nature research

Peer Review Information

Journal: Nature Cell Biology
Manuscript Title: Membrane-associated cytoplasmic granules carrying the Argonaute protein WAGO-3 enable paternal epigenetic inheritance in Caenorhabditis elegans
Corresponding author name(s): Professor René Ketting

Reviewer Comments & Decisions:

Decision Letter, initial version:

Subject: Decision on Nature Cell Biology submission NCB-K46345-T
Message:
*Please delete the link to your author homepage if you wish to forward this email to co-authors.

Dear Professor Ketting,

Your manuscript, "Paternal epigenetic inheritance through sperm cytoplasm", has now been seen by 3 referees, who are experts in epigenetic inheritance and c.elegans (referees 1 and 3) and granules in germ cells (referee 2). As you will see from their comments (attached below) they find this work of potential interest, but have raised substantial concerns, which in our view would need to be addressed with considerable revisions before we can consider publication in Nature Cell Biology.

Nature Cell Biology editors discuss the referee reports in detail within the editorial team, including the chief editor, to identify key referee points that should be addressed with priority, and requests that are overruled as being beyond the scope of the current study. To guide the scope of the revisions, I have listed these points below. We are committed to providing a fair and constructive peer-review process, so please feel free to contact me if you would like to discuss any of the referee comments further.

In particular, it would be essential to:

a) use a more standard assay such as RNAi heritance, rather than the complex genetic assay in fig 2, to demonstrate PEI granules are essential for epigenetic inheritance via the male germline, as noted by referee 2:

The weakest evidence is that PEI granules are essential for epigenetic inheritance via the male germline. This was shown primarily using a complex genetic assay (Fig. 2) that is not explained in sufficient detail (see point 6 below).

6. Line 119 – please define MIS phenotype and explain experiment shown in Fig. 2 in more detail. Stochastic silencing occurs in prg-1 animals able to generate sRNAs AND that are born with no small RNA memory?? This is a complex premise that requires explanation for non-experts. The authors may want to explain why they chose this assay? Would it be possible to use a more standard assay (such as inheritance of exogenous RNAi) to support the idea that PEI-1 mutants cannot transmit silencing via the male germline??

b) test whether mutations in pei-2 are also defective in epigenetic inheritance via sperm, as noted by referee 2:

The authors may also want to consider testing whether mutations in pei-2 are also defective in epigenetic inheritance via sperm, since this would provide more direct evidence that segregation of the granules into sperm is essential.

16. pei-2 mutants are fertile and could be tested for paternal inheritance? The prediction is that they should be defective, and this would reinforce the notion that segregation of PEI condensates to sperm is required for epigenetic inheritance via the male germline.

c) All other referee concerns pertaining to strengthening existing data, providing controls, methodological details, clarifications and textual changes, should also be addressed.

d) Finally please pay close attention to our guidelines on statistical and methodological reporting (listed below) as failure to do so may delay the reconsideration of the revised manuscript. In particular please provide:

- a Supplementary Figure including unprocessed images of all gels/blots in the form of a multi-page pdf file. Please ensure that blots/gels are labeled and the sections presented in the figures are clearly indicated.

- a Supplementary Table including all numerical source data in Excel format, with data for different figures provided as different sheets within a single Excel file. The file should include source data giving rise to graphical representations and statistical descriptions in the paper and for all instances where the figures present representative experiments of multiple independent repeats, the source data of all repeats should be provided.

We would be happy to consider a revised manuscript that would satisfactorily address these points, unless a similar paper is published elsewhere, or is accepted for publication in Nature Cell Biology in the meantime.

When revising the manuscript please:

- ensure that it conforms to our format instructions and publication policies (see below and https://www.nature.com/nature/for-authors).

- provide a point-by-point rebuttal to the full referee reports verbatim, as provided at the end of this letter.

- provide the completed Reporting Summary (found here https://www.nature.com/documents/nr-reporting-summary.pdf). This is essential for reconsideration of the manuscript will be available to editors and referees in the event of peer review. For more information see http://www.nature.com/authors/policies/availability.html or contact me.

When submitting the revised version of your manuscript, please pay close attention to our href="https://www.nature.com/nature-research/editorial-policies/image-integrity">Digital Image Integrity Guidelines. and to the following points below:

-- that unprocessed scans are clearly labelled and match the gels and western blots presented in figures.
-- that control panels for gels and western blots are appropriately described as loading on sample processing controls
-- all images in the paper are checked for duplication of panels and for splicing of gel lanes.

Finally, please ensure that you retain unprocessed data and metadata files after publication, ideally archiving data in perpetuity, as these may be requested during the peer review and production process or after publication if any issues arise.

Nature Cell Biology is committed to improving transparency in authorship. As part of our efforts in this direction, we are now requesting that all authors identified as 'corresponding author' on published papers create and link their Open Researcher and Contributor Identifier (ORCID) with their account on the Manuscript Tracking System (MTS), prior to acceptance. ORCID helps the scientific community achieve unambiguous attribution of all scholarly contributions. You can create and link your ORCID from

the home page of the MTS by clicking on 'Modify my Springer Nature account'. For more information please visit please visit www.springernature.com/orcid.

This journal strongly supports public availability of data. Please place the data used in your paper into a public data repository, or alternatively, present the data as Supplementary Information. If data can only be shared on request, please explain why in your Data Availability Statement, and also in the correspondence with your editor. Please note that for some data types, deposition in a public repository is mandatory - more information on our data deposition policies and available repositories appears below.

Please submit the revised manuscript files and the point-by-point rebuttal to the referee comments using this link:

[REDACTED]

*This url links to your confidential home page and associated information about manuscripts you may have submitted or be reviewing for us. If you wish to forward this email to co-authors, please delete the link to your homepage.

We would like to receive a revised submission within six months.

We hope that you will find our referees' comments, and editorial guidance helpful. Please do not hesitate to contact me if there is anything you would like to discuss.

Best wishes,

Jie Wang

Jie Wang, PhD
Senior Editor
Nature Cell Biology

Tel: +44 (0) 207 843 4924
email: jie.wang@nature.com

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:

The authors report the identification of the PEI-1/2 complex that retains WAGO-3 22G small RNAs in C. elegans sperm. The paternal epigenetic inheritance of these small RNAs is required for germline immortality.

Overall I found this an interesting study because how (specific) smallRNAs are retained in sperm is very poorly understood.

Comments

The authors refer to PEI granules as sperm-specific 'condensates'. But are they condensates, aggregates or membrane-localised complexes? The presented data suggest they don't have liquid-like properties. But what are they? I think a bit more clarity on this and more precise use of language would be interesting and also help to avoid confusion.

"PEI-like proteins are also expressed during human spermatogenesis, suggesting that the here identified mechanism of subcellular organization may be broadly conserved."
This is an interesting discussion point but I think it is a bit out of place in the abstract.

"show that segregation of PEI granules is coupled to the myosin-driven transport of sperm-specific secretory vesicles via S-palmitoylation of PEI-2"
This is a strong statement - does the authors' data actually show that it is S-palmitoylation of PEI-2 that guides transport?

Reviewer #2:
Remarks to the Author:
This manuscript describes a new cytoplasmic condensate that mediates paternal epigenetic inheritance in C. elegans. The study is very thorough and reports on:
1) the proteins responsible for condensate assembly ("scaffolds") and the key Argonaute required for paternal epigenetic inheritance ("client")
2) the protein domains required for condensate assembly and client recruitment
3) a novel mechanism involving palmitoylation that mediates segregation of the new condensates with membranous organelles in sperm

Overall, I find this story extremely novel and compelling. Epigenetic inheritance via maternal transmission of cytoplasmic condensates that contain Argonautes has been well documented. This study presents evidence for a parallel mechanism operating in sperm and is sure to generate lots of interest. The proteins that scaffold the new sperm granules may be conserved (Fig. S11); thus, this study is likely to have a broad impact beyond C. elegans. The data are of high quality and sufficient to support most major claims. The weakest evidence is that PEI granules are essential for epigenetic inheritance via the male germline. This was shown primarily using a complex genetic assay (Fig. 2) that is not explained in sufficient detail (see point 6 below). The authors may also want to consider testing whether mutations in pei-2 are also defective in epigenetic inheritance via sperm, since this would provide more direct evidence that segregation of the granules into sperm is essential.

Specific comments:

1. There are several abbreviations throughout the manuscript which are unnecessary and confusing for the non-expert. For example, paternal epigenetic inheritance should be spelled out to avoid confusion with gene names etc…
2. Line 68: the text refers to a comparison between wt and wago-3 mutant, but the figure compares prg-1 vs prg-1;wago-3. The use of prg-1 is not explained. The percent reactivation is very low and given the low N (50), it is not clear whether these results are significant??? It would be preferable to replace bar graphs with scatter plots that distinguish between experimental replicates.
3. Lines- 80-89: The characterization of WAGO-3-bound small RNAs as targeting primarily sperm transcripts and transposable elements does not seem to match with figure S3b (most transcripts are gender neutral) and figure S3e (most transcripts are protein coding).
4. Mass spec data??
5. WAGO-3 IP in Fig. S4d should show H3 control in the FLAG-IP samples to demonstrate the specificity of the IP (H3 does not come down).
6. Line 119 – please define MIS phenotype and explain experiment shown in Fig. 2 in more detail. Stochastic silencing occurs in prg-1 animals able to generate sRNAs AND that are born with no small RNA memory?? This is a complex premise that requires explanation for non-experts. The authors may want to explain why they chose this assay? Would it be possible to use a more standard assay (such as inheritance of exogenous RNAi) to support the idea that PEI-1 mutants cannot transmit silencing via the male germline??
7. Fig. 3b and h-n Please use same names to refer to various deletions
8. Fig. 3c what is OK1050
9. What do the small letters in Fig. 3c-f mean – groups of data that are not statistically different???

10. Fig. 3d Why does PEI-1 missing both the BACK and IDR domains colocalize with WAGO-3 in residual body???
11. Fig. 3g – not sure what is the take home message here?

12. Fig. S10A : why is PEI-2 not immunoprecipitated by WAGO-3??
13. Please show domain organization of PEI-2
14. Line 205 “Large PEI-1 deletion” be more specific
15. The analysis of PEI-1/PEI-2 modifications is confusing as written – perhaps it would be best to present the PEI-2 data first since PIE-2 is the one most likely to be palmitoylated since spe-10 dependent.
16. pei-2 mutants are fertile and could be tested for paternal inheritance? The prediction is that they should be defective, and this would reinforce the notion that segregation of PEI condensates to sperm is required for epigenetic inheritance via the male germline.
17. Model figure: Are PEI condensates visible in newly fertilized embryos? The scale of sperm and oocyte does not seem accurate.

Reviewer #3:
Remarks to the Author:
I already read this manuscript when it was pre-printed, and think that it's an excellent contribution to the field which illuminates many aspects of small RNA inheritance that were not clear before. I don't have too many comments (although I read it very carefully, a few times!) because it's just a very good paper and I don't think they need to add/edit much (barely anything) for it to be published. The claims are supported by the experiments and they did a lot of work validating their results in many different ways.

Briefly, they found a mechanism that explains how the sperm can affect the mortal germline and Mutator-induced sterility (heritable) phenotypes (small RNA-related phenomena), despite the fact that in spermatogenesis much of the cellular content is extruded. They found that one argonaute survives the cytoplasmic reduction that occurs in spermatogenes (how it avoids getting to the residual body), unlike other Agos. They describe a new granule in the sperm (a very novel discovery!) and, unlike typical C. elegans papers about inheritance, combine also biochemistry (with elegant genetics) to really understand how it works, how the granule get segregated properly to the mature sperm (the paper also uses advanced beautiful microscopy and different measurements, for example to show that this granule could be more stable than other liquid-like granules). So again, just to summarise: a really great paper that I would LOVE to see published in NCB and that is sure to excite many!

I have just three comments/requests:

1) It would be nice to speculate more in the discussion about how the PEI granules might be selecting their clients. That's challenging to find experimentally and could be done in the future, but it would be nice to discuss their ideas.

2) In my opinion (and i think it's an important point), the title of the pre-print reflected better their discoveries: "A membrane-associated condensate drives paternal epigenetic inheritance in C. elegans". The current title "Paternal epigenetic inheritance through sperm cytoplasm" is less accurate and could be confusing. They find a mechanism for getting an Ago (WAGO-3) to the mature sperm, but the inheritance itself, to the next generation, could involve other cellular compartments. A previous paper by Shouhong Guang's lab had a better title for describing the involvement of a cytoplasmic Argonaute in epigenetic inheritance ("A Cytoplasmic Argonaute Protein Promotes the Inheritance of RNAi", it's subtle, "promotes" instead of "through"). Perhaps the authors could have a title that focuses on the fact that they found a new granule, the cytoplamic Ago that it carries, and the observation that it is required for inheritance of the Mutator-induced sterility phenotype via the sperm, for example "Paternal epigenetic inheritance is required for fertility and enabled by a novel gel-like cytoplasmic granule, which carries the Argonaute WAGO-3". I'm not saying this is the best wording, it's too long, but it captures what the paper shows more accurately.

3. The paper is very well written and flows nicely, it would be perfect if they could just use less acronyms (for example EI in the abstract) or at least remove them from the titles of the paragraphs (for example: "PEI-1 recruits WAGO-3 to PEI granules through its IDR")

Oded Rechavi

Author Rebuttal to Initial comments

We kindly thank all the reviewers for their constructive criticism, and of course we were very happy to see the overall enthusiasm for our manuscript. We will address the various issues point-by-point:

Reviewer 1:
Remarks to the Author:

The authors report the identification of the PEI-1/2 complex that retains WAGO-3 22G small RNAs in C. elegans sperm. The paternal epigenetic inheritance of these small RNAs is required for germline immortality.
Overall I found this an interesting study because how (specific) small RNAs are retained in sperm is very poorly understood.

The positive feedback is highly appreciated.

The authors refer to PEI granules as sperm-specific 'condensates'. But are they condensates, aggregates or membrane-localised complexes? The presented data suggest they don't have liquid-like properties. But what are they? I think a bit more clarity on this and more precise use of language would be interesting and also help to avoid confusion.

This is a good point, but also one that is hard to resolve. The reviewer is right that we do not show properties typically associated with condensates, such as liquid-like behavior. We do show FRAP, but this does not prove a status as 'condensate'. We have removed the term 'condensate when we specifically talk about PEI granules, and instead refer to the foci as granules. We added a sentence to the discussion to point the reader that this is an unresolved topic:

"We note, that the material state of the PEI granules has not been clarified. To examine if PEI granules have liquid character, are more gel-like or represent some other form of complex, experiments with purified proteins will be required."

"PEI-like proteins are also expressed during human spermatogenesis, suggesting that the here identified mechanism of subcellular organization may be broadly conserved."
This is an interesting discussion point but I think it is a bit out of place in the abstract.

We would in principle be happy to remove that statement, but we think that this sentence may attract a broader audience to read the paper, so we prefer to leave it in. However, we shortened the phrasing:

"PEI-like proteins are found in human, suggesting that the identified mechanism may be conserved."

"show that segregation of PEI granules is coupled to the myosin-driven transport of sperm-specific secretory vesicles via S-palmitoylation of PEI-2"
This is a strong statement - does the authors' data actually show that it is S-palmitoylation of PEI-2 that guides transport?

We have changed the sentence to:

"We further show that PEI granule segregation is coupled to transport of sperm-specific secretory vesicles via PEI-2, in an S-palmitoylation dependent manner."
Reviewer #2:
Remarks to the Author:
This manuscript describes a new cytoplasmic condensate that mediates paternal epigenetic inheritance in C. elegans. The study is very thorough and reports on:
1) the proteins responsible for condensate assembly ("scaffolds") and the key Argonaute required for paternal epigenetic inheritance ("client")

2) the protein domains required for condensate assembly and client recruitment
3) a novel mechanism involving palmitoylation that mediates segregation of the new condensates with membranous organelles in sperm
Overall, I find this story extremely novel and compelling. Epigenetic inheritance via maternal transmission of cytoplasmic condensates that contain Argonautes has been well documented. This study presents evidence for a parallel mechanism operating in sperm and is sure to generate lots of interest. The proteins that scaffold the new sperm granules may be conserved (Fig. S11); thus, this study is likely to have a broad impact beyond C. elegans. The data are of high quality and sufficient to support most major claims. The weakest evidence is that PEI granules are essential for epigenetic inheritance via the male germline. This was shown primarily using a complex genetic assay (Fig. 2) that is not explained in sufficient detail (see point 6 below). The authors may also want to consider testing whether mutations in pei-2 are also defective in epigenetic inheritance via sperm, since this would provide more direct evidence that segregation of the granules into sperm is essential.

Thank you for supporting our work and for the constructive comments. The issues of the inheritance assay and pei-2 mutants will be addressed at points 6 and 16 below, respectively.

Specific comments:

1. There are several abbreviations throughout the manuscript which are unnecessary and confusing for the non-expert. For example, paternal epigenetic inheritance should be spelled out to avoid confusion with gene names etc…

We have removed these as much as possible (also see reply to reviewer 3). The term PEI is now only used for gene and granule names. We also removed acronyms from section titles.

2. Line 68: the text refers to a comparison between wt and wago-3 mutant, but the figure compares prg-1 vs prg-1;wago-3. The use of prg-1 is not explained. The percent reactivation is very low and given the low N (50), it is not clear whether these results are significant??? It would be preferable to replace bar graphs with scatter plots that distinguish between experimental replicates.

This paragraph has been edited to explain the presence of prg-1 mutations:

“Mutants defective for epigenetic inheritance, such as hrde-1, often show a mortal germline phenotype (Mrt) 1, implying that fertility decreases over subsequent generations. We found that wago-3 mutants display a Mrt phenotype (Extended Data Fig. 1a), suggesting a role for WAGO-3 in epigenetic inheritance. To test this, we analyzed if WAGO-3 affects the heritability of a germline-specific mCherry::H2B transgene silenced RNAe 21-23,30–32. The RNAe status implies that the silencing was induced by PRG-1, but afterwards maintained in a prg-1 mutant background 21,23. Therefore, we

assessed the RNAe-associated silencing in prg-1 mutant strains. We found that wago-3;prg-1 double mutants, but not prg-1 single mutants, stochastically lost silencing (Extended Data Fig. 1b-c). As expected 21-23, mut-7;prg-1 double mutants directly lost all silencing (Extended Data Fig. 1b-c). These data show that WAGO-3 has a role specifically in the inheritance of RNAe-related silencing. ”

Regarding the statistical analysis of these results, we note the following: For each generation 10 plates, seeded with a single parent animal were started and 50 offspring were scored for activation of the transgene. Hence, the numbers are larger than appeared from the way we presented the data. We now present the data in a different manner in Extended Data Figure 1b, in order to clarify this. Statistical analysis is not applicable in this case, as the variability in the control groups is zero.

3. Lines- 80-89: The characterization of WAGO-3-bound small RNAs as targeting primarily sperm transcripts and transposable elements does not seem to match with figure S3b (most transcripts are gender neutral) and figure S3e (most transcripts are protein coding).

This issue likely stems from a mis-conception. We do not write that primarily transposons are targeted. The text reads as follows:

“In both sexes, many transposable elements were targeted by WAGO-3 (Extended Data Fig. 3c,e), including Tc1, consistent with WAGO-3’s role in Tc1 silencing 35,36. Interestingly, Tc3-derived 22G RNAs were consistently depleted from WAGO-3 in males (Extended Data Fig. 3c), suggesting sex-specific regulation of this element.”

Hence, we indicate that most transposons are targeted, which is not the same as stating that transposons are the primary targets of WAGO-3.

Similarly, concerning sperm transcripts we wrote:

“we found a significant overlap between WAGO-3 targets and previously determined sperm-derived 22G RNA targets 37 (Extended Data Fig. 3f), suggesting that WAGO-3 is present in sperm.”

This implies that 22G RNAs that have been identified in sperm are indeed almost all enriched in WAGO-3 IPs. Again, this does not mean that they represent most of the WAGO-3-bound 22G RNA pool. Indeed, the WAGO-3 pool is much larger, and likely rather complex due to the fact that WAGO-3 is expressed at all stages of germ cell development. At the same time, the term “sperm transcripts” only reflects the fact that they are found in sperm. They could still also be found in any other tissue and be gender neutral. Explicitly stating these things is something that would require too much space, so we hope that this rebuttal sufficiently addresses this fact. We did address the protein-coding issue textually by writing:

"Many of these are protein-coding transcripts that are known Mutator targets (Phillips et al., 2012), while their overlap with CSR-1 targets is minor (Claycomb et al., 2009) (Extended Data Fig. 3d)."

4. Mass spec data??

All mass spec data is provided in a repository, as indicated in the section "Data Availability".

5. WAGO-3 IP in Fig. S4d should show H3 control in the FLAG-IP samples to demonstrate the specificity of the IP (H3 does not come down).

We apologize for the omission. We have added these controls.

6. Line 119 – please define MIS phenotype and explain experiment shown in Fig. 2 in more detail. Stochastic silencing occurs in prg-1 animals able to generate sRNAs AND that are born with no small RNA memory?? This is a complex premise that requires explanation for non-experts. The authors may want to explain why they chose this assay? Would it be possible to use a more standard assay (such as inheritance of exogenous RNAi) to support the idea that PEI-1 mutants cannot transmit silencing via the male germline??

As discussed over e-mail (we append the letter we wrote to address this below), we have opted to explain the used experiment in more detail. We adapted one paragraph in the introduction and one paragraph in the results section to explain the system in more detail.

Introduction paragraph:

"Small RNAs, most notably short-interfering RNAs (siRNAs) and Piwi-interacting RNAs (piRNAs) 2, have been implicated in epigenetic inheritance. These molecules act as sequence-specific guides for Argonaute proteins, which in turn regulate gene expression 5–7. In the nematode C. elegans, siRNAs with an established role in epigenetic inheritance are the 22G RNAs 8–13. These are made in a process driven by the Mutator proteins MUT-16 and MUT-7 14–17, and are bound by members of the worm-specific Argonaute (WAGO) family, such as the cytoplasmic WAGO-4 and the nuclear HRDE-1 proteins 16–19, both of which have been implicated in maternal epigenetic inheritance 9,12,20. Also the Piwi protein PRG-1 is inherited via the oocyte, and maternal piRNAs can initiate WAGO-dependent silencing that can be inherited PRG-1-independently for many generations, in a process known as RNA-induced epigenetic silencing (RNAe) 21–23.

Interestingly, a second, Mutator-independent class of 22G RNAs also exists. These are bound by the Argonaute protein CSR-1, and are mostly derived from genes that are expressed and required in the germline 24. CSR-1-22G RNAs and Mutator-22G RNAs should not become mixed, lest important genes

become inappropriately silenced, and epigenetic inheritance plays an important role in this. When embryos have a functional Mutator system, but their parents lacked both Mutator-22G RNAs and piRNAs, the Mutator system starts to produce 22G RNAs that are normally restricted to CSR-1 10,11. As a result, WAGO proteins such as HRDE-1 are loaded with CSR-1-type 22G RNAs, leading to the silencing of CSR-1 target genes, which in turn results in sterility 10,11. This phenotype, known as Mutator-induced sterility (Mis), effectively reveals a prominent self-targeting potential of the Mutator-22G RNAs, and shows that parental 22G RNAs and piRNAs play an essential role in suppressing this dangerous auto-immune-like property of the Mutator system.
”

Results paragraph:

“The Mis phenotype 10,11 (see Introduction) allows us to probe the relevance and mechanisms of epigenetic inheritance. The precise set-up we used in the experiment is shown in Fig. 2a. We note that mut-7 and mut-16 mutants can be used interchangeably in both sexes, as they both result in Mutator system dysfunction 10,11,16. This set-up generates embryos that can make Mutator-22G RNAs. Depending on the specific cross, the mother, the father or neither parent can make Mutator-22G RNAs. All strains carry a prg-1 mutation in order to remove the partially redundant activity of inherited piRNAs in this system 10,11. Using this set-up, we found that maternal or paternal 22G RNAs were sufficient to prevent the Mis phenotype (Fig. 2b-c, top three bars), allowing us to dissect male- and female-specific contributions to the Mis phenotype. Following up on this, we probed the roles of WAGO-3 and PEI-1 in this process, and found that both PEI-1 and WAGO-3 were specifically required in the male (Fig. 2b), but not in the female (Fig. 2c). We conclude that PEI-1 and WAGO-3 play critical roles in paternal epigenetic inheritance.”

7. Fig. 3b and h-n Please use same names to refer to various deletions

This has been changed as requested.

8. Fig. 3c what is OK1050

This is the number of the pei-1 deletion allele. This has now been clarified.

9. What do the small letters in Fig. 3c-f mean – groups of data that are not statistically different???

The reviewers assumption was correct. We tried to describe this in the figure legend, but we realized the text may have been ambiguous. We re-phrased the legends as follows:

“Statistically significant differences were determined by one-way ANOVA ($p \leq 0.001$) followed by Tukey’s honestly significant difference post hoc test ($p \leq 0.05$). Different letters represent significant differences. The exact P values are provided as source data.”

10. Fig. 3d Why does PEI-1 missing both the BACK and IDR domains colocalize with WAGO-3 in residual body???

This is because both proteins are no longer present in foci but are diffuse. This leads to very good colocalization, even though it does not carry much meaning.
We changed the sentence that describes this to:

“Deletion of both the BACK and IDR domains did not further affect WAGO-3, but resulted in diffuse PEI-1 signal that accumulated in the residual body, together with WAGO-3 (Fig. 3d,m).”

11. Fig. 3g – not sure what is the take home message here?

We extracted the main result from Figure 3g, from the aggregated citation of a number of Figure panels to clarify this:

“In contrast, deletion of the PEI-1-IDR resulted in WAGO-3 which mostly localized to the residual body (Fig. 3e-f,l, Extended Data Fig. 5a-b). WAGO-3 signal was diffuse, as quantified by a loss of high-intensity pixels (Fig. 3g).”

12. Fig. S10A : why is PEI-2 not immunoprecipitated by WAGO-3??

In IPs of PEI-1 we see WAGO-3 and vice versa. Indeed, PEI-2 and WAGO-3 are not easily co-IPed under our conditions, but we note that this interaction is most likely indirect, and therefore harder to detect. We think buffer, or other experimental conditions prevent us from detecting such a secondary interaction from being maintained well enough to allow detection by our methods.

13. Please show domain organization of PEI-2

This is now shown in Figure 5g.

14. Line 205 “Large PEI-1 deletion” be more specific

Sentence changed to:

“…as visualized by the H15-Q558 PEI-1 deletion (Fig. 4a-b),…”

15. The analysis of PEI-1/PEI-2 modifications is confusing as written – perhaps it would be best to present the PEI-2 data first since PIE-2 is the one most likely to be palmitoylated since spe-10 dependent.

We have reorganized this paragraph. It now reads as follows:

“Western blotting showed a clear doublet band for PEI-2 (Fig. 6b), compatible with palmitoylation 53,54. Interestingly, the upper band of PEI-2 disappeared in spe-10 mutants (Fig. 6b), suggesting PEI-2 is a substrate of the SPE-10 enzyme. Our Western blotting also revealed evidence for PEI-1 modification, although this appears more as a smear than as a discrete band (Fig. 6c). PEI-1 modification did not depend on SPE-10 (Fig. 6c), suggesting PEI-1 may carry a different kind of modification, or that another palmitoyltransferase may act on PEI-1. Strikingly, PEI-1 and PEI-2 affected each other’s modification status: while PEI-2 was required for PEI-1 modification (Fig. 6c), PEI-1 rather inhibited PEI-2 modification (Fig. 6b). In a heterologous expression system PEI-1 and PEI-2 also appeared as doublets, and showed decreased stability upon palmitoylation inhibition (Extended Data Fig. 10h-i), similar to what has been reported for the palmitoylated protein PD-L1 55. We conclude that PEI-2 is a potential direct substrate of SPE-10, and that PEI-1 can also be modified, but only in the presence of PEI-2.”

16. pei-2 mutants are fertile and could be tested for paternal inheritance? The prediction is that they should be defective, and this would reinforce the notion that segregation of PEI condensates to sperm is required for epigenetic inheritance via the male germline.

The pei-2 mutant had been tested already in our system and showed a phenotype, as would be expected. Please see Figure 5m.

17. Model figure: Are PEI condensates visible in newly fertilized embryos? The scale of sperm and oocyte does not seem accurate.

Whether PEI granules can be seen in zygotes is something that we are still experimentally addressing. This is not easy, and may require the use of brighter tags and/or faster imaging. While extremely interesting to follow the PEI granules after fertilization, this is beyond the current scope though. The model is indeed not in scale. If drawn in scale many things would hardly be visible. This fact is now indicated in the legend to the new Figure 7.

Reviewer #3:
Remarks to the Author:
I already read this manuscript when it was pre-printed, and think that it's an excellent contribution to the field which illuminates many aspects of small RNA inheritance that were not clear before. I don't

have too many comments (although I read it very carefully, a few times!) because it's just a very good paper and I don't think they need to add/edit much (barely anything) for it to be published. The claims are supported by the experiments and they did a lot of work validating their results in many different ways.

Briefly, they found a mechanism that explains how the sperm can affect the mortal germline and Mutator-induced sterility (heritable) phenotypes (small RNA-related phenomena), despite the fact that in spermatogenesis much of the cellular content is extruded. They found that one argonaute survives the cytoplasmic reduction that occurs in spermatogenesis (how it avoids getting to the residual body), unlike other Agos. They describe a new granule in the sperm (a very novel discovery!) and, unlike typical C. elegans papers about inheritance, combine also biochemistry (with elegant genetics) to really understand how it works, how the granule get segregated properly to the mature sperm (the paper also uses advanced beautiful microscopy and different measurements, for example to show that this granule could be more stable than other liquid-like granules). So again, just to summarise: a really great paper that I would LOVE to see published in NCB and that is sure to excite many!

We thank the reviewer for the very supportive comments!

I have just three comments/requests:

1) It would be nice to speculate more in the discussion about how the PEI granules might be selecting their clients. That's challenging to find experimentally and could be done in the future, but it would be nice to discuss their ideas.

Even though we do not want to speculate too much, we have extended the first paragraph of the discussion with the following text:

“We speculate that the PEI-1 IDR may create a condensate that is selective for some feature of the WAGO-3 protein, or possibly non-permissive for certain features of depleted proteins, such as WAGO-1 and CSR-1. Such features could be sequence intrinsic, but could also relate to post-translational modifications. Further experiments, notably with purified proteins, will be required to test these ideas.”

2) In my opinion (and i think it's an important point), the title of the pre-print reflected better their discoveries: "A membrane-associated condensate drives paternal epigenetic inheritance in C. elegans". The current title "Paternal epigenetic inheritance through sperm cytoplasm" is less accurate and could be confusing. They find a mechanism for getting an Ago (WAGO-3) to the mature sperm, but the inheritance itself, to the next generation, could involve other cellular compartments. A previous paper by Shouhong Guang's lab had a better title for describing the involvement of a cytoplasmic Argonaute in epigenetic inheritance ("A Cytoplasmic Argonaute Protein Promotes the Inheritance of RNAi", it's subtle,

"promotes" instead of "through"). Perhaps the authors could have a title that focuses on the fact that they found a new granule, the cytoplasmic Ago that it carries, and the observation that it is required for inheritance of the Mutator-induced sterility phenotype via the sperm, for example "Paternal epigenetic inheritance is required for fertility and enabled by a novel gel-like cytoplasmic granule, which carries the Argonaute WAGO-3". I'm not saying this is the best wording, it's too long, but it captures what the paper shows more accurately.

We thank the reviewer for the suggestion. We agree. We have changed the title to:
“Membrane associated PEI granules enable paternal epigenetic inheritance in Caenorhabditis elegans”

3. The paper is very well written and flows nicely, it would be perfect if they could just use less acronyms (for example EI in the abstract) or at least remove them from the titles of the paragraphs (for example: "PEI-1 recruits WAGO-3 to PEI granules through its IDR")

We have removed the use of the term EI and spelled it out in full in all instances. We also removed acronyms from the titles of sections, as far as reasonable (spelling out CLEM would be too long, for instance).

Appendix: Letter sent to editor to explain the use of the complex genetic assay (Mis crosses) to score paternal epigenetic inheritance:

Dear Dr Wang,

With this letter I would like to better explain the reasons why we use the rather complex epigenetic inheritance assay described in the Schreier et al. manuscript, and how we would prefer to address the issue raised on this topic by reviewer 2.
The main reason we use this assay is that is reports on inheritance of endogenous small RNAs, and not some artificial trigger of RNAi. As such, it stays rather close to C. elegans physiology and germ cell biology. This assay is based on two papers published by us and Carolyn Philips in Dev. Cell in 2015. However, admittedly it can appear rather complex and we apparently did not explain the system well in our manuscript.

In essence, the assay reveals how parental small RNAs, in particular 22G RNAs and 21U RNAs, can prevent the accumulation of self-targeting 22G RNAs in the germline of a developing nematode. In the two 2015 papers it was shown that embryos that can themselves make 22G RNAs, but whose parents did not express 22G and 21U RNAs develop into sterile adults and that this is due to the targeting of germline-expressed genes by the Argonaute protein HRDE-1. The parental 22G and 21U RNAs act

redundantly in this aspect, and hence the specific effect of parental 22G RNAs can only be studied in mutants lacking 21U RNAs (we use a prg-1 mutation for this). In the current manuscript we use this system to show that WAGO-3, PEI-1, and also PEI-2, are required, specifically in the male, to prevent this inherent auto-immunity aspect of the 22G RNA system. Note that we did already test pei-2 mutants for inheritance defects in this assay (Figure S10m): pei-2 mutants are also defective, as would be expected. We believe that our data clearly show that the physical inheritance of 22G-loaded WAGO-3 through sperm is very important of this epigenetic inheritance system.

An important reason not to use GFP RNAi is that the typically used GFP-inheritance assays are based on a transgene that is not expressed in the male germline. Therefore, GFP-targeting 22G RNAs cannot accumulate in the male germline, and testing male inheritance in such a setting is futile. That said, we do see the advantage of having a simpler, GFP-based, male RNAi inheritance assay, and we are trying to develop an effective system, but whether this will work is still unclear; we note that triggering exogenous RNAi in males in not as robust as in hermaphrodites, and triggering RNAi by feeding does not work at all. The only established male RNAi inheritance assay uses RNAi against a gene named oma-1. However, this is also not a 'simple' RNAi assay, as it is based on a specific neomorphic allele of oma-1, which gives a dominant, lethal phenotype at elevated temperatures. This is then suppressed by RNAi. To use this, we would need to build quite some strains, and unfortunately pei-1 and oma-1 are rather closely linked. Even though it is certainly possible, I am not convinced that the rather limited gain of a simpler experiment would justify the significant time that would be required to implement this system.

All considered, I would propose that we address the identified (and well appreciated) issue by dedicating more text to its explanation (see appendix for a proposed paragraph). In case our male GFP inheritance assay starts to work in time, we will not hesitate to include that.
Finally, I would appreciate a short explanation by reviewer 2 on issue 4. Point 4 of the rebuttal just mentions "Mass spec data??". I assume that some text has gone missing here. I note that all mass spec data will be available in a repository, in case this was the issue.

Kind regards

René Ketting

Decision Letter, first revision:

Subject: NCB: Your manuscript, NCB-K46345A
Message:

Our ref: NCB-K46345A

8th November 2021

Dear Dr. Ketting,

Thank you for your patience as we've prepared the guidelines for final submission of your Nature Cell Biology manuscript, "Membrane associated PEI granules enable paternal epigenetic inheritance in Caenorhabditis elegans" (NCB-K46345A). Please carefully follow the step-by-step instructions provided in the attached file, and add a response in each row of the table to indicate the changes that you have made. Ensuring that each point is addressed will help to ensure that your revised manuscript can be swiftly handed over to our production team.

We would like to start working on your revised paper, with all of the requested files and forms, as soon as possible (preferably within one week). Please get in contact with us if you anticipate delays.

When you upload your final materials, please include a point-by-point response to any remaining reviewer comments.

If you have not done so already, please alert us to any related manuscripts from your group that are under consideration or in press at other journals, or are being written up for submission to other journals (see: https://www.nature.com/nature-research/editorial-policies/plagiarism#policy-on-duplicate-publication for details).

In recognition of the time and expertise our reviewers provide to Nature Cell Biology's editorial process, we would like to formally acknowledge their contribution to the external peer review of your manuscript entitled "Membrane associated PEI granules enable paternal epigenetic inheritance in Caenorhabditis elegans". For those reviewers who give their assent, we will be publishing their names alongside the published article.

Nature Cell Biology offers a Transparent Peer Review option for new original research manuscripts submitted after December 1st, 2019. As part of this initiative, we encourage our authors to support increased transparency into the peer review process by agreeing to have the reviewer comments, author rebuttal letters, and editorial decision letters published as a Supplementary item. When you submit your final files please clearly state in your cover letter whether or not you would like to participate in this initiative. Please note that failure to state your preference will result in delays in accepting your manuscript for publication.

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If you have any further questions, please feel free to contact me.

Best regards,

Ziqian Li
Editorial Assistant
Nature Cell Biology

On behalf of

Jie Wang, PhD
Senior Editor
Nature Cell Biology

Tel: +44 (0) 207 843 4924
email: jie.wang@nature.com

Reviewer #1:
Remarks to the Author:
The authors have addressed my comments.

Reviewer #2:
Remarks to the Author:

The authors have responded to the reviewers comments very thoroughly. The conclusions are well supported by the data and the authors did a nice job to clarify how they assayed for paternal vs maternal inheritance of small RNAs. This is an exciting and important paper describing a new condensates that mediates paternal epigenetic inheritance.

Final Decision Letter:

Subject: Decision on Nature Cell Biology submission NCB-K46345B
Message:

Dear Dr Ketting,

I am pleased to inform you that your manuscript, "Membrane-associated cytoplasmic granules carrying the Argonaute protein WAGO-3 enable paternal epigenetic inheritance in Caenorhabditis elegans", has now been accepted for publication in Nature Cell Biology.

Thank you for sending us the final manuscript files to be processed for print and online production, and for returning the manuscript checklists and other forms. Your manuscript will now be passed to our production team who will be in contact with you if there are any questions with the production quality of supplied figures and text.

Over the next few weeks, your paper will be copyedited to ensure that it conforms to Nature Cell Biology style. Once your paper is typeset, you will receive an email with a link to choose the appropriate publishing options for your paper and our Author Services team will be in touch regarding any additional information that may be required.

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Please feel free to contact us if you have any questions.

With kind regards,

Jie Wang, PhD
Senior Editor
Nature Cell Biology

Tel: +44 (0) 207 843 4924
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