Redistribution of centrosomal proteins by centromeres and Polo kinase controls partial nuclear envelope breakdown in fission yeast

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)
RE: Manuscript #E21-05-0239
TITLE: "Redistribution of centrosomal proteins by centromeres and Polo kinase controls partial nuclear envelope breakdown in fission yeast"

Dear Sue,

Thank you for transferring your manuscript from JCB. I have now carefully read your paper, the reviewers' comments and your response to the critiques. I agree with the referees that your paper contains information that should be of interest to the field and feel that you have adequately addressed the concerns that were raised in the initial reports of the experts. I am therefore pleased to accept your manuscript for publication in Molecular Biology of the Cell.

Congratulations to you and your colleagues and best wishes,
Karsten

Editor
Molecular Biology of the Cell

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Dear Dr. Jaspersen:

Congratulations on the acceptance of your manuscript.

A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at www.molbiolcell.org/toc/mboc/0/0 is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

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Sincerely,
Eric Baker
Journal Production Manager
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May 12, 2021

Dear Editors,

Please consider our manuscript, "Redistribution of centrosomal proteins by centromeres and polo kinase controls partial nuclear envelope breakdown in fission yeast", for publication in Molecular Biology of the Cell.

A long-standing question in cell biology is how a cell breaks down its nuclear envelope (NEBD) during mitosis to allow the centrosome access to the chromosomes inside the nucleus. This problem is even more complex in organisms or in developmental states where only a localized region of the NE is broken down, raising mechanistic questions as to how the NE is remodeled but also how disassembly is temporally and spatially regulated. The fission yeast Schizosaccharomyces pombe is an ideal system to investigate temporal and spatial control of partial NEBD, as NE fenestration under the spindle pole body (SPB, the yeast centrosome) is needed for entry into mitosis and bipolar spindle formation.

Using high-resolution structured-illumination microscopy, we discovered and characterized a novel ring structure that surrounds the SPB at mitotic entry and show that specific steps of its formation are connected with partial NEBD. Formation of this ring requires the conserved SUN protein, Sad1. We show that Sad1 drives ring formation and ultimately NEBD through its ability to attach to the centromere, which we demonstrate brings Polo kinase to the inner nuclear membrane to initiate partial NEBD. Interestingly, although ring assembly and partial NEBD is dependent on Polo kinase, we find it is independent of Cdk1/Cdc25 control.

A previous version of our manuscript was reviewed by The Journal of Cell Biology. We have asked that these reviews be transferred to MBoC to possibly expedite the publication of our paper. The reviewers critiqued our approaches and the overlap of our results with studies published previously across different papers. In the revised manuscript, we address these concerns in full by showing that ring formation occurs in asynchronously dividing cells grown under 'standard' conditions and through the addition of data to verify mutants have been inactivated. We have performed additional experiments suggested by the reviewers to strengthen and extend our conclusions. As indicated in the text and in the point-by-point response that follows, advances in imaging technology were key to our discovery of this novel ring structure in fission yeast. We have worked to place our results in the context of the published literature and to explain how our data extends previous observations in a significant manner through the identification of the ring and the demonstration that its assembly at the NE is centromere and Polo kinase dependent. As centromere-dependent Polo kinase recruitment to the NE by SUN proteins could be a conserved mechanism to regulate localized NEBD in other eukaryotes, we believe our findings will be of considerable interest to readers of MBoC.

Sophie Martin, Kerry Bloom or Amy Gladfelter would be suitable editors for our paper. Julie Cooper (julia.p.cooper@cuanschutz.edu), Snezhana Oliferenko (snezhka.oliferenko@crick.ac.uk), Sigurd Braun (sigurd.braun@bmc.med.lmu.de), Yasushi Hiraoka (hiraoka@fbs.osaka-u.ac.jp), Megan King (megan.king@yale.edu) or Chris McInerny (Chris.McInerny@glasgow.ac.uk) would be possible reviewers. Due to a possible
conflict of interest, we ask that the paper not be reviewed by Iain Hagan (Iain.Hagan@cruk.manchester.ac.uk).

Thank you for your consideration of our manuscript.

Sincerely,

Sue L. Jaspersen
Response to Reviewers Comments
Note, that in some cases numbers were added so that we could more easily refer to points raised by various reviewers. Our responses are in blue.

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

Bestul et al.

The study by Bestul et al exploits the cdc25.22 arrest release approach to synchronise mitotic progression and then study a number of spindle pole body and nuclear envelope components in a number of genetic backgrounds in order to study the relationship between these components, the insertion of the SPB and the formation of a ring like array of proteins around these SPBs as they insert. There is an assumption throughout the study that the Ppc89 SPB component does not redistribute during the insertion event so that the distribution of the other components relative to Ppc89 gives a read out of their distribution relative to the core SPB structure. In the second part of the study the impact of mutations in mitotic kinases upon the formation of the patterns is observed and conclusions made about the role played by polo kinase on the regulation of these events.

Based on the observations the authors describe a model for the hierarchy of the recruitment of components to the Sad1 ring structure and suggest that Polo kinase drives the insertion of the SPB from the centromeres.

While these observations are potentially of great interest to the field, the study is too preliminary to support publication in its current form. The points listed below, including a concern with the approach used to synchronise SPB insertion throughout the study are so extensive that it is not practical to support publication at this stage, although the authors should be encouraged to continue to explore the exciting potential of this biology in a future study.

The reviewer raises a number of technical concerns which we have addressed by addition experimentation, clarification of the text and integration of our data into that of the field. We provide details in the text and our response to clarify why some key differences are in fact expected based on our methodology (SIM) and how this new approach expands our understanding of SPB fenestration and its regulation that were unappreciation/unknown from studies using other techniques.

Major Concerns
1. One major concern is around the reliance upon the cdc25.22 arrest release approach. There are two issues. The first is reports of "double spindles" in around 35% of cdc25.22 cells upon release from the arrest, as judged by standard widefield microscopy (Hagan et al. Cold Spring Harb Protoc. 2016 Aug 1;2016(8). doi: 10.1101/pdb.prot091264). It is likely that this figure of 35% is an underestimate and the frequency at which four SPBs are producing
spindles upon release is likely to be much higher, in the eyes of this reviewer nearer to 100%. The obvious assumption is that the SPB duplication cycle is continuing at the cdc25.22 arrest point.

We do not know the mechanistic basis of the 35% ‘double spindles’ to which the reviewer is referring, and we have found no evidence in peer-reviewed literature that this occurs in 35%, much less 100%, of cells. However, we can say that we have looked at SPBs extensively in this arrest, including the distribution of ALL known SPB components. We do not see any evidence of SPB reduplication, such as new foci of Ppc89, Pcp1, Sid4 or any core SPB component that one would expect if the reviewer was indeed correct that the SPB duplication cycle is continuing. We have added data to Figure S1 in the revised manuscript showing Pcp1-GFP, Sid4-GFP along with Ppc89-mCherry. In previous work (Bestul et al. JCB 2017), we also used the cdc25.22 arrest with GFP-Sfi1, Sfi1-GFP and GFP-Cdc31, which are the components of the bridge structure needed to license a new round of SPB duplication. There was no evidence in any of these images to indicate SPB duplication continues. We feel strongly that this body of evidence sufficiently addresses this issue as far as it concerns the conclusions of this manuscript.

In meiotic cells, recent work suggests the spindle can form inside the nucleus by SPB-independent mechanisms so it is possible that ‘double spindles’ might form through this pathway IF it exists in mitotic cells. However, addressing this issue is beyond the scope of the current manuscript. From our data we are confident, based on the body of evidence that we do show in previous work and in the current paper, that the SPB does not reduplicate so other phenomena must be considered by the reviewer and the field.

2. The second concern here is the use of the cold shock from 36°C to 25°C to release the cells. This is highly likely to impact upon the membrane fluidity. Thus, it would be important to compare the findings of with cdc25.22 with a different way of synchronising SPB insertion to complement the cdc25.22 analysis. The wee1.as8 mutation developed by Tay et al. 2013 (Journal of Cell Science 131:5052) offers an excellent alternative. The addition of the ATP analogue to inhibit Wee1 relieves the inhibition of cell division arising from size control and so causes all of the non-dividing cells to commit to mitosis within minutes. This method avoids any heat shock and there has been no extensive cell cycle delay to support repeated rounds of SPB duplication.

We agree that changes in membrane fluidity after a temperature shift are a potential concern. However, as shown in Figure S1 of the revised manuscript, we observe the same changes in distribution for Sad1, Kms2, Cut12 and Cut11 in asynchronously grown cells that are maintained at constant temperature as we do in cdc25.22 cells released from 36°C to 25°C, ruling out changes in membrane composition during a temperature shift as a major contributor to protein redistribution. Because the rings are larger and easier to visualize in cdc25.22 cells and because it was important to synchronize cells in the cell cycle, we continued to use this approach throughout the manuscript.
Such complementary studies would enable the reader to be confident that the structures such as the cut12 rings in figure 1A are indeed rings around individual SPBs rather than four SPBs undergoing insertion and a diffusion of proteins through the use of a fairly mild form of fixation used to prepare the cells for microscopy. This is an important point to address because immunoelectron microscopy with both polyclonal antibodies to Cut12 and an epitope tag at the amino terminus of Cut12 have shown Cut12 throughout the main body of a serial sectioned interphase SPB just above the nuclear envelope. While the resolution of the Cut12 images in Figure 6 a-f of Bridge et al 1998 (Genes and Development 12:927-942) is at a higher level than the Ppc89 images in Figure 1 c of Rosenberg et al. (Molecular Biology of the Cell 17: 3793-3805), they do put Ppc89 in a distribution that is remarkably reminiscent of that of Cut12, to beg the question as to why Cut12 should be moving so much but Ppc89 is not. Thus, it is important to understand whether the SPB component Cut12 is indeed moving from a distribution throughout the core of the cytoplasmic body of the SPB to the periphery of the SPB upon mitotic commitment, or whether the images are showing four SPBs that are held near each other by their interactions with the centromeres within the nucleoplasm alongside some protein leakage from the SPB due to the light touch fixation of 4% formaldehyde in the environmental shock of 100 mM sucrose for 20 minutes.

Like the reviewer, we too were surprised that Cut12 redistributed into a ring given the results listed above. Several lines of evidence suggest that the Cut12 rings we observe are not an artifact of fixation or cell cycle arrest: 1) as shown in Figure S1, we observe Cut12 rings in asynchronously growing wild-type cells upon mitotic entry; 2) Cut12 ring formation is dependent on factors such as sad1+ (Figure 1C) and plo1+ (Figure 6B, D).

Two important considerations in terms of SIM and EM images are resolution and orientation. As shown in Figure S3B, the SPB ring diameter is less than 200 nm. Although EM has a higher spatial resolution than SIM and is easily able to resolve in x-y at this distance, a typical EM serial section is 60-90 nm. To observe a ring the SPB must be in an ‘en face’ orientation (looking down at the SPB) rather than the usual sideview (looking at SPB layers), as shown in Bridge (Cut12), Rosenberg (Ppc89), West et al. MBoC 1998, (Cut11, Figure 10), Jiang et al. Nat Meth 2020 (Sad1, Figure 4k). In a sideview, a ring protein would appear in the same plane or perhaps slightly below or above a core SPB component based on the location of the epitopes (see Bestul et al JCB 2017). Because of both orientation and the resolution from the thickness of a section, it is not surprising at all that none of these proteins, including those with membrane domains, were detected in a ring-like structure using immunoEM -- only a sideview slice directly thru the center of a large mitotic ring would show peripheral distribution of protein because of the 60-90 nm resolution of a slice.

SIM requires acquisition of multiple images over several seconds so it is critical that structures do not move during image acquisition. To achieve this, it is necessary to perform a gentle fixation. We have consistently found that the use of EM grade paraformaldehyde in the presence of an osmotic support agent leads to few, if any, artifacts compared to live cells and
it is similar to the fixation methods used for immunofluorescence protocols. Thus, it is what we commonly use during our SIM experiments. If Cut12 ring formation was an artifact of fixation, we should observe not only Cut12 but also its binding partners in SPB rings. In the thousands of SIM images we have acquired using Ppc89, we have never detected it in a ring, and we also do not observe other core components such as Sid4 and Pcp1 in rings (Figure S1). Cut12 also binds to Plo1, which is also not detected in a ring (Figure 5E).

Our data provides overwhelming support that Sad1, Cut12, Kms2 and Cut11 redistribute into rings at the SPB during mitosis. This is an important new observation, enabled by advances in imaging methods, and it provides insight into the process of SPB fenestration.

Similarly, avoiding a sole reliance upon cdc25.22 arrest release would also give confidence that the image in figure 5c is truly representative of two SPBs with a half bridge in the middle rather than an artifact of the SPB over duplication in the cdc25.22 background. These are significant concerns as the imaging is being done after a single form of fixation which has the potential to differentially impact upon the distribution of proteins.

See above. We see rings in both using both a cdc25.22 arrest release and in asynchronous cells.

3. Cell culture.
It is important to characterise the phenotype of all mutants used by the scoring of tubulin/chromatin used in all the original studies that isolated the mutants when growing the cells in the culture protocols used here. The approach used here deviates so much from the standard approaches used by the fission yeast community that the cdc25.22 arrest time has to be reduced from 4.25 hours to 3.5 hours. The reader needs to know that the temperature shifts used will indeed inactivate the target protein to the same degree as characterised in the original studies. Standard approaches to the cultivation of fission yeast of growth for at least 16 hours in the minimal medium before the imposition of temperature shift has been used to characterise the mutants, rather than the two hours used here. This is absolutely essential because of the impact of culture context upon events at the SPB. Activation of the stress response pathway by the shift from Rich to minimal media (Shiozaki and Russell 1995 Nature 378:739) stimulates TOR signalling to promote the recruitment of polo to the SPB to change SPB physiology (Petersen and Hagan 2005 Nature 435:507, Petersen and Nurse 2007 Nature Cell Biology 9:1263, Hartmuth and Petersen 2009 J. Cell Sci 122:1737, Halova and Petersen 2011 J. Cell Sci. 124 3441). Thus, SPB physiology in this study and the penetrance of any SPB mutant maybe completely different from anything reported here. As polo recruitment to the SPB has a direct impact upon its activity (Grallert et al. 2013 Current Biology 23:213), it is entirely possible that the phenotypes of the SPB mutants is enhanced or reduced by the new nutritional regimen introduced into this study. This is particular important because the authors rely upon the incompletely penetrant allele sad1.1 for many of their conclusions. The authors should clarify what impact allele has when cells are cultured in this way. This can be presented as tubulin/chromatin time course shifts as mutants are shifted.
from 25 to 36°C in the supplementary figures. Alternatively, the authors could repeat the analysis with the standard culture conditions for fission yeast outlined in Petersen and Russell (Cold Spring Harb Protoc. 2016 Mar 1;2016(3):pdb.top079764).

We have expanded our materials and methods to address why it was necessary to use an extra overnight growth step and a shorter incubation at 36°C to obtain high quality SIM images. In addition, to address the concerns over the differences in these conditions and those suggested by the reviewer, we repeated the cdc25.22 arrest with the 4.25 h arrest time and 'standard culture conditions' to show that identical protein distributions were observed (compare Figure 1 and Figure S1). For other mutants (sad1.1, sad1.2, cut11.1, cut12.1, 41nmt1-GFP-Kms2), we used the same growth conditions/inactivation time as was used in the original work describing each allele. While we performed experiments to validate the cells arrest as expected and the data is available upon request, adding this data to the manuscript would do nothing but confirm previously reported results.

4. Sad1.1
Many of the conclusions rely upon the sad1.1 allele which is not particularly penetrant. The authors could use the Sad1.2 allele published by Tamm et al to consolidate the findings with sad1.1. Note that sad1.2 published by Tamm et al 2011 is completely different allele from sad1.2 published by Fernandez-Alvarez 2016. It is completely unclear why Fernandez-Alvarez ignored the publication of sad1.2 by Tamm et al when they named their mutant sad1.2, however, sad1.2 from Tamm et al does offer an alternative allele to consolidate the conclusions drawn from the hypomorphic sad1.1 allele from Hagan and Yanagida 1995.

We cannot comment on the nomenclature of sad1+ alleles. However, the sad1.1 from Hagan and Yanagida 1995 and sad1.2 from Fernandez-Alvarez 2016 were particularly useful in the context of our work, and we have used the nomenclature used in both papers. sad1.1 largely (although perhaps not completely) abolished sad1+ function/protein so that we could understand how it functions in recruitment of other ring components and in nuclear envelope breakdown (Figure 1C, 2A-B). sad1.2 only abolishes interaction with centromeres (as shown by Fernandez-Alvarez 2016), allowing us to investigate how the protein is organized in the absence of centromere attachment and under forced centromere binding/Plo1 activity (Figure 4, 6F). It is unclear what we would learn from the sad1.2 allele published by Tamm et al 2011 that would advance our story in a significant manner.

5. Mitotic commitment in cdc25.22 release into a polo background
It is vital to monitor the degree of mitotic commitment of the cdc25.22 plo1.as8 return to 25C in the presence of ATP analogue. This is because Polo is part of the system that pushes the cell into division and so when the commitment is already compromised by the cdc25.22 mutation it is highly likely that the reason that the phenotypes that the authors are scoring are reduced is because fewer cells are getting into mitosis. The change in the frequency of phenotypes is simply a reflection of a weakened commitment to mitosis, rather than a specific requirement for polo function in the process being monitored. Fewer cells are in mitosis, so fewer cells
show the mitotic phenotype. The degree of release can be monitored by scoring the frequency
with which the interphase microtubule cytoskeleton is disassembled at time intervals after the
release.

We apologize for the confusion and have revised both the text and our illustration of this
experiment in Figure 6A. The reviewer’s point is accurate – and the point of doing this
experiment. If cdc25.22 plo1.as8 cells arrested cells are treated with ATP analog and
returned to 25°C, they will NOT progress into mitosis. We do not need to look at microtubules,
as we know from looking at the SPBs that SPB separation does not occur, Sad1 ring
formation is decreased, Cut12 never forms a ring and the nuclear membrane does not
breakdown. This indicates that Plo1 has a very early mitotic function.

6. Figure 2
The experiments that are shown in figure 2 panels 3-5 of panel A have all been published
before. Tallada et al 2009 (J Cell Biol 185:875) shows the data for cut12.1 and cut11.1 while
Tamm 2011 shows the data for brr6.ts8 (J Cell Biol . 195:467) It is unclear why these are
presented here as novel findings. The data for nmt81-Kms2 are inconclusive because, as the
authors point out, the thiamine is repressing the expression of the marker. This work should
be repeated with the constitutive expression of a nuclear integrity marker.

Because each paper used a different NLS reporter, it was difficult to determine the extent of
nuclear envelope breakdown in each of the previously reported experiments. Thus, we
compared all mutants with the same NLS reporter here. We have clarified the rationale for
including this figure in the revised manuscript as well as cited/compared results to the work
listed above. Thank you for pointing out this oversight.

7. Is Sad1.GFP reflecting the protein?
Data are presented that show that loss of cut12 has no impact upon Sad1-GFP. However, this
contrasts sharply with the major impact that the cut12.1 mutations have on untagged sad1
protein in immunofluorescence microscopy with polyclonal antibodies to Sad1 as the sad1
staining is asymmetric mainly associating with the non functional pole in Bridge et al 1998 and
Tallada et al 2009. A similar relationship is shown in Tallada et al where every cut11.1 cell in
the field of view in figure 2 is asymmetric. As the penetrance of this phenotype for cut11.1
here is less, it is unclear whether this is because of the atypical culture conditions applied
here, or that the fusion of GFP to Sad1 is impacting upon its function.

We have replaced the confocal images that the reviewer discussed here with high resolution
SIM images to specifically focus on ring formation with each protein and in each mutant
background as suggested by Reviewer #3, point #3. As noted in the revised manuscript, we
observe a higher fraction of cut12.1 mutants without a Sad1-GFP ring. Also, in many mutants,
particularly cut11.1, Sad1.GFP rings were not as full or complete as in wild-type cells,
suggesting that Cut12 and/or Cut11 may stabilize Sad1, possibly at the new SPB as
previously suggested.
However, we did see Sad1-GFP rings at both poles in a significant fraction of all mutants. While this is different than the antibody staining previously reported (both in terms of what was assay and potentially the result of seeing Sad1 at both poles), we do not think that atypical culture conditions or the GFP fusion to Sad1 are the cause of the discrepancy. As noted above, we used the same 4 h growth arrest for these experiments as previously reported, and we previously demonstrated that Sad1-GFP is functional (Bestul et al. 2017). Major advances in fluorescent protein technology and in image acquisition hardware in the last decade, including the high sensitivity sCMOS camera used by our SIM microscope, allow for unprecedented detection of proteins inside of cells. For example, we previously reported that the yeast Sad1 ortholog Mps3 localized exclusively to the SPB based on polyclonal antibody staining by widefield microscopy and immunoEM (Jaspersen et al 2002). Years later with improved instrumentation and more stable GFP fluorophores, we know that Mps3 also is present at the nuclear envelope (Jaspersen et al. 2006) and it is present at a ring at the SPB (Chen et al. 2019). Moreover, we are not quantitating the amount of protein at each pole but rather whether the protein present at that pole is present in a ring configuration or not. To the best of our knowledge none of the work references by this reviewer or other reviewers has tested ring formation, so our data is an important advance for the field that was enabled by new technology.

In the revised manuscript, we have worked to integrate our data into previous data from the field, noting the contributions (and limitations) of each approach and describe the important ways in which our data advances our understanding of SPB fenestration and possible roles for Sad1, Cut12 and Kms2.

Minor points
8. Plo1.24 is a hypomorphic allele. The authors may wish to consider using plo1.ts41 (Grallert et al 2013 Nature Cell Biology 15:88) which was isolated by a specific regime to ensure that it was a highly penetrant allele.

Thank you for the suggestion. We used the plo1.as8 mutants to follow-up our initial observations with plo1.24 mutants.

9. In the introduction the beautiful work from McIntosh is cited as showing that the cytoplasmic body of the SPB sits on top of the nuclear envelope, however, it was McCully and Robinow 1971 (J Cell Sci 94:133) who showed this first, and Kanbe and Tanaka 1986 (J Cell Sci 80:253) also deserve citation here for their improved resolution by freeze substition.

Added.

10. The authors cite the partial nuclear envelope breakdown in C. elegans and Drosophila, however, I was surprised to see that there was no mention of the numerous other systems that do it. Perhaps a reference to the iconic Brent Heath review could be used to show how
extensively this occurs throughout eukaryotes (Int. Rev. Cytol. 64:1-80. http://dx.doi.org/10.1016/S0074-7696(08)60235-1)

Added.

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

This manuscript outlines reorganization of several NE- and SPB-associated proteins into ring-like structure proposed to facilitate SPB insertion in the NE at the mitotic onset in the fission yeast S. pombe. The authors further suggest that the Polo kinase activity is required for proper organization of this structure. The data are overall of high quality, but I do have a few concerns and suggestions for improving the manuscript.

We thank the author for recognizing the importance of our findings and for the comments to improve our manuscript further.

Specific comments:

1. I find the term "nuclear envelope breakdown" inappropriate - misleading, in fact - when describing the closed mitosis of S. pombe (e.g Summary of this manuscript). The NE in this organism is remodeled, or "fenestrated", to insert the SPBs for spindle assembly. However, this process is profoundly different from the open mitosis of somatic cells of metazoans and plants or even "semi-open" or "semi-closed" mitoses of many fungi or some embryonic metazoan cell types. For instance, in the syncytial nuclear division cycles or embryonic neuroblast divisions in Drosophila, the nucleocytoplasmic compartmentalization is lost, concurrent with the appearance of breaks in the NE. This is in stark contrast with S. pombe mitosis, where the nucleus is functionally insulated from the cytoplasm throughout chromosome segregation. Is it a NE "breakdown" if the nuclear integrity is maintained? I appreciate that there is a continuum of strategies to remodel the NE and I fully support discussing how they might be related to each other. Yet, in my opinion, conflating the terms "NE fenestration" and "NEBD" is both unnecessary and confusing. At the very least, it should be qualified at all times, including the summaries, that this version of "NEBD" is localized and extremely transient.

We continued the term NEBD as it has been used by others in the field to describe the fission yeast NE fenestration driven by Sad1 and centromeres/telomeres as well as the loss in nuclear permeability caused by changes in RanGAP1 localization in anaphase B of meiosis I. However, we do certainly understand the reviewer's point as to mechanistic differences with NEBD in metazoans. In the revised manuscript, we have added an extra paragraph to the introduction to highlight these issues. We have also worked to ensure we carefully edited our vocabulary to draw attention to the partial NEBD that we are interested in understanding using fission yeast.
2. Fig. 2. The lack of NLS-GFP efflux in sad1 mutant cells had been previously shown (PMID: 27889481), leading to the conclusion that Sad1 function was required for NE fenestration, and also that Sad1 functioned upstream of Cut11. Similarly, the behavior of this nucleoplasmic marker was already characterized in cut12-1 and cut11-1 mutants (PMID: 19487457). This has to be clearly acknowledged when describing experiments related to Fig. 2.

See point #6 from Reviewer #1.

3. I would appreciate at least some "mechanistic" discussion on how the authors imagine reorganization of these proteins into 'rings' lead to SPB insertion. Ultimately, SPB insertion requires membrane remodeling - how could this work?

This is a great question and something that we are anxious to pursue in future work. We have added a speculative paragraph to the discussion on pages 36-37, which is based on changes in binding partners and/or conformation. We also believe that Lem2 and possibly Ima1 are involved at the membrane.

4. The authors allude that Sad1-GFP is not fully functional - but is it valid then to make conclusions about Sad1 behavior in mitosis based on the tag that is not quite functional? Have the authors performed SIM using Sad1 antibodies or a differently marked Sad1 that behaves more like the wild type protein? For instance, Sad1 was previously shown to frequently delocalize from one of the SPBs in cut12-1 mutant (PMID: 9531532). That result, obtained using specific antibodies, is different from the one shown in this manuscript, which utilizes Sad1-GFP.

We don’t believe that we made a claim that Sad1-GFP is non-functional; in fact, we previously tested the functionality of all tagged SPB components used in this paper and did not detect any obvious defect their function (Bestul et al. JCB 2017). The differences in Sad1 localization previously seen in cut12.1 and other mutants was also raised by Reviewer #1, point #7.

5. Furthermore, since mitotic activation of Plo1 is significantly reduced in cut12-1 mutant cells (PMID: 12815070), would it be fair to expect that the cut12-1 mutation could for instance lead to problems in Sad1 reorganization into double rings, based on the authors' results using plo1 mutants?

We agree that this is possible, however we think that Plo1 has a very early function in mitosis that is nucleoplasmic (Figure 5C), centromere-associated (Figure 5D, 6F-G) to trigger Sad1 reorganization. We hope that our explanation of Figure 6A-E, which we did not introduce well (see Reviewer#1, point #5), helps to clarify this issue. While we don’t have specific data, we certainly would anticipate that the feedback loops that help yeast commit to mitosis (that involve Plo1) enhance ring maturation/stability, etc.
6. The authors state that "the bulk of Plo1 at the same spot as the centromere" but the pattern of Plo1 localization shown in Fig. 5C is different from centromeres (Fig. S1B). Does Plo1 form ring-like arrangements like Sad1? Difficult to see how it relates to Sad1 ring(s) in the view shown in Fig. 5C.

Thanks for pointing this out. We have modified our description of Plo1 localization because when we looked at it using SIM to try to detect it in rings like we see for centromere binding proteins (Mis6, Csi1) and Sad1 (Figure S2B, 3C-D), we did not see Plo1-GFP in rings in any mutant background or at any stage of mitosis (Figure 5C-E). Thus, we believe that Plo1 is not a stoichiometric ring component but acts catalytically. This was surprising not only based on the role we propose at the centromere but also based on Plo1’s previously described cytoplasmic interactions with Kms2 and Cut12!

7. What is the evidence for Plo1 being "delivered to the nuclear face of the SPB by the centromere", as stated on page 32? For instance, where is Plo1 when the centromeres cannot contact the SPB?

In Figure 5D, we examine Plo1 distribution in sad1.2 mutants. These cells are partially defective in centromere attachment and undergo a progressive loss of centromeres at the SPB after prolonged incubation at 36°C (see Fernandez-Alvarez 2016). Already at 25°C we observed a shift in Plo1 from the bridge region seen in wild-type cells (Figure 5C) to beneath each SPB (Figure 5D). More importantly, we saw a progressive loss of Plo1-GFP from the SPBs with increased incubation at 36°C (Figure 5D). To test if Plo1 activity at the centromere was important for Sad1 ring formation, we analyzed the distribution of sad1.2-mT2 after recruitment of centromeres using the tethering system. If Plo1 kinase is inhibited, centromere tethering is no longer able to reorganize sad1.2-mT2. We therefore conclude that Plo1 is an important centromere-associated factor for Sad1 reorganization. We hope that the additional experiments and rephrasing of our conclusion help to clarify this point.

8. Fig. 1C. Cut11 enrichment at the separated SPBs (rather that coincidence with Pcp89 quantified in this panel) appears lower in sad1-1 cells. If this difference is significant, the implications need to be mentioned/discussed.

Fig. 1D. For cut11-1 data, it is not clear if Sad1 is present just on one of the duplicated SPBs in the same cell and how this relates to the SPB NE detachment phenotype observed in cut11-1 mutants. This is an easy experiment and would clarify the relationship between the two proteins.

As noted above in point #7 to Reviewer #1 and to point #3 to Reviewer #3, we have replaced the confocal images in the original manuscript with high resolution SIM images to specifically focus on changes in ring formation with each protein and in each mutant background.
9. Fig. 3, S2. Please provide median sizes of Sad1, Csi1 and Lem2 rings to support the claim that the Lem2 rings are significantly larger. Furthermore, in those structures that have both Sad1 and Csi1, Csi1 appears to occupy less volume, forming smaller rings or clusters, rather than form rings of the same size, as suggested by the authors. Quantitation is needed to clarify this point.

We have added the data on ring diameters to Figure S3B in the revised manuscript. We also compare Csi1 and Sad1 directly in Figure 3C, although it is important to note that differences in resolution of GFP and mCherry affect ring size/thickness.

10. What could be an explanation for the temperature dependency of csi1del cells in terms of Sad1 ring formation phenotype?

Hiraoka et al., 2011 previously reported temperature-dependent differences in both growth and nuclear morphology when they first analyzed a number of deletion mutants in INM components, including several shown here. Based on their observations, we looked at 25°C, 30°C and 36°C in our experiments and saw the temperature-dependent defect in csi1Δ cells. We do not know why it is temperature dependent but speculate it could be related to lipid composition, protein folding or the stress response. We have modified the text to include both our rationale for testing various temperatures based on the Hiraoka paper and to speculate why defects might occur at 36°C.

11. Fig. 1A. It would be helpful to provide a timeline on the panel itself, to relate the experimental setup to the redistribution of proteins of interest. It is not clear what is the rationale for selecting magnified images on the right of the panel. They are neither coming from the same timepoint, nor they are the magnified versions of boxed areas on the left. The authors should include an image of separated rings for Sad1, for comparison with other markers - in the provided image, it is not clear how a double ring is different from separated rings.

First, we have renamed the rings to avoid some confusion (see minor point #8 from Reviewer #3). In the double ring images, the SPBs have separated. Thus, to view ring morphology at both SPBs, it was necessary to show one of the two SPBs in an inset in some cases (it is not magnified and has the same scale as the SPB not in the inset). These double rings are from separated SPBs.

As to a timeline, we are hesitant to add this to the images as different ring morphologies co-occur at each time point. Presumably, this is due to cell-to-cell asynchrony in cdc25.22 release combined with the fact that all but the double rings are transient. While we have data on the fraction of each type of ring observed at time points following cdc25.22 release (below), the data in Figure 1B seemed to illustrate the point that Sad1 rings mature more rapidly than Kms2 and Cut12. Because Cut11 does not localize into a ring until the 30 min time point and it only forms double rings, we did not analyze it.
12. Page 14, The sentence "Consistent with this idea, we observed that the Sad1 rings co-localized with the centromere, which was marked with Mis6-GFP (Fig S1B). I do not see co-localization - would it be more accurate to say that rings were positioned close to the centromeres?"

Yes, we have revised the text.

13. Throughout the manuscript: It would be helpful to provide the dotted outlines of cells (for instance in color overlays for each image) to orient the reader.

In the revised manuscript, most images show magnified images around the SPB at a scale that is much smaller than the size of the cell, so this isn't feasible. We did add dotted lines to the NLS-GFP images, as suggested.

14. Related to Fig. 1A-B. What is the timing of entry into mitosis the cdc25-22 block and release experiments shown by the authors? The authors mention that Cut11 "did not localize to the SPBs until mitosis" - do they mean prometaphase rather than mitosis? My understanding is that cdc25-22 S. pombe cells released from the G2/M block enter mitosis (as judged by CDK activity) considerably earlier, around 10-15 min timepoint.

We have changed mitosis to prometaphase in a number of cases to more accurately reflect the cell cycle position. Thank you for pointing this out.

15. Fig. 4A. This strain is not a WT, it’s sad1-2 - please correct. Similarly, the double mutant should be clearly indicated.

Fixed

16. Page 25. A typo, should be "...contained a Sad1-GFP ring".

Fixed
17. Fig. 1B. Please put the color key in the figure.

Added

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

In this work submitted by Bestul et al., the authors use super resolution microscopy techniques and genetic manipulation to show that polo kinase is required for the redistribution of multiple SPB-associated proteins, and that this redistribution is dependent upon centromere localization to the LINC protein Sad1. The work is careful and the data beautiful; however, there are instances where the authors fail to elucidate how they came to their conclusions or explain what is novel vs. published findings. We suggest publication after addressing these points:

We thank the reviewer for their careful and thoughtful comments on the manuscript. We have worked to address what is novel vs. what is published and explain how we reached each novel conclusion.

Major points:
1. The authors refer to previously tagged GFP strains in the second paragraph of the results. If these are previously characterized, please cite the reference and evidence that these are functional as tagged; if not, please check functionality.

We added the reference to where we tested all (Bestul et al. JCB 2017) but Cut11-GFP, which was described by West et al. MBoC 1998.

2. Has it been previously shown that the cdc25-22 arrest has no effect on subsequent NE dynamics or SPB insertion events? If so, please explain. If not, it could be useful to test Sad1 redistribution in cells arrested with a different synchronization protocol (or confirm some results in an asynchronous population), or at least to discuss why the authors believe the cdc25-22 arrest has no effect on the probed phenotypes.

See Points #1 and #2 from Reviewer #1. As suggested by Reviewer #3, we confirmed results for all four ring proteins and three SPB core components in asynchronous cells. We see identical protein distribution as cells enter into mitosis in the absence of cdc25.22 so we are confident this reorganization occurs during mitotic entry.

3. In Fig 1A, the squares designated by blue dotted lines versus the white are confusing to understand/see. What are the white boxes? They are mentioned as "inserts" in the figure legend but it is not clear what this means. Is it an insert from a different image? If not, why does one of the Ppc89-mCherry (magenta) dots disappear from the white box the
Cut11 panel? The Cut11 pattern in 1A is in general difficult to interpret as Cut11 localizes to so many sites - please explain Cut11’s localization and how ring designations are derived from these images.

See Reviewer #2, point #11. We have removed the blue lines, renamed the rings and ensured that each protein represents an identical stage. The Cut11-GFP localization is distinct because it is present at NPCs throughout the cell cycle (the dots; West et al. MBoC 1998). It only forms rings at the SPB following SPB separation (double rings).

3. Figure 1C and 1D are difficult to interpret. For instance, the images are too low resolution/zoom to evaluate the authors’ claim that there is only modest modification of the Cut11 ring phenotype in sad1.1 (no ring phenotype is obvious from Figure 1C). We see value in showing fields of cells in addition to a representative image; thus, we suggest moving 1C to supplemental and reanalyzing the cells with high-resolution SPA-SIM.

We have taken the reviewer’s suggestion and replaced the images with SIM examples. This allows us to focus on ring localization and morphology for each protein and in each mutant background. See also point #7 to Reviewer #1, and point #8 to Reviewer #2.

4. Also in Figure 1C and 1D, there appears to be altered Sad1 localization in the 81nmt1-kms2 and cut11.1 cells, yet there is no mention of this in the text. How did the authors ensure lack of bias when quantifying signals (i.e. was an ImageJ plug-in used or was it investigator discretion?)? The Sad1 puncta appear larger/less distinct in these cells, making distinction of puncta difficult. Higher resolution images (point 3) might clarify this, but a mention in the text will also help readers trust the conclusions.

As the reviewer surmised, we could obtain more useful information with higher resolution images. (See point 3). We added addition details on quantification to the materials and methods.

5. Temporal conclusions about which proteins come first vs. second would be better supported if looking at the proteins simultaneously. For example, in Fig 1B, looking at Sad1 and Cut12 simultaneously would elucidate which is first vs. second.

This experiment was added to Figure S1B, supporting our temporal model.

6. 81nmt1 promoter is notoriously leaky. With this in mind, for the 81nmt-kms2 strain, how were the authors sure the Kms2 is gone? Was western analysis or microscopy of Kms2 performed in these strains to show loss?

We used an HA-tagged version of Kms2 that we confirmed by western was no longer detectable, as shown previously in Walde and King JCS 2006.
7. The authors need to specify what is known about whether Cut11 is inside or outside the nucleus. If the latter is true, it would be unlikely to directly interact with Sad1.

This is a great question that we wish we knew the answer to! To the best of our knowledge, the distribution of Cut11 on the inside or outside of the nucleus has not been tested in fission yeast. Based on what is known about Ndc1 in budding yeast and various metazoans, we would predict that it is present at the region of the nuclear membrane where the inner and outer nuclear membranes are contiguous.

8. There are multiple cases where the authors use the term "colocalize" without strong evidence. For example, Mis6 and Sad1 in Figure S1B only partially colocalize, and may even display adjacency, which would also be very interesting. Similarly, Sad1 and Csi1 only partial overlap. As it is already known centromeres are linked to Sad1, addressing the partial nature of the overlap could make this data more meaningful.

We have changed the text as the reviewer suggests.

9. In the previous paper introducing sad1.2, centromeres were shown to be released from association with the SPB increasingly over time at restrictive temperature. The authors should check their culturing conditions to see how severe centromere release is at both the 4 and 8 hour time points in figure 4 using a centromere marker, or at the very least, speculate that varying centromere loss over time is responsible for their increasing severity of phenotype and cite the references need. The authors also state that in sad1.2 cells 'only cells with an unattached centromere have a defect in mitotic progression" - this statement should be changed to clarify that all centromeres need to be detached in order for NEBD and cell cycle progression to be blocked.

Thank you for catching this. We use the progressive loss of centromere attachment seen by Fernandez-Alvarez 2016 in a number of experiments to correlate a phenotypes. In Figure 4A-B, we see progressive loss of sad1.2-GFP ring formation after 4 and 8 h time points; and in Figure 5D, we see a progressive loss of Plo1-GFP at the SPB in sad1.2 cells after 4 and 8 h.

10. The cdc2-M17as mutant is indeed less severe than the classic cdc2-as mutant. Without a phenotype, how are the authors sure they sufficiently suppressed Cdc2? Do the SPBs typically duplicate and separate when cdc2-M17as cells are treated with the concentrations of inhibitor used? If yes, the authors need to mention this and address how they know they sufficiently suppressed Cdc2. If no, the claims about cdc2 need to be lessened.

We have added Figure S4 to show the SPBs following inhibition of cdc2.asM17. Based on the lack of SPB separation we knew that the cells arrested early in mitosis. Analysis of Sad1-GFP distribution showed that most (77.5%) cells contained a Sad1 ring. In wild-type cells and in cdc2.asM17 treated with the non-analog solution, we only observed this configuration in ~20%
of cells.

11. If there were a way to differentiate and/or manipulate nuclear vs. cytoplasmic Polo, the model could be much further substantiated. Using a mutant either in Polo or in SPB that cannot bind Polo would be highly informative, though not required for publication.

We agree that this would be interesting but it is beyond the scope of this paper. However, in an effort to demonstrate that centromere-bound Plo1 is needed for Sad1 ring formation we tested the requirement for plo1+ activity when ring formation is induced by artificial tethering of centromeres (Figure 6F-G).

12. There are points where it is unclear what is previously published versus novel findings, including findings from authors' 2017 paper on Sad1 redistribution. Importantly, the fact that artificial tethering of centromeres fully rescues sad1.2 defects has previously been shown (Fernandez-Alvarez et al 2016). It was also shown in that paper that in mitosis, centromere attachment is necessary for NEBD and cell-cycle progression; this was not shown only for telomeres and meiosis as the authors describe. Adding phrases like "in accordance with previous findings" would help in some instances.

We have worked to amend the current manuscript so it both accurately and appropriately cites the work of Fernandez-Alvarez et al 2016 and others. Our work obviously builds on Fernandez-Alvarez et al 2016 and takes advantage of key reagents/tools developed in that study. However, our finding that Plo1 is likely the centromere/Sad1 kinase and our establishment of a ring formation pathway is needed for NEBD are novel.

Minor points:
1. On page 3, the word "the" is missing from the sentence ", fission yeast restricts NEBD to the region localized only underneath SPB".

Fixed

2. Page 11 has a typo in the sentence "Together, these data support our model that Sad1 is the first protein the redistribute..." (should say "to" instead of second "the").

Fixed

3. On page 4, the authors use "sad1.2" multiple times, without mentioning cells. (i.e. "in sad1.2, which displays a partial loss of centromere-binding function...").

Fixed

4. The authors need to be diligent with pombe versus cerevisiae nomenclature to avoid confusion. There are multiple examples in the text where pombe nomenclature is not used
(i.e. "sad1.2-GFP" on page 19). For correct pombe nomenclature, alleles are in lowercase and italicized while proteins are capitalized (at first letter only) and non-italicized.

We have worked to fix this using the style suggested by the reviewer. Thank you for bringing this to our attention!

5. The bottom of page 4 contains a typo making the sentence difficult to understand. "NEBD and SPB insertion is unknown, although it is thought that chromatin might help increase the localize critical mitotic regulators..." We assume the word localize just needs a "d".

6. The wild-type control in S1C is at a different stage than two of the mutants (lem2- and bqt4-). This makes making conclusions from this data difficult.

We have added an extra panel for controls for these mutants (now Figure S2C).

7. The labels on the figures are somewhat lacking. (i.e. a key on the model or label of what the circles under the SPB are (we are guessing it is chromatin), a key on Fig 1B showing what orange vs. yellow means, a label on Figure 2A showing what the magenta foci are.

We have remedied this in the revised manuscript. The magenta foci in Figure 2A are Ppc89-mCh, which has been added to the figure and legend. Yes, the circles were chromatin but we went with a more 'streamlined' view of DNA in the revised paper.

8. The term "double ring" is somewhat misleading. It is likely to be interpreted as two rings (like a figure 8). Yet, what the authors mean is a single ring encompassing both SPBs. We suggest calling it larger ring or double-SPB ring.

We have renamed the rings as the reviewer suggests.
Dear Sue,

Thank you for transferring your manuscript from JCB. I have now carefully read your paper, the reviewers’ comments and your response to the critiques. I agree with the referees that your paper contains information that should be of interest to the field and feel that you have adequately addressed the concerns that were raised in the initial reports of the experts. I am therefore pleased to accept your manuscript for publication in Molecular Biology of the Cell.

Congratulations to you and your colleagues and best wishes,
Karsten

Editor
Molecular Biology of the Cell

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