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Global characterization of the oocyte-to-embryo transition in C. elegans uncovers a novel mRNA clearance mechanism

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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. The original formatting of letters and referee reports may not be reflected in this compilation.)

Editor: Thomas Schwarz-Romond

1st Editorial Decision 19 May 2014

Thank you very much for the opportunity to consider your paper on the oocyte-to-embryo transition in C. elegans that unravels a putative novel mRNA clearance for publication in The EMBO Journal.

The comments (copied for your information below) reveal technical merits and general interest. You will easily recognize that both refs#1 and #3 suggests further reaching functional experiments. While certainly valuable to have, we recognize the challenging /partly time-consuming nature of such demands. While NOT essential at this point to wrap-up this already data-rich study, I strongly encourage careful consideration of the straightforward suggestions particularly from ref#3. I am certain you are actively pursuing such crucial experiments.

We would therefore be delighted if at least initial results along these functional lines would become shortly available and could be integrated into a revised version of the paper as to substantiate the significant implications from your study.

Overall, I am delighted to formally invite a revised manuscript for eventual publication at The EMBO Journal.

I am happy to discuss feasibility, amount and timeline of your revision, particularly also with regard to coordinating print-publication with your recently accepted, related study at The EMBO Journal.

REFEREE REPORTS

Referee #1:

The manuscript by Stoeckius et al. is an interesting piece of work that nicely complements the paternal contribution manuscript that is also under review at EMBO. They are still sufficiently distinct that they warrants a separate submissions.
The key finding described in this work is that at the oocyte-to-embryo transition (OET) there are substantial changes in both RNA and protein levels. In particular, the authors go on to identify a 3'-UTR motif (polyC) that can trigger decay upon OET, and they identify proteins that bind to these motifs. This is however, also where the main problem lies with this manuscript: the correlation between these polyC binding proteins, the polyC motif and destabilization is not explored functionally. Mutants in these genes should be used to address the functionality of these factors in OET, and/or polyC tracks should be removed from endogenous genes using crispr technology (I would think that at least some of the genes that have such a motif would have a suitable crispr site close by, enabling excision of the stretch). These are significant experiments (time-wise), but without them, only a correlation remains, in combination with a resource-like data-set, that to my taste would not fit well in EMBO J. This also relates to the issue that at no point the authors show that polyC stretch actually stabilizes (also see below). It could also correlate with higher expression.

Other concerns/remarks:
-on page 6, top paragraph, the authors do not mention paternal contribution, while this is pushed hard in the other manuscript. I do not understand this.
-Page 7: 'This indicates that genes containing a polyC motif are specifically stabilized during oogenesis...'. I do not follow this. These genes can also just be more strongly expressed, right?
-Page 7: protruding vulva does not need to be a germline-related defect. In fact, most often it is not.
-Please check the manuscript carefully for typos and references to figure panels (for example, page 13: Supplemental Figure 3E should be 3D and '...which in turn leads to destabilization of its BOUND mRNAs...'
-Figure 3: how comparable is the baseline expression between the compared transgenes? Even though these are single copy insertions into defined sites, germline expression from these insertions can vary quite a bit. This could result in artificially strong down-regulation of relatively weakly expressed transgenes.
-Figure 5B needs better explanation in the legends (these tend to be very short overall). Same for Figure S3A.
-Figure S4C (legend) should be S4B).

Referee #2:

This is a masterful RNA sequencing and proteomic characterization of the fine resolution time series of developmental events in C. elegans from oocyte to one cell to two celled embryo. It is careful and complete and the exposition of the very complex full genome analysis can be understood even by a non genomicist----it does not get stuck in the usual statistical purgatory of most genomics papers. The authors focus on the most highly down-regulated (but also nicely on the strange transcripts that are up regulated) transcripts and find a C rich motif that explains a lot. They prove that the C rich domain is sufficient to impart the same down regulation on an unrelated gene. So the paper goes from description to mechanism nicely. It is a very fine paper and needs zero modification. That is very rare in my reviewer experience.

Referee #3:

An excellent study on OET in C. elegans. The authors revisited this critical developmental transition with innovative technologies, appropriate statistical analyses, and rigorous interpretation. A technical tour-de-force in fact, in the preparation of the biological samples, and the care taken in validation.

This reviewer does not think that extensive revision experiments are necessary for publication, however one or the other of the additional experiments outlined below would substantiate the findings significantly.

Our main criticism on this manuscript is that important new findings presented are not pursued to their full potential, even though the tools to do so are available. This is the case both on the role of the poly-C-binding proteins, and on the unveiled implication of endogenous siRNAs in OET.

First, in the case of poly-C-binding elements, an in vivo cross-link followed by immunoprecipitation and qRT-PCR at least on some of the candidate mRNA targets, would demonstrate direct involvement.

Second, 22G endogenous siRNA defective mutant alleles (drh-3 hypomorphic alleles, or composite wago mutants etc), are available, and would be invaluable in implicating this pathway in OET, or at least confirm the author's model that they contribute in the clearance of some of the maternal mRNAs.

On a side-note, this reviewer appreciated the extent and the care taken in describing the materials and methods, something rarely seen nowadays.

1st Revision - authors' response 03 June 2014

Summary

We thank the referees for appreciating that our manuscript "is an interesting piece of work that nicely complements the paternal contribution manuscript also under review (referee 1)", "is a masterful RNA sequencing and proteomic characterization of the fine resolution time series of developmental events in C. elegans from oocyte to one cell to two celled embryo.…(referee 2), ".. goes from description to mechanism nicely. It is a very fine paper and needs zero modification (referee 2)", is an "excellent study on OET in *C. elegans*. (referee 3)".

We have inserted an additional reporter experiment (new Figure 3D) which backs up our main mechanistic insight: the polyC motif is not only functionally required for the thousands of mRNAs degraded during the OET, but also sufficient to degrade a mRNA during the OET. In this experiment, insertion of the polyC motif (but not of a mutated control motif) into the WT 3' UTR of the mes-2 mRNA (which is normally not degraded during the OET) induced 16-fold degradation during the OET.

We have thus (1) discovered that thousands of mRNAs are specifically degraded during the OET (2) identified a novel *cis*-regulatory motif (the polyC) which is mechanistically required *and sufficient* to explain this degradation (3) identified conserved RNA binding proteins which specifically bind the motif. Further mechanistic insights are topic of extensive follow up projects.

Point-by-point response

Referee #1:

The manuscript by Stoeckius et al. is an interesting piece of work that nicely complements the paternal contribution manuscript that is also under review at EMBO. They are still sufficiently distinct that they warrants a separate submissions.
The key finding described in this work is that at the oocyte-to-embryo transition (OET) there are substantial changes in both RNA and protein levels. In particular, the authors go on to identify a 3'-UTR motif (polyC) that can trigger decay upon OET, and they identify proteins that bind to these motifs. This is however, also where the main problem lies with this manuscript: the correlation between these polyC binding proteins, the polyC motif and destabilization is not explored functionally. Mutants in these genes should be used to address the functionality of these factors in OET, and/or polyC tracks should be removed from endogenous genes using crispr technology (I would think that at least some of the genes that have such a motif would have a suitable crispr site close by, enabling excision of the stretch). These are significant experiments (time-wise), but without

them, only a correlation remains, in combination with a resource-like data-set, that to my taste would not fit well in EMBO J. This also relates to the issue that at no point the authors show that polyC stretch actually stabilizes (also see below). It could also correlate with higher expression.

We agree with the reviewer that we do not exhaustively explore the function of the polyC motif and its cognate binding proteins. We have already stated this at the submission of the paper in the cover letter. We have performed single, double and triple RNAi experiments of the candidate proteins but failed to observe altered expression of the polyC motif containing genes. We allude to this in the discussion of the text where we postulate that we most likely do not achieve sufficient knockdown of all candidates or miss a potential cofactor.

However, taking wealth of the data and analysis in the manuscript into account, an in depth functional characterization of the motif and its binding proteins would be beyond the scope of this manuscript. This is part of ongoing follow up projects in the lab.

Other concerns/remarks:
-on page 6, top paragraph, the authors do not mention paternal contribution, while this is pushed hard in the other manuscript. I do not understand this.

This was an unfortunate mistake that happened when we split the original draft (which contained both manuscripts) into two manuscripts. We added one sentence to the discussion of the results in the manuscript that refers to the paternal RNA manuscript.

-Page 7: 'This indicates that genes containing a polyC motif are specifically stabilized during oogenesis...'. I do not follow this. These genes can also just be more strongly expressed, right?

We agree with the reviewer that polyC motif containing genes could be also among the most strongly expressed genes in the oocytes. In fact, we show in Supplementary Figure S5A that motif-containing genes are highly expressed in oocytes. Since oocytes are transcriptionally silent, this higher expression must occur early during oogenesis. A possible role of the polyC motif in this higher expression cannot be excluded. Although, when we knock-in the polyC motif into a gene that does not contain the motif we do not see a trend towards higher expression of the reporter with the motif compared to reporters with a mutated variant of the motif (revised Supplementary Figure S5C).

However, the crucial point is that according to our data it is not the high expression in the oocytes but rather that the polyC motif is ***sufficient*** to mediate down-regulation of mRNAs during the OET. In the new version of the manuscript (revised Figure 3D) **we demonstrate this effect with an additional knock-in reporter**: the reporter with the 3' UTR of *mes-2*, a mRNA that is not down-regulated during the OET, is after insertion of a polyC motif over 16-fold down-regulated in the embryo. Insertion of a mutated polyC motif does not induce degradation. Since the polyC motif is largely absent in mRNAs, which are not degraded during the OET, our data suggest that the polyC motif emerged in highly expressed oocyte genes to mark them for degradation upon fertilization.

Since our RNAi experiments against our PCBP homologs did not show a strong effect, perhaps due to insufficient knockdown or redundancy of these RNA binding proteins, we could not determine the precise mechanism of this down-regulation. Two possibilities remain: (1) the polyC motif stabilizes mRNas in the oocyte by a RBP which is degraded during the OET (2) during the OET, the polyC motif is bound by a RBP which induces degradation. Scenario (1) is favored since we identified conserved RBPs (PCBPs) which specifically bind the polyC motif in *C. elegans*. As we show in Fig. 4C, several of the PCBPs homologues are strongly down regulated during the OET. Moreover, since human PCBPs are believed to stabilize target expression (Liebhaber lab, cited in the manuscript) we favor scenario (1). We now discuss these issues more clearly in the results and discussion of the revised manuscript.

-Page 7: protruding vulva does not need to be a germline-related defect. In fact, most often it is not.

We agree with the reviewer that vulval phenotypes do not need to be germline-related, however, it was interesting to us that we observe an enrichment of these phenotypes and GO terms in polyC motif containing genes. This can potentially argue for the function of a subset of polyC motif

containing genes outside of the germline. Along these lines, PES-4, one of the proteins that binds the polyC motif *in vitro* is seemingly also expressed outside of the germline.
In any case, we stress the maternal sterile and embryonic lethal phenotype enrichment in the revised version of the manuscript.

-Please check the manuscript carefully for typos and references to figure panels (for example, page 13: Supplemental Figure 3E should be 3D and '...which in turn leads to destabilization of its BOUND mRNAs...'

We thank the reviewer for the comment. We corrected all typos in the revised version of the manuscript.

-Figure 3: how comparable is the baseline expression between the compared transgenes? Even though these are single copy insertions into defined sites, germline expression from these insertions can vary quite a bit. This could result in artificially strong down-regulation of relatively weakly expressed transgenes.

When we compare the expression of the polyC knock-in reporters in oocytes relative to highly expressed housekeeping genes that we use as normalizers, we do not observe that reporters with the polyC motif are more highly expressed than those reporters that contain a mutated variant. We have plotted this in the Supplementary Figure 5C of the revised version of the manuscript.

We actually agree with the reviewer that single copy insertions can have varying expression in the germline and that this can be problematic when comparing different insertion lines. However, in the manuscript we compare the expression change of the reporter mRNA between oocytes and zygotes **within** a reporter strain, and **not between** different reporter strains. Insertion of the polyC motif leads to down-regulation of the reporter mRNA at the OET and this is not observed when inserting a mutated variant of the motif.

-Figure 5B needs better explanation in the legends (these tend to be very short overall). Same for Figure S3A.
-Figure S4C (legend) should be S4B).

We thank the reviewer for the comment. We corrected this in the revised version of the manuscript and improved explanation of the figure legends in general.

Referee #2:

This is a masterful RNA sequencing and proteomic characterization of the fine resolution time series of developmental events in C. elegans from oocyte to one cell to two celled embryo. It is careful and complete and the exposition of the very complex full genome analysis can be understood even by a non genomicist----it does not get stuck in the usual statistical purgatory of most genomics papers. The authors focus on the most highly down-regulated (but also nicely on the strange transcripts that are up regulated) transcripts and find a C rich motif that explains a lot. They prove that the C rich domain is sufficient to impart the same down regulation on an unrelated gene. So the paper goes from description to mechanism nicely. It is a very fine paper and needs zero modification. That is very rare in my reviewer experience.

We thank the reviewer for acknowledging our work.

Referee #3:

An excellent study on OET in C. elegans. The authors revisited this critical developmental transition with innovative technologies, appropriate statistical analyses, and rigorous interpretation. A technical tour-de-force in fact, in the preparation of the biological samples, and the care taken in validation.

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however one or the other of the additional experiments outlined below would substantiate the findings significantly.

Our main criticism on this manuscript is that important new findings presented are not pursued to their full potential, even though the tools to do so are available. This is the case both on the role of the poly-C-binding proteins, and on the unveiled implication of endogenous siRNAs in OET.

First, in the case of poly-C-binding elements, an in vivo cross-link followed by immunoprecipitation and qRT-PCR at least on some of the candidate mRNA targets, would demonstrate direct involvement.

We thank the reviewer for the comment. As discussed in the comment to reviewer 1, we agree and are aware of the fact that the manuscript does not contain a full mechanistic analysis of the polyC motif and the role of the PCBPs. However, we think that this is beyond the scope of the manuscript. We politely note that this is, to our knowledge, (1) the first report of a wave of mRNA destruction after fertilization prior to zygotic genome activation, (2) the first report of a specific *cis*-regulatory motif whose presence in 3' UTRs tags these mRNAs for destruction, and (3) provides RBP candidates which specifically bind this motif.

Our attempts in double, triple and quadruple RNAi of putative polyC binding proteins have failed to alter polyC motif containing gene expression at the OET. The proposed crosslinking and IP experiment is currently difficult due to lack of antibodies recognizing the putative *C. elegans* polyC binding proteins.

Second, 22G endogenous siRNA defective mutant alleles (drh-3 hypomorphic alleles, or composite wago mutants etc), are available, and would be invaluable in implicating this pathway in OET, or at least confirm the author's model that they contribute in the clearance of some of the maternal mRNAs.

We agree with the reviewer that we do not extensively explore the role of small RNAs at the OET. However, our main conclusion is that we do not see an indication for miRNAs directed regulation and that endo-siRNAs only play a minor role at mRNA clearance at this transition.
The proposed experiment is possible, but likely difficult to interpret because endo-siRNA have several functions during development including chromosome segregation. Moreover, small RNA biogenesis pathway mutants are often sterile or embryonic lethal.

The manuscript mainly focuses on the polyC directed destruction of thousands of mRNAs.

On a side-note, this reviewer appreciated the extent and the care taken in describing the materials and methods, something rarely seen nowadays.

We thank the reviewer for acknowledging our Methods section.

End of point-by-point response.

