuscripts. All authors should be mentioned and designated by their first and middle initials and full surnames. We encourage use of the CRediT nomenclature (https://casrai.org/credit/).

12) ORCID IDs: ORCID IDs are unique identifiers allowing researchers to create a record of their various scholarly contributions in a single place. Please note that ORCID IDs are required for all authors. At resubmission of your final files, please be sure to provide your ORCID ID and those of all co-authors.

13) JCB requires authors to submit Source Data used to generate figures containing gels and Western blots with all revised manuscripts. This Source Data consists of fully uncropped and unprocessed images for each gel/blot displayed in the main and supplemental figures. Since your paper includes cropped gel and/or blot images, please be sure to provide one Source Data file for each figure that contains gels and/or blots along with your revised manuscript files. File names for Source Data figures should be alphanumeric without any spaces or special characters (i.e., SourceDataF#, where F# refers to the associated main figure number or SourceDataFS# for those associated with Supplementary figures). The lanes of the gels/blots should be labeled as they are in the associated figure, the place where cropping was applied should be marked (with a box), and molecular weight/size standards should be labeled wherever possible. Source Data files will be directly linked to specific figures in the published article.
Source Data Figures should be provided as individual PDF files (one file per figure). Authors should endeavor to retain a minimum resolution of 300 dpi or pixels per inch. Please review our instructions for export from Photoshop, Illustrator, and PowerPoint here: https://rupress.org/jcb/pages/submission-guidelines#revised

14) Journal of Cell Biology now requires a data availability statement for all research article submissions. These statements will be published in the article directly above the Acknowledgments. The statement should address all data underlying the research presented in the manuscript. Please visit the JCB instructions for authors for guidelines and examples of statements at (https://rupress.org/jcb/pages/editorial-policies#data-availability-statement).

B. FINAL FILES:

Please upload the following materials to our online submission system. These items are required prior to acceptance. If you have any questions, contact JCB's Managing Editor, Lindsey Hollander (lhollander@rockefeller.edu).

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-- High-resolution figure and MP4 video files: See our detailed guidelines for preparing your production-ready images, https://jcb.rupress.org/fig-vid-guidelines.

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Please contact the journal office with any questions at cellbio@rockefeller.edu.

Thank you for this interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.

Sincerely,

Arshad Desai, PhD
Monitoring Editor
Journal of Cell Biology

Dan Simon, PhD
Scientific Editor
Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

The authors have addressed all of my concerns and I now believe the manuscript is acceptable for publication.

Reviewer #2 (Comments to the Authors (Required)):

The authors have taken great care both to conduct revisions of the manuscript, add many additional experiments, and to describe them clearly and thoroughly in the response. I commend them highly for this effort and am highly supportive of the manuscripts publication.