Identification of a novel Bax-Cdk1 signalling complex that links activation of the mitotic checkpoint to apoptosis

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Review timeline

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Original submission

First decision letter

MS ID#: JOCES/2020/244152

MS TITLE: Identification of a novel Bax-Cdk1 signalling complex that links activation of the Mitotic Checkpoint to Apoptosis.

AUTHORS: Omeed Darweesh, Eman Al-Shehri, Hugo Falquez, Joachin Lauterwasser, Frank Edlich, and Rajnikant R Patel
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We have now reached a decision on the above manuscript.

To see the reviewers' reports 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 reviewers raise 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 reviewers.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. 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

Review of Darweesh et al., 2020 In my view this is a very interesting paper that addresses a significant knowledge gap: How the SAC communicates to the mitochondria to initiate the intrinsic apoptotic pathway so that cells that have been unable to correct maloriented chromosomes are eliminated. The information about the phosphorylation of the key anti-apoptotic proteins Bcl2 and Bcl x-l is not novel, but is corroboratory, however, how cdk1 accesses the mitochondria so that it can phosphorylate these proteins is new and important.
The authors hypothesise that cdk1 forms a complex with the pro-apoptotic proteins, Bax and Bak, which enables it to translocate to the mitochondria where it phosphorylates the anti-apoptotic proteins, Bcl2 and Bcl-xl thereby promoting cell death.
Introduction is clear, well written and informative, the results support their hypothesis. The work is not overstated and the title conveys the message very effectively. I recommend that the paper is published subject to some minor corrections. I also think that the results and discussion could be combined, some of the figures put into the supplemental material, and the paper published as a brief report.

Comments for the author

Below is a summary of my comments on each figure and changes that I would like to see:
Figure 1.
The authors use conformational specific commercially available antibodies to IP activated Bax and Bak and interrogate taxol (and nocodazole) arrested cells for cdk1 interaction. In figure 1 they show that a complex comprised of Bax Bak cdk1 and cyclin B “accumulates” in taxol arrested cells.
Figrue 1A could be put in the supplementary material.
Figure 1C – this should be additionally probed for Bak Figure 1D – this should be additionally probed for Bax.
Figure 1E – lovely blot, nicely presented. Should be probed for Bak. How many times was this done and can it be quantitated please.
Despite these comments, Figure 1 clearly shows that cdk1 and cyclin B enter the mitochondria of taxol arrested cells, which is a very interesting result.
Figure 2. Can the authors ensure consistency in the ordering of their blots.
(A) Why are there 2 bands in the IP and Bax and only 1 in the WCE?
Figure 3: (A-B) Here they have used two different apoptosis assays
(cytokeratin 18 cleavage and PARP cleavage) – both apt. Unsure why the former is presented as a percentage – I would prefer the raw data even in arbitrary units. (A) Why is there no cyclin B in lanes 5, 8. 9 and 10? – they are all taxol treated.
Next (C) they use a cdk1 inhibitor, C demonstrates that it is effective (this could be put in supplemental material).
The remaining experiments use siRNA to ask whether Bak, Bax or the two removed in combination, prevents phosphorylation of BCl2 and BCl xl.
Experiments are appropriate and well controlled but there are one or two revisions required:
Figure 3G: blot and graph (H) should be combined. Blot needs the following:
evidence of siRNA working in this experiment, a loading control and cdk1 phosphorylation on Y15 (as in Figure 4) should be included.
Figure 4: Same rationale as for Figure 3D-I but they use genetically engineered Hct116 double knock out (DKO) cells rather than siRNA and extend the work to a rescue experiment using GFP-Bax, which they find is sufficient to restore phosphorylation of the target proteins. Overall very nice but D needs to be quantified.

Reviewer 2

Advance summary and potential significance to field

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Comments for the author

This study by Darweesh and colleagues investigates how prolonged mitotic arrest leads to apoptosis, as the authors state this is an open question. The authors describe an interaction between activated BAX or BAK and CDK1, leading to the recruitment of CDK1 to the mitochondria. Based on inhibitor data they propose that activated BAK, BAX recruit CDK1 to the mitochondria where CDK1 phosphorylates anti-apoptotic BCL-2 proteins, leading to their inactivation and apoptosis. The data are somewhat supportive of the authors' conclusions however I feel, key controls are missing at several points, besides this I have a major conceptual problem with the model proposed. These are detailed below:

- the interaction between activated BAX, BAK and CDK1 is convincingly demonstrated. In terms of the model the authors propose, it is difficult to reconcile why activated BAX and BAK would require CDK1 to dephosphorylate anti-apoptotic BCL-2 proteins in order to engage mitochondrial permeabilization. At the point where active BAX, BAK can be detected (and thereby associate with CDK1), anti-apoptotic BCL-2 function presumably has already been neutralized? This in my view, is a key point overlooked by the authors, potentially the CDK1 interaction with BAX and BAK occurs post-MOMP, given that it appears to be activation specific.

- the use of CDK1 inhibitor RO3306 effectively blocks taxane induced death, this associates with inhibition of BCL-2 phosphorylation, leading the authors to propose a causal relationship between apoptosis and CDK1 mediated phosphorylation of BCL-2. The role of phosphorylation in inhibiting anti-apoptotic BCL-2 function is controversial, to definitively claim this one would need to delete anti-apoptotic BCL-2 proteins and replace with non-phosphorylatable mutants.

- presumably the CDK1 inhibitor could indirectly block taxane induced death by preventing cell cycle arrest, can the authors discriminate this possibility from a potential pro-death role of CDK1 at the mitochondria ?

- figure 1, the authors suggest that CDK1 exists in a complex of BAX or BAK and cyclin B1, however the expts. don't necessarily demonstrate this, they could all be independent CDK1 complexes.

- figure 1, there are some inconsistencies across cell lines, for instance even though more active BAX is immunoprecipitatedd in U20S cells after taxol, there is no increase in associated CDK1 (E) the same holds true for BAK in 1H, the authors should comment on this.

- figure 2, regarding the mitochondrial fractionations, it would support the authors model (e.g. BAX, BAK recruitment of CDK1 to the mitochondria) to use BAX, BAK deficient cells (HCT116 they use later) to demonstrate that CDK1 is not recruited to the mitochondria in the absence of BAX, BAK.

First revision

Author response to reviewers' comments

Reviewer 1

Based on the comments, we believe that Reviewer 1 is referring to Figure 2 in the remarks pertaining to Figure 1

Figure 2A could be put in the supplementary material – We have placed Fig 2A (Commassie-stained gel of the purified, recombinant proteins) in the supplementary material labelled Fig S1.

Figure 2C, this should be additionally probed for Bak – we have added the blot for Bak **(now Fig 2B).**

Figure 2D, this should be additionally probed for Bax – we have added the blot for Bax **(now Fig 2C).**

Figure 2E, add blot for Bak, add n number and quantitate the blot – We have re-numbered this figure **(now Fig 3A),** have added a blot for Bak, the number of times the experiment was performed (included in the legend to Figure 3) and quantitated the blot **(Fig 3B)**.

Figure 2, why are there 2 bands in the IP and Bax and only 1 in the WCE – we believe this is refers to the original Figure 2C **(now Fig 2B)**. We analysed the doublet of Bax protein by mass spectrometry (seen in the Bax IP of Taxol-treated cells) to determine whether there was any post-translational modification of the protein (eg phosphorylation). We did not detect any phosphorylation of Bax but did find that it was oxidised. We do not understand the functional significance of this modification at the moment.

*Figure 3A **(now Fig 4A)**, why is there no cyclin B in lanes 5,8,9 and 10 as the cells are all Taxol-treated?* – RO3306 (5μM) binds to active Cdk1 to inactivate its kinase activity and the Cdk1-bound cyclin B is degraded by the 26S proteasome. This phenomenon has been reported previously (Vassilev et al., 2006. Selective small-molecule inhibitor reveals critical mitotic functions of human CDK1. Proc Natl Acad Sci U S A. 2006 Jul 11; 103(28): 10660–10665).

*Figure 3B **(now Fig 4B)**, cytokeratin 18 cleavage assay. I would prefer the raw data, even in arbitrary units* – We feel that the data is easier to interpret when expressed as % apoptosis but have included the raw data in the legend to Figure 3B **(now Fig 4B)**.

*Figure 3C **(now Fig 4C)**, they use a Cdk1 inhibitor, C demonstrates that it is effective (this could be put in supplemental material)* – We feel it is important to retain Figure 3C **(now Fig 4C)** in the main results section as not only does it demonstrate that RO3306 is effective, but it also demonstrates Cdk1-dependent phosphorylation of Bcl-2 and Bcl-xl (observed as a mobility shift in Fig 3A in Taxol- treated cells) and that this phosphorylation (and apoptosis) can be inhibited by RO3306.

*Figure 3G **(now Fig 4G)**, blot and graph should be combined* – we have combined Figure 3G and 3I (**now Fig 4G).**

*Figure 3G **(now Fig 4G)**, blot needs evidence of siRNA working in this experiment, a loading control and Cdk1 phosphorylation on Y15* – this data is now included in Figure 3G **(now Fig 4G).**

*Figure 4D **(now Fig 5D**), needs to be quantified* – the quantitation for Figure 4D **(now Fig 5D)** is included **(shown in Fig 5E).**

Reviewer 2
In terms of the model the authors propose, it is difficult to reconcile why activated BAX and BAK would require CDK1 to dephosphorylate anti-apoptotic BCL-2 proteins in order to engage mitochondrial permeabilization. At the point where active BAX, BAK can be detected (and thereby associate with CDK1), anti-apoptotic BCL-2 function presumably has already been neutralized? This in my view, is a key point overlooked by the authors, potentially the CDK1 interaction with BAX and BAK occurs post-MOMP, given that it appears to be activation specific.

Authors response – we assume that Reviewer 1 is asking why activated Bax and Bak would require Cdk1 to **phosphorylate** anti-apoptotic BCL-2 proteins in order to engage mitochondrial permeabilization. Although our study does not directly address the mechanism of mitochondrial permeabilization, our data **(now Figure 4A and 4B)** suggests that the Cdk1/cyclin B interaction with Bax/Bak occurs prior to MOMP (as judged by the % of apoptotic cells). In Taxol-arrested mitotic cells (12h) we observe a low level of apoptotic cells (5%) but Bcl-2 and Bcl-xl are

phosphorylated (as observed by their mobility shift). This suggests delivery of the Cdk1/cyclin B complex to the outer mitochondrial membrane pre-MOMP. If the Cdk1/cyclin B/Bax complex is formed post-MOMP (assuming MOMP represents a point of no return) then it should not be possible to suppress apoptosis by inhibiting Cdk1 (using RO3306). Our results indicate inhibition of Cdk1 with RO3306, specifically in mitotically-arrested cells **(now Figure 4A and 4B)**, suppresses phosphorylation of Bcl-2 and Bcl-xl and inhibits apoptosis (and by inference MOMP).

The use of CDK1 inhibitor RO3306 effectively blocks taxane induced death, this associates with inhibition of BCL-2 phosphorylation, leading the authors to propose a causal relationship between apoptosis and CDK1 mediated phosphorylation of BCL-2. The role of phosphorylation in inhibiting anti-apoptotic BCL-2 function is controversial, to definitively claim this one would need to delete anti- apoptotic BCL-2 proteins and replace with non-phosphorylatable mutants.

Authors response - Reviewer 2 is correct that the role of Bcl-2 and Bcl-xl phosphorylation in regulating their function is still unclear. Studies performed by Eichhorn et al., (2013)(cited in our discussion) by overexpression of phospho-defective mutants of Bcl-2 and Bcl-xl were found to inhibit mitotic cell death while a phosphomimetic mutant of Bcl-xL was unable to block mitotic death.

While these studies were performed with a background of endogenous Bcl-2 and Bcl-xl proteins, the data support our finding that Cdk1-mediated phosphorylation inhibits BCL-2 anti-apoptotic protein function.

Presumably the CDK1 inhibitor could indirectly block taxane induced death by preventing cell cycle arrest, can the authors discriminate this possibility from a potential pro-death role of CDK1 at the mitochondria ?

Authors response – Reviewer 2 is correct in that the addition of RO3306, prior to pro-metaphase, can inhibit apoptosis by inhibiting the activation of Cdk1 and hence entry into mitosis. Therefore, we first blocked the cells in mitosis by Taxol treatment and then treated only the mitotically-arrested cells with RO3306 to demonstrate the pro-apoptotic role of Cdk1 at the mitochondria **(now Figure 4A and 4B)**.

Figure 1, the authors suggest that CDK1 exists in a complex of BAX or BAK and cyclin B1, however the expts. don't necessarily demonstrate this, they could all be independent CDK1 complexes.
Authors response – We agree that there could be independent Cdk1 complexes with Bax, Bak and cyclin B in non-mitotic cells. However, it is well-established that active Cdk1 (in mitotic cells) is a dimer of Cdk1 and Cyclin B. In our Cdk1 Ip's from Taxol-arrested mitotic cells we find Cdk1 in complex with cyclin B and Bax (**Figure 1C**), indicating the presence of a minimal trimeric complex.

Figure 1, there are some inconsistencies across cell lines, for instance even though more active BAX is immunoprecipitated in U20S cells after taxol, there is no increase in associated CDK1 (E) the same holds true for BAK in 1H, the authors should comment on this.
Authors response – Our primary aim was to demonstrate the presence of Cdk1 and Bax/Bak complexes across different cell types. However, we agree with reviewer 2 that the level of Cdk1 co- immunoprecipitating with active Bax in U2OS **(Figure 1E**) and RPE1 cells **(Figure 1H**) is not increased in mitotic cells. One possibility is that in both U2OS and RPE cells a constant level of Cdk1 is constitutively associated with Bax throughout the cell cycle. At mitosis, when Cdk1 is activated, cyclin B may bind to a fraction of the Cdk1/Bax complex. We have included this comment in the results section for Figure 1 **(line 149-153**).

Figure 2, regarding the mitochondrial fractionations, it would support the authors model (e.g. BAX, BAK recruitment of CDK1 to the mitochondria) to use BAX, BAK deficient cells (HCT116 they use later) to demonstrate that CDK1 is not recruited to the mitochondria in the absence of BAX, BAK.

Authors response – We have added the requested data (**Figure 3C and 3D)** and quantitated the data to show that Cdk1 is not recruited to mitochondria in Bax/Bak DKO cells.

Second decision letter

MS ID#: JOCES/2020/244152

MS TITLE: Identification of a novel Bax-Cdk1 signalling complex that links activation of the Mitotic Checkpoint to Apoptosis.

AUTHORS: Omeed Darweesh, Eman Al-Shehri, Hugo Falquez, Joachin Lauterwasser, Frank Edlich, and Rajnikant R Patel
ARTICLE TYPE: Research Article

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To see the reviewers' reports 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 reviewers gave favourable reports but raised some critical points that will require amendments to your manuscript. I hope that you will be able to carry these out, because I would like to be able to accept your paper.

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.

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Reviewer 1

Advance summary and potential significance to field

This is a great paper as it addresses a knowledge gap that has been neglected until now, namely how the SAC links to apoptotic activation. I have found this paper very insightful. It's a huge amount of work and I will refer to it frequently in my own work going forward.

Comments for the author

I am satisfied that all of my comments have been addressed; changes have been made in the majority of cases, retention of data in a figure in the original format, where they have not made a change has been adequately justified.
I just have two requests before it is accepted for publication 1. Vassilev et al., 2006. Selective small-molecule inhibitor reveals critical mitotic functions of human CDK1. Proc Natl Acad Sci U S A. 2006 Jul 11; 103(28): 10660–10665, is mentioned in the text and included in the reference list.
2. The possibility that the double band seen in the Bax IP: a note in the legend that it has been investigated, is not a phosphor event, but MS suggests could be oxidised should be added.

Reviewer 2

Advance summary and potential significance to field

The authors have comprehensively addressed all points I made from the initial review.

Comments for the author

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Second revision

Author response to reviewers' comments

Response to reviewer 1
1.Vassilev et al., 2006. Selective small-molecule inhibitor reveals critical mitotic functions of human CDK1. Proc Natl Acad Sci U S A. 2006 Jul1 1; 103(28): 10660–10665, is mentioned in the text and included in the reference list.

We have cited the Vassilev et al., (2006) reference in the text (line 198) and it is included in the reference list (line 663-665).

2.The possibility that the double band seen in the Bax IP: a note in the
legend that it has been investigated, is not a phosphor event, but MS suggests could be oxidised should be added.

We have added a note in the legend to Figure 2B to indicate that the doublet observed for the Bax IP was analysed by mass spectrometry to determine if the protein was phosphorylated. We state that Bax phosphorylation was not detected but that the protein was found to be oxidised (lines 455-457).

Third decision letter

MS ID#: JOCES/2020/244152

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AUTHORS: Omeed Darweesh, Eman Al-Shehri, Hugo Falquez, Joachin Lauterwasser, Frank Edlich, and Rajnikant R Patel
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.