Increased numbers of nucleoli in a genome-wide RNAi screen reveal proteins that link the cell cycle to RNA polymerase I transcription

Lisa Ogawa, Amber Buhagiar, Laura Abriola, Bryan Leland, Yulia Surovtseva, and Susan Baserga

Corresponding author(s): Susan Baserga, Yale University

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RE: Manuscript #E20-10-0670
TITLE: Increased numbers of nucleoli in a genome-wide RNAi screen reveal proteins that link the cell cycle to RNA polymerase I transcription

Dear Dr. Baserga,

The reviews are back on your paper, "Increased numbers of nucleoli in a genome-wide RNAi screen reveal proteins that link the cell cycle to RNA polymerase I transcription". As you can see, both reviewers found your paper interesting but have criticisms that need to be addressed before we can publish your work. If you think you can address these criticisms in a revised manuscript, we would be happy to publish your work.

Sincerely,

Jennifer Lippincott-Schwartz
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Baserga,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made, as described in the Monitoring Editor's decision letter above and the reviewer comments below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

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Thank you for submitting your manuscript to Molecular Biology of the Cell. We look forward to receiving your revised paper.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

Reviewer #1 (Remarks to the Author):

This manuscript represent part II of a large scale siRNA screen to identify proteins that influence the number of nucleoli in the human cell line MCF10A. In this case, the screen identifies proteins that when depleted give rise to a significant increase in the number of nucleoli (from 2 or 3 per cell to 5 or more). The screen is well performed and the hits are appropriately analysed. Although no clear mechanistic insights are obtained, links between RNA pol I transcription, nucleolar organisation and cell cycle progression are firmly established. The results presented represent a very useful resource for further investigation of these interesting links. Specific comments are presented below.

It would be useful if the authors comment more specifically on the acrocentric chromosomes and rDNA distribution in MCF10A cells, as this is directly relevant.

Can the authors explain the rationale for siRISC-free and siKIF11 as negative and positive controls.

Lines 199-201. I don't understand the argument that proteins with IDRs determine nucleolar number through LLPS behaviour. I am not aware of any direct evidence that fusion of chromosomally tethered nucleoli involves liquid like behaviour.

siKIF11 positive control cells not only have more nucleoli but are quite a bit larger than the negative control. This is observed in most of the hits shown in Fig 2A. At face value the cell cycle profiling shown in Fig 4 suggests ~60% of these cells are in G2/M. This is based purely on DNA content. In my view, a more thorough analysis of cell-cycle stage by means other than measuring DNA content, combined with karyotype analysis of these positive control cells and some of the hits is warranted.

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Reviewer #2 (Remarks to the Author):

The authors address a very interesting and relevant question in the field. It has been known for decades that cancer cells often display changes in nucleolar morphology and number. However, the underlying mechanisms controlling these features remain largely unclear. Identification of factors affecting the number of nucleoli per cell might even reveal targets relevant for cancer therapy. Here, the authors set out to mine and validate screening results of a previously performed genome-wide RNAi screen (Farley-Barnes et al., Cell Reports, 2018). While their previous analysis had focused on hits that cause a reduction in nucleolar number, the current manuscript reports on factors whose depletion cause an increase in nucleoli per cell.

The screening approach and the subsequent analysis are generally nicely described in the manuscript. However, one point of some concern lies in the nature of the chosen positive control siRNA for the screen, namely in the use siKIF11. It is not well explained why this particular factor was chosen. The authors should give the reader a rational. KIF11/Eg5 is a motor protein important for centrosome separation and spindle bipolarity in mitosis (Blangy et al., Cell, 1995). It is well established that depletion of KIF11 results in unfaithful cell division, cell cycle arrest and eventually apoptosis. KIF11 or other mitotic regulators, such as PLK1 or INCENP (one of the hits described here), are therefore frequently used as death controls in screening campaigns to quantify the general RNAi transfection efficiency (Nikolic et al., Nucleic Acid Research, 2017; Stechow et al. Mol Cell Biol, 2015; Zanin et al., Dev Cell, 2013). One therefore wonders whether these established defects of KIF11 depletion were also observed in the reported screen and to which extent. More importantly, the authors should address whether the reported increase in nucleolar number for KIF11 and other hits arises because of failures in cell division, which would just duplicate the number of NORs per cell. (This would also be consistent with the reported increase in DNA content for some of the identified mitotic factors). Given the example images provided in Figure 2 and the segmentation examples in Figure S1A, the nuclei shown for many hits are larger than those of the control cells, perhaps reflecting failures in previous cell divisions. It would be nice, if the authors would analyze the number of nucleoli relative to the nuclear size for the selected follow up hits. Despite the aforementioned questions, the paper highlights some intriguing hits such as ENY2 or H1-10, which show an increased number of nucleoli in normally sized cells according to the images presented in Figure 2, and these hits would be interesting candidates for a more thorough follow-up analysis.

Other major points:

1 The authors should consider and discuss whether the observed defects in RNA polymerase I transcription and translation (Figure 6) could be indirect effects of reduced cellular fitness induced by the reported cell cycle arrest.

2. The cell cycle analysis (Figure 4) was performed solely relying on high-content image analysis. However, the validation of some

hits by FACS would make this result more convincing, especially given that binucleation was obviously not considered in the image segmentation pipeline.

3. Figure 5 shows that prolonged inhibition of mitosis and DNA replication by different drugs (72h) has a drastic effect on the nucleolar number per nucleus. The figure would benefit if some representative images were shown, especially to evaluate nucleolar number relative to nuclear size.

Response to reviewers

We thank both of the reviewers for their thorough reading of our manuscript and their insightful comments. Responses to the reviewers' comments and concerns are included in this document in red. Referenced figure numbers refer to the figure numbers in the revised version, and may be different than those in the previously submitted manuscript. Revisions have been made throughout the manuscript and are highlighted in yellow, including the following new and/or revised figures/tables and legends:

-Figure 3 (minor revision)

-Figure 4 (new)

-Figure 5 (major revision)

-Figure 6 (major revision)

-Supplemental Figure S2 (minor revision)

-Supplemental Figure S6 (new)

-Table 1 (minor revision)

-Supplemental Table S1 (minor revision)

-Supplemental Table S3 (new)

-Supplemental Table S4 (major revision)

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It would be useful if the authors comment more specifically on the acrocentric chromosomes and rDNA distribution in MCF10A cells, as this is directly relevant.

We have added text to the Introduction to highlight this important point.

A previously reported karyotype analysis of the immortalized MCF10A cell line revealed minor rearrangements of chromosomes 3, 6, and 9, as well as an extra copy of both chromosomes 6 and 18. The rDNA loci (nucleolar organizing regions) in human cells are localized on chromosomes 13, 14, 15, 21, and 22, and therefore were not impacted in this cell line and, in part, formed the basis for the selection of this cell line for our screen (Soule *et al.*, 1990).

Can the authors explain the rationale for siRISC-free and siKIF11 as negative and positive controls.

We have added text to the Results that explains our rationale for these controls.

siRISC-free and siKIF11 were selected as the negative and positive controls, respectively, in large part due to the favorable and reproducible Z-prime values between the two conditions relative to the ≥5 nucleoli per nucleus phenotype.

The rationale for using siRISC-free as the negative control was that, at the time of the screen, it was a standard negative control to provide a baseline for the cellular response to transfection with lipid-RNA complexes. It is an siRNA that has been modified such that it cannot be loaded into the RNA-induced silencing complex (RISC) and therefore cannot modulate mRNA levels. By the time we performed the biochemical analyses in Figure 7 several years later, the siON-TARGET*plus* non-targeting control pool (siNT) had been developed to minimize off-target binding, and therefore we updated our negative control to a negative control that can be loaded into RISC, but that does not have cellular mRNA targets.

The rationale for using siKIF11 as the positive control was due to the reproducibility with which treatment led to an increase in the percentage of cells with ≥5 nucleoli per nucleus. While KIF11 is a kinesin motor protein required for spindle assembly and mitosis (Blangy *et al.*, 1995), it has also been reported as a driver in cancer pathogenesis (Venere *et al.*, 2015; Pei *et al.*, 2017; Pei *et al.*, 2019), and required for protein synthesis during interphase (Bartoli *et al.*, 2011). We confirmed this observation in our biochemical analyses (Figure 7C-D), and further identified a significant defect in RNAPI transcription upon KIF11 depletion (Figure 7B). Thus, while initially included in our screen development assays as a positive transfection control (Weil *et al.*, 2002; Zanin *et al.*, 2013; von Stechow *et al.*, 2015), because it reliably produced an increase in nucleolar number, we verified that it could be used as a positive control with which to identify other proteins that, when depleted, cause an increase in nucleolar number.

Lines 199-201. I don't understand the argument that proteins with IDRs determine nucleolar number through LLPS behaviour. I am not aware of any direct evidence that fusion of chromosomally tethered nucleoli involves liquid like behaviour.

We have removed this analysis from our manuscript.

We initially included this analysis in our manuscript given the interest surrounding liquid-liquid phase separation (LLPS) as a driver of nucleolar formation, including coalescence events [as reviewed in (Lafontaine *et al.*, 2020)]. While initial studies were performed in *Xenopus* oocytes (Brangwynne *et al.*, 2011), more recent studies have observed coalescence events in live human cells, with chromosomally tethered nucleoli (Caragine *et al.*, 2018, 2019).

While the mechanism underlying the increased nucleolar number that we observe remains unknown, one hypothesis is that nucleoli are failing to coalesce as they would under normal conditions. For this reason, we asked whether our screen hits overlap with proteins implicated in LLPS. Because intrinsically disordered regions (IDRs) are reported drivers of LLPS (Lafontaine *et al.*, 2020), we used the DisProt database to determine whether the proteins uncovered by our screen contained IDRs. Interestingly, however, we found that only 4/113 screen hits harbor intrinsically disordered regions (IDRs), which suggests that LLPS may not be a driving principle regulating nucleolar number as described in our manuscript.

While it still may be of interest to some readers, we understand that this may be a leap in that the mechanisms underlying the ≥5 nucleoli per nucleus phenotype remain unknown. We have therefore decided to remove this analysis from the manuscript.

siKIF11 positive control cells not only have more nucleoli but are quite a bit larger than the negative control. This is observed in most of the hits shown in Fig 2A.

Excellent point. We have now included an analysis of nuclear area using the images collected for screen validation by oligonucleotide deconvolution, including each hit pictured in Figure 2A (New Figure 4). The text has been revised appropriately to report this new analysis.

Our analysis revealed that we do in fact observe a significant increase in nuclear area in nuclei with ≥5 nucleoli (New Figure 4A-B). Interestingly, however, this result is observed not only when screen hits are depleted, but also in the negative control cells. We do observe some variability in the nuclear size increase (New Figure 4B). Notably, depletion of CDCA8 and INCENP resulted in a ≥2-fold increase in the nuclear area of nuclei with ≥5 nucleoli compared to siRISC-free. CDCA8 and INCENP are known mitotic inhibitors and their depletion may represent failures in previous cell divisions as pointed out by Reviewer #2. Yet, depletion of the majority of screen hits reveals an approximately 200 pixel increase in nuclear area in nuclei with ≥5 nucleoli, including siRISC-free treatment, and based on our cell cycle analysis this does not necessarily correlate with accumulation of cells in G2/M phase (Revised Figure 5A). Intriguingly, however, it has been reported that nuclear volume scales with cellular volume, which gradually increases through the cell cycle (Jorgensen *et al.*, 2007; Neumann and Nurse, 2007; Maeshima *et al.*, 2011; Cantwell and Nurse, 2019), thus these data are consistent with our observation that the ≥5 nucleoli per nucleus phenotype is largely observed in cells in G2/M phase (Revised Figure 5B).

At face value the cell cycle profiling shown in Fig 4 suggests ~60% of these cells are in G2/M. This is based purely on DNA content. In my view, a more thorough analysis of cell-cycle stage by means other than measuring DNA content, combined with karyotype analysis of these positive control cells and some of the hits is warranted.

We have revised the cell cycle figure to include representative histograms of the log2-transformed integrated Hoechst 33342 fluorescence intensities and quantified each replicate individually to perform statistical analyses on which to base our conclusions (Revised Figure 5A; Revised Supplemental Table S4). In re-analyzing these data, however, we identified an error in our reporting of nuclei with >4N DNA content. These were accidentally defined as those with an integrated DNA intensity >2.50, but also <0.5. This has been remedied. The text has been revised appropriately to accommodate these revisions.

Hoechst 33342 (or DAPI) is a blue, fluorescent dye that binds in a stoichiometric manner to the minor groove of double-stranded DNA. While Hoechst or propidium iodide staining followed by flow cytometry is the most common method used to determine cell cycle phase based on fluorescence intensity, methods using high-content imaging have been developed and shown to be comparable to data acquired by flow cytometry (Chan *et al.*, 2013; Roukos *et al.*, 2015; Gomes *et al.*, 2018). Additionally, performing cell cycle profiling on the same cells with which we quantified nucleolar number allowed us to conclude that the increased nucleolar numbers that we observe are largely in nuclei in G2/M phase (Revised Figure 5B), which lends important insight into potential mechanisms underlying this phenotype.

It is true that our cell cycle profiling data revealed that, on average, ~60% of KIF11-depleted cells accumulate in G2/M phase (72h depletion), with an additional ~12% of cells with a >4N DNA content. While this may seem like a large percentage of the total cell population, it is consistent with previously published literature on KIF11. In one study, siRNA-depletion of KIF11 in triple negative breast cancer cell lines (24-48h depletion) revealed ~36-40% of cells in G2/M phase using propidium iodide (PI) staining followed by flow cytometry (Jiang *et al.*, 2017). In another study, also using PI followed by flow cytometry, siRNA-depletion of KIF11 in hTERT cells revealed 64% of cells in G2/M (5d depletion), and an additional 12% with a >4N DNA content. Intriguingly, however, (Asbaghi *et al.*, 2017) also depleted KIF11 in HCT116 cells and only observed 18% in G2/M and 15% with a >4N DNA content (Asbaghi *et al.*, 2017). These

data suggest that while our data are largely consistent with published studies, results may be cell line dependent and/or, in part, related to the duration of knockdown.

With regards to a karyotype analysis of KIF11-depleted cells and other screen hits, while it would be interesting to understand the extent to which each of the screen hits impacts chromosome instability, given the diverse range of hits it is likely that the mechanisms underlying each hit's contribution to chromosome instability, if any, are different. Furthermore, if chromosome instability in the form of aneuploidy is in fact observed, and extra copies of the rDNA/NOR-bearing chromosomes are present, this may suggest a possible cause for increased numbers of nucleoli; however, this hypothesis would still need to be tested as more NORs doesn't necessarily mean active NORs/nucleoli. Finally, if aneuploidy were the leading mechanism underlying the increased nucleolar number phenotype, it is likely that the increased nucleolar numbers would be observed in all phases of the cell cycle, whereas we find that the increased nucleolar numbers are mostly observed in cells in G2/M phase, for both wildtype (siNT) and hit-depleted cells (Revised Figure 5B). For these reasons, a karyotype analysis alone would be insufficient to address the broader question of what contributes to the increased nucleolar numbers that we observe.

Lines 253-256. Given the issues raised above can this statement be made.

We have added clarifying text to these lines. The conclusions made in here are based on our cell cycle profiling that analyzed DNA content, that we have now strengthened with statistics.

Lines 324-327. Nucleolar localisation of INCENP etc is consistent with but not sufficient evidence for a direct effect on transcription. Can the authors be more precise about the localisation of these proteins. Are the GC, DFC or FC localised? This could strengthen or weaken the argument.

Great point. We have added clarifying text to the manuscript, references to the "nucleolar" column in Supplemental Table S1, and performed an additional analysis based on available published data. Additionally, review of these data has led to a correction. We had indicated that TAF1D localized to the nucleolus, but this protein, although associated with the RNAPI SL1 promoter complex, was not identified in any of the datasets used.

We did indicate that nucleolar localization *may* suggest direct regulation, however, it is true that more data is required to establish the mechanism by which INCENP and the other screen hits with effects on RNAPI transcription (nucleolar localized and not) regulate RNAPI.

As far as being more precise about the subcellular localization of the screen hits, we do understand the weaknesses of our localization analysis; however, due to the high-throughput nature of this study, we had to use existing datasets in order to broadly analyze all of our results.

In this study, we used data collected both by mass spectrometry (Leung *et al.*, 2006; Ahmad *et al.*, 2009; Jarboui *et al.*, 2011), as well as by immunofluorescence microscopy (Thul *et al.*, 2017; Thul and Lindskog, 2018), and were lenient in calling nucleolar proteins based on presence in just 1 of the datasets. In the data collected by immunofluorescence microscopy, the researchers had distinguished between proteins that localize to the nucleolus (staining through whole nucleolar area) and the nucleolar fibrillar center [punctate signal in the nucleolus; (Thul *et al.*, 2017)], and we combined these datasets in our analysis. Here, however, we have performed an additional analysis separating these datasets to see if we can pull out more specific localization information on the screen hits. Of the 11 hits that we identified as nucleolar localized based on the Human Protein Atlas immunofluorescence data, 10 localized to "nucleoli", and just one

(ANKEF1) localized to “nucleoli fibrillar centers”. INCENP was not included in this dataset (Thul *et al.*, 2017), but was identified as nucleolar localized in the other two (Leung *et al.*, 2006; Ahmad *et al.*, 2009; Jarboui *et al.*, 2011).

Reviewer #2 (Remarks to the Author):

The authors address a very interesting and relevant question in the field. It has been known for decades that cancer cells often display changes in nucleolar morphology and number. However, the underlying mechanisms controlling these features remain largely unclear. Identification of factors affecting the number of nucleoli per cell might even reveal targets relevant for cancer therapy. Here, the authors set out to mine and validate screening results of a previously performed genome-wide RNAi screen (Farley-Barnes et al., Cell Reports, 2018). While their previous analysis had focused on hits that cause a reduction in nucleolar number, the current manuscript reports on factors whose depletion cause an increase in nucleoli per cell.

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We have added text to the Results that explains our rationale for these controls.

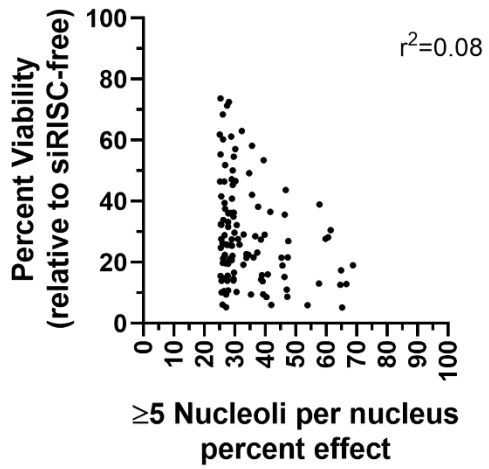
The rationale for using siKIF11 as the positive control was primarily due to the reproducibility with which treatment led to an increase in the percentage of cells with ≥5 nucleoli per nucleus. This was determined using Z-prime value monitoring between siRISC-free and siKIF11 during our assay development. Thus, while initially included in our screen development assays as a positive transfection control, because it reliably produced an increase in nucleolar number we decided that it could be used as a positive control for the screen with which to identify other proteins that, when depleted, cause an increase in nucleolar number.

Proteins required for mitosis, like KIF11, were in fact enriched among the screen hits (although other cellular processes were enriched as well, as shown in Figure 3). As a result, however, we performed a cell cycle analysis to identify whether mitotic inhibition was a unifying theme among the hits. Results from our analysis, as shown in Revised Figure 5A (Revised Supplemental Table S4), confirmed KIF11's role in mitosis with depletion leading to ≥2-fold accumulation of cells in G2/M phase; however, on investigation of a subset of 20 hits, only depletion of 8 caused ≥2-fold accumulation of cells in either G2/M or with a >4N DNA content (INCENP, TPX2, ABCE1, CDCA8, DYNC1H1, ENY2, INKA1, and RACGAP1). Furthermore, when taking into consideration whether an individual siRNA treatment that caused a cell cycle defect also resulted in an increase in the ≥5 nucleoli per nucleus percent effect, there are instances where a defect is observed, but no concomitant increase in nucleolar number is observed (e.g. DYNC1H1, INKA1, and others; Revised Supplemental Table S4). These data suggested to us that while failed mitosis may lead to the observed increase in nucleolar number with some of the screen hits, it is not likely the cause for increased nucleolar number for all of the screen hits.

We have also now evaluated whether an increased ≥5 nucleoli per nucleus percent effect is correlated with decreased viability (Response to reviewers Figure 1). Pearson r correlation analysis suggests there is no linear correlation between the ≥5 nucleoli per nucleus percent effect and viability (r^2=0.08). This analysis suggests that while siKIF11 and inhibition of other mitotic regulators are commonly used as death controls, this screen and the increase in percentage of cells with ≥5 nucleoli per nucleus does not simply report cell death.

Furthermore, while KIF11 is a kinesin motor protein required for spindle assembly and mitosis as you have described, it has also been reported as a driver in cancer pathogenesis (Venere *et al.*, 2015; Pei *et al.*, 2017; Pei *et al.*, 2019), and important for protein synthesis during interphase (Bartoli *et al.*, 2011). We confirmed a role for KIF11 in protein synthesis in our biochemical analyses (Figure 7C-D), and further identified a significant defect in RNAPI transcription upon KIF11 depletion (Figure 7B). In addition to KIF11, the other hits evaluated that caused an accumulation of cells in G2/M phase both caused a significant decrease in RNAPI transcription when depleted (INCENP and TPX2). Given the dynamic remodeling of the nucleolus during mitosis, we hypothesize that the identified proteins may not only be required for mitosis, but also for the nucleolar disassembly and assembly during mitosis through RNAPI regulation. Further intriguing and highlighted in Table 2, both KIF11 and INCENP were also identified in screens for regulators of nucleolar size. Taken together, these data necessitate further in-depth study to understand their role in nucleolar biology.

Thus, while KIF11 may seem like an odd selection for a positive control, the reproducibility with which depletion led to the increase in the percentage of cells with ≥5 nucleoli per nucleus allowed us to perform a reliable screen that led to the identification of 113 proteins that regulate nucleolar number and several that are required for RNAPI transcription.



Response to reviewers Figure 1. **There is no linear correlation between the ≥5 nucleoli per nucleus percent effect and viability.** For each of the 113 high confidence hits, the ≥5 nucleoli per nucleus percent effect (x-axis) and percent viability relative to siRISC-free (y-axis) are shown as a scatter plot. Pearson r correlation analysis of these data reveals r^2=0.08 and therefore no linear correlation between the two variables.

Given the example images provided in Figure 2 and the segmentation examples in Figure S1A, the nuclei shown for many hits are larger than those of the control cells, perhaps reflecting failures in previous cell divisions. It would be nice, if the authors would analyze the number of nucleoli relative to the nuclear size for the selected follow up hits.

Excellent observation. We have now included an analysis of nuclear area using the images collected for screen validation by oligonucleotide deconvolution, including each hit pictured in Figure 2A (New Figure 4). The text has been revised appropriately to report this new analysis.

As stated above in answer to Reviewer #1, we do in fact observe a significant increase in nuclear area in nuclei with ≥5 nucleoli (Figure 4A-B). Interestingly, however, this result is observed not only when screen hits are depleted, but also in the negative control cells. Yet, we do observe some variability in the nuclear size increase (Figure 4B). Notably, depletion of CDCA8 and INCENP resulted in a ≥2-fold increase in the nuclear area of nuclei with ≥5 nucleoli compared to siRISC-free. CDCA8 and INCENP are known mitotic inhibitors, and their depletion may represent failures in previous cell divisions. Yet, depletion of the majority of screen hits reveals an approximately 200 pixel increase in nuclear area in nuclei with ≥5 nucleoli, similar to siRISC-free, and based on our cell cycle analysis this does not necessarily correlate with accumulation of cells in G2/M phase (Revised Figure 5A; Revised Supplemental Table S4). Intriguing, however, it has been reported that nuclear volume scales with cellular volume, which gradually increases through the cell cycle (Jorgensen *et al.*, 2007; Neumann and Nurse, 2007; Maeshima *et al.*, 2011; Cantwell and Nurse, 2019), thus these data are consistent with our observation that the ≥5 nucleoli per nucleus phenotype is largely observed in cells in G2/M phase (Revised Figure 5B).

Despite the aforementioned questions, the paper highlights some intriguing hits such as ENY2 or H1-10, which show an increased number of nucleoli in normally sized cells according to the images presented in Figure 2, and these hits would be interesting candidates for a more thorough follow-up analysis.

Thank you. Additionally, we believe that several of the hits will be interesting to pursue in greater depth due to our discoveries in Figure 7, which revealed several required for the regulation of RNAPI transcription and protein translation.

Other major points:

1 The authors should consider and discuss whether the observed defects in RNA polymerase I transcription and translation (Figure 6) could be indirect effects of reduced cellular fitness induced by the reported cell cycle arrest.

We have added a new supplemental figure (New Supplemental Figure S6) and have modified the text appropriately to comment on this interesting point.

It is possible that cell cycle defects could lead to RNAPI transcription and translation defects through p53 repression of RNAPI (Beckerman and Prives, 2010). However, it is also possible that defects in RNAPI transcription and translation could lead to cell cycle arrest through both p53-mediated and p53-independent mechanisms (Rubbi and Milner, 2003; James *et al.*, 2014). Based on this understanding, we decided to perform a new analysis of p53 levels by western blot to determine the extent to which p53 is stabilized upon depletion of each hit included in Figure 7 and Table 1 in bold (New Supplemental Figure S6). While previous unpublished data from our laboratory suggests that the p53 nucleolar stress response is more muted in MCF10A cells, we do see a significant 2-fold increase in p53 when the ribosome biogenesis factor, NOL11, is depleted. Interestingly, these data reveal that depletion of only 2 proteins caused a significant increase in p53 levels (RFC1 and RACGAP1). Given these data, we conclude that p53 repression of RNAPI is not likely the cause for the RNAPI defects we observe. Thus, it is difficult to address the observed impacts on ribosome biogenesis and the cell cycle without

further in-depth analysis on each individual screen hit to determine the mechanism by which RNAPI transcription and translation are impacted and how that ties to the cell cycle defects we observe in some cases.

2. The cell cycle analysis (Figure 4) was performed solely relying on high-content image analysis. However, the validation of some hits by FACS would make this result more convincing, especially given that binucleation was obviously not considered in the image segmentation pipeline.

We have now revised our cell cycle figure to include representative histograms of the log2-transformed integrated Hoechst 33342 fluorescence intensities and quantified each replicate individually to perform statistical analyses on which to base our conclusions (Revised Figure 5A; Revised Supplemental Table S4). In re-analyzing these data, however, we identified an error in our reporting of nuclei with >4N DNA content. These were accidentally defined as those with an integrated DNA intensity >2.50, but also <0.5, and has now been remedied. The text has been revised appropriately to accommodate these revisions.

Hoechst 33342 (or DAPI) is a blue, fluorescent dye that binds in a stoichiometric manner to the minor groove of double-stranded DNA. While Hoechst or propidium iodide staining followed by flow cytometry is the most common method used to determine cell cycle phase based on fluorescence intensity, methods using high-content imaging have been developed and shown to be comparable to data acquired by flow cytometry (Chan *et al.*, 2013; Roukos *et al.*, 2015; Gomes *et al.*, 2018). Additionally, performing cell cycle profiling on the same cells with which we quantified nucleolar number allowed us to conclude that the increased nucleolar numbers that we observe are largely in nuclei in G2/M phase (Revised Figure 5B), which lends important insight into potential mechanisms underlying this phenotype.

As noted, and presented in Supplemental Figure S1A, while segmentation of annular nuclei was sometimes identified as a single nucleus rather than binuclear, when we performed our cell cycle analysis, we did identify that depletion of 8 hits led to an accumulation of cells with a >4N DNA content [INCENP, TPX2, ABCE1, CDCA8, DYNC1H1, ENY2, INKA1, and RACGAP1; (Revised Figure 5A)]. The images in Supplemental Figure S1A were from cells depleted of INCENP, and INCENP was included among these screen hits. Thus, we conclude that in some cases the increased nucleolar numbers that we observe may be due to failed cytokinesis revealed by increased nuclei with >4N DNA content in our cell cycle analysis, but this is not the case for all the screen hits analyzed. Furthermore, regardless of the mechanism(s) underlying the increase in nucleolar number, we still identified significant effects on ribosome biogenesis when screen hits were depleted, including with depletion of KIF11, INCENP, TPX2, ENY2, and RACGAP1 (ABCE1 and DYNC1H1 were not tested).

3. Figure 5 shows that prolonged inhibition of mitosis and DNA replication by different drugs (72h) has a drastic effect on the nucleolar number per nucleus. The figure would benefit if some representative images were shown, especially to evaluate nucleolar number relative to nuclear size.

Thank you for this suggestion. We have now revised Figure 6 to include representative images of the cells with the different drug treatments at the 72-hour time point (Revised Figure 6B). We have also performed an additional analysis to evaluate nuclear size relative to the number of nucleoli [(0-4 vs. ≥5; Revised Figure 6C-F]. Furthermore, based on the increased scrutiny of the images obtained from this experiment, we have removed data from the actinomycin D treatment. While we knew that after actinomycin D treatment viability was low for all time points (<4% relative to DMSO in 6/9 replicates), manual review of all 81 images revealed that many

fields of view failed to detect a single nucleus and therefore we do not feel comfortable reporting these results. As a follow-up, we performed a manual review of the remaining drug treatments that had a viability <10%, and did not have to make other changes to our reporting.

As we observed with siRNA treatment, in all conditions the nuclei of cells with ≥5 nucleoli are significantly larger, even in the DMSO control condition (Revised Figure 6C-D). We do, however, observe that the nucleus of cells with ≥5 nucleoli is even larger under some conditions [e.g. ICRF-193 (72h), hesperadin (48h), and hesperadin (72h); Revised Figure 6D]. Hesperadin, for example, is an Aurora B kinase-selective inhibitor with significantly larger nuclei (Revised Figure 6D). Aurora B kinase interacts with INCENP and CDCA8 during mitosis, and depletion of INCENP and CDCA8 also led to the largest nuclei in cells with ≥5 nucleoli. Thus, these data are consistent with what we observed in our analysis of nuclear area upon siRNA treatment (New Figure 4B). Furthermore, in only a subset of conditions does nuclear area significantly increase over time, and therefore nuclear area does not strictly correlate with the ≥5 nucleoli per nucleus percent effect we observe. The increase that we observe was in both nuclei with 0-4 nucleoli [(etoposide, ICRF-193, mitomycin C, and BMH-21); Revised Figure 6E], as well as in nuclei with ≥5 nucleoli [(hesperadin, etoposide, ICRF-193, mitomycin C, and 5-fluorouracil); Revised Figure 6F].

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RE: Manuscript #E20-10-0670R
TITLE: "Increased numbers of nucleoli in a genome-wide RNAi screen reveal proteins that link the cell cycle to RNA polymerase I transcription"

Dear Dr. Baserga,

The reviewers have gone through your revised paper and their comments are appended below. While reviewer 1 is satisfied with your revision, reviewer #2 requests that you add text in your discussion as to the possibility that some of your hits may be indirect (i.e., some hits might only affect nucleolar numbers because cells have become tetraploid (and have an increased nuclear size for this reason.) Once you have added this caveat to your discussion, I will be happy to accept your paper for publication in MBoC.

Sincerely,
Jennifer Lippincott-Schwartz
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Baserga,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript requires minor revisions before it can be published in Molecular Biology of the Cell, as described in the Monitoring Editor's decision letter above and the reviewer comments (if any) below.

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Reviewer #1 (Remarks to the Author):

This revised manuscript has comprehensively addressed all of this reviewers original comments. It would appear that it has similarly dealt with comments from the other reviewer (a number of which overlap). The authors should be applauded for the thoroughness of their responses. New data has been provided, some of the original data has been re-evaluated and there are many changes to the text. This revised manuscript represents a very valuable contribution to the field of nucleolar formation and undoubtedly we ignite further interest in links between nucleoli and the cell cycle.

Reviewer #2 (Remarks to the Author):

In the revised manuscript, the authors have nicely addressed the choice of si-KIF11 as the positive control for their analysis and convincingly explain why it was chosen.

In addition, they describe more clearly in the Results section that several of their hits might affect nucleolar numbers only as a secondary and indirect consequence of cell division defects. Yet, in the discussion the authors don't mention this caveat. Here, the authors should be more explicit and state that failure in cytokinesis will duplicate the number of NORs per cell and increase cell size, and that (some of the) hits affecting 'cell cycle progression' might not be direct regulators of ribosome synthesis or nucleolar numbers (lines 471-473).

The revised presentation of cell cycle profiles is more appealing and informative, yet it still faces the problem that it does not allow to differentiate between failures in cell division plus subsequent tetraploidization versus prior cell cycle arrest at G2/M. The discussion is mostly considering an effect of cell cycle hits on G2/M, but a number of 'hits' and some of the drugs tested in Figure 6 are known to cause cytokinesis defects (see also above).

Further points:
- Percentages in Figure 5 don't match percentages given in corresponding supplemental table. Both should be aligned.

Overall, the revised version has improved but the authors should be more explicit about the potential impact of cell division errors on the interpretation of their data, most importantly in the Discussion section.

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considering an effect of cell cycle hits on G2/M, but a number of 'hits' and some of the drugs tested in Figure 6 are known to cause cytokinesis defects (see also above).

We have now added this caveat to the first paragraph of the discussion that states failed cytokinesis in some cases could cause the increased numbers of nucleoli that we observe.

Further points:
- Percentages in Figure 5 don't match percentages given in corresponding supplemental table. Both should be aligned.

Thank you for your careful attention to these data. We have double-checked the percentages in Figure 5 compared to the corresponding Supplemental Table S4. We incorrectly labeled the INCENP histogram with siINCENP-04 rather than siINCENP-02. This has now been corrected. Otherwise, the percentages are accurate. The histograms are labeled in accordance with the Chan *et al.* (2013) protocol that we followed; however, this may have led to some confusion because we defined % G2/M as nuclei with DNA content from 1.75-2.25 *and* 2.25-2.5. Additionally, % >4N includes just the nuclei with DNA content >2.5, and the percent of cells labeled in the figure include not only those >2.5, but also <0.75. We have modified the figure legend to make this more clear and we have removed from the histogram the percentage listed for the cells “outside the dotted pink.”

Overall, the revised version has improved but the authors should be more explicit about the potential impact of cell division errors on the interpretation of their data, most importantly in the Discussion section.

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