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Maternal mRNA deadenylation and allocation via Rbm14 condensates facilitates vertebrate blastula development

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(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. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Thank you for submitting your manuscript for consideration by The EMBO Journal. We have now received a full set of referee reports on your manuscript, which are included below for your information.

As you will see from the comments, all reviewers find the study of interest and appreciate its quality, while also indicating a number of important and partially overlapping concerns and potential alternative explanations of the data that would have to be addressed before they can support publication of the manuscript. From my side, I find these points generally reasonable. Therefore, I would like to invite you to address the concerns raised by the reviewers in a revised manuscript.

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, please contact me as soon as possible upon publication of any related work to discuss the appropriate course of action. Should you foresee a problem in meeting this three-month deadline, please contact me to discuss an extension.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess. Please also see the attached instructions for further guidelines on preparation of the revised manuscript.

Please feel free to contact me if you have any further questions regarding the revision. I would be happy to discuss the revision in more detail via email or phone/videoconferencing.

Thank you for the opportunity to consider your work for publication. I look forward to receiving the revised manuscript.

Referee #1:

In this manuscript, Xiao et al. found that cytoplasmic Rbm14 condensates regulate blastula-to-gastrula development by deadenylating m6A-modified maternal mRNAs. Rbm14 deficiency leads to severe blastula arrest in zebrafish and mouse. The authors combined the information of deadenylation, RNA modifications, and phase separation to further unravel MZT process. Overall, this manuscript provides comprehensive information and is generally well-written. There are several important issues need to be addressed:
1. zRbm14a condensates mainly appeared at 2 hpf (Fig 1D). Does it mean that zRbm14a-m6A-independent mechanism contributes to the regulation of early development mainly before ZGA? It seems confused that Rbm14 protects maternal RNAs

from decay as concludes from the first half of the article, whereas after 4 hpf, Rbm14 depletion impedes RNA degradation (Fig. 6C). Does Rbm14 play dual roles during MZT?
2. zRbm14 condensates modulate mitotic segregations for either asymmetric or symmetric division. The authors should explain why the assembling of condensates leads to two different types of division or in which phenomenon it subjects to asymmetric or symmetric way.
3. The authors proposed that zRbm14 undergoes LLPS with maternal mRNAs (Fig 3), but they didn't mention the definition of maternal mRNAs here, only referred in Fig 6. How they associated zRbm14 with maternal mRNAs in LLPS formation did not show in the main text.
4. The conclusions of "Their formation (LLPS) was dose-dependently stimulated by m6A, but repressed by m5C" is insufficiently supported by the present data. Better to provide direct interaction between m6A/m5C-modified RNA and Rbm14, neither using Co-IP, crystallization, nor high-throughput sequencing of enzymes knockout samples. In addition, only one example gene (cap1) was used to test whether m5C could impact LLPS of zRbm14. They jumped to conclusions for the negative role of m5C in LLPS formation. Meanwhile, how m6A and m5C affect LLPS could be speculated in the Discussion section.
5. In Fig 7, the author illustrated that zRbm14 condensates protect their content poly(A)-less mRNAs from premature degradation leading to proper cell cycle and differentiation. The authors should perform ChIP or Tail-Seq to further verify the role of zParn, or provide the reasonable interpretation of current results.
6. The authors have delivered that Rbm14 paralogues capable of both LLPS and cell fate regulation previously, as well as in this article. How about other RBM family members? Do they have the potential binding ability of modified-RNA to form condensates subsequently, considering that they have similar recognition motifs?

Minor Points:
1. Several factors have been indicated to be essential in regulating maternal mRNA decay including microRNA miR-430, suboptimal codon usage, RNA m6A and m5C, ploy-A/U etc. Authors should add those background since they aim to unravel that Rbm14 and maternal mRNAs co-phase separate into cytoplasmic condensates to ensure vertebrate blastula-to-gastrula development through regulating maternal mRNA decay. Additionally, the concept of phase separation should be briefly referred in Introduction section. Please prefer to cite the references from the past three years.
2. It seems that mixed cells were used to perform the immunoblotting of zRbm14a in Fig 1B. Thus, it is not surprise that the expression level of zRbm14a increased as development progresses during which the number of cells also increased. Meanwhile, how to explain that zRbm14a slightly upregulated form 0.75-1.75 hpf whereas greatly increased after 1.75 hpf in Fig 1C?
3. The significance test is missing in many places, such as Fig 1J, Fig 8H. The p value needs to be consistent marked either in the main text or figures. For example, in Fig 6E "P < 2.2E-16" and in Fig 6D "P = 1.3E-181".
4. Some figures did not have icons or titles of scale marks, such as Fig 1K, Fig 2F, Fig 3K, Fig 8E-G, etc.
5. The altered transcripts were not defined in the main text (Fig 6B).
6. There are several works related to RNA methylation-dependent phase separation should be taken into consideration in Discussion section.

Referee #2:

The authors of this manuscript, Xiao, Chen et al., investigate the role of the RNA-binding protein RBM14 in maternal mRNA localization and clearance, using zebrafish as a model system.
Using antibody staining and fluorescent protein localization, the authors determine that RBM14 paralogs localize in the cytoplasm near the spindle poles during early stages of embryogenesis, to later translocate to the nucleus. The authors use elegant and complementary in vivo and in vitro approaches to demonstrate that RBM14 co-localizes with gamma-tubulin, mRNA (even with m6A but not m5C modifications) and PARN via liquid-liquid phase transitions.

The main message of this work is that RBM14, thanks to its subcellular localization and its partners, regulates part of maternal mRNA clearance by setting the stage for the deadenylation of its target genes. While the data clearly suggests an important role of RBM14 in RNA regulation during early embryogenesis, the authors do not elaborate in other potential scenarios that can explain the same data and hence, better pinpoint the exact role of RBM14. For instance, RBM14 is known for its role in splicing. Precisely in the model that the authors are testing, lack of splicing of part the early zygotic genes will impair further zygotic genome activation (PMID: 24056933) and in turn fail to deploy the components of the zygotic program of maternal mRNA clearance. This scenario will justify the results by the authors showing that loss of RBM14 function leads to impaired mRNA clearance and delayed zygotic genome activation.

In summary, the question addressed in the present manuscript is relevant to the fields of Development and RNA regulation. The data is solid and many aspects are tested with orthogonal approaches, increasing rigor. Therefore, this manuscript is potentially of interest for the audience of EMBO Journal. However, the manuscript cannot be published in the present form, as the authors should address the central concern:

- The authors should analyze if splicing is disrupted in the RBM14 morphant embryos. Splicing disruption of early zygotic genes is compatible with the data presented in this manuscript and may explain part of the phenotypes derived from the loss of RBM14. One possible approach would be to analyze their RNA-Seq data for intron retention. This approach would allow the

authors to discriminate which mRNAs are targets of RBM14 for destabilization and which ones require RBM14 for splicing. The same slicing analysis should be done for the mouse data, where RBM14 is a constitutive member of the paraspeckle bodies in the nucleus. These paraspeckles are in charge of regulating splicing, among other functions.

Referee #3:

In the manuscript "Rbm14 condensates deadenylate and allocate maternal mRNAs to ensure vertebrate blastula Development", Xiao and colleagues set out to understand the function of an RNA-binding protein, zRbm14, in regulating the deadenylation and clearance of maternal mRNAs during early zebrafish development. They conclude that zRbm14 forms condensates with m6A modified maternal RNAs and zParn, which localize to spindle poles. These condensates are thought to distribute maternal RNAs asymmetrically. Within the condensates - zParn gradually deadenylates RNAs, leading to an increase in m5C cap concentration, resulting in the dissolution of condensates to release maternal mRNAs which are degraded. While this is an appealing story, with lots of interesting data, some parts of the story are not well-supported by data and these will need to be addressed prior to publication.

Major concerns
I do not think that the data supports the statement (title) that Rbm condensates 'allocate maternal RNA'. While the authors show that Rbm condensates are asymmetrically segregated, they do not show this for RNA specifically, and even if RNA would be segregated asymmetrically, it is not clear that this distributes RNA for a specific purpose. In that context, I find it also hard to understand whether the authors expect that a difference in the segregation of bulk maternal RNA causes cell fate differences, or whether they think that it is specific RNAs that are allocated asymmetrically. If the latter, how would that work?

I similarly do not think the data supports that (title) deadenylation and allocation of maternal RNA ensure vertebrate blastula development. The authors convincingly show that Rbm14 condensates are required for development, but whether that is due to their role in deadenylation and allocation of RNA is not shown

It is unclear from the data that is presented whether the effect of Rbm14 MO on gene expression is a direct effect of Rbm14 loss, or an indirect effect of developmental delay.

To claim that 'deadenylase Parn co-phase separated with the condensates to actively deadenylate the mRNAs in early blastomeres' (abstract) one would at least like to see Parn localize to the condensates in vivo.

Other concerns

General
- There are quite some typos.
- Legends should contain more information (like, whether images are MIPs or Z-slices, ...) to facilitate interpretation of results.
- Why do the authors use zRbm14a in Figures 1 and 2, and then switch to zRbm14b?

Figure 1.
Panel D - higher magnification would be helpful to see cytoplasmic condensates.
Panel J - I do not understand this text: "Quantifications on 8-cell embryos indicated that their immunofluorescent intensities at poles close to the midline differed by 2-fold or more from those at corresponding poles away from the midline in 20.3% of the blastomeres (median = 1.5-fold) (Fig. 1J)".
Panel J - use either cell number or hpf.
Panel K - slightly unclear what is supposed to be taken from this schematic, especially the different timepoints.

Figure 2.
Panel C - Can the authors comment on why tubulin levels are not affected by protK?
Panel F - As there is no analysis of cell fate differences related to asymmetrical distribution of Rbm14 condensates, this schematic is misleading. I would also be interested, as indicated above, what exactly the authors think is the effect of asymmetric Rbm14 distribution on RNA distribution, especially related to bulk RNA or specific RNAs...

Figure 3.
Panels 3D-F - I am surprised that m5C do not form droplets with Rbm14 (3D,E) but can enter existing droplets and dissociate them (3F). Or is the idea that deadenylation/degradation results in a relative increase in m5C? Can the authors elaborate?
Panel H - injecting 800 or 1200 pg RNA into the embryo is a lot. Do these embryos develop normally? Related, this image looks quite different from what we see for zRbm14a in Figure 1 (same timepoint), which may suggest that these big condensates are an artifact. Can the authors explain the difference?
P9 - The header "zRbm14 undergoes LLPS in vivo with maternal RNAs" is misleading, as there is no evidence for the statement that zRbm14 only associates with maternal RNAs.

Panel I - can 3D information be provided? Or is this a Z-slice? What thickness?

Figure 4.
Panel D - the cell number is derived from the number of nuclei observed in a 10um z-depth. Is this a reliable representation of the total number of cells in the embryo? Would it be better to use something like Ki67 to measure the proliferation index?

Figure 5
Panel B - embryos should be shown in same orientation to allow for comparison and, once possible, using standard orientation.
Panel D - the use of hexanediol is aspecific and therefore not very meaningful. Other condensates in the embryo will also be affected and therefore one cannot conclude anything about the importance of Rbm14 condensates.

Figure 6
Since Rbm14 MO embryos are delayed (see Figure 5) compared to control MO embryos, the differences in maternal and zygotic transcripts may be a mere consequence of the different developmental stages that are analyzed for the two conditions. While this delay is a consequence of the lack of Rbm14 (Figure 5), it is difficult to know whether the changes in gene expression are directly or indirectly due to lack of Rbm14. The experiment presented in panels H and I is not sensitive enough to remedy this issue. Since the authors propose that m6A containing transcripts are specifically enriched in Rbm condensates, they may wanna look whether they are specifically affected, especially in the maternal set.

Figure 7
Panel B - If Parn can co-phase separate with just the IDR of Rbm14, which protein do the authors think recruits the RNAs to the condensate?
Panel D - Does zParn colocalize with zRbm14 in vivo? Since there is an antibody available, this seems like smth the authors should do to strengthen their hypothesis.

Figure 8
Panel B - Higher magnification would be useful to see cytoplasmic punctae (and lack thereof in later stages)
Panel B -more than n=1 is needed to conclude that Rbm14 localizes to spindle pole in mouse.

Point-to-point response to reviewers' comments

Referee #1:

In this manuscript, Xiao et al. found that cytoplasmic Rbm14 condensates regulate blastula-to-gastrula development by deadenylating m6A-modified maternal mRNAs. Rbm14 deficiency leads to severe blastula arrest in zebrafish and mouse. The authors combined the information of deadenylation, RNA modifications, and phase separation to further unravel MZT process. Overall, this manuscript provides comprehensive information and is generally well-written. There are several important issues need to be addressed:

Response:

We thank our reviewer for recognizing our efforts. We would also like to sincerely thank our reviewer for helping us to improve the manuscript. We have carefully addressed the concerns and hope our reviewer would find the revised manuscript satisfactory.

1. zRbm14a condensates mainly appeared at 2 hpf (Fig 1D). Does it mean that zRbm14a-m6A-independent mechanism contributes to the regulation of early development mainly before ZGA? It seems confused that Rbm14 protects maternal RNAs from decay as concludes from the first half of the article, whereas after 4 hpf, Rbm14 depletion impedes RNA degradation (Fig. 6C). Does Rbm14 play dual roles during MZT?

Response:

We are sorry for the confusions. As Fig 1D (and also 1F) is intended to show the dynamics of the condensates following the embryonic development in addition to their subcellular localizations, the images were acquired using similar laser powers, so that immunofluorescent intensities in cells of different developmental stages can be compared. This leads to the conclusion that the condensates peak at 2 hpf. We found that we only included the sentence "exposures were kept the same during the imaging for comparison" in the legend of Fig 1F. In the revised manuscript, we have included this sentence in the legend of Fig 1D as well to avoid confusing. Even in these images, zRbm14a condensates are prominent at 1.5 hpf and visible at 1.25 hpf (Fig 1D, F). Condensates in 1.25-hpf (8-cell) embryos are also shown in Fig 1I-J. As the images in Fig 1I are intended to emphasize the asymmetric segregation of the condensates, the 8-cell and 16-cell embryos were imaged using laser powers optimized for each situation.

Our results indeed suggest that zRbm14 plays dual roles: its condensates enriched zParn to promote the initial deadenylation of its target maternal mRNAs and then sequestered the deadenylated mRNAs from premature degradation till they were released. The impaired maternal mRNA clearance in maternal *zRbm14* morphants (Fig 6) is thus attributed to the lack of their initial deadenylation. During the revision, following the request of our reviewer, we performed poly(A) length assays on transcripts of *org*, *trip10a*, and *dnajc5ga*, which were known to be accumulated in maternal *zRbm14* morphants (Fig 6F, G). We found that their transcripts in maternal *zRbm14* morphants were indeed abundant in poly(A)-containing ones as compared to control morphants (Figs 7A-C and EV4A-B, revised manuscript). We also show that zParn co-localized with zRbm14 condensates in early embryos (Fig 7G-H, revised manuscript). These results further strengthen our model, which we have also modified for increased the clarity (Fig 7M, revised manuscript).

2. zRbm14 condensates modulate mitotic segregations for either asymmetric or symmetric division. The authors should explain why the assembling of condensates leads to two different types of division or in which phenomenon it subjects to asymmetric or symmetric way.

Response:

We are sorry for having not clearly explained the rationales. Symmetric and asymmetric cell divisions are known to govern the self-renewal and differentiation of pluripotent stem cells by equally or differentially allocating cell fate determinants such as signaling molecules and even centrosomes through proper spindle orientation and positioning (Knoblich, 2001; Lechler and Mapelli, 2021; Venkei and Yamashita, 2018). As centrosome pairs in mitotic blastomeres of 8-cell and 16-cell zebrafish embryos are both asymmetric in size and oriented in a polarized fashion when labeled with γ-tubulin (Rathbun et al., 2020), the tight co-localization of zRbm14a condensates with γ-tubulin-positive puncta prompted us to speculate that the asymmetric mitotic centrosomes might also serve as carriers to partition zRbm14a condensates unequally to regulate cell fates. In the revised manuscript, we have modified the main text for this part of results in Fig 1 to improve the clarity. Accordingly, we removed redundant sentences in the discussion section (paragraph 3, initial manuscript). How zRbm14 condensates achieve their tight associations with γ-tubulin puncta is currently unknown. We have stated in the discussion that this remains to be clarified in the future.

3. The authors proposed that zRbm14 undergoes LLPS with maternal mRNAs (Fig 3), but they didn't mention the definition of maternal mRNAs here, only referred in Fig 6. How they associated zRbm14 with maternal mRNAs in LLPS formation did not show in the main text.

Response:

We show in Fig 2 that zRbm14 condensates in zebrafish cells contained maternal RNAs (Fig 2A), including m^6A-modified RNAs (Fig 2D) and the *ccnb1* mRNA (cyclin B1 mRNA) (Fig 2E). These results prompted us to examine the interplay between zRbm14b and RNAs through in-vitro assays (Fig 3A-F).

To increase the clarity of presentation, we have included a sentence to define maternal mRNAs in Introduction (1st paragraph, 1st sentence) of the revised manuscript. We have also included rationales to link maternal mRNAs to potential cell fate-regulatory functions of zRbm14 condensates in early embryos before describing the results in Fig 2. Accordingly, we removed redundant sentences in the discussion section (paragraph 3, initial manuscript). We hope our reviewer would find that the clarity of the manuscript is largely improved through these revisions.

4. The conclusions of "Their formation (LLPS) was dose-dependently stimulated by m6A, but repressed by m5C" is insufficiently supported by the present data. Better to provide direct interaction between m6A/m5C-modified RNA and Rbm14, neither using Co-IP, crystallization, nor high-throughput sequencing of enzymes knockout samples. In addition, only one example gene (cap1) was used to test whether m5C could impact LLPS of zRbm14. They jumped to conclusions for the negative role of m5C in LLPS formation. Meanwhile, how m6A and m5C affect LLPS could be speculated in the Discussion section.

Response:

We thank our reviewer for the comments. During the revision we further examined how the LLPS of zRbm14b was affected by different stoichiometry of m^6A and m^5C modifications when the total concentration of RNA was kept constant (1 μM) and have presented the results in Figs 3E and EV1F in the revised manuscript. We observed that $1m^6A$-cy3 and $1m^5C$-cy3 mixed at a 1 : 3 stoichiometry still failed to induce the LLPS in 30 min, similar to $1m^5C$-cy3 alone, whereas the mixture of a 1 : 1 stoichiometry induced the LLPS in 20 min. Moreover, the mixture of a 3 : 1 stoichiometry induced the LLPS in 10 min, similar to $1m^6A$-cy3 alone. As we also used $0m^5C$-cy3 and $1m^5C$-cy3 as controls in these experiments (Figs 3E and EV1F, revised manuscript), the data presented in Fig 3E of the initial manuscript are removed to avoid redundancy. We hope that our reviewer would agree that these new data, together with those presented in Fig 3D and F, clearly indicate a dose-dependent antagonizing effect of m^6A and m^5C modifications on the LLPS of zRbm14b. On the other hand, as $1m^6A$ and $10m^6A$ displayed increasingly prominent LLPS-promoting effect on zRbm14b over the unmodified RNA fragment ($0m^6A$) at the same (1 μM) RNA concentration (Figs 3A-C and EV1D, revised manuscript), we believe that it is also appropriate to conclude a dose-dependent promoting effect of the m^6A modification.

Our results suggest that the LLPS-promoting effect of RNA is largely sequence-independent. The RNA sequences that we named as $0m^6A$ or $0m^5C$ share no sequence homology. We chose them simply because they were used in literature as unmodified controls (Ries et al., 2019; Yang et al., 2019). Yet, they displayed similar efficacy in our assays, i.e., inducing LLPS of zRbm14b in 20 min (Figs 3B, E and EV1D, F, revised manuscript; also refer to Fig 3E of the initial manuscript). Therefore, the distinct properties of $1m^5C$, $1m^6A$, and $10m^6A$ are attributed to the specific modifications, rather than the differences in RNA sequences. This, however, does not mean that we object our reviewer's request about using an additional m^5C-modified sequence. The experiments are simple to us if we had another m^5C-RNA. The entire Shanghai, however, was unfortunately locked down for more than two months between April and June due to the pandemic of COVIC19. So was our institute. We have thus been working hard on essential experiments to avoid extensive delay of the revision, though we have asked for an extension of additional two weeks. As our m^5C-caprin 1 fragment was a gift from other researcher (Yang et al., 2019), to synthesize another m^5C-modified RNA we need to purchase m^5-CTP and wait for the arrival of the imported reagent from abroad, the process of which is largely indefinite due to the current unstable situation of COVID19 pandemic. We thus hope that our reviewer would agree that such an experiment is not essential to our manuscript.

We demonstrate that $0m^6A$, $1m^6A$, $10m^6A$, and $0m^5C$ all co-phase separated with zRbm14b into the condensates (Figs 3A, C, D, and EV1E, F, revised manuscript). Together with the failure of $10m^6A$ to promote the LLPS of zIDR (Figs 3A, B and EV1D), we believe that these results sufficiently indicate that RNA exerts its LLPS-promoting effect through direct interactions with the RNA-binding domain (the RRM region) of zRbm14b. In comparison, we feel that co-IP (we believe that our reviewer meant to use "either… or" instead of "neither…nor" in the comment) is a relatively indirect assay for RNA-Rbm14 interactions as compared to our direct visualization of LLPS and partitioning of cy3-labeled RNAs into the condensates. In addition, co-IP is usually performed at low temperatures (e.g., 0°C to 4°C) that do not favor the LLPS of zRbm14b (please refer to our earlier studies on BuGZ and abLIM1 for temperature-dependent effects on LLPS (Jiang et al., 2015; Yang et al., 2022), whether the results could reflect the situation under phase separation is also unclear. In this context, using co-IP would complex the issue.

We fully agree with our reviewer that our current results do not sufficiently provide detailed molecular mechanisms on RNA-Rbm14 interactions and on interplays between m^6A and m^5C modifications. Nevertheless, we also hope our reviewer would agree that such detailed molecular mechanisms are beyond the focus of our manuscript and can be left for future researches. Despite this, we carefully considered the proposed crystallization and high-throughput sequencing of enzymes knockout samples. As LLPS-induced condensates are usually supramolecular networks based on weak multivalent intermolecular interactions among their component macromolecules (Alberti and Hyman, 2021; Banani et al., 2017; Zhang et al., 2020), how to stabilize and crystalize the RNA-Rbm14 complex will be a challenge. Therefore, researches in this direction should be considered as a long-term project for an expert in crystallography, rather than experiments to be finished in the limited time of a revision. In addition, we found that it is unlikely practicable to obtain enzymes knockout samples, even if the time required for such experiments are not considered. As maternal transcripts of thousands of genes are already m^6A-modified in zebrafish oocytes and the level of m^6A modifications further increases after fertilization (Zhao et al., 2017), to completely abolish the m^6A modification of maternal mRNAs, one needs to knock out the gene encoding the m^6A methyltransferase (*Mettl3*) from germlines. Germ line-knockout of *Mettl3*, however, represses gamete maturation in both zebrafish and mouse (Xia et al., 2018; Xu et al., 2017), thus precluding the availability of knockout embryonic samples for the proposed sequencing analysis.

When going through the main text for the results in Fig 3 during the revision, we realized that our descriptions might not be clear enough for our reviewer to catch all the information. In the revised manuscript, we have modified the text to improve the clarity. We have also discussed potential mechanisms underlying the antagonizing effect of m^6A and m^5C in Discussion, following the request of our reviewer.

5. In Fig 7, the author illustrated that zRbm14 condensates protect their content poly(A)-less mRNAs from premature degradation leading to proper cell cycle and differentiation. The authors should perform ChIP or Tail-Seq to further verify the role of zParn, or provide the reasonable interpretation of current results.

Response:

We thank our reviewer for the insightful comments. As omics assays such as ChIP or Tail-Seq will inevitably cause extensive delay of the revision due to the long-time lockdown of Shanghai and our institute, we performed poly(A) length assays on maternal transcripts of *org*, *trip10a*, and *dnajc5ga*, which were known to be accumulated in maternal *zRbm14* morphants (Fig 6F-G). We found that they displayed obvious deadenylation defects in zRbm14-depleted embryos (Figs 7A-C and EV4A-B, revised manuscript). In addition, we also noticed that, in control embryos, deadenylated transcripts of these genes can be selectively stabilized and polyadenylated following embryonic development (Figs 7B, C and EV4A-B, revised manuscript). We also show that zParn co-localized with zRbm14 condensates in early embryos (Fig 7G-H, revised manuscript). These results provide further evidence for the proposed roles of zRbm14 condensates (Fig 7M). We have accordingly revised the main text. We hope our reviewer would agree that the revised manuscript is significantly improved.

6. The authors have delivered that Rbm14 paralogues capable of both LLPS and cell fate regulation previously, as well as in this article. How about other RBM family members? Do they have the potential binding ability of modified-RNA to form condensates subsequently, considering that they have similar recognition motifs?

Response:

We thank our reviewer for the insightful question. Lots of RNA-binding proteins containing intrinsically disordered regions (IDRs) are able to undergo LLPS with RNA to form biological condensates important for various aspects of mRNA metabolisms, cell cycle regulation, and differentiation (Hentze et al., 2018; Rhine et al., 2020; Roden and Gladfelter, 2021). Among those containing the RNA-recognition motif (RRM), many have been shown to be capable of LLPS in vitro (Wang et al., 2018). Interestingly, hnRNPA2B1, hnRNPC, and hnRNPG have also been shown to preferentially bind to RNAs containing m^6A modifications (Liu et al., 2015; Liu et al., 2017; Wu et al., 2018; Zaccara et al., 2019). Among them, only hnRNPA2B1 is documented to undergo LLPS (Wang et al., 2018). Whether modified RNAs could impact its LLPS, however, has not been documented to the best of our knowledge. Interestingly, the m^6A modification is shown to enhance the accessibility of RNA to hnRNPC and hnRNPG by inducing RNA conformational changes, or structural switches (Liu et al., 2015; Liu et al., 2017). A similar mechanism might underly the effect of m6A modifications on the LLPS of zRbm14. In this context, the m^6C modification might reverse the process to make RNAs less accessible to zRbm14, therefore impairing multivalent interactions among zRbm14b and RNA molecules to repress their co-phase separation. Future studies, however, are required to verify these speculations. We have accordingly modified Introduction and Discussion of the revised manuscript.

Minor Points:

1. Several factors have been indicated to be essential in regulating maternal mRNA decay including microRNA miR-430, suboptimal codon usage, RNA m6A and m5C, ploy-A/U etc. Authors should add those background since they aim to unravel that Rbm14 and maternal mRNAs co-phase separate into cytoplasmic condensates to ensure vertebrate blastula-to-gastrula development through regulating maternal mRNA decay. Additionally, the concept of phase separation should be briefly referred in Introduction section. Please prefer to cite the references from the past three years.

Response:

We thank our reviewer for these instructions. We have included the requested information in Introduction of the revised manuscript.

2. It seems that mixed cells were used to perform the immunoblotting of zRbm14a in Fig 1B. Thus, it is not surprise that the expression level of zRbm14a increased as development progresses during which the number of cells also increased. Meanwhile, how to explain that zRbm14a slightly upregulated form 0.75-1.75 hpf whereas greatly increased after 1.75 hpf in Fig 1C?

Response:

As pointed out in the legend for Fig 1B-C, we used lysates from whole embryos (5 embryos each lane) for the immunoblotting. Our reviewer might not realize that cleavage-stage zebrafish embryos are basically transcription-silenced and need to solely depend on maternal mRNAs for protein synthesis

(please use the illustration in Fig 1A as a reference). As the total maternal mRNAs are divided into daughter blastomeres following each cell division (cleavage), daughter blastomeres always contain less amount of maternal mRNAs than their parental blastomeres do. Protein levels thus do not necessarily correlate with the number of blastomeres in the embryos. Furthermore, blastomeres should not be considered as discrete cells because their cytosols are still connected. The whole embryo is thus a multinucleated syncytium capable of exchanging proteins. This is why they display synchronized cell cycle progression. The increased levels of zRbm14a (please also refer to Fig 7G, revised manuscript, for zParn) thus suggest globally increased translation of the protein from its maternal mRNA, though detailed regulatory mechanisms still require future investigations.

3. The significance test is missing in many places, such as Fig 1J, Fig 8H. The p value needs to be consistent marked either in the main text or figures. For example, in Fig 6E "P < 2.2E-16" and in Fig 6D "P = 1.3E-181".

Response:

We have included the *t*-test result in Fig 1J in the revised manuscript. We also quantified fluorescent intensities in Fig 8H as requested and have provided the data along with significance test results in Fig 8I in the revised manuscript.

The different appearance of *P* values is due to the use of different software. The *P* values in Fig 6C and E were calculated by using two-sided Wilcoxon and Mann-Whitney tests, whereas the *P* values in Fig 6D were calculated by using Short Time-series Expression Miner (STEM) software. We have provided the information in the figure legend of the revised manuscript. We prefer to keep these values as they are to allow direct comparisons by other researchers using the same software. As generally a *P* value of ≤ 0.05 is considered statistically significant, we can change them all to $P < 0.001$ if our reviewer insists.

4. Some figures did not have icons or titles of scale marks, such as Fig 1K, Fig 2F, Fig 3K, Fig 8E-G, etc.

Response:

In the revised manuscript, we have stated in the figure legends of Figs 1A, 1K, 2F, 3K, and 6H that the schematic diagrams are not drawn to scale. We have also fixed the missing icons in Fig 8F-G. The PCA analysis in Fig 8E, however, does not have titles for scale marks (Jolliffe and Cadima, 2016).

5. The altered transcripts were not defined in the main text (Fig 6B).

Response:

Altered transcripts were those with absolute fold change >2 between ctrl-MO and 14-tMO samples at each stage. We have included such a statement in both the main text and methods in the revised manuscript.

6. There are several works related to RNA methylation-dependent phase separation should be taken into consideration in Discussion section.

Response:

As described earlier in our responses, we have included additional references and discussed possible mechanisms underlying the antagonizing effect of m^6A and m^5C modifications on the LLPS of zRbm14 in the revised manuscript.

Referee #2:

The authors of this manuscript, Xiao, Chen et al., investigate the role of the RNA-binding protein RBM14 in maternal mRNA localization and clearance, using zebrafish as a model system.
Using antibody staining and fluorescent protein localization, the authors determine that RBM14 paralogs localize in the cytoplasm near the spindle poles during early stages of embryogenesis, to later translocate to the nucleus. The authors use elegant and complementary in vivo and in vitro approaches to demonstrate that RBM14 co-localizes with gamma-tubulin, mRNA (even with m6A but not m5C modifications) and PARN via liquid-liquid phase transitions.

The main message of this work is that RBM14, thanks to its subcellular localization and its partners, regulates part of maternal mRNA clearance by setting the stage for the deadenylation of its target genes. While the data clearly suggests an important role of RBM14 in RNA regulation during early embryogenesis, the authors do not elaborate in other potential scenarios that can explain the same data and hence, better pinpoint the exact role of RBM14. For instance, RBM14 is known for its role in splicing. Precisely in the model that the authors are testing, lack of splicing of part the early zygotic genes will impair further zygotic genome activation (PMID: 24056933) and in turn fail to deploy the components of the zygotic program of maternal mRNA clearance. This scenario will justify the results by the authors showing that loss of RBM14 function leads to impaired mRNA clearance and delayed zygotic genome activation.

In summary, the question addressed in the present manuscript is relevant to the fields of Development and RNA regulation. The data is solid and many aspects are tested with orthogonal approaches, increasing rigor. Therefore, this manuscript is potentially of interest for the audience of EMBO Journal. However, the manuscript cannot be published in the present form, as the authors should address the central concern:

Response:

We thank our reviewer for recognizing our efforts and would also like to express our sincere thankfulness to our reviewer for helping us to improve the manuscript. We have carefully addressed the concerns, which we hope our reviewer would find to be satisfactory.

- The authors should analyze if splicing is disrupted in the RBM14 morphant embryos. Splicing disruption of early zygotic genes is compatible with the data presented in this manuscript and may explain part of the phenotypes derived from the loss of RBM14. One possible approach would be to analyze their RNA-Seq data for intron retention. This approach would allow the authors to discriminate which mRNAs are targets of RBM14 for destabilization and which ones require RBM14 for splicing. The same slicing

analysis should be done for the mouse data, where RBM14 is a constitutive member of the paraspeckle bodies in the nucleus. These paraspeckles are in charge of regulating splicing, among other functions.

Response:

We thank our reviewer for the insightful comments. Following the request, we analyzed differential alternative splicing events for both zebrafish and mouse and have presented the results in Figs EV3C-D and EV5D-E in the revised manuscript. We found that the gene number of transcripts with retained intron(s) (RI), which are prone to degradation (Ge and Porse, 2014), are low comparing with that of down-regulated transcripts (Figs EV3D and EV5E). Furthermore, only a small portion of the genes overlapped with the down-regulated genes (Figs EV3D and EV5E). These results suggest that the global downregulation of zygotic transcripts (Figs 6D-E and 8F-G) is mainly due to defects in MZT.

Referee #3:

In the manuscript "Rbm14 condensates deadenylate and allocate maternal mRNAs to ensure vertebrate blastula Development", Xiao and colleagues set out to understand the function of an RNA-binding protein, zRbm14, in regulating the deadenylation and clearance of maternal mRNAs during early zebrafish development. They conclude that zRbm14 forms condensates with m6A modified maternal RNAs and zParn, which localize to spindle poles. These condensates are thought to distribute maternal RNAs asymmetrically. Within the condensates - zParn gradually deadenylates RNAs, leading to an increase in m5C cap concentration, resulting in the dissolution of condensates to release maternal mRNAs which are degraded. While this is an appealing story, with lots of interesting data, some parts of the story are not well-supported by data and these will need to be addressed prior to publication.

Response:

We appreciate the positive feedback of our reviewer and are grateful to our reviewer for helping us to substantially improve the manuscript. We hope our reviewer would find that the concerns are sufficiently addressed in the revised manuscript.

Major concerns
I do not think that the data supports the statement (title) that Rbm condensates 'allocate maternal RNA'. While the authors show that Rbm condensates are asymmetrically segregated, they do not show this for RNA specifically, and even if RNA would be segregated asymmetrically, it is not clear that this distributes RNA for a specific purpose. In that context, I find it also hard to understand whether the authors expect that a difference in the segregation of bulk maternal RNA causes cell fate differences, or whether they think that it is specific RNAs that are allocated asymmetrically. If the latter, how would that work?

Response:

We appreciate the comments. Our reviewer might have missed some results in Fig 2, in which we showed that maternal mRNAs (m^6A-modified RNAs and *ccnb1* mRNA) nicely colocalized with mitotic spindle pole-associated γ-tubulin puncta and appeared to be encapsulated in zRbm14 condensates. Together with our immunostaining results (Fig 1), co-phase separation assays (Fig 3), rescue experiments (Fig 5), and omics analysis (Fig 6), we propose that the segregation of zRbm14 condensates in mitosis is also accompanied with the allocation of their content maternal mRNAs (Fig 7M). Our results suggest that

the condensates segregate the bulk of their associated RNAs, instead of only a specific one or subset. Firstly, our in-vitro assays suggest that the LLPS-promoting effect of RNA is largely sequence-independent: although the RNA sequences that we named as $0m^6A$ or $0m^5C$ share no sequence homology, they displayed similar efficacy in our assays, i.e., inducing LLPS of zRbm14b in 20 min (Figs 3B, E and EV1D, F, revised manuscript; also refer to Fig 3E of the initial manuscript). Furthermore, the condensate formation is preferentially promoted by m^6A-modified RNAs (Figs 3A-E and EV1D-F, revised manuscript). Secondly, thousands of different maternal transcripts were accumulated in 6-hpf embryos deprived of zRbm14 (Figs 6D-G and EV3F, revised manuscript). Even if a portion of these transcripts were targets of zRbm14 condensates, the condensates would contain many different transcripts.

We are sorry for having not clearly explained how asymmetric segregation of bulk maternal mRNAs could impact cell fates. In transcription-silenced blastomeres, maternal transcriptomes are the major cell fate determinants. As maternal transcriptomes are hierarchically aliquoted into blastomeres by serial embryonic cleavages, progeny blastomeres inherited with equal or different aliquots of the transcriptome would theoretically assume distinct developmental potentials. We thus reason that the polarized asymmetric segregation of zRbm14 condensates in 8-cell and 16-cell embryos (Fig 1I-K) (Rathbun et al., 2020) would accordingly enable daughter blastomeres close to the midline to acquire more copies of the condensates-associated maternal transcripts, forming a bilateral mRNA gradient. Subsequent asymmetric and symmetric segregations of zRbm14 condensates, as exampled in 64-cell embryos (Fig 1I-K), would further increase the difference in maternal mRNA contents among blastomeres to assume their different fates. Therefore, we propose that the deadenylation, sequestration, mitotic allocation, and stepwise release of maternal mRNAs by zRbm14 condensates collectively facilitate cell cycle, differentiation, and MZT of zebrafish embryos (Fig 7M). Details, however, still await future investigations. In the revised manuscript, we have modified the discussion section similarly to improve the clarify of our presentation.

I similarly do not think the data supports that (title) deadenylation and allocation of maternal RNA ensure vertebrate blastula development. The authors convincingly show that Rbm14 condensates are required for development, but whether that is due to their role in deadenylation and allocation of RNA is not shown.

Response:

As explained before, we showed that maternal mRNAs (m^6A-modified RNAs and *ccnb1* mRNA) nicely colocalized with mitotic spindle pole-associated γ-tubulin puncta and appeared to be encapsulated in zRbm14 condensates (Fig 2). Together with our immunostaining results (Fig 1), co-phase separation assays (Fig 3), rescue experiments (Fig 5), and omics analysis (Fig 6), we propose that the segregation of zRbm14 condensates in mitosis is also accompanied with the allocation of their content maternal mRNAs (Fig 7M).

In the revised manuscript, we have provided two lines of evidence to substantially strengthen our model. Firstly, we performed poly(A) length assays on transcripts of *org*, *trip10a*, and *dnajc5ga*, which were initially chosen to indicate that the zRbm14 depletion-induced maternal mRNA retention was not due to an inhibition of a specific clearance pathway (Fig 6F-G). We observed that their transcripts in zRbm14-depleted embryos were obviously defective in deadenylation (Figs 7A-C and EV4A-B, revised manuscript), supporting our proposed role of zRbm14 condensates in the deadenylation of their associated mRNAs. In addition, we also noticed that, in control embryos, deadenylated transcripts of these genes can

be selectively stabilized and polyadenylated following embryonic development (Figs 7B, C and EV4A, B, revised manuscript). These results also allow us to present our findings more smoothly in the revised manuscript to improve the readability and clarity. Secondly, we show that zParn is indeed enriched in zRbm14 condensates (Fig 7H, revised manuscript). We hope our reviewer would agree that the revised manuscript is significantly improved with these newly included results.

It is unclear from the data that is presented whether the effect of Rbm14 MO on gene expression is a direct effect of Rbm14 loss, or an indirect effect of developmental delay.

Response:

Our results collectively suggest that zRbm14 functions primarily in inactivation (through Parn-mediated deadenylation) and protection (through condensates-mediated sequestration) of maternal mRNAs and then their timely release (through zParn downregulation and m^5C modification) for re-activation or clearance (Fig 7M, revised manuscript). Accordingly, the depletion of zRbm14 leads to a failure in maternal RNA decay, which in turn results in MZT defect and embryonic arrest in mid-blastula stage. Consistently, proper maternal mRNA clearance is known to be critical for full activation of zygotic genome (MZT) that produce enough zygotic transcripts to sustain the embryonic development (Eckersley-Maslin et al., 2018; Vastenhouw et al., 2019).

We have provided three additional sets of results to strengthen the causal link in the revised manuscript, following requests of our reviewers. Firstly, we show that zParn was indeed enriched in zRbm14 condensates (Fig 7H, revised manuscript). Secondly, we performed poly(A) length assays on maternal transcripts of *org*, *trip10a*, and *dnajc5ga*, which accumulated in zRbm14 depleted embryos (Fig 6F-G, revised manuscript), and found that they displayed obvious deadenylation defects in zRbm14-depleted embryos (Figs 7A-C and EV4A-B, revised manuscript). In addition, we also noticed that, in control embryos, deadenylated transcripts of these genes can be selectively stabilized and polyadenylated following embryonic development (Figs 7B, C and EV4A, B, revised manuscript). Thirdly, analysis on differential alternative splicing (Fig EV3C-D; please also refer to Fig EV5D-E for mouse embryos) further shows that the massive down-regulation of zygotic transcripts in the zRbm14-depleted embryos (Figs 6D-E and Fig 8F-G) is indeed mainly attributed to MZT defects, instead of splicing defect-induced zygotic mRNA degradation. These results provide further evidence for the proposed roles of zRbm14 condensates (Fig 7M). In the revised manuscript, we have accordingly revised the main text to improve the clarity of our presentation. We hope our reviewer would agree that the revised manuscript is significantly improved.

To claim that 'deadenylase Parn co-phase separated with the condensates to actively deadenylate the mRNAs in early blastomeres' (abstract) one would at least like to see Parn localize to the condensates in vivo.

Response:

We thank our reviewer for the constructive comment. We performed immunostaining with the anti-human PARN antibody that was previously used for immunoblotting to show the expression profile of zParn in zebrafish embryos (Fig 7D in the initial manuscript, which has been moved to Fig EV4G in the

revised manuscript) but did not detect clear immunofluorescent signals in embryos, possibly due to its relative low affinity to zebrafish Parn. We thus generated a rabbit antibody using purified zParn as antigen. Immunoblotting confirmed that this antibody recognized the same band (Fig 7G, revised manuscript) as the anti-human PARN antibody did (Fig EV4G). Furthermore, its immunofluorescent signals tightly co-localized with those for centrosomal γ-tubulin, which was used as a marker for zRbm14 condensates because the anti-zRbm14a antibody was also from rabbit, in both interphase and mitotic blastomeres of 16-cell and 32-cell embryos (Fig 7H, revised manuscript). In 64-cell embryos, however, zParn no longer displayed such a localization (Fig 7H, revised manuscript), consistent with its downregulation at this stage (Figs 7G and EV4G). These results further strengthen the proposed role of zParn. As the immunoblotting results with anti-zebrafish Parn and anti-human PARN antibodies are independent ones that validate the expression profile of zParn and the antibody specificity, we have kept both in the revised manuscript.

Other concerns

General
- There are quite some typos.

Response:

We are sorry for this and have carefully proofread the revised manuscript to avoid typos.

- Legends should contain more information (like, whether images are MIPs or Z-slices, ...) to facilitate interpretation of results.

Response:

In the revised manuscript, we have provided such information in the legends as requested. Please note that sometimes the information is provided in “data information” of figure legends.

- Why do the authors use zRbm14a in Figures 1 and 2, and then switch to zRbm14b?

Response:

We have previously shown that zRbm14a and zRbm14b are functionally redundant (Xiao et al., 2019). We chose to use zRbm14b for in-vitro assays because we have previously expressed and purified His-GFP-tagged zRbm14b and its phase-separation region (zIDR) from *E. coli* and characterized their phase separation properties (Xiao et al., 2019).We have modified the manuscript to clarify this. Similarly, the zRbm14b and related constructs used in rescue experiments (Fig 5A-C) have also been validated previously (Xiao et al., 2019).

Figure 1.
Panel D - higher magnification would be helpful to see cytoplasmic condensates.

Response:

We agree with our reviewer that images with higher magnifications can provide more details and have kept this in mind when preparing our figures. Considering the limited print size of a figure and based on our past experiences, we feel that the magnification we used for the insets is largely appropriate because cytoplasmic condensates are clearly visible on our displayers. It is thus possible that the image quality in the pdf version for reviewing purpose was compromised. If the insets were further magnified by 2 times, for instance, each inset would reach a print size of over 4 cm × 4 cm. Such large images would be hard to be arranged properly and the figure would be oversized. Therefore, we choose not to change the magnification. Despite this, we can magnify the insets if our reviewer insists.

Panel J - I do not understand this text: "Quantifications on 8-cell embryos indicated that their immunofluorescent intensities at poles close to the midline differed by 2-fold or more from those at corresponding poles away from the midline in 20.3% of the blastomeres (median = 1.5-fold) (Fig. 1J)".

Response:

In the revised manuscript, we have modified the text as follows:

Quantifications on 8-cell embryos indicated that, in 90.8% of blastomeres (n = 153), immunofluorescent intensity of zRbm14a condensates at the spindle pole close to the embryonic midline exceeded that at the opposite pole (Fig 1J). Furthermore, the difference was over 1.5-fold and 2-fold in 50% and 20.3% of the blastomeres, respectively (Fig 1J).

Panel J - use either cell number or hpf.

Response:

We have changed “2 hpf” to “64-cell” in the panel.

Panel K - slightly unclear what is supposed to be taken from this schematic, especially the different timepoints.

Response:

The schematic is aimed to show segregation patterns of zRbm14 condensates in mitotic blastomeres. Condensates in 8-cell and 16-cell embryos are segregated mainly in an asymmetric pattern identical to that of centrosomes (Rathbun et al., 2020), i.e., larger centrosome contains more zRbm14 condensates than the smaller centrosome does in a cell and is located closer to the embryonic midline. In 64-cell embryos, blastomeres display both asymmetric and symmetric segregations, though the distribution pattern of these cells is currently unclear. We have included these descriptions in the legend in the revised manuscript. To increase the clarity of our presentation, we have also modified the main text for this part of results.

Figure 2.
Panel C - Can the authors comment on why tubulin levels are not affected by protK?

Response:

In our pilot experiments, the γ-tubulin signals were eliminated upon longer PK digestions (e.g., 15 min). Therefore, γ-tubulin is only more resistant to the protease than zRbm14. The difference might be due to their different conformations. Phase-separated zRbm14 molecules are expected to assume open conformations due to extensive intermolecular interactions among their IDRs. Such conformations would be more susceptible to the PK digestion. In contrast, γ-tubulin forms tight complex with other proteins (γ-tubulin ring complex) and might thus be more resistant to the PK digestion.

Panel F - As there is no analysis of cell fate differences related to asymmetrical distribution of Rbm14 condensates, this schematic is misleading. I would also be interested, as indicated above, what exactly the authors think is the effect of asymmetric Rbm14 distribution on RNA distribution, especially related to bulk RNA or specific RNAs...

Response:

In our responses to the major concerns of our reviewer, we have explained why we propose a link between asymmetric allocation of bulk maternal mRNAs and cell fates. The schematic in Fig 2F is provided as a speculation model to aid readability. We have indicated this in the manuscript.

The developmental arrest (Fig 4) and results of PCA (principal component analysis) and GO (gene ontology) enrichment analysis (Figs 6A, B and EV3E, revised manuscript) have consistently indicated differentiation defects in zebrafish embryos deprived of zRbm14. During the revision, we analyzed our RNA deep sequencing data for expression profiles of differentiation-related transcripts. We found that multiple markers of endoderm, mesoderm, and ectoderm were largely down-regulated in 6-hpf zRbm14-depleted morphants as compared to control embryos (Fig EV3G, revised manuscript), further confirming developmental defects in germ layer differentiations. We propose that deadenylation, sequestration, mitotic allocation, and stepwise release of maternal mRNAs by zRbm14 condensates collectively facilitate cell cycle, differentiation, and MZT of zebrafish embryos (Fig 7M). Detailed contributions of the each aspect will need future investigations.

Figure 3.
Panels 3D-F - I am surprised that m5C do not form droplets with Rbm14 (3D,E) but can enter existing droplets and dissociate them (3F). Or is the idea that deadenylation/degradation results in a relative increase in m5C? Can the authors elaborate?

Response:

Our results suggest that the LLPS-promoting effect of RNA is largely sequence-independent. The RNA sequences that we named $0m^6A$ or $0m^5C$ for simplicity were from literature (Ries et al., 2019; Yang et al., 2019) and share no sequence homology. Yet, they displayed similar efficacy in our assays, i.e., induced LLPS of zRbm14b in 20 min (Figs 3B, E and EV1D, F, revised manuscript). Therefore, zRbm14b primarily interplays with RNA, whereas m^5C and m^6A modifications function to modulate the strength of the interactions in an antagonizing manner. In this context, it would not be difficult to understand why $1m^5C$ was also recruited into pre-formed condensates containing m^6A-RNA. As phase-

separated condensates are supramolecular networks (Alberti and Hyman, 2021), m^5C modifications would also require to be in the networks to impair or disrupt them.

RNA-recognition motif (RRM)-containing proteins hnRNPA2B1, hnRNPC, and hnRNPG have been documented to preferentially bind to m^6A-modified RNAs as well (Liu et al, 2015; Liu et al, 2017; Wu et al, 2018; Zaccara et al, 2019). Furthermore, m^6A modifications are shown to enhance the accessibility of RNA to hnRNPC and hnRNPG by inducing RNA conformational changes, or structural switches (Liu et al., 2015; Liu et al., 2017). A similar mechanism might underly the effect of m^6A modifications on the LLPS of zRbm14. In this context, the m^6C modification might reverse the process to make RNAs less accessible to zRbm14, therefore impairing multivalent interactions among zRbm14b molecules and RNAs to repress their co-phase separation. Future studies, however, are required to verify these speculations. We have included these in Discussion of the revised manuscript.

Panel H - injecting 800 or 1200 pg RNA into the embryo is a lot. Do these embryos develop normally? Related, this image looks quite different from what we see for zRbm14a in Figure 1 (same timepoint), which may suggest that these big condensates are an artifact. Can the authors explain the difference?

Response:

In rescue experiments we injected 800 pg of RNA per egg and found that embryos injected with zRbm14b mRNA developed to 24 hpf (Figs 5A-C and EV2G). We observed that embryos injected with 1200 pg of the RNA developed to 6 hpf but we did not check for a longer time.

Centrosomal γ-tubulin is markedly diminished at 4 hpf as compared to 2 hpf or earlier (Rathbun et al., 2020), so was centrosomal zRbm14a condensates (Fig 1D). Their nice co-localization (Fig 1G and I) further indicates that zRbm14 condensates are enriched to the centrosome by associating with γ-tubulin puncta. When GFP-Rbm14b was expressed to low levels by injecting 400 pg of its mRNA, it was enriched at the centrosome/spindle poles in cells of 4-hpf embryos, similar to the endogenous zRbm14a (Fig 1H vs Fig 1D and F). We reason that, when GFP-Rbm14b was expressed to relatively high levels by injecting 800 pg or 1200 pg of its mRNA, excessive amount of GFP-Rbm14b condensates formed artificially in 4-hpf cells. These condensates largely exceeded the capacity of centrosomal γ-tubulin puncta, thus becoming dispersed.

We purposely overexpressed the protein because the aim of the experiments was to clarify whether zRbm14 condensates in cells were indeed co-phase-separated with mRNAs. Although the results in Fig 2 are strong evidence for the presence of maternal mRNAs in zRbm14 condensates, one might still argue that the RNAs we observed might be located in other compartments showing similar distribution patterns as the condensates, such as the γ-tubulin puncta. The enrichment of the RNA-specific dye Pyronin Y (please also refer to Fig 2B) into GFP-zRbm14b condensates induced through artificial high-level expression (Fig 2H) thus serves as an evidence for the enrichment of RNA into zRbm14 condensates in vivo. The fusion of condensates (Fig 3I) further confirms their liquid property, a hallmark of LLPS.

P9 - The header "zRbm14 undergoes LLPS in vivo with maternal RNAs" is misleading, as there is no evidence for the statement that zRbm14 only associates with maternal RNAs.

Response:

As Pyronin Y staining does not distinguish different RNAs and that cells in 4-hpf embryos contain both maternal and zygotic RNA, we have modified the heading into "zRbm14b undergoes LLPS *in vivo* with RNA".

Panel I - can 3D information be provided? Or is this a Z-slice? What thickness?

Response:

Images in Fig 3I were maximum intensity-projected images (we have indicated this in the legend following the request of our reviewer). They were projected from 7 z-slices of 1-μm intervals.

We verified that the fusion event is a real one through 3D-movies, whose representative image sequences are shown in Figure 1 for reviewers below (please note that the orientation of the 3D images is different from that of the images in Fig 3I). As the increased volume (size) of the fused condensate and its singleness over time already validated the fusion event (Fig 3I), we choose not to present the 3D movie in the manuscript. We can present the movie if our reviewer requests this.

Figure 1 for reviewers

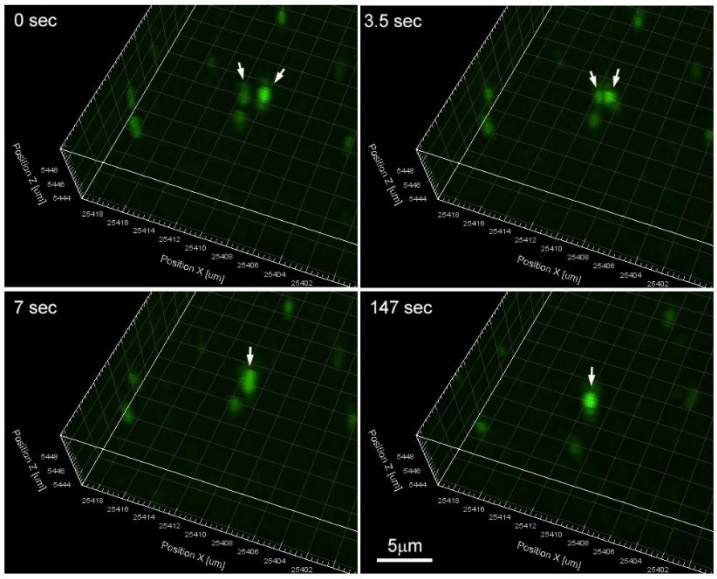


Figure 4.
Panel D - the cell number is derived from the number of nuclei observed in a 10um z-depth. Is this a reliable representation of the total number of cells in the embryo? Would it be better to use something like Ki67 to measure the proliferation index?

Response:

Embryonic cells at the animal pole occupy a thickness of approximately 500 μm. Such a thickness (plus the thickness of the supporting substratum) is beyond the working distance of the oil lens we used. Although the ideal way is to count all cells in an embryo, practically people in the field usually image a

10-µm z-depth from the animal pole and measure cells in the area for comparisons during early embryonic stages (Meier et al., 2018; Menon et al., 2020; Sato et al., 2019; Zhang et al., 2014). We thus also chose to follow this.

We appreciate the suggestion but Ki67 is expressed in all cells undergoing the cell cycle (G1-to-M). Only quiescent (G0) cells do not express Ki67. It is thus widely used as a proliferation marker to discriminate cycling cells from quiescent (G0) cells. In early embryos, however, all the embryonic cells are cycling cells.

Figure 5
Panel B - embryos should be shown in same orientation to allow for comparison and, once possible, using standard orientation.

Response:

We have displayed the embryos in standard orientation as requested.

Panel D - the use of hexanediol is aspecific and therefore not very meaningful. Other condensates in the embryo will also be affected and therefore one cannot conclude anything about the importance of Rbm14 condensates.

Response:

We agree with our reviewer on the specificity of Hex. We point out in the manuscript that we used it to induce acute disassembly of intracellular liquid condensates, including those of zRbm14, to assess the importance of LLPS in the blastula-to-gastrula development. It is usually more difficult to demonstrate the physiological importance of LLPS than to characterize the phase separation property of condensates due to the lack of tools. Although Hex does not just influence zRbm14 condensates, if Hex had no effect, the results would argue against a role of LLPS of any sort of condensates, including the zRbm14 ones. In the revised manuscript, we have modified the last sentence of the paragraph as "the high sensitivity of the embryos to Hex strengthens the importance of liquid condensates in the blastula-to-gastrula development".

Figure 6
Since Rbm14 MO embryos are delayed (see Figure 5) compared to control MO embryos, the differences in maternal and zygotic transcripts may be a mere consequence of the different developmental stages that are analyzed for the two conditions. While this delay is a consequence of the lack of Rbm14 (Figure 5), it is difficult to know whether the changes in gene expression are directly or indirectly due to lack of Rbm14. The experiment presented in panels H and I is not sensitive enough to remedy this issue. Since the authors propose that m6A containing transcripts are specifically enriched in Rbm condensates, they may wanna look whether they are specifically affected, especially in the maternal set.

Response:

As described earlier in our response, our results collectively suggest that zRbm14 functions primarily in inactivation (through Parn-mediated deadenylation) and protection (through condensates-mediated sequestration) of maternal mRNAs and their timely release (through zParn downregulation and m^5C modification) for re-activation and clearance (Fig 7M, revised manuscript). Accordingly, the depletion of

zRbm14 leads to a failure in maternal RNA decay, which in turn results in MZT defect. As described earlier, we have provided three additional sets of results to strengthen the causal link in the revised manuscript.

Following the request of our reviewer, we analyzed how many maternal transcripts accumulated in 4-hpf and/or 6-hpf maternal zRbm14 morphants ($\geqslant$ 2-fold) overlapped with m^{6}A-modified transcripts reported previously (Zhao et al., 2017). We found that 33.7% of them (1,876 out of 5,566) overlapped with the reported 5,994 m^{6}A-modified transcripts (Fig 2 for reviewers), further supporting a relationship between zRbm14 and m^{6}A-RNAs. We have described the results in the main text related to Fig EV3B in the revised manuscript.

Figure 2 for reviewers

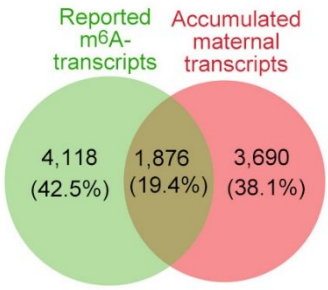


Figure 7
Panel B - If Parn can co-phase separate with just the IDR of Rbm14, which protein do the authors think recruits the RNAs to the condensate?

Response:

We demonstrate that RNAs, including unmodified RNA (0m^{6}A) and m^{6}A-modified RNA (10m^{6}A), directly co-phase separated with zRbm14b and concomitantly promoted the phase separation (Fig 3A-C). Nevertheless, even 10m^{6}A, the most potent RNA in our hands, was unable to induce the phase separation of zIDR (Fig 3A-B). Therefore, in sharp contrast to zParn, RNA interacts with the RNA-binding domain (the RRM-containing region) of zRbm14b (please refer to the schematic in Fig 2A) to stimulate the formation of zRbm14b-RNA condensates, possibly by promoting the multivalent interactions among zRbm14b and RNA molecules.

Panel D - Does zParn colocalize with zRbm14 in vivo? Since there is an antibody available, this seems like smth the authors should do to strengthen their hypothesis.

Response:

We thank our reviewer for the question. As described earlier in our response, we performed immunostaining with the anti-human PARN antibody that was previously used for immunoblotting but did not detect clear immunofluorescent signals in embryos, possibly due to its relative low affinity to zebrafish Parn. We thus generated a rabbit antibody using purified zParn as antigen. Immunoblotting confirmed that this antibody recognized the same band (Fig 7G, revised manuscript) as did the anti-human PARN antibody (Fig EV4G). Furthermore, its immunofluorescent signals tightly co-localized with those for centrosomal γ-tubulin, which was used as a marker for zRbm14 condensates because the anti-zRbm14a antibody was also from rabbit, in both interphase and mitotic blastomeres of 16-cell and 32-cell embryos (Fig 7H, revised manuscript). In 64-cell embryos, however, zParn no longer displayed such a localization (Fig 7H, revised manuscript), consistent with its downregulation at this stage (Figs 7G and EV4G). These results further strengthen the proposed role of zParn. As the immunoblotting results with

anti-zebrafish Parn and anti-human PARN antibodies are independent ones that validate the expression profile of zParn and the antibody specificity, we have kept both in the revised manuscript.

Figure 8
Panel B - Higher magnification would be useful to see cytoplasmic punctae (and lack thereof in later stages)

Response:

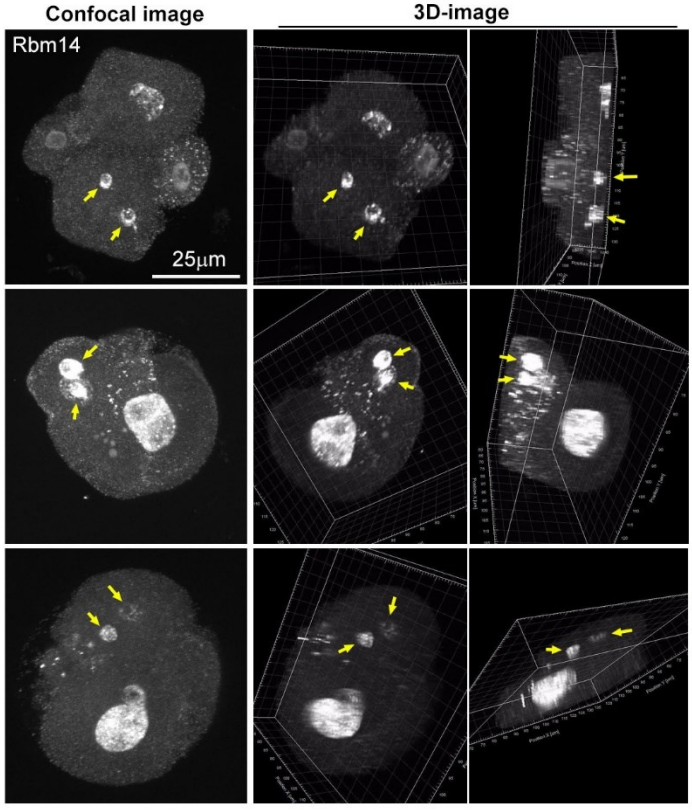
Following the request, we have included magnified insets in Fig 8B in the revised manuscript. We have also provided a 3D movie for the embryo containing the spindle pole-enriched Rbm14 (Movie EV4).

Panel B -more than n=1 is needed to conclude that Rbm14 localizes to spindle pole in mouse.

Response:

During the revision, we examined additional 200 2-cell mouse embryos and found three additional mitotic cells, all of them containing spindle pole-enriched Rbm14 similar to the one in Fig 8B. We have provided the images of these embryos and their selective snap shots of 3D-reconstructed images in Figure 3 for reviewers below (arrows point to spindle poles). Please note that, for the mitotic cell in the third (bottom) embryo, one of the two spindle poles was only partly imaged.

Figure 3 for reviewers



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Thank you for submitting a revised version of your manuscript. The study has now been seen by all original referees. While both reviewers appreciate the added information, reviewers #2 and #3 also indicate that the manuscript still contains several overstatements, and a more balanced interpretation of the results and discussion of possible alternative interpretations is needed before they can accept publication of the manuscript. Furthermore, referee #3 finds that additional data quantification is required. Therefore, I would like to invite you to address the remaining referee comments and the following editorial issues:

1) Please add a short Table of Contents at the beginning of the Appendix file.
2) We are missing the ORCID iD for the co-corresponding author Naihe Jing. In order to link the ORCID iD to the account in our manuscript tracking system, the author in question has to do the following:
- Click the 'Modify Profile' link at the bottom of your homepage in our system.
- On the next page you will see a box halfway down the page titled ORCID*. Below this box is red text reading 'To Register/Link to ORCID, click here'. Please follow that link: you will be taken to ORCID where you can log in to your account (or create an account if you don't have one)
- You will then be asked to authorise Wiley to access your ORCID information. Once you have approved the linking, you will be brought back to our manuscript system.
Unfortunately, we cannot do this linking on the author's behalf for security reasons.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess.

Please feel free to contact me if have any further questions regarding this final revision. Thank you again for giving us the chance to consider your manuscript for The EMBO Journal. I look forward to receiving the revised version.

Referee #1:

The authors have addressed most of my major concerns and I recommend its publication.

Referee #2:

This is a revised version of the original manuscript by Xiao, Chen et al., where they study the role of RBM14 in the clearance of maternal mRNA during early embryogenesis.
My main concern with the data in the previous version was that part of the data about the role of RBM14 creating a

deadenylation center to trigger mRNA degradation could be explained by an alternative hypothesis. This alternative hypothesis involved that loss of RBM14 interfered with splicing of de novo synthetized transcripts, a known role for RBM14 in other organisms. Lack of splicing of certain mRNAs would prevent the translation of proteins that are directly involved in the zygotic program of maternal mRNA degradation, which in turn will results in stabilization of certain maternal mRNAs. The authors followed my suggestion to explore how splicing is affected by the loss of RBM14. However, they interpret that aberrant splicing may be the direct cause of mRNA degradation and focused their reply on the study of intron retention, that can lead to mRNA instability. They don't explore the main question of my concern: the possibility that among all the alternative splicing events that occur in RBM14 morphants compared to controls, one of them could disrupt the production of an essential zygotic gene involved in maternal mRNA degradation (PMID: 24056933). With over 7,000, 9,000 and 15,000 alternative splicing events between RBM14 morphants and controls at 2.5, 4 and 6 hpf, respectively, it is conceivable that a key factor of the maternal clearance pathway may be affected, and that would provide an alternative explanation to the data than the one provided by the authors.

I still consider that to warrant publication, the authors should contemplate splicing disruption as a possible justification of the data. However, since demonstrating that none of the splicing targets of RBM14 participate in mRNA clearance will be hard to demonstrate, the authors at least should acknowledge this possibility in the discussion and then the paper will merit publication in EMBO Journal.

Referee #3:

The revised manuscript "Rbm14 condensates deadenylate and allocate maternal mRNAs to ensure vertebrate blastula development" has much improved and I am happy to, in principle, recommend it for publication. I do still think, however, that there are some statements in the text that are not supported by the data. Before publications, these should be addressed, or statements should be changed to fit the data.

***
I am still uncomfortable with the title "Rbm14 condensates deadenylate and allocate maternal mRNAs to ensure vertebrate blastula development" and related statements throughout the MS.

While I agree that they show colocalization of m6A RNA and ccnd1 RNA with Rbm condensates, there is no quantification of the asymmetric nature of it. Images should be quantified, and number of replicates indicated.

On the second part of the title, the authors convincingly show that condensates are important for development, and also that condensates are required for deadenylation and (perhaps, see above) allocation of maternal RNA. While this makes it likely that they are connected, there is no proof that they are. To write 'to ensure...' in the title is therefore misleading.

Related, there is no evidence that the (potential) asymmetric distribution of RNA directly results in cell fate changes as is mentioned in the abstract.

To prove both, one would have to follow cells and connect more/less maternal RNA with different cell fates. I appreciate that this is difficult and do not think it is essential for the paper, but if not done, statements and Figure panels should be adjusted to reflect that this is a hypothesis.

***
It is still unclear to me whether we can be sure that the effect of Rbm14 MO on gene expression is the consequence of Rbm14 loss, or the consequence of developmental delay.

In my previous comments I wrote "Since Rbm14 MO embryos are delayed (see Figure 5) compared to control MO embryos, the differences in maternal and zygotic transcripts may be a mere consequence of the different developmental stages that are analyzed for the two conditions."

While the authors have provided their rationale for the mechanism by which Rbm14 MO might impact maternal and zygotic transcripts, I am more concerned about the developmental delay that is caused by the Rbm14 MO and the effect that has on the RNA seq results. Especially because the time is indicated is in hpf and not stage. Can the authors be sure this is not a problem?

Related - is the overlap with m6A RNA that was added to the MS more than expected by chance?

***
I am still concerned about Figure 3, panel H.
At 800pg I see no colocalization, and at 1200pg the droplets look very different which makes me suspicious. So there is no actual proof for co-localization with m6A RNA.

***

The authors should rigorously go through their legends to indicated Ns and ns, which are missing throughout.

Point-to-point response to reviewers' comments

Referee #1:
The authors have addressed most of my major concerns and I recommend its publication.

Response:
We sincerely thank our reviewer for spending his/her precious time and helping us to improve the manuscript.

Referee #2:
This is a revised version of the original manuscript by Xiao, Chen et al., where they study the role of RBM14 in the clearance of maternal mRNA during early embryogenesis.
My main concern with the data in the previous version was that part of the data about the role of RBM14 creating a deadenylation center to trigger mRNA degradation could be explained by an alternative hypothesis. This alternative hypothesis involved that loss of RBM14 interfered with splicing of de novo synthetized transcripts, a known role for RBM14 in other organisms. Lack of splicing of certain mRNAs would prevent the translation of proteins that are directly involved in the zygotic program of maternal mRNA degradation, which in turn will results in stabilization of certain maternal mRNAs. The authors followed my suggestion to explore how splicing is affected by the loss of RBM14. However, they interpret that aberrant splicing may be the direct cause of mRNA degradation and focused their reply on the study of intron retention, that can lead to mRNA instability. They don't explore the main question of my concern: the possibility that among all the alternative splicing events that occur in RBM14 morphants compared to controls, one of them could disrupt the production of an essential zygotic gene involved in maternal mRNA degradation (PMID: 24056933). With over 7,000, 9,000 and 15,000 alternative splicing events between RBM14 morphants and controls at 2.5, 4 and 6 hpf, respectively, it is conceivable that a key factor of the maternal clearance pathway may be affected, and that would provide an alternative explanation to the data than the one provided by the authors.

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Response:

We sincerely thank our reviewer for helping us to improve the clarity of the manuscript.

We agree with our reviewer on this possibility. In addition, reviewer #3 pointed out that different developmental stages of the two groups of embryos that were analyzed might also contribute to the differences in maternal transcripts. We have thus added a paragraph of the following sentences to clarify these issues in the revised manuscript:

"It should be noted that maternal mRNAs accumulated in maternal *zRbm14* morphants (Fig 6E) may not all be the direct targets of zRbm14. Some of them may only be due to the developmental delay of the morphants (Fig 4). Furthermore, as thousands of alternative splicing

events occurred in the morphants (Fig EV3C), splicing defects of zygotic transcripts encoding proteins involved in the zygotic program of maternal mRNA clearance (Despic & Neugebauer, 2018; Lee et al, 2013) might also contribute to the accumulation of some maternal mRNAs. Future studies will thus be required to further discriminate *bona fide* target maternal mRNAs of zRbm14 and understand how the regulations on them impact early embryogenesis" (Discussion, page 23).

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The revised manuscript "Rbm14 condensates deadenylate and allocate maternal mRNAs to ensure vertebrate blastula development" has much improved and I am happy to, in principle, recommend it for publication. I do still think, however, that there are some statements in the text that are not supported by the data. Before publications, these should be addressed, or statements should be changed to fit the data.

Response:

We thank our reviewer for the positive feedback and valuable comments. We have carefully modified the wording of the manuscript following the requests.

***

I am still uncomfortable with the title "Rbm14 condensates deadenylate and allocate maternal mRNAs to ensure vertebrate blastula development" and related statements throughout the MS.

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Related, there is no evidence that the (potential) asymmetric distribution of RNA directly results in cell fate changes as is mentioned in the abstract.

To prove both, one would have to follow cells and connect more/less maternal RNA with different cell fates. I appreciate that this is difficult and do not think it is essential for the paper, but if not done, statements and Figure panels should be adjusted to reflect that this is a hypothesis.

Response:

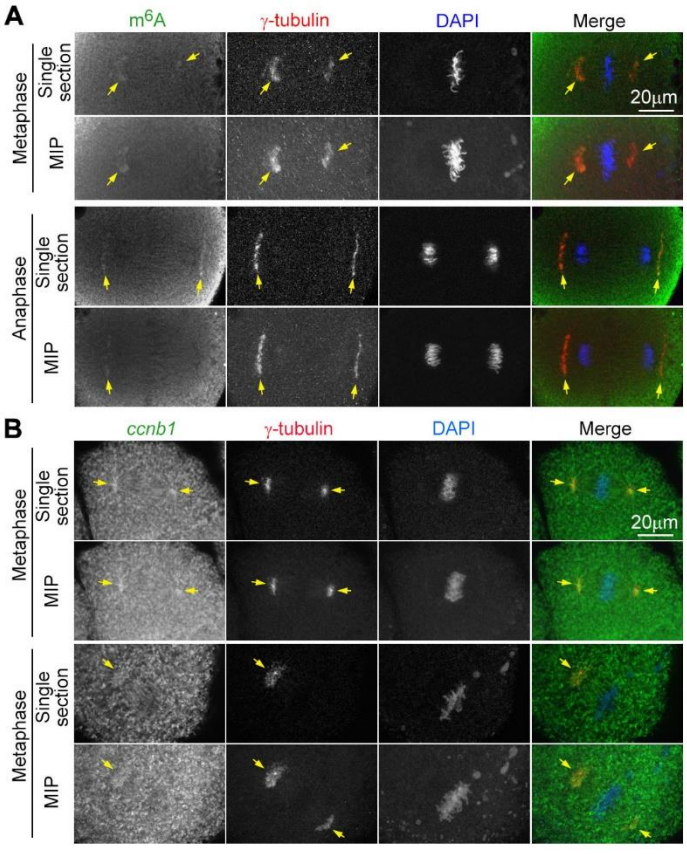
We have changed the title into "Rbm14 condensates deadenylate and allocate maternal mRNAs to facilitate vertebrate blastula development" and modified the main text accordingly.

We agree with our reviewer on the importance of quantification. In this particular case, however, we do not think that quantification results from single-section images can properly indicate the levels of spindle pole-associated RNAs. For spindles that were not positioned parallel to the imaging plane, quantification results from a single section apparently cannot reflect the real

situation. Even for well-aligned spindles, whether the quantification results can represent spindle-pole signals in entire cells is still unclear.

The reason we chose to present single optical sections in Fig 2D-E is because the m^{6}A immunofluorescent images and *ccnb1* FISH images were of high background, probably due to high levels of dispersed RNAs in the cytoplasm. The background signals tended to obscure the spindle-pole signals in maximum intensity-projected (MIP) images (please refer to Fig 1 for reviewers below). Sometimes RNA signals at one pole or even both poles became invisible or only barely visible in MIP images. The poor signal-to-background ratio and uneven backgrounds of these projected images also make the reliability of quantification results doubtful. Due to these technical difficulties, we only use the single-section images (Fig 2D-E) as evidence of spindle pole-associated m^{6}A-RNAs and *ccnb1* mRNAs in our manuscript and strictly keep asymmetric distribution of mRNAs as a speculation. We hope our reviewer would agree that detailed quantification of the RNA signals can be left for future studies. In the revised manuscript, we have explained in the legend of Fig 2D-E why single optical sections are presented: "As the high background fluorescent signals, probably due to high levels of dispersed RNAs in the cytoplasm, tended to obscure the spindle-pole RNA signals in maximum intensity-projected images, confocal images of a representative single optical section are shown".

Fig 1 for reviewers



Following the request of our reviewer, we have modified the manuscript to clearly indicate asymmetric allocation of mRNAs and its role in cell fate regulation as hypotheses or speculations:

(1) Abstract: we have modified “In zebrafish, the condensates were highly abundant in blastomeres...” as “In zebrafish, Rbm14 condensates were highly abundant in blastomeres...”.

(2) Fig 2F legend: we have modified “Summarizing illustrations for possible cell fate-regulatory functions of zRbm14 condensates...” as “Speculative illustrations for possible cell fate-regulatory functions of zRbm14 condensates...”.

(3) Discussion: we have modified “We thus reason that the polarized asymmetric segregation of zRbm14 condensates in 8-cell and 16-cell embryos (Fig 1I-K) (Rathbun et al., 2020) would accordingly enable daughter blastomeres close to the midline to acquire more copies of the condensates-associated maternal transcripts, forming a bilateral mRNA gradient. Subsequent asymmetric and symmetric segregations of zRbm14 condensates, as exampled in 64-cell embryos (Fig 1I-K), would further increase the difference in maternal mRNA contents among blastomeres to assume different fates” (paragraph 3) as “We thus speculate that the polarized asymmetric segregation of zRbm14 condensates in 8-cell and 16-cell embryos (Fig 1I-K) (Rathbun et al., 2020) might accordingly enable daughter blastomeres close to the midline to acquire more copies of the condensates-associated maternal transcripts, forming a bilateral mRNA gradient. Subsequent asymmetric and symmetric segregations of zRbm14 condensates, as exampled in 64-cell embryos (Fig 1I-K), might further increase the difference in maternal mRNA contents among blastomeres to assume different fates”.

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In my previous comments I wrote "Since Rbm14 MO embryos are delayed (see Figure 5) compared to control MO embryos, the differences in maternal and zygotic transcripts may be a mere consequence of the different developmental stages that are analyzed for the two conditions."

While the authors have provided their rationale for the mechanism by which Rbm14 MO might impact maternal and zygotic transcripts, I am more concerned about the developmental delay that is caused by the Rbm14 MO and the effect that has on the RNA seq results. Especially because the time is indicated is in hpf and not stage. Can the authors be sure this is not a problem?

Response:

We chose to use hpf here because this is the objective condition of the samples. As control embryos developed normally (Fig 4A-B), their developmental stage at a certain time point was similar to the stage of intact embryos (Fig 1A) (Kimmel *et al*, 1995). For maternal *zRbm14* morphants, however, the apparent stage at a certain time was only a phenotype based on relatively subjective judgement and could be inaccurate or even wrong. For instance, although 2.5-hpf and 4-hpf 14-tMOs embryos were morphologically similar to 2.5-hpf and 4-hpf ctrl-MO embryos, respectively (Fig 4A-B), the PCA analysis (Fig 6A) revealed that they were different from the control ones. Similarly, 6-hpf 14-tMOs embryos also differed from 4-hpf ctrl-MO embryos in PCA analysis (Fig 6A), despite their similar morphologies (Fig 4A-B). Therefore, we feel that marking maternal *zRbm14* morphants with their apparent stages would be biased and cause confusions in description.

We understand the logic of our reviewer's concern: if the zRbm14 depletion-induced developmental delay (Fig 4) was caused by defects in processes other than maternal RNA metabolism, transcriptome differences between the control and *zRbm14* morphants (Fig 6) would solely be consequences of the developmental delay. We bear in mind the same concern during our research. This is why we performed rescue experiments with various zRbm14b constructs (Fig 5A-C) prior to the RNA seq analyses to verify that zRbm14 functions in the blastula-to-gastrula development through both RNA binding and phase separation (Fig 5A-C). These results also further linked the function of zRbm14 to cytoplasmic zRbm14-RNA condensates (Figs 1-3).

Our follow-up mechanistic studies (Figs 7 and EV4) also obtained consistent results. For instance, maternal mRNAs accumulated in the 6-hpf *zRbm14* morphants, such as those of *org*, *trip10a*, and *dnajc5ga* (Fig 6F-G), displayed deadenylation defects at 2.5 hpf and/or 4 hpf (Figs 7A-C and EV4A-B). At 4 hpf, the zRbm14-depleted embryos had not morphologically shown observable developmental delay (Fig 4A-B). At 2.5 hpf, even differences in cell size, number, and nuclear size were not yet observed (Fig 4C-F). As deadenylation sensitizes mRNAs to degradation, the lack of deadenylation is expected to stabilize these mRNAs and result in their accumulations. If the effect of Rbm14 MO on gene expression (Fig 6) were solely the consequence of developmental delay, these accumulated maternal mRNAs would have displayed similar poly(A) status as those in control embryos at 4 hpf and, especially, 2.5 hpf. Our results on zParn (Fig 7D-L) further explained why the depletion of zRbm14 results in deadenylation defects in the early embryos.

Nevertheless, we fully agree with our reviewer that some of the altered maternal transcripts may only be the consequence of developmental delay. In addition, our reviewer #2 also pointed out that splicing defects of zygotic transcripts encoding proteins involved in the zygotic program of maternal mRNA clearance may also contribute to the accumulation of maternal mRNAs. We have thus added a paragraph of the following sentences to clarify these issues in the revised manuscript:

"It should be noted that maternal mRNAs accumulated in maternal *zRbm14* morphants (Fig 6E) may not all be the direct targets of zRbm14. Some of them may only be due to the developmental delay of the morphants (Fig 4). Furthermore, as thousands of alternative splicing events occurred in the morphants (Fig EV3C), splicing defects of zygotic transcripts encoding proteins involved in the zygotic program of maternal mRNA clearance (Despic & Neugebauer, 2018; Lee et al, 2013) might also contribute to the accumulation of some maternal mRNAs. Future studies will thus be required to further discriminate *bona fide* target maternal mRNAs of zRbm14 and understand how the regulations on them impact early embryogenesis" (Discussion, page 23).

Zygotic transcription is known to be essential to the blastula-to-gastrula development of zebrafish (Kane *et al*, 1996; Vejnar *et al*, 2019). Therefore, the global downregulation of zygotic transcripts (Fig 6E) is expected to impair the embryonic development. We also agree with our reviewer that the downregulation of some zygotic transcripts may be a result, but not the cause, of the developmental delay. As it is beyond our current scope to elaborate the roles of zygotic transcripts, we only generally attribute the downregulation to impaired MZT for simplicity (pages 16-17).

Related - is the overlap with m6A RNA that was added to the MS more than expected by chance?

Response

These data were presented in Fig S4D of the initial manuscript and moved to Fig EV3B in the revised manuscript. They were thus not newly added to the manuscript. As our accumulated transcripts only occupied 10.3% (4 hpf) or 39.8% (6 hpf) of total transcripts, whereas the accumulated transcripts in the *zYthdf2*$^{-/-}$ embryos only occupy 10.5% (4 hpf) or 9.3% (6 hpf) of total transcripts (Zhao *et al*, 2017), the overlaps appeared to be more than expected by chance. This might suggest that Rbm14 and Ythdf2 shared some common target mRNAs but requires to be clarified in future studies.

To be accurate, we have modified the sentences "zRbm14 also regulated different sets of m6A-modified maternal mRNAs as zYthdf2 does" (page 23, line 4) and "zRbm14 and zYthdf2 were responsible for the decay of different mRNAs" (Fig EV3B legend) as "zRbm14 also mainly regulated different sets of m6A-modified maternal mRNAs as zYthdf2 does" and "zRbm14 and zYthdf2 were largely responsible for the decay of different mRNAs".

***

I am still concerned about Figure 3, panel H.

At 800pg I see no colocalization, and at 1200pg the droplets look very different which makes me suspicious. So there is no actual proof for co-localization with m6A RNA.

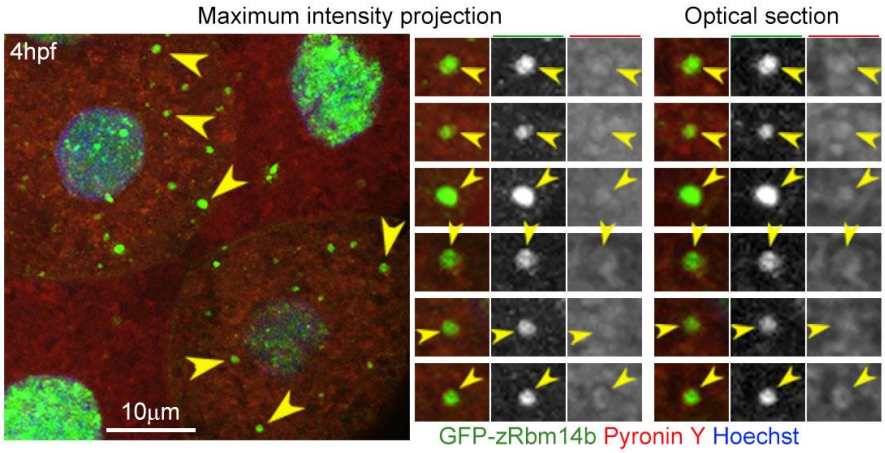
Response

These experiments were performed to investigate whether zRbm14b could undergo LLPS *in vivo* to form cytoplasmic condensates with RNA. As described in the manuscript, we performed the experiments at 4 hpf due to relatively high-level expression of exogenous GFP-Rbm14b at this time (Fig 3G). Another reason was that, to achieve our experimental purposes, we had to induce ectopic GFP-zRbm14b condensate formation through overexpression, otherwise it would be difficult to attribute the cause of condensate formation to zRbm14b. At 4 hpf, endogenous cytoplasmic zRbm14 condensates were no longer abundant and mainly appeared as one or two juxtanuclear puncta (presumably at the centrosomal region) in interphase cells (Fig 1D). Ectopic GFP-zRbm14b condensates could thus be easily identified. Nevertheless, as the total level of endogenous zRbm14a persistently increased (Fig 1B), the lack of endogenous zRbm14 condensates at 4 hpf might be due to reduced m^{6}A modifications and/or increased m^{5}C modifications of mRNAs (Yang *et al*, 2019; Zhao *et al.*, 2017). Therefore, a disadvantage could be that RNA signals in the ectopic condensates might be weak.

We tested what expression levels of GFP-zRbm14b could form ectopic condensates and found that GFP-zRbm14b expressed from 400 pg of injected mRNA mainly displayed similar localizations as endogenous zRbm14a (Fig 1H vs Fig 1D and F). Ectopic condensates formed at 800-pg mRNA and were generally much larger in size at 1,200-pg mRNA (Fig 3H), suggesting a dose-dependent effect. The condensates formed at 800-pg mRNA were positively stained with Pyronin Y, as demonstrated in magnified insets of Fig 2 for reviewers below. The Pyronin signals were clearer in single optical sections (Fig 2 for reviewers) because in maximum intensity-

projected images the high cytoplasmic background, attributing to abundant RNAs in the cytoplasm, could obscure some of the condensate-specific signals. Large condensates were more prominent for the Pyronin signals (Fig 3H), probably due to their large volumes: for spherical condensates, volumes are a cubic function of the radius. For instance, when the diameter differed by 2 times, the volume (and molecules within) would differ by 8 times.

Fig 2 for reviewers



In the revised manuscript, we have added the magnified maximum intensity-projected insets of Pyronin Y signals to Fig 3H to facilitate the visualization of the weak signals. We have also modified the main text (page 11) and the figure legend (page 46) to improve the clarify of the presentation.

***

The authors should rigorously go through their legends to indicated Ns and ns, which are missing throughout.

Response

"ns" was indicated in the "Data information" section at the end of figure legends. Nevertheless, we do find that the legend of Fig EV4 lacks "ns, no significance; * $P < 0.05$; ** $P < 0.01$" and have included the information into the revised manuscript.

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Zhao BS, Wang X, Beadell AV, Lu Z, Shi H, Kuuspalu A, Ho RK, He C (2017) m(6)A-dependent maternal mRNA clearance facilitates zebrafish maternal-to-zygotic transition. *Nature* 542: 475-478

Editor accepted the revised manuscript.

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Reporting Checklist for Life Science Articles (updated January 2022)

This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your manuscript.

Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:

  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Yes	Materials and Methods, Appendix Table S1

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Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Appendix Table S1

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Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Not Applicable	
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes	Materials and Methods

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If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	Acknowledgements

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Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Materials and Methods, Figure legends
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Not Applicable	
Include a statement about **blinding** even if no blinding was done.	Yes	Materials and Methods: Statistical analysis
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Figure legends, Materials and methods

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In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	Figure legends
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Figure legends

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Studies involving experimental **animals**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Yes	Materials and Methods: Animal use and care
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Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Yes	Data Availability Section: RNA-seq data was deposited to the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo).
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Yes	Reference list: Zhao BS, Wang X, Beadell AV, Lu Z, Shi H, Kuuspalu A, Ho RK, He C (2017) m(6)A-dependent maternal mRNA clearance facilitates zebrafish maternal-to-zygotic transition. Nature 542: 475-478