Ring canal formation in the Drosophila testis occurs via a midbody-like intermediate

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Abstract

Cytokinesis normally proceeds to completion at the end of mitosis. However, during male and female gametogenesis in various invertebrate and vertebrate species, cytokinesis is incomplete, occuring in the absence of abscission, to yield cells connected by a shared cytoplasmic intercellular bridge. These bridges, called ring canals in Drosophila, are membrane-attached cytoskeletal structures that stabilize the cytoplasmic opening between cells and are comprised of several contractile ring components suggesting that ring canals are derived from the contractile ring; however, the mechanism that transforms a contractile ring into a ring canal is not known. We aim to uncover the timeline of ring canal formation in the Drosophila testis and understand how ring canal formation is coordinated with the inhibition of abscission.

Time-lapse imaging of GFP-tagged ring canal components in the male germline has revealed novel insights into the mechanism of ring canal biogenesis. In contrast to proposed models of contractile ring arrest, we find that ring canals are formed from a midbody-like intermediate. Live imaging of Pavarotti::GFP (Pav/MKLP1/Klf23), a subunit of the centralspindlin complex and major component of ring canals, shows that Pav-labeled cleavage furrows constrict to a dense midbody-like focus that resolves into an open ring over the course of one hour. We find that known midbody ring and ring canal components localize in a ring around the Pav-labeled focus, similar to the localizations of midbody ring proteins during cytokinesis. Ring canal formation occurs in the absence of multinucleation, polyploidization, events associated with midbody formation and function. The ESCRT-associated protein ALIX::GFP is enriched at intercellular bridges, but not mature ring canals, whereas downstream ESCRT components are not. These data suggest a model wherein components/protein modifications at the “midbody” inhibit membrane abscission to facilitate the formation of ring canals. We are employing a combination of proteomics, genetics, and imaging approaches to identify and validate new targets for future investigation.

1. Germ cells develop in a syncytium and are connected by ring canals (RCs) throughout Drosophila spermatogenesis

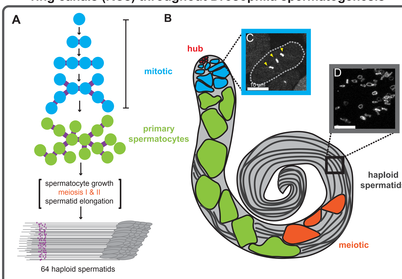


Figure 1. (A) Schematic of syncytial germline development during spermatogenesis. Male germ cells undergo four mitotic divisions (in blue) giving rise to a 16-cell primary spermatocyte cluster (green). Primary spermatocytes divide twice meiotically to yield 64 haploid spermatids (dark grey). Ring canals (marked in purple) are present throughout to connect all cells within a cyst (B) Schematic of the Drosophila testis, displaying the spatiotemporal development of germline cysts with (C) showing ring canals (arrowhead) in a 4-cell cyst and (D) ring canals in haploid spermatids.

2. RCs allow movement of proteins between syncytial cells

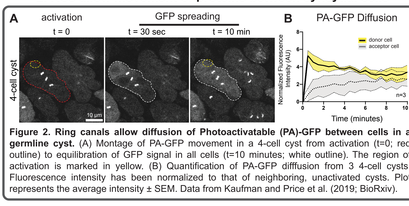
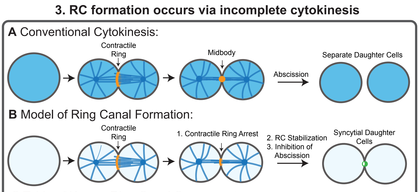


Figure 2. Ring canals allow diffusion of Photoactivatable (PA)-GFP between cells in a germline cyst. (A) Montage of PA-GFP movement in a 4-cell cyst from activation (t=0; red outline) to equilibration of GFP signal in all cells (t=10 minutes; white outline). The region of activation is marked in yellow. (B) Quantification of PA-GFP diffusion from 3 4-cell cysts. Fluorescence intensity has been normalized to that of neighboring, unactivated cysts. Plot represents the average intensity ± SEM. Data from Kaufman and Price et al. (2019; BioRxiv).

3. RC formation occurs via incomplete cytokinesis



C Table of Known Ring Canal Components:

	Contractile Ring	Male RCs	Female RCs
cytoskeleton	actomyosin	septin	actin
key components	Anillin septin MKLP1/KIF23/Pav	Anillin septin MKLP1/KIF23/Pav Vsg	MKLP1/KIF23/Pav Vsg Filamin HtsRC
size (µm)	variable	1-1.5 µm	0.5-10 µm

Figure 3. (A) and (B) depict the major differences between cytokinesis that results in physically separate daughter cells versus the incomplete cytokinesis that accompanies ring canal formation. (B) A model for ring canal formation can be inferred from the literature, but the mechanism has yet to be understood. (C) Male ring canals contain contractile ring components, suggesting they are derived from the contractile ring. Differences in protein composition suggest there may also exist different mechanisms of ring canal formation between male and female germlines.

4. RCs form from a midbody-like intermediate

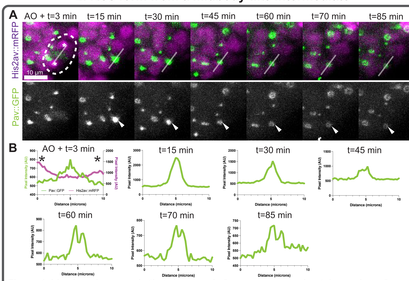


Figure 4. Live imaging reveals that ring canals form over approximately one hour from a midbody-like intermediate. (A) Montage of images from time-lapse movie of Pav::GFP-labeled ring canals; time is relative to anaphase onset (AO). (Top) Merge of Pav::GFP (green) and His2av::mRFP (magenta). Dashed white line marks the dividing cell; asterisks mark the anaphase chromosomes. Translucent line in merged images denotes the line scan represented in (B). The first indication of a ring canal lumen is at t=45 minutes, but is clearly visible by t=60 minutes. Concomitant with ring canal formation is an overall reduction in fluorescence intensity from midbody-like focus to ring canal. Quantification of Pav::GFP confirms that the reduction in fluorescence intensity is not due to photobleaching (not shown).

5. The “midbody”-to-RC transition is accompanied by a reduction in fluroscence intensity and growth in RC diameter

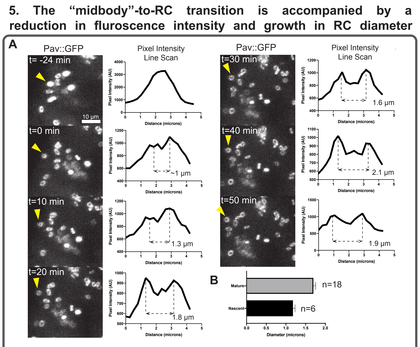


Figure 5. Ring canal formation is accompanied by a reduction in fluorescence intensity in the lumen of nascent ring canals as well as a short period of expansion before reaching final size. (A) Stills from a time-lapse movie showing the midbody-to-ring canal transition with accompanying quantification of pixel intensities from the ring canal marked with yellow arrowhead. Time is relative to the first appearance of a ring canal lumen. Ring canal diameter was determined by taking the difference in distance between the two peaks of the line scan. Over the course of the movie, the distance between peaks grew larger as the pixel intensity between the peaks was reduced. Fluctuation in diameter is likely due to shifting focal planes during imaging due to movement of testes (B) Additional ring canal diameters were measured in nascent and mature ring canals (from four testes). (C) Electron micrographs obtained using Focused Ion Beam Scanning Electron Microscopy allow for high resolution volumetric views of developing ring canals; micrographs depict all three ring canals from a 4-cell cyst with the relative z positions marked. Ring canals are marked by electron density (false colored in orange; top), which was used for measurement. The left and right ring canals are smaller than the center ring canal that is the oldest.

6. RCs contain midbody ring proteins

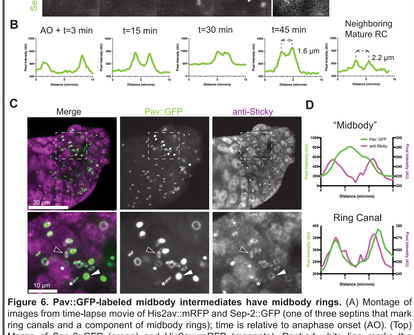


Figure 6. Pav::GFP-labeled midbody intermediates have midbody rings. (A) Montage of images from time-lapse movie of His2av::mRFP and Sep-2::GFP (one of three septins that mark ring canals and a component of midbody rings); time is relative to anaphase onset (AO). (Top) Merge of Sep-2::GFP (green) and His2av::mRFP (magenta). Dashed white line marks the dividing cell; asterisks mark the anaphase chromosomes. Translucent line in merged images marks the result of the line scan shown in (B). (Bottom) Sep-2::GFP channel; arrowheads mark an individual contractile ring and subsequent ring canal in all time frames. In contrast to Pav::GFP, which forms a compact focus at t=15 minutes, Sep-2::GFP retains a ring structure throughout ring canal formation. Furthermore, Sep-2::GFP-labeled ring canals appear to exhibit the same reduction in fluorescence intensity and expansion of ring canal diameter as seen in Figure 5. (C) Pav::GFP-expressing testes were fixed and stained with anti-Sticky antibody (magenta). (Top) Low magnification micrograph showing a recently divided 16-cell cyst (outlined with dashed white line) identified by the number of Pav-GFP “midbody” foci. Concomitant with “midbody” formation is the localization of Sticky/Citron Kinase, a known midbody ring component, mediator of midbody ring formation, and Pav interaction partner, in a ring encircling each Pav::GFP focus. (Bottom) Higher magnification micrograph of the region marked in the top panel. While every Pav::GFP focus has a ring of Sticky protein (closed arrowhead), only a subset of ring canals are marked with Sticky (open arrowhead); those ring canals unaccompanied by anti-Sticky signal are denoted with asterisks. (D) Quantification of either “midbody” or ring canal from micrographs in (C) demonstrating the extent of colocalization. The finding that Sticky is associated with a subset of ring canals suggests that Sticky localizes transiently to ring canals and might facilitate the transition from “midbody” to ring canal.

7. Spindle midbody microtubules appear stable during the “midbody”-to-RC transition

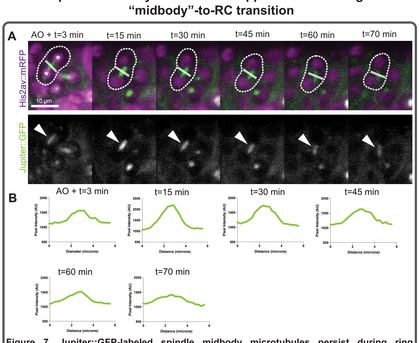


Figure 7. Jupiter::GFP-labeled spindle midbody microtubules persist during ring formation. (A) Montage of stills from a time-lapse movie of His2av::mRFP (magenta) and Jupiter::GFP (green), a microtubule binding protein used to visualize microtubules; time is relative to anaphase onset (AO). (Top) Merge of Jupiter::GFP and His2av::mRFP; white dashed line marks the dividing cell and asterisks mark the anaphase chromosomes. Translucent line marks the pixel intensity line scan graphed in (B). (Bottom) Jupiter::GFP single channel; white arrowhead marks the same spindle throughout all time points. (B) Quantification of pixel intensity across the spindle midzone. Interestingly, microtubules do not appear to be severed, however the pixel intensity is reduced over time. These data, taken together with the timing of ring canal formation from imaging of Pav::GFP, reveal that ring canals form before the midzone is dissassembled and suggest that while a “midbody” preceeds ring canal formation, it does not appear to effect microtubule disassembly or severing in the spindle midzone.

8. The ESCRT-associated protein ALIX is found on a subset of “midbodies” and RCs

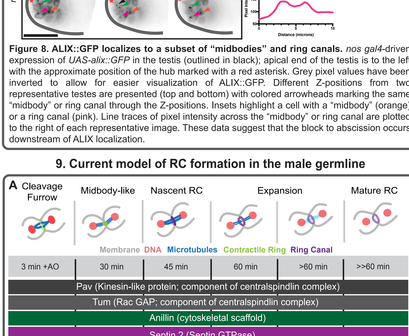


Figure 8. ALIX::GFP localizes to a subset of “midbodies” and ring canals. *nos gal4*-driven expression of *UAS-alix::GFP* in the testis (outlined in black); apical end of the testis is to the left with the approximate position of the hub marked with a red asterisk. Grey pixel values have been inverted to allow for easier visualization of ALIX::GFP. Different Z-positions from two representative testes are presented (top and bottom) with colored arrowheads marking the same “midbody” or ring canal through the Z-positions. Insets highlight a cell with a “midbody” (orange) or a ring canal (pink). Line traces of pixel intensity across the “midbody” or ring canal are plotted to the right of each representative image. These data suggest that the block to abscission occurs downstream of ALIX localization.

9. Current model of RC formation in the male germline

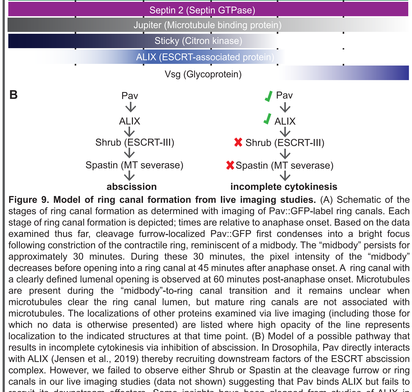


Figure 9. Model of ring canal formation from live imaging studies. (A) Schematic of the stages of ring canal formation as determined with imaging of Pav::GFP-label ring canals. Each stage of ring canal formation is depicted; times are relative to anaphase onset. Based on the data examined thus far, cleavage furrow-localized Pav::GFP first condenses into a bright focus following constriction of the contractile ring, reminiscent of a midbody. The “midbody” persists for approximately 30 minutes. During these 30 minutes, the pixel intensity of the “midbody” decreases before opening into a ring canal at 45 minutes after anaphase onset. A ring canal with a clearly defined lumenal opening is observed at 60 minutes post-anaphase onset. Microtubules are present during the “midbody”-to-ring canal transition and it remains unclear when microtubules clear the ring canal lumen, but mature ring canals are not associated with microtubules. The localizations of other proteins examined via live imaging (including those for which no data is otherwise presented) are listed where high opacity of the line represents localization to the indicated structures at that time point. (B) Model of a possible pathway that results in incomplete cytokinesis via inhibition of abscission. In Drosophila, Pav directly interacts with ALIX (Jensen et al., 2019) thereby recruiting downstream factors of the ESCRT abscission complex. However, we failed to observe either Shrub or Spastin at the cleavage furrow or ring canals in our live imaging studies (data not shown) suggesting that Pav binds ALIX but fails to recruit its downstream effectors. Some insights have been gleaned from studies of ALIX in multivesicular budding; phosphorylation of the proline-rich C terminal region is necessary for interaction of ALIX with ESCRT components (Sun et al., 2016). Regulatory phosphorylation of ALIX may also be important during ring canal formation and will be an area of future study.

10. Future Directions

1. Is there a role for actin in the expansion of nascent ring canals as is seen in the female germline? (Figure 5)
2. What is the mechanism that mediates release of Pav from midbody microtubules during ring canal formation? (Figure 7)
3. How is ALiX differentially regulated during incomplete cytokinesis resulting in an inhibition in abscission? (Figure 8)

To address these questions, we will continue the use of live cell imaging and genetics approaches to address the roles of candidate proteins in ring canal biogenesis. We are also utilizing proteomics approaches to identify novel regulatory components that might function in the inhibition of abscission that accompanies ring canal formation.

11. Acknowledgements and Funding

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