JCB

centrocortin RNA localization to centrosomes is regulated by FMRP and facilitates error-free mitosis

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Review Timeline:

Submission Date:	2020-04-14
Editorial Decision:	2020-05-10
Revision Received:	2020-09-12
Editorial Decision:	2020-10-05
Revision Received:	2020-10-13

Monitoring Editor: Arshad Desai

Scientific Editor: Melina Casadio

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

DOI: https://doi.org/10.1083/jcb.202004101

May 10, 2020

Re: JCB manuscript #202004101

Dr. Dorothy Anne Lerit
Emory University School of Medicine
615 Michael Street
435 Whitehead Building
Atlanta, GA 30033

Dear Dr. Lerit,

Thank you for submitting your manuscript entitled "The coordinated localization of mRNA to centrosomes facilitates error-free mitosis". Your manuscript has been assessed by expert reviewers, whose comments are appended below. Although the reviewers express potential interest in this work, significant concerns unfortunately preclude publication of the current version of the manuscript in JCB.

As you will see, the reviewers find the work to be of interest but they have significant concerns about a number of conclusions presented (Reviewer #1 is most critical but concerns are echoed by the other reviewers). Given the many reviewer concerns, all of which related to key aspects of the manuscript, we believe that any revision will need to address all of these comments in full. While specific key experiments may need to be repeated/extended, a number of the criticisms should be addressable through more careful writing, statistical analysis, and better matching of the data to the conclusions. The reviewers have done a thorough job of highlighting the issues and any re-submitted manuscript will be re-evaluated by all 3 reviewers.

Please let us know if you are able to address the major issues outlined above and wish to submit a revised manuscript to JCB. Note that a substantial amount of additional experimental data likely would be needed to satisfactorily address the concerns of the reviewers. 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 the 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.

If you choose to revise and resubmit your manuscript, please also attend to the following editorial points. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:
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Figures: Your manuscript may have up to 10 main text figures. To avoid delays in production, figures

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Supplemental information: There are strict limits on the allowable amount of supplemental data. Your manuscript may have up to 5 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

If you choose to resubmit, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

Regardless of how you choose to proceed, we hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised. You can contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for thinking of JCB as an appropriate place to publish your work.

Sincerely,

Arshad Desai, PhD
Editor, Journal of Cell Biology

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

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

In this manuscript, Ryder and colleagues investigate the regulation of centrosomally localized RNAs in early Drosophila embryos. They adapted single-molecule fluorescent in situ hybridization (smFISH) and developed an image analysis pipeline to re-assess the distribution of five RNAs (cyc B, plp, pins, sov, and cen) reported to be enriched at the mitotic apparatus in a 2007 study (Lécuyer et al., 2007). Using this new pipeline, the authors reach similar conclusions reported in the 2007 study, but gain additional information on their differential regulation in a cell-cycle dependent manner. The authors then focus on studying cen RNA, which shows the most striking centrosomal enrichment among these five RNAs. Using a series of immunofluorescence, biochemical pulldowns, and genetic approaches, the authors conclude that the fly fragile-X mental retardation protein (FMRP) homolog regulates multiple aspects of cen RNA metabolism. Finally they show that mis-targeting cen RNA to the anterior pole impairs nuclear division and causes other mitosis-related defects.

One advance of this study is the development of the smFISH and its subsequent imaging analysis

pipeline. However, this pipeline falls short at the last step, a statistical analysis of the processed data. In their experiments, the difference between different conditions is often modest, some with a wide range of variations. Without proper statistical analysis, it is unknown whether the conclusions are supported by the data. Another concern is the failure to consider alternative interpretations for some of their results. For example, the Lécuyer group recently published an extensive characterization of cen RNA distribution in fly embryos, including its cell cycle-dependent centrosomal enrichment and the mechanism of centrosomal enrichment (Bergalet et al., 2020). The authors should expand the discussion and reconcile the data from both studies.

My concerns and suggestions are detailed below (roughly figure by figure). The authors should consider these points to make this manuscript suitable for publication.

Figure 1 and its supplement figures:

- Definition of interphase versus metaphase: The first 13 mitotic divisions of fly embryos do not have gap phases, cycling between M and short S-phases. How did the authors define "interphase" (i.e., S phase) here? Can some of the "interphase" actually include sub-phases of M phase other than the metaphase? Along this line, can the wider variation in the "interphase" data (e.g., lines 187-191) in part due to the potential mixed cell cycle stages in the "interphase" dataset, other than just reflecting the dynamic properties of the interphase centrosome?

- When assessing RNA distribution, the authors plotted cumulative % RNA as a function of distance to the centrosome. Using cumulative % RNA loses the information of RNA abundance at a given distance relative to others (i.e., the information of RNA distribution). This seems to defeat the purpose of plotting the data as a function of distance. Plotting fractions of RNA as a function of distance would preserve this information and make the centrosomal enrichment (or lack of it) easier to appreciate (e.g., a peak within the 1-µm range would indicate centrosomal enrichment).

- Statistical analysis is needed to assess whether there is a significant difference between the tested RNA relative to the control (gapdh RNA). For example, is the 1.6- vs. 1.3-fold difference in centrosomal enrichment of plp RNA between interphase and metaphase statistically significant (lines 162-164, Figure 1I)? The n numbers should be noted on the plots, not in a separate table (Figure 1 Supplemental Table 2), or at least the table should be placed with the main figures. Similarly, in line 172, what does "modest enrichments" really mean? Is the enrichment significantly different from the gapdh data or not? Again doing statistical analysis is needed to draw conclusions.

Figure 2 and its supplement figures:

- Same quantification issues as in Figure 1. For example, is the 15% to 20% increase (lines 221-223) or 12% to 18% increase (lines 223-225) statistically significant? Without statistical analysis, the conclusion that there is a difference in the centrosomal enrichment of cen RNA between interphase and metaphase is not well supported, especially in this case, where the variations are particularly large in the metaphase data (Figure 2G, H).

Figure 3A, B and Figure 3 Supplement 1:

- Lines 232-249: Regarding the cen granule and its translation. The Lécuyer group recently demonstrates that cen RNAs are accumulated at the centrosome as part of cen translating

polysomes since disrupting polysomes dissolves cen RNA granules (Bergalet et al., 2020). Together with the data presented in this manuscript, the centrosomal enrichment of cen RNA granules is likely the result of active cen translation, followed by its targeting to the centrosome as cen polysomes, and eventually its retention at the centrosome through the Cnn-Cen protein-protein interaction (Kao and Megraw 2009). Cen translation thus acts upstream of cen granule formation. Lines 247-248, "cen granule is not required for cen translation" is thus not the best conclusion. All the data seem to point to the model that translation of cen mRNA is required for cen granule formation.

Figure 3C-F and Figure 3 Supplement 2 and Figure 5

- Lines 258-259 (Figure 3 Supplement 2E, F): FMRP puncta are widespread, but only a small fraction of them overlaps with centrosomes and cen RNAs. In the zoom-out merged image (Figure 3 Supplement 2F), many cen granules appear to be FMRP-negative. The authors should perform co-localization analysis between these two signals to quantify the data. To further control for random co-localization events, the authors should also shuffle the cen and FMRP channels from different images (or turn one of the channels 90{degree sign}) and repeated the co-localization analysis (as done recently in the Bergalet et al., 2020 study).

- PLA experiments: How are the different conditions in Figure 3F compared? What are the "n.s" and the "****" results compared to? Without primary antibodies is also not a very good negative control because it is not testing the specificity of the antibodies. A good negative control would be to perform PLA in cen or fmr1 null embryos (both lines available to the authors) using both anti-Cen and anti-FMRP antibodies. The authors should also discuss why only a small fraction of centrosomes showed Cen/FMRP-positive PLA signals (Figure 3E).

- Figure 4 and its supplements: The difference in centrosomal enrichment of cen granules between the WT and fmr1 null embryos is modest (especially in NC10) and is not assessed by statistical analysis.

- The authors conclude that "FMRP negatively regulates cen localization to centrosomes" (line 306), "FMRP contributes to cen RNA turnover and translational repression" (line 324), and "FMRP contributes to multiple aspects of cen RNA post-transcriptional regulation, either directly or indirectly (lines 327-328)." However, an alternative interpretation is that FMRP only negatively regulates steady-state cen RNA levels (shown in Figure 5C) so that more cen RNAs are localized to the centrosome and more Cen proteins are made. The data support that FMRP regulates cen RNA turnover, but not necessarily translational repression, because controlling RNA levels alone could affect protein abundance. In short, affecting cen RNA levels alone appears to be sufficient to explain the changes in cen RNA localization and protein expression observed in Figures 4 and 5; FMRP may not contribute to multiple aspects of cen RNA regulation.

- Figure 5F-I (lines 345-356): The immunofluorescence images are not informative. Showing the quantifications of spindle defects along with the figures would be a better way to present the data. The process of quantification also needs to be clarified. What does the "N" mean? How many mitotic spindles were quantified per embryo? Is "76.1% N=16/21" vs. "48.1% N=13/27" significantly different? Statistical analysis should be performed to draw a conclusion (e.g., p values and variations between biological replicates).

Figure 6
- Lines 377-381: The data show that the enlarged cen RNAs are still associated with centrosomes

in cen-bcd-3'UTR embryos (Figure 6C and Figures 6 Supplement 1B). Therefore, native cen 3'UTR is NOT required for cen RNA localization, an opposite of what the authors conclude.

- In cen-bcd-3'UTR embryos, both cen RNA and Cen protein are concentrated at the anterior pole in the cen null background. It is thus unclear why the authors characterized the mitotic spindle phenotypes at ~50% egg-length in cen-bcd-3'UTR embryos, a region where both cen RNA and Cen protein are depleted.

- Since about 3-fold more Cen protein is made in cen-bcd-3'UTR embryos, this would complicate the interpretation of the phenotypes (i.e., local overexpression of Cen, cen RNA mis-targeting, or both, could contribute to the phenotypes). Constructing a cen-bcd-3'UTR transgene expressing Cen comparable to the WT level could be a better experimental system to address the proposed questions.

Minor points
- General comments on the flow and figure presentations: (1) The description often starts with the presentation of supplementary figures. The back and forth between the main and supplementary figures makes it hard for the readers to follow the story. (2) The immunofluorescence images could be larger while the quantification plots could be smaller. It is particularly hard to see the signals in the zoom-out fields.

- Line 147: the claim of higher order RNA structures is not well supported. At this magnification under light microscopy, individual RNA molecules may still be separated from one another instead of forming higher-order RNA structures.

- The cyc B smFISH images appear to have a low signal-to-noise ratio. If the signals are all true, cyc B RNA would be much more abundant than gapdh RNA. Could this be possible?

- Line 173: "However, for the purposes of this study, all measurements were made in the somatic region at approximately 50% egg-length unless otherwise noted." What are the "purposes" of this study? What is the rationale of choosing this particular location?

- Line 197: It is the Cen protein, not cen RNA, that was previously shown to be required for normal nuclear division in fly early embryos. This sentence is thus not completely correct.

- Figure 3I: It is not clear the purpose of showing this blot in a separate panel. It only shows that GFP-FMRP can be pulled down. Did the authors probe for endogenous Cen protein, i.e., a reciprocal IP of the result shown in Figure 3G?

- Figure 5 uses hours instead of nuclear cycles to stage embryos. The authors may want to unify the staging units so it is easier for the readers to follow.

- Figure 5F-I (lines 345-356): What is the purpose of labeling centrosomes with both anti-Cnn and anti-Asl antibodies?

- The description of the imaging processing pipeline is very technical (Methods session). Without a coding background, it is hard to follow.

- Bergalet et al., 2020 reports that the cen 3'UTR-mediated centrosomal targeting of ik2 RNA is functionally important in vivo. Therefore, in cen-bcd-3'UTR embryos, ik2 localization is likely also

perturbed due to the lack of cen 3'UTR. This may thus in part contribute to the observed phenotypes in cen-bcd-3'UTR embryos. The authors may want to incorporate the data such as this from the Bergalet et al., 2020 study more into their discussion/interpretation.

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

While the notion that centrosomes are DNA-containing organelles was put to rest twenty years ago, there are numerous accounts of mRNAs residing at or near centrosomes but no studies to address the functional importance of centrosomal mRNAs.
This work examines the functions of mRNA localization at centrosomes in early Drosophila embryos. The authors evaluated the localization of five mRNAs that were shown in a previous screen (Lecuyer et al 2007) to localize at or near centrosomes. They found two mRNAs at centrosomes (cen, sov), and two others that localize in the proximity of centrosomes (cyc B, plp). The authors go farther to show that FMRP negatively regulates the timing and quantity of cen mRNA into granules, and that heterozygous mutant cen embryos suppressed the partial lethality associated with Fmr1 mutant maternal effect embryos.
The paper is well written and presented, and the data are of high quality. The title might be overstated as it suggests a generalized requirement for mRNAs at centrosomes to coordinate embryonic mitosis, but only centrocortin was demonstrated. One element not provided, despite the quantification and statistical analysis, was evaluation of significance for the localizations of the mRNAs and therefore some conclusions were not well supported by the data, or quantification was needed in order to make the conclusions. These points are highlighted below.
Overall, this works makes an important advance to our understanding of localized mRNA function at centrosomes and its regulation by FMRP.

Major points:
1. It is important for the authors to ascribe significance (p values) to the measurements of mRNA levels and quantification of granules made for the five candidate mRNAs relative to gapdh, and for the effect of Fmr1 mutant on cen mRNA granule formation. The quantification data provided in the tables should make this straightforward.
Related to that, the data in Figure 1 and its supplements show that cyc B, sov, and plp mRNAs localize in the proximity of centrosomes in early embryos, consistent with Lecuyer et al 2007, but that pins mRNA did not. Some comment about this difference, or acknowledgement of it, should be included if this is a correct interpretation of your data - that pins mRNA does not localize in proximity to centrosomes. It is described as 'modest' on p. 7 but the authors should indicate if it is significant or not.
2. It is probably not fair to state that the cen mRNA granule is dispensable for Cen translation based on the data in Figure 3 and supplements. While evident that the granules are significantly reduced in the cnnB4 mutant and that Cen levels are not significantly different, the levels of Cen protein are probably mostly contributed maternally and perhaps most translation of cen mRNA may not occur at granules, but the data in Bergalet et al 2020 show that translation does occur at centrosomes. So both might be true: Cen can be translated without granules, and Cen is translated at centrosomal granules. It seems the authors considered this in the Discussion, but the header in the results on line 232 should be tempered and it seems to contradict their model in Figure 7.
3. The co-localization of FMRP with Cen granules, shown in Figure 3 Supplement 2, is not clear. The example that is highlighted does not appear to be representative, and quantitative analysis is required to conclude whether colocalization is significant. From the images, FMRP puncta appear uniformly distributed. FMRP does not have to localize at granules to negatively affect their

formation and the association of FMRP with Cen might occur outside of the granules. Likewise, the association of FMRP with cen mRNA shown in Figure 3 might also occur outside of the granule. One experiment to test the potential association of FMRP with Cen mRNA granules is to look at its recruitment to the oversized granules produced by the bcd 3'UTR fusion as in Figure 6.
4. The data in Figure 4 show that Fmr1 negatively regulates cen mRNA granule formation. What about the other mRNAs (cyc B, plp, and sov), were they likewise affected or is this effect specific to cen mRNAs? Accordingly, the title of the manuscript is too broad and should specify centrocortin mRNA.
5. The header for Figure 4 (Fmr1 regulates cen granule formation and size) should be adjusted, since the data here do not show a quantification of granule size. Instead, it shows that there is more cen mRNA in granules near the centrosome. The legend to Figure 4 refers to "cen" but should specify that it is cen mRNA. The conclusion from these data, that FMRP negatively regulates cen mRNA localization holds, but probably more accurate to state that it regulates its levels at centrosomes.

Minor points:
6. In the introduction, the authors refer to the essential functions for centrosomes in the early embryo, but of the citations for this statement (Deák et al., 1997; Freeman, Nüsslein-Volhard, & Glover, 1986; Sunkel & Glover, 1988) only the Sunkel & Glover citation addresses centrosome functions (polo mutant embryos). Citations of papers that directly address the functions of centrosomes would be more appropriate.
7. The tables are not labeled, and reviewers have to infer which table is which from context. The excel files should have a title within them like 'Table 1: quantification of RNA proximity to centrosomes', etc.
8. Figure 1E does not look like metaphase; please check it.
9. The text often refers to cen mRNA as 'cen'; need to add 'mRNA' to this description throughout the manuscript to differentiate it from the cen gene or Cen protein (eg on lines 306, 359, 446, etc).
10. Check the statement and citation on line 309 regarding transcriptional inactivity in the early embryo. There are some genes expressed during the early embryo, the gap genes being an example.
11. It is not clear how the paragraph that begins on line 373 culminates in the conclusion that "These data suggest that the normal temporal and spatial pattern of cen RNA localization requires sequence or structural elements encoded within the native cen 3'UTR. ".
12. In the text, the authors alternate between 'fmr1' and 'fmr'. Flybase refers to it as 'Fmr1', which is recommended.

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

The manuscript by Ryder et al reports that cen mRNA, an important component of centrosomes is required for proper mitoses during early embryogenesis and is a target of FMRP. The authors make important and elegant observations of mRNA localization at/near centromere during the cell cycle in Drosophila syncytia. They examine a few candidate mRNAs, then focus on cen. They go on to show that FMRP but not other RNA binding proteins associate with centrosomes in vivo and cen mRNA co-IPs with FMRP using an FMRP-GFP line. Furthermore, FMRP is required for the formation of cen granules and this is cell cycle dependent. Indeed, by NC13, loss of FMRP causes an increase in cen mRNA and Cen protein, suggesting a role in mRNA stability and translation, consistent with FMRP's known role as a translation inhibitor. Overall, this is an interesting paper that convincingly identifies a new mRNA target of FMRP and a role in ensuring proper mitoses for cen and FMRP. The title

seems a bit more general than the actual conclusions and there are some issues that I detail below.

1. The experiments in Figure 1 are reporting observations of mRNA localization and the results do not show a causative relationship between granule formation and cycB mRNA localization. Thus it is unclear why the authors conclude that cycB localization to centrosomes is dictated by granule formation (line 154).
2. The observation that cen mRNA is increased in its association with centrosomes during interphase compared to metaphase in NC13 is interesting. An interesting dynamic behavior is also reported for NC10. Do the authors think this reflects solely the dynamic behavior of cen mRNA localization, translation or both? Do the authors have qPCR and/or total protein data from these cycles to address this?
3. The authors nicely show that cnn is required for the formation of cen granules. However the conclusion that cen granules are not required for Cen translation is premature. While this is the most obvious explanation of cnn mutations causing no changes in Cen protein levels, the authors cannot eliminate possible effects on Cen protein stability. Therefore, in my opinion the current interpretation needs to be adjusted. Also, the western blot shown for Cen protein is not as convincing, although the accompanying quantification supports the "no change" reported result.
4. Although the 5A11 antibody has been published it would be useful if the authors checked its specificity in their hands using fmr1 nulls.
5. The RNA IPs support the conclusion that cen mRNA co-IPS with FMRP specifically but the authors should show the whole blot. Also related to RNA IPs, the authors should test for the presence of a nontarget mRNA. Finally, the FMRP-GFP is a useful tool but it would be more convincing if endogenous FMRP from wild-type embryos would pull down cen mRNA as well. Or perhaps the authors have data that FMRP levels in the FMRP-GFP line are similar to wild-type embryos.

Minor comments:
Lines 118, 119 - define all candidate mRNAs
"fmr" should read "fmr1" throughout

EMORY UNIVERSITY SCHOOL OF MEDICINE

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10 Sept 2020

Dear Dr. Desai and Reviewers,

Please accept a revised submission of our manuscript (202004101) for consideration for publication in *JCB*. The original title for this manuscript was "*The coordinated localization of mRNA to centrosomes facilitates error-free mitosis*," however, in response to reviewer comments, we revised the title. Our current submission is entitled, "*centrocortin* RNA localization to centrosomes is regulated by FMRP and facilitates error-free mitosis."

We were pleased to learn of the reviewers' potential interest in the work. In the following point-by-point response letter, we detail our efforts to address all of the comments provided by the reviewers. We note our responses using blue font.

In the text itself, we also highlight changes from the original submission using blue font.

Thank you for reconsidering this work for publication in *JCB*.

Reviewer #1 (Comments for Authors (Required)):
In this manuscript, Ryder and colleagues investigate the regulation of centrosomally localized RNAs in early Drosophila embryos. They adapted single-molecule fluorescent in situ hybridization (smFISH) and developed an image analysis pipeline to re-assess the distribution of five RNAs (cyc B, plp, pins, sov, and cen) reported to be enriched at the mitotic apparatus in a 2007 study (Lécuyer et al., 2007). Using this new pipeline, the authors reach similar conclusions reported in the 2007 study, but gain additional information on their differential regulation in a cell-cycle dependent manner. The authors then focus on studying cen RNA, which shows the most striking centrosomal enrichment among these five RNAs. Using a series of immunofluorescence, biochemical pulldowns, and genetic approaches, the authors conclude that the fly fragile-X mental retardation protein (FMRP) homolog regulates multiple aspects of cen RNA metabolism. Finally they show that mis-targeting cen RNA to the anterior pole impairs nuclear division and causes other mitosis-related defects.

One advance of this study is the development of the smFISH and its subsequent imaging analysis pipeline. However, this pipeline falls short at the last step, a statistical analysis of the processed data. In their experiments, the difference between different conditions is often modest, some with a wide range of variations. Without proper statistical analysis, it is unknown whether the conclusions are supported by the data. Another concern is the failure to consider alternative interpretations for some of their results. For example, the Lécuyer group recently published an extensive characterization of cen RNA distribution in fly embryos, including its cell cycle-dependent centrosomal enrichment and the mechanism of centrosomal enrichment (Bergalet et al., 2020). The authors should expand the discussion and reconcile the data from both studies.

We thank the reviewer for these comments. While working on revisions for this paper, we consulted a biostatistician and, paired with this reviewer's concern about showing cumulative distribution plots, we significantly revised our data visualization to facilitate statistical analyses. Specifically, we now present quantification of RNA residing at 0 μm from the centrosome, highlighting data where RNA and centrosome signals overlap. This approach streamlines the paper and allows us to focus discussion to our main point of interest: RNA at the centrosome. We intend for these changes to improve the clarity of

the work.

Our revised work also expands discussion of the Bergalet paper in the following ways. First, we draw reference to the work throughout the results section when we note complementary findings. Second, as suggested by the reviewer, we expanded our discussion (lines 400–416) to illuminate findings that are complementary to the Bergalet study, and also to highlight how our work furthers our understanding on the formation, dynamics, composition, regulation, and function of *cen* mRNA granules.

My concerns and suggestions are detailed below (roughly figure by figure). The authors should consider these points to make this manuscript suitable for publication.

Figure 1 and its supplement figures:

- Definition of interphase versus metaphase: The first 13 mitotic divisions of fly embryos do not have gap phases, cycling between M and short S-phases. How did the authors define "interphase" (i.e., S phase) here? Can some of the "interphase" actually include sub-phases of M phase other than the metaphase? Along this line, can the wider variation in the "interphase" data (e.g., lines 187-191) in part due to the potential mixed cell cycle stages in the "interphase" dataset, other than just reflecting the dynamic properties of the interphase centrosome?

The reviewer is correct that *Drosophila* embryos proceed through abridged S- and M-phases without intervening gap phases for the first 2 hours of development. We now note this in our introduction (lines 61–3) at the outset, before further introducing the embryo system. Further, on lines 134–137, we define how interphase versus metaphase embryos were selected. Because all of our interphase embryos have round nuclei and lack condensed chromosomes, they do not represent M-phase samples.

- When assessing RNA distribution, the authors plotted cumulative % RNA as a function of distance to the centrosome. Using cumulative % RNA loses the information of RNA abundance at a given distance relative to others (i.e., the information of RNA distribution). This seems to defeat the purpose of plotting the data as a function of distance. Plotting fractions of RNA as a function of distance would preserve this information and make the centrosomal enrichment (or lack of it) easier to appreciate (e.g., a peak within the 1-μm range would indicate centrosomal enrichment).

Thank you for this suggestion. We now highlight RNA localization at 0 μm from the centrosome (i.e., where RNA and centrosome signals overlap). This recommendation streamlines the paper and allows us to focus solely on RNA at the centrosome.

- Statistical analysis is needed to assess whether there is a significant difference between the tested RNA relative to the control (gapdh RNA). For example, is the 1.6- vs. 1.3-fold difference in centrosomal enrichment of plp RNA between interphase and metaphase statistically significant (lines 162-164, Figure 1I)? The n numbers should be noted on the plots, not in a separate table (Figure 1 Supplemental Table 2), or at least the table should be placed with the main figures. Similarly, in line 172, what does "modest enrichments" really mean? Is the enrichment significantly different from the gapdh data or not? Again doing statistical analysis is needed to draw conclusions.

We recognize the need for robust statistical analysis, and in the revised work, we present side-by-side statistical comparisons for each RNA versus *gapdh* and for RNAs at interphase versus metaphase. These changes are reflected throughout the text and figures.

As suggested, we revised our prior table and now include in our main text Table 1, tabulating numbers of embryos, centrosomes, and RNA objects quantified per condition.

Figure 2 and its supplement figures:

- Same quantification issues as in Figure 1. For example, is the 15% to 20% increase (lines 221-223) or 12% to 18% increase (lines 223-225) statistically significant? Without statistical analysis, the conclusion that there is a difference in the centrosomal enrichment of cen RNA between interphase and metaphase is not well supported, especially in this case, where the variations are particularly large in the metaphase data (Figure 2G, H).

These graphs are now revised and include statistical analysis.

Figure 3A, B and Figure 3 Supplement 1:

- Lines 232-249: Regarding the cen granule and its translation. The Lécuyer group recently demonstrates that cen RNAs are accumulated at the centrosome as part of cen translating polysomes since disrupting polysomes dissolves cen RNA granules (Bergalet et al., 2020). Together with the data presented in this manuscript, the centrosomal enrichment of cen RNA granules is likely the result of active cen translation, followed by its targeting to the centrosome as cen polysomes, and eventually its retention at the centrosome through the Cnn-Cen protein-protein interaction (Kao and Megraw 2009). Cen translation thus acts upstream of cen granule formation. Lines 247-248, "cen granule is not required for cen translation" is thus not the best conclusion. All the data seem to point to the model that translation of cen mRNA is required for cen granule formation.

Precisely when *cen* mRNA versus protein accumulates at centrosomes is still unclear; however, loss of the Cnn scaffold (cnn^{B4} mutation) blocks *cen* mRNA granule formation. We temper our conclusion in the revised text, saying (lines 210–214):

> *We observed no difference in the levels of Cen protein in wild-type (WT) versus cnn^{B4} mutant 0–2h embryos (Fig. S2B, B'). These data suggest that the cen mRNA granule is not required for normal steady state levels of Cen protein; however, an important caveat is that maternal deposition of Cen may obscure changes resulting from granule loss.*

In the discussion, we restate (lines 396–400):

> *However, disruption of cen granule formation, as in cnn^{B4} mutants, does not impair total Cen protein levels. This finding raises the possibility that Cen may be translated at alternate sites or that maternal stores of Cen obscure changes resulting from cen mRNA granule loss. These models are not mutually exclusive, and cen mRNA may be translated at multiple locales.*

Figure 3C-F and Figure 3 Supplement 2 and Figure 5

- Lines 258-259 (Figure 3 Supplement 2E, F): FMRP puncta are widespread, but only a small fraction of them overlaps with centrosomes and cen RNAs. In the zoom-out merged image (Figure 3 Supplement 2F), many cen granules appear to be FMRP-negative. The authors should perform co-localization analysis between these two signals to quantify the data. To further control for random co-localization events, the authors should also shuffle the cen and FMRP channels from different images (or turn one of the channels 90{degree sign}) and repeated the co-localization analysis (as done recently in the Bergalet et al., 2020 study).

We agree the FMRP immunofluorescence does not provide a clear picture, and we now show only a single image of FMRP with Cnn. These data are in revised Supplemental Fig 2G and G' as part of a panel showing embryos co-stained for Cnn and a variety of RNA-binding proteins, of which FMRP was one we decided to follow-up on. In the text, we do not make conclusions other than to say (lines 224–6), "Among these, FMRP appeared predominantly cytoplasmic with a subset of puncta overlapping with centrosomes (arrowheads, Fig. S2G'). We selected FMRP for further analysis."

- PLA experiments: How are the different conditions in Figure 3F compared? What are the "n.s" and the

"****" results compared to? Without primary antibodies is also not a very good negative control because it is not testing the specificity of the antibodies. A good negative control would be to perform PLA in cen or fmr1 null embryos (both lines available to the authors) using both anti-Cen and anti-FMRP antibodies. The authors should also discuss why only a small fraction of centrosomes showed Cen/FMRP-positive PLA signals (Figure 3E).

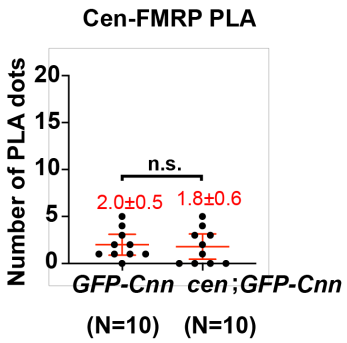

Fig. 1 Cen-FMRP PLA shows no difference between WT (*GFP-Cnn*) and *cen* null (*cen;GFP-Cnn*) embryos.

The reviewer is correct, we did use the no-antibodies sample as a negative control and basis for statistical comparison in the original submission, but we agree the *cen* or *Fmr1* nulls are better controls. This suggestion set us off on a series of PLA experiments.

As suggested, we sought to compare Cen-FMRP PLA signals in control embryos expressing *GFP-Cnn* versus *cen* null (*cen*; *GFP-Cnn)* embryos. By eye, we noted great variability in signals; in both cases, some embryos had several bright PLA puncta, other embryos had few or none. In short, we saw no statistical difference. We show in Fig. 1 representative data from this experiment.

At first, we assumed this was experimenter error, as the COVID-19 pandemic coupled with university restrictions necessitated other personnel to take over these experiments. We decided to take a step back and revisit the Cen-Puro PLA experiments as described in Bergalet et al., 2020. Using their published methods, we repeated PLA experiments using anti-Cen and anti-Puro antibodies in *GFP-Cnn* versus *cen* null (*cen*; *GFP-Cnn)* embryos. We still saw great embryo-to-embryo variability and no statistical difference. We purchased a fresh PLA kit, thinking the assay could be optimized, but this made no difference.

To quantify these data objectively, we performed two types of analyses in parallel. First, we created a custom ImageJ macro to threshold, segment, and then count puncta in batch. Second, we blinded the experimenter to genotype, and then manually counted the puncta. We observed similar trends from either approach. That is, we saw no statistical difference (Fig. 2A, B). Several attempts to repeat the experiment produced the same, obfuscating results.

We then added another control. We pre-treated *GFP-Cnn* embryos with anisomycin, using the same protocol described in the Bergalet et al. 2020 paper. Anisomycin blocks ribosome assembly, so pretreatment with this drug should block translation (that is, we predicted no PLA signals). To our surprise, we noted no difference in PLA signals from anisomycin versus untreated *GFP-Cnn* or *cen; GFP-Cnn* embryos. We show in Fig. 2 representative data from these trials.

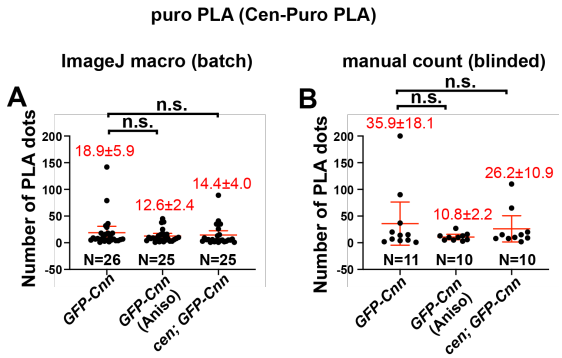

Fig. 2 Cen-Puro PLA (puro-PLA for Cen) shows no difference between WT (GFP-Cnn), cen null, or anisomycin pre-treated embryos. (A) A custom ImageJ macro was designed to batch analyze the data. (B) Some of the same images (experimenter was blinded to condition/genotype) were manually analyzed. N, # of embryos counted. Mean±S.D. are displayed (red text).

These experiments prompted us to pull the PLA studies from our submission, as we no longer trust those data. We are grateful the reviewer suggestion prompted us to revisit this assay.

While our revised manuscript no longer includes PLA assays to demonstrate Cen-FMRP interactions, we do add new data further confirming their association.

Our original work included the finding Cen pulls down FMRP (shown now in revised Fig. 3C). We now include data showing co-immunoprecipitation (FMRP pulls down Cen), and we show the large *cen* RNPs that form in *cen-bcd-3'UTR* embryos are sufficient to recruit FMRP, both experiments suggested by Reviewer 2 and presented in revised Fig. 3F and Fig. 9A–C, respectively.

- Figure 4 and its supplements: The difference in centrosomal enrichment of cen granules between the WT and fmr1 null embryos is modest (especially in NC10) and is not assessed by statistical analysis.

These graphs are now revised and include statistical analysis. For the *Fmr1* analysis, we did increase the number of embryos examined (reflected in our revised Table 1) to ensure statistical power was reached. These data are presented in revised Figures 4 and 5.

- The authors conclude that "FMRP negatively regulates cen localization to centrosomes" (line 306), "FMRP contributes to cen RNA turnover and translational repression" (line 324), and "FMRP contributes to multiple aspects of cen RNA post-transcriptional regulation, either directly or indirectly (lines 327-328)." However, an alternative interpretation is that FMRP only negatively regulates steady-state cen RNA levels (shown in Figure 5C) so that more cen RNAs are localized to the centrosome and more Cen proteins are made. The data support that FMRP regulates cen RNA turnover, but not necessarily translational repression, because controlling RNA levels alone could affect protein abundance. In short, affecting cen RNA levels alone appears to be sufficient to explain the changes in cen RNA localization and protein expression observed in Figures 4 and 5; FMRP may not contribute to multiple aspects of cen RNA regulation.

While preparing our revision, we hoped to include puro-PLA experiments using *Fmr1* mutants to more directly test whether Cen translation events (anti-Cen and anti-Puro antibody pairs) increase in the absence of FMRP. Given our confounding PLA issues, described above, we decided against these plans. To the point that changes in RNA may account for the increase in Cen protein, this is noted in the revised text on lines 277–83:

> Thus, both *cen* mRNA and protein levels are increased in later-stage *Fmr1* embryos. Taken together, these data suggest that FMRP may contribute to *cen* mRNA turnover and/or translational repression. While *cen* mRNA localization and levels may be coupled, such that increased *cen* mRNA content accounts for augmented *cen* mRNA localization to centrosomes and translation in *Fmr1* mutants, we cannot rule out the possibility that FMRP contributes to multiple aspects of *cen* mRNA post-transcriptional regulation, either directly or indirectly.

- Figure 5F-I (lines 345-356): The immunofluorescence images are not informative. Showing the quantifications of spindle defects along with the figures would be a better way to present the data. The process of quantification also needs to be clarified. What does the "N" mean? How many mitotic spindles were quantified per embryo? Is "76.1% N=16/21" vs. "48.1% N=13/27" significantly different? Statistical analysis should be performed to draw a conclusion (e.g., p values and variations between biological replicates).

We now include quantification of the spindle defects paired with the immunofluorescence images, as recommended, and presented in revised Fig. 7.

Figure 6
- Lines 377-381: The data show that the enlarged cen RNAs are still associated with centrosomes in cen-bcd-3'UTR embryos (Figure 6C and Figures 6 Supplement 1B). Therefore, native cen 3'UTR is NOT required for cen RNA localization, an opposite of what the authors conclude.

We agree the *cen* CDS is necessary for centrosome association, and this is clearly stated in the text (lines 329–33). In our original submission, we argued the native *cen* 3'UTR regulated aspects of *cen* granule regulation (specifically, the timing of their formation and organization) because we note *cen* granules form precociously and are substantially larger in *cen-bcd-3'UTR* embryos. In our revised work, we eliminate this speculation, as the relative over-expression of *cen* and the presence of the *bcd*-3'UTR make it challenging to ascribe functions to the native 3'UTR.

- In cen-bcd-3'UTR embryos, both cen RNA and Cen protein are concentrated at the anterior pole in the cen null background. It is thus unclear why the authors characterized the mitotic spindle phenotypes at ~50% egg-length in cen-bcd-3'UTR embryos, a region where both cen RNA and Cen protein are depleted.

We regret this was unclear in the original submission. The reviewer is correct that the embryo mid-region is essentially depleted of *cen* mRNA and protein; indeed, this allowed us to test the effect of local *cen* depletion in an embryo that still expressed *cen* mRNA and protein.

The revised text now reads (lines 321–4):

> Given its restricted localization to the anterior pole, *the cen-bcd-3'UTR* transgene allowed us to simultaneously test opposing effects of *cen* dosage. We examined the effect of excess *cen* by visualizing the anterior pole, and we examined the effect of local *cen* mRNA depletion by visualizing the embryo mid-region.

and on line 336:

> The restricted localization of *cen* mRNA and protein to the anterior pole within *cen-bcd-3'UTR* embryos allowed us to test whether *cen* mRNA was required locally for error-free mitosis. Examination of mitotic spindles at ~50% egg-length, an area devoid of *cen* mRNA and protein, revealed an increased rate of microtubule spindle defects…

- Since about 3-fold more Cen protein is made in cen-bcd-3'UTR embryos, this would complicate the interpretation of the phenotypes (i.e., local overexpression of Cen, cen RNA mis-targeting, or both, could contribute to the phenotypes). Constructing a cen-bcd-3'UTR transgene expressing Cen comparable to the WT level could be a better experimental system to address the proposed questions.

Please note our response above; excess *cen* within the anterior region of *cen-bcd-3'UTR* embryos allowed us to examine if local *cen* dosage impacted spindle assembly. Indeed, it did (Fig. 7E shows quantification).

Minor points

- General comments on the flow and figure presentations: (1) The description often starts with the presentation of supplementary figures. The back and forth between the main and supplementary figures makes it hard for the readers to follow the story. (2) The immunofluorescence images could be larger while the quantification plots could be smaller. It is particularly hard to see the signals in the zoom-out fields.

We regret the original organization and have significantly simplified the manuscript to make it more enjoyable to read and improve clarity. Many of the prior supplemental items have been moved into main-text and figures expanded. We've reduced the space occupied by plots and increased the size of immunofluorescence images; enlarged insets are provided for many figures.

- Line 147: the claim of higher order RNA structures is not well supported. At this magnification under light microscopy, individual RNA molecules may still be separated from one another instead of forming higher-order RNA structures.

We define a granule as a higher-order RNA structure consisting of 4 or more overlapping RNA signals using the same quantitative parameters previously established (Mueller, Senecal et al. 2013) and (Little, Sinsimer et al., 2015). These RNA granules are computationally defined based on deviations in object size - an "object" being a segmented RNA molecule or cluster of RNA molecules. A single mRNA molecule is an object of 50-100 pixels; therefore, RNA objects bigger than 100 pixels contain more than one RNA molecule. For our work, we count RNA granules containing 4 or more RNA molecules, in accordance with Little et al. Our approach to single molecule normalization is described on line 575-84.

In the revised work, we discuss RNA granules and the relative amount of RNA within granules only for *cen* mRNA and the control *gapdh*, as RNA granules represent a prominent and important feature of *cen* mRNA. We omitted discussion about RNA granules for the other transcripts we examined.

- The cyc B smFISH images appear to have a low signal-to-noise ratio. If the signals are all true, cyc B RNA would be much more abundant than gapdh RNA. Could this be possible?

We also noted *cyc B* signals had lower signal-to-noise, which was still evident even after increasing the stringency of embryo blocking. In contrast, the germline (pole plasm) signals are quite bright. Based on this reviewer comment, we revisited *cyc B* versus *gapdh* expression levels as annotated on FlyBase.org from the modENCODE data. Indeed, there is nearly 3-times more *cyc B* mRNA expressed in 0-2 hr embryos than *gapdh* (linear, scaled expression modENCODE_mRNA-Seq_U Temporal Expression Data).

- Line 173: "However, for the purposes of this study, all measurements were made in the somatic region at approximately 50% egg-length unless otherwise noted." What are the "purposes" of this study? What is the rationale of choosing this particular location?

We revised the text to now read (lines 131-3):

> To standardize measurements of mRNA enriched near somatic centrosomes across samples, we imaged embryos at approximately 50% egg-length unless otherwise noted.

- Line 197: It is the Cen protein, not cen RNA, that was previously shown to be required for normal nuclear division in fly early embryos. This sentence is thus not completely correct.

We revised the text to read (lines 169-70):

> We next investigated the localization of *cen* mRNA. Cen was previously shown to be required for normal nuclear divisions in *Drosophila* embryos (Kao and Megraw 2009).

- Figure 3I: It is not clear the purpose of showing this blot in a separate panel. It only shows that GFP-FMRP can be pulled down. Did the authors probe for endogenous Cen protein, i.e., a reciprocal IP of the result shown in Figure 3G?

In the revised work, Fig. 3F (middle blot) shows endogenous Cen is immunoprecipitated from FMRP-GFP.

- Figure 5 uses hours instead of nuclear cycles to stage embryos. The authors may want to unify the staging units so it is easier for the readers to follow.

In the revised work, our text and legends now provide the approximate NC stage corresponding to the developmental time. We felt it prudent to leave the figure labels unchanged for the following reasons. 1) A pooled embryo collection (e.g., 0-1 hour) represents a diverse collection of stages, mostly enriched for

nuclear cycle (NC) stages 1–7. However, it is not possible to precisely synchronize these collections. A 0-1 hour collection may also include unfertilized embryos and even a few embryos older than NC 7. 2) When we show a NC 13 interphase embryo, or talk about data from that stage, those are embryos we manually identify based on nuclear morphology and density. In those cases, we can be more precise about developmental stage.

We appreciate the reviewer's point, and try to clarify developmental staging in the text and figure legends. For example, on line 269-70, we now state:

> We found no significant change in *cen* mRNA levels in *Fmr1* vs. WT 0–1h embryos, a period encompassing up to NC 7 (p=0.07 by unpaired t-test; Fig. 6A) (Foe, Odell et al. 1993).

- Figure 5F-I (lines 345-356): What is the purpose of labeling centrosomes with both anti-Cnn and anti-Asl antibodies?

Revised Fig. 7 no longer includes Asl signals.

- The description of the imaging processing pipeline is very technical (Methods session). Without a coding background, it is hard to follow.

We simplified the pipeline description provided in the "RNA detection and measurements" section of the Methods and also added a "Data Availability" section to the Methods (lines 681–685) for readers wishing to access code and more detail about the approach.

- Bergalet et al., 2020 reports that the cen 3'UTR-mediated centrosomal targeting of ik2 RNA is functionally important in vivo. Therefore, in cen-bcd-3'UTR embryos, ik2 localization is likely also perturbed due to the lack of cen 3'UTR. This may thus in part contribute to the observed phenotypes in cen-bcd-3'UTR embryos. The authors may want to incorporate the data such as this from the Bergalet et al., 2020 study more into their discussion/interpretation.

We highlight the role of the *cen* 3'UTR to recruit *ik2* mRNA and discuss possible contributions to spindle morphogenesis on lines 340-3:

> Together with recent work showing the native *cen* 3'UTR recruits *I-kappaB kinase ε* (*IKKε* or *ik2*) mRNA to centrosomes (Bergalet, Patel et al. 2020), these data suggest that *cen* mRNA functions locally to support spindle integrity, perhaps in concert with *ik2* mRNA.

And in the discussion on lines 413-6:

> As the *cen* 3'UTR recruits *ik2* mRNA to centrosomes, the mitotic defects observed following *cen* perturbation may result from indirect effects via *ik2* mRNA (Bergalet, Patel et al. 2020). Nonetheless, *cen* mRNA dosage must be properly regulated for mitotic fidelity.

Finally, *ik2* mRNA is noted in our revised model, Fig. 9E and legend.

Reviewer #2 (Comments for Authors (Required)):

While the notion that centrosomes are DNA-containing organelles was put to rest twenty years ago, there are numerous accounts of mRNAs residing at or near centrosomes but no studies to address the functional importance of centrosomal mRNAs.

This work examines the functions of mRNA localization at centrosomes in early Drosophila embryos. The authors evaluated the localization of five mRNAs that were shown in a previous screen (Lecuyer et al 2007) to localize at or near centrosomes. They found two mRNAs at centrosomes (cen, sov), and two others that localize in the proximity of centrosomes (cyc B, plp).

The authors go farther to show that FMRP negatively regulates the timing and quantity of cen mRNA into granules, and that heterozygous mutant cen embryos suppressed the partial lethality associated with Fmr1 mutant maternal effect embryos.

The paper is well written and presented, and the data are of high quality. The title might be overstated as it suggests a generalized requirement for mRNAs at centrosomes to coordinate embryonic mitosis, but only centrocortin was demonstrated. One element not provided, despite the quantification and statistical analysis, was evaluation of significance for the localizations of the mRNAs and therefore some conclusions were not well supported by the data, or quantification was needed in order to make the conclusions. These points are highlighted below.

Overall, this works makes an important advance to our understanding of localized mRNA function at centrosomes and its regulation by FMRP.

We thank the reviewer for this overall positive assessment.

Major points:

1. It is important for the authors to ascribe significance (p values) to the measurements of mRNA levels and quantification of granules made for the five candidate mRNAs relative to gapdh, and for the effect of Fmr1 mutant on cen mRNA granule formation. The quantification data provided in the tables should make this straightforward.

Related to that, the data in Figure 1 and its supplements show that cyc B, sov, and plp mRNAs localize in the proximity of centrosomes in early embryos, consistent with Lecuyer et al 2007, but that pins mRNA did not. Some comment about this difference, or acknowledgement of it, should be included if this is a correct interpretation of your data - that pins mRNA does not localize in proximity to centrosomes. It is described as 'modest' on p. 7 but the authors should indicate if it is significant or not.

We simplified how we displayed the quantification and include statistical analysis, as noted above. Regarding *pins*, our revised text eliminates descriptive terms like “modest,” and now presents in Supplemental Fig. 1E a plot showing significantly more *pins* mRNA localized to (interphase) centrosomes than *gapdh*. The numbers are lower, for example, only ~5% of *pins* mRNA overlaps with centrosomes versus ~11% of *plp* mRNA at the same developmental stage, but it is significant. In the revised text we state:

> We similarly analyzed the localization of *pins* and *sov* mRNAs to centrosomes. Relative to *gapdh*, significantly more *pins* mRNA localized to interphase centrosomes (Fig. S1C–E).

2. It is probably not fair to state that the cen mRNA granule is dispensable for Cen translation based on the data in Figure 3 and supplements. While evident that the granules are significantly reduced in the cnnB4 mutant and that Cen levels are not significantly different, the levels of Cen protein are probably mostly contributed maternally and perhaps most translation of cen mRNA may not occur at granules, but the data in Bergalet et al 2020 show that translation does occur at centrosomes. So both might be true: Cen can be translated without granules, and Cen is translated at centrosomal granules. It seems the authors considered this in the Discussion, but the header in the results on line 232 should be tempered and it seems to contradict their model in Figure 7.

The revised text now further tempers interpretation of those results. We now similarly note potential caveats in the results section (lines 212–214), we’ve changed the sub-header to read “The *cen* mRNA granule contains Cen protein and requires the centrosome scaffold,” and we expand the discussion (lines 396-400) to state Cen may be translated at multiple sites.

3. The co-localization of FMRP with Cen granules, shown in Figure 3 Supplement 2, is not clear. The example that is highlighted does not appear to be representative, and quantitative analysis is required to conclude whether colocalization is significant. From the images, FMRP puncta appear uniformly

distributed. FMRP does not have to localize at granules to negatively affect their formation and the association of FMRP with Cen might occur outside of the granules. Likewise, the association of FMRP with cen mRNA shown in Figure 3 might also occur outside of the granule. One experiment to test the potential association of FMRP with Cen mRNA granules is to look at its recruitment to the oversized granules produced by the bcd 3'UTR fusion as in Figure 6.

We agree the images were not too clear, and we eliminated those data except for Fig. S1G, G' to show we included FMRP in our initial survey of RNA-binding proteins. We expanded our biochemical analyses, and now include the co-IP showing Cen and FMRP co-immunoprecipitated (revised Fig. 3F, middle).

We also took this reviewer's suggestion and looked at FMRP localization in control versus cen-bcd-3'UTR embryos (presented in Fig. 9A–C). These data show mostly dispersed FMRP signals in controls (quantification in C shows overlap of Cen and FMRP is non-random, however, as compared to a 180^{o} rotated control). More importantly, FMRP is robustly recruited to the massive Cen RNPs (inset, Fig 9B). Quantification shows a Pearson's correlation of about 0.8, indicating strong correlation. This overlap is specific, as the correlation drops when we rotate the channels 180^{o}. These data suggest the massive Cen RNPs are sufficient to recruit FMRP. We thank the reviewer for this informative suggestion.

4. The data in Figure 4 show that Fmr1 negatively regulates cen mRNA granule formation. What about the other mRNAs (cyc B, plp, and sov), were they likewise affected or is this effect specific to cen mRNAs? Accordingly, the title of the manuscript is too broad and should specify centrocortin mRNA.

In our present work, we only examine the relationship of FMRP with *cen* mRNA, and did not explore the other mRNAs (i.e., *cyc B, plp, sov, or pins*). We clarify this in the discussion on line 391, stating:

> Whether FMRP similarly regulates other centrosome-localized mRNAs is an interesting question for future study.

The revised title is "*centrocortin* RNA localization to centrosomes is regulated by FMRP and facilitates error-free mitosis."

5. The header for Figure 4 (Fmr1 regulates cen granule formation and size) should be adjusted, since the data here do not show a quantification of granule size. Instead, it shows that there is more cen mRNA in granules near the centrosome. The legend to Figure 4 refers to "cen" but should specify that it is cen mRNA. The conclusion from these data, that FMRP negatively regulates cen mRNA localization holds, but probably more accurate to state that it regulates its levels at centrosomes.

The reviewer is correct that we do not present data evaluating RNA granule size. In the revised text (lines 245–61) we minimized use of descriptive terms when comparing *cen* mRNA granules between controls and *Fmr1* mutants. For example, when talking about NC 10 (now presented in revised Fig. 4), we state:

> Quantification confirmed that *cen* mRNA localization to centrosomes and residence within RNA granules were both significantly higher in *Fmr1* embryos relative to WT (Fig. 4E,F). These data suggest that FMRP regulates *cen* mRNA granule formation and localization to centrosomes.

On Line 259:

> In sum, loss of FMRP is associated with more *cen* mRNA localized to granules, which reside closer to and are more likely to overlap with interphase centrosomes.

The conclusion that FMRP regulates granule formation is supported by the finding that there is significantly more *cen* mRNA within pericentrosomal granules in *Fmr1* mutants versus controls. We propose that it is the formation of *cen* mRNA granules that contributes to a bulk enrichment of RNA to centrosomes.

Throughout the revised text and legend headers, we added "mRNA" to help specify our reference to *cen*

mRNA, as opposed to the *cen* gene.

Minor points:
6. In the introduction, the authors refer to the essential functions for centrosomes in the early embryo, but of the citations for this statement (Deák et al., 1997; Freeman, Nüsslein-Volhard, & Glover, 1986; Sunkel & Glover, 1988) only the Sunkel & Glover citation addresses centrosome functions (polo mutant embryos). Citations of papers that directly address the functions of centrosomes would be more appropriate.

We updated the references on line 68.

7. The tables are not labeled, and reviewers have to infer which table is which from context. The excel files should have a title within them like 'Table 1: quantification of RNA proximity to centrosomes', etc.

We regret this confusion. The revised text contains fewer tables: Table 1 in the main text lists all objects quantified and Supplemental Table 1 lists all smFISH probes used in our study. Each table now includes a clear title within them.

8. Figure 1E does not look like metaphase; please check it.

The original image included some pro-metaphase nuclei. We replaced this image with a new Fig 1E.

9. The text often refers to cen mRNA as 'cen'; need to add 'mRNA' to this description throughout the manuscript to differentiate it from the cen gene or Cen protein (eg on lines 306, 359, 446, etc).

We revised the text and legend headers by adding “mRNA” as appropriate.

10. Check the statement and citation on line 309 regarding transcriptional inactivity in the early embryo. There are some genes expressed during the early embryo, the gap genes being an example.

On line 264, we revised the text to clearly state “most” genes are “maternally endowed.”

11. It is not clear how the paragraph that begins on line 373 culminates in the conclusion that "These data suggest that the normal temporal and spatial pattern of cen RNA localization requires sequence or structural elements encoded within the native cen 3'UTR. ".

This text is now revised and we no longer discuss possible contributions of the native *cen* 3’UTR.

12. In the text, the authors alternate between 'fmr1' and 'fmr'. Flybase refers to it as 'Fmr1', which is recommended.
The revised text uses the preferred Flybase gene name, *Fmr1*.

Reviewer #3 (Comments for Authors (Required)):

The manuscript by Ryder et al reports that cen mRNA, an important component of centrosomes is required for proper mitoses during early embryogenesis and is a target of FMRP. The authors make important and elegant observations of mRNA localization at/near centromere during the cell cycle in Drosophila syncytia. They examine a few candidate mRNAs, then focus on cen. They go on to show that FMRP but not other RNA binding proteins associate with centrosomes in vivo and cen mRNA co-IPs with FMRP using an FMRP-GFP line. Furthermore, FMRP is required for the formation of cen granules and this is cell cycle dependent. Indeed, by NC13, loss of FMRP causes an increase in cen mRNA and Cen protein, suggesting a role in mRNA stability and translation, consistent with FMRP's known role as a

translation inhibitor. Overall, this is an interesting paper that convincingly identifies a new mRNA target of FMRP and a role in ensuring proper mitoses for cen and FMRP. The title seems a bit more general than the actual conclusions and there are some issues that I detail below.

We thank the reviewer for this overall positive assessment. As suggested, we revised the title to "*centrocortin* RNA localization to centrosomes is regulated by FMRP and facilitates error-free mitosis."

1. The experiments in Figure 1 are reporting observations of mRNA localization and the results do not show a causative relationship between granule formation and cycB mRNA localization. Thus it is unclear why the authors conclude that cycB localization to centrosomes is dictated by granule formation (line 154).

In the revised work, we limit our discussion of RNA localization to granules in our discussion of *cen* mRNA and the control, *gapdh*.

2. The observation that cen mRNA is increased in its association with centrosomes during interphase compared to metaphase in NC13 is interesting. An interesting dynamic behavior is also reported for NC10. Do the authors think this reflects solely the dynamic behavior of cen mRNA localization, translation or both? Do the authors have qPCR and/or total protein data from these cycles to address this?

We agree it would be exciting to compare stage-by-stage changes in RNA and protein over time, but we have not done single stage qPCR or western blots. In our discussion, we do speculate that changes in RNA localization to centrosomes may correlate with translational status. For example, on lines 410-11 we state:

> Translational repression or derepression may be coupled to *cen* mRNA granule centrosome proximity, which decreases as embryos enter mitosis.

We are careful not to extend the speculation too far, as we believe live imaging RNA transport and local translation in situ will be needed to rigorously test these models.

3. The authors nicely show that cnn is required for the formation of cen granules. However the conclusion that cen granules are not required for Cen translation is premature. While this is the most obvious explanation of cnn mutations causing no changes in Cen protein levels, the authors cannot eliminate possible effects on Cen protein stability. Therefore, in my opinion the current interpretation needs to be adjusted. Also, the western blot shown for Cen protein is not as convincing, although the accompanying quantification supports the "no change" reported result.

In the revised work, we tempered our conclusions regarding *cen* mRNA granule loss and steady Cen protein levels. In the results and discussion, we mention (e.g., line 213) the "important caveat is that maternal deposition of Cen may obscure changes resulting from granule loss." We measured protein bands from replicated experiments, and no significant difference was detected. All immunoblots are now available for readers to visualize (FigShare links provided below in response to MP5).

4. Although the 5A11 antibody has been published it would be useful if the authors checked its specificity in their hands using fmr1 nulls.

We include a new immunoblot, Fig. 3D, showing levels of FMRP as detected with the monoclonal 5A11 antibody in control (WT), *Fmr1* null, and the FMRP-GFP bac line. The antibody detects FMRP at the expected molecular weight, and is absent in *Fmr1* null embryo (and ovary, not shown) extracts. FMRP-GFP migrates on SDS-PAGE with the expected upshift in molecular weight given the presence of a GFP tag.

5. The RNA IPs support the conclusion that cen mRNA co-IPS with FMRP specifically but the authors

should show the whole blot. Also related to RNA IPs, the authors should test for the presence of a nontarget mRNA. Finally, the FMRP-GFP is a useful tool but it would be more convincing if endogenous FMRP from wild-type embryos would pull down cen mRNA as well. Or perhaps the authors have data that FMRP levels in the FMRP-GFP line are similar to wild-type embryos.

All uncropped DNA gels and immunoblots may be visualized on FigShare. We provide links to these data in our Methods section in a new "Data Availability" section (lines 681–85) and are copied here: doi.org/10.6084/m9.figshare.12821564 and doi.org/10.6084/m9.figshare.12821579

In figure legends, we also note for the reader that they can see the full blots/gels in FigShare, and we direct them to the Methods. The revised work (and FigShare files) includes the non-target RNA control, *His 3.3B* (revised Fig. 3G, bottom).

For FMRP-GFP, we confirmed by immunoblot the bac construct is not over-expressed. Fig. 3D shows levels of FMRP-GFP are lower than endogenous FMRP.

Minor comments:
Lines 118, 119 - define all candidate mRNAs

We clarified this sentence, which now reads (line 116-9):

> Among the candidate RNAs reported to localize near spindle poles (i.e., *Bsg25D*, *cen*, *cyc B*, *plp*, *small ovary (sov)*, and *partner of inscuteable (pins*) mRNAs), we selected five for investigation based on prior data implicating their protein products in centrosome regulation and/or cell division: *cyc B*, *cen*, *plp*, *sov*, and *pins* (Lécuyer et al., 2007).

From the original Lécuyer paper, only *Bsg25D* was excluded from our survey because we noted it is not centrosomal in all stages we examined. Work describing that RNA distribution is currently in preparation.

"fmr" should read "fmr1" throughout

To avoid confusion, the revised text uses the preferred Flybase gene name, *Fmr1*.

We thank the reviewers for their time and these thoughtful comments and suggestions. We appreciate the opportunity to improve the quality of our manuscript. We hope the revised manuscript is now suitable for publication in *JCB*.

Sincerely,

Dorothy A. Lerit, Ph.D.
Assistant Professor
Department of Cell Biology
Emory University School of Medicine

October 5, 2020

RE: JCB Manuscript #202004101R

Dr. Dorothy Anne Lerit
Emory University School of Medicine
Cell Biology
615 Michael Street
435 Whitehead Building
Atlanta, GA 30033

Dear Dr. Lerit,

Thank you for submitting your revised manuscript entitled "centrocortin RNA localization to centrosomes is regulated by FMRP and facilitates error-free mitosis". You will see that the reviewers continue to be supportive of the study and praise the changes made in revision. We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below). Please also consider the remaining reviewer points, which are reasonable and could be mentioned in the manuscript text/discussion.

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

1) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends.
Please indicate n/sample size/how many experiments the data are representative of: S2B'

2) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions in the text for readers who may not have access to referenced manuscripts.
- Please include database/vendor IDs for all constructs, plasmids, cDNAs, fly strains (e.g., Addgene, BDSC, FlyBase, etc) or if they are not available, please include a basic description of their genetic features, even if described in other published work or received as gifts from other investigators.
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4) A summary paragraph of all supplemental material should appear at the end of the Materials and methods section.
Please include one brief sentence per item.

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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,

Arshad Desai, PhD
Editor, Journal of Cell Biology

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

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

Overall, I am satisfied with this revised manuscript. The authors have made a considerable effort to improve their manuscript. In particular, the new statistical analyses strengthen their conclusion on the cell cycle- and developmental stage-dependent centrosomal localization of several mRNAs. The reciprocal IPs between Cen and FMRP also support that they are in the same complex. The new data also show that the massive cen-bcd-3'UTR RNP complex recruits FMRP, thus supporting the functional relationship (although the caveat here is that this recruitment could be bcd-3'UTR-dependent, not cen mRNA-dependent).

However, I still think that an alternative model in which translation of cen mRNA dictates its ultimate centrosomal localization and granule formation should be discussed (the authors only discussed this co-translational mechanism for sov mRNA, which was not characterized in depth in this study), especially when a similar finding has been reported for the same cen mRNA in the same Drosophila system (Bergalet et al., 2020). Recent studies from the Edouard Bertrand group further suggest that such a translation-dependent RNA localization mechanism could be widespread. Notably their studies include several examples of centrosomally localized mRNAs (https://doi.org/10.1016/j.devcel.2020.07.010 and doi: https://doi.org/10.1101/2020.09.04.282038). Based on these other studies and the data presented here, I feel that this alternative model warrants further discussion (e.g., in the paragraph starting line 404) and should be incorporated into the proposed model (Figure 9E).

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

The authors have done a very good job with the revisions and have addressed all the concerns. For the new data shown in Figure 9B where FMRP and Cen are localized to the oversized Cen mRNA particles, Cen is clearly higher at the periphery, while FMRP appears uniformly distributed. It is not a major point, but the authors should comment on this.

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

The authors did an outstanding job revising this manuscript. I have no further concerns.