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Membrane rupture and independent extension of sister membranes drive cytokinesis in *C. elegans* embryos

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eLife Assessment

In this manuscript, based on electron microscopy observations of *C. elegans* embryos, the authors make the bold claim that the plasma membrane ruptures during cell division and that closure of this opening by membrane extension contributes to cytokinesis. Although the findings are potentially **valuable**, the evidence in support of the authors' claims is **inadequate**.

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Abstract

Cell division, the physical separation of a cell into two daughter cells, is classically thought to require continuous plasma membrane invagination driven by equatorial contractile forces. Here, we challenge this paradigm by uncovering an unexpected mechanism in *C. elegans* embryos: the plasma membrane ruptures as early as metaphase. Instead of invaginating as a continuous sheet, the separated membranes extend independently to partition the cytoplasm. The membrane rupture and independent extension of sister membranes create membrane discontinuities—widely observed in electron microscopy (EM) sections. Without plasma membrane coverage, the exposed cytoplasmic regions are shielded either by plasma membrane from neighboring, likely kinship, cells or extracellular matrix components, preventing massive leakage. Our findings overturn the long-standing assumption that cytokinesis requires persistent membrane integrity, and offer fresh insights into cell division, particularly in fast dividing embryonic cells.

Introduction

The plasma membrane integrity is the corner stone of cell biology. Based upon, , current models further propose that cell division should also occur with uninterrupted membrane integrity and the invagination of this intact membrane, driven by a contractile ring of actin filaments, myosin II, and regulatory proteins (Pollard and O'Shaughnessy, 2019 [↗](#)), with vesicle fusion supplying new membrane to accommodate shape change (Gerien and Wu, 2018 [↗](#)), progressively deepens until pinching the cell into two daughter cells (Figure 1A [↗](#)). Critically, the foundational premise of uninterrupted membrane integrity during cytokinesis has never been rigorously tested until our unexpected discovery in *C. elegans* embryos.

After fertilization, the single cell *C. elegans* embryo begins a series of highly stereotyped cell divisions. During this phase, cells undergo rapid division (15-30 minutes per cycle), with each cycle alternating between S (DNA synthesis) and M (mitosis and cytokinesis) phases while bypassing G1 and G2 gap phases until the 28-cell stage (Edgar and McGhee, 1988 [↗](#); Deppe et al., 1978 [↗](#); Sulston et al., 1983 [↗](#); Bao et al., 2008 [↗](#); Pintard and Bowerman, 2019 [↗](#)). Around this stage, the worm embryos are laid by the mother. Subsequent development continues outside the mother, encapsulated within a protective, multi-layer extracellular matrix known as the eggshell (Stein,

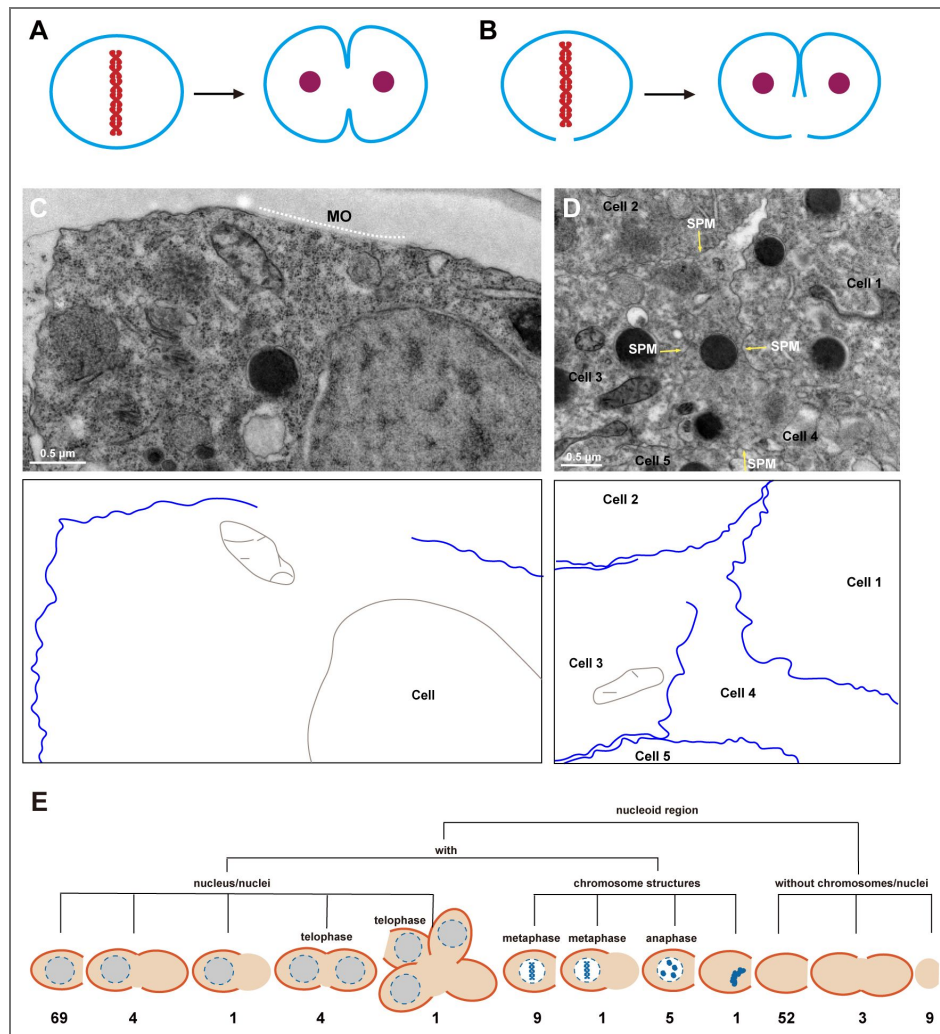


Figure 1. Membrane discontinuities in *C. elegans* embryonic cells.

A, Schematic drawing of a metaphase cell and the classical membrane invagination model. B, Schematic drawing of a metaphase cell with a ruptured plasma membrane and a cytokinesis model showing that the detached sister membranes mediate membrane extension in *C. elegans* embryonic cells. C, A membrane opening (MO, indicated by white dashed line) in an embryonic cell at the embryo's periphery. D, Membrane openings in the embryo's central regions. Yellow arrows point to single plasma membranes (SPM) traversing the cytoplasm. Plasma membranes are in blue. E, Summary of the single EM section survey of plasma membrane continuity. Orange lines outline the plasma membranes. Solid gray circles/ellipses enclosed by dashed lines represent evenly stained nucleoid regions. Condensed chromosome structures are colored gray-blue. Light brown areas represent cytoplasmic regions.

2018). This impermeable eggshell is established earlier, by the end of anaphase II of meiosis, and undergoes its final modifications during the embryo's first few mitotic divisions (Bembenek et al., 2007; Zhang et al., 2005).

During routine ultrastructural analysis (Zhu et al., 2022), we found that rather than maintaining membrane continuity, the dividing *C. elegans* embryonic cells routinely exhibit plasma membrane discontinuities. Further analysis revealed that the plasma membrane of the embryonic cells ruptures as early as metaphase and sister membrane primarily extend independent from each other to achieve cytoplasmic partition (Figure 1B), a mechanism never before documented. This discovery challenges the canonical view of cytokinesis at its core, revealing an unanticipated plasticity in cell division mechanisms and prompting a re-evaluation of how membrane dynamics are regulated across species.

Results

Widespread membrane discontinuities in dividing *C. elegans* embryonic cells

Our study was initially prompted by the curious observation that the plasma membrane of embryonic cells in *C. elegans* does not appear to be continuously intact. We refer to these membrane discontinuities as membrane openings (MOs). They are particularly evident at the embryo's periphery. As shown in Figure 1C, the plasma membrane is absent in the region marked by the white dashed line. Membrane interruptions are also observed in the embryo's central regions. For instance, Figure 1D shows that Cell 3 contains a MO facing both Cell 1 and Cell 2. Additionally, we noted instances where a single plasma membrane (SPM) appears to traverse the cytoplasm, seemingly partitioning it into separate compartments across adjacent cells (Figure 1D) (yellow arrows). According to the conventional view, the plasma membrane should remain intact throughout the cell cycle—whether during division or interphase—and each cell should be enclosed by its own membrane. Consequently, between two adjacent cells, we would expect to observe two distinct plasma membranes. The presence of a single continuous membrane, however, suggests the existence of regions lacking a complete boundary. For instance, Cell 4 in Figure 1D clearly contains such membrane discontinuities. Collectively, these findings challenge the established notion that the plasma membrane remains intact over the entire lifetime of a cell.

To rule out potential artifacts inherent to electron microscopy (EM) techniques, we performed a series of control experiments. First, we prepared four independent sample batches. Membrane discontinuities were consistently observed in all batches, indicating that the phenomenon is not specific to a single preparation. Second, we examined a publicly available historical collection of *C. elegans* embryo EM images (Wormatlas) and identified MOs in several instances, particularly at the embryonic periphery. This confirmation in an independent dataset suggests that MOs are genuine cellular structures rather than procedural artifacts. Third, we analyzed embryos across various developmental stages. MO-containing cells were observed in both intrauterine and already laid embryos at early stages (Figure S1). However, as embryos reached the comma stage (Figure S2)—a period characterized by the onset of elongation and a decline in active cell proliferation—the incidence of MOs dropped dramatically (observed in 0/13, 0/17, 0/30, and 0/30 cells examined, respectively). These results demonstrate that plasma membrane discontinuities are associated with specific stages of *C. elegans* embryogenesis and are not a product of random membrane tearing.

The early stage of *C. elegans* embryonic cells is under rapid and extensive cell division. Within approximately 100 minutes, a two-cell embryo develops into an embryo containing nearly 30 cells (Bao et al., 2008). Given this dynamic proliferation, we suspected that the formation of membrane discontinuities might be associated with cell division events. Therefore, to systematically examine the formation of membrane discontinuities, we collected EM images of 60 early embryos from 9 gravid adults. By examining the entire circumference of 425 cells in single high-pressure freezing transmission electron microscopy (HPF-TEM) sections (Figure S2)

(Modern Electron Microscopy Methods for *C. elegans*, 2012 [↗](#)), we found that MOs in 159 cells distributed across 57 embryos, representing 95% of the total sampled embryos (Figure 1E [↗](#)). Among these 159 affected cells, 89% (141/159) exhibited a single membrane discontinuity, while 13 cells had two openings and 5 cells had three.

We analyzed the 159 cells exhibiting MOs based on nuclear and cytoplasmic morphology (Figure 1E [↗](#)). Of these, 64 cells appeared in EM sections containing only cytoplasmic regions—9 of which completely lacked discernible plasma membranes. The remaining 95 cells contained one or more nuclei or condensed chromosomes. Among them, 10 cells were clearly in metaphase stage (defined by a single chromosomal cluster). As shown in Figure 2A [↗](#), a representative metaphase cell (MC) displayed a MO (dashed white line) spanning roughly one-third of the cell's circumference. High-magnification imaging revealed that in contrast to the wrinkled plasma membranes (Figure 2A [↗](#), enlarged boxed area displayed in 1), the large MO exhibited a smoother surface morphology, and the internal cytoplasm remained enclosed within the cell (Figure 2A [↗](#), enlarged boxed areas displayed in 2 and 3). MOs were also observed in anaphase cells (AC) (n=5; characterized by <10 chromosomal clumps; Figure 2B [↗](#), enlarged in 2) and telophase cells (n=5; defined by two nuclei sharing cytoplasm). Intriguingly, one telophase-like cell contained three nuclei within a common cytoplasm (Figure S2 [↗](#), marked by &).

Because EM sectioning captures cells at random stages of the cell cycle, we sought to systematically assess the relationship between mitosis and MOs. To do so, we analyzed 20 cells with unambiguous division features, selected based on nuclear morphology—such as aligned chromosomes (metaphase) or discrete clusters (anaphase)—and cytoplasmic sharing, indicated by two or more nuclei within a common cytoplasm (telophase). Strikingly, 19 out of 20 cells (95%) revealed MOs upon tracing the plasma membrane in single EM sections (Figure S3 [↗](#)). This near-ubiquitous association strongly suggests that membrane discontinuities are an integral feature of cell division in early *C. elegans* embryos.

Membrane rupture in cells at metaphase

The observed membrane discontinuities suggest that the plasma membrane of early *C. elegans* embryos may rupture during cell division. However, because TEM provides only static, two-dimensional images, we could not determine the three-dimensional nature of these ruptures. To better understand this phenomenon, we acquired continuous serial sections and performed 3D reconstruction.

Since membrane discontinuities appear at metaphase—well before nuclear or cytoplasmic division—we performed continuous EM sectioning to trace the entire plasma membrane of a metaphase cell (Figures 2C [↗](#)). Through 3D reconstruction, we identified a continuous plasma membrane rupture extending over 8,940 nm, which is more than two-thirds of the cell's circumference (Figure 2D [↗](#)). Notably, one end of the membrane rupture appeared long and narrow, while the other was wide and exhibited cytoplasmic leakage (Figure 2D [↗](#) and Video S1). Further analysis revealed that the leaked cytoplasm remained confined to a relatively localized region (Video S1). During metaphase, chromosomes align at the equatorial plane, a key region for subsequent segregation. To explore the spatial relationship between membrane rupture and chromosomal organization, we further reconstructed the entire condensed chromosome region (Figures 2C [↗](#)). We found that this large-scale membrane rupture largely encircled the chromosomal disc (Figure 2D [↗](#) and Video S1), spatially aligning with the future segregation zone.

The detachment of sister membranes

According to the conventional model, cytokinesis in animal cells begins with the formation of a membrane invagination. As this invagination deepens, it gives rise to two back-to-back membrane layers, ultimately leading to the physical separation of the two daughter cells. In the context of membrane rupture, however, plasma membrane disruption results in the separation of two putative sister membranes. We then asked how the subsequent cytokinesis process proceeds with detached sister membranes. To address this question, we examined 51 dividing cells. In each of these cells, two well-separated nuclei were observed, indicating that they were at telophase. We

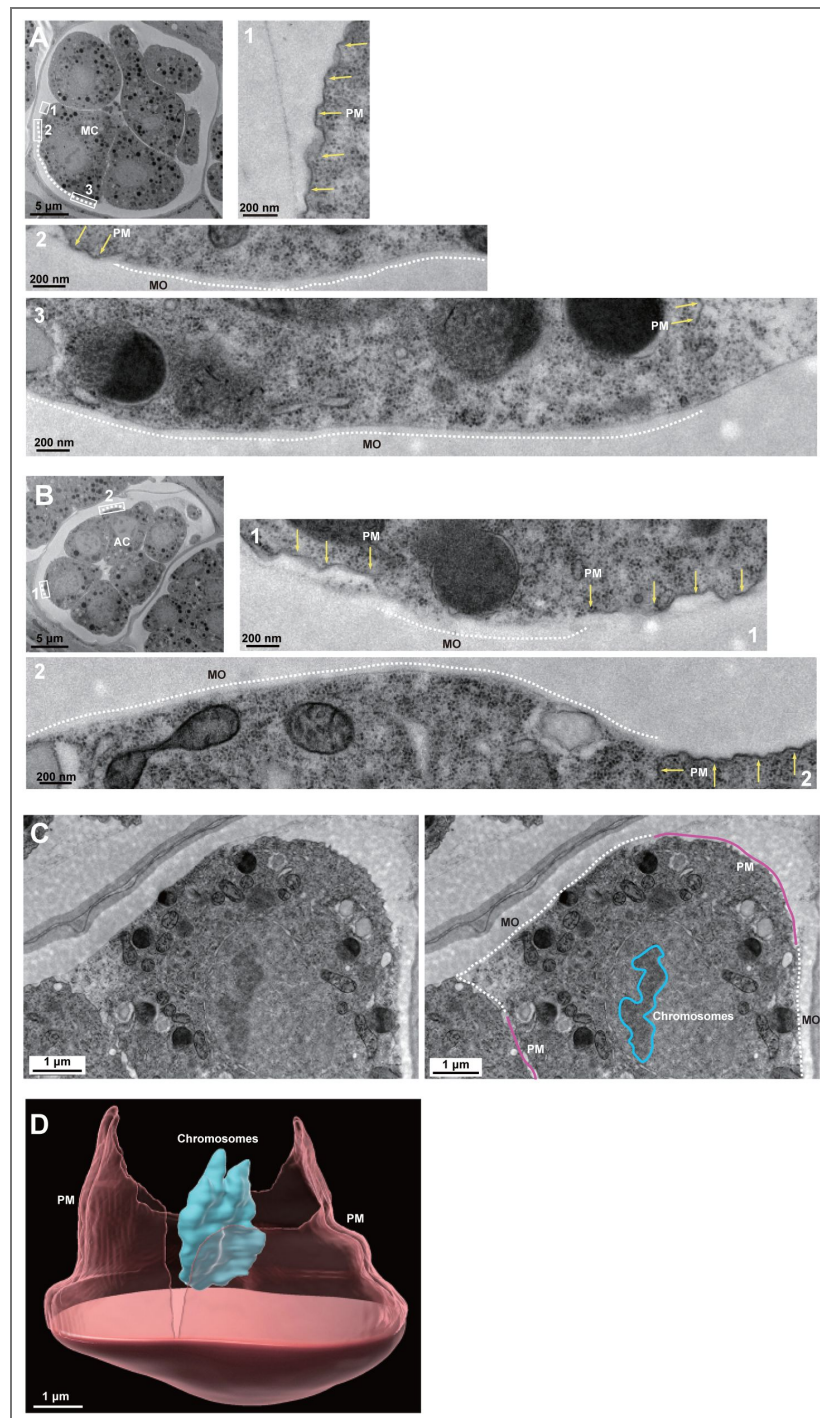


Figure 2. Metaphase membrane rupture.

A, Membrane discontinuities or openings (MO) (white dashed line) in a metaphase cell (MC). Enlarged views of the boxed regions (1, 2 and 3) are shown. Yellow arrows indicate the plasma membrane (PM). B, MOs (marked by 1 and 2) are detected in anaphase (AC) and other early embryonic cells; enlarged views of the MOs are shown (white dashed lines). All yellow arrows point from the interior of the cell toward the plasma membrane. C and D, Three-dimensional (3D) reconstruction of a metaphase cell. Representative EM images are shown in (C). Purple lines outline the plasma membranes. White dashed lines outline the exposed cytoplasmic region. Blue lines outline the condensed chromosome region. D, The 3D reconstruction of the metaphase cell. The plasma membrane (PM) and condensed chromosome region are displayed.

found that while a minority of cells (6/51) exhibited classical membrane invagination (Figure 3A), including one embryo transitioning from the one-cell to the two-cell stage, the vast majority (45/51) displayed a striking detached membrane configuration (Figure 3B). In these cases, sister membrane tips were completely separated from each other (Figures 3B and 3C, marked with *), forming distinct, non-contiguous structures that defy conventional models of cytokinesis. This predominant pattern suggests that membrane detachment—rather than continuous invagination—may represent the primary mode of cytoplasmic separation in *C. elegans* embryos. Of note, the membrane rupture simultaneously generates individual sister membranes with severed edges. Indeed, as shown in Figure 3B, the tips of both free-ended edges (*) of Cell 1 are visible in the EM section.

In the detached membrane configuration, cytoplasmic components—such as glycogen granules and ribosomal particles—were clearly visible surrounding the severed edges of the two sister membranes (Figure 3C). This observation is particularly significant because the classical invagination model cannot generate single plasma membrane barriers between adjacent cytoplasmic domains. Instead, independent extension of severed sister membranes could explain the formation of cytoplasm-immersed membranes. To further test this hypothesis, we traced 12 cytoplasm-embedded membranes, including those separating Cell 1 from Cells 2 and 3, and those separating Cell 4 from 3 (displayed on Figure 3D, enlarged in 1 and 2). In all cases, the cytoplasmic regions flanking these membranes belonged to cytosolically interconnected sister cells. Together, these results demonstrate that *C. elegans* embryonic cytokinesis relies on detached sister membrane extension rather than invagination mediated by intact membranes.

Tracing from the severed edges back to their origins, we found that the sister membranes were frequently anchored side-by-side near the cytosol-immersed region (Figures 4A–4D). Within this region, gap junction plaques were often observed (57 in 94 cases) at membrane contact sites (Figure 4B), though their potential role in sister membrane cohesion is unclear. These contact sites alternated with regions where the two sister membranes separated, revealing lightly stained intercellular spaces in EM images. Beyond the anchored region, the separation between sister membranes became evident and each membrane exhibited the characteristic features of a typical plasma membrane—with one side facing the cytosol and the other exposed to the extracellular environment (Figure 4D).

Cellular vesicles were observed near free membrane edges (Figures 4A–4C). Some cytosol-immersed membrane tips appeared connected to vesicles (Figure 4A), with instances of direct contact (Figure 4B) and possible fusion events (Figures 4B and 4C). Vesicles also clustered along the anchoring area, as well as the separated sister plasma membranes (Figure 4D). EM analysis revealed no morphological differences between vesicles associated with the cytosol-immersed region and those near conventional regions of the sister membranes (Figures 4B and 4D).

Analysis of a tri-nucleated cell during cytokinesis

To gain more comprehensive insight into membrane organization during cytokinesis, we further applied the AutoCUTS-SEM (automatic collector of ultrathin sections scanning electron microscopy) workflow (Li et al., 2017) to acquire serial EM sections of a telophase cell. Through continuous tracing, we discovered that this cell actually contained three independent nuclei. We color-coded each membrane region according to its associated nucleus, with relative sizes estimated from EM sections to ensure proportional representation (Figure 5A). The 3D model demonstrated that the division plane between the yellow and blue cells was roughly perpendicular to that between the purple cell and the other two (Figure 5A). The presence of three nuclei within a shared cytoplasm suggests that one of the daughter cells entered the next round of cell division without completing the previous one. This phenomenon—concurrent divisions—appears to be common in early *C. elegans* embryos, as our EM analysis identified more than 10 cells with three or four nuclei. For example, Figure 3D shows an EM section of a cell containing four nuclei.

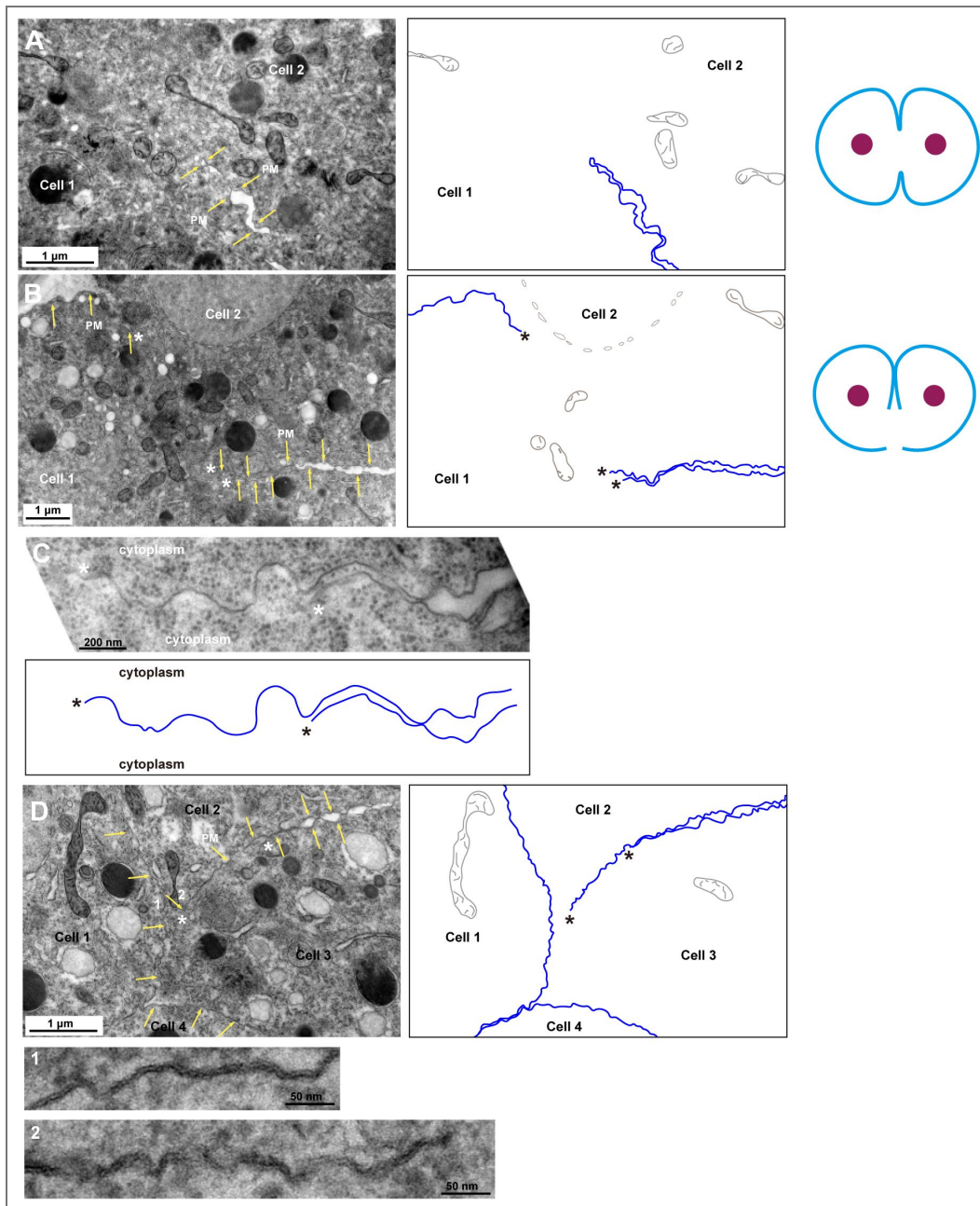


Figure 3. The detached severed sister membranes.

A, Representative image of a classical back-to-back membrane arrangement. B, Representative image of detached severed sister membranes. Diagrams of the corresponding cytokinesis models are displayed on the right. C, Tips of two detached sister membranes. D, Cytosol-immersed single membranes separating the cytoplasmic regions of two adjacent cells. Enlarged views of regions 1 and 2 are shown below. Plasma membranes (PM) are indicated by yellow arrows (A, B and D) or blue lines. Asterisks indicate membrane tips.

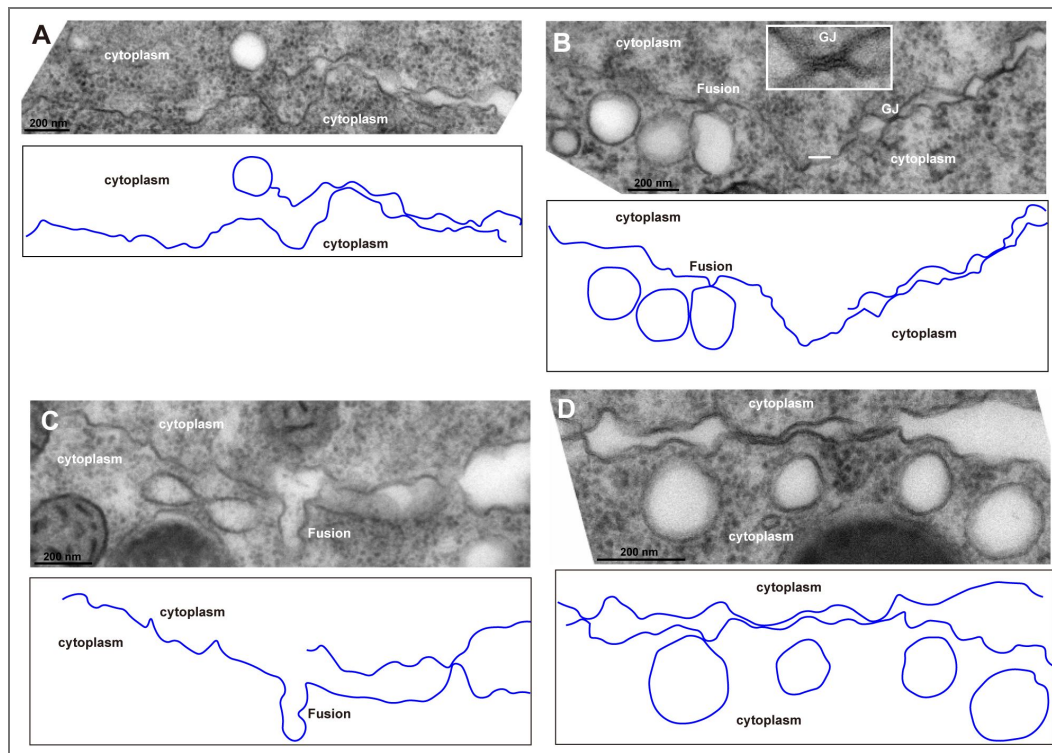


Figure 4. Cellular vesicles on detached sister membranes.

A, A vesicle localized at the tip of a cytosol-immersed membrane. B, Vesicles clustering near the cytosol-immersed region of sister membranes. Contact or fusion between vesicles and membranes is indicated. The rectangular inset shows an enlarged view of a gap junction (GJ) plaque. C, Putative fusion events between vesicles and free-ended membranes. D, Vesicles found near the separated sister membranes. Plasma membranes are in blue.

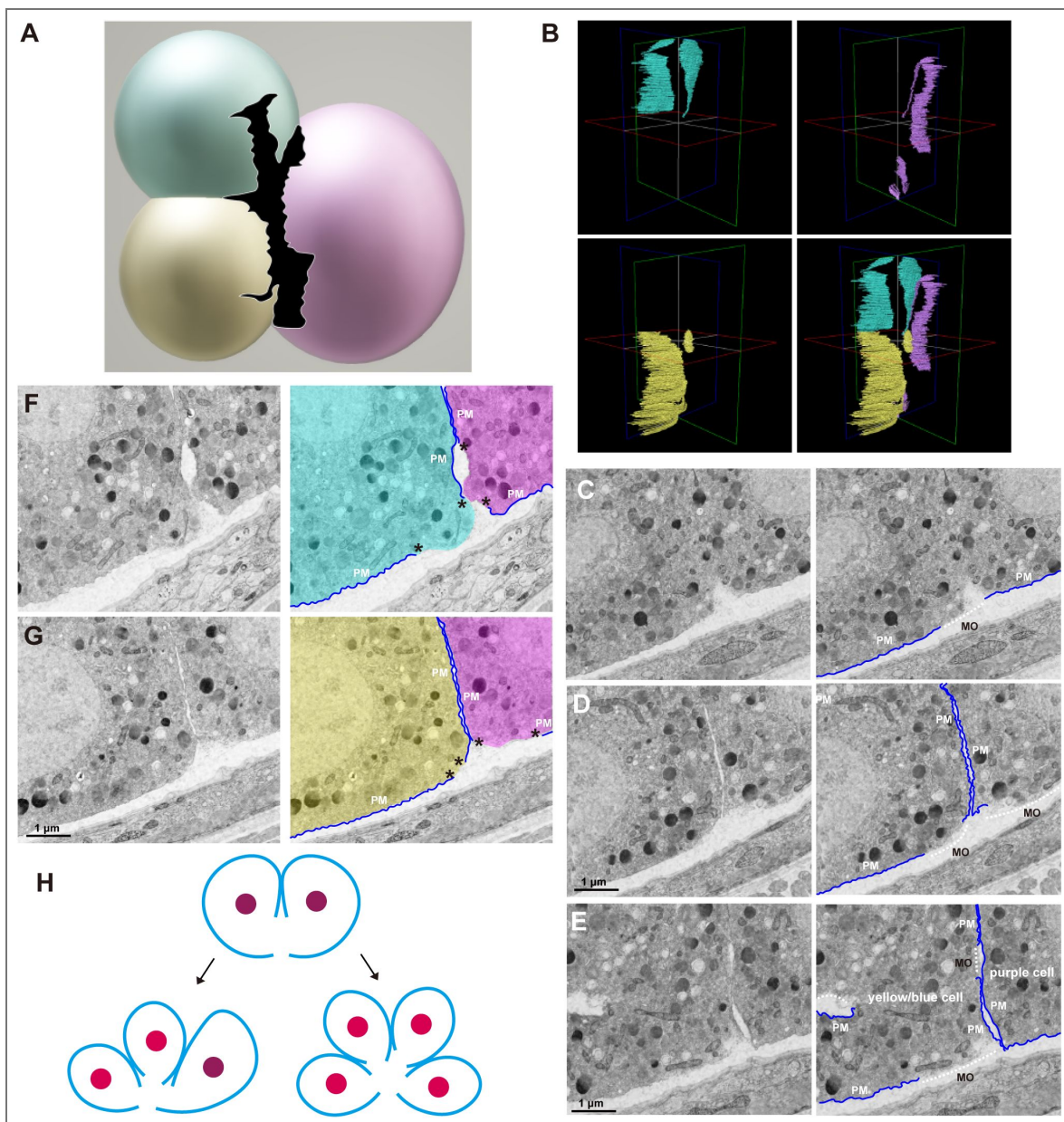


Figure 5. Membrane rupture on a tri-nucleated cell.

A, The 3D model of a tri-nucleated cell showing the membrane rupture (black). B, Reconstruction of the membrane ruptures and adjacent plasma membranes. Individual membranes, each associated with a distinct nucleus, are color-coded (blue, yellow, and purple). C and D, Membrane openings (MO, dashed white lines) arise either from separation between sister membranes (C) or between the severed membrane edges of the same cell (D). E, In a single EM section, the multiple openings originate from the same rupture. F and G, The opposing severed edges are close to each other in the yellow, purple and blue cells. Plasma membranes (PM) are indicated in blue. Asterisks indicate membrane tips. H, Schematic diagram of the formation of cells with three or four nuclei.

Using consecutive EM sections, we traced membrane integrity in this tri-nucleated cell. We found that this tri-nucleated cell was enveloped by three distinct plasma membranes, each containing a single rupture site. These three ruptures converged to form a “Y”-shaped exposed cytoplasmic region spanning >351 sections (21,060 nm; [Figures 5A](#) and [5B](#) and [Video S2](#)). Throughout these 351 consecutive sections, we detected no continuity between adjacent membranes, indicating that membrane independence had been established by this stage. Consecutive tracing further showed that the MOs—frequently observed in single EM sections ([Figures 1](#) and [2](#))—could arise from either the separation between sister membranes ([Figure 5C](#)) or from separated edges of the plasma membrane within the same cell ([Figure 5D](#)). Additionally, multiple openings within a single cell might represent different cross-sectional planes of a common membrane rupture ([Figure 5E](#)).

In all three cells (where cytoplasmic separation was incomplete), the free membrane edges were in close proximity (marked by *; [Figures 5F](#) and [5G](#)), suggesting they were in late cytokinesis and that membrane extension mediated by detached membranes could persist into this stage. Taken together, these findings demonstrate that this tri-nucleated cell was undergoing concurrent cytokinesis, with three detached membranes independently extending toward a common cytoplasmic region to achieve cytoplasm individualization ([Figure 5H](#)). A similar mechanism may operate in four-nucleated cells ([Figure 3D](#), for instance), in which two detached daughter membranes—without completing the last round of cell division—undergo further rupture, forming four granddaughter membranes. These membranes then extend independently to complete cell division ([Figure 5H](#)).

Capturing the membrane rupture and independent extension of detached sister membranes in living embryos

While high resolution HPF-TEM analysis revealed widespread membrane discontinuities in early *C. elegans* embryos, this static imaging approach cannot capture dynamic membrane behavior. To investigate whether optical microscopy can visualize membrane ruptures and the movement of severed sister membranes, we employed a fluorescence-labeled membrane marker, GFP::PH(PLC181) (see Methods and Materials for details). Our live imaging detected both putative membrane ruptures ([Figure 6A](#)) and free-ended sister membrane structures ([Figures 6B](#) and [6C](#)), providing additional evidence that membrane rupture and independent extension of detached sister membranes underlie cytokinesis in *C. elegans* embryos (see Discussion).

Sealing the exposed cytosol by neighboring membranes and extracellular matrix layers

The integrity of the plasma membrane is essential for cell survival, as its disruption can lead to cytoplasmic leakage and, if severe, result in cell death. However, widespread cytoplasmic leakage has not been observed in wild-type *C. elegans* embryos. Based on our EM observations, we propose two mechanisms through which dividing embryonic cells may seal or cover exposed cytosolic regions.

First, the sharing of plasma membrane with neighboring cells may protect the exposed cytosol. As repeatedly shown in EM images in this study ([Figures 1](#), [3](#) and [4](#)), a single elongated free-ended membrane from one cell consistently helps define boundaries with adjacent cells. These neighboring cells may be direct sister cells derived from the same mother cell, or cousin cells originating from mother cells that were once sisters. In cases where three nuclei share a common cytosol, the adjacent cell could even be an aunt or nephew cell.

In addition, eggshell components may seal cytoplasmic regions lacking membrane coverage. The *C. elegans* eggshell is composed of six distinct extracellular matrix layers ([Olson et al., 2012](#); [Benenati et al., 2009](#); [Rappleye et al., 1999](#); [Schierenberg and Junkersdorf, 1992](#); [Stein, 2018](#)). Among these, the fifth permeability barrier layer (PBL), a lipid-rich structure, serves as the osmotic and permeability barrier for the developing embryo ([Olson et al., 2012](#); [Benenati et al., 2009](#)). Normally, the PBL encloses the peri-embryonic layer (PEL), which separates it from

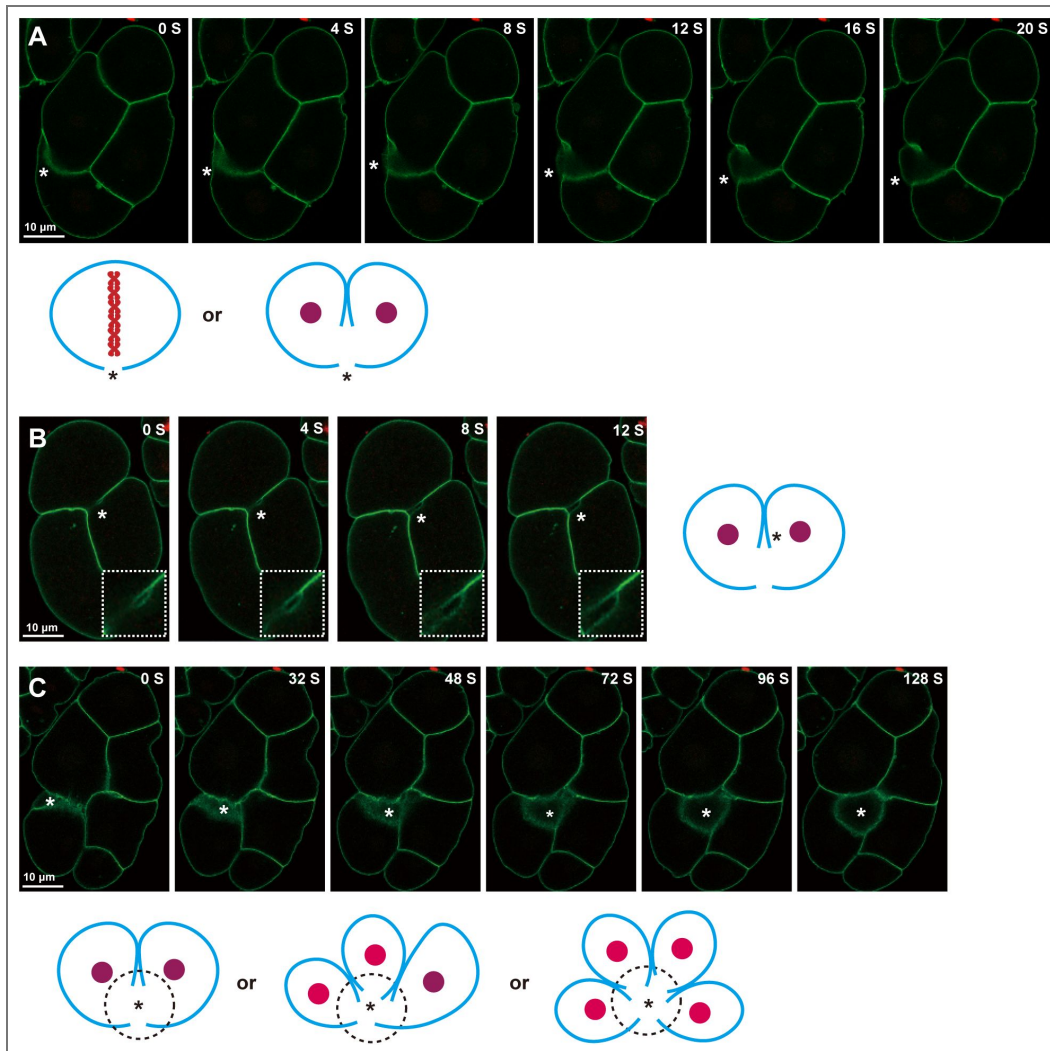


Figure 6. Detection of membrane disruptions by optical microscopy.

A, The putative membrane rupture and extension (*). B, Two extending free-ended sister membranes (*). Insets are enlarged views. C, Possible free-ended edges of detached membranes, probably from multiple cells (*). Below are proposed models illustrating the membrane disruptions that could explain the anomalous GFP signals. Images were taken at 4-second intervals for (A and B) and 8-second intervals for (C). Green signal is GFP::PH(PLC1δ1).

the embryonic plasma membrane (Olson et al., 2012 [↗](#); Benenati et al., 2009 [↗](#)) (Figures 7A [↗](#) and 7B [↗](#)). In contrast, in regions where the plasma membrane was absent, we observed the PBL adhering directly to the exposed cytoplasm (red triangles and dashed red lines, Figures 7B [↗](#) and 7C [↗](#)), bypassing the PEL. This phenomenon was also evident in other EM images (Figures 1C [↗](#) and Figure 2 [↗](#), box 1). PBL-mediated sealing is not always complete. When the PBL was not accessible or only loosely covered membrane-free regions, minor cytoplasmic leakage occurred (Figure 7D [↗](#)). In cases where PBL sealing was ineffective or absent, cytoplasmic material appeared to be retained within a confined region, likely by the PEL (Figure 7D [↗](#)). However, the mechanisms by which extracellular matrix components protect the exposed cytoplasmic region remain unknown.

Discussion

In this study, we discovered that the plasma membrane of *C. elegans* embryonic cells undergo rupture at metaphase. Following this rupture, the detached sister membranes extend rather independently from each other to complete cytokinesis. To date, the ultrastructural dynamics of plasma membrane integrity during cell division remained unexplored in other species. We anticipate that our findings will catalyze comparative studies across different species or under various pathological conditions, to determine whether this mechanism represents a fundamental paradigm or a context-specific strategy. Our work established the first mechanistic framework to guide such future investigations.

The advantage of membrane rupture and independent sister membrane extension

Cell division involves both nuclear and cytoplasmic division. However, due to the difficulty of visualizing cell boundaries in living embryos, previous studies in *C. elegans* have largely emphasized nuclear events. Here, we observed that early *C. elegans* embryonic cells can contain three or four nuclei within a shared cytoplasm, indicating that cytoplasmic division can be uncoupled from nuclear division. This phenomenon is not unique to *C. elegans*—it has also been reported in other species such as *Drosophila*, where the early embryo undergoes 13 rapid nuclear divisions without cytokinesis before cellularization occurs. It has been proposed that by minimizing or delaying cytokinesis, embryos can accelerate nuclear multiplication, thereby supporting the rapid pace of early development. In this context, the embryonic cells of *C. elegans* appear to adopt a notably rapid—though seemingly risky—strategy for cell proliferation, yet one with clear developmental benefits.

First, membrane rupture may help alleviate the rise in intracellular pressure during division (Stewart et al., 2011 [↗](#); Hiramoto, 1963 [↗](#)). We observed that the size of membrane discontinuities appeared to peak during metaphase, primarily around the equatorial plane—consistent with localized pressure surges at this stage. Notably, ruptures were also detected in tri-nucleated cell clusters, where all three cells exhibited membrane discontinuities during late cytokinesis. This suggests that even though the relative size of the ruptures decreases as mitosis proceeds, membrane discontinuities may persist throughout the cell cycle. Maintaining an opening in the plasma membrane during division likely reduces internal pressure, thereby conserving energy and resources that would otherwise be expended on resisting membrane tension. These conserved resources could then be redirected toward essential processes such as chromosome segregation and membrane synthesis—a particular advantage for rapidly dividing embryonic cells.

Of note, 3D reconstruction revealed that the metaphase rupture does not fully bisect the membrane (Figure 2 [↗](#)). However, given the limitations of our EM sampling—neither random nor continuous imaging provides complete coverage—we cannot exclude the possibility that some ruptures completely sever the membrane. Regardless of the extent of separation, the rupture produces two distinct membrane domains. Since this occurs as early as metaphase, before nuclear or cytoplasmic division, the physical independence of the plasma membranes may serve as an early marker of nascent daughter cells, preceding other cellular landmarks.

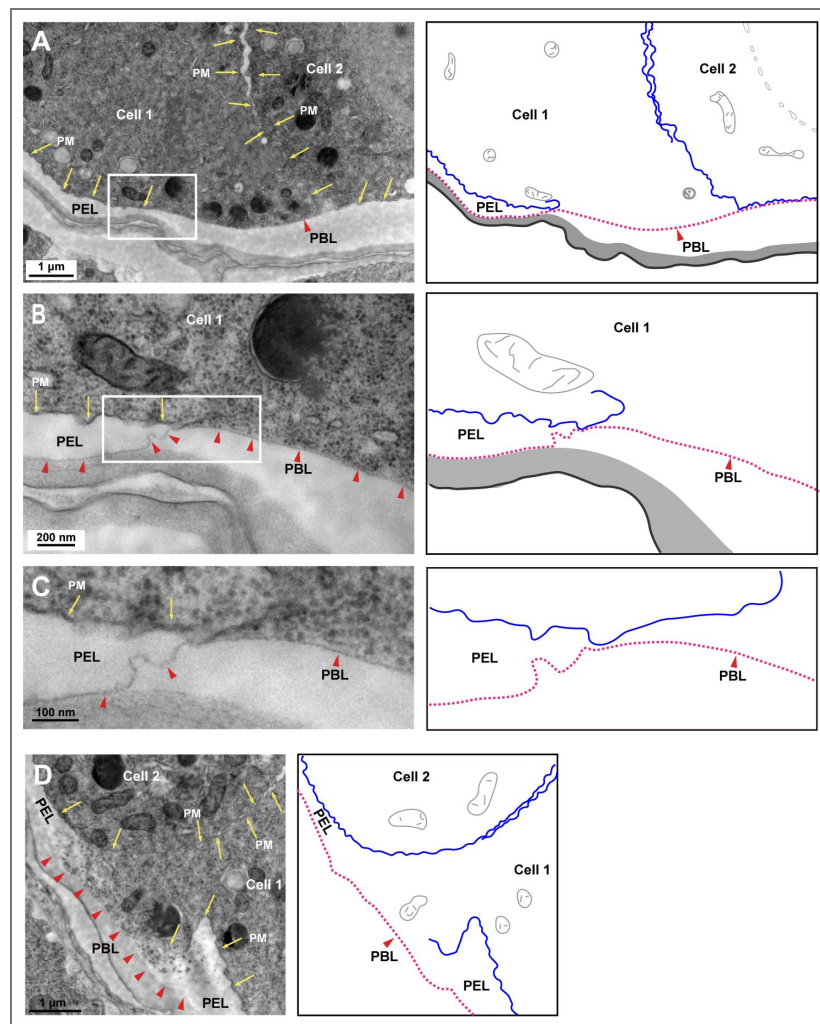


Figure 7. The exposed cytoplasmic regions are covered by extracellular matrix layers.

A-C, The exposed cytoplasmic region is covered by the permeability barrier layer (PBL, red triangles and red dashed lines). PM, plasma membrane (yellow arrows and blue lines). The boxed region in (A) is enlarged in (B), and the boxed region in (B) is enlarged in (C). D, The exposed cytoplasmic region can also be confined by the peri-embryonic layer (PEL).

Following membrane rupture, the prospective sister membranes become separated, raising the question of how cytokinesis proceeds with severed membranes. Analysis of single EM sections revealed classical membrane invagination in only a few cases (6 out of 51; [Figure 3A](#)). Notably, one such instance occurred during the transition from a one-cell to a two-cell embryo. This suggests that while a subset of *C. elegans* embryonic cells employs the membrane invagination and extension model, the majority do not. Interestingly, membrane discontinuities were observed in one-cell embryos ([Figure S1](#)), indicating that membrane rupture can co-occur with invagination. Alternatively, in dividing cells, the back-to-back invagination may be transient and may subsequently transition to free-ended extension. At present, distinguishing between these hypotheses remains challenging. A definitive resolution will likely require advanced imaging techniques capable of high-resolution, real-time visualization at the EM level.

Unlike a continuous bilayer, a free-ended membrane offers greater flexibility during cytokinesis. Without being physically constrained by its sister, each membrane can extend in different directions as needed. This independence may be particularly advantageous in accommodating asynchrony between nuclear and cytoplasmic division, as seen in early *C. elegans* embryos where multinucleated cells form through incomplete cytokinesis. In such cases, independent membrane growth from two directions facilitates cytoplasmic separation more effectively than a single continuous membrane would allow. Such autonomous movement may also explain the divergent orientations of sister membranes frequently observed in EM sections.

The expansion of the plasma membrane is generally driven by vesicle fusion. In this study, vesicles seem observed randomly distributed beneath the extending sister membranes. EM analysis further showed no morphological differences between vesicles near cytosol-immersed regions and those at conventional membrane sites. Since vesicles can fuse with the membrane at various locations, we speculate that membrane growth may be enhanced under these conditions—potentially accelerating cell cleavage during early embryogenesis. However, this remains speculative and requires further experimental validation.

Finally, when the separated membrane edges meet within daughter cells, full cytoplasmic separation is achieved. In the classical invagination-extension model, cytokinesis requires both membrane scission and fusion. In the detached-membrane mode described here, however, cytokinesis proceeds without a continuous membrane structure. This raises important questions: Is membrane scission dispensable in this context? Could bypassing scission accelerate cell separation? If so, might such a mechanism offer a selective advantage during rapid embryonic development? These questions remain open and merit further investigation.

Limitations and perspectives

In this study, the application of HPF-TEM has enabled unprecedented ultrastructural visualization of dividing cell surfaces. Our findings reveal a cytoplasmic division mechanism that challenges current models and raises important new questions regarding the plasticity of cytokinesis.

HPF-TEM offers a significant advantage for membrane studies by capturing cellular structures in a near-native state. Through ultra-rapid freezing under high pressure, it suppresses ice crystal formation and preserves water in a vitreous state, thereby minimizing chemical artifacts and maintaining the integrity of lipids and proteins. This method provides a faithful snapshot of membrane architecture and dynamics ([Moor et al., 1980](#)). In our experiments, the consistent observation of limited number of membrane discontinuities—generally one per dividing cell—that are closely associated with cell division strongly suggests these are genuine biological structures rather than preparation-induced artifacts. While one might argue that such features could reflect EM-related artifacts specific to the plasma membrane during division, the correlative evidence presented here supports their biological relevance.

Using fluorescence-labeled membrane markers, we identified membrane ruptures and free-ended sister membrane structures in live embryos ([Figure 6](#)). Similar “abnormal” or “fuzzy” membrane signals have in fact been reported previously ([Harrell and Goldstein, 2011](#)). However, interpreting these structures has remained challenging for both technical and conceptual

reasons. Technically, the rapid kinetics of membrane extension—about 10–30 seconds during metaphase and less than 3 minutes during cytokinesis—combined with cell motility, introduce inherent spatiotemporal ambiguities. Conceptually, the longstanding dominance of the membrane invagination model has likely shaped—and potentially constrained—interpretations of membrane dynamics. This paradigm may have led researchers to prioritize observations consistent with invagination, while dismissing ambiguous structures such as free-ended extensions as artifacts, rather than considering them as potential elements of alternative mechanisms. Furthermore, theoretical and computational models of cytokinesis have largely been built on invagination-centric assumptions, further narrowing the conceptual framework.

A key limitation is our current inability to capture 3D dynamics of these membrane structures in real time, a challenge that awaits imaging technologies with sufficient spatiotemporal resolution at near-EM levels.

Therefore, while our findings propose a transformative model for cytokinesis, they open a new field of inquiry. Fully validating this mechanism and exploring its conservation and implications will require an integrated approach combining advanced live-cell imaging, biophysical modeling, and genetic tools.

Methods and Materials

Examination of *C. elegans* embryonic cells

Wild-type N2 worms were cultured on OP50-seeded NGM plates at 22°C (Brenner, 1974 [↗](#)). To obtain images of early embryos, gravid hermaphrodites were collected and sectioned. At low magnification ($\times 4000$), individual embryonic cells were identified by the presence of a nucleus. In sections where the nucleus was not visible, cytoplasmic regions—whether covered or not covered by plasma membrane—were counted as individual cells if they were separated from surrounding cells. A total of 425 cells were examined (summarized in Figure 1C [↗](#)), with one EM section analyzed per cell. For embryos with fewer than 10 cells (one or two rows of cells), every cell in the given section was examined. For embryos with three or more rows of cells, only cells at the periphery were analyzed. At higher magnification ($\times 30,000$), the entire surface of each cell was examined, revealing 159 cells with one or more membrane openings. Higher magnification improved the resolution of cytoplasmic continuity, reducing potential misidentification of multinucleated cells or interconnected cytoplasmic regions that might have been miscounted at lower magnification. A total of 60 early embryos were serially sectioned as completely as possible, with each embryo yielding at least 20 sections. While Figure 1C [↗](#) presents summary data, images from additional sections were also used for further analyses. Since these additional sections may capture different cells, the total number of embryonic cells examined likely exceeds 425.

Additionally, we examined eight embryos laid outside the worm. Among these, four pre-comma stage embryos each contained at least one MO-positive cell. In another pre-comma stage embryo, no MOs were detected after examining 17 cells. Analysis of one comma-stage embryo (19 cells examined) and two 1.5-fold-stage embryos (>30 cells analyzed each) revealed no MOs.

Identification of MOs

The entire cell surface of individual embryonic cells was examined using electron microscopy at $30,000\times$ magnification. The isolated cytoplasmic regions without plasma membrane coverage were defined as MOs. Sites where the plasma membrane terminated and reappeared were marked as putative MOs. In periphery region of worm embryos, in the non-plasma-membrane-covering regions, the PEL was generally absent, while the PBL typically extended over the exposed cytoplasmic surface. Plasma membranes often appeared wrinkled, whereas cytoplasmic regions covered by the PBL exhibited a smoother appearance. Smooth, non-plasma-membrane-covered regions overlaid by the PBL are readily identifiable as MOs. When exposed cytoplasm was not tightly covered by the PBL, cytoplasmic leakage—still confined to a specific area by the PEL—could also indicate a MO. Given the variable morphology and frequent cytoplasmic leakage, membrane discontinuity size could not serve as a reliable criterion for defining MOs. However, to ensure

robustness in our analysis, we considered only linear membrane discontinuities exceeding 200 nm as genuine openings. Each MO was examined independently at least three times. When necessary, adjacent sections were also reviewed to conform an MO's presence, and identification was finalized only after all three assessments were positive.

High-pressure freezing and transmission electron microscopy

Basic protocols for high-pressure freezing of a range of organisms, including *C. elegans*, have been described (Weimer, 2006 [↗](#); McDonald, 2007 [↗](#); Manning and Richmond, 2015 [↗](#)). Briefly, adult worms were loaded into carriers and cryofixed using a Leica Microsystems EM ICE high-pressure freezer, followed by automatic cooling in liquid nitrogen. The carriers used for freezing (Leica Microsystems, Germany; catalog nos. 16770153 and 16770152) were coated with a non-stick layer of 1-hexadecene (Sigma-Aldrich). After high-pressure freezing (HPF), the samples were transferred under liquid nitrogen to a Leica AFS-2 freeze-substitution unit and incubated at -90°C for 72 h in a freeze-substitution solution consisting of acetone with 2% (wt/vol) osmium tetroxide. The temperature was then raised at a rate of $8^{\circ}\text{C}/\text{h}$ for 4 h, held at -60°C for 12 h, increased again at $5^{\circ}\text{C}/\text{h}$ for 6 h, held at -30°C for 10 h, and finally raised to 10°C at a rate of $4^{\circ}\text{C}/\text{h}$ for 10 h. Samples were washed four times in acetone, stained with 1% uranyl acetate for 1 h, and rinsed three times with pure acetone. They were then infiltrated stepwise in increasing concentrations of SPI-Chem™ resin (2:1 resin:acetone for 3 h, 1:1 for 5 h), followed by two incubations in 100% fresh resin (4 h each). The samples were placed in fresh resin in embedding molds and polymerized in a 60°C oven for 3 days. Ultrathin sections (60 nm) were cut using a diamond knife (Diatome) on an ultramicrotome (Leica Ultracut UC7) and collected on slot copper grids (EMS). The sections were visualized using a Hitachi HT7700 transmission electron microscope operating at 80 kV. Images were recorded with a Gatan 832 ($4\text{k} \times 2.7\text{k}$) CCD camera. Data from four independent preparations were analyzed in this study.

3D reconstruction

The metaphase 3D model was prepared using the following steps. First, resin blocks containing gravid adults were prepared via high-pressure freezing and freeze substitution. Continuous ultrathin sections (60 nm thick) were manually collected using a Leica UC-7 ultramicrotome and mounted on slot grids for TEM imaging. A total of 112 serial sections were collected, though 3 sections were lost due to obstruction, resulting in 822 TEM images. Among these, 105 sections contained metaphase-stage cell information. To examine the plasma membrane integrity, high-magnification imaging was performed. Since a single high-magnification image could not cover the entire cell, multiple overlapping images were captured per section and stitched together using ImageJ to generate a complete composite image of the cellular morphology at each section. In total, 292 images were stitched. The 105 stitched images were then aligned using ImageJ, and 99 of these aligned images were used for 3D reconstruction using Imaris software. About a quarter of the cell lacked membrane discontinuities. For this portion, we estimated its size and shape based on EM sections to ensure proportional representation. Of note, single MOs smaller than 200 nm that appeared continuously across more than 3 consecutive sections were considered the endpoint of the membrane rupture. Based on this, the actual length of the membrane rupture could be underestimated at one end, while the other end was clearly defined.

The ultrastructural 3D study of the tri-nucleated cell was performed using an AutoCUTS device (Li et al., 2017 [↗](#)) and a Helios Nanolab 600i dual-beam scanning electron microscope (FEI). Adult worms were treated with high-pressure freezing, freeze-substitution, and embedding as described above. Subsequently, 351 serial sections (60 nm thick) were collected using the AutoCUTS device, and scanning electron microscopy imaging was performed. All datasets were analyzed using IMARIS 8 software. After Z-stacks were aligned, the membrane rupture was manually traced and reconstructed.

Live imaging of plasma membrane dynamics using GFP::PH

The plasma membrane was labeled using GFP::PH(PLC181). The strain carrying this marker was obtained from the Caenorhabditis Genetics Center and was originally generated in Dr. Karen Faye Oegema's laboratory. Gravid adult hermaphrodites expressing this marker were collected and washed with M9 buffer. Embryos were then released from the gravid adult worms using a 1 mL syringe needle and transferred onto a 2% agarose pad. Fluorescence imaging was performed on a Zeiss LSM-980 microscope with a 63×/1.42 oil objective. Only one embryo at a time was imaged, starting at the 2- to 4-cell stage. Images were acquired at 4 or 8-second intervals, with total imaging duration typically kept under 30 minutes to ensure embryo viability.

Supplementary Figures

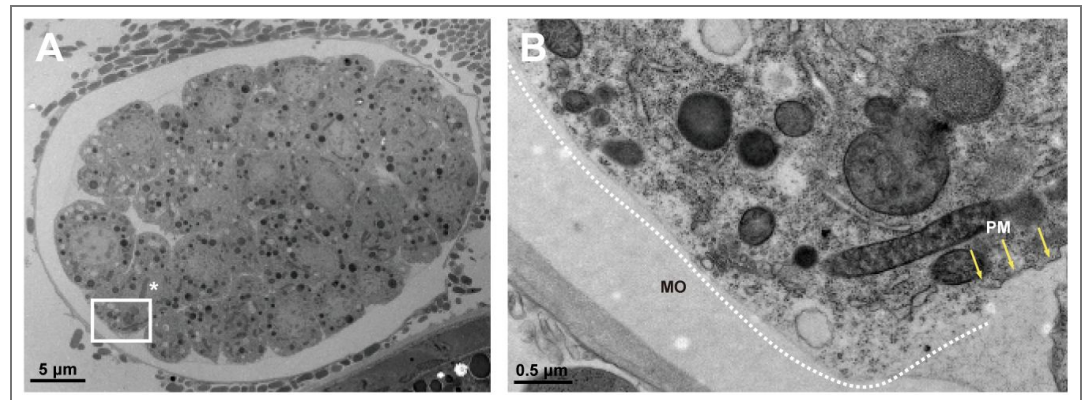


Figure S1. Membrane discontinuities in externally laid *C. elegans* embryos. A, An externally laid embryos with a membrane discontinuity (white box). B, Higher-magnification view of the membrane opening (MO) (white dashed line). Yellow arrow, plasma membrane; asterisk (*), condensed chromosome.

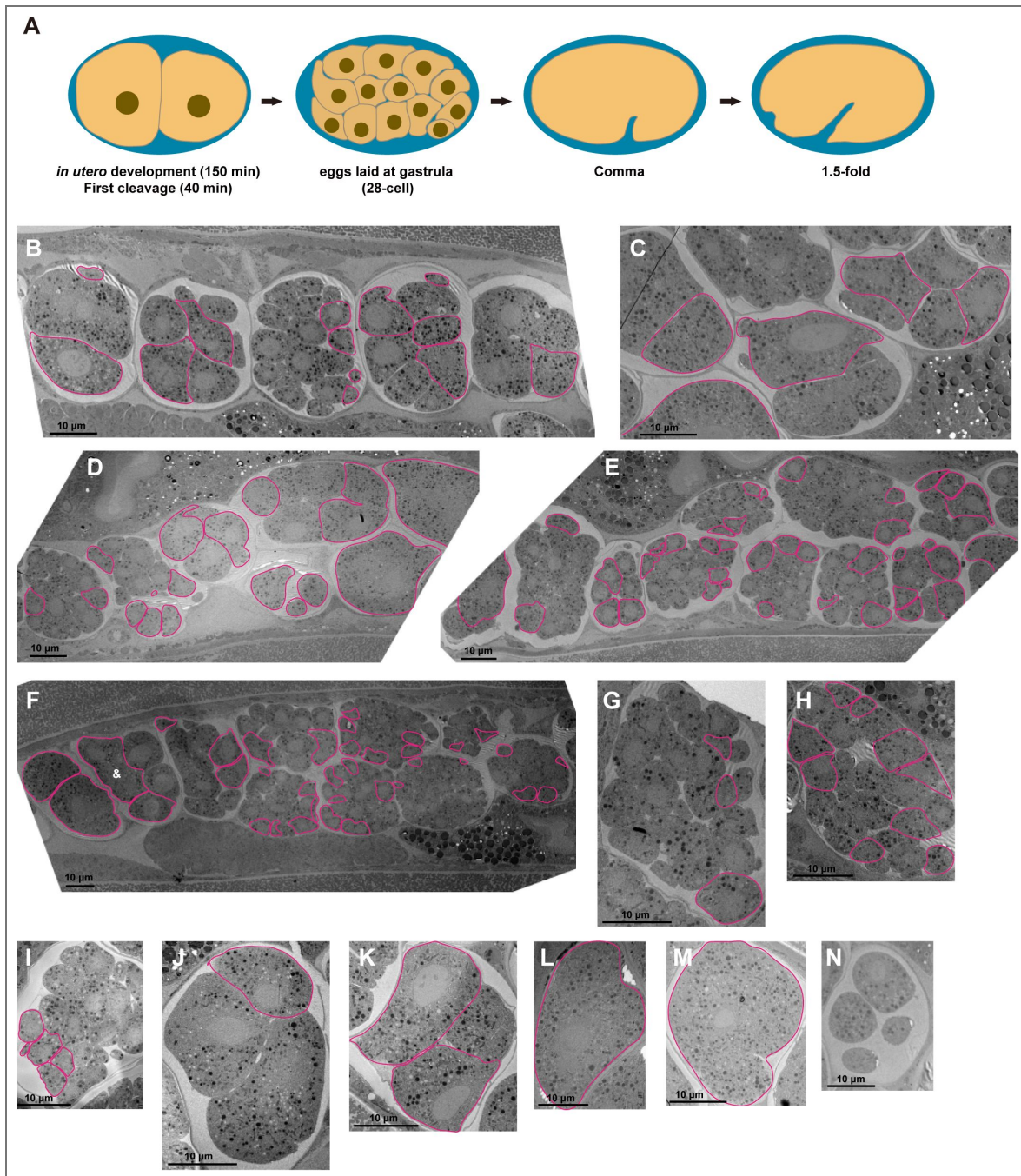


Figure S2. The wide distribution of cells with membrane discontinuities in early *C. elegans* embryos.

A, Schematic diagram of *C. elegans* embryogenesis. B-N, Representative low-magnification EM images of early embryos within adult hermaphrodites. Red lines outline cells contain membrane openings. In (F), a tri-nucleated cell is marked with &. (L) and (M) show embryos during the transition from the one-cell to the two-cell stage.

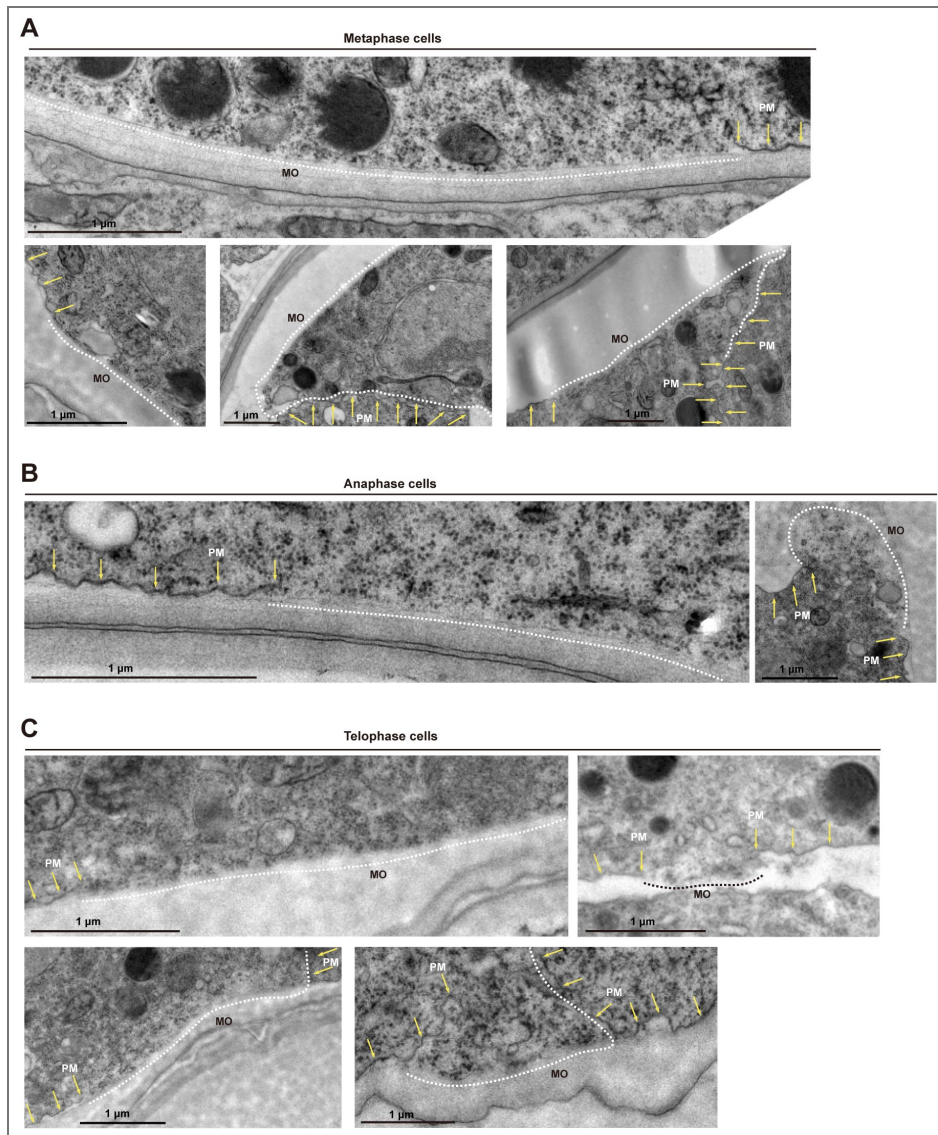


Figure S3. Membrane discontinuities in cells during mitosis.

A-C, Membrane discontinuities or openings (MO, white or black dashed lines) in cells at metaphase (A), anaphase (B) or telophase (C). Yellow arrows points to plasma membranes (PMs).

Data availability

The current manuscript is mainly based on EM images and all data are available in the manuscript.

Acknowledgements

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Additional information

Author contributions

Study concept and design: MD, JL. EM analyses: JL, TH, MD. Drafting and major editing of original manuscript: MD, JL, TH, XH. All authors reviewed, revised, and approved the final version of the paper.

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Additional files

Video S1. [3D reconstruction of the metaphase cell in Figure 2.](#) The plasma membrane is shown in red, the condensed chromosome is in blue, and exposed cytoplasm is in green.

Video S2. [3D reconstruction of the tri-nucleated cell in Figure 5.](#) 3D reconstruction of membrane ruptures and adjacent plasma membranes. Individual membranes, each associated with a distinct nucleus, are color-coded (purple, yellow, or blue).

References

Bao Z., Zhao Z., Boyle T.J., Murray J.I., Waterston R.H. (2008) Control of cell cycle timing during *C. elegans* embryogenesis. *Developmental Biology* **318**:65-72

<https://doi.org/10.1016/j.ydbio.2008.02.054>

Bembenek J.N., Richie C.T., Squirrell J.M., Campbell J.M., Eliceiri K.W., Poteryaev D., Spang A., Golden A., White J.G. (2007) Cortical granule exocytosis in *C. elegans* is regulated by cell cycle components including separase. *Development* **134**:3837-3848 <https://doi.org/10.1242/dev.011361>

Benenati G., Penkov S., Müller-Reichert T., Entchev E.V., Kurzchalia T.V. (2009) Two cytochrome P450s in *Caenorhabditis elegans* are essential for the organization of eggshell, correct execution of meiosis and the polarization of embryo. *Mechanisms of Development* **126**:382-393

<https://doi.org/10.1016/j.mod.2009.02.001>

- Brenner S (1974) The genetics of *Caenorhabditis elegans*. *Genetics* **77**:71-94 <https://doi.org/10.1093/genetics/77.1.71>
- Deppe U., Schierenberg E., Cole T., Krieg C., Schmitt D., Yoder B., Von Ehrenstein G. (1978) Cell lineages of the embryo of the nematode *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. U.S.A* **75**:376-380 <https://doi.org/10.1073/pnas.75.1.376>
- Edgar L.G., McGhee J.D. (1988) DNA synthesis and the control of embryonic gene expression in *C. elegans*. *Cell* **53**:589-599 [https://doi.org/10.1016/0092-8674\(88\)90575-2](https://doi.org/10.1016/0092-8674(88)90575-2)
- Gerien K.S., Wu J.-Q. (2018) Molecular mechanisms of contractile-ring constriction and membrane trafficking in cytokinesis. *Biophys Rev* **10**:1649-1666 <https://doi.org/10.1007/s12551-018-0479-3>
- Harrell J.R., Goldstein B. (2011) Internalization of multiple cells during *C. elegans* gastrulation depends on common cytoskeletal mechanisms but different cell polarity and cell fate regulators. *Developmental Biology* **350**:1-12 <https://doi.org/10.1016/j.ydbio.2010.09.012>
- Hiramoto Y (1963) Mechanical properties of sea urchin eggs. I. Surface force and elastic modulus of the cell membrane. *Experimental Cell Research* **32**:59-75 [https://doi.org/10.1016/0014-4827\(63\)90069-7](https://doi.org/10.1016/0014-4827(63)90069-7)
- Li X., Ji G., Chen X., Ding W., Sun L., Xu W., Han H., Sun F. (2017) Large scale three-dimensional reconstruction of an entire *Caenorhabditis elegans* larva using AutoCUTS-SEM. *Journal of Structural Biology* **200**:87-96 <https://doi.org/10.1016/j.jsb.2017.09.010>
- Manning L., Richmond J. (2015) High-Pressure Freeze and Freeze Substitution Electron Microscopy in *C. elegans*. *Methods in Molecular Biology* **1327**:121-140 https://doi.org/10.1007/978-1-4939-2842-2_10
- McDonald K (2007) Cryopreparation Methods for Electron Microscopy of Selected Model Systems. *Methods in Cell Biology* **107**:23-56 [https://doi.org/10.1016/S0091-679X\(06\)79002-1](https://doi.org/10.1016/S0091-679X(06)79002-1)
- Rothman D. H., Singson A. (2012) Modern Electron Microscopy Methods for *C. elegans*. In: Hall D. H., Hartweg E., Nguyen K. C. Q. (Eds). *Methods in Cell Biology* Elsevier. pp. 93-149 <https://doi.org/10.1016/B978-0-12-394620-1.00004-7>
- Moor H., Bellin G., Sandri C., Akert K. (1980) The influence of high pressure freezing on mammalian nerve tissue. *Cell Tissue Res* **209** <https://doi.org/10.1007/BF00237626>
- Olson S.K., Greenan G., Desai A., Müller-Reichert T., Oegema K. (2012) Hierarchical assembly of the eggshell and permeability barrier in *C. elegans*. *Journal of Cell Biology* **198**:731-748 <https://doi.org/10.1083/jcb.201206008>
- Pintard L., Bowerman B. (2019) Mitotic Cell Division in *Caenorhabditis elegans*. *Genetics* **211**:35-73 <https://doi.org/10.1534/genetics.118.301367>
- Pollard T.D., O'Shaughnessy B. (2019) Molecular Mechanism of Cytokinesis. *Annu. Rev. Biochem* **88**:661-689 <https://doi.org/10.1146/annurev-biochem-062917-012530>
- Rappleye C.A., Paredes A.R., Smith C.W., McDonald K.L., Aroian R.V. (1999) The coronin-like protein POD-1 is required for anterior-posterior axis formation and cellular architecture in the nematode *Caenorhabditis elegans*. *Genes & Development* **13**:2838-2851 <https://doi.org/10.1101/gad.13.21.2838>
- Schierenberg E., Junkersdorf B. (1992) The role of eggshell and underlying vitelline membrane for normal pattern formation in the early *C. elegans* embryo. *Roux's Arch Dev Biol* **202**:10-16 <https://doi.org/10.1007/BF00364592>
- Stein K.K (2018) The *C. elegans* eggshell. *WormBook* 1-36 <https://doi.org/10.1895/wormbook.1.179.1>
- Stewart M.P., Helenius J., Toyoda Y., Ramanathan S.P., Muller D.J., Hyman A.A. (2011) Hydrostatic pressure and the actomyosin cortex drive mitotic cell rounding. *Nature* **469**:226-230 <https://doi.org/10.1038/nature09642>
- Sulston J.E., Schierenberg E., White J.G., Thomson J.N. (1983) The embryonic cell lineage of the nematode *Caenorhabditis elegans*. *Developmental Biology* **100**:64-119 [https://doi.org/10.1016/0012-1606\(83\)90201-4](https://doi.org/10.1016/0012-1606(83)90201-4)

Weimer R.M (2006) Preservation of *C. elegans* Tissue Via High-Pressure Freezing and Freeze-Substitution for Ultrastructural Analysis and Immunocytochemistry. *Methods in Molecular Biology* **351**:203-221 <https://doi.org/10.1385/1-59745-151-7:203>

Zhang Y., Foster J.M., Nelson L.S., Ma D., Carlow C.K.S. (2005) The chitin synthase genes *chs-1* and *chs-2* are essential for *C. elegans* development and responsible for chitin deposition in the eggshell and pharynx, respectively. *Developmental Biology* **285**:330-339 <https://doi.org/10.1016/j.ydbio.2005.06.037>

Zhu J., Lam S.M., Yang L., Liang J., Ding M., Shui G., Huang X. (2022) Reduced phosphatidylcholine synthesis suppresses the embryonic lethality of seipin deficiency. *Life Metabolism* **1**:175-189 <https://doi.org/10.1093/lifemeta/loac021>

Peer reviews

Reviewer #1 (Public review):

Summary:

Using electron microscopy, the authors report discontinuities in the plasma membrane of *C. elegans* embryos. They associate these discontinuities with cell division and speculate that membrane rupture and subsequent resealing contribute to cytokinesis. They further discuss the proximity of these sites to vesicles and propose a role for vesicle-mediated membrane extension.

Weaknesses:

(1) The possibility that the membrane discontinuity is an artifact

Although the authors focus on discontinuities in the plasma membrane, similar discontinuities are also observed in mitochondria, the nuclear envelope, and yolk granules. This raises concerns about whether the electron micrographs presented are suitable for assessing membrane continuity.

Electron micrographs result from a lengthy sample preparation process, including high-pressure freezing, freeze substitution in acetone containing OsO₄, gradual warming, uranyl acetate staining, resin embedding, and ultrathin sectioning. In general, lipids are soluble in acetone at temperatures above -30 {degree sign}C, and preservation of membrane structures relies heavily on efficient OsO₄ fixation. Insufficient OsO₄ treatment would be expected to reduce membrane contrast.

C. elegans embryos are encapsulated by an eggshell that forms at fertilization and gradually develops during the first few cell divisions. It is unclear how efficiently OsO₄ in acetone penetrates the eggshell during freeze substitution, raising further concern about plasma membrane preservation under the conditions used.

(2) Lack of evidence linking membrane discontinuity to cell division

The reported plasma membrane discontinuities are not specific to mitotic cells. If this were a physiological process playing an important role in cytokinesis, it should occur in a temporally and spatially coordinated manner with nuclear division. However, it remains unclear at what stage of the cell cycle the membrane rupture occurs and where it is located relative to chromosomes and the mitotic spindle.

(3) Lack of evidence for extension of the separated membrane

Although the authors speculate that resealing of the ruptured membrane occurs via extension of the separated membrane, no direct evidence supporting this mechanism is

presented. Proximity to vesicles alone does not demonstrate that membrane extension occurs through vesicle fusion. More direct evidence is required to support this claim.

(4) Inconsistency with published work

Numerous studies have examined cell division in developing *C. elegans* embryos using the GFP::PH(PLC181) marker expressed from the *ltIs38* transgene [pAA1; *pie-1*::GFP::PH(PLC181) + *unc-119(+)*], generated by the Oegema lab (https://wormbase.org/species/c_elegans/transgene/WBTransgene00000911#01--10). To date, no study has reported membrane ruptures of the magnitude described here. The complexity of cell surface morphology from the 8- to 12-cell stages onward has been well documented, for example, by Fu et al. (2016) using light-sheet microscopy and 3D reconstruction (doi:10.1038/ncomms11088).

Supplementary Movies 5, 6, and 10 of this paper illustrate how single-plane images can easily produce apparent membrane discontinuities, for example, due to membrane orientations nearly parallel to the imaging plane.

The three single-plane images from only three embryos presented in Figure 6 are insufficient to support the authors' strong conclusions. Raw 3D data should be provided.

<https://doi.org/10.7554/eLife.109984.1.sa3>

Reviewer #2 (Public review):

Summary:

Liang et al. explore an unusual observation of membrane discontinuities in dividing *C. elegans* embryonic cells. This report is the first to demonstrate that, instead of the classical invagination of membranes during cytokinesis, cells in the early embryos of *C. elegans* exhibit separation of sister membranes that extend independently. TEM images of high-pressure-frozen samples provide strong evidence for the presence of Membrane Openings (MOs) in cells at various stages of the cell cycle, predominantly during mitosis. High-resolution images (x 30,000) clearly show the wrinkled plasma membrane and smooth MOs.

The electron microscopy data are supported by the live cell imaging of strains with fluorescently tagged membrane markers. This study opens up the possibility of tracking MOs at other stages of *C. elegans* development, and also asks if it might be a common phenomenon in other species that exhibit rapid embryonic growth and divisions.

Strengths:

(1) Thorough verification of Membrane Openings (MO) by several methods:

(a) 4 independent sample batches.

(b) Examined historical collections.

(c) Analysed embryos at different stages of development. The absence of MOs in later stages (comma) serves as a negative control and gives confidence that MOs are genuine and not technical artifacts.

(2) Live cell imaging of strain with fluorescently labelled membranes provides real-time dynamics of membrane rupture.

(3) After observing the membrane rupture, the next obvious question is - what prevents the cytosol from leaking out? The EM images showing PBL and PEL - extracellular matrix serving as barriers for the cytosol are convincing.

Weakness:

(1) The association of membrane discontinuities with cell division is not convincing, as there are 159 cells out of 425 showing MOs, but it is not mentioned clearly how many of these are undergoing cell division. Also, it's not clear whether the 20 dividing cells analysed for MOs are a part of the 159 cells or a separate dataset. A graphical representation of the number of samples and observed frequencies would be helpful to understand the data collection workflow.

(2) In Figures 3A and 3B, the resolution of the images is not enough to verify 3A as classical membrane invagination and 3B as detached sister membranes.

(3) Figure 6 lacks controls. How does the classical invagination look in this strain? Also, adding nuclear dye would be informative, in order to correlate the nuclear division with membrane rupture, as claimed.

<https://doi.org/10.7554/eLife.109984.1.sa2>

Reviewer #3 (Public review):

Summary:

In this manuscript, the authors challenge a dogma in cell biology, namely that cells are at any time point engulfed by a continuous plasma membrane. Liang et al. find that during *C. elegans* embryogenesis, a high number of cells are not entirely surrounded by a plasma membrane but show membrane openings (MOs). These openings are enriched at the embryo's periphery, towards the eggshell. The authors propose that plasma membrane discontinuities emerge during metaphase of mitosis and that independent extension of "sister membranes" engulfs the daughter cells.

Strengths:

On the positive side, the authors find plasma membrane discontinuities not only by electron microscopy but also by fluorescence microscopy and provide information about the dynamics of membrane openings and their emergence. While this is assuring, the authors conclude that MOs emerge during metaphase. From what the authors show, this particular information cannot be deduced, as there is no dynamic capture of a membrane scission event together with a chromatin marker that would indicate mitosis. The authors could, however, attempt to find such events in live movies, given the high incidence of MOs reported from their EM data.

Weaknesses:

In order to convincingly demonstrate the absence of any plasma membrane in the respective regions of the embryonic periphery or between cells of the embryo, the authors would have to show consecutive serial TEM sections where MOs are detected over more z-planes, beyond the mere 3D reconstructions. Although the authors state in the methods section that continuous ultrathin sections were cut for the metaphase sample (page 21, line 472), consecutive sections are never shown in TEM. While we do see the 3D reconstructions, better documentation of the underlying TEM data is missing. It would be necessary to show a membrane opening in consecutive z sections. Alternatively, the authors could seek the possibility to convincingly back up their claims with volume imaging by focused ion beam scanning EM (FIBSEM), where cellular volumes can be sectioned in almost isotropic resolution.

Another critical issue concerns the detection of the membrane discontinuities in electron micrographs, which, in my opinion, is ambiguous. How do the authors reliably discriminate

in their TEM images whether there is a plasma membrane or not? The absence - or weak appearance - of the stain of the electron dense material at membranes, which seems to be their criterion for MOs, is also apparent at other, intracellular membranes, like at the NE or at the ER (for example, see Figure 1C). Also, the plasma membrane itself appears unevenly stained in regions that the authors delineate as intact (for example, Figure 1C, 2B/1).

<https://doi.org/10.7554/eLife.109984.1.sa1>

Author response:

Public Reviews:

Reviewer #1 (Public review):

Summary:

*Using electron microscopy, the authors report discontinuities in the plasma membrane of *C. elegans* embryos. They associate these discontinuities with cell division and speculate that membrane rupture and subsequent resealing contribute to cytokinesis. They further discuss the proximity of these sites to vesicles and propose a role for vesicle-mediated membrane extension.*

Weaknesses:

(1) The possibility that the membrane discontinuity is an artifact

Although the authors focus on discontinuities in the plasma membrane, similar discontinuities are also observed in mitochondria, the nuclear envelope, and yolk granules. This raises concerns about whether the electron micrographs presented are suitable for assessing membrane continuity.

Electron micrographs result from a lengthy sample preparation process, including high-pressure freezing, freeze substitution in acetone containing OsO₄, gradual warming, uranyl acetate staining, resin embedding, and ultrathin sectioning. In general, lipids are soluble in acetone at temperatures above -30 {degree sign}C, and preservation of membrane structures relies heavily on efficient OsO₄ fixation.

Insufficient OsO₄ treatment would be expected to reduce membrane contrast.

**C. elegans* embryos are encapsulated by an eggshell that forms at fertilization and gradually develops during the first few cell divisions. It is unclear how efficiently OsO₄ in acetone penetrates the eggshell during freeze substitution, raising further concern about plasma membrane preservation under the conditions used.*

We thank the reviewer for raising this important technical concern. We have taken this question seriously since first observing membrane discontinuities six years ago, and we have since conducted extensive controls to rule out fixation artifacts. Below, we present multiple lines of evidence—ranging from technical reproducibility to orthogonal imaging approaches—that collectively demonstrate the biological reality of these structures.

(1) Technical expertise and standard protocols

Our laboratory has extensive experience with electron microscopy across diverse biological systems, including neurons, muscle cells, and hypodermis in *C. elegans*, as well as tissues from *Drosophila*, mouse, bacteria, and cultured cells (Chen et al., 2013; Ding et al., 2018; Guan et al., 2022; Y. Li et al., 2018; Miao et al., 2024; Qin et al., 2014; Wang et al., 2026; J. Xu et al., 2022; M. Xu et al., 2021; L. Yang et al., 2020; X. Yang et al., 2019; Zhu et al., 2022). Importantly, we did not introduce any novel or unconventional steps in our EM preparation; all protocols

were standard and well-established. Thus, the observed membrane discontinuities are unlikely to stem from technical inexperience or idiosyncratic methods.

In addition to membrane discontinuities, we would like to emphasize that a large number of single plasma membranes separating adjacent cytoplasmic domains were also detected under EM (Figure 1, 3 and 4, for instance). This observation is particularly significant because the invagination model cannot generate single plasma membrane barriers between adjacent cytoplasmic domains. Instead, independent extension of detached sister membranes could explain the formation of cytoplasm-enclosed membranes. Furthermore, as the morphology and continuity of these single cytoplasm-immersed membrane structures are well preserved, this indicates successful EM processing and argues against inefficient fixation or other technical issues.

(2) Reproducibility across independent preparations and techniques

To test whether the discontinuities were preparation-specific, we examined four independent sample batches collected in the lab over the years. Membrane discontinuities, as well as cytoplasm-immersed membranes, on embryonic cells were consistently observed across all batches, indicating that the phenomenon is not dependent on a single preparation method. Furthermore, we validated our findings using two EM techniques: transmission electron microscopy (HPF-TEM) and dualbeam scanning electron microscopy (SEM). Membrane discontinuities were clearly identifiable with both techniques, further supporting their robustness.

(3) Validation using an independent public dataset

We examined the publicly available *C. elegans* embryo EM collection (WormAtlas). In several instances, particularly at the embryonic periphery where plasma membrane discontinuities are more readily visualized (<https://www.wormimage.org/image.php?id=140265&page=1>), we identified similar structures. The presence of these features in an independent dataset generated by different researchers confirms that they are not artifacts unique to our sample preparation.

(4) Developmental regulation of membrane discontinuities

We analyzed embryos across multiple developmental stages. Membrane discontinuities were observed in both intrauterine and laid embryos at early stages. However, as embryos reached the comma stage—a period marked by the onset of elongation and reduced cell proliferation—the incidence of discontinuities dropped dramatically (0/13, 0/17, and 0/30 cells examined). This developmental specificity argues strongly against a general fixation artifact, which would be expected to occur randomly across stages. Additionally, the eggshell is present throughout the embryonic stage of *C. elegans*; therefore, the dramatic reduction of membrane discontinuities in comma-stage of embryo argues against the possibility that the eggshell poses a fixation problem.

(5) Rigorous criteria for identifying membrane discontinuities

To ensure unbiased analysis, we systematically collected images from early embryonic cells using the following criteria:

(1) Random section selection: For each sample, we randomly selected one section containing the largest number of embryos or cells (Sup Figure 2) for initial analysis. We found membrane discontinuities in 159 cells distributed across 57 embryos, representing 95% of the total sampled embryos. This portion of the data is summarized in Figure 1.

(2) Whole-membrane examination: Each putative membrane discontinuity was identified only after examining the entire plasma membrane of the cell on a given section. Importantly,

aside from the discontinuity, the remainder of the plasma membrane remained intact. Moreover, in most cells, only a single discontinuity was present per section, arguing against random, widespread membrane tearing during preparation.

(3) Neighboring section verification: Because EM preparation yields serial sections, we verified nearly all membrane discontinuities by examining adjacent sections. Again, the same membrane discontinuity was confirmed only after inspecting the entire plasma membrane on those neighboring sections as well. We will include this verification protocol in the revised Methods and additional imaging of consecutive sections would be provided if needed.

(4) Serial section reconstruction: To further determine whether a dividing cell indeed contains one membrane rupture, we performed two serial reconstruction experiments.

First, we used HPF-TEM to analyze 105 consecutive sections of a metaphase cell, reconstructing the entire plasma membrane and chromosome configuration. We found that one membrane rupture largely encircled the chromosomal disc (Figure 2 and Video S1), spatially aligning with the future segregation zone. Second, we used AutoCUTS-SEM to collect approximately 600 sections covering ~95% of a telophase cell containing three nuclei sharing a common cytoplasm. This tri-nucleated cell was enclosed by three distinct plasma membranes, each harboring a single rupture site. These three ruptures converged to form a Y-shaped exposed cytoplasmic region spanning >351 sections (Figure 5). Collectively, these reconstructions demonstrate that each cell contains only one discontinuity from a 3D point of view, further supporting that the phenomenon is not due to random sample preparation damage.

(6) Orthogonal validation by live imaging: In addition to EM, we performed live imaging of plasma membrane dynamics. While live imaging provides important temporal context, we recognize its limitations in resolving membrane ultrastructure. The rapid kinetics of membrane extension (approximately 20–30 seconds for metaphase and less than 3 minutes for cytokinesis), combined with embryo motility, introduces spatiotemporal ambiguities. To capture dynamic membrane events, our live imaging using the GFP::PH membrane marker was performed at 4-second intervals, approaching the practical limit for single-section scanning of the embryo. With single-plane live imaging, nevertheless, both membrane ruptures and free-ended sister membrane structures could be detected (Figures 6), providing additional evidence that membrane rupture and independent extension of detached sister membranes underlie cytokinesis in *C. elegans* embryos. Notably, 3D membrane dynamics analysis using light-sheet microscopy (Fu et al. Imaging multicellular specimens with real-time optimized tiling light-sheet selective plane illumination microscopy. *Nature Communications*. 2016. DOI:10.1038/ncomms11088) revealed membrane ruptures in dividing early *C. elegans* embryonic cells, including during telophase or metaphase. Therefore, live imaging further validates the membrane rupture phenomena in dividing embryonic cells in *C. elegans*.

While future advances in imaging technology may enable real-time visualization at near-EM resolution, our extensive, multi-year effort to test the artifact hypothesis has convinced us that these membrane discontinuities are genuine biological features of dividing *C. elegans* embryonic cells.

We are confident that the cumulative evidence presented here addresses the reviewer's concerns and demonstrates that the observed membrane discontinuities, as well as cytoplasm-immersed membranes, are not procedural artifacts but rather reflect a previously underappreciated aspect of plasma membrane dynamics during embryonic cell division.

(2) Lack of evidence linking membrane discontinuity to cell division

The reported plasma membrane discontinuities are not specific to mitotic cells. If this

were a physiological process playing an important role in cytokinesis, it should occur in a temporally and spatially coordinated manner with nuclear division. However, it remains unclear at what stage of the cell cycle the membrane rupture occurs and where it is located relative to chromosomes and the mitotic spindle.

Thank you for this insightful comment. We agree that establishing a direct link between plasma membrane discontinuities and mitotic progression is critical, and we appreciate the opportunity to clarify this point.

In *C. elegans* embryos, the early stages of development are characterized by rapid and extensive cell division. Within approximately 100 minutes, a two-cell embryo develops into an embryo containing nearly 30 cells. The majority of the electron microscopy analyses in our study were performed on embryos at stages with fewer than 30 cells, where most cells are actively dividing. Thus, it is reasonable to infer that the cells exhibiting membrane discontinuities are predominantly mitotic cells.

Supporting this notion, as embryos reached the comma stage—a period marked by the onset of elongation and reduced cell proliferation—the incidence of membrane discontinuities dropped dramatically (0/13, 0/17, and 0/30 cells examined). This developmental specificity strongly suggests that membrane discontinuities are tightly linked to cell division.

Importantly, mitotic features such as metaphase chromosomes aligned at the equatorial plane or two (or more) nuclei sharing common cytoplasm can be identified in EM images. In our single random EM section analysis, we captured membrane discontinuities in cells at metaphase, anaphase (characterized by fewer than 10 chromosomal clumps), and telophase (defined by two nuclei sharing cytoplasm). Hence, membrane discontinuities are indeed present on mitotic cells. In addition, a published work by Fu et al (Fu et al. Imaging multicellular specimens with real-time optimized tiling light-sheet selective plane illumination microscopy. *Nature Communications*. 2016. DOI:10.1038/ncomms11088) using light-sheet microscopy captured similar membrane discontinuities in cells displaying classical mitotic features, including anaphase or telophase.

To further investigate the spatial relationship between membrane ruptures and chromosome organization, we performed three-dimensional reconstructions on a metaphase cell. As shown in Figure 2 and Video S1, the membrane discontinuities largely encircled the condensed chromosome disc and were spatially aligned with the future segregation zone, further revealing the relative location of membrane discontinuities to chromosomes, at least at metaphase.

We further collected 3D information for a telophase cell containing three nuclei. This tri-nucleated cell was enclosed by three distinct plasma membranes, each harboring a single rupture site that merged to form a single rupture. The observation that membrane ruptures are present in a tri-nucleated cell is particularly informative. The tri-nucleated feature indicates that this cell underwent two rounds of cell division and that both divisions were at telophase. The presence of a single membrane rupture suggests that membrane discontinuities may persist throughout the cell cycle, as the second cell cycle began from a mother cell that still shared cytoplasm with its sister cell and already had one membrane rupture. Therefore, in addition to the mitotic phase, membrane discontinuities—at least in this context—also exist during the DNA synthesis stage.

(3) Lack of evidence for extension of the separated membrane

Although the authors speculate that resealing of the ruptured membrane occurs via extension of the separated membrane, no direct evidence supporting this mechanism is presented. Proximity to vesicles alone does not demonstrate that membrane extension occurs through vesicle fusion. More direct evidence is required to support this claim.

Thank you for raising this important point. We appreciate the opportunity to clarify our conclusion.

In our study, EM analysis revealed the presence of cellular vesicles in close proximity to both free membrane edges and the already separated sister plasma membranes (Figure 4). However, we acknowledge that without advanced live-cell imaging, it is not possible to conclusively determine whether the extension of these separated sister membranes occurs through vesicle fusion.

We realize that a statement in the Discussion section—“The expansion of the plasma membrane is generally driven by vesicle fusion”(page 16)—may have inadvertently led the reviewer to interpret this as our own conclusion regarding the mechanism of membrane extension in this context. In fact, that statement was intended to reflect the current general understanding of membrane expansion, not to imply that we had demonstrated such a mechanism for the free-ended sister membranes. As we subsequently noted, “However, this remains speculative and requires further experimental validation.”

To avoid any misunderstanding, we will revise this section to clearly state that the mechanism by which the separated sister membranes extend remains unknown and that further investigation is needed to determine how existing models of membrane expansion may apply to or be adapted for this novel context.

We thank the reviewer again for their thoughtful comment, which has helped us improve the clarity of our manuscript

(4) Inconsistency with published work

*Numerous studies have examined cell division in developing *C. elegans* embryos using the GFP::PH(PLC1 δ 1) marker expressed from the *ItIs38* transgene [pAA1; pie-1::GFP::PH(PLC1 δ 1) + *unc-119(+)*], generated by the Oegema lab (https://wormbase.org/species/c_elegans/transgene/WBTransgene00000911#01--10). To date, no study has reported membrane ruptures of the magnitude described here. The complexity of cell surface morphology from the 8- to 12-cell stages onward has been well documented, for example, by Fu et al. (2016) using light-sheet microscopy and 3D reconstruction (doi:10.1038/ncomms11088).*

Supplementary Movies 5, 6, and 10 of this paper illustrate how single-plane images can easily produce apparent membrane discontinuities, for example, due to membrane orientations nearly parallel to the imaging plane.

The three single-plane images from only three embryos presented in Figure 6 are insufficient to support the authors' strong conclusions. Raw 3D data should be provided.

Thank you for this important comment. We fully agree that the GFP::PH(PLC1 δ 1) marker, generated by the Oegema lab, has been widely and effectively used to study various aspects of *C. elegans* embryonic development. In fact, we also employed this same marker in our study to assess membrane integrity.

However, while live imaging provides invaluable temporal resolution, its limitations in resolving membrane ultrastructure are substantial. In *C. elegans* embryos, early development is marked by rapid and extensive cell divisions. Within approximately 100 minutes, a two-cell embryo develops into one containing nearly 30 cells. During this fast-dividing stage, the rapid kinetics of membrane extension—approximately 20–30 seconds during metaphase and less than 3 minutes during cytokinesis—combined with embryo motility, introduce considerable spatiotemporal ambiguities. Furthermore, the longstanding invagination model of cytokinesis has shaped interpretations in the field, which may have led to ambiguous structures such as

free-ended extensions being dismissed as potential artifacts rather than recognized as alternative morphological features. Theoretical and computational models have largely been built upon invagination-centric assumptions, which may have further constrained conceptual frameworks. Therefore, fluorescence protein-based live imaging analysis alone could not serve as a convincing approach to challenge the current dogma of cell division, nor did we intend it to.

However, when reexamined in light of our findings, previous studies using this same GFP marker have in fact revealed membrane discontinuities that went unnoticed. For example, Fu et al (Fu et al. Imaging multicellular specimens with real-time optimized tiling light-sheet selective plane illumination microscopy. *Nature Communications*. 2016. DOI:10.1038/ncomms11088) using light-sheet microscopy and 3D reconstruction, captured membrane discontinuities in cells undergoing mitotic phases such as anaphase or telophase. Similarly, an earlier study by Harrell and Goldstein (Harrell and Goldstein. 2011. Internalization of multiple cells during *C. elegans* gastrulation depends on common cytoskeletal mechanisms but different cell polarity and cell fate regulators. *Developmental Biology*. DOI:10.1016/j.ydbio.2010.09.012) showed regions where the GFP::PH signal appeared fuzzy and discontinuous.

Nevertheless, given the inherent limitations of fluorescence microscopy in resolving membrane ultrastructure, high-resolution electron microscopy—supported by rigorous controls and serial section analysis—remains the gold standard for definitively identifying such membrane discontinuities.

We acknowledge that our findings are surprising. We did not set out to challenge the long-held view of membrane integrity during cell division. In fact, this study began when our dedicated EM technician, Jingjing Liang, first observed membrane discontinuity phenomena in control samples—wild-type embryos. Had she not come across this observation, we likely would never have pursued this line of inquiry.

We appreciate the opportunity to clarify these points and thank the reviewer for thoughtful engagement with our work.

Reviewer #2 (Public review):

Summary:

*Liang et al. explore an unusual observation of membrane discontinuities in dividing *C. elegans* embryonic cells. This report is the first to demonstrate that, instead of the classical invagination of membranes during cytokinesis, cells in the early embryos of *C. elegans* exhibit separation of sister membranes that extend independently. TEM images of high-pressure-frozen samples provide strong evidence for the presence of Membrane Openings (MOs) in cells at various stages of the cell cycle, predominantly during mitosis. High-resolution images (x 30,000) clearly show the wrinkled plasma membrane and smooth MOs.*

*The electron microscopy data are supported by the live cell imaging of strains with fluorescently tagged membrane markers. This study opens up the possibility of tracking MOs at other stages of *C. elegans* development, and also asks if it might be a common phenomenon in other species that exhibit rapid embryonic growth and divisions.*

Strengths:

(1) Thorough verification of Membrane Openings (MO) by several methods:

(a) 4 independent sample batches.

(b) Examined historical collections.

(c) Analysed embryos at different stages of development. The absence of MOs in later stages (comma) serves as a negative control and gives confidence that MOs are genuine and not technical artifacts.

(2) Live cell imaging of strain with fluorescently labelled membranes provides realtime dynamics of membrane rupture.

(3) After observing the membrane rupture, the next obvious question is - what prevents the cytosol from leaking out? The EM images showing PBL and PEL - extracellular matrix serving as barriers for the cytosol are convincing.

Thanks to the reviewer for the encouragement. Highly appreciated.

Weakness:

(1) The association of membrane discontinuities with cell division is not convincing, as there are 159 cells out of 425 showing MOs, but it is not mentioned clearly how many of these are undergoing cell division. Also, it's not clear whether the 20 dividing cells analysed for MOs are a part of the 159 cells or a separate dataset. A graphical representation of the number of samples and observed frequencies would be helpful to understand the data collection workflow.

We sincerely thank the reviewer for raising this important question and appreciate the opportunity to clarify these points.

(1) Relationship between membrane discontinuities and cell division

In *C. elegans* embryos, early development is characterized by rapid and extensive cell division: within approximately 100 minutes, a two-cell embryo develops into one containing nearly 30 cells. Most of our electron microscopy (EM) analyses were performed on embryos at stages with fewer than 30 cells, in which the majority of cells are actively dividing. Therefore, it is reasonable to infer that the cells exhibiting membrane discontinuities (MOs) are predominantly mitotic. Supporting this, as embryos reached the comma stage—when cell proliferation declines and elongation begins—the incidence of MOs dropped sharply (0/13, 0/17, and 0/30 cells examined). This developmental specificity strongly links MOs to cell division.

Moreover, in single random EM sections, we observed MOs in cells displaying clear mitotic features, such as metaphase chromosomes aligned at the equatorial plate, or anaphase/telophase configurations (fewer than 10 chromosomal clumps or two nuclei sharing common cytoplasm). Thus, MOs are indeed present in mitotic cells.

From our 3D reconstruction (Figure 5), we identified a telophase cell containing three nuclei, each enclosed by its own plasma membrane, with each membrane harboring a single rupture that converged into a single opening. This tri-nucleated configuration indicates that the cell had undergone two rounds of division and was at telophase in both. The presence of a single membrane rupture in this context suggests that MOs can persist beyond mitosis, as the second cell cycle initiated from a mother cell that already shared cytoplasm with its sister and already contained a rupture. Thus, in this case, MOs were also present during DNA synthesis stage.

(2) Clarification of sample numbers and datasets

In Figure 1, we present results from a single EM section per embryonic cell, with sections randomly selected per embryo as detailed in Sup Figure 2. This initial dataset (425 cells)

forms the basis of Figure 1.

From the same pool of 425 cells, we used additional EM sections—distinct from those shown in Sup Figure 2—to locate 20 dividing cells for analysis of membrane discontinuities. Thus, while these 20 cells originated from the same set of embryos, they were not derived from the sections used in Figure 1 or Sup Figure 2.

A graphical summary of sample numbers from the single-section analysis is already provided in Figure 1. Notably, cells with two clearly visible nuclei are more likely to be sectioned through or near their maximal diameter. In contrast, the randomly selected sections used for Figure 1 captured cells at variable planes, reducing the likelihood of observing MOs. Consistent with this, in the three embryos where no MOs were detected (one example is Sup Figure 2N), the sections likely passed through peripheral regions of the cells. Consequently, the frequency of MOs in randomly sectioned cells (Figure 1) is not directly comparable to that observed in the 20 dividing cells, which were analyzed using sections more likely to capture cells near their maximal diameter. These 20 dividing cells should therefore be considered a separate analysis. We will add detailed explanations in the Methods section to ensure this distinction is clearly understood.

We are grateful for the reviewer's thoughtful feedback and believe these clarifications will improve the clarity and rigor of the manuscript.

(2) In Figures 3A and 3B, the resolution of the images is not enough to verify 3A as classical membrane invagination and 3B as detached sister membranes.

Thank you for your valuable comment. In the revised manuscript, we will provide additional images at higher magnification to better illustrate the classical membrane invagination in Figure 3A and the detached sister membranes in Figure 3B.

(3) Figure 6 lacks controls. How does the classical invagination look in this strain? Also, adding nuclear dye would be informative, in order to correlate the nuclear division with membrane rupture, as claimed.

Thank you for this important comment. As we addressed how we correlated nuclear division with membrane rupture in response to weakness (1), below we will focus on how we may distinguish classical invagination from membrane rupture.

While live imaging provides invaluable temporal resolution, its limitations in resolving membrane ultrastructure are substantial. In *C. elegans* embryos, early development is marked by rapid and extensive cell divisions. Within approximately 100 minutes, a two-cell embryo develops into one containing nearly 30 cells. During this fast-dividing stage, the rapid kinetics of membrane extension—approximately 20–30 seconds during metaphase and less than 3 minutes during cytokinesis—combined with embryo motility, introduce considerable spatiotemporal ambiguities. Furthermore, the longstanding invagination model of cytokinesis has shaped interpretations in the field, which may have led to ambiguous structures such as free-ended extensions being dismissed as potential artifacts rather than recognized as alternative morphological features. Theoretical and computational models have largely been built upon invagination-centric assumptions, which may have further constrained conceptual frameworks. Therefore, fluorescence protein-based live imaging analysis alone could not serve as a convincing approach to challenge the current dogma of cell division, nor did we intend it to.

However, when reexamined in light of our findings, previous studies using GFP::PH or similar markers have in fact revealed membrane discontinuities that went unnoticed. For example, using light-sheet microscopy and 3D reconstruction, Fu et al captured membrane discontinuities in cells undergoing division such as anaphase or telophase (Fu et al. Imaging

multicellular specimens with real-time optimized tiling light-sheet selective plane illumination microscopy. *Nature Communications*. 2016.DOI:10.1038/ncomms11088)

Similarly, an earlier study by Goldstein et al. (Harrell and Goldstein. 2011. Internalization of multiple cells during *C. elegans* gastrulation depends on common cytoskeletal mechanisms but different cell polarity and cell fate regulators. *Developmental Biology*. DOI:10.1016/j.ydbio.2010.09.012) showed regions where the GFP::PH signal appeared fuzzy and discontinuous.

Here, to capture dynamic membrane events, our live imaging using the GFP::PH membrane marker was performed at 4-second intervals, approaching the practical limit for single-section scanning of the embryo. With single-plane live imaging, both membrane ruptures and free-ended sister membrane structures (Figures 6) could be detected, providing additional evidence that membrane rupture and independent extension of detached sister membranes underlie cytokinesis in *C. elegans* embryos.

However, given the inherent limitations of fluorescence microscopy in resolving membrane ultrastructure, high-resolution electron microscopy—supported by rigorous controls and serial section analysis—remains the gold standard for definitively distinguishing invagination from membrane discontinuities.

While future advances in imaging technology may enable real-time visualization at near-EM resolution, our extensive, multi-year effort to test the artifact hypothesis has convinced us that these membrane discontinuities are genuine biological features of dividing *C. elegans* embryonic cells.

Reviewer #3 (Public review):

Summary:

*In this manuscript, the authors challenge a dogma in cell biology, namely that cells are at any time point engulfed by a continuous plasma membrane. Liang et al. find that during *C. elegans* embryogenesis, a high number of cells are not entirely surrounded by a plasma membrane but show membrane openings (MOs). These openings are enriched at the embryo's periphery, towards the eggshell. The authors propose that plasma membrane discontinuities emerge during metaphase of mitosis and that independent extension of "sister membranes" engulfs the daughter cells.*

Strengths:

On the positive side, the authors find plasma membrane discontinuities not only by electron microscopy but also by fluorescence microscopy and provide information about the dynamics of membrane openings and their emergence. While this is assuring, the authors conclude that MOs emerge during metaphase. From what the authors show, this particular information cannot be deduced, as there is no dynamic capture of a membrane scission event together with a chromatin marker that would indicate mitosis. The authors could, however, attempt to find such events in live movies, given the high incidence of MOs reported from their EM data.

Thanks to the reviewer for the encouragement. Highly appreciated.

Weaknesses:

In order to convincingly demonstrate the absence of any plasma membrane in the respective regions of the embryonic periphery or between cells of the embryo, the authors would have to show consecutive serial TEM sections where MOs are detected over more z-planes, beyond the mere 3D reconstructions. Although the authors state in the methods section that continuous ultrathin sections were cut for the metaphase

sample (page 21, line 472), consecutive sections are never shown in TEM. While we do see the 3D reconstructions, better documentation of the underlying TEM data is missing. It would be necessary to show a membrane opening in consecutive z sections. Alternatively, the authors could seek the possibility to convincingly back up their claims with volume imaging by focused ion beam scanning EM (FIBSEM), where cellular volumes can be sectioned in almost isotropic resolution

We Thank the reviewer for raising these important technical concerns. We have taken this question seriously since first observing membrane discontinuities six years ago, and we have since conducted extensive controls to rule out fixation artifacts.

First of all, in addition to membrane discontinuities, we would like to highlight that a large number of single plasma membranes separating adjacent cytoplasmic domains were detected by EM (Figure 1, 3 and 4). This observation is particularly significant because the invagination model cannot account for the formation of single plasma membrane barriers between adjacent cytoplasmic domains. Instead, independent extension of detached sister membranes offers a plausible explanation for the generation of cytoplasm-immersed membranes. Furthermore, the morphology and continuity of these single cytoplasm-immersed membrane structures are well preserved, indicating successful EM processing and arguing against potential issues such as inadequate fixation or other technical limitations.

Second, we applied rigorous criteria for identifying membrane discontinuities:

(1) To test whether the discontinuities were preparation specific, we examined four independent sample batches and validated our findings using two EM techniques: transmission electron microscopy (HPF-TEM) and dual-beam scanning electron microscopy (SEM).

(2) We analyzed embryos across multiple developmental stages. Membrane discontinuities were observed in both intrauterine and laid embryos at early stages. However, as embryos reached the comma stage—a period marked by the onset of elongation and reduced cell proliferation—the incidence of discontinuities dropped dramatically (0/13, 0/17, and 0/30 cells examined). This developmental specificity argues strongly against a general fixation artifact, which would be expected to occur randomly across stages. Additionally, the eggshell is present throughout the embryonic stage of *C. elegans*; therefore, the dramatic reduction of membrane discontinuities in comma-stage of embryo argues against the possibility that the eggshell poses a fixation problem.

(3) Each putative membrane discontinuity was identified only after examining the entire plasma membrane of the cell on a given section. Importantly, aside from the discontinuity, the remainder of the plasma membrane remained intact. Moreover, in most cells, only a single discontinuity was present per section, arguing against random, widespread membrane tearing during preparation. Because EM preparation yields serial sections, we verified nearly all membrane discontinuities by examining adjacent sections. Again, the same membrane discontinuity was confirmed only after inspecting the entire plasma membrane on those neighboring sections as well. We will include this verification protocol in the revised Methods and additional imaging of consecutive sections would be provided if needed.

To further determine whether a dividing cell indeed contains one membrane rupture, we performed two serial reconstruction experiments using consecutive sections, as the reviewer suggested. First, we used HPF-TEM to analyze 105 consecutive sections of a metaphase cell, reconstructing the entire plasma membrane and chromosome configuration. We found that one membrane rupture largely encircled the chromosomal disc (Figure 2 and Video S1), spatially aligning with the future segregation zone. Second, we used AutoCUTS-SEM to collect approximately 600 sections covering ~95% of a telophase cell containing three nuclei sharing a common cytoplasm. This tri-nucleated cell was enclosed by three distinct plasma

membranes, each harboring a single rupture site. These three ruptures converged to form a Yshaped exposed cytoplasmic region spanning >351 sections (Figure 5). Collectively, these reconstructions demonstrate that each cell contains only one discontinuity from a 3D point of view, further supporting that the phenomenon is not due to random sample preparation damage.

(4) In addition to EM, we performed live imaging of plasma membrane dynamics. While live imaging provides important temporal context, we recognize its limitations in resolving membrane ultrastructure. The rapid kinetics of membrane extension (approximately 20–30 seconds for metaphase and less than 3 minutes for cytokinesis), combined with embryo motility, introduces spatiotemporal ambiguities. To capture dynamic membrane events, our live imaging using the GFP::PH membrane marker was performed at 4-second intervals, approaching the practical limit for single-section scanning of the embryo. With single-plane live imaging, nevertheless, both putative membrane ruptures (Figure 6A) and free-ended sister membrane structures could be detected (Figures 6B and 6C), providing additional evidence that membrane rupture and independent extension of detached sister membranes underlie cytokinesis in *C. elegans* embryos. Notably, 3D membrane dynamics analysis using light-sheet microscopy (Fu et al. Imaging multicellular specimens with real-time optimized tiling light-sheet selective plane illumination microscopy. *Nature Communications*. 2016. DOI:10.1038/ncomms11088). revealed membrane ruptures in dividing early *C. elegans* embryonic cells, including during telophase and metaphase. Therefore, live imaging further validates the membrane rupture phenomena in dividing embryonic cells in *C. elegans*.

We are confident that the cumulative evidence presented here addresses the reviewer's concerns and demonstrates that the observed membrane discontinuities, as well as cytoplasm-immersed membranes, are not procedural artifacts but rather reflect a previously underappreciated aspect of plasma membrane dynamics during embryonic cell division.

Another critical issue concerns the detection of the membrane discontinuities in electron micrographs, which, in my opinion, is ambiguous. How do the authors reliably discriminate in their TEM images whether there is a plasma membrane or not? The absence - or weak appearance - of the stain of the electron dense material at membranes, which seems to be their criterion for MOs, is also apparent at other, intracellular membranes, like at the NE or at the ER (for example, see Figure 1C). Also, the plasma membrane itself appears unevenly stained in regions that the authors delineate as intact (for example, Figure 1C, 2B/1).

We thank the reviewer for raising this important concern.

First, our laboratory has extensive experience with electron microscopy across diverse biological systems, including neurons, muscle cells, and hypodermis in *C. elegans*, as well as tissues from *Drosophila*, mouse, bacteria, and cultured cells (Chen et al., 2013; Ding et al., 2018; Guan et al., 2022; Y. Li et al., 2018; Miao et al., 2024; Qin et al., 2014; Wang et al., 2026; J. Xu et al., 2022; M. Xu et al., 2021; L. Yang et al., 2020; X. Yang et al., 2019; Zhu et al., 2022). Importantly, we did not introduce any novel or unconventional steps in our EM preparation; all protocols were standard and well established. Thus, the observed membrane discontinuities are unlikely to result from technical inexperience or idiosyncratic methods.

Second, because EM preparation yields serial sections, we verified nearly all membrane discontinuities by examining adjacent sections. Specifically, a membrane discontinuity was confirmed only after inspecting the entirety of the plasma membrane in neighboring sections. We will include this verification protocol in the revised Methods section, and additional images of consecutive sections can be provided if needed.

Third, in addition to membrane discontinuities, a large number of single plasma membranes separating adjacent cytoplasmic domains were detected by EM (Figure 1, 3 and 4). This

observation is particularly significant because the invagination model cannot account for the formation of single plasma membrane barriers between adjacent cytoplasmic domains. Instead, independent extension of detached sister membranes offers a plausible explanation for the generation of cytoplasm-immersed membranes. Furthermore, the morphology and continuity of these single cytoplasm-immersed membrane structures are well preserved, indicating successful EM processing and arguing against potential issues such as inadequate fixation or other technical limitations.

EM-related publications by Jingjing Liang:

Chen D, Jian Y, Liu X, Zhang Y, Liang J, Qi X, Du H, Zou W, Chen L, Chai Y, Ou G, Miao L, Wang Y, Yang C. 2013. Clathrin and AP2 Are Required for Phagocytic Receptor-Mediated Apoptotic Cell Clearance in *Caenorhabditis elegans*. *PLoS Genetics* 9:e1003517. DOI: <https://doi.org/10.1371/journal.pgen.1003517>

Ding L, Yang X, Tian H, Liang J, Zhang F, Wang G, Wang Y, Ding M, Shui G, Huang X. 2018. Seipin regulates lipid homeostasis by ensuring calcium-dependent mitochondrial metabolism. *The EMBO Journal* 37:e97572. DOI: <https://doi.org/10.15252/embj.201797572>

Guan L, Yang Y, Liang J, Miao Y, Shang A, Wang B, Wang Y, Ding M. 2022. ERGIC2 and ERGIC3 regulate the ER-to-Golgi transport of gap junction proteins in metazoans. *Traffic* 23:140–157. DOI: <https://doi.org/10.1111/tra.12830>

Li Y, Zhang Y, Gan Q, Xu M, Ding X, Tang G, Liang J, Liu K, Liu X, Wang X, Guo L, Gao Z, Hao X, Yang C. 2018. *C. elegans*-based screen identifies lysosome-damaging alkaloids that induce STAT3-dependent lysosomal cell death. *Protein & Cell* 9:1013–1026. DOI: <https://doi.org/10.1007/s13238-018-0520-0>

Miao Y, Du Y, Wang B, Liang J, Liang Y, Dang S, Liu J, Li D, He K, Ding M. 2024. Spatiotemporal recruitment of the ubiquitin-specific protease USP8 directs endosome maturation. *eLife* 13:RP96353. DOI: <https://doi.org/10.7554/eLife.96353>


Qin J, Liang J, Ding M. 2014. Perlecan Antagonizes Collagen IV and ADAMTS9/GON-1 in Restricting the Growth of Presynaptic Boutons. *Journal of Neuroscience* 34:10311–10324. DOI: <https://doi.org/10.1523/JNEUROSCI.5128-13.2014>


Wang Z, Zhang L, Zhou B, Liang J, Tian Y, Jiang Z, Tao J, Yin C, Chen S, Zhang W, Zhang J, Wei W. 2026. A single MYB transcription factor GmMYB331 regulates seed oil accumulation and seed size/weight in soybean. *Journal of Integrative Plant Biology* 68:470–485. DOI: <https://doi.org/10.1111/jipb.70101>

Xu J, Chen S, Wang W, Man Lam S, Xu Y, Zhang S, Pan H, Liang J, Huang Xiahe, Wang Yu, Li T, Jiang Y, Wang Yingchun, Ding M, Shui G, Yang H, Huang Xun. 2022. Hepatic CDP-diacylglycerol synthase 2 deficiency causes mitochondrial dysfunction and promotes rapid progression of NASH and fibrosis. *Science Bulletin* 67:299–314. DOI: <https://doi.org/10.1016/j.scib.2021.10.014>

Xu M, Ding L, Liang J, Yang X, Liu Y, Wang Y, Ding M, Huang X. 2021. NAD kinase sustains lipogenesis and mitochondrial metabolism through fatty acid synthesis. *Cell Reports* 37:110157. DOI: <https://doi.org/10.1016/j.celrep.2021.110157>

Yang L, Liang J, Lam SM, Yavuz A, Shui G, Ding M, Huang X. 2020. Neuronal lipolysis participates in PUFA-mediated neural function and neurodegeneration. *EMBO reports* 21:e50214. DOI: <https://doi.org/10.15252/embr.202050214>

Yang X, Liang J, Ding L, Li X, Lam S-M, Shui G, Ding M, Huang X. 2019. Phosphatidylserine synthase regulates cellular homeostasis through distinct metabolic mechanisms. *PLOS Genetics* 15:e1008548. DOI: <https://doi.org/10.1371/journal.pgen.1008548> 

Zhu J, Lam SM, Yang L, Liang J, Ding M, Shui G, Huang X. 2022. Reduced phosphatidylcholine synthesis suppresses the embryonic lethality of seipin deficiency. *Life Metabolism* 1:175–189. DOI: <https://doi.org/10.1093/lifemeta/loac02> 

<https://doi.org/10.7554/eLife.109984.1.sa0>