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An ATM-Chk2-INCENP pathway activates the abscission checkpoint

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September 10, 2020

Re: JCB manuscript #202008029

Dr. George Zachos
University of Crete
Department of Biology
Vassilika Vouton
Heraklion, Crete 70013
Greece

Dear Dr. Zachos,

Thank you for submitting your manuscript entitled "An ATM-Chk2-INCENP pathway activates the abscission checkpoint in human cells" and thank you for your patience with the peer review process. 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.

As you will see, all three reviewers are enthusiastic about the topic and the mechanism for abscission checkpoint regulation that is emerging from your work. However, all three reviewers also have some concerns that they would like to see addressed prior to publication. Experimentally, it will be important to address points #3-5 of Reviewer #1, which would include repeating key experiments after acute rather than long-term inhibition of Chk2, and also repeating key experiments using live imaging employing phase contrast microscopy to assess abscission timing.

Many of the other reviewer concerns are requests for clarification and/or additional explanation or discussion that can be addressed by additional analysis of existing data and/or changes to the text. We do think it is important, as highlighted by Reviewer #1 (points #1 & #2) to cite the prior literature to provide context for your experiments, address how your results compare to prior results in the field, and discuss/explain the reasons for significant differences. It is also essential, as highlighted by Reviewer #2, to be clear on which cell line is being used for each experiment and what concentration of inhibitors. For this, I would recommend including schematic outlines of how key experiments were performed either in the main or supplemental figures.

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, acknowledgments, and figure legends. Count does not include materials and methods, 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,

http://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.

As you may know, the typical timeframe for revisions is three to four months. However, we at JCB realize that the implementation of social distancing and shelter in place measures that limit spread of COVID-19 also pose challenges to scientific researchers. Lab closures especially are preventing scientists from conducting experiments to further their research. Therefore, JCB has waived the revision time limit. We recommend that you reach out to the editors once your lab has reopened to decide on 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 or call (212) 327-8588.

Sincerely,

Karen Oegema, PhD
Monitoring Editor, Journal of Cell Biology

Melina Casadio, PhD
Senior Scientific Editor, Journal of Cell Biology

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

Defective activation of the abscission checkpoint in mammalian cells leads either to bridge instability/binucleation or chromosome breakage, depending on the studies. This checkpoint delays abscission in response to entrapped DNA in the intercellular bridge and depends on the Aurora B kinase. The authors previously reported that Aurora B's full activation relies on Clk kinases in the context of the abscission checkpoint. However, it is unclear how DNA bridges are detected as well as the pathway that relays chromatin bridges to Aurora B activation. Here, Petsalaki and Zachos addressed this issue and proposed a multi-step pathway that goes from DNA bridge detection to

Aurora B activation.

There is a huge amount of work (rather amazing for a single experimentalist), most of the data being convincing. The rescue by targeting INCENP (wt of mutant) to the midbody bulge is particularly elegant. In my opinion, this manuscript is very interesting and a strong candidate for publication in JCB. Nevertheless, additional experiments should be carried out to clarify a number of points.

Major points

1- Previous literature regarding the role of Chk2 in cytokinesis (PMID 25904336) should be cited and discussed. The bottom line of this previous paper is that Chk1 but not Chk2 is involved in the abscission checkpoint. In particular, Fig. 5 in PMID 25904336 argues that neither the inhibition of ATM or Chk2 accelerates abscission in HeLa cells in absence of stress, based on live cell imaging. This is the opposite in the present manuscript. The reason of this discrepancy should be carefully addressed. Is it due to the assay (see also below about imaging quality)? A difference in the cell type? Or something else?

2- The abscission checkpoint is a complex and a relatively new field that is far from being understood. The first sentence of the abstract does not reflect the results obtained by others. It is actually in contradiction with the founding abscission checkpoint paper in mammals (Steigemann et al. 2009) but also with other papers that could be cited (e.g. PMID 24814515, 26929449 and 32029597). In these studies, inactivating the checkpoint leads to late furrow regression and binucleation, not premature abscission and chromosome breakage. This could be due to the exact cell type used in the different studies or the component of the checkpoint that has been inactivated. However, in Fig. 5D-E, the authors used the HeLa cell line previously characterized by Steigemann et al. 2009 and argue in the manuscript that Chk2 inhibition inhibits Aurora B activation. Thus, I would have expected to see binucleated cells and not premature abscission after acute Chk2 or ATM inhibition. Did the authors observe binucleation? Any differences with previous work should be clearly discussed in the discussion. In addition, can the authors really show using LAP-GFP cell lines from Steigemann et al. and live cell imaging a breakage of the chromatin after acute Aurora B, ATM and Chk2 inhibition?

3- Chk2 has been previously involved in the spindle checkpoint. Most of the experiments in this manuscript have been carried out after long term depletion of Chk2 by RNAi. Thus, the authors cannot formally exclude that the observed results are an indirect consequence of the spindle checkpoint inhibition. The authors should reproduce the key results of this study (e.g. Fig; 2G, Fig. 2K, Fig. 5C, Fig. 5F, Fig. 5I/J) by acutely inhibiting Chk2 in cells synchronized after mitotic exit. It could be also reassuring to show that inhibiting the spindle checkpoint by alternative means does not lead to defective activation of the abscission checkpoint.

4- How could the authors obtain late cytokinetic bridges after MKLP2, INCENP or Aurora B depletion? These experimental conditions indeed lead either to furrow ingression defects or bridge instability, and result in binucleation. Did the authors do partial depletions? And how did binucleation influence the results obtained on fixed cells? For instance, it is argued that INCENP RNAi accelerates abscission based on fixed cells (Fig. 1c and page 4 "indicating premature abscission"). Without live cell imaging, this is not a convincing conclusion. Indeed, bridge regression after Aurora B/INCENP/MKLP2 depletion will induce a loss of midbody stage in fixed cells, but this is not due to premature abscission.

The authors should first provide the % of binucleated cells each time they used siRNAs. They should also confirm their main conclusions regarding abscission timing after RNAi (including swapping wt by S91A) by live cell imaging using phase contrast.

5- The Mre11 results are particularly exciting since it could help to resolve the long-standing question of the sensing of the chromatin bridges. In order to back up the proposed model, the authors should clarify the following points: What is the exact % (or intensity) of Mre11-positive midbodies in the presence and in the absence of LAP2-positive bridges? Is the pAuroraB intensity diminished in chromatin bridges after Mre11 depletion? Furthermore, it would be very informative to investigate whether the DNA binding site of Mre11 is required for its localization at the midbody, and discuss whether Mre11 is the sensor of chromatin bridges? Perhaps DNA bridges are stretched and nuclear rupture leads to endonuclease entry and double strand breaks that direct the

minor points

1- Liu et al (PMID 31189537) mentioned cytokinetic defects after ATM inhibition. How does this relate to the current manuscript?

2- Could they authors explain or at least discuss why inhibiting Chk2 inhibits MKLP2 recruitment at the midbody center (Fig. S4K/M)? This result seems at odd with the proposed model.

3- The quality of the live cell imaging is rather poor. Contrary to PMID 25904336, PMID 19203582 or others, the microtubule cut is not clearly seen. On the contrary, it seems that there is a gradual disappearance of the tubulin signal, perhaps due to bleaching (see the loss of cytoplasmic signal in Fig. 1A). This should be ruled out. Furthermore, this assay is not an actual measure of abscission. Phase contrast movies are much more convincing (Fig. 5D). The key experiment in Fig. 4B (acute ATM inhibition) should be confirmed using live cell imaging and phase contrast.

3- In the second part of the manuscript (presence of chromatin bridges), most of the data is based on DAPI staining and fixed images. Could the authors provide the % of LAP2-positive bridges that are DNA (DAPI)-negative? If it is different from 0, how does it influence the results in the first part of the manuscript (absence of chromatin bridges)? Quantification in Fig. 5G, 5H etc. would actually be more convincing if the authors had used LAP2 as a DNA bridge marker.

4- Specificity of the antibody staining should be confirmed by RNAi in Fig. 4A and S3J

5- The authors should further explain in the "Quantification of fluorescence signal" section what is exactly the normalization that they mentioned and that likely explains why all the relative means equal 1 in control conditions. The authors should also clearly explain how they quantified the "relative midbody center intensity". Is it the ratio between the signal at the midbody center and the signal at the arms? Or the ratio between the signal at the midbody center and the signal outside the intercellular bridge? The authors should rather provide the real ratio between the center:arms signals, which is clearly different from 1 in the provided control pictures, and that would be much more informative.

6- Related to the previous point but might be irrelevant depending on the clarification above: many conclusions are based on quantifications of "relative midbody center intensity". Is it really what matters for activating the checkpoint? What about the absolute level at the midbody center? For instance, a condition that strongly decreases the staining both at the midbody arms and at the center in the same proportion would make the relative intensity look like as "unchanged" or

"normal" although it would likely disturb the checkpoint. The authors should justify and discuss why they quantified the relative intensity. They should also provide the absolute intensities in a suppl. figure for comparison.

7- It has been shown that the C-term region deleted in MKLP2 overlaps with a myosin II-binding site. Thus, this deletion potentially disrupts the interaction between MKLP2 and CEP55, as well as MKLP2 and myosin II. Would this change the conclusions? Is there a way to selectively disrupt the MKLP2/CEP55 interaction? This should be at least discussed. Furthermore, a cartoon describing the domains of MKLP2 interacting with INCENP, CEP55, Myosin II etc might be useful.

8- Results in Fig. S2D-E and S8G are key results. The authors should consider to transfer them in the main figures (and perhaps transfer less important data to suppl. Figures). In addition, there is a huge amount of data [thus despite hours spent on reading this manuscript, it is possible that some of the experiments requested above have actually been carried out!]. But it is really perturbing that some of the Figures/Suppl. Figures are not cited in the chronological order. As it would greatly facilitate the reading, the authors should consider to rearrange some of the figures.

9- In Fig. 1E (absence of chromatin bridges), INCENP localizes both to the MB center and the MB arms. In contrast, in Fig. 6E (presence of chromatin bridges), INCENP localizes only at the MB center. Is it the same for MKLP2? Could the authors discuss this important finding?

10- It should be clearly stated in the Figure legends (or in the Mat and Meth) which cells (HeLa vs BE) have been used for each panel.

11- Hu et al. 2012 described aa 456-961 (not 456-858, p5) as a MB targeting domain. Is it a typo? Alternatively, was there a reason to use 456-858?

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

In this paper, the authors investigate the mechanism through which the abscission checkpoint is activated in mammalian cells. A proper regulation of abscission timing prevents chromosome breakage that can arise from chromosome bridges present in the intracellular canal. The authors find that ATM or CHK2 inhibition alters abscission timing by preventing the phosphorylation of INCENP on late midbodies. Mechanistically, Chk2 phosphorylates INCENP on Ser91 which creates a docking site with the Mklp2 kinesin and mediates the association with the Flemming body. Altering abscission timing, through ATM and CHK2 depletion/inhibition, leads to chromatin bridges breakage and reduces cell viability. Importantly, in absence of chromatin bridges, the ATM-dependent canonical CHK2 activation does not require the MRN complex at midbodies, shedding lights on novel avenues for ATM signaling activation during mitosis/cytokinesis. Ultimately, this paper uncovers a pathway for regulation of the abscission checkpoint by regulating CPC localization to the midbody through ATM-Chk2-INCENP.

Overall, this is a provocative paper that should have an important impact in the field. The data are of high quality, with elegant microscopy and cell biology. The conclusions are interesting and reveal an intriguing non-canonical role for ATM and CHK2 in the abscission checkpoint. Since the results are quite provocative, some of the more unexpected claims, such as MRN-independent activation of ATM in the midbody center, would benefit from a defined molecular mechanism, which is currently missing from the paper. But I agree that addressing this point is probably beyond the scope of this

paper, which is already massive in data. While I don't think major additional experiments are required, the manuscript could still be significantly improved on its presentation, especially in the text, which is extremely dense, and constantly proceeds at a frenetic pace without properly situating the reader on some key background information and important experimental details. In addition, the discussion is very short, and given that several exciting findings are being presented, it would be useful to have a longer discussion where the authors can elaborate on potential models, implications and future directions. For example:

1. Concerning ATM activation, the proposed model of ATM activation in "normally segregating cells" is intriguing and warrants further discussion. The authors state that ATM activation is independent of the MRN complex and dependent on Aurora B activity when chromatin bridges are not present. The authors don't really discuss how they envision that ATM could be recruited to the midbody in the absence of the MRN complex. Also, there is no discussion on how the MRN complex would be recruited and required for ATM activation in the presence of chromatin bridges.

2. CHK2 is not an essential gene, and chk2 knockout mice are close to normal. Can the authors explain why a protein important for abscission checkpoint may not be important during development?

3.Moreover, in page 7 the authors mention that the role of CHK2 in the abscission checkpoint is important for cell proliferation, but cells lacking CHK2 or with inhibited CHK2 don't display any proliferation defects. This author's statement seems inconsistent.
4. Figure legends lack important experimental information such as what cell line was used in each experiment and concentrations of inhibitors, etc. This information needs to be included in the revised version of the manuscript.

Additional Points
• Figure 1: please show western blots of CHK2 depletion.
• Figure 2B: it would be nice to include a control with the CHK2 inhibitor to make sure the signal is not due to contaminating kinases.
• Figure 2E-G: it would be nice to include a validation of INCENP-S91 phospho-antibody specificity. The authors could IP INCENP wt and INCENP-S91A and blot with the home raised antibody.
• Figure S3K-L: the data that CHK2 depletion does not affect INCENP-S91 phosphorylation on early midbodies is confusing and worrisome. It is true that kinase promiscuity is a frequent event, but it is just confusing why two different kinases should target the same INCENP site in early and late midbodies. Is this implying that this phospho-site is undergoing rapid/dynamic phospho-cycling with the involvement of a phosphatase?
• Figure 2H-K: please show western blots of INCENP depletion and complementation and CHK2 depletion.

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

Aurora B, the enzymatic component of the Chromosomal Passenger Complex (CPC) regulates the abscission checkpoint to prevent DNA damage during cytokinesis in the presence of a DNA bridge. Aurora B controls the recruitment of the ESCRT-III protein Chmp4C to the midbody center, which inhibits/delays abscission. Here, Petsalaki and Zachos identify the upstream signaling pathway that regulates recruitment of Aurora B to the midbody center and thereby the abscission checkpoint. The authors show that the DNA damage response kinases ATM and Chk2 control recruitment of

the CPC to the midbody center. ATM, at the midbody center, phosphorylates and activates Chk2, which in turn phosphorylates the CPC subunit INCENP on S91. INCENP-pS91 mediates the interaction of INCENP with Mklp2, resulting in the interdependent recruitment toCep55 at the midbody center where the abscission checkpoint is regulated. Strikingly, in the presence of a chromatin bridge, this pathway is now initiated by the MRN complex, presumably delivered by the bridge, which activates ATM and the downstream cascade to delay abscission and prevent chromatin bridge breakage.

In general, the experiments appear technically sound and the findings are of interest, highlighting how the activity of several DNA damage response kinases span multiple cell cycle stages to ensure cells maintain a stable genome. I have only a few minor comments, mainly regarding how midbodies may differ in the presence of chromatin bridges (points 4-5).

Main points:

1) Aurora B mediates ATM activity, governing Chk2 phosphorylation and thus INCENP phosphorylation and consequently CPC-Mklp2 recruitment to the Flemming body. Could this perhaps explain the interdependence of Mklp2 and CPC recruitment to the Flemming body? Does the interdependence CPC-Mklp2 dependent on CPC activity or protein? Alternatively, it is worth mentioning that Adriaans et al. 2020 have shown that the binding of the CPC to Mklp2 influences Mklp2 processivity on microtubules, which could contribute to their interdependence.
2) Since the CPC and Mklp2 are interdependent for their localization to the midbody, can the authors exclude a role of Mklp2 in any of the processes described, beyond targeting of the CPC to the midbody? Importantly, is Mklp2 absent from midbodies upon expression of GFP:INCENP(FB)?
3) Page 10, "In contrast, Aurora B-depletion diminished localization of phosphorylated ATM-S1981 and Chk2-T68, but not total ATM, to late midbodies (Figure 4N, O and Figure S6H, K-N), suggesting Aurora B is required for ATM activation in normally segregating cells (Figure 4P, dashed arrow)." I think a comment is in line here that this data is in line with previously published data that show (active) ATM phosphorylated by AurB is found at the midbody where it colocalizes with Aurora B, including the reference to Yang et al. 2011.
4) The morphology of the midbody appears different in the presence of a chromatin bridge (based on several of the presented stainings), and so the authors use Mklp1 as a marker for a midbody structure. Does this pool of Mklp1 and the other marks found there like pATM, pChk2, INCENP, etc correspond to the "midbody center/ Flemming body" identified in normal late midbodies? This distinction is important since, for example, loss of Chk2 activity led to a specific loss of the Flemming body pool of Aurora B-pS331, causing accumulation/mislocalization on the midbody arms (see Fig. S2D) which is not apparent in the presence of a chromatin bridge.
5) It is striking that ATM activity regulated in two different ways in more or less the same process, either through Aurora B or MRN. This suggests that cytokinesis/midbody in the presence of a bridge could be different, see also point 4. Is Aurora B ever seen to accumulate on the midbody arms (adjacent to the Flemming body) in the presence of a chromatin bridge? Perhaps at early midbodies? If no pool of the CPC is formed adjacent to the Flemming body in the presence of a bridge perhaps that is why another signal is required to initiate/control Flemming body recruitment of the abscission checkpoint machinery such as the described MRN complex. This would also argue that the Aurora B-ATM-Chk2-INCENP-Chmp4C pathway is not so much a checkpoint but more of an abscission timer (as it does not function in the presence of an actual bridge), whereas the MRN-ATM-Chk2-INCENP-Chmp4C pathway, activated in the presence of a bridge, constitutes the actual checkpoint. I think this requires more thorough discussion.

JCB manuscript #202008029

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

Defective activation of the abscission checkpoint in mammalian cells leads either to bridge instability/binucleation or chromosome breakage, depending on the studies. This checkpoint delays abscission in response to entrapped DNA in the intercellular bridge and depends on the Aurora B kinase. The authors previously reported that Aurora B's full activation relies on Clk kinases in the context of the abscission checkpoint. However, it is unclear how DNA bridges are detected as well as the pathway that relays chromatin bridges to Aurora B activation. Here, Petsalaki and Zachos addressed this issue and proposed a multi-step pathway that goes from DNA bridge detection to Aurora B activation.

There is a huge amount of work (rather amazing for a single experimentalist), most of the data being convincing. The rescue by targeting INCENP (wt of mutant) to the midbody bulge is particularly elegant. In my opinion, this manuscript is very interesting and a strong candidate for publication in JCB. Nevertheless, additional experiments should be carried out to clarify a number of points.

Major points

1- Previous literature regarding the role of Chk2 in cytokinesis (PMID 25904336) should be cited and discussed. The bottom line of this previous paper is that Chk1 but not Chk2 is involved in the abscission checkpoint. In particular, Fig. 5 in PMID 25904336 argues that neither the inhibition of ATM or Chk2 accelerates abscission in HeLa cells in absence of stress, based on live cell imaging. This is the opposite in the present manuscript. The reason of this discrepancy should be carefully addressed. Is it due to the assay (see also below about imaging quality)? A difference in the cell type? Or something else?

Inhibition of ATM or Chk2 did not accelerate midbody resolution in the absence of stress in a previous study, based on live cell imaging of a HeLa cell line (Mackay and Ullman, 2015). This appears to be at odds with findings from our study showing that ATM or Chk2-inhibition, or expression of a non phosphorylatable S91A INCENP accelerates abscission compared with control cells, as judged by timing of midbody disassembly and cleavage of the intercellular canal in HeLa and BE cells by time-lapse microscopy, and by midbody index analysis of fixed cells. Although the reason for the difference between the results of Mackay and Ullman (2015) and our study is unknown, it could be due to genomic differences in the cell lines used resulting in varying levels of ATM or Chk2-inhibition (Liu et al., 2019). This is now discussed in paragraph 2, page 16 of the revised manuscript.

2- The abscission checkpoint is a complex and a relatively new field that is far from being understood. The first sentence of the abstract does not reflect the results obtained by others. It is actually in contradiction with the founding abscission checkpoint paper in mammals (Steigemann et al. 2009) but also with other papers that could be cited (e.g. PMID 24814515, 26929449 and 32029597). In these studies, inactivating the checkpoint leads to late furrow regression and binucleation, not premature abscission and chromosome breakage. This could be due to the exact cell type used in the different studies or the component of the checkpoint that has been inactivated. However, in Fig. 5D-E, the authors used the HeLa cell line previously characterized by Steigemann et al. 2009 and argue in the manuscript that Chk2 inhibition inhibits Aurora B activation. Thus, I would have expected to see binucleated cells and not premature abscission after acute Chk2 or ATM inhibition. Did the authors observe binucleation? Any differences with previous work should be clearly discussed in the discussion. In addition, can the authors really show using LAP-GFP cell lines from Steigemann et al. and live cell imaging a breakage of the chromatin after acute Aurora B, ATM and Chk2 inhibition?

The first sentence of the abstract now reads: “In response to chromatin bridges, the Chromosomal Passenger Complex (CPC) delays abscission to prevent chromosome

breakage or tetraploidization", to reflect that abscission checkpoint-failure can also lead to binucleation (page 2 of the revised manuscript). Furthermore, the papers by Steigemann et al 2009, Thorensen et al 2014, and Bai et al 2020 are cited in paragraph 1, page 3 of the Introduction and elsewhere, as suggested by the reviewer.

We did not observe binucleation in HeLa LAP2b:RFP cells after ATM or Chk2-inhibition. It is should perhaps be noted that Steigemann *et al* had observed binucleation instead of chromatin breakage after Aurora B-inhibition by chemical inhibitors using the same HeLa LAP2b:RFP cell line (Steigemann et al., 2009). Because Chk2 regulates CPC localization to the midbody centre in late cytokinesis, but not in early midbodies, one possibility is that potent Aurora B inhibition at relatively early midbodies with chromatin bridges in the study by Steigemann *et al* destabilizes the anchoring of the plasma membrane to the midbody leading to binucleation, whereas impaired CPC localization to the midbody remnant in late midbodies after Chk2-inhibition in our study leads to premature abscission and chromatin breakage. Better understanding of the midbody morphology in cytokinesis with chromatin bridges is required to address these possibilities. This is now discussed in paragraph 4, page 15 and in paragraph 1, page 16 of the revised manuscript.

We also confirmed our findings from monitoring breakage of the intercellular LAP2b-positive canal by showing breakage of LAP2b:RFP bridges after ATM or Chk2-inhibition by live-cell fluorescence microscopy, as requested by the reviewer (Fig. S4A-C; Videos 8 and 10; paragraph 1, page 12 and paragraph 1 page 13 of the revised manuscript).

3- Chk2 has been previously involved in the spindle checkpoint. Most of the experiments in this manuscript have been carried out after long term depletion of Chk2 by RNAi. Thus, the authors cannot formally exclude that the observed results are an indirect consequence of the spindle checkpoint inhibition. The authors should reproduce the key results of this study (e.g. Fig; 2G, Fig. 2K, Fig. 5C, Fig. 5F, Fig. 5I/J) by acutely inhibiting Chk2 in cells synchronized after mitotic exit. It could be also reassuring to show that inhibiting the spindle checkpoint by alternative means does not lead to defective activation of the

abscission checkpoint.

We reproduced the results showing impaired localization of phospho-INCENP-S91 at the midbody centre, reduced frequency of cells at the midbody stage after expression of S91A INCENP and rescue of the midbody index in Chk2-deficient cells after expression of S91D, but not WT, INCENP by acutely inhibiting Chk2 in cells synchronized after mitotic exit, as requested by the reviewer. These data are shown in Fig. 3L and Fig. S3A; paragraph 2, page 7 and paragraph 2, page 8 of the revised manuscript. Because chromatin bridges can last for 24 h in control cells (perhaps even more) as judged by live-cell microscopy in HeLa cells (our unpublished results) we did not synchronize cells after mitotic exit for studying chromatin bridges, as we felt we wouldn't be able to distinguish between chromatin bridges arising after synchronization or pre-existing ones. Instead, we treated cells with chromatin bridges in cytokinesis with Chk2 inhibitor II immediately before filming and monitored them by live-cell microscopy (Fig. 8E, F and Fig. S4A, C).

Also, inhibition of the spindle checkpoint kinase Mps1 by 10 μM AZ3146 that fully inhibits Mps1 catalytic activity did not induce broken chromatin bridges, suggesting inhibition of the mitotic spindle checkpoint does not lead to defective activation of the abscission checkpoint (Fig. 8B; paragraph 3, page 11 of the revised manuscript).

4- How could the authors obtain late cytokinetic bridges after MKLP2, INCENP or Aurora B depletion? These experimental conditions indeed lead either to furrow ingression defects or bridge instability, and result in binucleation. Did the authors do partial depletions? And how did binucleation influence the results obtained on fixed cells? For instance, it is argued that INCENP RNAi accelerates abscission based on fixed cells (Fig. 1c and page 4 "indicating premature abscission"). Without live cell imaging, this is not a convincing conclusion. Indeed, bridge regression after Aurora B/INCENP/MKLP2 depletion will induce a loss of midbody stage in fixed cells, but this is not due to premature abscission. The authors should first provide the % of binucleated cells each time they used siRNAs. They should also confirm their main conclusions regarding abscission

timing after RNAi (including swapping wt by S91A) by live cell imaging using phase contrast.

The percentage of binucleated cells each time we use siRNAs and measure mitotic indices in fixed cells is now provided as requested (Fig. S1E, and S1N; Fig. S2P; Fig. S3Q; and Fig. 6H). We did not observe an increase in binucleation after protein-depletion by siRNAs compared with controls, perhaps because the remaining protein was sufficient for furrow ingression to be completed.

We confirmed our main conclusions regarding abscission timing by live-cell phase contrast microscopy as requested by the reviewer: HeLa tubulin:GFP cells treated with Chk2 inhibitor II or with the ATM-inhibitor KU-55933 in cytokinesis exhibited faster cleavage of the intercellular canal compared with controls by phase contrast live-cell imaging, indicating premature abscission (Fig. S1A, B; Fig. S3N, O; Videos 3-5; paragraph 1, page 5 and paragraph 2, page 10 of the revised manuscript). Furthermore, expression of S91A V5/His-INCENP accelerated cleavage of the intercellular canal compared with the WT V5/His-INCENP in cytokinesis by time-lapse microscopy (Fig. S2N, O; paragraph 2, page 8).

5- The Mre11 results are particularly exciting since it could help to resolve the long-standing question of the sensing of the chromatin bridges. In order to back up the proposed model, the authors should clarify the following points: What is the exact % (or intensity) of Mre11-positive midbodies in the presence and in the absence of LAP2-positive bridges? Is the pAuroraB intensity diminished in chromatin bridges after Mre11 depletion? Furthermore, it would be very informative to investigate whether the DNA binding site of Mre11 is required for its localization at the midbody, and discuss whether Mre11 is the sensor of chromatin bridges? Perhaps DNA bridges are stretched and nuclear rupture leads to endonuclease entry and double strand breaks that direct the

The intensity of Mre11 at the midbody in control cells with chromatin bridges compared with normally segregating cells is now shown in Fig. 10G; paragraph 2, page 13 of the revised manuscript.

Depletion of Mre11 reduced localization of phosphorylated Aurora B-S331 at the midbody compared with control cells (Fig. S5C, D; paragraph 2, page 13).

The mechanism by which the MRN recognizes chromatin bridges in cytokinesis is incompletely understood. One possibility is that nuclear rupture leads to endonuclease entry and generation of ds DNA breaks that are recognized by the MRN complex, as suggested by the reviewer. However, the whole purpose of the abscission checkpoint is to avoid chromatin breakage in control cells; furthermore, we don't detect any γ-H2AX staining in control bridges and Mre11-nuclease activity is dispensable for the checkpoint. We therefore favor an alternative theory:

It was previously reported that continuous immobilization of MRN components to chromatin can activate the ATM-Chk2 signaling pathway in the absence of damaged DNA (Soutoglou and Misteli, 2008). Therefore, one possibility is that chromatin remodeling enzymes acting to resolve the intertwined DNA strands can promote stable binding of the MRN complex to the chromatin bridge and subsequent ATM activation at the midbody (Chan et al, 2007). We believe a more thorough investigation of this mechanism is perhaps better suited for a separate paper in the future, but discuss some of the above possibilities in paragraph 2, page 18 of the revised manuscript.

minor points

1- Liu et al (PMID 31189537) mentioned cytokinetic defects after ATM inhibition. How does this relate to the current manuscript?

Liu *et al* (PMID 31189537) proposed that, in response to radiation-induced DNA damage, ATM activity on the DNA must be switched-off after DNA repair to avoid

cytokinesis-failure, binucleation and cellular senescence. We took into consideration the reviewers' suggestion and included the above findings in our papers' Discussion:

Although unchecked ATM activity on the DNA after DNA repair may lead to cytokinesis-defects (Liu et al, 2019), our results show that localized ATM activity on the midbody is required for proper abscission-timing in the absence of DNA damage (paragraph 3, page 16).

2- Could they authors explain or at least discuss why inhibiting Chk2 inhibits MKLP2 recruitment at the midbody center (Fig. S4K/M)? This result seems at odd with the proposed model.

Inhibition of Chk2 impairs CPC or Mklp2 localization inside the Flemming body in late midbodies; furthermore, expression of non-phosphorylatable INCENP-S91A that does not efficiently bind to Mklp2 impairs INCENP or Mklp2 localization to the midbody arms and the midbody centre in early or late midbodies. These results are consistent with previous reports that CPC and Mklp2 mutually depend on each other for midzone/midbody localization, by Mklp2 transporting CPC along central spindle microtubules and CPC-binding promoting the microtubule processivity of Mklp2 in cytokinesis (Adriaans et al., 2020; Hummer and Mayer, 2009; Kitagawa et al., 2013; Serena et al., 2020). Of note however, INCENP-binding to Mklp2 is not required for Mklp2 interaction with the midbody protein Cep55 by GST pull-downs when proteins can freely interact in cell extracts, indicating the CPC-Mklp2 complex localizes to the midbody centre through Mklp2-binding to Cep55 as proposed in our model. This is now discussed in paragraph 3, page 14 of the revised manuscript.

3- The quality of the live cell imaging is rather poor. Contrary to PMID 25904336, PMID 19203582 or others, the microtubule cut is not clearly seen. On the contrary, it seems that there is a gradual disappearance of the tubulin signal, perhaps due to bleaching (see the loss of cytoplasmic signal in Fig. 1A). This should be ruled out. Furthermore, this assay is not an actual measure of abscission. Phase contrast movies are much more convincing (Fig. 5D). The key

experiment in Fig. 4B (acute ATM inhibition) should be confirmed using live cell imaging and phase contrast.

Phase contrast movies monitoring cleavage of the intercellular canal in HeLa cells after Chk2 or ATM-inhibition as a direct measure of abscission are now shown as requested by the reviewer (Fig. S1A; Fig. S3N; and Videos 3-5; please also see the response to reviewer 1, main point #4 above). These results are in agreement with the results obtained by monitoring the kinetics of midbody disassembly by fluorescence live-cell imaging (paragraph 1, page 5 and paragraph 2, page 10 of the revised manuscript).

3- In the second part of the manuscript (presence of chromatin bridges), most of the data is based on DAPI staining and fixed images. Could the authors provide the % of LAP2-positive bridges that are DNA (DAPI)-negative? If it is different from 0, how does it influence the results in the first part of the manuscript (absence of chromatin bridges)? Quantification in Fig. 5G, 5H etc. would actually be more convincing if the authors had used LAP2 as a DNA bridge marker.

We did not observe telophase cells exhibiting LAP2b-positive bridges that were DAPI-negative in our analysis (n>300; please also see Fig. 8G).

4- Specificity of the antibody staining should be confirmed by RNAi in Fig. 4A and S3J

Depletion of ATM by siRNA abolished phospho-ATM-S1981 staining by immunofluorescence, confirming the antibody is specific (Fig. S3M; paragraph 2, page 10). Also, depletion of Chk2 by siRNA abolished phospho-Chk2-T68 and phospho-Chk2-T383 staining, indicating the antibodies were specific (Fig. S2G, H; paragraph 1, page 7).

5- The authors should further explain in the "Quantification of fluorescence signal" section what is exactly the normalization that they mentioned and that likely explains why all the relative means equal 1 in control conditions. The authors should also clearly explain how they quantified the "relative midbody center intensity". Is it the ratio between the signal at the midbody center and the signal at the arms? Or the ratio between the signal at the midbody center and the signal outside the intercellular bridge? The authors should rather provide the real ratio between the center:arms signals, which is clearly different from 1 in the provided control pictures, and that would be much more informative.

Fluorescence intensity signals at midbodies were quantified using the LASX polygon tool by analyzing an image area of 2 μm^2 around each Flemming body and intensity values were normalized versus background values obtained by analyzing an identical area within the cell immediately adjacent on the midbody, by subtracting the background-signal value from the midbody value (Petsalaki and Zachos, 2016; Waters, 2009). After subtraction of the background, the average values from control or mutant midbodies were calculated and were then all divided with the control midbodies-average value to obtain the relative midbody intensity values plotted (i.e. relative to control = 1), for comparison reasons (paragraph 2, page 25 of the revised manuscript).

6- Related to the previous point but might be irrelevant depending on the clarification above: many conclusions are based on quantifications of "relative midbody center intensity". Is it really what matters for activating the checkpoint? What about the absolute level at the midbody center? For instance, a condition that strongly decreases the staining both at the midbody arms and at the center in the same proportion would make the relative intensity look like as "unchanged" or "normal" although it would likely disturb the checkpoint. The authors should justify and discuss why they quantified the relative intensity. They should also provide the absolute intensities in a suppl. figure for

comparison.

As explained in our response to the reviewers' minor point #5 above, after subtraction of the background, the average values from control or mutant Flemming bodies were calculated and were then all divided with the control Flemming bodies-average value to obtain the relative midbody centre intensity values plotted (i.e. relative to control = 1). Therefore, the midbody-centre fluorescence intensity values plotted represent the fluorescence signal inside the Flemming body and NOT the ratio between center:arms signals.

7- It has been shown that the C-term region deleted in MKLP2 overlaps with a myosin II-binding site. Thus, this deletion potentially disrupts the interaction between MKLP2 and CEP55, as well as MKLP2 and myosin II. Would this change the conclusions? Is there a way to selectively disrupt the MKLP2/CEP55 interaction? This should be at least discussed. Furthermore, a cartoon describing the domains of MKLP2 interacting with INCENP, CEP55, Myosin II etc might be useful.

Deletion of human Mklp2 (amino acids 800-890) C-terminal region diminishes the Mklp2-Cep55 interaction in cell extracts and *in vitro* and impairs INCENP-localization to the midbody centre. Because Mklp2 (amino acids 800-890) overlaps with a myosin-II binding site (Fig. S3J; Kitagawa et al., 2013), a potential role for myosin-II in stabilizing the Mklp2-Cep55 interaction in cell extracts cannot be formally excluded and requires further investigation. This is now discussed in paragraph 3, page 14 of the revised manuscript. Also, a cartoon describing the amino acid regions of Mklp2 interacting with INCENP, Cep55 and myosin-II is now shown in Fig. S3J as requested.

8- Results in Fig. S2D-E and S8G are key results. The authors should consider to transfer them in the main figures (and perhaps transfer less important data to suppl. Figures). In addition, there is a huge amount of data [thus despite hours

spent on reading this manuscript, it is possible that some of the experiments requested above have actually been carried out!]. But it is really perturbing that some of the Figures/Suppl. Figures are not cited in the chronological order. As it would greatly facilitate the reading, the authors should consider to rearrange some of the figures.

Previous Fig. S2D, E showing localization of phosphorylated Aurora B-S331 at late midbodies, and previous Fig. S8G showing the frequency of broken chromatin bridges in cells expressing GFP:INCENP(FB) are now transferred in the main figures as requested (revised Fig. 2A, B and Fig. 9K, respectively). We also rearranged Figures/ Supplemental Figures so that data are largely presented in the chronological order they are cited in the text, as suggested by the reviewer.

9- In Fig. 1E (absence of chromatin bridges), INCENP localizes both to the MB center and the MB arms. In contrast, in Fig. 6E (presence of chromatin bridges), INCENP localizes only at the MB center. Is it the same for MKLP2? Could the authors discuss this important finding?

Similar to INCENP, Mklp2 localizes to both the midbody arms and the midbody centre in control cells in the absence of chromatin bridges (revised Fig. 4I), but does not exhibit localization to “midbody arms” in cytokinesis with chromatin bridges (revised Fig. S4J). This is now discussed in paragraph 3, page 17:

“The morphology of the midbody appears different in the presence of a chromatin bridge compared with “unperturbed” midbodies in normally segregating cells, based for example on CPC proteins or Mklp2 colocalizing with Mklp1 as a single dot on the midbody remnant and not exhibiting “midbody arms”-localization in control cells with chromatin bridges. One possibility is that, in cytokinesis with chromatin bridges exhibiting a relatively long and narrow intercellular canal, midbody arms-microtubules are disassembled giving rise to midbody remnants that correspond to the midbody centre/ Flemming body (Connell et al., 2009). Although a more systematic analysis of the midbody structure in the presence of chromatin bridges is required, our

results are consistent with the midbody pool of ATM, Chk2, Mklp2, or CPC proteins in the presence of a chromatin bridge corresponding to the “midbody centre/ Flemming body” identified in unperturbed late midbodies. Such differences in midbody structure may also explain why the mislocalization of the Flemming body pool of phosphorylated Aurora B-S331 or phospho-INCENP-S91 on the midbody arms after Chk2-inhibition that is observed in unperturbed late midbodies is not apparent in the presence of a chromatin bridge”.

10- It should be clearly stated in the Figure legends (or in the Mat and Meth) which cells (HeLa vs BE) have been used for each panel.

The cell line used is now clearly stated in the Figure legends for each panel, as requested.

11- Hu et al. 2012 described aa 456-961 (not 456-858, p5) as a MB targeting domain. Is it a typo? Alternatively, was there a reason to use 456-858?

The Mklp1 plasmid we used (Addgene plasmid #70154) is coding for human Mklp1 isoform 2 that is slightly shorter (858 amino acids long). We therefore PCRed up Mklp1 (456-858) for our GFP:INCENP(FB) construct. This is now mentioned in the Materials and Methods, paragraph 2, page 20 of the revised manuscript.

JCB manuscript #202008029

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

In this paper, the authors investigate the mechanism through which the abscission checkpoint is activated in mammalian cells. A proper regulation of abscission timing prevents chromosome breakage that can arise from chromosome bridges present in the intracellular canal. The authors find that ATM or CHK2 inhibition alters abscission timing by preventing the phosphorylation of INCENP on late midbodies. Mechanistically, Chk2 phosphorylates INCENP on Ser91 which creates a docking site with the Mklp2 kinesin and mediates the association with the Flemming body. Altering abscission timing, through ATM and CHK2 depletion/inhibition, leads to chromatin bridges breakage and reduces cell viability. Importantly, in absence of chromatin bridges, the ATM-dependent canonical CHK2 activation does not require the MRN complex at midbodies, shedding lights on novel avenues for ATM signaling activation during mitosis/cytokinesis. Ultimately, this paper uncovers a pathway for regulation of the abscission checkpoint by regulating CPC localization to the midbody through ATM-Chk2-INCENP.

Overall, this is a provocative paper that should have an important impact in the field. The data are of high quality, with elegant microscopy and cell biology. The conclusions are interesting and reveal an intriguing non-canonical role for ATM and CHK2 in the abscission checkpoint. Since the results are quite provocative, some of the more unexpected claims, such as MRN-independent activation of ATM in the midbody center, would benefit from a defined molecular mechanism, which is currently missing from the paper. But I agree that addressing this point is probably beyond the scope of this paper, which is already massive in data. While I don't think major additional experiments are required, the manuscript could still be significantly improved on its presentation, especially in the text, which is extremely dense, and constantly proceeds at a frenetic pace without properly situating the reader on some key background information and important experimental details. In addition, the discussion is very short, and given that several exciting findings are being presented, it would

be useful to have a longer discussion where the authors can elaborate on potential models, implications and future directions. For example:

1. Concerning ATM activation, the proposed model of ATM activation in "normally segregating cells" is intriguing and warrants further discussion. The authors state that ATM activation is independent of the MRN complex and dependent on Aurora B activity when chromatin bridges are not present. The authors don't really discuss how they envision that ATM could be recruited to the midbody in the absence of the MRN complex. Also, there is no discussion on how the MRN complex would be recruited and required for ATM activation in the presence of chromatin bridges.

A more detailed mechanism by which ATM is activated at the midbody requires further investigation. Aurora B activates ATM in mitosis through ATM-S1403-phosphorylation (Yang et al., 2011). Therefore, one possibility is that midbody proteins function as scaffold to promote or regulate the Aurora B-ATM interaction in cytokinesis in normally segregating cells. And how does the MRN activate ATM in cytokinesis with chromatin bridges? It was previously reported that continuous immobilization of MRN components to chromatin can activate the ATM-Chk2 signaling pathway in the absence of damaged DNA (Soutoglou and Misteli, 2008). One possibility is that chromatin remodeling enzymes acting to resolve the intertwined DNA strands can promote stable binding of the MRN complex to the chromatin bridge and subsequent ATM activation at the midbody (Chan et al., 2007). This is now discussed in paragraph 2, page 18 of the revised manuscript.

2. CHK2 is not an essential gene, and chk2 knockout mice are close to normal. Can the authors explain why a protein important for abscission checkpoint may not be important during development?

One possibility is that Chk2 is required for the abscission checkpoint only in somatic cells, and that the abscission checkpoint is wired differently in mouse embryonic cells. As an example, embryonic stem cells do not exhibit a Chk2-mediated G1 arrest

in response to DNA damage and Chk2 kinase is not intranuclear as in somatic cells, but is sequestered at centrosomes in embryonic stem cells (PMID:15452351). This is now discussed in paragraph 3, page 18 of the revised manuscript.

3.Moreover, in page 7 the authors mention that the role of CHK2 in the abscission checkpoint is important for cell proliferation, but cells lacking CHK2 or with inhibited CHK2 don't display any proliferation defects. This author's statement seems inconsistent.

Expression of S91A V5/His-INCENP reduces cell proliferation and increases cell death compared with WT or S91D V5/His-INCENP. Chk2-deficient cells exhibit relatively mild proliferation defects compared with cells expressing INCENP-S91A (Rainey et al., 2008); however, this could be due to Chk2 regulating S91-phosphorylation only in the late stages of cytokinesis.

4. Figure legends lack important experimental information such as what cell line was used in each experiment and concentrations of inhibitors, etc. This information needs to be included in the revised version of the manuscript.

The cell lines used and the concentrations of the inhibitors are now clearly stated in the Figure legends for each panel.

Additional Points

22; Figure 1: please show western blots of CHK2 depletion.

This is shown in Fig. S1C of the revised manuscript.

22; Figure 2B: it would be nice to include a control with the CHK2 inhibitor to make sure the signal is not due to contaminating kinases.

This control is now included in Fig. 3D:

Recombinant Chk2 phosphorylated human GST-INCENP (1-120 amino acids) compared with other GST-INCENP fragments or GST-alone *in vitro* and this phosphorylation was diminished when Chk2 inhibitor II was included in the kinase reaction (Fig. 3C, D; paragraph 1, page 7 of the revised manuscript).

22; Figure 2E-G: it would be nice to include a validation of INCENP-S91 phospho-antibody specificity. The authors could IP INCENP wt and INCENP-S91A and blot with the home raised antibody.

Unfortunately, our phospho-specific INCENP-S91 antibody does not work in western blots. Perhaps it recognizes a conformational (native) epitope. However, we have validated this antibody in immunofluorescence:

Phosphorylated-S91 staining was impaired after incubation of the anti-pINCENP-S91 antiserum with the phosphorylated (phospho-S91) peptide compared with the unphosphorylated (S91) synthetic peptide by immunofluorescence, showing that this reagent is specific for the phosphorylation (Fig. S2J-L). Depletion of INCENP diminished phospho-S91 staining compared with control cells (Fig. 3J, K; paragraph 2, page 7 of the revised manuscript).

22; Figure S3K-L: the data that CHK2 depletion does not affect INCENP-S91 phosphorylation on early midbodies is confusing and worrisome. It is true that kinase promiscuity is a frequent event, but it is just confusing why two different kinases should target the same INCENP site in early and late midbodies. Is this implying that this phospho-site is undergoing rapid/dynamic phospho-cycling with the involvement of a phosphatase?

Chk2-deficient cells exhibit impaired INCENP-S91 phosphorylation inside the Flemming body; however, Chk2 is dispensable for S91 phosphorylation on the midbody arms, suggesting a different kinase phosphorylates INCENP-S91 in early midbodies. Because Chk2 localizes to the midbody centre in both early and late midbodies, this division of labor likely reflects different intracellular compartments in which the S91-targeting kinases localize and/or are activated. This kinase promiscuity is perhaps reminiscent of the Aurora B-S331 phosphorylation at kinetochores by two different kinases (Chk2 and Chk1) in, respectively, early or late prometaphase (Petsalaki et al., 2011; Petsalaki and Zachos, 2014). It also implies that S91 undergoes a rapid phospho-cycling, perhaps with the involvement of a phosphatase. Recruitment of phosphatases to the midbody during abscission has been previously reported (Bhowmick et al., 2019; Fung et al., 2017) and this notion is consistent with our using of the phosphatase inhibitor microcystin during cell fixation to visualize S91 phosphorylation by immunofluorescence (please also see Materials and methods). This is now discussed in paragraph 2, page 15 of the revised manuscript.

22; Figure 2H-K: please show western blots of INCENP depletion and complementation and CHK2 depletion.

This is now shown in Fig. S2Q; paragraph 2, page 8 of the revised manuscript.

JCB manuscript #202008029

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

Aurora B, the enzymatic component of the Chromosomal Passenger Complex (CPC) regulates the abscission checkpoint to prevent DNA damage during cytokinesis in the presence of a DNA bridge. Aurora B controls the recruitment of the ESCRT-III protein Chmp4C to the midbody center, which inhibits/delays abscission. Here, Petsalaki and Zachos identify the upstream signaling pathway that regulates recruitment of Aurora B to the midbody center and thereby the abscission checkpoint. The authors show that the DNA damage response kinases ATM and Chk2 control recruitment of the CPC to the midbody center. ATM, at the midbody center, phosphorylates and activates Chk2, which in turn phosphorylates the CPC subunit INCENP on S91. INCENP-pS91 mediates the interaction of INCENP with Mklp2, resulting in the interdependent recruitment toCep55 at the midbody center where the abscission checkpoint is regulated. Strikingly, in the presence of a chromatin bridge, this pathway is now initiated by the MRN complex, presumably delivered by the bridge, which activates ATM and the downstream cascade to delay abscission and prevent chromatin bridge breakage.

In general, the experiments appear technically sound and the findings are of interest, highlighting how the activity of several DNA damage response kinases span multiple cell cycle stages to ensure cells maintain a stable genome. I have only a few minor comments, mainly regarding how midbodies may differ in the presence of chromatin bridges (points 4-5).

Main points:

1) Aurora B mediates ATM activity, governing Chk2 phosphorylation and thus INCENP phosphorylation and consequently CPC-Mklp2 recruitment to the Flemming body. Could this perhaps explain the interdependence of Mklp2 and CPC recruitment to the Flemming body? Does the interdependence CPC-Mklp2 dependent on CPC activity or protein? Alternatively, it is worth mentioning that

Adriaans et al. 2020 have shown that the binding of the CPC to Mklp2 influences Mklp2 processivity on microtubules, which could contribute to their interdependence.

Inhibition of Chk2 impairs CPC or Mklp2 localization inside the Flemming body in late midbodies; furthermore, expression of non-phosphorylatable INCENP-S91A that does not efficiently bind to Mklp2 impairs INCENP or Mklp2 localization to the midbody arms and the midbody centre in early or late midbodies. These results are consistent with previous reports that CPC and Mklp2 mutually depend on each other for midzone/midbody localization, by Mklp2 transporting CPC along central spindle microtubules and CPC-binding promoting the microtubule processivity of Mklp2 in cytokinesis (Adriaans et al., 2020; Hummer and Mayer, 2009; Kitagawa et al., 2013; Serena et al., 2020). We also show that Aurora B is required for ATM and Chk2 activation at the midbody in normally segregating cells. Our results suggest that Aurora B catalytic activity is required for the interdependent Mklp2 and CPC recruitment to the midbody (Kitagawa et al., 2013) by Aurora B governing Chk2-activation thus imposing INCENP-S91 phosphorylation at the Flemming body. These points are now discussed in paragraph 3, page 14 and paragraph 3, page 15 of the revised manuscript.

2) Since the CPC and Mklp2 are interdependent for their localization to the midbody, can the authors exclude a role of Mklp2 in any of the processes described, beyond targeting of the CPC to the midbody? Importantly, is Mklp2 absent from midbodies upon expression of GFP:INCENP(FB)?

Because the CPC and Mklp2 are interdependent for their localization to the midbody, a direct role for Mklp2 in the abscission delay, beyond targeting of the CPC to the midbody, cannot be excluded (Fung et al., 2017). However, because expression of midbody-targeted GFP:INCENP(FB) rescues the frequency of cells at midbody stage after Chk2-depletion and GFP:INCENP(FB) exhibits impaired binding to Mklp2, we believe accelerated abscission in Chk2-deficient cells can be attributed to impaired

CPC localization to the midbody centre. This is now discussed in paragraph 1, page 15 of the revised manuscript.

3) Page 10, "In contrast, Aurora B-depletion diminished localization of phosphorylated ATM-S1981 and Chk2-T68, but not total ATM, to late midbodies (Figure 4N, O and Figure S6H, K-N), suggesting Aurora B is required for ATM activation in normally segregating cells (Figure 4P, dashed arrow)." I think a comment is in line here that this data is in line with previously published data that show (active) ATM phosphorylated by AurB is found at the midbody where it colocalizes with Aurora B, including the reference to Yang et al. 2011.

We also show that Aurora B is required for ATM and Chk2 activation at the midbody in normally segregating cells. (…), these findings are in agreement with previous data showing that active ATM phosphorylated by Aurora B localizes to the midbody where it colocalizes with Aurora B (Yang et al., 2011). This comment is now included in paragraph 3, page 15 of the revised manuscript, as requested by the reviewer.

4) The morphology of the midbody appears different in the presence of a chromatin bridge (based on several of the presented stainings), and so the authors use Mklp1 as a marker for a midbody structure. Does this pool of Mklp1 and the other marks found there like pATM, pChk2, INCENP, etc correspond to the "midbody center/ Flemming body" identified in normal late midbodies? This distinction is important since, for example, loss of Chk2 activity led to a specific loss of the Flemming body pool of Aurora B-pS331, causing accumulation/mislocalization on the midbody arms (see Fig. S2D) which is not apparent in the presence of a chromatin bridge.

The morphology of the midbody appears different in the presence of a chromatin bridge compared with “unperturbed” midbodies in normally segregating cells, based for example on CPC proteins or Mklp2 colocalizing with Mklp1 as a single dot on the

midbody remnant and not exhibiting “midbody arms”-localization in control cells with chromatin bridges. One possibility is that, in cytokinesis with chromatin bridges exhibiting a relatively long and narrow intercellular canal, midbody arms-microtubules are quickly disassembled giving rise to midbody remnants that correspond to the midbody centre/ Flemming body (Connell et al., 2009). Although a more systematic analysis of the midbody structure in the presence of chromatin bridges is required, our results are consistent with the midbody pool of ATM, Chk2, Mklp2, or CPC proteins in the presence of a chromatin bridge corresponding to the “midbody centre/ Flemming body” identified in unperturbed late midbodies. Such differences in midbody structure may also explain why the mislocalization of the Flemming body pool of phosphorylated Aurora B-S331 or phospho-INCENP-S91 on the midbody arms after Chk2-inhibition that is observed in unperturbed late midbodies is not apparent in the presence of a chromatin bridge. This is now discussed in paragraph 3, page 17 of the revised manuscript.

5) It is striking that ATM activity regulated in two different ways in more or less the same process, either through Aurora B or MRN. This suggests that cytokinesis/midbody in the presence of a bridge could be different, see also point 4. Is Aurora B ever seen to accumulate on the midbody arms (adjacent to the Flemming body) in the presence of a chromatin bridge? Perhaps at early midbodies? If no pool of the CPC is formed adjacent to the Flemming body in the presence of a bridge perhaps that is why another signal is required to initiate/control Flemming body recruitment of the abscission checkpoint machinery such as the described MRN complex. This would also argue that the Aurora B-ATM-Chk2-INCENP-Chmp4C pathway is not so much a checkpoint but more of an abscission timer (as it does not function in the presence of an actual bridge), whereas the MRN-ATM-Chk2-INCENP-Chmp4C pathway, activated in the presence of a bridge, constitutes the actual checkpoint. I think this requires more thorough discussion.

It is perhaps striking that ATM can be activated in two different ways at the midbody, by the MRN complex or Aurora B kinase, depending on whether there is a chromatin

bridge inside the intercellular canal or not. Because there is no pool of “midbody arms”-CPC adjacent to the midbody remnant in the presence of a chromatin bridge as discussed in the previous paragraph, a different signal (generated by the MRN complex) may be required to control recruitment of the checkpoint machinery to the Flemming body compared with the unperturbed midbody. Our results argue that the Aurora B-ATM-Chk2-INCENP-Chmp4c pathway functions as an abscission timer (as it does not function in the presence of a chromatin bridge), whereas the MRN-ATM-Chk2-INCENP-Chmp4c pathway, activated in the presence of a bridge, generates a robust abscission-delay signal and constitutes the actual checkpoint. This is now discussed in paragraph 4, page 17 and paragraph 1, page 18 of the revised manuscript.

November 18, 2020

RE: JCB Manuscript #202008029R

Dr. George Zachos
University of Crete
Department of Biology
Vassilika Vouton
Heraklion, Crete 70013
Greece

Dear Dr. Zachos,

Thank you for submitting your revised manuscript entitled "An ATM-Chk2-INCENP pathway activates the abscission checkpoint in human cells". As you will see, your revised manuscript has been read by two of the three original reviewers, who appreciate the effort put into the revision and are now highly supportive of publication. Reviewer #3 offers a few additional minor points that you can address in your final submission. In light of their very positive comments, we would be happy to publish your paper in JCB pending these changes and 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.

1) Title: Please consider the following revision suggestions aimed at increasing the accessibility of the work for a broad audience and non-experts.

Title: An ATM-Chk2-INCENP pathway activates the abscission checkpoint
(We can emphasize that the work is done in human cultured cell lines in the abstract instead)

Abstract: (word count is OK - we can help edit the abstract in the system if you encounter an issue)
During cell division, in response to chromatin bridges, the Chromosomal Passenger Complex (CPC) delays abscission to prevent chromosome breakage or tetraploidization. Here, we show that inhibition of ATM or Chk2 kinases impairs CPC-localization to the midbody centre, accelerates midbody resolution in normally segregating cells, and correlates with premature abscission and chromatin breakage in cytokinesis with trapped chromatin. In cultured human cells, ATM activates Chk2 at late midbodies. In turn, Chk2 phosphorylates human INCENP-S91 to promote INCENP-binding to Mklp2 kinesin and CPC-localization to the midbody centre through Mklp2 association with Cep55. Expression of truncated Mklp2 that doesn't bind to Cep55 or non-phosphorylatable INCENP-S91A impairs CPC midbody-localization and accelerates abscission. In contrast, expression of phosphomimetic INCENP-S91D or a chimeric INCENP protein that is targeted to the midbody centre rescues the abscission-delay in Chk2-deficient or ATM-deficient cells. Furthermore, the Mre11-Rad50-Nbs1 complex is required for ATM activation at the midbody in cytokinesis with chromatin bridges. These results identify an ATMChk2-INCENP pathway that imposes the abscission checkpoint by regulating CPCmidbody localization.

2) Figure formatting: Scale bars must be present on all microscopy images, including inset

magnifications.
- Please add scale bars to 1E (magnifications and individual image planes), 1GIKM (Mags), 1ACDEFJ (mags), 3AGHJLN (mags), 4F, 4ABI (mags), 5BCE (mags), 6ABEFIKMO (mags), 7AEFJK (mags), 8JKL (mags), 9ACDEILMNO (mags), 10ABFHI (mags), S1GIK (mags), S2ACDGHJK (mags), S3CGM (mags), S4FGIJLMQ (mags), S5ACFHLKMNOP (mags)
Molecular weight or nucleic acid size markers must be included on all gel electrophoresis.
- Please make sure unit labels are present on all blots.

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. 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 indicate n/sample size/how many experiments the data are representative of: 8D, S1N

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 in the text for readers who may not have access to referenced manuscripts.
- 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*
- Please include species and source for all antibodies, including secondary, as well as catalog numbers/vendor identifiers if available.
- Sequences should be provided for all oligos: primers, si/shRNA, gRNAs, etc.
- 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.).

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.**

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).

-- An editable version of the final text (.DOC or .DOCX) is needed for copyediting (no PDFs).

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Please contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

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

Sincerely,

Karen Oegema, PhD
Monitoring Editor, Journal of Cell Biology

Melina Casadio, PhD
Senior Scientific Editor, Journal of Cell Biology

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

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Minor points

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Figure 5K: The + and - above the Western blot don't appear to be correct.

JCB manuscript #202008029RR

Editorial Comments

Dear Dr. Zachos,

Thank you for submitting your revised manuscript entitled "An ATM-Chk2-INCENP pathway activates the abscission checkpoint in human cells". As you will see, your revised manuscript has been read by two of the three original reviewers, who appreciate the effort put into the revision and are now highly supportive of publication. Reviewer #3 offers a few additional minor points that you can address in your final submission. In light of their very positive comments, we would be happy to publish your paper in JCB pending these changes and 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.

1) Title: Please consider the following revision suggestions aimed at increasing the accessibility of the work for a broad audience and non-experts.

Title: An ATM-Chk2-INCENP pathway activates the abscission checkpoint (We can emphasize that the work is done in human cultured cell lines in the abstract instead)

The manuscript title has been changed as suggested by the editors.

Abstract: (word count is OK - we can help edit the abstract in the system if you encounter an issue)

During cell division, in response to chromatin bridges, the Chromosomal Passenger Complex (CPC) delays abscission to prevent chromosome breakage or tetraploidization. Here, we show that inhibition of ATM or Chk2 kinases impairs CPC-localization to the midbody centre, accelerates midbody resolution in normally segregating cells, and correlates with premature abscission and chromatin breakage in cytokinesis with trapped chromatin. In cultured human cells, ATM activates Chk2 at late midbodies. In turn, Chk2 phosphorylates human INCENP-S91 to promote INCENP-binding to Mklp2 kinesin and CPC-localization to the midbody centre through Mklp2 association with Cep55. Expression of truncated Mklp2 that doesn't bind to Cep55 or non-phosphorylatable INCENP-S91A impairs CPC midbody-localization and accelerates abscission. In contrast, expression of phosphomimetic INCENP-S91D or a chimeric INCENP protein that is targeted to the midbody centre rescues the abscission-delay in Chk2-deficient or ATM-deficient cells. Furthermore, the Mre11-Rad50-Nbs1 complex is required for ATM activation at the midbody in cytokinesis with chromatin bridges. These results identify an ATMChk2-INCENP pathway that imposes the abscission checkpoint by regulating CPCmidbody localization.

We revised the abstract as suggested by the editors.

2) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications.
- Please add scale bars to 1E (magnifications and individual image planes), 1GIKM (Mags), 1ACDEFJ (mags), 3AGHJLN (mags), 4F, 4ABI (mags), 5BCE (mags), 6ABEFIKMO (mags), 7AEFJK (mags), 8JKL (mags), 9ACDEILMNO (mags), 10ABFHI (mags), S1GIK (mags), S2ACDGHJK (mags), S3CGM (mags), S4FGIJLMQ (mags), S5ACFHLKMNOP (mags)
Molecular weight or nucleic acid size markers must be included on all gel electrophoresis.
- Please make sure unit labels are present on all blots.

Insets show 1.6x magnification of the midbodies. Individual image planes in Fig. 1E show 3.5x magnification of the midbodies. This information has been added in Figure legends and Supplementary Figure legends as appropriate.

Unit labels (kD) have also been included in all blots, as requested.

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. 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 indicate n/sample size/how many experiments the data are representative of: 8D, S1N

Figure 8D: Mean ± s.d. from three independent experiments (n>150).

Figure S1N: Mean ± s.d. from three independent experiments (n>90).

This information has been included in the Figure legends, as requested.

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 in the text for readers who may not have access to referenced manuscripts.
- 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*

Human colon carcinoma BE cells are diploid cells that contain an oncogenic Kras-G13D mutation as well as the BRAF-G463V oncogenic mutation (paragraph 2, page 23 of the revised manuscript).

- Please include species and source for all antibodies, including secondary, as well as catalog numbers/vendor identifiers if available.
- Sequences should be provided for all oligos: primers, si/shRNA, gRNAs, etc.
- 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.).

Secondary antibodies are now described in paragraph 3, page 19 and paragraph 1, page 20 of the revised manuscript. Primary antibodies (page 19), siRNA sequences (page 22) and microscope image acquisition details (page 24) are also described in Materials and Methods.

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.**

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A cover image and caption was also submitted for consideration by the editors.

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GFP:INCENP(FB) binds to Aurora B in cell extracts, but does not bind to Mklp2 or Borealin compared with GFP:INCENP (Fig. S1O; paragraph 2, page 6 of the revised manuscript).

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Figure 5K has now been corrected.