Micronuclei in Kif18a mutant mice form stable nuclear envelopes and do not promote tumorigenesis

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Review #1

1. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Less than 1 month

2. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

In the present work Stumpff, Reinholdt and co-workers investigate the mechanism by which micronuclei contribute to tumorigenesis. Micronuclei are classic markers of genomic instability widely used in the diagnosis
of cancer, but whether they work as drivers of the process has recently attracted significant attention due to their link with chromothripsis. Here, the Stumpf/Reinhold labs have explored an interesting model to test some ideas about the role of micronuclei as drivers of tumorigenesis, based on Kif18A/p53 double KO mice. They confirm the formation of micronuclei in these animals, but find no substantial increase in survival and tumor incidence relative to p53 KO animals, despite higher incidence of micronuclei in Kif18A/p53 KO tumors. They conclude that, per se, micronuclei do not have the capacity to form tumors, regardless of p53 status. This was surprising, given the well-established role of p53 in preventing the proliferation of micronucleated cells. To shed light into this apparent paradox, they compared micronuclei from Kif18A KO cells with micronuclei generated by a number of other experimental conditions that promote formation of anaphase lagging chromosomes or generates acentric fragments. They found that micronuclei derived from Kif18A are intrinsically different from micronuclei generated by those other means and essentially showed increased accumulation of lamin B, were more resistant to rupture and preserved the capacity to expand as cells exited mitosis. Of note, they find a correlation between chromosome proximity to the poles/main chromosome mass and the different features that characterize micronuclei from Kif18A KO cells, compared with the other experimental conditions in which late lagging chromosomes are more frequent. Overall, I find this study extremely interesting, well designed and executed in a rigorous way that characterizes the consistent solid work from these laboratories over the years. I have just few minor points that I recommend to be addressed prior to publication.

1-Abstract and main text lines 70 and 100: the authors indicate that Kif18A mutant mice produce micronuclei due to unaligned chromosomes. This is correct, but at the same time misleading. The authors should clarify that although micronuclei derive from compromised congression, I was convinced from previous works (Fonseca et al., JCB, 2019) that it was their asynchronous segregation in anaphase that led to micronuclei formation. As is, a less familiar reader may conceive that misaligned chromosomes directly result in micronuclei, for example by being detached from the main chromosome mass.

2-Page 2, line 59: "cells entering cell division...become fragmented". It is not the cells, but the chromosomes that fragment. Please correct.

3-Page 4, line 149: "reduced survival in the Kif18A null, p53 mice". P53 what? KO, WT? Please clarify.

4-Page 5, line 212: the authors refer that micronuclei were scored for absence of lamin A/C, but previously they scored it as
"continuous/discontinuous". Please clarify.

5-Page 6, line 243: "Kif18A is not required for micronuclear envelope rupture". Shouldn't it be micronuclear envelope "integrity"?

6-One of the most interesting results of the paper is the correlation between envelope formation in micronuclei with their respective position relative to the poles/midzone. Could the authors try to investigate causality? For instance, the authors refer to works from other labs in which MT bundles and a midzone Aurora B activity gradient might play a role in the different features associated with micronuclei envelope formation, depending on their origin. Could the authors manipulate this gradient and investigate whether it changes the outcome in terms of nuclear envelope assembly properties on micronuclei? Are there any detectable features in midzone MT organization in Kif18A KO cells that would justify the observed differences?

3. Significance:

Significance (Required)

Kif18A plays a key role in chromosome alignment, without apparently affecting kinetochore-microtubule attachments in non-transformed cells. Because they cannot establish a proper metaphase plate Kif18A KO cells enter anaphase with highly asynchronous segregation due to non-uniform chromosome distribution along the spindle axis. Consequently, some "delayed" chromosomes form micronuclei, in cell culture and in vivo. Interestingly, prior art has failed to detect any increased signs of genomic instability in Kif18A KO cells and mice, and, contrary to what would be expected based on current trends, these mice do now show any signs of increased incidence of tumors, in fact they even show some protective effect to induced colitis-associated colorectal cancer. Noteworthy, all previous experimental works pointing to a role of micronuclei as key intermediates of genomic instability in cancer relied on models in which the tumor suppressor protein p53 had been inactivated. In the present work, the authors explore the relationship between micronuclei formation and p53 inactivation by investigating
tumor formation in Kif18A/p53 double KO animals (1 or 2 alleles of p53 inactivated. The reported results are timely and will attract the interest of a broad readership, while decisively contributing to shed light into an ongoing debate. I am therefore all in favor for the publication of this work in any journal affiliated with review commons, pending some minor revisions.

Review #2

1. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 1 and 3 months

2. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)
Sepaniac and colleagues use in vivo and in vitro approaches to examine why micronuclei generated by lack of KIF18A activity do not promote tumorigenesis. The authors conclude that micronuclei in KIF18A depleted cells form stable micronuclear envelopes, which may be a result from lagging chromosomes being closer to the spindle pole when the micronuclear envelope forms. The authors further conclude that the stability of the micronuclei arising from lack of KIF18A can explain why Kif18a mutant mice do not develop tumors. These results also suggest that the consequences of micronuclei and their possible contribution to tumorigenesis depend on the context of their genesis. While the mouse model data and characterization of the stability of micronuclei generated by different insults support the conclusions, the lagging chromosome positioning data could be improved. Moreover, a number of other issues should be addressed prior to publication.

**Major issues:**

1. Line 153-155. The authors conclude that the slight reduction in overall survival is "due to a reduced ability of Kif18a mutants to cope with rapid tumorigenesis," but it is unclear why this would be the case. There is also an increase in micronucleated cells in thymic lymphomas from Kif18a/p53 homozygous mice (Fig. 2B)—could this not contribute? In Fig. 3C, the authors show that micronuclear rupture is similar in both Kif18a WT and mutant mice, so it seems possible that the increase in the frequency of micronuclei (Fig. 2B) coupled with a similar frequency of micronuclear rupture (Fig. 3C) could lead to the reduced survival. Then, in the discussion, the authors downplay this finding by saying (line 371) "loss of Kif18a had modest or no effect on survival of Trp53 homozygotes and heterozygotes." Why then speculate earlier in the text that loss of Kif18a reduces the ability to cope with tumorigenesis?

2. Related to the point above, the authors show in figure 3 that the micronuclei found in healthy tissues display infrequent membrane rupture (panel B). However, micronuclear membrane rupture in tumor tissues is much more frequent (panel C). How do the authors explain this? Do they hypothesize that the micronuclei in the tumors originate by mechanisms other than the misalignment caused by lack of KIF18A? Does KIF18A depletion cause aneuploidy due to segregation of two sisters to the same pole? If so, one could expect the tumors to be aneuploid (is this the case?) and aneuploidy has been shown by numerous groups to cause genomic instability. Such genomic instability could then explain the difference in membrane rupture.
The authors conclude that lagging chromosomes in KIF18A KO cells are found closer to the main chromatin mass. The Stumpff lab showed in a 2019 JCB paper that KIF18 KO cells have a chromosome alignment defect and as a result during anaphase the chromosomes can be scattered rather than forming the tight, uniform mass that is observed in WT cells. The scattering of kinetochores resulting from this phenotype could affect the value of "Avg Chromosomes Distances" in Fig 7B and the normalized distance in the KIF18A KO cells. Therefore, live-cell imaging experiments would be helpful to resolve this and possibly strengthen this conclusion. RPE1 cells with fluorescently tagged CENP-A and centrin could be used to ensure that the lagging chromosomes will not rejoin the main nucleus. Moreover, these cells could be used for correlative live-fixed cell experiments in which fixed cell analysis following micronucleus formation could be used to show that chromosomes that lag farther away from the spindle pole are more likely to have defective micronuclear envelopes.

Based on the Fonseca et al. 2019 JCB paper (video 2), micronuclei from KIF18A KO do not exclusively arise from lagging chromosomes. Instead, chromosomes can also escape the main chromatin mass after segregation and subsequently be excluded from the main nucleus. It would be important to know what fraction of the micronuclei in KIF18A KO cells arise via lagging chromosomes. Since Aurora B and/or bundled microtubules at the spindle midzone are believed to prevent proper nuclear envelope formation, chromosomes that properly segregate but later become separated from the main nucleus would be more likely to form proper micronuclear envelopes than those arising from lagging chromosomes. The correlative microscopy experiment suggested in the previous point could allow differentiation between these two routes to micronucleus formation.

**Minor issues:**

1. Some parts of the manuscript are excessively wordy and some sentences are unclear or convoluted (e.g., lines 148-153 and 238-239).

2. Lines 59-61. This sentence is formulated incorrectly. First of all, the subject of the sentence is "cells" and the verb is "can become fragmented." However, the authors mean that the DNA in the micronucleus can become fragmented (not the cells). Moreover, the way the sentence is currently formulated seems to suggested that the fragmentation occurs
during cell division. However, this is not the case. Please, revise the text to make it more accurate.

3. Lines 114-115. Please, provide references in support of this statement.

4. Line 153. The authors refer to Fig. 1C, but I think they mean Fig. 1B.

5. Line 324. The authors find that RPE1 KIF18A KO cells have lagging chromosomes in ana/telophase 9% of the time, then say that this shows that lagging chromosomes are rare in KIF18A KO cells. However, this is a large increase compared to normal RPE1 cells, which only have 1-2% frequency of lagging chromosomes. So, they should revise the text here to say that the rates of lagging chromosomes from KIF18A KO are lower compared to the rates induced by nocodazole washout.

6. Line 383. The references listed here should be moved earlier and specifically after the statement summarizing the results of the studies instead of being listed after the authors' conclusion/interpretation of the data. The same issue was noted in other parts of the manuscript.

7. Figure 1A. In the text, the authors say they cross a Kif18a heterozygous mutant mouse with a p53 heterozygous mutant mouse, but the two mice in this figure are already heterozygous for both. Please, revise the text or depict the previous additional cross necessary to obtain the double heterozygous.

8. Figure 3A. Arrows or dotted circles outlining the micronuclei in the insets of the middle and bottom rows would be helpful since the DAPI signal in the micronuclei is low and somewhat difficult to see.

9. Figure 3B. Error bars should be added to the graph. Moreover, the authors noted that the differences are not significant. However, this seems surprising, given that in some cases there is a three- to five-fold difference between certain pairs. Indeed, a chi-square test using the numbers from table S1 indicated p values <0.05 for several pairwise comparisons.
10. Figure 5G. When referring to this figure (lines 292-294), the authors talk about correlation. However, the points in this graph seem to be scattered a bit randomly.

11. Figure 6B-D. The Y-axis titles of the three graphs are a bit confusing. Please, consider revising.

12. In Figure 7 and the text, the authors use the terms "late-lagging" and "lagging" chromosomes interchangeably, which is somewhat confusing in this context because lagging chromosome distance from the main chromosome mass is thought to contribute to defective assembly of micronuclear envelopes. It is not clear whether the authors intend to indicate, with this term, that the lagging chromosome is farther away from the main chromosome mass or that the lagging chromosome is in a "late" anaphase cell. Because this is confusing, I suggest just using the term "lagging chromosome" consistently. It could be useful to include representative images of lagging chromosomes located at different distances from the main chromosome mass. And certainly, the authors should include an example of a lagging chromosome in the KIF18A KO cells.

13. Figure S2A. The example in the bottom right image looks more like a chromosome bridge than a lagging chromosomes. Kinetochore staining is necessary to unequivocally identify lagging chromosomes.

3. Significance:

Significance (Required)

Based on the previous knowledge on the factors that cause abnormal assembly of the micronuclear membrane, the results presented in this study were somewhat predictable. However, these findings will add to the knowledge of how micronuclei form and the potential factors that lead to micronuclear membrane rupture. Previous studies investigating micronucleus behavior have focused on micronuclei arising via merotelic kinetochore mis-attachments. These mis-attachments lead to formation of micronuclei close to the spindle midzone. In the present study, instead,
the micronuclei arising from lack of KIF18A activity form farther away from the spindle midzone. The results presented here suggest that the positioning of these micronuclei farther away from the midzone enables assembly of a more stable micronuclear membrane that will be less likely to rupture during the following cell cycle. A recent study showed that the microtubule bundles in the spindle midzone interfere with micronuclear membrane assembly. Based on this, it is not surprising that micronuclei forming away from the spindle midzone (like those resulting from lack of KIF18A activity) assemble more normal membranes. Although somewhat expected, this study provides the actual data in support of this phenomenon. This study will be of interest to cell biologists interested in cell division and genomic instability. My research has focused on cell division, aneuploidy, and chromosomal instability for nearly thirty years. Therefore, I believe I am fully qualified to evaluate this manuscript.

**Referees cross-commenting**

My areas of expertise do not include nuclear membrane structure and function. Therefore, I encourage the authors to consider the comments of reviewer #3 for issues related to reliable quantification of micronuclear membrane rupture.

Review #3

1. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)
Sepaniac et al demonstrate that loss of KIF18a, a motor protein required for proper chromosome congression and chromatin compaction during mitosis, is insufficient to drive tumor development in mice although it does increase the frequency of micronuclei (MN), nuclear compartments that form around broken or missegregated chromosomes, in both normal and tumor tissue. MN are thought to increase genome instability and metastasis by undergoing DNA damage and activating innate immune signaling after irreparable nuclear membrane rupture. The authors use a non-transformed human cell line, hTERT-RPE-1, with KIF18a knocked out to demonstrate that MN formed as a result of KIF18a loss have more stable nuclear membranes than MN generated by other methods. They go on to correlate this increased stability with increased chromosome proximity to the main chromatin mass during nuclear envelope assembly and increased chromatin decompaction by a combination of fixed and live cell imaging.

1. This study relies heavily on the use of lamin A loss or discontinuity to identify ruptured micronuclei. Although the authors validate this marker against "leakage" of the soluble nuclear protein mCherry-NLS,
there are several lines of evidence suggesting that lamin A loss or disruption is not a reliable reporter. In figure S3C, the top two panels of intact MN in the KIF18KO appear ruptured based on the gH2AX labeling, yet have significant levels of lamin A and are labeled as intact. In figure 4D, the rate of MN rupture after nocodazole release (60% ruptured in 2 hours) is much faster than that reported in other papers (40-60% in 16-18 hours, Liu et al; 60% in 16 hours; Hatch et al). In addition, images in Hatch et al, 2013 show lamin A localizing to both intact and ruptured MN and anecdotal information in the field suggests that lamin A localization is not a reliable reporter.

These discrepancies may be due to how the authors' define "mCherry-NLS leakage", which needs to be defined in the methods as previous studies have demonstrated that MN frequently have delayed or reduced nuclear import even though the membrane is intact. Regardless, the authors need to provide compelling independent evidence that lamin A loss and disruption faithfully recognize ruptured MN by either validating this marker against additional rupture reporters, such as Lap2, LBR, or emerin accumulation, or by repeating key experiments in cells expressing mCherry-NLS.

1. In figure 6A, it is unclear when the videos start and how micronuclei are selected for analysis. Do the micronuclei have to be continuously visible from the time they missegregate? Do the videos all start at the
same time point during mitosis or is it contingent on when the MN appears separated from the main nucleus? One concern is that a consistent delay in micronucleus appearance in the nocodazole treated cells could artificially decrease the amount of MN expansion observed.

2. In figure 7A, it is difficult to identify the "lagging" chromosome in the top panel. It would be helpful to label the chromosome that becomes the MN, or ideally, to include a video or still images to demonstrate how micronuclei form in the KIF18A KO cells.

3. The two image panels in figure 7A are imaged at significantly different times during anaphase (early anaphase on bottom versus late anaphase/telophase on top). A better comparison would be between two cells at the same time point in anaphase.

3. Significance:

Significance (Required)

In this study, the authors identify chromatin decondensation in micronuclei as a new predictor of membrane stability. Although these results are correlative, if their micronucleus rupture results can be validated as described in major comment 1, this study would advance our understanding of the micronucleus rupture mechanism by linking mitotic spindle location, chromatin decondensation, and lamin B1 protein recruitment. This would provide needed support to a current model in the field that micronucleus stability is largely determined during nuclear envelope assembly. In addition, if KIF18a loss generates stable micronuclei at high frequency, it will become a critical system for testing MN rupture hypotheses in the field. Thus, this work would be of significant interest to cell biologists working on nuclear envelope structure and function, chromosome organization, and mitosis. I include myself in this group as a cell biologist studying nuclear envelope structure and function with an expertise in membrane dynamics.
The authors also find that mice mutant for KIF18a have increased micronucleation in normal tissues but not increased tumor initiation. They hypothesize that this is due to the low rupture frequency of KIF18a-induced MN, however their data cannot reject the null hypothesis that the small increase in MN they see in KIF18a mutant mice would be insufficient to induce tumorigenesis even if rupture frequency was high. Thus the significance of their finding that micronucleation is not sufficient for cancer progression is unclear. However, the thorough analysis of micronucleation and rupture in several healthy tissues as well as a tumor model in KIF18 mutant mice would be of interest to both pathologists and cancer researchers focused on mechanisms of genome instability. These types of experiments are critical to determine how micronuclei contribute to cancer progression and the quantifications presented in this paper are truly impressive.

**Referees cross-commenting**

I agree with the concerns raised by the other 2 reviewers, especially their comments about the need to clarify the mechanism of chromosome lagging versus chromosome congression and compaction.

I think that all of these suggestions, though, are contingent on them being able to reproduce their micronucleus rupture results with a better marker of nucleus integrity. I strongly believe that additional validation of lamin A as a micronucleus rupture marker will demonstrate that it is unreliable, based both on our own observations in RPE-1 cells and the images they show.
We would like to thank the reviewers for taking the time to carefully evaluate our manuscript. The paper will be significantly improved by their suggestions, and we are grateful for their perspectives.

To address the reviewers’ concerns, we will complete additional control experiments and revise the manuscript as detailed below.

Reviewer #1 (Evidence, reproducibility and clarity (Required)):

In the present work Stumpff, Reinholdt and co-workers investigate the mechanism by which micronuclei contribute to tumorigenesis. Micronuclei are classic markers of genomic instability widely used in the diagnosis of cancer, but whether they work as drivers of the process has recently attracted significant attention due to their link with chromothripsis. Here, the Stumpff/Reinhold labs have explored an interesting model to test some ideas about the role of micronuclei as drivers of tumorigenesis, based on Kif18A/p53 double KO mice. They confirm the formation of micronuclei in these animals, but find no substantial increase in survival and tumor incidence relative to p53 KO animals, despite higher incidence of micronuclei in Kif18A/p53 KO tumors. They conclude that, per se, micronuclei do not have the capacity to form tumors, regardless of p53 status. This was surprising, given the well-established role of p53 in preventing the proliferation of micronucleated cells. To shed light into this apparent paradox, they compared micronuclei from Kif18A KO cells with micronuclei generated by a number of other experimental conditions that promote formation of anaphase lagging chromosomes or generates acentric fragments. They found that micronuclei derived from Kif18A are intrinsically different from micronuclei generated by those other means and essentially showed increased accumulation of lamin B, were more resistant to rupture and preserved the capacity to expand as cells exited mitosis. Of note, they find a correlation between chromosome proximity to the poles/main chromosome mass and the different features that characterize micronuclei from Kif18A KO cells, compared with the other experimental conditions in which late lagging chromosomes are more frequent. Overall, I find this study extremely interesting, well designed and executed in a rigorous way that characterizes the consistent solid work from these laboratories over the years. I have just few minor points that I recommend to be addressed prior to publication.

1-Abstract and main text lines 70 and 100: the authors indicate that Kif18A mutant mice produce micronuclei due to unaligned chromosomes. This is correct, but at the same time misleading. The authors should clarify that although micronuclei derive from compromised congression, I was convinced from previous works (Fonseca et al., JCB, 2019) that it was their asynchronous segregation in anaphase that led to micronuclei formation. As is, a less familiar reader may conceive that misaligned chromosomes directly result in micronuclei, for example by being detached from the main chromosome mass.

We thank the reviewer for raising this point. We agree that micronuclei form in the absence of KIF18A due to chromosome alignment defects, which reduces interchromosomal compaction and leads to asynchronous arrival of chromosomes at spindle poles during anaphase. As the reviewer suggests, micronuclei form around chromosomes that travel longer distances and arrive late to the poles. We have revised the manuscript to clarify this (Lines 12-13, 72-73, 102).
2-Page 2, line 59: "cells entering cell division...become fragmented". It is not the cells, but the chromosomes that fragment. Please correct.

   We have revised this wording to indicate it is the chromosomes within micronuclei which fragment (Line 60-63).

3-Page 4, line 149: "reduced survival in the Kif18A null, p53 mice". P53 what? KO, WT? Please clarify.

   We have revised this wording as suggested, to read: “reduced survival in the Kif18a<sup>gcd2/gcd2</sup>, p53<sup>-/-</sup> mice,” (Line 158).

4-Page 5, line 212: the authors refer that micronuclei were scored for absence of lamin A/C, but previously they scored it as "continuous/discontinuous". Please clarify.

   Thank you for raising this question. When we scored lamin A/C, we noted cases where lamin A/C signal was incompletely present (not fully co-localizing with the micronuclear area, as indicated by DAPI). In these infrequent cases, micronuclei were identified as having “discontinuous” lamin A/C signal and were binned with those micronuclei lacking lamin A/C, for purposes of creating a binary readout of the micronuclear envelope: either 1) “intact” (having full, completely continuous lamin A/C signatures) or 2) “ruptured” (lacking a complete micronuclear signal of lamin A/C). We will update the text and the methods to more clearly reflect this categorization (Lines 221-225; 603-607).

5-Page 6, line 243: "Kif18A is not required for micronuclear envelope rupture". Shouldn't it be micronuclear envelope "integrity"?

   We apologize for the confusion here. The experiment performed was designed to distinguish whether micronuclear envelopes are more stable in KIF18A KO cells or if KIF18A itself is somehow required for the rupture of all micronuclear envelopes to occur. Since nocodazole-induced micronuclei were able to rupture in KIF18A KO cells at similar frequencies to those seen in control cells, the data indicate that KIF18A is not required for the process of micronuclear envelope rupture. We modified the text to improve clarity (lines 252-253).

6-One of the most interesting results of the paper is the correlation between envelope formation in micronuclei with their respective position relative to the poles/midzone. Could the authors try to investigate causality? For instance, the authors refer to works from other labs in which MT bundles and a midzone Aurora B activity gradient might play a role in the different features associated with micronuclear envelope formation, depending on their origin. Could the authors manipulate this gradient and investigate whether it changes the outcome in terms of nuclear envelope assembly properties on micronuclei? Are there any detectable features in midzone MT organization in Kif18A KO cells that would justify the observed differences?

   We agree that this result is very interesting. However, we feel the proposed experiments would repeat previous work and are somewhat outside the purview of the present study. Elegant experiments to address Aurora’s role in preventing micronucleus formation have already been
performed using genetic approaches in Drosophila neuroblasts and small molecule inhibitors in mammalian cells and Drosophila S2 cells (PMIDs: 24925910, 25877868, and 29986897). Interpreting effects of Aurora B inhibition are complicated by the many critical roles Aurora B plays in ensuring proper and faithful chromosome segregation. Thus, experiments to precisely test Aurora’s effect on micronuclear envelope stability require addition of Aurora B inhibitors on a cell-by-cell basis, administered within a narrow window of minutes during anaphase. It would require significant effort to obtain enough cells from different experimental conditions to make a meaningful comparison.

The suggestion to investigate detectable differences or features in midzone MT organization in KIF18A KO cells is also appreciated. We have not observed gross differences in midzone microtubules in KIF18A KO cells, but we will quantitatively evaluate this and add these results to the revised manuscript.

Reviewer #1 (Significance (Required)):

Kif18A plays a key role in chromosome alignment, without apparently affecting kinetochore-microtubule attachments in non-transformed cells. Because they cannot establish a proper metaphase plate Kif18A KO cells enter anaphase with highly asynchronous segregation due to non-uniform chromosome distribution along the spindle axis. Consequently, some "delayed" chromosomes form micronuclei, in cell culture and in vivo. Interestingly, prior art has failed to detect any increased signs of genomic instability in Kif18A KO cells and mice, and, contrary to what would be expected based on current trends, these mice do now show any signs of increased incidence of tumors, in fact they even show some protective effect to induced colitis-associated colorectal cancer. Noteworthy, all previous experimental works pointing to a role of micronuclei as key intermediates of genomic instability in cancer relied on models in which the tumor suppressor protein p53 had been inactivated. In the present work, the authors explore the relationship between micronuclei formation and p53 inactivation by investigating tumor formation in Kif18A/p53 double KO animals (1 or 2 alleles of p53 inactivated). The reported results are timely and will attract the interest of a broad readership, while decisively contributing to shed light into an ongoing debate. I am therefore all in favor for the publication of this work in any journal affiliated with review commons, pending some minor revisions.

Reviewer #2 (Evidence, reproducibility and clarity (Required)):

Sepaniac and colleagues use in vivo and in vitro approaches to examine why micronuclei generated by lack of KIF18A activity do not promote tumorigenesis. The authors conclude that micronuclei in KIF18A depleted cells form stable micronuclear envelopes, which may be a result from lagging chromosomes being closer to the spindle pole when the micronuclear envelope forms. The authors further conclude that the stability of the micronuclei arising from lack of KIF18A can explain why Kif18a mutant mice do not develop tumors. These results also suggest that the consequences of micronuclei and their possible contribution to tumorigenesis depend on the context of their genesis. While the mouse model data and characterization of the stability of micronuclei generated by different insults support the conclusions, the lagging chromosome
positioning data could be improved. Moreover, a number of other issues should be addressed prior to publication.

**Major issues:**

1. Line 153-155. The authors conclude that the slight reduction in overall survival is "due to a reduced ability of Kif18a mutants to cope with rapid tumorigenesis," but it is unclear why this would be the case. There is also an increase in micronucleated cells in thymic lymphomas from Kif18a/p53 homozygous mice (Fig. 2B)-could this not contribute? In Fig. 3C, the authors show that micronuclear rupture is similar in both Kif18a WT and mutant mice, so it seems possible that the increase in the frequency of micronuclei (Fig. 2B) coupled with a similar frequency of micronuclear rupture (Fig. 3C) could lead to the reduced survival. Then, in the discussion, the authors downplay this finding by saying (line 371) "loss of Kif18a had modest or no effect on survival of Trp53 homozygotes and heterozygotes." Why then speculate earlier in the text that loss of Kif18a reduces the ability to cope with tumorigenesis?

We thank the reviewer for pointing out this issue. Our goal here was to try and explain why the Kif18a/p53 mutant homozygotes display a small but significant reduction in survival compared to p53 mutants, while the Kif18a mutation does not impact survival of p53 heterozygotes, which could be considered a more sensitive model for detecting decreased survival. Kif18a homozygous mutants do display a small reduction in survival shortly after birth compared to heterozygote and wild type littermates (PMID: 25824710). Thus, we can't exclude the possibility that incompletely penetrant, postnatal lethality might be coincident with reduced fitness in surviving mutants, thus naming them more sensitive to loss of p53 loss of function. We have removed this statement form the revised text.

However, the reviewer's point that the combination of increased micronuclei in Kif18a/p53 homozygous mutants combined with a similar rupture rate seen in p53 mutants could also underlie or at least contribute to reduced survival is a good one. We have softened our conclusion in the Results section regarding the reduced survival of double homozygous mice (lines 158-164). We also agree that the way in which this point is addressed in the results and discussion sound contradictory. Thus, we have edited the language in the Discussion to improve consistency (lines 393-399).

2. Related to the point above, the authors show in figure 3 that the micronuclei found in healthy tissues display infrequent membrane rupture (panel B). However, micronuclear membrane rupture in tumor tissues is much more frequent (panel C). How do the authors explain this? Do they hypothesize that the micronuclei in the tumors originate by mechanisms other than the misalignment caused by lack of KIF18A? Does KIF18A depletion cause aneuploidy due to segregation of two sisters to the same pole? If so, one could expect the tumors to be aneuploid (is this the case?) and aneuploidy has been shown by numerous groups to cause genomic instability. Such genomic instability could then explain the difference in membrane rupture.

We agree that this is an interesting question. We plan to investigate several possible contributors to increased rupture in tumor cells in a separate study. As outlined in the Discussion (lines 443-458), we hypothesize that rupture could increase in tumor tissue due to changes in lamin expression or cytoskeletal forces in these cells. However, as the reviewer notes, differences
in aneuploidy could also potentially explain the differences in membrane rupture observed in healthy (non-tumorous) and thymic lymphoma tissues. For example, an increase in chromosome number could lead to lagging chromosomes being positioned closer to the midzone in Kif18a mutant cells or, as the reviewer suggests, the micronuclei could occur in aneuploid tumors due mitotic defects other than misalignment. This may be difficult to determine unequivocally in primary cell or tissue samples. However, we do have a limited quantity of primary thymic lymphoma-derived cells and we will use these to initially investigate aneuploidy in the two genotypes. The results of these studies will be added to the final revised manuscript. In addition, we will incorporate a discussion of how aneuploidy may increase rupture frequency in tumors into the revised manuscript.

3. The authors conclude that lagging chromosomes in KIF18A KO cells are found closer to the main chromatin mass. The Stumpff lab showed in a 2019 JCB paper that KIF18 KO cells have a chromosome alignment defect and as a result during anaphase the chromosomes can be scattered rather than forming the tight, uniform mass that is observed in WT cells. The scattering of kinetochores resulting from this phenotype could affect the value of "Avg Chromosomes Distances" in Fig 7B and the normalized distance in the KIF18A KO cells. Therefore, live-cell imaging experiments would be helpful to resolve this and possibly strengthen this conclusion. RPE1 cells with fluorescently tagged CENP-A and centrin could be used to ensure that the lagging chromosomes will not rejoin the main nucleus. Moreover, these cells could be used for correlative live-fixed cell experiments in which fixed cell analysis following micronucleus formation could be used to show that chromosomes that lag farther away from the spindle pole are more likely to have defective micronuclear envelopes.

The reviewer’s concern that the unalignment phenotype, characteristic of KIF18A KO cells, may impact the value of average chromosome distances used to set a threshold for chromosomes meeting our definition of lagging is valid. To address this, we analyzed the standard deviations for chromosome-to-pole distances within half spindles of KIF18A KO and nocodazole-washout treated anaphase cells as a way to compare chromosome scattering in these two conditions. This analysis revealed no significant difference between the standard deviations of chromosome positions in the two groups, suggesting that scattering is similar in nocodazole treated and KIF18A KO cells. We have included these data in the manuscript (Line 351-356, and additional data added to Figure S2C).

In order to further strengthen this conclusion, we are certainly willing to attempt the live cell imaging experiments suggested by the reviewer. We would like to point out that the frequency of micronucleus formation in the KIF18A KO cells is relatively low compared to the frequency seen after other experimental treatments (~7% of divisions result in a micronucleus). Thus, a large number of individual cells would need to be imaged with relatively high temporal resolution to make conclusions about the effects of chromosome position on micronuclear envelope formation (such analyses are not possible with the live data sets we currently have, where cells were imaged every 2 minutes). This difficulty led us to perform these measurements in synchronized and fixed cells to begin with.

4. Based on the Fonseca et al. 2019 JCB paper (video 2), micronuclei from KIF18A KO do not exclusively arise from lagging chromosomes. Instead, chromosomes can also escape the main
chromatin mass after segregation and subsequently be excluded from the main nucleus. It would be important to know what fraction of the micronuclei in KIF18A KO cells arise via lagging chromosomes. Since Aurora B and/or bundled microtubules at the spindle midzone are believed to prevent proper nuclear envelope formation, chromosomes that properly segregate but later become separated from the main nucleus would be more likely to form proper micronuclear envelopes than those arising from lagging chromosomes. The correlative microscopy experiment suggested in the previous point could allow differentiation between these two routes to micronucleus formation.

The reviewer is correct that we did occasionally see chromosomes escape the main chromatin mass after segregation in the Fonseca et al., 2019 study referenced. We did not quantify the frequency of these events in that study, but they were rare. To address this quantitatively, we have measured the incidence of micronuclear formation around lagging chromosomes and chromosomes that escape the main chromatin mass after segregation in videos of KIF18A KO cells. We find that when micronuclei form in these cells, they form around lagging chromosomes 98% (46 out of 47 events) of the time. These data were derived from 4 live cell imaging experiments. This information has been added to the Results section (line 328-330).

**Minor issues:**

1. Some parts of the manuscript are excessively wordy and some sentences are unclear or convoluted (e.g., lines 148-153 and 238-239).

   Thank you for this feedback. We have revised the text in these two locations to improve clarity (lines 159-162 and 247-248 in the revised manuscript).

2. Lines 59-61. This sentence is formulated incorrectly. First of all, the subject of the sentence is "cells" and the verb is "can become fragmented." However, the authors mean that the DNA in the micronucleus can become fragmented (not the cells). Moreover, the way the sentence is currently formulated seems to suggest that the fragmentation occurs during cell division. However, this is not the case. Please, revise the text to make it more accurate.

   We appreciate this point and have revised this text to reflect more precise language to describe this model. It is certainly the micronucleated chromatin which may become fragmented, and this fragmentation occurs as a result of replication stress, including replication fork collapse, after an existing micronucleated cell enters a subsequent round of S or G2 phase (PMIDs: 22258507, 26017310).

3. Lines 114-115. Please, provide references in support of this statement.

   The statement in question: "This arrest was at least partially dependent on p53, consistent with other reports of cell cycle arrest following micronucleation," shares the same references as the sentence that follows it (Sablina 1998, Thompson and Compton, 2010; Fonseca et al., 2019). We have updated the references to appear after the first statement to make this clear.
4. Line 153. The authors refer to Fig. 1C, but I think they mean Fig. 1B.

   Thank you, we have updated the text to read Fig 1B.

5. Line 324. The authors find that RPE1 KIF18A KO cells have lagging chromosomes in ana/telophase 9% of the time, then say that this shows that lagging chromosomes are rare in KIF18A KO cells. However, this is a large increase compared to normal RPE1 cells, which only have 1-2% frequency of lagging chromosomes. So, they should revise the text here to say that the rates of lagging chromosomes from KIF18A KO are lower compared to the rates induced by nocodazole washout.

   This is an important distinction. We have removed this confusing statement from the revised text (lines 336-338).

6. Line 383. The references listed here should be moved earlier and specifically after the statement summarizing the results of the studies instead of being listed after the authors' conclusion/interpretation of the data. The same issue was noted in other parts of the manuscript.

   We have corrected this error (Lines 402-408). Before final submission, we will further amend the style of the manuscript throughout to cite relevant papers after the statement summarizing the results of those studies, rather than after our interpretation of the studies.

7. Figure 1A. In the text, the authors say they cross a Kif18a heterozygous mutant mouse with a p53 heterozygous mutant mouse, but the two mice in this figure are already heterozygous for both. Please, revise the text or depict the previous additional cross necessary to obtain the double heterozygous.

   We thank the reviewer for catching this discrepancy. We have revised the text to describe the crosses necessary to obtain the double heterozygous mice shown in the figure (lines 121-123). The gcd2 mutation in Kif18a was named due to the “germ cell depleted” phenotype it causes. These homozygous mice are therefore infertile (Czechanski et al., 2015). For this reason, heterozygous mice for each gene were crossed to achieve the necessary homozygous progeny.

8. Figure 3A. Arrows or dotted circles outlining the micronuclei in the insets of the middle and bottom rows would be helpful since the DAPI signal in the micronuclei is low and somewhat difficult to see.

   We have updated these figures as suggested to more clearly indicate the micronuclear area.

9. Figure 3B. Error bars should be added to the graph. Moreover, the authors noted that the differences are not significant. However, this seems surprising, given that in some cases there is a three- to five-fold difference between certain pairs. Indeed, a chi-square test using the numbers from table S1 indicated p values <0.05 for several pairwise comparisons.
We appreciate this feedback on the statistical tests and comparisons among these data. The main point of these analyses is to demonstrate that tissues other than blood form micronuclei in vivo in the absence of Kif18a function and that the majority of these micronuclear envelopes are completely surrounded by Lamin A/C. The data presented in Figure 3B were obtained by counting several tissue types from a single mouse of each genotype. Thus, we do not believe that error bars are appropriate in this context. To avoid confusion, we have also removed the statistical bars which had indicated no significant differences in rupture frequency among the genotypes in each sampled tissue, as these are also probably inappropriate.

We understand the reviewer’s point that some pairwise comparisons of the data in Table S1 indicate that they are significantly different. We originally used a Chi-square test to compare the data from all three genotypes for each tissue. Because these data did not rise to the threshold of significance necessary to reject the null hypothesis across all three genotypes within each individual tissue type, we did not think performing pairwise comparisons between only two of those genotypes was appropriate (Whitlock and Schluter, The Analysis of Biological Data, 2009). Specifically, analyses of rupture frequency for spleen, liver, and thymus tissue gave p-values above 0.05 (spleen, p = 0.35; liver, p = 0.056; thymus, p = 0.052). Thus, we did not proceed with pairwise comparisons. In contrast, the analyses of p53 effects on micronucleus levels in peripheral blood in Fig 1D utilized samples from 8 individual mice for each genotype, and are therefore more amenable to statistical comparisons. If the reviewer believes any of the details of this approach are incorrect, we are happy to revise the analyses.

10. Figure 5G. When referring to this figure (lines 292-294), the authors talk about correlation. However, the points in this graph seem to be scattered a bit randomly. To address this concern, we performed a Pearson’s correlation test on the data in Figure 5G. As suspected by the reviewer, this analysis did not indicate a significant correlation, and we have removed this plot from the manuscript.

11. Figure 6B-D. The Y-axis titles of the three graphs are a bit confusing. Please, consider revising. We have updated the Y-axis titles for these graphs to more accurately represent what is displayed on each plot.

12. In Figure 7 and the text, the authors use the terms "late-lagging" and "lagging" chromosomes interchangeably, which is somewhat confusing in this context because lagging chromosome distance from the main chromosome mass is thought to contribute to defective assembly of micronuclear envelopes. It is not clear whether the authors intend to indicate, with this term, that the lagging chromosome is farther away from the main chromosome mass or that the lagging chromosome is in a "late" anaphase cell. Because this is confusing, I suggest just using the term "lagging chromosome" consistently. It could be useful to include representative images of lagging chromosomes located at different distances from the main chromosome mass. And certainly, the authors should include an example of a lagging chromosome in the KIF18A KO cells.

We agree with the reviewer’s concern regarding confusion of these terms. We have updated the text to use the term “lagging chromosome” consistently, as the reviewer suggests.
We have also updated Figure 7A to include a representative image of a lagging chromosome in a KIF18A KO cell.

13. Figure S2A. The example in the bottom right image looks more like a chromosome bridge than a lagging chromosomes. Kinetochore staining is necessary to unequivocally identify lagging chromosomes.

    We agree with the reviewer that kinetochore staining is necessary to precisely identify lagging chromosomes. We had used these images to quickly and crudely assess the presence and frequency of potentially lagging chromosomes, observed in late-anaphase cells by eye, and for subsequent experiments where lagging chromosomes were measured, repeated these experiments with proper staining of poles and kinetochores to make precise, quantifiable assessments.

Reviewer #2 (Significance (Required)):

Based on the previous knowledge on the factors that cause abnormal assembly of the micronuclear membrane, the results presented in this study were somewhat predictable. However, these findings will add to the knowledge of how micronuclei form and the potential factors that lead to micronuclear membrane rupture. Previous studies investigating micronucleus behavior have focused on micronuclei arising via merotelic kinetochore mis-attachments. These mis-attachments lead to formation of micronuclei close to the spindle midzone. In the present study, instead, the micronuclei arising from lack of KIF18A activity form farther away from the spindle midzone. The results presented here suggest that the positioning of these micronuclei farther away from the midzone enables assembly of a more stable micronuclear membrane that will be less likely to rupture during the following cell cycle. A recent study showed that the microtubule bundles in the spindle midzone interfere with micronuclear membrane assembly. Based on this, it is not surprising that micronuclei forming away from the spindle midzone (like those resulting from lack of KIF18A activity) assemble more normal membranes. Although somewhat expected, this study provides the actual data in support of this phenomenon. This study will be of interest to cell biologists interested in cell division and genomic instability. My research has focused on cell division, aneuploidy, and chromosomal instability for nearly thirty years. Therefore, I believe I am fully qualified to evaluate this manuscript.

**Referees cross-commenting**

My areas of expertise do not include nuclear membrane structure and function. Therefore, I encourage the authors to consider the comments of reviewer #3 for issues related to reliable quantification of micronuclear membrane rupture.
Reviewer #3 (Evidence, reproducibility and clarity (Required)):

**Summary**

Sepaniac et al demonstrate that loss of KIF18a, a motor protein required for proper chromosome congression and chromatin compaction during mitosis, is insufficient to drive tumor development in mice although it does increase the frequency of micronuclei (MN), nuclear compartments that form around broken or missegregated chromosomes, in both normal and tumor tissue. MN are thought to increase genome instability and metastasis by undergoing DNA damage and activating innate immune signaling after irreparable nuclear membrane rupture. The authors use a non-transformed human cell line, hTERT-RPE-1, with KIF18a knocked out to demonstrate that MN formed as a result of KIF18a loss have more stable nuclear membranes than MN generated by other methods. They go on to correlate this increased stability with increased chromosome proximity to the main chromatin mass during nuclear envelope assembly and increased chromatin decompaction by a combination of fixed and live cell imaging.

**Major Comments**

1. This study relies heavily on the use of lamin A loss or discontinuity to identify ruptured micronuclei. Although the authors validate this marker against "leakage" of the soluble nuclear protein mCherry-NLS, there are several lines of evidence suggesting that lamin A loss or disruption is not a reliable reporter. In figure S3C, the top two panels of intact MN in the KIF18KO appear ruptured based on the gH2AX labeling, yet have significant levels of lamin A and are labeled as intact. In figure 4D, the rate of MN rupture after nocodazole release (60% ruptured in 2 hours) is much faster than that reported in other papers (40-60% in 16-18 hours, Liu et al; 60% in 16 hours; Hatch et al). In addition, images in Hatch et al, 2013 show lamin A localizing to both intact and ruptured MN and anecdotal information in the field suggests that lamin A localization is not a reliable reporter.

These discrepancies may be due to how the authors' define "mCherry-NLS leakage", which needs to be defined in the methods as previous studies have demonstrated that MN frequently have delayed or reduced nuclear import even though the membrane is intact. Regardless, the authors need to provide compelling independent evidence that lamin A loss and disruption faithfully recognize ruptured MN by either validating this marker against additional rupture reporters, such as Lap2, LBR, or emerin accumulation, or by repeating key experiments in cells expressing mCherry-NLS.

Our decision to use lamin A/C as a reporter was based on its use as a marker for micronuclear envelope presence in prior studies (Hatch, 2013; Liu, 2018). We were unaware of
anecdotal information in the field that suggests that lamin A localization may not be a reliable reporter.

However, we think we understand the reviewer's point to be that although it is clear from prior studies that gaps in the nuclear lamina are a known predictor of micronuclear rupture, these gaps can persist for some time before rupture has actually occurred. We agree that this is an important distinction and thank the reviewer for raising these questions.

As the reviewer notes, we performed control experiments to address this issue and validate the use of lamin A/C as a marker of micronuclear envelope rupture. Our approach involved correlating lamin staining with the localization of mCherry-NLS signal to the micronucleus (Figure S1). We found that these signals correlated well. As the reviewer points out, this analysis in fixed cells could be misleading in cases where nuclear import is reduced, but the micronuclear envelope is intact. If this were a significant contributor, we may have expected to see greater instances of micronuclei that exhibit continuous lamin A/C signal but lack nuclear localization of mCherry-NLS. However, we found this combination was rare among the KIF18A and RPE1 nocodazole washout treated cells (2%, or 1 of 46 micronuclei had continuous lamin A/C while lacking mCherry-NLS). We admit that this assumption may be oversimplified though.

The reviewer's point about the timing of nocodazole treatment and washout something we have definitely considered. We note that prior studies have used differing time points after nocodazole treatment and release. For Hatch et al., 2013: U2OS cells were treated for 6 hours with nocodazole and then subjected to mitotic shakeoff, 48% of micronuclei were ruptured after 6 hours and ~60% were ruptured after 16 hours. Similarly, in Liu et al., 2018 60% of micronuclei were ruptured 16 hours post mitotic shake off and nocodazole release. While these results suggest that rupture increases with time after mitosis, it isn’t clear how early rupture may occur. In other words, does it take several hours in G2 before nearly half of micronuclei rupture or do many of these rupture shortly after cell division?

We note that other explanations could also potentially contribute to the differences in rupture rates reported in our study compared to those in previous publications. For example, we used a short nocodazole treatment (2 hrs) compared to the longer treatments (6 hrs) used in previous studies. We did this originally in order to produce a similar percentage of micronucleated cells as is seen in KIF18A KO cell populations. However, the difference in nocodazole treatment length could potentially influence the types and frequencies of kinetochore microtubule attachments formed. For example, if centrosomes stay closer together in mitotic cells after short nocodazole treatments, this could increase the number of abnormal attachments (e.g. PMID: 22130796). Such an effect would be expected to increase the frequency of lagging chromosomes and/or potentially produce more lagging chromosomes within the anaphase midzone.

The best way to address this issue would be to repeat our analyses of mcherry-NLS in live cells to track the formation and rupture of micronuclei. We did attempt these live imaging experiments previously and have found this experiment challenging due to: 1) the low frequency of micronuclear formation in KIF18A KO cell population; 2) a low transfection/expression efficiency for the mCherry-NLS plasmid in RPE1 cells, and 3) photobleaching of the mCherry-NLS plasmid. For these reasons, we transitioned into fixed cell experiments for the mCherry-NLS reporter. However, we propose to troubleshoot this assay and attempt to obtain the data necessary to determine when rupture is occurring. In addition, we will use additional markers to investigate micronuclear envelope stability, as the reviewer has suggested.
Regardless of the outcome of these experiments, we have measured a clear difference between the lamin deposition within micronuclear envelopes of KIF18A KO cells compared to those formed following other insults. Lamin recruitment is well established as a predictor of nuclear envelope stability. If necessary, we could alter the text to indicate that the presence of lamin A/C and B within micronuclear envelopes of KIF18A KO cells are indicative of nuclear envelope stability, and that this is distinct from the lamin profiles of micronuclei in cells subjected to nocodazole-washout.

2. Micronuclei in tumor sections and other dense tissues can appear very similar to other types of chromatin, including blebs from adjacent nuclei and dead cells. To verify that the quantified structures are bona fide micronuclei, the authors need to include a marker for the cell boundary. This is especially critical in the lamin a stained tumor sections with heterogenous lamin A protein expression.

We appreciate the point this reviewer raises and we carefully considered accurate identification of micronuclei in tissues. Three optical sections were collected from each sample. During analyses, we scrolled through the ~2-micron thick sections to exclude chromatin bodies connected to an out-of-plane nucleus or nuclear bleb. We have a limited number of sectioned and preserved thymic lymphoma tissues remaining. We will use these samples to reassess micronuclear frequency in the presence of a cell boundary marker.

3. Figure 4 compares MN rupture frequency between cells treated with different inducers of micronuclei - KIF18A KO, nocodazole release, and irradiation. These treatments have different effects on the cell cycle: KIF18A causes minor delays, nocodazole arrests cells in mitosis, and g-IR likely causes delays in S and G2. Since MN rupture frequency increases with the duration of interphase, the authors need to assess rupture frequency at similar time points after mitosis for all three conditions. One way to accomplish this would be to repeat this experiment and analyze cells collected by mitotic cells by shake-off prior to fixation and labeling.

We appreciate this point regarding differences in mitotic timing. Since micronuclear rupture frequency increases with time in interphase, we would expect the MN in KIF18A KO cells to exhibit the highest level of rupture if cell cycle timing were the primary variable affecting stability in our experiments. KIF18A KO cells are asynchronously dividing, and the micronuclei examined in populations of those cells could have been generated at any time. We do not have the same type of temporal control of these events as we do with drug treatment. In contrast, the vast majority of the MN in nocodazole washout cells would not have been in interphase for more than 1.5 hours in our experiments, yet showed increased lamin A/C defects. RPE1 cells treated with MAD2 siRNA knockdown, which do not experience mitotic delays (PMID: 9606211; 15239953), also showed greater frequencies of micronuclear envelopes which lacked lamin A/C compared to those arising in KIF18A KO cells.

To further address this question, we could attempt a mitotic shake-off assay, however, we believe that the formation of micronuclei, as a percentage in the population of KIF18A KO cells, will be limiting in these experiments.

As an alternative, we propose to use live cell imaging to follow micronuclear formation and rupture, as described above in reference to point 1.
1. In figure 6A, it is unclear when the videos start and how micronuclei are selected for analysis. Do the micronuclei have to be continuously visible from the time they missegregate? Do the videos all start at the same time point during mitosis or is it contingent on when the MN appears separated from the main nucleus? One concern is that a consistent delay in micronucleus appearance in the nocodazole treated cells could artificially decrease the amount of MN expansion observed.

   We thank the reviewer for these questions. The individual micronuclei did not need to be continuously visible from the time that they missegregated, though the majority were. When a micronucleus was not sufficiently in the plane of focus for an accurate area measurement, the individual measurement at that time point was not collected. In cases where one or more frames which were not measurable, a micronucleus was only included in the final data set if it was 1) the only micronucleus present in the daughter cell or 2) easily identifiable to be the same micronucleus. Measurements were taken until the micronuclear area reached an equilibrium for several frames. Final fold change in area was established by dividing final area measurements by initial measurements.

   The initial measurement for each micronucleus taken from the videos all start at the same relative point during mitosis, which is just after chromosome segregation has occurred.

2. In figure 7A, it is difficult to identify the "lagging" chromosome in the top panel. It would be helpful to label the chromosome that becomes the MN, or ideally, to include a video or still images to demonstrate how micronuclei form in the KIF18A KO cells.

   We have updated the images in Figure 7A to include an example of a lagging chromosome in a KIF18A KO cell. We will also include a more explicit reference to our previous study (Fonseca et al., 2019), which described how micronuclei form around lagging chromosomes in KIF18A KO RPE1 cells.

3. The two image panels in figure 7A are imaged at significantly different times during anaphase (early anaphase on bottom versus late anaphase/telophase on top). A better comparison would be between two cells at the same time point in anaphase.

   We have updated the images in Figure 7A to compare cells at similar stages of anaphase. In our quantification of lagging chromosomes, we also accounted for anaphase-timing differences by normalizing all measurements within each half-spindle.

Reviewer #3 (Significance (Required)):

In this study, the authors identify chromatin decondensation in micronuclei as a new predictor of
membrane stability. Although these results are correlative, if their micronucleus rupture results can be validated as described in major comment 1, this study would advance our understanding of the micronucleus rupture mechanism by linking mitotic spindle location, chromatin decondensation, and lamin B1 protein recruitment. This would provide needed support to a current model in the field that micronucleus stability is largely determined during nuclear envelope assembly. In addition, if KIF18a loss generates stable micronuclei at high frequency, it will become a critical system for testing MN rupture hypotheses in the field. Thus, this work would be of significant interest to cell biologists working on nuclear envelope structure and function, chromosome organization, and mitosis. I include myself in this group as a cell biologist studying nuclear envelope structure and function with an expertise in membrane dynamics.

The authors also find that mice mutant for KIF18a have increased micronucleation in normal tissues but not increased tumor initiation. They hypothesize that this is due to the low rupture frequency of KIF18a-induced MN, however their data cannot reject the null hypothesis that the small increase in MN they see in KIF18a mutant mice would be insufficient to induce tumorigenesis even if rupture frequency was high. Thus the significance of their finding that micronucleation is not sufficient for cancer progression is unclear. However, the thorough analysis of micronucleation and rupture in several healthy tissues as well as a tumor model in KIF18 mutant mice would be of interest to both pathologists and cancer researchers focused on mechanisms of genome instability. These types of experiments are critical to determine how micronuclei contribute to cancer progression and the quantifications presented in this paper are truly impressive.

We appreciate this reviewer’s enthusiasm for our work and acknowledge that we cannot definitively conclude that micronuclear envelope stability explains why Kif18a mutant mice do not form tumors. However, it is interesting to note that the micronuclear loads measured using a peripheral erythrocyte assay are similar in Kif18agcd2/gcd2 mutant mice (0.6% micronucleated erythrocytes, of total erythrocytes) and ATM^{tm1 Awb/tm1 Awb} mutant mice (0.6% of micronucleated erythrocytes, of total) (Fonseca et al., 2019). Yet, the tumor frequency in these two models is dramatically different: Kif18agcd2/gcd2 mutant mice do not spontaneously form tumors – while the majority of ATM^{tm1 Awb/tm1 Awb} mutant mice do develop thymic lymphoma tumors between 2 and 4 months (Barlow, 1996). It is not clear how much micronuclei contribute to tumorigenesis in the ATM mutant model, but this comparison does suggest that the increase in MN seen in Kif18a mutants may be physiologically relevant. We have added this information to the revised text (lines 125-130).

**Referees cross-commenting**

I agree with the concerns raised by the other 2 reviewers, especially their comments about the need to clarify the mechanism of chromosome lagging versus chromosome congression and compaction.

I think that all of these suggestions, though, are contingent on them being able to reproduce their micronucleus rupture results with a better marker of nucleus integrity. I strongly believe that additional validation of lamin A as a micronucleus rupture marker will demonstrate that it is unreliable, based both on our own observations in RPE-1 cells and the images they show.
February 5, 2021

Re: JCB manuscript #202101165T

Dr. Jason Stumpff
University of Vermont
149 Beaumont Avenue
HSRF 118
Burlington, Vermont 05405

Dear Dr. Stumpff,

Thank you for submitting your manuscript entitled "Micronuclei arising due to loss of KIF18A form stable micronuclear envelopes and do not promote tumorigenesis". We editorially assessed the manuscript along with the expert reviews obtained at Review Commons. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

The relationship between micronuclei and tumorigenesis is fascinating and of high interest to our audience. Editorially, we feel that the work has the potential to provide an important advance in our understanding of the determinants controlling micronucleus stability and fate as well as the fate of micronucleus-containing cells. We however also feel that the reviewers' points are valid and need to be addressed thoroughly in revision. In particular, the remarks from Reviewer #2 and Reviewer #3 need to be addressed in full. Reviewer #2 requested stronger lagging chromosome analyses and stressed that live cell imaging was needed. We checked in with Reviewer #2 before making our decision and the reviewer maintained that these analyses, albeit challenging, would be important to strengthen your interpretation. Further, we agree with Rev#3 that the inclusion of other markers to monitor envelope integrity is essential.

Convincing new evidence resolving the referees' concerns will be needed for publication in JCB. Upon resubmission to JCB, the revision will go back to the original revs. As you know, JCB policy limits all papers to one round of major experimental revision. For publication in JCB, we'd expect all reviewers to be convinced of the key conclusions without the need for additional major experimentation. We are happy to discuss further if that is helpful or if you anticipate any issues with the revision.

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.

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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.

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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.

Thank you for this interesting contribution to the Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

Roger Greenberg, MD, PhD
Monitoring Editor, Journal of Cell Biology

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

---------------------------------------------------------------------------
We would like to thank the reviewers for taking the time to carefully evaluate our manuscript. The paper will be significantly improved by their suggestions, and we are grateful for their perspectives.

To address the reviewers’ concerns, we completed additional control experiments and revised the manuscript as detailed below. Major additions are highlighted in red text in the revised manuscript.

Reviewer #1 (Evidence, reproducibility and clarity (Required)):

In the present work Stumpff, Reinholdt and co-workers investigate the mechanism by which micronuclei contribute to tumorigenesis. Micronuclei are classic markers of genomic instability widely used in the diagnosis of cancer, but whether they work as drivers of the process has recently attracted significant attention due to their link with chromothripsis. Here, the Stumpff/Reinhold labs have explored an interesting model to test some ideas about the role of micronuclei as drivers of tumorigenesis, based on Kif18A/p53 double KO mice. They confirm the formation of micronuclei in these animals, but find no substantial increase in survival and tumor incidence relative to p53 KO animals, despite higher incidence of micronuclei in Kif18A/p53 KO tumors. They conclude that, per se, micronuclei do not have the capacity to form tumors, regardless of p53 status. This was surprising, given the well-established role of p53 in preventing the proliferation of micronucleated cells. To shed light into this apparent paradox, they compared micronuclei from Kif18A KO cells with micronuclei generated by a number of other experimental conditions that promote formation of anaphase lagging chromosomes or generates acentric fragments. They found that micronuclei derived from Kif18A are intrinsically different from micronuclei generated by those other means and essentially showed increased accumulation of lamin B, were more resistant to rupture and preserved the capacity to expand as cells exited mitosis. Of note, they find a correlation between chromosome proximity to the poles/main chromosome mass and the different features that characterize micronuclei from Kif18A KO cells, compared with the other experimental conditions in which late lagging chromosomes are more frequent. Overall, I find this study extremely interesting, well designed and executed in a rigorous way that characterizes the consistent solid work from these laboratories over the years. I have just few minor points that I recommend to be addressed prior to publication.

1-Abstract and main text lines 70 and 100: the authors indicate that Kif18A mutant mice produce micronuclei due to unaligned chromosomes. This is correct, but at the same time misleading. The authors should clarify that although micronuclei derive from compromised congression, I was convinced from previous works (Fonseca et al., JCB, 2019) that it was their asynchronous segregation in anaphase that led to micronuclei formation. As is, a less familiar reader may conceive that misaligned chromosomes directly result in micronuclei, for example by being detached from the main chromosome mass.

We thank the reviewer for raising this point. We agree that micronuclei form in the absence of KIF18A due to chromosome alignment defects, which reduces interchromosomal compaction and leads to asynchronous arrival of chromosomes at spindle poles during anaphase. As the reviewer suggests, micronuclei form around chromosomes that travel longer distances and arrive late to the poles. We have revised the manuscript to clarify this (Lines 13-14, 68-69, 105).
2-Page 2, line 59: "cells entering cell division...become fragmented". It is not the cells, but the chromosomes that fragment. Please correct.

We have revised this wording to indicate it is the chromosomes within micronuclei which obtain structural defects (Line 57-59).

3-Page 4, line 149: "reduced survival in the Kif18A null, p53 mice". P53 what? KO, WT? Please clarify.

We have revised the text to indicate the precise genotype being referred to in this context (Lines 143-150).

4-Page 5, line 212: the authors refer that micronuclei were scored for absence of lamin A/C, but previously they scored it as "continuous/discontinuous". Please clarify.

Thank you for raising this question. When we scored lamin A/C, we noted cases where lamin A/C signal was incompletely present (not fully co-localizing with the micronuclear area, as indicated by DAPI). In these infrequent cases, micronuclei were identified as having "discontinuous" lamin A/C signal and were binned with those micronuclei lacking lamin A/C for purposes of creating a binary readout of the micronuclear envelope: either 1) "intact" (having completely continuous lamin A/C signatures) or 2) "ruptured" (lacking a complete micronuclear signal of lamin A/C). We have updated the text and the methods to more clearly reflect this categorization (Lines 171-173; 217-218; 633-636).

5-Page 6, line 243: "Kif18A is not required for micronuclear envelope rupture". Shouldn't it be micronuclear envelope "integrity"?

We apologize for the confusion here. The experiment performed was designed to distinguish whether micronuclear envelopes are more stable in KIF18A KO cells or if KIF18A itself is somehow required for the rupture of all micronuclear envelopes to occur. Since nocodazole-induced micronuclei were able to rupture in KIF18A KO cells at similar frequencies to those seen in control cells, the data indicate that KIF18A is not required for the process of micronuclear envelope rupture. We modified the text to improve clarity (Lines 251-261).

6-One of the most interesting results of the paper is the correlation between envelope formation in micronuclei with their respective position relative to the poles/midzone. Could the authors try to investigate causality? For instance, the authors refer to works from other labs in which MT bundles and a midzone Aurora B activity gradient might play a role in the different features associated with micronuclei envelope formation, depending on their origin. Could the authors manipulate this gradient and investigate whether it changes the outcome in terms of nuclear envelope assembly properties on micronuclei? Are there any detectable features in midzone MT organization in Kif18A KO cells that would justify the observed differences?

We agree that this result is very interesting. However, we feel the proposed experiments would repeat previous work and are somewhat outside the purview of the present study. Elegant experiments to address Aurora's role in preventing micronucleus formation have already been
performed using genetic approaches in Drosophila neuroblasts and small molecule inhibitors in mammalian cells and Drosophila S2 cells (PMIDs: 24925910, 25877868, and 29986897). Interpreting effects of Aurora B inhibition are complicated by the many critical roles Aurora B plays in ensuring proper and faithful chromosome segregation. Thus, experiments to precisely test Aurora’s effect on micronuclear envelope stability require addition of Aurora B inhibitors on a cell-by-cell basis, administered within a narrow window of minutes during anaphase. We feel these experiments are beyond the scope of the current manuscript. This question was also recently readdressed in a preprint from the Maiato lab:

https://www.biorxiv.org/content/10.1101/2021.02.26.433009v1.

Reviewer #1 (Significance (Required)):

Kif18A plays a key role in chromosome alignment, without apparently affecting kinetochore-microtubule attachments in non-transformed cells. Because they cannot establish a proper metaphase plate Kif18A KO cells enter anaphase with highly asynchronous segregation due to non-uniform chromosome distribution along the spindle axis. Consequently, some "delayed" chromosomes form micronuclei, in cell culture and in vivo. Interestingly, prior art has failed to detect any increased signs of genomic instability in Kif18A KO cells and mice, and, contrary to what would be expected based on current trends, these mice do now show any signs of increased incidence of tumors, in fact they even show some protective effect to induced colitis-associated colorectal cancer. Noteworthy, all previous experimental works pointing to a role of micronuclei as key intermediates of genomic instability in cancer relied on models in which the tumor suppressor protein p53 had been inactivated. In the present work, the authors explore the relationship between micronuclei formation and p53 inactivation by investigating tumor formation in Kif18A/p53 double KO animals (1 or 2 alleles of p53 inactivated). The reported results are timely and will attract the interest of a broad readership, while decisively contributing to shed light into an ongoing debate. I am therefore all in favor for the publication of this work in any journal affiliated with review commons, pending some minor revisions.

Reviewer #2 (Evidence, reproducibility and clarity (Required)):

Sepaniac and colleagues use in vivo and in vitro approaches to examine why micronuclei generated by lack of KIF18A activity do not promote tumorigenesis. The authors conclude that micronuclei in KIF18A depleted cells form stable micronuclear envelopes, which may be a result from lagging chromosomes being closer to the spindle pole when the micronuclear envelope forms. The authors further conclude that the stability of the micronuclei arising from lack of KIF18A can explain why Kif18a mutant mice do not develop tumors. These results also suggest that the consequences of micronuclei and their possible contribution to tumorigenesis depend on the context of their genesis. While the mouse model data and characterization of the stability of micronuclei generated by different insults support the conclusions, the lagging chromosome positioning data could be improved. Moreover, a number of other issues should be addressed prior to publication.
**Major issues:**

1. Line 153-155. The authors conclude that the slight reduction in overall survival is "due to a reduced ability of Kif18a mutants to cope with rapid tumorigenesis," but it is unclear why this would be the case. There is also an increase in micronucleated cells in thymic lymphomas from Kif18a/p53 homozygous mice (Fig. 2B)—could this not contribute? In Fig. 3C, the authors show that micronuclear rupture is similar in both Kif18a WT and mutant mice, so it seems possible that the increase in the frequency of micronuclei (Fig. 2B) coupled with a similar frequency of micronuclear rupture (Fig. 3C) could lead to the reduced survival. Then, in the discussion, the authors downplay this finding by saying (line 371) "loss of Kif18a had modest or no effect on survival of Trp53 homozygotes and heterozygotes." Why then speculate earlier in the text that loss of Kif18a reduces the ability to cope with tumorigenesis?

   We thank the reviewer for pointing out this issue. Our goal here was to try and explain why the Kif18a/p53 mutant homozygotes display a small but significant reduction in survival compared to p53 mutants, while the Kif18a mutation does not impact survival of p53 heterozygotes, which could be considered a more sensitive model for detecting decreased survival. Kif18a homozygous mutants do display a small reduction in survival shortly after birth compared to heterozygote and wild type littermates (PMID: 25824710). Thus, we can’t exclude the possibility that incompletely penetrant, postnatal lethality might be coincident with reduced fitness in surviving mutants, thus making them more sensitive to loss of p53 loss of function. We have removed this statement from the revised text.

   However, the reviewer’s point that the combination of increased micronuclei in Kif18a/p53 homozygous mutants combined with a similar rupture rate seen in p53 mutants could also underlie or at least contribute to reduced survival is a good one. We have softened our conclusion in the Results section regarding the reduced survival of double homozygous mice (lines 157-158). We also agree that the way in which this point is addressed in the results and discussion sound contradictory. Thus, we have edited the language in the Discussion to improve consistency (lines 390-400).

2. Related to the point above, the authors show in figure 3 that the micronuclei found in healthy tissues display infrequent membrane rupture (panel B). However, micronuclear membrane rupture in tumor tissues is much more frequent (panel C). How do the authors explain this? Do they hypothesize that the micronuclei in the tumors originate by mechanisms other than the misalignment caused by lack of KIF18A? Does KIF18A depletion cause aneuploidy due to segregation of two sisters to the same pole? If so, one could expect the tumors to be aneuploid (is this the case?) and aneuploidy has been shown by numerous groups to cause genomic instability. Such genomic instability could then explain the difference in membrane rupture.

   We agree that this is an interesting question. We plan to investigate several possible contributors to increased rupture in tumor cells in a separate study. As outlined in the Discussion (lines 438-449), we hypothesize that rupture could increase in tumor tissue due to changes in lamin expression or cytoskeletal forces in these cells. However, as the reviewer notes, differences in aneuploidy could also potentially explain the differences in membrane rupture observed in healthy (non-tumorous) and thymic lymphoma tissues. For example, an increase in chromosome
number could lead to lagging chromosomes being positioned closer to the midzone in Kif18a mutant cells or, as the reviewer suggests, the micronuclei could occur in aneuploid tumors due to mitotic defects other than misalignment. This may be difficult to determine unequivocally in primary cell or tissue samples. However, we did retain a limited quantity of primary thymic lymphoma-derived cells and to address these questions, we took advantage of these samples to investigate aneuploidy in the two genotypes. The results of these studies suggest that tumor cells from both genotypes are similarly aneuploid (new Figure S1, Lines 155-156). In addition, we have incorporated a discussion of how aneuploidy may increase rupture frequency in tumors (Lines 439-441).

3. The authors conclude that lagging chromosomes in KIF18A KO cells are found closer to the main chromatin mass. The Stumpff lab showed in a 2019 JCB paper that KIF18 KO cells have a chromosome alignment defect and as a result during anaphase the chromosomes can be scattered rather than forming the tight, uniform mass that is observed in WT cells. The scattering of kinetochores resulting from this phenotype could affect the value of "Avg Chromosomes Distances" in Fig 7B and the normalized distance in the KIF18A KO cells. Therefore, live-cell imaging experiments would be helpful to resolve this and possibly strengthen this conclusion. RPE1 cells with fluorescently tagged CENP-A and centrin could be used to ensure that the lagging chromosomes will not rejoin the main nucleus. Moreover, these cells could be used for correlative live-fixed cell experiments in which fixed cell analysis following micronucleus formation could be used to show that chromosomes that lag farther away from the spindle pole are more likely to have defective micronuclear envelopes.

The reviewer’s concern that the unalignment phenotype, characteristic of KIF18A KO cells, may impact the value of average chromosome distances used to set a threshold for chromosomes meeting our definition of lagging is valid. To address this, we analyzed the standard deviations for chromosome-to-pole distances within half spindles of KIF18A KO and nocodazole-washout treated anaphase cells as a way to compare chromosome scattering in these two conditions. This analysis revealed no significant difference between the standard deviations of chromosome positions in the two groups, suggesting that scattering is similar in nocodazole treated and KIF18A KO cells. We have included these data in the manuscript (Line 335-337, and additional data added to Figure S4C).

In order to further strengthen this conclusion, we imaged live RPE1 cells expressing GFP-CENP-A, GFP-Centrin-1, GFP-NLS, and mCherry-H2B following nocodazole washout. These markers allowed us to measure the distances between lagging chromosomes and spindle poles at the end of mitosis. We subsequently followed any resulting micronuclei over several hours and assayed for nuclear envelope rupture. Analyses of 81 cells from 14 experiments revealed a clear trend where lagging chromosomes that form micronuclei that rupture are significantly further from the pole as telophase begins than those that form micronuclei that did not rupture during the experiment. These data have been added to the manuscript in Figure 8 and Lines 346-363.

4. Based on the Fonseca et al. 2019 JCB paper (video 2), micronuclei from KIF18A KO do not exclusively arise from lagging chromosomes. Instead, chromosomes can also escape the main chromatin mass after segregation and subsequently be excluded from the main nucleus. It would be important to know what fraction of the micronuclei in KIF18A KO cells arise via lagging...
chromosomes. Since Aurora B and/or bundled microtubules at the spindle midzone are believed to prevent proper nuclear envelope formation, chromosomes that properly segregate but later become separated from the main nucleus would be more likely to form proper micronuclear envelopes than those arising from lagging chromosomes. The correlative microscopy experiment suggested in the previous point could allow differentiation between these two routes to micronucleus formation.

The reviewer is correct that we did occasionally see chromosomes escape the main chromatin mass after segregation in the Fonseca et al., 2019 study referenced. We did not quantify the frequency of these events in that study, but they were rare. To address this quantitatively, we have measured the incidence of micronuclear formation around lagging chromosomes and chromosomes that escape the main chromatin mass after segregation in videos of KIF18A KO cells. We find that when micronuclei form in these cells, they form around lagging chromosomes 98% (46 out of 47 events) of the time. These data were derived from 4 live cell imaging experiments. This information has been added to the Results section (line 315-317).

**Minor issues:**

1. Some parts of the manuscript are excessively wordy and some sentences are unclear or convoluted (e.g., lines 148-153 and 238-239).

   Thank you for this feedback. We have revised the text in these two locations and have throughout the manuscript to improve clarity.

2. Lines 59-61. This sentence is formulated incorrectly. First of all, the subject of the sentence is "cells" and the verb is "can become fragmented." However, the authors mean that the DNA in the micronucleus can become fragmented (not the cells). Moreover, the way the sentence is currently formulated seems to suggested that the fragmentation occurs during cell division. However, this is not the case. Please, revise the text to make it more accurate.

   We appreciate this point and have revised this text to reflect more precise language to describe this model. It is certainly the micronucleated chromatin which may become fragmented, and this fragmentation occurs as a result of replication stress, including replication fork collapse, after an existing micronucleated cell enters a subsequent round of S or G2 phase (PMIDs: 22258507, 26017310).

3. Lines 114-115. Please, provide references in support of this statement.

   The statement in question: “This arrest was at least partially dependent on p53, consistent with other reports of cell cycle arrest following micronucleation,” shares the same references as the sentence that follows it (Sablina 1998, Thompson and Compton, 2010; Fonseca et al., 2019). We have updated the references to appear after the first statement to make this clear.

4. Line 153. The authors refer to Fig. 1C, but I think they mean Fig. 1B.
Thank you, we have updated the text to read Fig 1B.

5. Line 324. the authors find that RPE1 KIF18A KO cells have lagging chromosomes in ana/telophase 9% of the time, then say that this shows that lagging chromosomes are rare in KIF18A KO cells. However, this is a large increase compared to normal RPE1 cells, which only have 1-2% frequency of lagging chromosomes. So, they should revise the text here to say that the rates of lagging chromosomes from KIF18A KO are lower compared to the rates induced by nocodazole washout.

This is an important distinction. We have removed this confusing statement from the revised text (lines 323-324).

6. Line 383. The references listed here should be moved earlier and specifically after the statement summarizing the results of the studies instead of being listed after the authors' conclusion/interpretation of the data. The same issue was noted in other parts of the manuscript.

We have corrected this error (Lines 403-407) and amended the style of the manuscript throughout to cite relevant papers after the statement summarizing the results of those studies, rather than after our interpretation of the studies.

7. Figure 1A. In the text, the authors say they cross a Kif18a heterozygous mutant mouse with a p53 heterozygous mutant mouse, but the two mice in this figure are already heterozygous for both. Please, revise the text or depict the previous additional cross necessary to obtain the double heterozygous.

We thank the reviewer for catching this discrepancy. We have revised the text to describe the crosses necessary to obtain the double heterozygous mice shown in the figure (lines 113-115). The gcd2 mutation in Kif18a was named due to the "germ cell depleted" phenotype it causes. These homozygous mice are, therefore, infertile (Czechanski et al., 2015). For this reason, heterozygous mice for each gene were crossed to achieve the necessary homozygous progeny.

8. Figure 3A. Arrows or dotted circles outlining the micronuclei in the insets of the middle and bottom rows would be helpful since the DAPI signal in the micronuclei is low and somewhat difficult to see.

We have updated these figures as suggested to more clearly indicate the micronuclear area.

9. Figure 3B. Error bars should be added to the graph. Moreover, the authors noted that the differences are not significant. However, this seems surprising, given that in some cases there is a three- to five-fold difference between certain pairs. Indeed, a chi-square test using the numbers from table S1 indicated p values <0.05 for several pairwise comparisons.

We appreciate this feedback on the statistical tests and comparisons among these data. The main point of these analyses is to demonstrate that tissues other than blood form micronuclei.
in vivo in the absence of Kif18a function and that the majority of these micronuclear envelopes are completely surrounded by Lamin A/C. The data presented in Figure 3B were obtained by counting several tissue types from a single mouse of each genotype. Thus, we do not believe that error bars are appropriate in this context. To avoid confusion, we have also removed the statistical bars which had indicated no significant differences in rupture frequency among the genotypes in each sampled tissue.

We understand the reviewer’s point that some pairwise comparisons of the data in Table S1 indicate that they are significantly different. We originally used a Chi-square test to compare the data from all three genotypes for each tissue. Because these data did not rise to the threshold of significance necessary to reject the null hypothesis across all three genotypes within each individual tissue type, we did not think performing pairwise comparisons between only two of those genotypes was appropriate (Whitlock and Schluter, The Analysis of Biological Data, 2009). Specifically, analyses of rupture frequency for spleen, liver, and thymus tissue gave p-values above 0.05 (spleen, p = 0.35; liver, p = 0.056; thymus, p = 0.052). Thus, we did not proceed with pairwise comparisons. In contrast, the analyses of p53 effects on micronucleus levels in peripheral blood in Fig 1D utilized samples from 8 individual mice for each genotype, and are therefore more amenable to statistical comparisons. If the reviewer believes any of the details of this approach are incorrect, we are happy to revise the analyses.

10. Figure 5G. When referring to this figure (lines 292-294), the authors talk about correlation. However, the points in this graph seem to be scattered a bit randomly.

To address this concern, we performed a Pearson’s correlation test on the data in Figure 5G. As suspected by the reviewer, this analysis did not indicate a significant correlation, and we have removed this plot from the manuscript.

11. Figure 6B-D. The Y-axis titles of the three graphs are a bit confusing. Please, consider revising.

We have updated the Y-axis titles for these graphs to more accurately represent what is displayed on each plot.

12. In Figure 7 and the text, the authors use the terms "late-lagging" and "lagging" chromosomes interchangeably, which is somewhat confusing in this context because lagging chromosome distance from the main chromosome mass is thought to contribute to defective assembly of micronuclear envelopes. It is not clear whether the authors intend to indicate, with this term, that the lagging chromosome is farther away from the main chromosome mass or that the lagging chromosome is in a "late" anaphase cell. Because this is confusing, I suggest just using the term "lagging chromosome" consistently. It could be useful to include representative images of lagging chromosomes located at different distances from the main chromosome mass. And certainly, the authors should include an example of a lagging chromosome in the KIF18A KO cells.

We agree with the reviewer’s concern regarding confusion of these terms. We have updated the text to use the term “lagging chromosome” consistently, as the reviewer suggests. We have also updated Figure 7A to include a representative image of a lagging chromosome in a KIF18A KO cell.
13. Figure S2A. The example in the bottom right image looks more like a chromosome bridge than a lagging chromosomes. Kinetochore staining is necessary to unequivocally identify lagging chromosomes.

We agree with the reviewer that kinetochore staining is necessary to precisely identify lagging chromosomes. We had used these images to quickly and crudely assess the presence and frequency of potentially lagging chromosomes, observed in late-anaphase cells by eye. For subsequent experiments where lagging chromosomes were measured, poles and kinetochores were labeled to make permit precise, quantifiable assessments.

Reviewer #2 (Significance (Required)):

Based on the previous knowledge on the factors that cause abnormal assembly of the micronuclear membrane, the results presented in this study were somewhat predictable. However, these findings will add to the knowledge of how micronuclei form and the potential factors that lead to micronuclear membrane rupture. Previous studies investigating micronucleus behavior have focused on micronuclei arising via merotelic kinetochore mis-attachments. These mis-attachments lead to formation of micronuclei close to the spindle midzone. In the present study, instead, the micronuclei arising from lack of KIF18A activity form farther away from the spindle midzone. The results presented here suggest that the positioning of these micronuclei farther away from the midzone enables assembly of a more stable micronuclear membrane that will be less likely to rupture during the following cell cycle. A recent study showed that the microtubule bundles in the spindle midzone interfere with micronuclear membrane assembly. Based on this, it is not surprising that micronuclei forming away from the spindle midzone (like those resulting from lack of KIF18A activity) assemble more normal membranes. Although somewhat expected, this study provides the actual data in support of this phenomenon. This study will be of interest to cell biologists interested in cell division and genomic instability. My research has focused on cell division, aneuploidy, and chromosomal instability for nearly thirty years. Therefore, I believe I am fully qualified to evaluate this manuscript.

**Referees cross-commenting**

My areas of expertise do not include nuclear membrane structure and function. Therefore, I encourage the authors to consider the comments of reviewer #3 for issues related to reliable quantification of micronuclear membrane rupture.

Reviewer #3 (Evidence, reproducibility and clarity (Required)):
**Summary**

Sepaniac et al demonstrate that loss of KIF18a, a motor protein required for proper chromosome congression and chromatin compaction during mitosis, is insufficient to drive tumor development in mice although it does increase the frequency of micronuclei (MN), nuclear compartments that form around broken or missegregated chromosomes, in both normal and tumor tissue. MN are thought to increase genome instability and metastasis by undergoing DNA damage and activating innate immune signaling after irreparable nuclear membrane rupture. The authors use a non-transformed human cell line, hTERT-RPE-1, with KIF18a knocked out to demonstrate that MN formed as a result of KIF18a loss have more stable nuclear membranes than MN generated by other methods. They go on to correlate this increased stability with increased chromosome proximity to the main chromatin mass during nuclear envelope assembly and increased chromatin decompaction by a combination of fixed and live cell imaging.

**Major Comments**

1. This study relies heavily on the use of lamin A loss or discontinuity to identify ruptured micronuclei. Although the authors validate this marker against "leakage" of the soluble nuclear protein mCherry-NLS, there are several lines of evidence suggesting that lamin A loss or disruption is not a reliable reporter. In figure S3C, the top two panels of intact MN in the KIF18KO appear ruptured based on the gH2AX labeling, yet have significant levels of lamin A and are labeled as intact. In figure 4D, the rate of MN rupture after nocodazole release (60% ruptured in 2 hours) is much faster than that reported in other papers (40-60% in 16-18 hours, Liu et al; 60% in 16 hours; Hatch et al). In addition, images in Hatch et al, 2013 show lamin A localizing to both intact and ruptured MN and anecdotal information in the field suggests that lamin A localization is not a reliable reporter.

These discrepancies may be due to how the authors' define "mCherry-NLS leakage", which needs to be defined in the methods as previous studies have demonstrated that MN frequently have delayed or reduced nuclear import even though the membrane is intact. Regardless, the authors need to provide compelling independent evidence that lamin A loss and disruption faithfully recognize ruptured MN by either validating this marker against additional rupture reporters, such as Lap2, LBR, or emerin accumulation, or by repeating key experiments in cells expressing mCherry-NLS.

Our decision to use lamin A/C as a reporter was based on its use as a marker for micronuclear envelope presence in prior studies (Hatch, 2013; Liu, 2018). We were unaware of anecdotal information in the field that suggests that lamin A localization may not be a reliable reporter.
However, we think we understand the reviewer’s point to be that although it is clear from prior studies that gaps in the nuclear lamina are a known predictor of micronuclear rupture, these gaps can persist for some time before rupture has actually occurred. We agree that this is an important distinction and thank the reviewer for raising these questions.

As the reviewer notes, we performed control experiments to address this issue and validate the use of lamin A/C as a marker of micronuclear envelope rupture. Our approach involved correlating lamin staining with the localization of mCherry-NLS signal to the micronucleus (Figure S1). We found that these signals correlated well. To further extend these analyses, we have also now validated our findings using an additional rupture reporter (LAP2), as suggested by the reviewer. These data are included in the new Figure S3.

As the reviewer points out, this analysis in fixed cells could be misleading in cases where nuclear import is reduced, but the micronuclear envelope is intact. If this were a significant contributor, we may have expected to see greater instances of micronuclei that exhibit continuous lamin A/C signal but lack nuclear localization of mCherry-NLS. However, we found this combination was rare among the KIF18A and RPE1 nocodazole washout treated cells (2%, or 1 of 46 micronuclei had continuous lamin A/C while lacking mCherry-NLS). We admit that this assumption may be oversimplified though.

The reviewer’s point about the timing of nocodazole treatment and washout is something we have definitely considered. We note that prior studies have used differing time points after nocodazole treatment and release. In Hatch et al., 2013: U2OS cells were treated for 6 hours with nocodazole and then subjected to mitotic shakeoff, which led to 48% of micronuclei exhibiting rupture after 6 hours and ~60% exhibiting rupture after 16 hours. Similarly, in Liu et al., 2018 60% of micronuclei were ruptured 16 hours post mitotic shake off and nocodazole release. While these results suggest that rupture increases with time after mitosis, it isn’t clear how early rupture may occur. In other words, does it take many hours before nearly half of micronuclei rupture or do many of these micronuclei rupture shortly after cell division, which is what our fixed cell data suggest?

To address this question, we performed live imaging of RPE1 cells expressing NLS-EGFP and mCherry-H2B, which allowed us to follow micronucleus formation and rupture in real time. In order to get a reasonable data set for assessing micronucleus stability, we performed 12 independent experiments. We imaged 34 micronuclei that formed following nocodazole washout and 36 that formed in dividing KIF18A KO cells. Consistent with our fixed cell data, 53% of micronuclei (18/34) ruptured during the course of the experiment, with an average time to rupture of 2.2 hours (Figure S3E). We noted that the majority of micronuclei ruptured within 2 hours after formation and a few remained intact for 4-6 hours before rupturing, which increased the average time. Although fewer micronuclei ruptured in KIF18A KO cells (5/36, 14%), a similar trend of rupture timing was observed (Figure S3E-F). These data suggest that micronuclear rupture often occurs shortly after completion of mitosis in this experimental system. These results (described in Lines 238-248) are consistent with the conclusion that the majority of the micronuclei exhibiting loss of lamin A/C in our fixed cell experiments being ruptured.

We did also observe a small fraction of micronuclei (6% or 5/80) that showed a delayed import of NLS-EGFP signal after mitosis. These micronuclei were not included in our measurements of micronucleus rupture timing. This information is included in the revised Results section (Lines 244-246).
We note that other explanations could also potentially contribute to the differences in rupture rates reported in our study compared to those in previous publications. For example, we used a short nocodazole treatment (2 hrs) compared to the longer treatments (6 hrs) used in previous studies. We did this originally in order to produce a similar percentage of micronucleated cells as seen in KIF18A KO cell populations. However, the difference in nocodazole treatment length could potentially influence the types and frequencies of kinetochore microtubule attachments formed. For example, if centrosomes stay closer together in mitotic cells after short nocodazole treatments, this could increase the number of abnormal attachments (e.g. PMID: 22130796). Such an effect would be expected to increase the frequency of lagging chromosomes and/or potentially produce more lagging chromosomes within the anaphase midzone.

In summary, we have measured a clear difference between the lamin deposition within micronuclear envelopes of KIF18A KO cells compared to those formed following other insults. Lamin recruitment is well established as a predictor of nuclear envelope stability and correlates well with rupture frequency in our live imaging experiments. In addition to including new supporting data, we have also revised the text to more precisely distinguish between experiments that measure lamin A/C and B defects within micronuclear envelopes and those that directly assess nuclear envelope rupture.

2. Micronuclei in tumor sections and other dense tissues can appear very similar to other types of chromatin, including blebs from adjacent nuclei and dead cells. To verify that the quantified structures are bona fide micronuclei, the authors need to include a marker for the cell boundary. This is especially critical in the lamin a stained tumor sections with heterogeneous lamin A protein expression.

We appreciate the point this reviewer raises, and we carefully considered accurate identification of micronuclei in tissues. Three optical sections were collected from each sample. During analyses, we scrolled through the ~2-micron thick sections to exclude chromatin bodies connected to an out-of-plane nucleus or nuclear bleb. However, to further verify micronuclei, we stained thymic lymphoma sections with antibodies against the cell membrane protein ezrin. We evaluated a number of antibodies for this analysis, including those that specifically recognize cadherin, e-cadherin, caveolin, claudin-1, β-catenin, ezrin, and Zo-1. We found that anti-ezrin antibodies provided the most distinct cell boundary signal in murine thymus tissue. Analyses of micronuclear load and lamin A/C localization to micronuclei in ezrin-labeled lymphoma samples from three mice of each genotype were consistent with our original measurements (new Figure S2, Lines 138-141). Thus, we conclude that loss of KIF18A does increase the percentage of micronucleated cells in thymic lymphoma but does not alter the frequency of lamin A/C defects.

3. Figure 4 compares MN rupture frequency between cells treated with different inducers of micronuclei - KIF18A KO, nocodazole release, and irradiation. These treatments have different effects on the cell cycle: KIF18A causes minor delays, nocodazole arrests cells in mitosis, and g-IR likely causes delays in S and G2. Since MN rupture frequency increases with the duration of interphase, the authors need to assess rupture frequency at similar time points after mitosis for all three conditions. One way to accomplish this would be to repeat this experiment and analyze cells collected by mitotic cells by shake-off prior to fixation and labeling.
We appreciate this point regarding differences in mitotic timing. Since micronuclear rupture frequency increases with time in interphase, we would expect the MN in KIF18A KO cells to exhibit the highest level of rupture if cell cycle timing were the primary variable affecting stability in our experiments. KIF18A KO cells are asynchronously dividing, and the micronuclei examined in populations of those cells could have been generated at any time. We do not have the same type of temporal control of these events as we do with drug treatment. In contrast, the vast majority of the MN in nocodazole washout cells would not have been in interphase for more than 1.5 hours in our experiments, yet showed increased lamin A/C defects. RPE1 cells treated with MAD2 siRNA knockdown, which do not experience mitotic delays (PMID: 9606211; 15239953), also showed greater frequencies of micronuclear envelopes which lacked lamin A/C compared to those arising in KIF18A KO cells.

To further address the timing of micronucleus rupture in RPE1 cells, we performed live imaging studies of cells treated with nocodazole or lacking KIF18A activity, as described in Point 1 above. These analyses indicate that rupture primarily occurs in both contexts shortly after completion of mitosis and is more prevalent in nocodazole treated cells than in KIF18A KO cells (Figure S3E-F, Lines 238-248). These data further support the conclusion that micronuclei rupture more frequently in nocodazole treated cells than in KIF18A KO cells.

**Minor Comments**

1. In figure 6A, it is unclear when the videos start and how micronuclei are selected for analysis. Do the micronuclei have to be continuously visible from the time they missegregate? Do the videos all start at the same time point during mitosis or is it contingent on when the MN appears separated from the main nucleus? One concern is that a consistent delay in micronucleus appearance in the nocodazole treated cells could artificially decrease the amount of MN expansion observed.

We thank the reviewer for these questions. The individual micronuclei did not need to be continuously visible from the time that they missegregated, though the majority were. When a micronucleus was not sufficiently in the plane of focus for an accurate area measurement, the individual measurement at that time point was not collected. In cases where one or more frames which were not measurable, a micronucleus was only included in the final data set if it was 1) the only micronucleus present in the daughter cell or 2) easily identifiable to be the same micronucleus. Measurements were taken until the micronuclear area reached an equilibrium for several frames. Final fold change in area was established by dividing final area measurements by initial measurements.

The initial measurement for each micronucleus taken from the videos all start at the same relative point during mitosis, which is just after chromosome segregation has occurred.

2. In figure 7A, it is difficult to identify the "lagging" chromosome in the top panel. It would be helpful to label the chromosome that becomes the MN, or ideally, to include a video or still images to demonstrate how micronuclei form in the KIF18A KO cells.
We have updated the images in Figure 7A to include an example of a lagging chromosome in a KIF18A KO cell. We will also include a more explicit reference to our previous study (Fonseca et al., 2019), which described how micronuclei form around lagging chromosomes in KIF18A KO RPE1 cells.

3. The two image panels in figure 7A are imaged at significantly different times during anaphase (early anaphase on bottom versus late anaphase/telophase on top). A better comparison would be between two cells at the same time point in anaphase.

We have updated the images in Figure 7A to compare cells at similar stages of anaphase. In our quantification of lagging chromosomes, we also accounted for anaphase-timing differences by normalizing all measurements within each half-spindle.

Reviewer #3 (Significance (Required)):

In this study, the authors identify chromatin decondensation in micronuclei as a new predictor of membrane stability. Although these results are correlative, if their micronucleus rupture results can be validated as described in major comment 1, this study would advance our understanding of the micronucleus rupture mechanism by linking mitotic spindle location, chromatin decondensation, and lamin B1 protein recruitment. This would provide needed support to a current model in the field that micronucleus stability is largely determined during nuclear envelope assembly. In addition, if KIF18a loss generates stable micronuclei at high frequency, it will become a critical system for testing MN rupture hypotheses in the field. Thus, this work would be of significant interest to cell biologists working on nuclear envelope structure and function, chromosome organization, and mitosis. I include myself in this group as a cell biologist studying nuclear envelope structure and function with an expertise in membrane dynamics.

The authors also find that mice mutant for KIF18a have increased micronucleation in normal tissues but not increased tumor initiation. They hypothesize that this is due to the low rupture frequency of KIF18a-induced MN, however their data cannot reject the null hypothesis that the small increase in MN they see in KIF18a mutant mice would be insufficient to induce tumorigenesis even if rupture frequency was high. Thus the significance of their finding that micronucleation is not sufficient for cancer progression is unclear. However, the thorough analysis of micronucleation and rupture in several healthy tissues as well as a tumor model in KIF18 mutant mice would be of interest to both pathologists and cancer researchers focused on mechanisms of genome instability. These types of experiments are critical to determine how micronuclei contribute to cancer progression and the quantifications presented in this paper are truly impressive.

We appreciate this reviewer’s enthusiasm for our work and acknowledge that we cannot definitively conclude that micronuclear envelope stability explains why Kif18a mutant mice do not form tumors. However, it is interesting to note that the micronuclear loads measured using a peripheral erythrocyte assay are similar in Kif18a<sup>pcd2</sup> mutant mice (0.6% micronucleated erythrocytes, of total erythrocytes) and ATM<sup>mt1</sup> Hang/mt1<sup>mt1</sup> Hang mutant mice (0.6% of micronucleated
erythrocytes, of total) (Fonseca et al., 2019). Yet, the tumor frequency in these two models is dramatically different: Kif18a^gcd2/gcd2 mutant mice do not spontaneously form tumors – while the majority of ATM^tm1 Awb/tm1 Awb mutant mice do develop thymic lymphoma tumors between 2 and 4 months (Barlow, 1996). It is not clear how much micronuclei contribute to tumorigenesis in the ATM mutant model, but this comparison does suggest that the increase in MN seen in Kif18a mutants is physiologically relevant in the context of cancer progression. We have added this information to the revised text (lines 118-123).

**Referees cross-commenting**

I agree with the concerns raised by the other 2 reviewers, especially their comments about the need to clarify the mechanism of chromosome lagging versus chromosome congression and compaction.

I think that all of these suggestions, though, are contingent on them being able to reproduce their micronucleus rupture results with a better marker of nucleus integrity. I strongly believe that additional validation of lamin A as a micronucleus rupture marker will demonstrate that it is unreliable, based both on our own observations in RPE-1 cells and the images they show.
July 26, 2021

RE: JCB Manuscript #202101165R

Dr. Jason Stumpff
University of Vermont
149 Beaumont Avenue
HSRF 118
Burlington, Vermont 05405

Dear Dr. Stumpff,

Thank you for submitting your revised manuscript entitled "Micronuclei in Kif18a mutant mice form stable nuclear envelopes and do not promote tumorigenesis." Your revised manuscript was evaluated by two of the original Review Commons referees, whose comments are appended to this letter. We would be happy to publish your paper in JCB pending final revisions necessary to address the final comments from reviewers and to meet our formatting guidelines (see details below). Please note that all the requested changes to the text and figures should be done prior to submission of your final files. When submitting the final version, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

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

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

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

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

2) Figures limits: Articles may have up to 10 main text figures.

3) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis.

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

5) Abstract and title: The summary should be no longer than 160 words and should communicate the significance of the paper for a general audience. The title should be less than 100 characters including spaces. Make the title concise but accessible to a general readership.

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

7) 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 be sure to provide the sequences for all of your oligos: primers, si/shRNA, RNAi, gRNAs, etc. in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers/vendor identifiers (where appropriate) for all of your antibodies, including secondary.

8) 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.).

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

10) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures and 10 videos. Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section. Please include one brief sentence per item.

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

12) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements
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13) A separate author contribution section is required following the Acknowledgments in all research manuscripts. All authors should be mentioned and designated by their first and middle initials and full surnames. We encourage use of the CRediT nomenclature (https://casrai.org/credit/).

14) ORCID IDs: ORCID IDs are unique identifiers allowing researchers to create a record of their various scholarly contributions in a single place. At resubmission of your final files, please consider providing an ORCID ID for as many contributing authors as possible.

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

Re-review of Sepaniac et al.

I appreciate the work the authors put into their revisions, and their patience, and think that they have made the paper much stronger. They have addressed all the concerns from my review. There are still some data that are confusing or contradictory, but I think they can all be addressed by additional analysis of their existing data, additional explanation of their image analysis criteria, or discussing additional interpretations in their text. These specific minor issues are outlined below.

1. The new data in Fig. S3G-F analyzing MN rupture by live-cell imaging of GFP-NLS are the strongest indicator that MN rupture frequency is different between nocodazole and KIF18A KO MN. These data validate the correlation of lamin A absence with mCherry-NLS absence in Fig. S3D and address my concern about cell cycle differences between nocodazole release and KIF18A asynchronous cells. These data could be further strengthened by an additional plot of the existing data analyzing the proportion of intact versus ruptured MN in videos where the MN is visible for X number of hours (whatever time point makes sense for a solid n value).

2. The new lap2 data are more confusing than helpful. Willan et al. 2019 describe Lap2 accumulation in ruptured MN (Fig. 4H), whereas here the authors use loss of Lap2 to identify ruptured MN. If Lap2 accumulation on ruptured MN cannot be replicated in this cell line, then it does not make sense to include it as a marker for MN integrity, and I apologize for a dud of an experimental suggestion.

3. gH2AX accumulation strongly correlates with MN rupture, specifically in MN that rupture in S or G2 phase (Hatch et al., 2013; Zhang et al., 2015). The gH2AX signal the authors show in Fig. S5C (row 1, 2, and 4) is consistent with what is observed for ruptured MN, based on its appearance versus the nucleus labeling, and row 1 and 2 MN are lamin A/C +, suggesting a disconnect between the lamin A/C and gH2AX rupture markers. If gH2AX accumulation is the criteria for gH2AX+ MN in Fig. S5D, then the graph suggests that over 45% of KIF18A KO MN are ruptured rather than 20% (Fig. 4D). This disconnect would appear if all gH2AX foci are being counted as positive, since mitotic delays can increase gH2AX signal at telomeres (Hayashi et al., Nat. Struct. And Mol. Biol., 2012; Hain et al., Sci. Rep. 2016), or if the population is enriched in G2 cells, where even intact MN tend to accumulate gH2AX (Umbreit et al., 2020). A brief discussion of how gH2AX+ MN were scored in the material and methods or whether cells are likely to be enriched in G2, would be helpful to resolve the discrepancy. If neither of these situations are the case, a sentence about other potential sources of the disconnect could be added to the discussion.

4. The low frequency of ACA+ MN after nocodazole release (Fig. S5D) and the larger population of
small MN after nocodazole treatment versus after KIF18A KO (Fig. S5A) are still surprising results and should be addressed in the text. They suggest that nocodazole treatment is generating a lot of DNA fragments that become micronucleated, which is different than the expected result of an enrichment of whole chromosome 1 and 2 in the MN (Worrall et al., Cell Reports, 2018). Based on their data in Fig. S5, the authors should discuss recent data demonstrating that small MN have lower lamin B1 levels and more frequent and earlier membrane rupture as part of their interpretation of their nocodazole results (Kneissig et al., eLife, 2019; Xia et al., JCB, 2019, Mammel et al., bioRxiv 2021).

5. To aid in understanding the visual criteria used to identify ruptured MN, it would be great if the authors added colors or lines to the existing triangle labels in all the figures to differentiate between the intact and ruptured MN. In some cases, especially the tissue sections, it can be hard to determine how different MN would be scored (e.g. Fig.s 3A, S3E, Fig. S2).

6. Fig. 5D is not referenced in the text.

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

The authors have revised their manuscript following a round of reviews through Review Commons. I very much appreciate the effort the authors put into revising their manuscript. I believe the manuscript is greatly improved. However, there are still a few issues that I believe should be rectified prior to publication.

1. In response to my major point #3, the authors performed live-cell imaging experiments. This was an important addition to the study. However, in the images presented in Figure 8, I cannot discern the centrin signals, which raises questions about the accuracy of the measurements. It will be important to add images in which the spindle poles are visible and label the figures in a way that illustrates how the measurements were taken. The authors could consider adding zoomed-in views with clearly visible centrin marks, a super-imposed line demarcating the distance measurement, and a measurement label. As an additional note, I also checked the videos related to Figure 8 and I cannot see the centrin signals there either. So, I am not sure how the authors actually collected the "distance from the spindle pole" measurements reported in Figure 8A. I was unable to find a detailed description of this in the methods section of the manuscript or in the figure legend. This information needs to be added and if the distance measured was not from the actual pole (which cannot be determined in these images), then the authors need to explain what point was set as the pole.

2. In response to my minor point 13, the authors stated: "We had used these images to quickly and crudely assess the presence and frequency of potentially lagging chromosomes, observed in late-anaphase cells by eye. For subsequent experiments where lagging chromosomes were measured, poles and kinetochores were labeled to permit precise, quantifiable assessments." Whereas, I am generally fine with the authors using the images in the way they described, I still think that the way the data are presented is inaccurate and misleading. The image at the bottom-right corner of Figure S4A is clearly a chromatin bridge. Even without kinetochore staining, this is very obvious. Therefore, including these numbers in a graph (Figure S4B) in which the Y-axis title reads "Percent late anaphase cells with mid-zone lagging chromosome" is not fine. Perhaps, the Y-axis title could be changed to "Percent late anaphase cells with mid-zone missegregating chromosomes." Moreover, the figure legend currently reads "Lagging chromosomes indicated via white arrowhead,"
which again is incorrect and misleading. The figure legend should say something like "Representative late anaphase RPE1 cells treated with nocodazole washout or containing KIF18A KO mutations. Misregulating chromosomes in the midzone were quantified in late anaphase cells by eye and included both lagging chromosomes/DNA (white arrowhead) and chromatin bridges (yellow arrowhead)." Please, note that I am suggesting the phrase "lagging chromosomes/DNA" because without kinetochore staining it is not possible to discriminate lagging chromosomes from chromosome fragments. I also suggest using different color arrowheads for the two different types of misregulation.

3. Scale bars are missing from the following figures: 4C, 5A, 6A, 7A, 8B, S1A, S2A, S3A, S3C, S3E, S4A, S5C.
We would like to again thank the reviewers for taking the time to carefully evaluate our manuscript. The paper will be further improved by their additional suggestions, and we are grateful for their perspectives. We have provided point-by-point responses to address each concern raised by Reviewer #2 and Reviewer #3 below.

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

I appreciate the work the authors put into their revisions, and their patience, and think that they have made the paper much stronger. They have addressed all the concerns from my review. There are still some data that are confusing or contradictory, but I think they can all be addressed by additional analysis of their existing data, additional explanation of their image analysis criteria, or discussing additional interpretations in their text. These specific minor issues are outlined below.

1. The new data in Fig. S3G-F analyzing MN rupture by live-cell imaging of GFP-NLS are the strongest indicator that MN rupture frequency is different between nocodazole and KIF18A KO MN. These data validate the correlation of lamin A absence with mCherry-NLS absence in Fig. S3D and address my concern about cell cycle differences between nocodazole release and KIF18A asynchronous cells. These data could be further strengthened by an additional plot of the existing data analyzing the proportion of intact versus ruptured MN in videos where the MN is visible for X number of hours (whatever time point makes sense for a solid n value).

We have reanalyzed the live imaging data described (now found in Fig. S3C-D) to create an additional plot as this reviewer suggested. This plot is now found in Fig. S3E, and shows the percent of micronuclei that lose envelope integrity (indicated by loss of EGFP-NLS) and remain visible for at least 2.5 hours. This is a nice additional data set to include. Thank you for this suggestion.

2. The new lap2 data are more confusing than helpful. Willan et al. 2019 describe Lap2 accumulation in ruptured MN (Fig. 4H), whereas here the authors use loss of Lap2 to identify ruptured MN. If Lap2 accumulation on ruptured MN cannot be replicated in this cell line, then it does not make sense to include it as a marker for MN integrity, and I apologize for a dud of an experimental suggestion.

While we did observe Lap2 recruitment to micronuclear envelopes in these experiments, which corresponded to recruitment of the other core nuclear envelope component lamin A/C, we note that we did not observe the “enrichments of Lap2” which were observed in the ruptured micronuclear envelopes characterized in the Willan et al. 2019 publication. Instead, the Lap2 signal which we observed at micronuclei was predominantly consistent with the signal intensity and accumulation observed across primary nuclear envelopes within the same cell. However, we agree with the reviewer that the Lap2 data could be more confusing than helpful, and we have removed these data from the final revised manuscript.
3. gH2AX accumulation strongly correlates with MN rupture, specifically in MN that rupture in S or G2 phase (Hatch et al., 2013; Zhang et al., 2015). The gH2AX signal the authors show in Fig. S5C (row 1, 2, and 4) is consistent with what is observed for ruptured MN, based on its appearance versus the nucleus labeling, and row 1 and 2 MN are lamin A/C +, suggesting a disconnect between the lamin A/C and gH2AX rupture markers. If gH2AX accumulation is the criteria for gH2AX+ MN in Fig. S5D, then the graph suggests that over 45% of KIF18A KO MN are ruptured rather than 20% (Fig. 4D). This disconnect would appear if all gH2AX foci are being counted as positive, since mitotic delays can increase gH2AX signal at telomeres (Hayashi et al., Nat. Struct. And Mol. Biol., 2012; Hain et al., Sci. Rep. 2016), or if the population is enriched in G2 cells, where even intact MN tend to accumulate gH2AX (Umbreit et al., 2020). A brief discussion of how gH2AX+ MN were scored in the material and methods or whether cells are likely to be enriched in G2, would be helpful to resolve the discrepancy. If neither of these situations are the case, a sentence about other potential sources of the disconnect could be added to the discussion.

Thank you, we have added text to the discussion to address this potential disconnect (Lines 427-433) and have included a more detailed description of our γH2AX quantification within the methods (Lines 659-662). For the purposes of scoring γH2AX signal, we included any positive signal within the micronucleus, regardless of signal intensity or size of the focus, as γH2AX-positive. The point the reviewer makes about G2-arrested cells could be relevant here. We previously observed a G2-enrichment of KIF18A mutant MEFs (Czechanski et al., 2015). Alternatively, micronuclei in KIF18A loss of function cells may accumulate damage without rupturing their nuclear envelopes. We have updated the discussion with these possibilities (Lines 427-433).

4. The low frequency of ACA+ MN after nocodazole release (Fig. S5D) and the larger population of small MN after nocodazole treatment versus after KIF18A KO (Fig. S5A) are still surprising results and should be addressed in the text. They suggest that nocodazole treatment is generating a lot of DNA fragments that become micronucleated, which is different than the expected result of an enrichment of whole chromosome 1 and 2 in the MN (Worral et al., Cell Reports, 2018). Based on their data in Fig. S5, the authors should discuss recent data demonstrating that small MN have lower lamin B1 levels and more frequent and earlier membrane rupture as part of their interpretation of their nocodazole results (Kneissig et al., eLife, 2019; Xia et al., JCB, 2019, Mammel et al., bioRxiv 2021).

We have added text to the discussion to address the low frequency of ACA+ micronuclei after nocodazole release (Lines 433-436). One possibility is that this could be a result of the shorter nocodazole treatment chosen to generate a similar frequency of micronucleus formation to that observed in KIF18A KO cells. For example, if the shorter treatment keeps centrosomes closer together and allows more abnormal kinetochore-microtubule attachments to occur following drug washout, this could in turn promote more chromosome damage after washout. This is pure speculation at this point, so we kept this discussion brief in the manuscript.

While we appreciate the suggestion to consider the effects of micronucleus size and lamin B1 recruitment on rupture frequency, we did not detect a difference in micronuclear area in cells
treated with nocodazole compared to those lacking KIF18A function (Fig S5A- no significance between conditions). Thus, we did not incorporate this consideration into the discussion.

5. To aid in understanding the visual criteria used to identify ruptured MN, it would be great if the authors added colors or lines to the existing triangle labels in all the figures to differentiate between the intact and ruptured MN. In some cases, especially the tissue sections, it can be hard to determine how different MN would be scored (e.g. Fig.s 3A, S3E, Fig. S2).

We have updated the arrowhead colors in all figures to differentiate between intact and ruptured MN, with particular attention to Figures 3A, S3E, and S2.

6. Fig. 5D is not referenced in the text.

Thank you for catching this oversight. We have corrected this omission (Lines 285-287).

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

The authors have revised their manuscript following a round of reviews through Review Commons. I very much appreciate the effort the authors put into revising their manuscript. I believe the manuscript is greatly improved. However, there are still a few issues that I believe should be rectified prior to publication.

1. In response to my major point #3, the authors performed live-cell imaging experiments. This was an important addition to the study. However, in the images presented in Figure 8, I cannot discern the centrin signals, which raises questions about the accuracy of the measurements. It will be important to add images in which the spindle poles are visible and label the figures in a way that illustrates how the measurements were taken. The authors could consider adding zoomed-in views with clearly visible centrin marks, a super-imposed line demarcating the distance measurement, and a measurement label. As an additional note, I also checked the videos related to Figure 8 and I cannot see the centrin signals there either. So, I am not sure how the authors actually collected the "distance from the spindle pole" measurements reported in Figure 8A. I was unable to find a detailed description of this in the methods section of the manuscript or in the figure legend. This information needs to be added and if the distance measured was not from the actual pole (which cannot be determined in these images), then the authors need to explain what point was set as the pole.

The centrin signal in some of the stable RPE1 cells used is faint in comparison to the signal at the centromeres. In those cases, we had to adjust brightness contrast to a point where the centromere signal was saturated in order to determine the position of the centrin foci. In addition, it was often difficult to keep the centrin and lagging chromosome centromere signals in focus at all times. We actually manually adjusted the focus throughout each movie to maintain information about the position of the centrioles and centromere of interest. Due to the length of the movies required to track lagging chromosomes and then determine the fate of the micronucleus, we found that acquiring multiple z-sections often damaged the cells and
prevented normal division. We have added these details to the methods to make the process clearer (Lines 627-631). We chose the cell shown in Fig 8 because it had two lagging chromosomes at different distances from the pole, one that eventually ruptured and one that did not. Unfortunately, the centrin signal is difficult to see in this cell. To improve the clarity of Figure 8, we have added annotated images below the original ones that indicate the position of the centrin foci and an example line to indicate how distance measurements were made. We have also now included another example movie of a cell with a lagging chromosome that forms a micronucleus that has much more visible centrin foci (new Supplemental Video 1).

2. In response to my minor point 13, the authors stated: “We had used these images to quickly and crudely assess the presence and frequency of potentially lagging chromosomes, observed in late-anaphase cells by eye. For subsequent experiments where lagging chromosomes were measured, poles and kinetochores were labeled to permit precise, quantifiable assessments.” Whereas, I am generally fine with the authors using the images in the way they described, I still think that the way the data are presented is inaccurate and misleading. The image at the bottom-right corner of Figure S4A is clearly a chromatin bridge. Even without kinetochore staining, this is very obvious. Therefore, including these numbers in a graph (Figure S4B) in which the Y-axis title reads “Percent late anaphase cells with mid-zone lagging chromosome” is not fine. Perhaps, the Y-axis title could be changed to “Percent late anaphase cells with mid-zone missegregating chromosomes.” Moreover, the figure legend currently reads “Lagging chromosomes indicated via white arrowhead,” which again is incorrect and misleading. The figure legend should say something like “Representative late anaphase RPE1 cells treated with nocodazole washout or containing KIF18A KO mutations. Missegregating chromosomes in the midzone were quantified in late anaphase cells by eye and included both lagging chromosomes/DNA (white arrowhead) and chromatin bridges (yellow arrowhead).” Please, note that I am suggesting the phrase “lagging chromosomes/DNA” because without kinetochore staining it is not possible to discriminate lagging chromosomes from chromosome fragments. I also suggest using different color arrowheads for the two different types of missegregation.

We have updated the y-axis, as the reviewer has suggested, to more accurately describe the types of lagging chromosomes/DNA which were scored. The figure legend has also been updated as suggested.

3. Scale bars are missing from the following figures: 4C, 5A, 6A, 7A, 8B, S1A, S2A, S3A, S3C, S3E, S4A, S5C.

Scale bars have been added to all microscopy images, as well as enlarged inserts.