C. elegans pronuclei fuse after fertilization through a novel membrane structure

Mohammad Rahman, Irene Chang, Adam Harned, Richa Maheshwari, Kwabena Amoateng, Kedar Narayan, and Orna Cohen-Fix

Corresponding Author(s): Orna Cohen-Fix, NIH/NIDDK and Kedar Narayan, CMM, CCR, NCI, NIH

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Dear Dr. Cohen-Fix,

Thank you for submitting your manuscript entitled "C. elegans pronuclei fuse after fertilization through a novel membrane structure" to the Journal of Cell Biology. We received reviews from three experts in the field, whose comments are appended below.

You will see that all referees find the results striking and praise the high quality of the structural work. Reviewer #2 comments on the limited mechanistic insights, and given Rev#2's high level of general expertise, we have taken their views very seriously. However, the work was submitted as a Report and therefore does not require the same depth of significant mechanistic insight as a full Article. Instead, the requirement is for "observations of outstanding interest that have the potential to open up new avenues of research". Both Revs #3 and #1 consider that the results meet this requirement, and we editorially fully agree. However, we feel that the reviewers make good points that should be addressed to improve the data presentation and discussion, including Rev#2's interesting discussion point, "it remains possible that these "3-way sheet junctions" actually originate as points of tubular membrane fusion, a process that occurs continuously during remodeling of the ER in interphase", which could be addressed by textual changes.

We would be happy to publish your paper in JCB pending revisions to address the reviewers' points and revisions necessary to meet our formatting guidelines (see details below).

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

1) eTOC summary: A 40-word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person.

Suggested revised eTOC: (I would suggest including the approach to stress the advance more generally)
Rahman et al analyze the first zygotic division in C. elegans embryos via focused ion beam scanning electron microscopy and identify novel membrane structures, including a 3-way sheet junction, underlying the fusion of the pronuclei containing the parental genomes.

2) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Please add scale bars to 1D (left, magnification), 3E, 5C

3) Statistical analysis: Error bars on graphic representations of numerical data must be clearly
described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends.

Please indicate n/sample size/how many experiments the data are representative of: 1F, 3G

4) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions in the text for readers who may not have access to referenced manuscripts.
- Please include database IDs for C elegans strains (e.g., from WormBase if available).
- Please include sequences for all siRNA oligos, including negative controls if those were made available to you from the manufacturer.
- Please include more information about siRNA procedures.
- Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
d. Imaging medium
e. Fluorochromes
f. Camera make and model
g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstructions, surface or volume rendering, gamma adjustments, etc.).

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

Full guidelines are available on our Instructions for Authors page, http://jcb.rupress.org/submission-guidelines#revised. **Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.**

B. FINAL FILES:

Please upload the following materials to our online submission system. These items are required prior to acceptance. If you have any questions, contact JCB's Managing Editor, Lindsey Hollander (lhollander@rockefeller.edu).

-- An editable version of the final text (.DOC or .DOCX) is needed for copyediting (no PDFs).

-- High-resolution figure and video files: See our detailed guidelines for preparing your production-ready images, http://jcb.rupress.org/fig-vid-guidelines.

-- Cover images: If you have any striking images related to this story, we would be happy to consider them for inclusion on the journal cover. Submitted images may also be chosen for highlighting on the journal table of contents or JCB homepage carousel. Images should be uploaded as TIFF or EPS files and must be at least 300 dpi resolution.

**It is JCB policy that if requested, original data images must be made available to the editors.**
A central part of development is when two pronuclei meet in the newly fertilized embryo and then enter the first mitosis. However, very little is known about what happens to the 4 membranes of the two pronuclear envelopes when the two pronuclei first meet. This manuscript presents a tour de force in FIB-SEM microscopy of the one-cell C elegans embryo to see beautiful images of the four membranes at the interface of the two pronuclei during the first mitosis. It is incredibly innovative and novel. The FIB-SEM microscopy is absolutely beautiful. New membrane structures are seen for the first time and surprising new structures are described. It is well written and easy to read. All the FIB-SEM are well quantified and backed by analysis in multiple embryos. They even go on to describe at the EM level a mutant interface. It should be published without delay and featured prominently. There are many images that would make great cover shots. The fist image on Figure 1C would be ideal for a December or January issue!

I will list a couple of the novel findings. When pronuclei meet, each of the nuclear envelopes begin to make small fenestrations during prophase, but they don't line up. The magic happens between prophase and metaphase. The four membranes become two. This was a surprising result and has implications for how pronuclei fuse. The FIB-SEM beautifully caught two types of junctions that could explain how the four membranes became two, including novel three-way junctions. They do have some mechanism, in that a plk-1 mutant, these junctions fail to form at the FIB-SEM level (which goes nicely with previously published light images in plk-1 mutants). It is rare when such elaborate EM so clearly document a mutant phenotype at the level of how membranes are interacting with each other. After describing the novel membrane junctions, they attempt to address the whether the two remaining membranes are an inner and outer from the same pronucleus or two inners. They stain with SUN and KASH proteins. The data are consistent with it being an inner and outer from the same pronucleus. The data aren't completely convincing, but
they are written in a way that leaves open a possibility that they are making the wrong conclusion. So, I am happy with the way it is presented.

In summary, this is a beautiful manuscript with thoroughly described and analyzed FIB-SEM images from many embryos. Its technical innovation allows them to examine an essential process in development that has never been looked at in such detail in any multicellular organism. It is an example of studying Cell Biology with EM at its best. It should be published without delay at JCB.

I have only two really minor comment. In Figure 1A, I was a bit confused at the timing, until I noticed the first panel was -100 sec. Can you move the text, as the "-" symbol is hard to see over the background fluorescence. In the discussion of three-way junctions, a typo "Outer-outer junctions were former by..." Should be formed.

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

Here Rahman et al use high pressure freezing and FIB-SEM methods to examine pronuclear fusion during M-phase after fertilization in C. elegans zygotes, with focus on nuclear envelope (NE) dynamics. They show striking images and 3D reconstructions that give snapshots of this process, which involves formation of large fenestrations in the NE in the space between fusing pronuclei. The membranes surrounding these fenestrations contain apparent intermediates in the NE reorganization process, including 3-way junctions that are interpreted to consist of a fused outer nuclear membrane flanked by inner nuclear membranes from the two fusing pronuclei. This is described as a 3-way sheet junction, which would be similar to structures that have been reported previously in Drosophila embryos during insertion of cytoplasmic annulate lamellae in the NE. However, it remains possible that these "3-way sheet junctions" actually originate as points of tubular membrane fusion, a process that occurs continuously during remodeling of the ER in interphase. Different approaches would be needed to address the latter question.

Notwithstanding the high quality structural work in this manuscript, the study doesn't provide much new mechanistic insight on the process of pronuclear membrane fusion, other than PLK is not needed for fenestrae formation but is required for membrane fusion. Questions arise as to how the initial steps of apparent outer nuclear membrane fusion occur, and/or whether the interphase tubular ER fusion machinery or additional fusion proteins (such as yeast Kar5) play a role here. For these reasons, I don't think the manuscript has the substantive depth that is comparable to similar types of structure-oriented Reports that appear in JCB.

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

This study by Rahman and colleagues explores how pronuclear membranes are ruptured to permit the merging of the parental chromosomes during mitosis in the one-cell C. elegans embryo. The authors studied the configuration of the pronuclear membranes from pro-metaphase to metaphase using focused ion beam scanning electron microscopy (FIB-SEM). They observe that the two pronuclei are linked by different types of membrane structures, including a novel 3-way sheet junction. They suggest that the membrane junctions are necessary for the merging of the parental chromosomes. Although the study is potentially interesting, a number of points should be carefully addressed before it can be considered for publication in the JCB.
Major points
I think the last sentence of the abstract somewhat overstates the results. While the authors report the identification of a novel type of membrane junction, they have no evidence that it requires a specific membrane fusion machinery. The two membranes might for example fuse "passively" after NPC components and other proteins have been removed from the NE. In the plk-1 mutant, although the membrane junctions appear absent, the literature suggest that NPC and possibly other NE proteins are still present. The defect in membrane fusion in plk-1 ts might merely result from a defect in NPC disassembly. This should be clearly stated.

I appreciate that it is necessary to "sell" one's results to the fullest in this competitive age, however I feel that in many cases the authors overdo it with excessive wording such as "for the first time" etc... and often it is not really justified. (e.g) End of Page 4: "To our knowledge, this kind of systematic nanoscale analysis of the first mitosis after fertilization has not been done for any organism". It is not true! Please check König et al. JCB 2017, or Laband et al. Nat Com 2017).

Page 5 Figure 2: It has already been reported that NEBD occurs earlier at the interface between the pronuclei in one-cell C. elegans embryos than at the periphery.

Figure 1.
Panels A and B are too small; it is difficult for a non-initiated reader to see the membrane gap. Please, increase the size of the images or show insets.
Panel D: The arrow indicates the position of an NPC perforating the double membrane, but careful examination of the membrane above the NE, which is probably the ER, presents the same type of fenestrations. How can the authors be sure that the fenestrations correspond to the NPC? Panel F: The authors should fuse the graphs by using a Y axis break so that they can include the largest hole area. Comparatively to the images, the graphs are too large.

Figure 2.
Panel C: Again fuse the graphs, with a Y axis break to include the largest hole area.
Panel D: It has been shown previously that NPC disassembly occurs at the interface of the pronuclei before it occurs at the periphery. This panel should be move to Figure 1 together with panel A to introduce the current understanding of NEBD in the one-cell stage embryos.

Figure 3:
Panel B: The authors should present these data as in Figure 2 panel B: by guiding the reader through the Figure.
Panel D: As presented on this graph, the NE hole area is the same between prometaphase and metaphase (compare graphs Figures 2C and 3D). However, the authors show in Figure 1F that the NE hole area increases during metaphase.

Figure 4: It would helpful to guide the reader through the Figure without having to read the entire Figure legend first.

Figure 5: Panels B and C could be moved to supplementary information. This analysis does not bring any useful information. At this resolution, the authors can't discriminate between inner and outer membranes just by looking at ZYG-12 and SUN-1 localization.
Panel F. The analysis of plk-1ts embryo is incomplete. Include the graph of the NE hole area of the plk-1ts metaphase embryos. It would also be helpful if the authors included a consecutive SEM
images of the plk-1ts pronuclear membranes to highlight the absence of junctions. It would be important to add a small discussion describing the differences between the wt and the plk-1ts pronuclear membranes.

Minor points
Reference(s) missing in the text Introduction 1st paragraph "NEBD requires the activity of various kinases, including cyclin-dependent kinase 1 (Cdk1) and Polo-like kinase (Plk1), which drive the disassembly of the NPCs and nuclear lamina.
An extra "(" in the 3rd paragraph of the introduction lane 3.
(Cohen et al.) Missing a «T»
Response to the Editors’ and Reviewers’ comments:
(verbatim comments are italicized)

Editor comments
“Reviewer #2’s interesting discussion point “it remains possible that these "3-way sheet junctions" actually originate as points of tubular membrane fusion, a process that occurs continuously during remodeling of the ER in interphase”.

The reviewer is suggesting that 3-way sheet junctions originate from points of tubular membrane fusion. There are two ways to interpret this suggestion: First, that 3-way sheet junctions form as a series of tubule fusion events, such as the outer-outer junctions we observe in prometaphase and metaphase. We fully agree with this possibility, and on page 7 we noted that “The similar size of (the 3-way sheet junction) tubes and the tubes in the outer-outer junctions suggests that they are structurally related”. We also added the following discussion: “Our observations raise two related questions: how do the 3-way sheet junctions form, and from where did the two remaining membranes at the metaphase interface originate? We propose that the first step is the formation of outer-outer junctions, possibly in a process that is analogous to atlastin-driven ER tubule fusion”. We then go on to propose models through which 3-way sheet junctions might form (page 7 and Figure 5A and B). It should also be noted that the “process that occurs continuously during remodeling of the ER in interphase” is only known to involve tubules, and here we have a situation that the starting material is sheets, which is novel and interesting.

If, however, the reviewer is suggesting that 3-way sheet junctions form directly from the fusion of ER tubules, then this possibility seems unlikely because of the location of these junctions: 3-way sheet junctions are only observed at metaphase, at a considerable distance from the interface edge. At this position, in prometaphase, which is immediately prior to 3-way sheet junction appearance, we detect various types of structures between the two pronuclei but none of them are ER tubules (Figure 2E). Rather, the membranes at the location where the 3-way sheet junction are detected were previously membrane sheets: the inner and outer membranes of the two pronuclei. It is therefore unlikely that the 3-way sheet junction could have formed directly through fusion of ER tubules.

Specific points:
1) The eTOC summary is now as proposed by the editor, with minor modifications.
2) Scale bars: Please add scale bars to 1D (left, magnification), 3E, 5C
   Scale bars have been added to all panels. In case of multi-panelled panels, the scale bar applies to all images (indicated in the Figure legend)
3) Statistical analyses: The type of statistical test used is noted in all cases. Error bars are included where distributions of hole sizes in a specific size range are compared (see new Supplemental Figure S1). Otherwise, the entire distribution of hole sizes measured is shown. Since there are different classes of hole sizes, calculating the overall average does not provide a
biologically meaningful value. In all experiments, all the embryos from which the data were derived are indicated.

Please indicate n/sample size/how many experiments the data are representative of: 1F, 3G

In the case of peripheral NE hole size (Figure 1F), the number and size of areas used to measure hole size in each embryo is now indicated in the Figure legend. In Figure 3G, all intranuclear membranous structures in the 9 embryos shown were included: 2 interphase, prophase and prometaphase embryos, and 3 metaphase embryos.

4) Material and Methods:
- All methods are described in detail. References are included to give credit where credit is due.
- C. elegans strains: All strains were generated in house and are mentioned by their name (OCF+ number). These names will eventually appear on WormBase (OCF3 already does).
- siRNA: siRNA was not used in this study (plk-1 down-regulation was done using a temperature sensitive allele)
- Microscopes and image acquisition: all the required information is provided.

Reviewer comments
Reviewer #1:
A central part of development is when two pronuclei meet in the newly fertilized embryo and then enter the first mitosis. However, very little is known about what happens to the 4 membranes of the two pronuclear envelopes when the two pronuclei first meet. This manuscript presents a tour de force in FIB-SEM microscopy of the one-cell C elegans embryo to see beautiful images of the four membranes at the interface of the two pronuclei during the first mitosis. It is incredibly innovative and novel. The FIB-SEM microscopy is absolutely beautiful. New membrane structures are seen for the first time and surprising new structures are described. It is well written and easy to read. All the FIB-SEM are well quantified and backed by analysis in multiple embryos. They even go on to describe at the EM level a mutant interface. It should be published without delay and featured prominently. There are many images that would make great cover shots. The first image on Figure 1C would be ideal for a December or January issue!

We thank the reviewer for his/her enthusiasm. Following the reviewer’s suggestion we submitted the “snowman pronuclei” for the cover image.

I will list a couple of the novel findings. When pronuclei meet, each of the nuclear envelopes begin to make small fenestrations during prophase, but they don’t line up. The magic happens between prophase and metaphase. The four membranes become two. This was a surprising result and has implications for how pronuclei fuse. The FIB-SEM beautifuly caught two types of junctions that could explain how the four membranes became two, including novel three-way junctions. They do have some mechanism, in that a plk-1 mutant, these junctions fail to form at the FIB-SEM level (which goes nicely with previously published light images in plk-1 mutants). It is rare when such elaborate EM so clearly document a mutant phenotype at the level of how membranes are interacting with each other. After describing the novel membrane junctions, they attempt to address the whether the two remaining membranes are an inner and outer
from the same pronucleus or two inners. They stain with SUN and KASH proteins. The data are consistent with it being an inner and outer from the same pronucleus. The data aren’t completely convincing, but they are written in a way that leaves open a possibility that they are making the wrong conclusion. So, I am happy with the way it is presented.

We thank the reviewer for his/her positive assessment. We now moved the SUN-1/ZYG-12 experiment to Supplemental Figure S3. We still believe it is a valid approach, but one must bear in mind the potential caveats, which are detailed in the text as they were in the original version of the manuscript.

In summary, this is a beautiful manuscript with thoroughly described and analyzed FIB-SEM images from many embryos. Its technical innovation allows them to examine an essential process in development that has never been looked at in such detail in any multicellular organism. It is an example of studying Cell Biology with EM at its best. It should be published without delay at JCB.

Thanks!

I have only two really minor comment. In Figure 1A, I was a bit confused at the timing, until I noticed the first panel was -100 sec. Can you move the text, as the "." symbol is hard to see over the background fluorescence.

Corrected.

In the discussion of three-way junctions, a typo "Outer-outer junctions were former by...." Should be formed.

Corrected.

Reviewer #2:

Here Rahman et al use high pressure freezing and FIB-SEM methods to examine pronuclear fusion during M-phase after fertilization in C. elegans zygotes, with focus on nuclear envelope (NE) dynamics. They show striking images and 3D reconstructions that give snapshots of this process, which involves formation of large fenestrations in the NE in the space between fusing pronuclei. The membranes surrounding these fenestrations contain apparent intermediates in the NE reorganization process, including 3-way junctions that are interpreted to consist of a fused outer nuclear membrane flanked by inner nuclear membranes from the two fusing pronuclei.

That is one interpretation, but it was not the one presented in the original version of the manuscript. Based on this and other comments we now present two possible models for the structure of the 3-way sheet junctions (Figure 5A and B): In the first the remaining two membranes are the inner + outer of one of the pronuclei (as was described in the original
version of the manuscript), and in the second they are the two inner membranes, as assumed by the reviewer. The latter model was suggested to us by Dr. Michael Kozlov (Tel Aviv University). It was not included in the original version because the SUN-1/ZYG-12 experiment is consistent with former model. However, given the stated caveats associated with the SUN-1/ZYG-12 experiment, we thought it best to present both models in the revised manuscript. Dr. Koslov is now mentioned in the Acknowledgement section.

This is described as a 3-way sheet junction, which would be similar to structures that have been reported previously in Drosophila embryos during insertion of cytoplasmic annulate lamellae in the NE.

Actually, Hampoelz et al., 2016, did not explicitly state that the intermediate between annulate lamellae (AL) and NE fusion is a 3-way sheet junction; that is our suggestion. Their study does not explore structural properties of the junction between the AL and NE, and in particular the extent of contact between the AL and the NE: whether it’s a single tube (which is how ER typically connects with the NE), a series of tubes (as we observe here), or fusion along the entire AL sheet, as suggested in their model. I could not find how many AL-NE junctions were segmented in the Hampoelz et al study, and I expect that it is not easy to find these junctions. I’m guessing that for this reason Hampoelz et al speculated about the nature of the AL-NE junction, and rightly so as this wasn’t the focus of their study. In our study, in contrast, the 3-way sheet junctions are extensive, encircling the entire 2-membrane interface in all metaphase embryos examined, and allowing us to generate robust 3-D reconstructions (Figure 4 and supplemental Figure S2). Here we demonstrate the existence of a novel structure; Hampoelz et al speculated about the nature of theirs.

However, it remains possible that these "3-way sheet junctions" actually originate as points of tubular membrane fusion, a process that occurs continuously during remodeling of the ER in interphase. Different approaches would be needed to address the latter question.

The reviewer is suggesting that 3-way sheet junctions originate from points of tubular membrane fusion. There are two ways to interpret this suggestion: First, that 3-way sheet junctions form as a series of tubule fusion events, such as the outer-outer junctions that we observe in prometaphase and metaphase. We fully agree with this possibility, and on page 7 we noted that “The similar size of (the 3-way sheet junction) tubes and the tubes in the outer-outer junctions suggests that they are structurally related”. We also added the following discussion: “Our observations raise two related questions: how do the 3-way sheet junctions form, and from where did the two remaining membranes at the metaphase interface originate? We propose that the first step is the formation of outer-outer junctions, possibly in a process that is analogous to atlastin-driven ER tubule fusion”. We then go on to propose models through which three-way junctions might form (page 7 and Figure 5).

If, however, the reviewer is suggesting that 3-way sheet junctions form directly from ER tubule fusion, this possibility seems unlikely because of the location of these junctions: 3-way sheet junctions are only observed at metaphase, at a considerable distance from the interface edge.
At this position, in prometaphase, immediately prior to three-way junction appearance, we do detect various types of structures between the two pronuclei but none of them are ER tubules (Figure 2E). Rather, the membranes at the location where the 3-way sheet junction are detected were previously membrane sheets: the inner+outer membranes of each of the two pronuclei. It is therefore unlikely that the three-way sheet junction could have formed directly through fusion of ER tubules.

_Notwithstanding the high quality structural work in this manuscript, the study doesn’t provide much new mechanistic insight on the process of pronuclear membrane fusion, other than PLK is not needed for fenestrae formation but is required for membrane fusion._

We respectfully disagree: the prevailing notion is that parental chromosome mix as a result of the complete disappearance of nuclear membranes. We show that at least in _C. elegans_, this is not the case, and that the mechanism by which the two parental genomes mix involves membrane fusion through novel types of junctions. It is true that we haven’t uncovered the entire mechanism, namely the proteins that drive this fusion, but we did identify a key step in this process, based on which we and others can now pursue further insight. We also propose that membrane junctions are needed for efficient removal of membrane between the two pronuclei. This can be considered as a little bit of mechanism.

_Questions arise as to how the initial steps of apparent outer nuclear membrane fusion occur, and/or whether the interphase tubular ER fusion machinery or additional fusion proteins (such as yeast Kar5) play a role here._

As noted in the original manuscript, we and others have not been able to identify a Kar5-like protein in _C. elegans_. We agree that fusion proteins are likely to be involved, but their identity is currently unknown. We are examining proteins such as atlastin, but for FIB-SEM analysis the efficiency of inactivation has to be extremely high and temporally regulated (otherwise the ER will be too deformed to support pronuclei formation) and we’re not there yet.

_For these reasons, I don’t think the manuscript has the substantive depth that is comparable to similar types of structure-oriented Reports that appear in JCB._

**Reviewer #3:**

_This study by Rahman and colleagues explores how pronuclear membranes are ruptured to permit the merging of the parental chromosomes during mitosis in the one-cell _C. elegans_ embryo. The authors studied the configuration of the pronuclear membranes from pro-metaphase to metaphase using focused ion beam scanning electron microscopy (FIB-SEM). They observe that the two pronuclei are linked by different types of membrane structures, including a novel 3-way sheet junction. They suggest that the membrane junctions are necessary for the merging of the parental chromosomes. Although the study is potentially interesting, a number of points should be carefully addressed before it can be considered for publication in the JCB._
Major points

I think the last sentence of the abstract somewhat overstates the results. While the authors report the identification of a novel type of membrane junction, they have no evidence that it requires a specific membrane fusion machinery. The two membranes might for example fuse "passively" after NPC components and other proteins have been removed from the NE.

We understand the objection to speculating on the involvement of a fusion machinery. Therefore, the last sentence of the Abstract now reads: “In the plk-1 mutant, where parental genomes fail to merge, these junctions are absent, suggesting that 3-way sheet junctions are needed for formation of a diploid genome.”

In the plk-1 mutant, although the membrane junctions appear absent, the literature suggest that NPC and possibly other NE proteins are still present. The defect in membrane fusion in plk-1 ts might merely result from a defect in NPC disassembly. This should be clearly stated.

We completely agree with the reviewer that the involvement of PLK-1 in pronuclear fusion may be indirect. In fact, we stated in the original version of the manuscript that “… (PLK-1 may be required) indirectly, for example by promoting pronuclear juxtaposition and/or NPC disassembly (Rahman et al., 2015; Martino et al., 2017; Linder et al., 2017) that may be a prerequisite for junction formation.” This statement is also included in the revised manuscript.

I appreciate that it is necessary to "sell" one’s results to the fullest in this competitive age, however I feel that in many cases the authors overdo it with excessive wording such as “for the first time” etc... and often it is not really justified. (e.g) End of Page 4: "To our knowledge, this kind of systematic nanoscale analysis of the first mitosis after fertilization has not been done for any organism”. It is not true! Please check Konig et al. JCB 2017, or Laband et al. Nat Com 2017).

We do apologize- we did not mean to imply that no one has previously analyzed the 1-cell embryo in 3D at EM-resolution. What we meant to say was that the process of pronuclear fusion was not previously analyzed in this level of detail. Konig et al examined the process of cytokinesis/abscission, whereas Laband et al studied chromosome segregation in the oocyte. Neither of these studies, as well as other very elegant studies from Muller-Reichert lab, have examined the fate of the pronuclei after fertilization. That said, our statement was poorly phrased and it was removed in the revised version. The only places where we now comment on the novelty of our findings are in relation to the 3-way sheet junction. In the abstract we state that this is a “novel membrane structure”, and on page 7 we state that “To our knowledge, this type of membrane configuration has not been demonstrated before.”

Page 5 Figure 2: It has already been reported that NEBD occurs earlier at the interface between the pronuclei in one-cell C. elegans embryos than at the periphery.
The reviewer is correct. The panels showing NPP-1 disassembly from the interface at prometaphase were removed and the following references were added: Schetter et al., 2006; Galy et al., 2007; Hachet et al., 2012; Martino et al., 2017.

**Figure 1.**
Panels A and B are too small; it is difficult for a non-initiated reader to see the membrane gap. Please, increase the size of the images or show insets.

The size of the images was increased.

**Panel D:** The arrow indicates the position of an NPC perforating the double membrane, but careful examination of the membrane above the NE, which is probably the ER, presents the same type of fenestrations. How can the authors be sure that the fenestrations correspond to the NPC?

The arrows in panel D, which shows the peripheral nuclear membrane at metaphase, are pointing to fenestrations that are larger than normal NPC openings. The legend in the original version stated that “White arrows in the “metaphase” panel point to holes that are larger than the expected size for NPC holes”. That is exactly the point: the membrane formerly known as the nuclear membrane becomes highly fenestrated at metaphase, with large openings. Holes that correspond to NPCs are shown in the inset on the left: they appear as a narrow central line flanked by high curvature (U-shaped) membrane where the inner and outer nuclear membranes meet. The white arrows are pointing to membrane holes that are both wider than NPCs and lack the central line. We do speculate that these holes may have resulted from expansion of membrane fenestrations left behind after NPC disassembly (page 4): “We speculate that the smaller metaphase fenestrations are the result of the enlargement of the membrane holes left behind after NPC disassembly, consistent with a model proposed by (Terasaki et al., 2001). The large fenestrations could be a result of enlargement and/or fusion of smaller fenestrations.”.

The astute reviewer noticed that there are extra layers of membrane around the metaphase pronuclei. We noticed this as well and we see it in other metaphase embryos. Interestingly, earlier mitotic stages do not exhibit this level of “ER wrapping”, if you will, around the nuclear membrane. In fact, there are a number of interesting membrane structures in the vicinity of the larger fenestrations. Due to space limitations, and because we don’t yet understand this phenomenon, we did not elaborate on these structures. Our datasets will be available to anyone who wishes to pursue this observation further.

**Panel F:** The authors should fuse the graphs by using a Y axis break so that they can include the largest hole area. Comparatively to the images, the graphs are too large.

I confess that I was skeptical about fusing the graphs, but it worked beautifully. We used segmented Y axes to show all the data points at reasonable resolution and moved the inset graphs to the supplemental material (Supplemental Figure S1) for the comparison of small-sized
holes (<0.04 µm²) from different stages. In cases where the Y axis is segmented, the graph background for each segment is shown in a different color, to emphasize the different scales.

Figure 2.
Panel C: Again fuse the graphs, with a Y axis break to include the largest hole area.

Done

Panel D. It has been shown previously that NPC disassembly occurs at the interface of the pronuclei before it occurs at the periphery. This panel should be move to Figure 1 together with panel A to introduce the current understanding of NEBD in the one-cell stage embryos.

Following the reviewer’s comment we found a number of studies with very similar images. As noted above, we removed this panel altogether and these studies are now cited.

Figure 3:
Panel B: The authors should present these data as in Figure 2 panel B: by guiding the reader through the Figure.

We thank the reviewer for this useful suggestion. The section discussing these data now begins as follows:” The membrane interfaces of two metaphase embryos were segmented (Figure 3A). This allowed us to determine the sizes of the interface membrane holes (Figure 3B), as was done above for the prometaphase interface. It also allowed us to examine the position of the chromosomes relative to the membrane (Figure 3C) and to determine the configuration of the interface membranes at this stage (see below). As in prometaphase, the metaphase interface had both large and small holes (Figure 3D).” The rest of the section is the same.

Panel D: As presented on this graph, the NE hole area is the same between prometaphase and metaphase (compare graphs Figures 2C and 3D). However, the authors show in Figure 1F that the NE hole area increases during metaphase.

We thank the reviewer for this comment as it highlights an important conclusion that we didn’t explain properly: In Figure 1F the hole sizes were measured at the nuclear periphery while in Figure 3D the hole sizes were measured in the interface. Interestingly, there is a difference between the prometaphase and metaphase hole sizes at the periphery (where the metaphase holes are larger) but not at the interface. This suggests that the regulation and/or mechanism of hole size expansion in the periphery and interface may be distinct. This is now shown in Supplemental Figure S1B and mentioned in the text (page 6): “Interestingly, the size of the small fenestrations (< 0.04 µm²) was the same in prometaphase and metaphase (Supplemental Figure S1B). At the pronuclear periphery, on the other hand, the metaphase small holes were significantly larger than the prometaphase ones (Supplemental Figure S1A). This suggests that the regulation and/or mechanism of hole size expansion in the periphery and interface may be distinct.”
Figure 4: It would helpful to guide the reader through the Figure without having to read the entire Figure legend first.

In the original version of the manuscript, the discussion of the two junctions types, the outer-outer junctions and the 3-way sheet junctions, was intermingled, which is likely why the reviewer felt that he/she needed to read the Figure legend in order to understand the nature of each junctions. We have now separated the discussion of the two junctions (middle of page 6-middle of page 7) and we guide the reader through Figure 4 in order of data appearance. We hope that this clarifies things.

Figure 5: Panels B and C could be moved to supplementary information. This analysis does not bring any useful information. At this resolution, the authors can't discriminate between inner and outer membranes just by looking at ZYG-12 and SUN-1 localization.

As suggested by the reviewer, panels B and C of the original manuscript were moved to the Supplemental Material. However, the reviewer is mistaken regarding the need to discriminate between inner and outer nuclear membranes at this resolution. This statement would have correct had we been looking at the interface “from the side”, where, for example, the inner and outer nuclear membranes appear as parallel lines. In this case, the reviewer is correct that one cannot discriminate between the two membranes at the resolution of a confocal microscope. In our experiment, however, we are looking at the interface face on, at the plane that is shown, for example, in Figure 3B. In this case, the two membranes of the interface (i.e. the inner and outer membranes) are one above the other (think a stack of two pancakes viewed from the top). The point isn’t whether we can tell SUN-1 (inner) and ZYG-12 (outer) apart. The question is whether ZYG-12 is even there. The purpose of this experiment is to distinguish between two models that are now shown in Figures 5A and 5B. What distinguishes these models is the composition of the two-membrane at the interface, namely the membrane immediately adjacent to the large holes: in the first model the interface would be composed of inner + outer membranes, while in the second it will be two inner membranes. The fact that not only do we see ZYG-12 signal at the interface but that it overlaps almost completely with SUN-1 suggests that the first model is correct.

Panel F. The analysis of plk-1ts embryo is incomplete. Include the graph of the NE hole area of the plk-1ts metaphase embryos.

Done. See new Figure 5F. We also included an image of the plk-1ts interface itself in new Figure 5E.

It would also be helpful if the authors included a consecutive SEM images of the plk-1ts pronuclear membranes to highlight the absence of junctions.

It is not possible to show consecutive SEM images of the interface as one would need dozens of images to do so. Instead, we now include 2 movies that span over 700 nm of a wild type
interface (Supplemental Movie S3) and a \textit{plk-1}ts interface (Supplemental Movie S4). I think the reviewer will agree that the difference is striking.

\textit{It would be important to add a small discussion describing the differences between the wt and the plk-1ts pronuclear membranes.}

In new Figure 5D we now show the \textit{plk-1} interface membrane at metaphase and the difference between \textit{plk-1} and wild type is apparent: in the \textit{plk-1} embryos there are many large holes (i.e. larger than expected for NPCs) but no giant holes as seen the wild type metaphase embryos. Based on this we conclude that junctions are needed for efficient removal of membrane between the two pronuclei. This is discussed on page 8.

\textit{Minor points}

Reference(s) missing in the text Introduction 1st paragraph "NEBD requires the activity of various kinases, including cyclin-dependent kinase 1 (Cdk1) and Polo-like kinase (Plk1), which drive the disassembly of the NPCs and nuclear lamina."

References were added

\textit{An extra "(" in the 3rd paragraph of the introduction lane 3.}

Corrected

\textit{(Cohen et al.) Missing a «T»}

Corrected