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# Assembly of ring canals in the male germ line from structural components of the contractile ring

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## SUMMARY

**Stable intercellular bridges called ring canals form following incomplete cytokinesis, and interconnect mitotically or meiotically related germ cells. We show that ring canals in *Drosophila melanogaster* males are surprisingly different from those previously described in females. Mature ring canal walls in males lack actin and appear to derive directly from structural proteins associated with the contractile ring. Ring canal assembly in males, as in females, initiates during cytokinesis with the appearance of a ring of phosphotyrosine epitopes at the site of the contractile ring. Following constriction, actin and myosin II disappear. However, at least four proteins present at the contractile ring remain: the three septins (Pnut, Sep1 and Sep2) and anillin. In sharp contrast, in ovarian ring canals, septins have not been detected, anillin is lost from mature ring canals and filamentous actin is a major component. In both males and females, a highly branched vesicular structure, termed the fusome, interconnects developing germ cells via the ring canals and is thought to coordinate mitotic germ cell divisions. We show that, in males, unlike females, the fusome persists and enlarges following cessation of the mitotic divisions, developing additional branches during meiosis. During differentiation, the fusome and its associated ring canals localize to the distal tip of the elongating spermatids.**

Key words: *Drosophila*, Ring canal, Fusome, Phosphotyrosine, Cytokinesis, Meiosis, Spermatogenesis, Anillin, Septins, Cytoplasmic myosin II, Actin

## INTRODUCTION

Syncytial development of male germ cell cysts is conserved from *Drosophila* to mammals (Burgos and Fawcett, 1955; Fawcett, 1961; King and Akai, 1971; Tates, 1971; Rasmussen, 1973). Cytoplasmic bridges, termed ring canals, allow sharing of cytoplasm among all members of a cyst, yet maintain the individuality of each cell by preventing mixing of nuclei and organelles (Braun et al., 1989). Cytoplasmic connections between clonally related male germ cells may be important for their synchronous differentiation (Fawcett, 1961). In mammals, intercellular connections between meiotic products may ensure that the haploid spermatids have access to the products of post-meiotically expressed X-linked genes during the complex morphological changes of spermiogenesis (Braun et al., 1989; Caldwell and Handel, 1991).

Ring canals form following incomplete cytokinesis. Cytokinesis in *Drosophila* and other animal cells is thought to proceed via an actinomyosin-mediated constriction of a contractile ring (reviewed in Satterwhite and Pollard, 1992). A number of *Drosophila* proteins that localize to the contractile ring have been identified (Miller and Kiehart, 1995). In addition to actin and cytoplasmic myosin II, these include Pnut, Sep1, Sep2 and anillin (Neufeld and Rubin, 1994; Fares et al., 1995; Field and Alberts, 1995; Field et al., 1996). Pnut, Sep1 and Sep2 are members of the family of septins, homologs of proteins that form the bud neck filaments in yeast (Neufeld and Rubin, 1994; Fares et al., 1995; Field et al., 1996; reviewed in Sanders and Field, 1994; Longtine et al., 1996). Anillin, initially identified as an actin-binding protein in *Drosophila* embryo extracts, localizes to the nucleus in interphase cells and colocalizes with actin, myosin and septins in invaginating pseudocleavage and cleavage furrows during embryogenesis and in dividing cells (Miller et al., 1989; Neufeld and Rubin, 1994; Fares et al., 1995; Field and Alberts, 1995).

In *Drosophila* males and females, gametes develop as syncytia of clonally related cells (King, 1970; King and Akai, 1971; Rasmussen, 1973; Lindsley and Tokuyasu, 1980). A founder gonial cell (in males) or cystoblast (in females) is produced by asymmetric division of a germ-line stem cell. The founder cell initiates four rounds of mitotic division to produce a cyst of sixteen germ cells. Cytokinesis is incomplete during these gonial mitotic divisions, resulting in 16 spermatocytes (in males) or 15 nurse cells and a single oocyte (in females) connected by 15 cytoplasmic bridges (see Fuller, 1993; Spradling, 1993 for reviews).

Although the cells within each germ cell cyst undergo nearly synchronous cell-cycle divisions and differentiation, very little is known about the mechanisms that coordinate syncytial germ cell development. The fusome, a region of highly vesiculated

and specialized cytoplasm, has been implicated in the regulation of germ cell division (Lin et al., 1994). The fusome is rich in membranous structures, spectrin and the adducin-like product of the *hu-li tai shao* (*hts*) gene (Lin et al., 1994). In females, fusome material passing through the intercellular bridges connects spindle poles in neighboring cells during mitosis, suggesting that the fusome may play a role in orienting the mitotic spindles (Lin et al., 1994). In addition, mutations in *hts* that affect female fertility cause defects in both the structure of the fusome and the number of cell divisions within a cyst (Lin et al., 1994). In females, the fusome regresses following formation of the 16-cell cyst, suggesting that its function is limited to the mitotic divisions (Lin et al., 1994).

Ring canal assembly in *Drosophila* females involves the sequential addition of proteins at the site of the arrested cleavage furrow. After the gonial mitotic divisions, the walls of each arrested cleavage furrow become lined with a ring of tyrosine-phosphorylated epitopes (Robinson et al., 1994). Anillin, an actin-binding protein present in contractile rings, is also found in these early ring canals (Field and Alberts, 1995). Accumulation of phosphotyrosine epitopes is followed by recruitment of an ovary-specific product of *hts* and a robust array of inner rim F-actin fibers. Subsequently, the product of the *kelch* gene is incorporated into the ring canal walls (Robinson et al., 1994), followed by the disappearance of anillin (Field and Alberts, 1995). Female ring canals enlarge in diameter, length and thickness as differentiation of the nurse cells and oocyte proceeds (Robinson et al., 1994; Tilney et al., 1996).

Both the components of ring canal walls and the number of ring canals per cyst in *Drosophila* males differ from those in the female. The products of the *kelch* and *hts* genes do not appear to be incorporated into ring canal walls in males (Robinson et al., 1994). Also, the alleles of *kelch* and *hts* that affect female ring canals have no discernible effect on male fertility (Yue and Spradling, 1992; Xue and Cooley, 1993; Robinson et al., 1994). During female gametogenesis, no post-mitotic cell divisions occur and the total number of ring canals formed by each cyst is 15. In the male, in contrast, two additional rounds of ring canal formation occur, as the 16 spermatocytes in each cyst undergo two meiotic divisions to form 64 haploid spermatids interconnected by 63 cytoplasmic bridges.

In this paper, we explore the mechanism of ring canal formation in male germ-line cysts. We show that, as in females, a ring of phosphotyrosine-containing protein accumulates around male ring canal walls following the gonial mitotic divisions. Rings of tyrosine-phosphorylated epitopes also form after each of the two meiotic divisions. However, the structure of mature ring canal walls is strikingly different in males from that previously described in females. In males, unlike females, a robust array of filamentous actin does not appear to be recruited to the ring canal wall. At least four proteins commonly present in contractile rings, Pnut, Sep1, Sep2 and anillin, form stable components of the mature ring canal walls in males. Thus male ring canals appear to derive directly from the arrested contractile ring. In addition, we show that fusomes in the male, in contrast to those in the female, persist and enlarge following mitosis and are further elaborated during meiosis. Fusome material and ring canals localize to the distal tip of elongating spermatids where they may play a role in differentiation.

## MATERIALS AND METHODS

For all experiments, *Oregon R* flies were raised at 25°C in humidified incubators on a standard cornmeal medium.

For immunofluorescence staining, testes and ovaries from young (0-2 day old) flies were dissected in TB-1 buffer (Kemphues et al., 1980) and flattened under a siliconized coverslip on a microscope slide pre-treated with 10% polylysine solution (Sigma). Slides were quick frozen in liquid nitrogen and the coverslips removed prior to extraction in chilled 95% ethanol for 10 minutes. Samples were then fixed in 4% formaldehyde in phosphate-buffered saline (PBS) for 7 minutes at room temperature, washed twice for 15 minutes per wash in PBS containing 0.3% Triton X-100 and 0.3% sodium deoxycholate, once in PBT (PBS with 0.1% Triton X-100) for at least 10 minutes and blocked for at least 30 minutes in PBTB (PBT with 3% bovine serum albumin) at room temperature or at 37°C. Slides were incubated for 16 hours at 4°C in one or more of the following: a 1:200 dilution of rabbit anti-cytoplasmic myosin II antibody (Kiehart and Feghali, 1986; gift of D. Kiehart), a 1:100 dilution of mouse anti-phosphotyrosine antibody (Upstate Biotechnology Incorporated), a 1:4 dilution of mouse anti-Pnut antibody (Neufeld and Rubin, 1994; gift of T. Neufeld and G. Rubin), a 1:1,000 dilution of rabbit anti-anillin (Field and Alberts, 1995; gift of C. Field), a 1:200 dilution of rabbit anti-α-spectrin (Pesacreta et al., 1989; courtesy of D. Branton), a 1:1 dilution of mouse anti-adducin (ADD-87) monoclonal 1B1 (Zaccai and Lipshitz, 1996; gift of H. Lipshitz), or a 1:10 dilution of mouse anti-α-tubulin monoclonal 4A1 (Piperno and Fuller, 1985), a 1:20 dilution of affinity-purified anti-Sep1 or anti-Sep2 rabbit polyclonal antisera (Fares et al., 1995; Field et al., 1996; gift of O. Al Awar and J. Pringle), washed four times in PBTB (15 minutes each), then incubated in a 1:200 dilution of fluorochrome-conjugated secondary antibody (from Vector or from Jackson Labs) in PBTB for 1 hour at 37°C. If desired, rhodamine phalloidin (Molecular Probes) was included with the secondary antibody at 20 units/ml (as described by Molecular Probes). Slides were washed four times 10 minutes with PBTB, with 1 μg/ml DAPI (Sigma) included in the second wash.

Samples were mounted for microscopy in 9:1 Citifluor:PBS containing 100 μg/ml *p*-phenylenediamine (Johnson and Noguira-Araujo, 1981), and examined using epifluorescence on a Zeiss Axiophot microscope. Images were captured using a Photometrics cooled CCD camera connected to a Zeiss Axiophot microscope (kindly made available by Bruce Baker) or a Bio-Rad MRC100 confocal imaging system connected to a Zeiss Axioskop microscope (kindly made available by Matthew Scott). Images of separate fluorochromes from multiply stained tissues were collected individually and combined using Adobe Photoshop.

## RESULTS

### Male ring canals, like those in the female, are marked by the presence of one or more tyrosine-phosphorylated proteins

In males, as in females, one of the earliest signs of ring canal formation is the appearance of phosphotyrosine epitopes at the site of the constricting cleavage furrow. Immunostaining of wild-type testes with an antibody directed against phosphotyrosine revealed that phosphotyrosine-containing epitopes accumulate on, and line, ring canal walls, becoming apparent following the gonial divisions in males (Fig. 1A,B, arrowheads), as in females (Fig. 1C, arrowheads; Robinson et al., 1994). Phosphotyrosine epitopes were found in the somatic hub at the tip of the testis (Fig. 1A, arrow), as well as in the contractile rings and ring canal walls. The phosphotyrosine rings in males persisted throughout much of the spermatocyte growth period. However, the rings of phosphotyrosine-con-

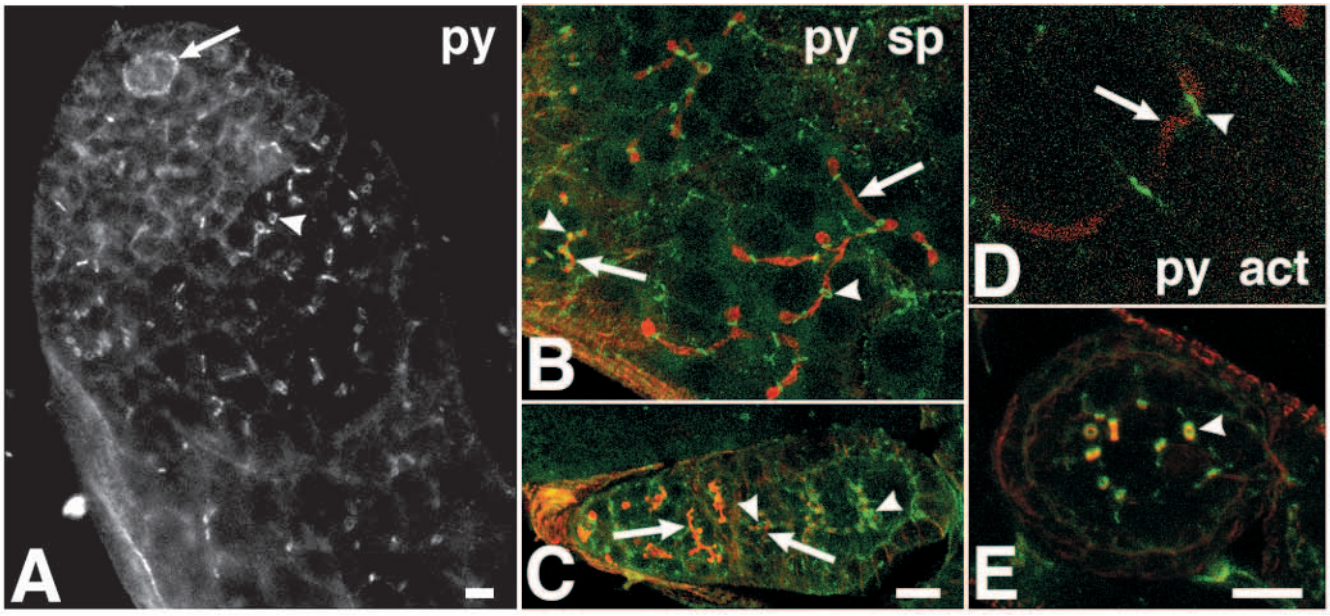


**Fig 1.** Ring canals and fusomes in males and females exhibit similarities and differences in their structure and behavior. Fluorescence micrographs of cells stained for phosphotyrosine (py), α-spectrin (sp) and actin (act). (A-E) Stacked confocal images of (A,B,D) male and (C,E) female differentiating germ cells. Arrowheads, ring canals. (A) Testis tip stained with anti-phosphotyrosine antibody. Arrowhead, ring canal in post-mitotic cyst of primary spermatocytes. Arrow, somatic hub cells at apical tip of testis. (B,C) Ring canals stained with anti-phosphotyrosine antibody (green). Fusomes stained with anti-α-spectrin antibody (red). (B) In primary spermatocytes, fusomes (arrows) and phosphotyrosine-containing ring canals (arrowheads) persist and increase in size with spermatocyte growth (compare early stages (left arrow, arrowhead) with older spermatocytes (right arrow, arrowhead)). Apical tip of testis to left of this field. (C) Fusomes and ring canals in the germarium. Fusome material is evident at the tip of the germarium and connects cells within each cyst (left arrow). Phosphotyrosine epitopes are visible in ring canals by region 1 and are easily seen by region 2 (left arrowhead). Fusome starts to disappear by region 2b (right arrow). Phosphotyrosine-containing ring canals grow in size (compare younger (left arrowhead) and older (right arrowhead) cysts). (D,E) Ring canals stained with anti-phosphotyrosine antibody (green). Fusome (D) and ring canals (E) stained with rhodamine phalloidin to visualize actin (red). (D) Thin confocal section showing that ring canals connecting primary spermatocytes contain phosphotyrosine epitopes (arrowhead) but not filamentous actin. Actin-containing fusome material persists in spermatocytes (arrow). (E) Ring canals from a stage 2-3 egg chamber. Yellow color indicates co-localization of phosphotyrosine epitopes with filamentous actin in ovarian ring canals (arrowhead). Bars: A-E, 10 μm (B,C, same magnification; D,E, same magnification).

taining material were not static in structure during spermatogenesis, increasing in diameter as the spermatocytes increased in volume (Fig. 1B, arrowheads; compare left (early) with right (late)). The distribution of anti-phosphotyrosine-stained epitopes appeared to change shape into plaque-like structures as spermatocytes matured (data not shown).

### Both the structure of ring canals and the behavior of the fusome differ between males and females

Ring canal walls have different structural components in males (Fig. 1D) versus females (Fig. 1E). Although both male and female ring canals contain phosphotyrosine epitopes (green; Fig. 1D,E, arrowheads), F-actin (red), a major component of ovarian ring canals, did not appear to concentrate in ring canal walls in males (compare Fig. 1D,E, arrowheads; yellow color indicates co-localization of actin and phosphotyrosine in females, Fig. 1E). Likewise, two ovarian ring canal proteins, hu-li tai shao and kelch, have not been found in testes (Robinson et al., 1994). In addition to sex-specific differences in ring canal formation, males and females exhibited differences in fusome behavior. Fusomes in primary spermatocytes contained F-actin (Fig. 1D, arrow), which was not easily detected in female fusomes (data not shown). α-spectrin (Fig. 1B,C, arrows; Lin et al., 1994) and at least one adducin-like protein (data not shown) were present in fusomes in males, as in females. Fusomes did not break down after the fourth gonial division in males (Fig. 1B, arrows), as they do in females (Fig. 1C, arrows; compare fusome in early post-mitotic cyst (left) with that in older cyst (right)). Instead, fusomes in males persisted and enlarged throughout the primary spermatocyte growth period (Fig. 1B, arrows; compare early cyst (left) with older cyst (right)).

### Major structural components of male ring canal walls appear to derive from proteins that mark the site of the contractile ring during cytokinesis

Male ring canal walls contain proteins associated with contractile rings during cytokinesis. Ring canals in males contained anillin, an actin-binding protein (Fig. 2A,B, arrows) and at least three septins - Pnut (Fig. 2C, arrow), Sep1 and Sep2 (data not shown). Anillin and Pnut co-localized in mitotic ring canal walls, which interconnect primary spermatocytes (compare Fig. 2B,C). Anillin localized to nuclei of primary spermatocytes (Fig. 2A,B, arrowheads), as well as to ring canal walls. In other cell types studied, anillin cycles between the interphase nucleus and the site of the contractile ring (Field and Alberts, 1995). Persistence of anillin at cytoplasmic bridges throughout interphase in primary spermatocytes may reflect stabilization of contractile ring anillin to form a structural component of ring canal walls.

The male ring canal proteins anillin, Pnut, Sep1 and Sep2 are present in cleavage furrows of germ-line cells undergoing mitotic division at the apical tip of the testis. This is similar to what has been shown previously for dividing cells, pseudocleavage and cleavage furrows in several other *Drosophila* tissues (Neufeld and Rubin, 1994; Fares et al., 1995; Field and Alberts, 1995; Field et al., 1996; Longtine et al., 1996). Pnut, Sep1 and Sep2 localized to both the somatic hub at the tip of the testis (Fig. 2D, arrow) and to the mitotic contractile rings (Fig. 2D, arrowhead; Sep1 and Sep2 data not shown). Anillin localized to contractile rings and not nuclei in cysts of male germ-line cells undergoing gonial mitotic divisions (Fig. 2E, arrowhead points to cyst undergoing fourth mitotic division). Anillin failed to stain the nuclei of cells in the somatic hub at the tip of the testis (Fig. 2E, arrow). Actin and cytoplasmic myosin II, which are presumably present in the mitotic contractile rings, were difficult to visualize at the testis tip due to the intense staining of the muscular sheath surrounding the developing germ line.

One of the primary differences in cell division patterns between male and female gametogenesis is that male germ cells undergo two additional rounds of cell division and ring canal formation prior to their final differentiation. Ring canals formed following meiosis I and meiosis II appeared similar in structure to those formed after the mitotic divisions (see below). As cytokinesis is easier to observe during meiosis than during the germ-line mitotic divisions, we examined in detail the sequence of events leading to ring canal formation during each of the two meiotic divisions.

Cytokinesis during male meiosis involves assembly and constriction of a contractile ring containing F-actin, septins, anillin and cytoplasmic myosin II. During late anaphase of both meiosis I and II, a circumferential band of actin filaments (Fig. 3A,C,D,F) coincident with Pnut (Fig. 3D,E), anillin (Fig. 3P,Q; actin not shown), Sep1, Sep2 and cytoplasmic myosin II (data not shown) formed at the cell periphery equidistant between the two separating daughter nuclei. At early stages, Pnut and anillin localized to tighter bands at the site of the presumptive contractile rings than did F-actin (Fig. 3D-F; anillin and actin data not shown). Cleavage furrows were often observed centered on the contractile ring, although cell membranes frequently did not remain attached in the squashed preparations (see also Cenci et al., 1994). In both meiosis I and II, contractile rings of actin, Pnut, myosin II, anillin, Sep1 and Sep2, which encircled the cell periphery in early telophase, constricted as the cleavage furrow moved inward (Fig. 3G-I, meiosis I; Fig. 3J-O,S,T, meiosis II; Sep1 and Sep2 data not shown). During constriction, Pnut and actin (Fig. 3G-I), actin and myosin II (Fig. 3J-L) and Pnut and anillin (Fig. 3M-O) co-localized at the site of the contractile ring. At the time of meiosis, anillin was no longer nuclear (Fig. 3M,O-Q), and was found, together with the septins, in both the meiotic contractile rings and the previously formed mitotic ring canals (data not shown).

During meiosis I and II, fusomes lost their characteristic branched structure, first appearing fragmented (Fig. 3P,R), then becoming globular (Fig. 3S,U), with only thin wisps of material appearing to penetrate either post-mitotic ring canals or meiotic contractile rings (Fig. 3S,U; ring canals not shown).

### Ring canal assembly during meiosis involves accumulation of tyrosine-phosphorylated epitopes, loss of F-actin and myosin II and formation of a stable structure containing anillin and septin proteins

Meiotic ring canals appear to derive from modification of the contractile ring. The first sign of ring canal formation was the accumulation of phosphotyrosine-containing epitopes at the

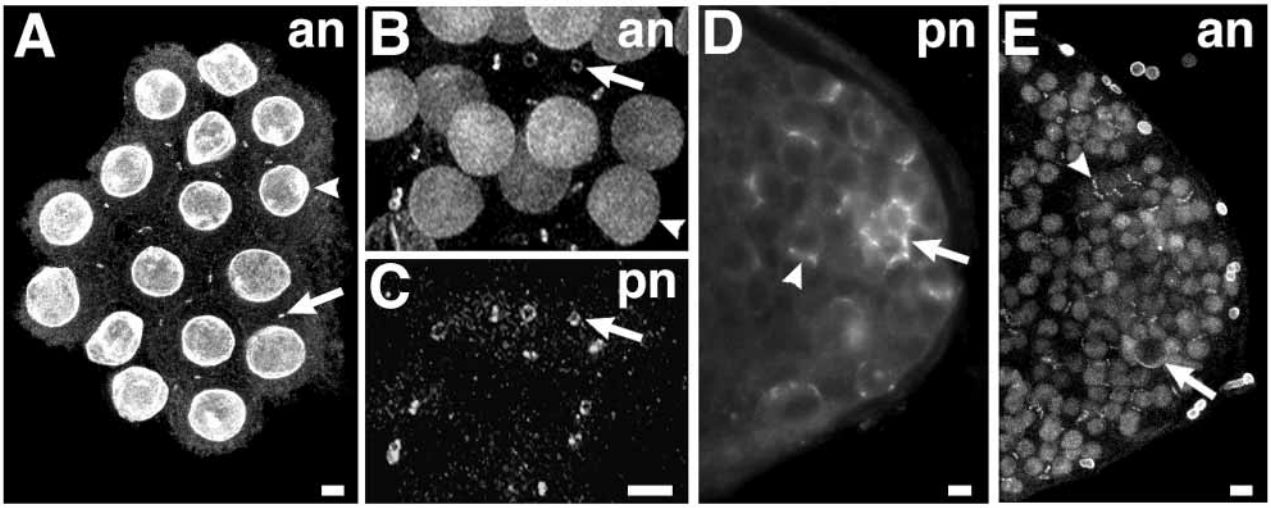


**Fig. 2.** Post-mitotic ring canals and mitotic contractile rings in the male germ line contain Pnut (pn) and anillin (an). (A-C) Stacked confocal images of indirect immunofluorescence of primary spermatocytes. (A) Cyst of 16 primary spermatocytes stained with anti-anillin antibody, showing simultaneous staining of interphase nuclei (arrowhead) and ring canals (arrow). (B,C) Higher magnification of cyst of primary spermatocytes stained with antisera directed against anillin (B) and Pnut (C), showing co-localization of anillin and Pnut in post-mitotic ring canals (B,C, arrows), as well as localization of anillin to interphase nuclei (B, arrowhead). (D) CCD image of testis tip stained with anti-Pnut antibody. Pnut stains mitotic contractile rings (arrowhead) as well as somatic hub cells (arrow) at apical tip of testis. (E) Thin confocal section showing anti-anillin staining of testis tip. Anillin is present in contractile rings but not nuclei of mitotic cyst (arrowhead). Anillin fails to stain nuclei of somatic hub (arrow). Bars: A-E, 10 μm (B,C, same magnification).

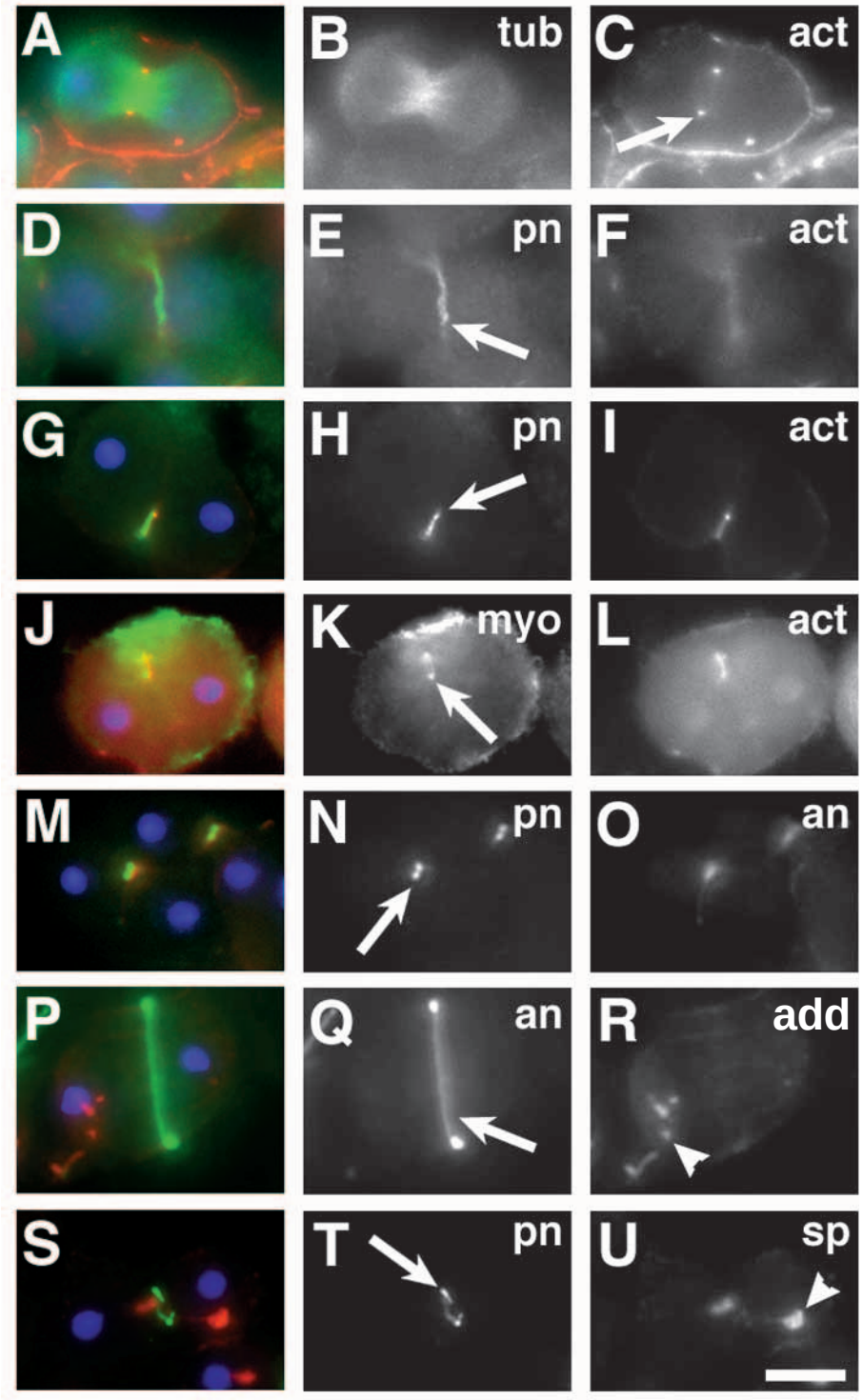


**Fig. 3.** Constricting contractile rings in meiotic cells contain actin (act), Pnut (pn), cytoplasmic myosin II (myo) and anillin (an). (A-U) CCD images of cells undergoing meiosis I (A-I) or meiosis II (J-U). (A,D,G,J,M,P,S) Superimposed CCD images. DNA visualized by staining with DAPI (blue). Arrows (C,E,H,K,N,Q,T), contractile rings. (A-C) Cells stained with anti-α-tubulin (tub, green) and rhodamine phalloidin to visualize actin (red). Two dots of actin (A,C) are visible in the plane of focus of the spindle (B) as the contractile ring constricts. (D,E,F,G,H,I) Pnut protein (green; D,E,G,H) co-localizes with actin (red; D,F,G,I) in constricting contractile rings (D,E,F, early telophase; G,H,I, late telophase). (J,K,L) Cytoplasmic myosin II (green; J,K) colocalizes with actin (red; J,L) in contractile ring. Note that the filter set used for this experiment permitted DAPI staining of DNA to bleed into the rhodamine channel (J,L). (M,N,O) Pnut (green; M,N) colocalizes with anillin (red; M,O) in constricted contractile rings. Fusomes in meiotic cells are fragmented and globular. (P-U) Fusomes stained for hts adducin domain (hts) or α-spectrin (sp) (P-R) Meiotic cell in early telophase stained with anti-anillin (green; P,Q) and anti-adducin-like domain to visualize fusome (red; P,R). The fusome is fragmented (R, arrowhead). The anti-adducin-like domain antibody also detects cell surface staining perpendicular to the contractile ring (P,R). (S-U) Mitotic cell in late telophase stained with anti-Pnut (green; S,T) and anti-α-spectrin (red; S,U). The fusome has coalesced into two irregular spheres (U, arrowhead), connected by a thin fiber that penetrates the constricted contractile ring. Note that preparation of cells for immunofluorescence often results in detachment of the plasma membrane from the contractile ring (A-C, J-L) and in bending of the spindle (G-I, J-L, M-O). Bar, 10 μm.

site of contractile rings in late telophase of both meiosis I (Fig. 4A-F) and meiosis II (Fig. 4G-L). Immunofluorescence staining with anti-phosphotyrosine antibody initially showed punctate dots along the site of the contractile ring, appearing approximately midway through constriction (Fig. 4A,B,G,H, arrows). By late anaphase, a band of phosphotyrosine appeared coincident with the maximally constricted ring of filamentous actin (Fig. 4D-F,J-L, arrows). The previously formed mitotic ring canals were also marked with phosphotyrosine epitopes at the completion of each meiotic division in an identical fashion to the nascent ring canal walls (Fig. 4A,B, arrowhead).

Like ring canals formed following the mitotic divisions, post-meiotic male ring canals contained a subset of the proteins found in contractile rings. Ring canals interconnecting haploid spermatids contained anillin (Fig. 5A), Pnut (Fig. 5B), Sep1 and Sep2 (data not shown) yet lacked detectable F-actin (Fig. 6B,F; note lack of ring canal staining) or myosin II (data not shown). In early haploid spermatids, the anti-anillin antibody

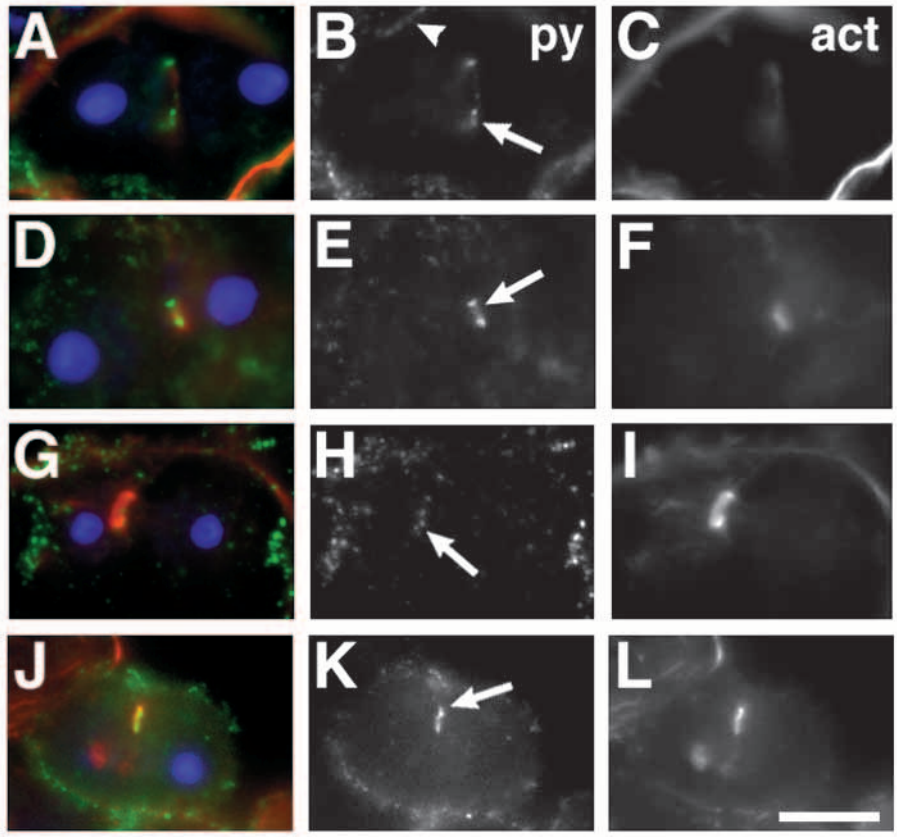


**Fig. 4.** Meiotic ring canals accumulate phosphotyrosine epitopes. (A-L) Meiotic cells stained with anti-phosphotyrosine antibody (py, green), rhodamine phalloidin to visualize actin (act, red) and DAPI to visualize DNA (blue). (A,D,G,J) Superimposed CCD images, with (C,F,I,L) actin and (B,E,H,K) phosphotyrosine staining shown separately. (Arrows) Phosphotyrosine epitopes in constricting contractile rings. (A-C) Early cytokinesis in cell undergoing meiosis I. Phosphotyrosine epitopes accumulate in a punctate pattern along contractile rings during constriction (B, arrow). Phosphotyrosine epitopes on previously assembled mitotic ring canal (B, arrowhead). (D-F) Late cytokinesis in wild-type cell undergoing meiosis I. Phosphotyrosine epitopes accumulate in a ring coincident with the constricted contractile ring (E, arrow). (G-I) Mid-cytokinesis in cell undergoing meiosis II. Phosphotyrosine epitopes accumulate in region of constricting contractile ring (H, arrow). (J-L) Late constriction of meiosis II contractile ring showing co-localization of phosphotyrosine (K, arrow) and actin (L). (J) Nucleus to the left is out of the plane of focus. Actin can be seen in fusome (L, arrowhead). Bar, 10 μm.

initially faintly stained nuclei in addition to the more brightly staining ring canal walls (Fig. 5A, arrow). Anillin staining was absent from spermatid nuclei during the later elongation stages, with the exception of a single prominent small dot of staining associated with each nucleus (data not shown). Throughout germ-line development, anillin stained the interphase nuclei of the pair of somatic cyst cells associated with each cyst (Fig. 6E, arrow).

### Fusomes in males persist following meiosis and associate with ring canals at the distal tip of elongating spermatids

The fusome undergoes dramatic morphological changes during spermatid differentiation. In spermatid cysts, filamentous actin (Fig. 6B, arrow) initially co-localized with α-spectrin (Fig. 6A, arrow) and at least one adducin-like protein (data not shown) in highly branched early post-meiotic fusomes. As spermatid bundles elongated, fusomes (Fig. 6C, arrow) and ring canals (Fig. 6D, arrowhead) clustered toward the distal end, opposite the spermatid nuclei (Fig. 6D, arrow). As the spermatids elongated, actin no longer co-localized with the fusome, but instead was distributed along the length of the elongating spermatids (compare Fig. 6E (ring canals),F (actin) with Fig. 6G (ring canals),H (α-spectrin)). Early during elongation, fusome branches extending through the ring canals (Fig. 6G, arrowhead) appeared irregularly shaped and somewhat nebulous (Fig. 6H, arrow). Later, fusome material containing α-spectrin and adducin-like proteins took on a highly ordered hexagonal arrangement (Fig. 6I, arrows; adducin-like protein staining not shown), appearing as a web knitting together the ring canals (not shown) at the extreme distal tip of each spermatid bundle. Mature ring canals in elongating spermatids contained the septins, Pnut (Fig. 6C,D,G), Sep1 and Sep2 (data not shown), and anillin (Fig. 6E).

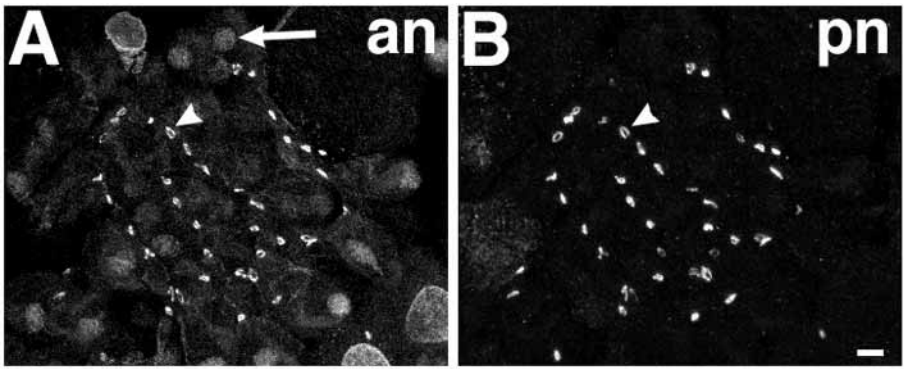


**Fig. 5.** Post-meiotic ring canal walls contain anillin (an) and Pnut (pn). (A,B) Stacked confocal images of cyst of early haploid spermatids stained for anillin (A) and Pnut (B), showing co-localization of these two proteins in ring canals (arrowheads). Anillin can also be observed in haploid nuclei (A, arrow). Bar, 10 μm.

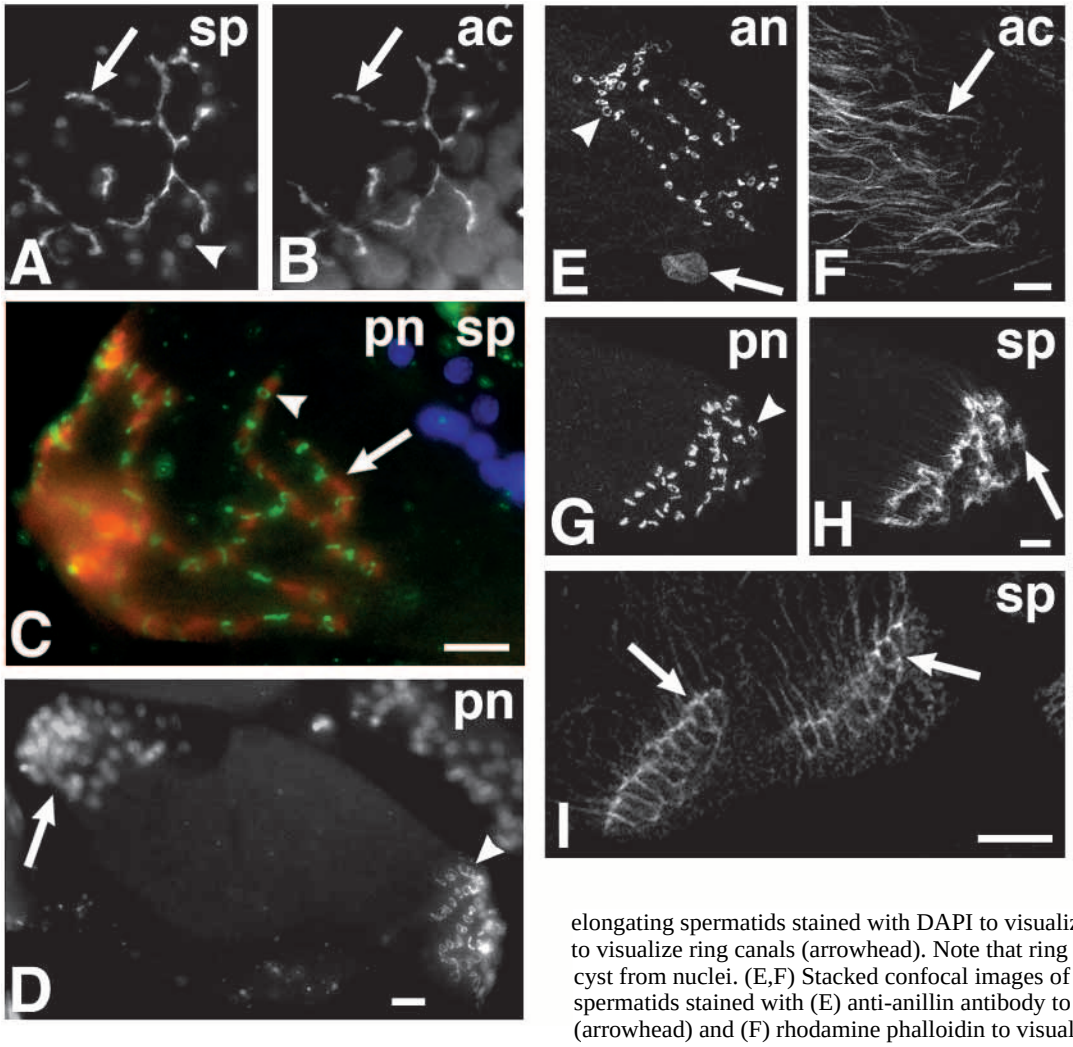


**Fig. 6.** Fusomes and ring canals persist and localize to the distal tip of elongating spermatids. Cysts of cells stained for actin (act), α-spectrin (sp), Pnut (pn) and anillin (an). (A,B) CCD images of part of a cyst of early post-meiotic spermatids stained with anti-α-spectrin (A, arrow), DAPI to visualize DNA (A, arrowhead) and rhodamine phalloidin to visualize actin (B). α-spectrin and actin co-localize in early post-meiotic fusomes (A,B). (C) Combined CCD image of early elongating cyst of spermatids stained with anti-α-spectrin to visualize the fusome (red, arrow), anti-Pnut to visualize ring canals (green, arrowhead) and DAPI to visualize DNA (blue). Note that ring canals line up along the branches of the fusome (arrow, arrowhead) as they move away from the haploid nuclei (upper right). (D) Combined CCD image of later stage cyst of elongating spermatids stained with DAPI to visualize DNA (arrow) and anti-Pnut to visualize ring canals (arrowhead). Note that ring canals are at opposite end of cyst from nuclei. (E,F) Stacked confocal images of a single cyst of elongating spermatids stained with (E) anti-anillin antibody to visualize ring canals (arrowhead) and (F) rhodamine phalloidin to visualize F-actin (arrow). Note that actin is not closely associated with the ring canals at this late stage of elongation. Anillin stains somatic cyst cell nucleus (E, arrow). (G,H) Stacked confocal images of a single cyst of elongating spermatids stained with (G) anti-Pnut antibody to visualize ring canals (arrowhead) and (H) anti-α-spectrin to visualize the fusome (arrow). In merged image (not shown), the α-spectrin-containing fusome can be seen to pass through the ring canals. (I) Thin confocal section of a late-stage cyst of elongating spermatids showing fusome stained with anti-α-spectrin antibody. The fusome appears to assume a honeycomb-like pattern (arrows). Bars: A-I, 10 μm (A,B,C, same magnification; E,F, same magnification; G,H, same magnification).

## DISCUSSION

*Drosophila* males and females display both similarities and profound differences in the assembly and composition of the ring canals that interconnect clonally related germ cells within a cyst (Fig. 7). In both sexes, the initial stages of ring canal wall assembly appear to occur on a template provided by contractile-ring- or cleavage-furrow-associated proteins. In males and females, appearance of a ring of phosphotyrosine-containing protein(s) along the inner wall of the intercellular bridges formed by incomplete cytokinesis is one of the earliest signs of ring canal wall assembly (Fig. 1A-C, arrowheads, Fig. 4, arrows; Robinson et al., 1994). The circumferential array of filamentous actin and myosin II that provides the actual contractile machinery for cytokinesis is lost from the site of the arrested cleavage furrow subsequent to the appearance of the phosphotyrosine epitope ring in males (Fig. 6B,F; data for myosin II not shown) and probably also in females (Robinson et al., 1994), although subsequent re-recruitment of filamentous actin to ring canal walls in the female somewhat obscures the prior loss of the contractile ring filamentous actin array.

After the initial stages, the structural composition of ring canal walls becomes quite different in the two sexes. In females, maturation of ring canal walls begins soon after generation of the 16-cell cyst with addition of an ovary-specific form of the hu-li tai shao protein (Robinson et al., 1994) and recruitment of a robust circumferential array of actin filaments (Fig. 1E, arrowhead), followed by addition of kelch protein (Robinson et al., 1994; Tilney et al., 1996). Although anillin is initially present in female ring canals, it is lost prior to budding of the egg chamber from the germarium (Field and Alberts, 1995). In contrast, neither actin (Figs 1D, 6B,F) nor hu-li tai shao and kelch (Robinson et al., 1994) were detected in ring canal walls in males. Instead, post-mitotic and post-meiotic ring canal walls in the developing male germ line contain

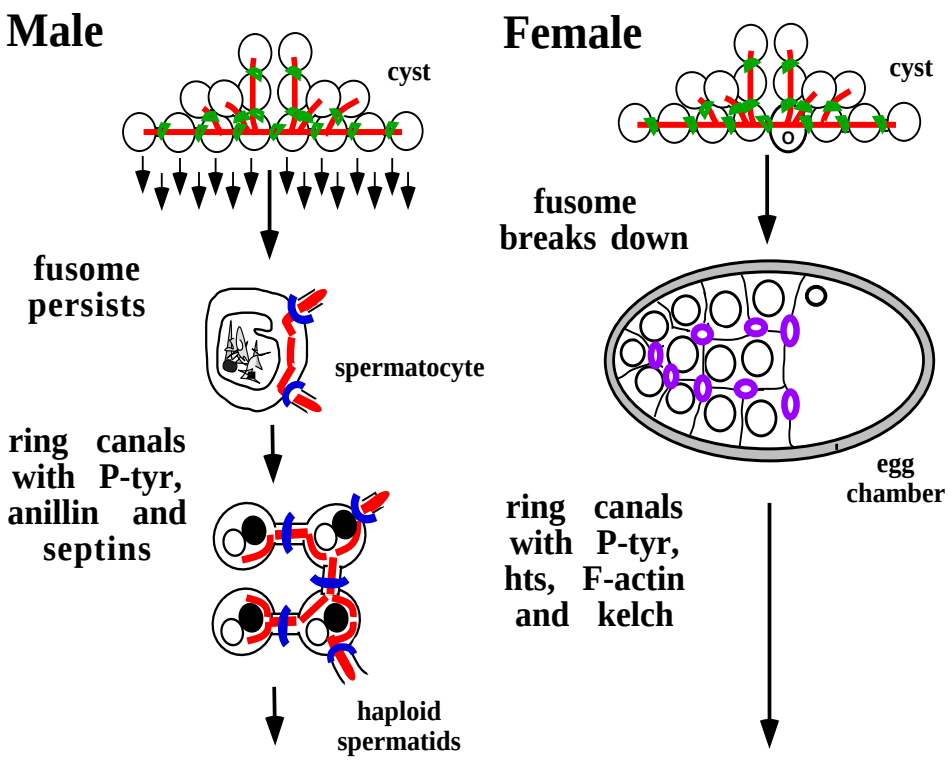


**Fig. 7.** Sex-specific differences in ring canals and fusomes. (Left) Male germ line. Shown: Cyst of 16 primary spermatocytes, single spermatocyte, four haploid spermatids. (Right) Female germ line. Shown: Cyst of 15 nurse cells and single oocyte, stage 10 egg chamber. Fusome material (red) is shown connecting clonally related cells via intercellular bridges. In males and females, phosphotyrosine epitopes accumulate on the walls of intercellular bridges following the fourth mitotic division (green). In males, the fusome remains following mitosis (this work), whereas the fusome breaks down in the female (Lin et al., 1994). Mature male ring canals (blue) formed following mitotic and meiotic germ cell divisions contain phosphotyrosine epitopes, anillin and septins (this work), but lack actin, hts and kelch (Robinson, et al., 1994). Mature female ring canals (magenta) contain phosphotyrosine, actin, hts and kelch (Robinson et al., 1994), and lack both anillin and septin proteins (Fares et al., 1995; Field and Alberts, 1995). Note that only 8 of 15 egg chamber ring canals are shown.

anillin and at least three septins (Figs 2, 5, 6 and data not shown).

Ring canal assembly appears to be under different genetic control in males and females. Ovarian ring canal assembly requires the products of the *hu-li tai shao* and *kelch* genes (Yue and Spradling, 1992; Xue and Cooley, 1993; Robinson et al., 1994). Male ring canal assembly does not require *hts* or *kelch*. However, at least two genes required for formation of stable post-meiotic intercellular bridges in males, *ms(2)3R* and *four wheel drive*, have been identified (Romrell et al., 1972; J. A. Brill, G. R. Hime, N. Wolf and M. T. Fuller, unpublished data).

The robust ring of F-actin, hu-li tai shao and kelch proteins characteristic of mature ring canals in *Drosophila* females may be a specialization of ring canal wall structure required for the massive directional transport of specific mRNAs and cytoplasmic components from the nurse cells into the oocyte during *Drosophila* oogenesis. In the male germline, ring canals serve the dual functions of permitting cell-cell communication while maintaining a physical separation between developing gametes. However, there is no obvious need known for directional transport between male germ cells.

Mature male ring canals appear to be derived from structural proteins that mark the site of contractile ring assembly. Although actin and myosin II disappear following constriction of the contractile ring, at least four contractile ring proteins, Pnut, Sep1, Sep2 and anillin, remain and tightly co-localize to mature male ring canal walls (Figs 2, 5, 6 and data not shown). The *peanut* gene (encoding Pnut) is required for cytokinesis (Neufeld and Rubin, 1994). Anillin, Pnut, Sep1 and Sep2 are found at nearly all sites of cytokinesis, pseudocleavage and cleavage furrow formation that have yet been examined (Neufeld and Rubin, 1994; Fares et al., 1995; Field and Alberts, 1995), with the exception that septins have not been detected in contractile rings in the female germ line (Fares et al., 1995; J. A. Brill and M. T. Fuller, unpublished observations). In contrast, two of the major structural proteins of ring canals in the female, hu-li tai shao and kelch, have not been found in ovarian contractile rings (Robinson et al., 1994). Anillin, Pnut, Sep1 and Sep2 in mature male ring canals co-localize in a stable cytoskeletal structure lacking detectable filamentous actin. Septins have been shown to form stable filamentous structures lacking actin in vitro (Field et al., 1996; reviewed in Longtine et al., 1996). However, to our knowledge, this is the first description of a stable actin-deficient structure containing both septins and anillin.

The structure of male ring canals suggests a model for their assembly from contractile rings. At the onset of cytokinesis, septins and anillin may localize to and mark the site of the nascent cleavage furrow (Fig. 3), perhaps via interactions with each other, the plasma membrane (Neufeld and Rubin, 1994; Fares et al., 1995; Field and Alberts, 1995; Field et al., 1996) or membrane-associated proteins. Anillin protein may then utilize its actin-binding properties (Field and Alberts, 1995) to recruit or organize the filamentous actin that forms part of the actual contractile machinery for cytokinesis. Cytokinesis in differentiating germ-line cysts is specialized in that constriction of the contractile ring halts, leaving a cytoplasmic bridge. As cytokinesis progresses toward this point in the specialized germ-line divisions, structural components of the contractile ring or associated proteins may become phosphorylated on tyrosine residues, leading to appearance of the characteristic ring of phosphotyrosine epitopes (Figs 1, 4).

The accumulation of phosphotyrosine epitopes at the site of the nascent ring canal wall indicates a possible role for tyrosine kinases in regulating ring canal assembly. Tyrosine phosphorylation has been shown to play a role in regulating the

assembly and disassembly of the actin cytoskeleton at sites of cell-cell contact. In many cases, tyrosine phosphorylation of junctional complex proteins, for example, plakoglobin or β-catenin at adherens junctions, disrupts cytoskeletal-membrane interactions (reviewed in Cowin and Burke, 1996; Craig and Johnson, 1996). Perhaps tyrosine phosphorylation of targets in the constricting contractile ring leads to dissociation of filamentous actin from the contractile ring and cessation of cleavage. Alternatively, or in addition, tyrosine phosphorylation might serve to stabilize structural proteins present at the cleavage furrow (for example, anillin or one or more of the septins) to form the nascent ring canal walls. Tyrosine phosphorylation could also stabilize interactions between nascent ring canal walls and the plasma membrane. Consistent with the latter hypothesis, flies mutant for *four wheel drive* form fully constricted meiotic contractile rings that exhibit little or no tyrosine phosphorylation. The interaction between the nascent ring canal walls and the plasma membrane appears to be destabilized, resulting in fusion of the daughter cells and formation of multinucleate spermatids (J. A. Brill, G. R. Hime, N. Wolf and M. T. Fuller, unpublished data).

The presence of septins in *Drosophila* male ring canals parallels the structure of bud neck filaments in yeast. Like ring canals in the male germ line, bud necks of *Saccharomyces cerevisiae* are stable cytoskeletal structures that permit sharing of cytoplasm between lineally related cells. The yeast bud neck forms a ring with a diameter of approximately 0.5 μm connecting mother and daughter cells prior to cytokinesis. Bud neck filaments contain septins encoded by *CDC3*, *CDC10*, *CDC11* and *CDC12* (reviewed in Longtine et al., 1996; Sanders and Field, 1994). Male ring canals contain at least three septins, Pnut, Sep1 and Sep2 (Figs 2, 5, 6 and data not shown), which have been shown to interact biochemically to form filaments in vitro (Field et al., 1996). Taken together, these similarities imply that yeast bud necks and male ring canals are likely to have in common aspects of their higher order structure.

In addition to their structural similarities, bud necks and male ring canals appear to share at least one regulatory protein in common. In yeast, *BNI1* encodes a protein of the vertebrate *limb deformity* family required for the proper structure of the bud neck; in many *bni1* mutant cells, the diameter of the bud neck is increased and cytokinesis is defective, although the mutants are viable (Jansen et al., 1996; H. Fares and J. Pringle, personal communication). In *Drosophila*, weak alleles of *diaphanous*, a *BNI1* homolog, cause male sterility and exhibit a phenotype similar to *four wheel drive*, suggesting a defect in meiotic cytokinesis (Castrillon and Wasserman, 1994). Although it remains to be seen whether this failure to separate the products of meiosis is due to a defect in the contractile ring or in ring canal assembly, strong alleles of *diaphanous* are cytokinesis defective, strongly implying a role for *diaphanous* in contractile ring function (Castrillon and Wasserman, 1994). Thus, the BNI1 family of proteins likely regulates the assembly or function of septins in multiple organisms.

As with intercellular bridges, the behavior of the fusome is profoundly different in males and females. In females, fusomes break down soon after formation of the 16-cell cyst (Fig. 1C). In males, in contrast, the fusome does not break down after completion of the mitotic divisions but remains throughout the mature primary spermatocyte stage (Fig. 1B). The fusome network is further enlarged during male meiosis such that it interconnects the 64 early post-meiotic spermatids (Fig. 6A,B). The function of fusomes in either sex is poorly understood. Fusomes have been proposed to coordinate cell division and orient mitotic spindles in members of a germ-line cyst (Lin and Spradling, 1995; McKearin and Ohlstein, 1995). Persistence of the fusome in primary spermatocytes may reflect a similar requirement for fusome function during the two additional rounds of cell division, meiosis I and meiosis II, that occur in male but not female gametogenesis.

The fusome persists during male gametogenesis well into the later stages of spermatid elongation, when it appears to gather the ring canals to the extreme distal end of elongating spermatid bundles (Fig. 6C,H,I). We have designated the structure that interconnects ring canals at the distal tip of elongating spermatids as fusome based on two criteria: (1) it stains with antisera directed against α-spectrin (Fig. 6A,C,H,I) and the adducin-like protein (not shown); and (2) it clearly evolves from the branched fusome structures present in early post-meiotic spermatids, which resemble the characteristic branched structures seen in mitotic fusomes (compare Figs 2B, 6A,B). Post-meiotic fusomes in the male are enriched in actin in early spermatids, but not apparently in the later stages of spermatid elongation (compare Fig. 6F,H). The fusome and its associated ring canals may be required post-meiotically for some aspect of differentiation, perhaps serving to organize and align elongating spermatid flagella within a cyst.

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