Reciprocal regulation of p21 and Chk1 controls the cyclin D1-RB pathway to mediate senescence onset after G2 arrest

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DOI: 10.1242/jcs.259114

Editor: David Glover

Review timeline
Original submission: 12 July 2021
Editorial decision: 13 September 2021
First revision received: 15 February 2022
Accepted: 18 March 2022

Original submission

First decision letter

MS ID#: JOCES/2021/259114

MS TITLE: Reciprocal regulation of p21 and Chk1 controls the Cyclin D1-RB pathway to mediate senescence onset after G2 arrest

AUTHORS: Gerald Lossaint, Andela Horvat, Veronique Gire, Katarina Bacevic, Karim Mrouj, Fabienne Charrier-Savournin, Virginie Georget, Daniel Fisher, and Vjekoslav Dulic
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewer's report and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewer raises a number of substantial criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewer.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The authors have demonstrated that p21 inhibits Cyclin D1-CDK (CDK2 and CDK4) complex, resulting in hypo-phosphorylation of RB pocket family proteins, at the G2/M transition during DDR. This hypo-phosphorylation presumably inhibits the expression of several genes involved in the G2/M transition; this phenomenon is likely required for permanent cell-cycle exit (cellular senescence). Conversely, Chk1 activation in response to DDR maintains G2 arrest, which prevents permanent cell-cycle exit. The study was well designed and carefully done. In addition, this study is useful to understand how cells decide senescence in response to DDR.

Comments for the author

However, there are several points that the authors should address before publication.
1. In order to show RB hypo-phosphorylation in the immunoblotting, the authors demonstrated the anti-RB bands including hyper- and hypo-phosphorylated bands. Figs. 1A and 6E are good because the hypo-phosphorylated RB band is clearly detected. On the other hand, Figs. 2A, 4H, S1E, and S7B are not so good because only Rb expression appears to be reduced. If so, the authors should state this fact in the manuscript.
In addition, the authors should indicate the position of hyper- or hypo-phosphorylated band(s) in the first Figure.
2. In Fig. 1I, it appears that p21 depletion reduces only Cyclin D1 expression rather than the complex formation of Cyclin D1 and CDK2/4/6. The authors should change the interpretation. Alternatively, the authors should adjust the equal level of anti-Cyclin D1 in the immunoprecipitate(s) between control and sip21 groups.
3. In Fig. 2A, the level of Chk2 or Chk2-pT68 at 24h after IR irradiation appears to be significant changed. If so, the authors should describe the reason(s) why.
4. The authors checked the involvement of Chk2 in the manuscript. However, the authors only checked the level of p53 phosphorylation at Ser15 (an ATM/ATR phosphorylation site). The authors should also check the level of p53 phosphorylation at Ser20 (a Chk2 phosphorylation site) in Figs. S3C, S4C-S4E, and S5B.

Minor points:
1. In Fig. S6, Fig. S6G is unlikely to exit.
2. In the legend of Fig. 1A, “Sen.” appears to mean “Senescent cells”. Please clarify it so that general readers are easy to understand.
3. Page 6, line 156: Please check the reference style.

First revision

Author response to reviewers' comments

Response to reviewers

As mentioned in the Cover letter to the editor, in order to comply with the JCS guidelines, we had to reduce the number of supplementary figures to keep their number equal to that of main figures. For that reason, we added another main figure (Figure 2) and transferred some of the results into remaining figures. Also, we redistributed the results into 7 supplementary figures from 9 that were present in the first version.
However, we kept almost all the results presented in the first version, except where experiments

were repeated to improve the analysis according to the reviewer's suggestions.

Comments to the Author

Reviewer 1 Advance Summary and Potential Significance to Field:
The authors have demonstrated that p21 inhibits Cyclin D1-CDK (CDK2 and CDK4) complex, resulting in hypo-phosphorylation of RB pocket family proteins, at the G2/M transition during DDR. This hypo-phosphorylation presumably inhibits the expression of several genes involved in the G2/M transition; this phenomenon is likely required for permanent cell-cycle exit (cellular senescence). Conversely, Chk1 activation in response to DDR maintains G2 arrest, which prevents permanent cell-cycle exit. The study was well designed and carefully done. In addition, this study is useful to understand how cells decide senescence in response to DDR.

We thank the reviewer for appreciating our work and for constructive criticism that will definitely improve understanding of the manuscript.

However, there are several points that the authors should address before publication.

1. In order to show RB hypo-phosphorylation in the immunoblotting, the authors demonstrated the anti-RB bands including hyper- and hypo-phosphorylated bands. **Figs.1A and 6E** are good because the hypo-phosphorylated RB band is clearly detected. On the other hand, **Figs. 2A, 4H, S1E, and S7B** are not so good because only Rb expression **appears to be reduced**. If so, the authors should state this fact in the manuscript. In addition, the authors should indicate the position of hyper- or hypo-phosphorylated band(s) in the first Figure.

Answer:
1.1. In all relevant figures we have added bars indicating RB phosphorylation shift (Figs. 1A; S1E now 1E, 2A now 3A; 2I; 4H now 5H; S4D; 5B; S7B now S6B)
1.2. In most figures we also added P^{S780}-RB immunoblots to better document Cyclin D- specific RB phosphorylation, which is highly relevant for our model (Figs. 1A; S1E now 1E, 2A now 3A; 2I; 4H now 5H; S4D; 6D). These results strengthen our hypothesis that p21 inhibits Cyclin D-specific RB phosphorylation.
1.3. As suggested, we commented reduced RB levels after cell cycle exit and provided a reference that explains relevant mechanisms of p21-dependent RB degradation (Broude et al. Oncogene 2007). Regrettably, we neglected this aspect of RB behaviour during the cell cycle exit. Moreover, we added to most of the figures immunoblots of p130, another member of RB family whose levels increase during the cell cycle exit, to document the specificity of RB downregulation.
1.4. Finally, to better document CycD1-specific RB phosphorylation, we added a lane showing that CycD1 downregulation in the absence of serum results in apparition of hypo-phosphorylated RB (Fig. 1A). This provides evidence that CycD1 is sensor for growth factors beyond G1/S transition.

Please note that sentences that directly address the reviewer's comments are in red.

2. In Fig. 1I, it appears that p21 depletion **reduces only Cyclin D1 expression** rather than the complex formation of Cyclin D1 and CDK2/4/6. The authors should change the interpretation. Alternatively, the authors *should adjust the equal level of anti-Cyclin D1 in the immunoprecipitate(s) between control and sip21 groups*.

A: Fig1I is now Fig. 2H.
The reviewer is correct: the knockdown of p21 inhibits augmentation of CycD1-CDK complexes upon DNA damage probably by preventing the increase in expression of cyclin D rather than by affecting complex formation *per se*, and it appears that we had not worded this correctly if the reviewer did not understand. We stated that p21 KD prevents **accumulation** of CycD1-CDK complexes, which to us implied that it reduces CycD1 induction by DNA damage (as shown by immunofluorescence and immunoblotting). Since Cyclin D1 is a major p21 partner (Chen et al. Mol Cell 2013), p21 depletion prevents increase of CycD1 (but not CycA) levels that we show now in **Fig. 2G**-(cf. also Fig. 7B, 7E, S7B, S7G). However, as the reviewer suggested, we now state this more explicitly.

Conversely, as shown by newly-added results, CycD1 depletion downregulates p21 upregulation after DNA damage (Fig. 2I).

The 2nd suggestion is rather complicated to do. However, we show p27 levels in CycD1 IPs that do not change upon p21 KD, conveniently serving as a loading control.

3. In Fig. 2A, the level of Chk2 or Chk2-pT68 at 24h after IR irradiation appears to be significant changed. If so, the authors should describe the reason(s) why.

A: Fig. 2A is now Fig3A. The reason of changes seen in the level of Chk2 at 24 hours after irradiation was unequal loading. We repeated this experiment and this is corrected in our new version.

4. The authors checked the involvement of Chk2 in the manuscript. However, the authors only checked the level of p53 phosphorylation at Ser15 (an ATM/ATR phosphorylation site). The authors should also check the level of p53 phosphorylation at Ser20 (a Chk2 phosphorylation site) in Figs. S3C, S4C-S4E, and S5B.

A: This is a relevant question, but one of the reasons why we did not do it earlier is that unlike other phospho-specific antibodies, several p53-S20ph (P^{S20}-p53) antibodies we've tried produced a very weak signal with often a lot of background. Nevertheless, we attempted to monitor P^{S20}-p53 in all relevant experiments, which usually required repeating the experiments. We apologize for the quality of some blots as indeed these antibodies are not outstanding. However, this did not modify the conclusions of the paper.

In Fig 3A (former 2A) we added a bleomycin-treated sample as a positive control as it elicits stronger DNA damage than □□radiation. As shown in Fig S3A, these cells also arrested in G2.

Note that we removed p53 data in former Fig. S3C (now S2A), as they are not relevant for the message, but they are included in new Fig, S3C where we analysed all p53-relevant results (former S4C). We removed p53-relevant blots from S4D (now S3D) and we added P^{S20}-p53 in Fig.S3F (former S4E) and in S5B. We also added P^{S15}/P^{S20}-p53 immunoblots in Fig. 3A (former 2A) to complete DNA damage signalling analysis.

In addition, to further explore the role of Chk2 role in p53 phosphorylation and activation, we now show P^{S20}-p53 and P^{S15}-p53 in U2OS cells in which either Chk1 or Chk2 were down-regulated by siRNA-mediated knock-down (Fig. S6C). Although in U2OS cells strong P^{S20}-p53 phosphorylation correlates with stronger Chk2 phosphorylation (Fig. S4B), Chk2 KD did not alter this phosphorylation (Fig. S6C).

We also show that Chk2 KD did not diminish p53-mediated induction of CDK inhibitor p21 after DNA damage neither in cancer U2OS cell line (Fig. 6D and S6B) nor in non-transformed human fibroblasts (Fig. S6D and E). Moreover, in these cells p21 remain induced even after double Chk1/ Chk2 knockdown, suggesting that Chk1 does not compensate for the absence of Chk2. These results do not support idea that Chk2 plays a major role in p53 activation, at least in these cells. Although the role of Chk2 as an activator is well accepted (Matthews et al. 2022), note that this role was also challenged in the literature (Chen and Poon, 2008).

Minor points:

1. In Fig. S6, Fig. S6G is unlikely to exit.

A: We are not sure to understand the comment. Fig. S6G (now S5G) shows p21 upregulation in G2-arrested U2OS after DNA damage. We are not sure to understand the comment.

2. In the legend of Fig. 1A, "Sen." appears to mean "Senescent cells". Please clarify it so that general readers are easy to understand.
A: A good remark. We've corrected this. This is now Fig. 1B

3. Page 6, line 156: Please check the reference style.

A: Corrected. Thanks for the remark.

Second decision letter

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ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

Lossaint et al.
The authors have almost completely addressed my concerns or corrected my misinterpretation.

Comments for the author

Lossaint et al.
The authors have almost completely addressed my concerns or corrected my misinterpretation.