Germline cyst formation and incomplete cytokinesis during Drosophila melanogaster oogenesis

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A R T I C L E   I N F O

Article history:
Received for publication 18 May 2009
Revised 10 October 2009
Accepted 12 October 2009
Available online 19 October 2009

Keywords:
Incomplete cytokinesis
Oogenesis
Ring canal
Germline cyst
Gametogenesis
Drosophila

A B S T R A C T

Germline cyst formation via incomplete cytokinesis (IC) is necessary to generate functional eggs and sperm in various organisms. Drosophila melanogaster oogenesis is an ideal system for studying IC. 29 stages of germline cyst formation can be identified in D. melanogaster oogenesis. We have defined necessary terminology to describe IC and have developed a method to measure the sizes of contractile rings and ring canals. Time course study of germline cyst formation demonstrates that contractile ring constriction proceeds to a defined end point unique for each mitotic division. Contractile rings constrict to a greater degree, resulting in smaller ring diameters, for each subsequent round of mitotic division. Contrary to conventional wisdom, ring canal growth is not initiated until well after the fourth mitotic division. Ring canals grow, in an orderly manner, with ring canals derived from the first mitotic division enlarging first followed by those from the second, then those from the third, and finally those from the fourth mitotic division. This work establishes a foundation for identifying genes specific for IC and for elucidating the molecular mechanism underlying this aspect of germline cyst formation.

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Introduction

Cytokinesis is the final step of cell division. In animal cells, during complete cytokinesis (CC), commonly known as cytokinesis, the contractile ring constricts and eventually closes, forming two separate cells (Eggert et al., 2006). In contrast, during incomplete cytokinesis (IC) the contractile ring does not fully close. It is instead subsequently modified to become a stable intercellular bridge, or ring canal. In this manner, all newly generated cells remain interconnected, thus facilitating the exchange of proteins, mRNAs, and organelles between interconnected cells (Braun et al., 1989; Ventela et al., 2003). A lack of ring canals, and thus a lack of intercellular communication, results in sterility and may cause germline neoplasia in humans (Gondos, 1987; Gondos, 1993).

The conservation of IC in gametogenesis across diverse metazoans (Guo and Zheng, 2004; Robinson and Cooley, 1996), including insects, birds, and mammals, suggests that it is a fundamentally important process and that the propagation of a variety of organisms throughout the animal kingdom relies on it. Ring canals also play an important role in somatic cells where they coordinate cell division, migration, differentiation, and hormone release (Robinson and Cooley, 1996). However, the underlying mechanism that mediates IC remains an enigma.

Drosophila melanogaster oogenesis occurs continuously in adult females. D. melanogaster females have a pair of ovaries, each of which contains approximately 16 ovarioles that function as egg assembly lines (Fig. 1A) (King, 1970; King, 1975; Mahowald and Kambsells, 1980; Spradling, 1993). Egg chambers (or follicles) are formed from the progeny of germline stem cells (GSCs) and somatic stem cells in the gerarium, which is located at the anterior tip of each ovariole. The gerarium is divided into 3 regions (Fig. 1B). Region 1 lies just behind a stack of somatic cells known as the terminal filament (TF) and contains GSCs (green), as well as cytosplasts (blue), and mitotically active cysts of 2, 4, or 8 cells (red). The GSCs are located at the very anterior of the gerarium and make direct contact with the niche cap cells (dark turquoise) that are located right behind the TF. Region 2 begins with the first 16-cell cyst, i.e., after the fourth and final mitotic division is completed. Region 2 is further divided into 2a and 2b. Region 2b begins from where a cyst spans the entire width of the gerarium. In region 2b the 16 connected cystocytes (yellow green) become enclosed together by somatically derived follicle cells (sky blue) to form an egg chamber. Region 3 contains a budding (i.e., stage 1) egg chamber.

Both CC and IC occur in region 1. Each GSC undergoes CC to form a new stem cell and a cystoblast. The former remains in contact with the GSC niche cells, while the latter is displaced at least one cell away from the niche cells (Fig. 1C and Brown and King, 1964). The cystoblast divides mitotically four times, via IC, in a fixed, characteristic pattern. The first mitotic division generates one M1 ring (M1 for short); the second division generates two M2s; the third division generates four M3s; and the fourth division generates eight M4s. During each round of mitosis, the daughter cell closer to the interior of a cyst receives all of the pre-existing ring canals. After the final mitotic division, a 16-cell
A cyst is formed with two cells containing four ring canals, two cells with three ring canals, four cells with two ring canals, and eight cells with one ring canal. The two cells with four ring canals are termed pro-oocytes, one of which will become the oocyte, and the other a nurse cell.

Ring canals appear differently when viewed via fluorescent microscopy, depending on the plane of section and the orientation of the rings (Fig. 1D). A ring perpendicular to the Z-axis appears as an “o”, and a ring parallel to the Z-axis appears as a “:” or a “bar” depending on the position of the ring. A tilted ring appears as an “oval”. When the plane of focus covers only part of the oval-shaped ring, the ring appears C-shaped.

The ring canals increase in size according to the age of the 16-cell clusters bearing them (Koch and King, 1969; Mahowald and Strassheim, 1970; Tilney et al., 1996); however, it has not previously been determined exactly when ring canal growth initiates. Mahowald and Strassheim observed, via transmission electron microscopy (TEM), that, in region 2a, all the ring canals are less than 1 μm in diameter, except for the M1 ring canal that joins the two pro-oocytes (Mahowald and Strassheim, 1970). In regions 2b and 3, an M1 ring is approximately 2 μm in diameter, while the other ring canals vary from below 1 μm to about 1.5 μm in diameter.

Several molecules are localized to the contractile rings and/or ring canals. Both anillin and pavarotti (Pav) are integral components of the contractile rings and nascent ring canals, though only Pav stays in the ring canals throughout oogenesis (Field and Alberts, 1995; Straight et al., 2005). Pav is the D. melanogaster member of the mitotic kinesin-like protein 1 (Minestrini et al., 2002). Anillin is an actin and myosin binding protein. It is expressed in actively dividing cells and its localization changes with the cell cycle (Field and Alberts, 1995; Straight et al., 2005). A glycoprotein, mucin-D, with unknown molecular nature, is transiently localized to the nascent ring canals (Kramerova and Kramerov, 1999). Both anillin and mucin-D decrease and eventually disappear from the ring canals as the ring canals mature. After the third mitotic division, an unidentified protein(s) containing phosphotyrosine is recruited into the ring canals. This
protein, together with anillin, Pav (discussed below), and probably unidentified molecules, constitutes the outer rims of the ring canals (Robinson et al., 1994). When all four mitotic divisions are completed, as the ring canals mature, flamin (cheerio) (Li et al., 1999; Robinson et al., 1997; Sokol and Cooley, 1999), aducin-like protein Ovhts-RC and filamentous actin are recruited to form the inner rims of the ring canals (Petrella et al., 2007; Robinson et al., 1994; Yue and Spradling, 1992). At the same time, phosphotyrosine proteins are also detected in the inner rims. Src64 and Tec29 are responsible for the majority of the phosphotyrosine staining, though it is not clear whether they are the phosphotyrosine proteins localized in the ring canals or they are the kinases that phosphorylate ring canal components (Dodson et al., 1998; Guarnieri et al., 1998; Roulier et al., 2007). Later, the inner rims are further stabilized by the actin bundling protein kelch (Kelso et al., 2002; Xue and Cooley, 1993).

Another landmark for D. melanogaster germline cell divisions is the fusome, a membranous cytoplasmic organelle and part of a continuous endoplasmic reticulum (de Cuevas et al., 1996; de Cuevas and Spradling, 1998; Lin et al., 1994; Snapp et al., 2004). Several membrane skeletal proteins, including α-spectrin, β-spectrin, ankyrin, and aducin-like protein Ovhts-Fus, are components of the fusome (de Cuevas et al., 1996; Lin et al., 1994; Petrella et al., 2007). The ring canal-specific Ovhts-RC and the fusome-specific Ovhts-Fus are the two cleavage products of a polypeptide Ovhts (Petrella et al., 2007). Loss-of-function mutations of α-spectrin or Ovhts result in aberrant fusome formation and a decrease in the number of mitotic divisions that a cystoblast normally undergoes. This in turn leads to defective egg chambers that are generally absent an identifiable oocyte and do not contain the full compliment of nurse cells (de Cuevas et al., 1996; Yue and Spradling, 1992). Ring canals also appeared abnormal and lacked associated actin rings in the Ovhts mutants (Yue and Spradling, 1992), although those in the α-spectrin mutants appeared normal based on phosphotyrosine and phalloidin immunostaining (de Cuevas et al., 1996).

The amount and the morphology of the fusome changes as the germline cyst develops (de Cuevas and Spradling, 1998). A GSC at late interphase or at mitosis contains a spherical fusome, termed a fusome. After mitosis, the spectrosome migrates posteriorly towards the cleavage furrow. Meanwhile, a plug of fusomel material forms in the transient ring canal and connects the newly generated GSC and its sister cystoblast. The spectrosome and the fusome plug eventually fuse. The resulting fusome grows and is divided unequally into the two daughter cells when the transient ring canal closes. This occurs in the G2 phase of following cell cycle; thus, the stem cell and its sister cystoblast enter and exit S-phase together. At the end of each of the four mitotic divisions of the cystoblast, a fusome plug is also formed and will fuse with the pre-existing fusome. Cyst cell divisions differ from the GSC division in that only one of the two sister cells receives the fusome and the ring canal does not close during cyst cell divisions.

To elucidate the mechanism of IC, we have characterized the stages of germline cyst formation in D. melanogaster oogenesis, using various molecular markers. We have also developed a method to accurately measure the sizes of contractile rings and ring canals. Our ring measurements demonstrate that the maximum constrictions of contractile rings are different for each mitotic division. At the point of maximum constriction the largest contractile rings are produced following the first mitotic division (M1) of cystoblasts and the smallest following the fourth mitotic division (M4). Strikingly, the ring canals that are derived from the contractile rings do not expand in size until cysts have migrated to late region 2a of the germarium, well after the fourth mitotic division. In contrast to conventional wisdom, our observations indicate that the size difference between nascent M1, M2, M3, and M4 ring canals is not due to ring canal growth. Rather, it is a result of the contractile rings constricting further before arresting for each subsequent mitotic division.

Results

Terminology and methods for studying IC

Anillin, an indicator for both cell cycle progression and the age of germline cysts

To follow the progression of IC in germ cells, we immunostained fly ovaries with antibodies for anillin (green in the confocal images)

![Fig. 2. Germaria are composed of clusters of cells with distinct nuclear anillin levels and fusomal structures. (A, B) Single-plane confocal images of a gerarium co-immunostained with antibodies against anillin (A) and α-spectrin (B) in rainbow palettes. The rainbow palettes indicate the intensity of fluorescence signals; blue is the lowest and red the highest (see the rainbow palette scale bar in the insert of panel A). (C) An overlay of panels A and B (green: anillin, red: α-spectrin). Dashed white lines outlines clusters of cells. Note that cells in the same cluster (a cyst) have the same level of nuclear anillin, while neighboring cysts do not. In addition, fusomes in different cysts differ both in morphology and in the levels of α-spectrin immunostaining. (D) A projection of several confocal planes of the same gerarium. Note that region 1 and region 2a overlaps. For instance, a 16-cell cyst is located next to a germline stem cell and two cystoblasts and adjacent to, instead of behind or posterior to, an 8-cell cyst. Same magnification for all images. Scale bars: 10 μm.](image-url)
and blue in the schematics in Figs. 2–8, red in Fig. 9 also rainbow palette in Figs. 2A and 8) and α-spectrin (red in the confocal images and yellow in the schematics in Figs. 2–5, also rainbow palette in Figs. 2B and 3). The stained germaria were imaged with a confocal microscope. Strikingly, each germarium was composed of various distinct clusters of cells with different levels of anillin in the nuclei and on the ring canals as well as different amounts of α-spectrin (Fig. 2 and Supplementary Movies 1–3). Consequently, even though most cells were not stained on the cell membrane, the boundaries of cysts could be easily determined because cells in a cyst enter the cell cycle synchronously and possess the same levels of anillin nuclear staining, while neighboring cysts were at different points of the cell cycle and thus contained distinct levels of anillin (Fig. 2A, Supplementary Movies 1 and 3). In addition, each cyst contained a unique fusome around which all cells in the cyst were organized (Figs. 2B–D, Supplementary Movies 2 and 3). In addition to germ cells, anillin and α-spectrin are also expressed in somatic follicle cells (Fig. 2 and Supplementary Movies 1–3). For the convenience of discussion, cysts referenced in the text are outlined with dashed lines.

We find that the levels and the localization of anillin changed not only with the cell cycle as previously reported (Field and Alberts, 1995; Straight et al., 2005) but also with the age of germline cysts. During cytokinesis, anillin is localized to the contractile rings and in nascent ring canals (Fig. 3). The anillin signal is very strong in constricting contractile rings (M4 in Fig. 3A) as well as other rings in Figs. 3B–E). Anillin remains in the contractile rings and their derived ring canals in the germarium. The ring canal staining of anillin disappears eventually as egg chambers develop in the vitellarium.

When the cystocytes enter interphase, anillin accumulates inside the nuclei (Figs. 4 and 5). In the first three mitotic divisions, nuclear anillin level increases with the progression of the cell cycle (Fig. 4, from top to bottom in each column). When the nuclear envelope breaks down, anillin moves to the cell cortex and then to the contractile ring when cytokinesis starts. In newly formed 16-cell cysts, nuclear anillin staining first increases (Figs. 5A–E), peaks (Fig. 5E), and then gradually decreases (Figs. 5F–G) to undetectable levels (Figs. 5H–M). This progressive change in anillin staining both in contractile...
rings and in nuclei is one criterion we used to define the age or stages of cysts. An additional criterion for defining stages is the morphology of the fusome.

**Fusome development and progression of IC**

The morphology and intensity of the fusome, revealed by α-spectrin immunostaining, also varies with the cell cycle and the age of cysts. During each mitotic division, when contractile rings are actively constricting, no fusome exists in the nascent rings (Fig. 3A). After constriction arrest, a fusome plug forms in each nascent ring canal (Fig. 3B, and de Cuevas and Spradling, 1998). For clarity, we use the fusome plugs as a physical marker to distinguish contractile rings from ring canals; once a fusome plug is formed inside a contractile ring, we regard this ring as a ring canal.

The fusome plug gradually fuses with the pre-existing fusome (marked with asterisks in Fig. 3) within a cyst (Fig. 3C). When the fusome plug is fused with the pre-existing fusome it becomes a fusome bud (Fig. 3D), which will grow to become a mature fusome, also termed a full fusome (Fig. 3E). The distal tip of a fusome bud is dome shaped and abuts the nascent ring. The distal tip of a fully grown fusome is cylindrical and prominently protrudes from the nascent ring. The intensity of fusome staining increases with the progression of the cell cycle (Fig. 3, fusome intensity increases from A to E).

The GSCs undergo CC with a transient ring canal that fully closes after the fusome matures (Figs. 5N–Q, detailed below). This is the only germ cell division in which a fully grown fusome is separated into two parts, of which the nascent stem cell receives the bigger portion, before subsequent mitotic division.

In dividing cysts, the daughter cell closer to the interior of a cyst receives all of the pre-existing fusome during each round of mitosis, similar to the inheritance of ring canals. The fusing and growing of the fusome leads to the formation of a fusome network, a branched fusome structure (Figs. 3–5). Each ring canal associates with one specific branch of the fusome network. Once a cyst has stopped dividing, the fusome begins to break down (Figs. 5F–M) and usually disappears soon after the cyst leaves the gerarium.

**Stages of D. melanogaster female germline cyst development**

The *D. melanogaster* gerarium has conventionally been divided into three regions. This is not sufficient for studying the progress of IC. It is necessary to stage cyst development in far greater detail, especially in region 1 and early region 2a where IC occurs.

We have divided the *D. melanogaster* female germline cyst formation into 29 stages. The first 20 stages detail the four mitotic divisions of cystoblasts, followed by nine stages of 16 cell cysts maturing into a stage 1 egg chamber. This staging is based on the

**Fig. 4. Cyst stages for the first three mitotic divisions.** Five stages of germine cyst formation can be recognized in each of the first three mitotic divisions based on immunostaining of anillin (green, blue in the schematics) and α-spectrin (red, yellow in the schematics). For each stage, on the left is a projection of several confocal planes (not all have the same numbers of planes) and on the right a schematic drawing. Pink arrows identify M1 rings. Those nuclei that are not seen in the chosen planes were not drawn in the schematics. (A–E) Two cell stages: stages I-a to I-e as indicated. (F–J) Four cell stages: stages II-a to II-e. (K–O) Eight cell stages: stages III-a to III-e.
levels and distribution of α-spectrin and anillin immunostaining (Figs. 4 and 5 and Table 1).

- Each mitotic division is identified with a prefix—I, II, III, or IV, and is divided into five stages according to the presence (or absence) and the morphology of the fusome in the nascent rings (Figs. 4 and 5):
  - stage a: No fusome has formed in the contractile rings. Anillin staining in the nascent rings is very bright and may mask the pre-existing ring canals.
  - stage b: At least one nascent ring canal has a fusome plug, but none of the newly formed fusome plugs are connecting or have connected with the pre-existing fusome.
  - stage c: At least one fusome plug is connecting, but not all have fused with the pre-existing fusome.
  - stage d: All fusome plugs have fused, or fully connected, with the pre-existing fusome to form one body, but the cyst contains at least one fusome bud.
  - stage e: The fusome has fully grown.

- After the 4th mitotic division, nine more stages are distinguished in region 2a and region 2b based on the intensity and integrity of the fusome, the level of anillin nuclear staining, and the shapes of the cysts (Fig. 5):
  - stage IV-f: The fusome, whose staining intensity increases from stages IV-c to IV-e and peaks at stage IV-e, starts to degrade at stage IV-f, forming what appears to be “valleys” between ring canals. Stage IV-f and late stage IV-c are similar in that both stages have valleys in their fusomes and might be mis-assigned.
  - stage IV-g: Fusomes are broken and gaps appear in the fusome, between ring canals. The fusome is more condensed at stage IV-g than at stage IV-f and becomes heterogeneous. Furthermore, at stage IV-g, the intensity of anillin staining in ring canals is distinctly higher than that in nuclei.
  - stage nona (no nuclear anillin)-2a1: From this stage onward, anillin is no longer detectable in the cystocyte nuclei, thus the name nona. The nuclear anillin intensity first increases from stages IV-a to IV-c, peaks at stage IV-e, decreases from stage IV-f, and disappears from stage nona-2a1. Nona stage consists of six sub-stages: two in region 2a and four in region 2b. At stage nona-2a1, the residual α-spectrin-stained fusome still allows for distinguishing the mitotic origins of most ring canals.

Late stage IV-c is the period in which the M4 rings of a cyst have either connecting or connected fusome but not all of the M4 rings have fully connected fusome. Several criteria can be used to distinguish stages IV-f and late stage IV-c. (1) Thickness and brightness of the fusome: α-spectrin-stained fusomes at stage IV-c are thicker and less bright, while those at stage IV-f are skinner and more condensed. (2) Morphology of fusome in M4 rings: At stage IV-f, all M4 rings have mature fusomes, while at stage IV-c, the M4 rings, at least some of them, have a connecting fusome, or a fusome bud. (3) Relative levels of anillin immunostaining in ring canals vs. nuclei: The intensity of anillin staining in ring canals is similar to the staining in nuclei at stage IV-c but distinctly higher than that in nuclei at stage IV-f.
○ stage nona-2a2: The α-spectrin-stained fusome is largely disintegrated so that the mitotic origins of most ring canals cannot be discerned by the morphology of the α-spectrin-stained fusome.

○ stage nona-2b1 to 2b4 (schematics in Fig. 1B, confocal images in Figs. 5J–M): Cysts span the entire width of the gerarium. Stages nona-2b1 to 2b4 cysts are defined according to their shapes and correspond to the straightening, straightening, bulging, and budding stages of Grieder et al. (2000). A stage nona-2b1 cyst (Fig. 1B type b1 and Fig. 5J) is convex on both anterior and posterior sides. A stage nona-2b2 cyst (Fig. 1B type b2 and Fig. 5K) appears flat or straight. A stage nona-2b3 cyst (Fig. 1B type b3 and Fig. 5L) bulges posteriorly with its oocyte at the posterior. A stage nona-2b4 cyst (Fig. 1B type b4 and Fig. 5M) rounds up and will next become a stage 1 egg chamber in region 3.

Stem cell division also goes through similar stages, beginning with the formation of a fusome plug (Fig. 5N), followed by a connecting fusome (data not shown), formation of a fusome bud (Fig. 5O), and generation of a mature fusome (Fig. 5P). However, the ring canal formed during stem cell division is transient and closes completely to form two separate cells: a new germline stem cell and a cystoblast (Fig. 5Q, outlined with yellow and white dashed line, respectively). Concurrently, the mature fusome is divided asymmetrically into two parts with the stem cell receiving the larger portion (Fig. 5Q, asterisk).

The frequency of finding different staged cysts varies greatly (Fig. 6). Not all stages are observed in a single gerarium at the same time. In fact, at any given time, many stages were lacking, reflecting the highly dynamic nature of germline cyst development. Some stages, for instance, stages a to c (except IV-c), were very rare, indicating that the time a cyst spends in those stages is quite short.

It is worth noting that region 2a often overlaps with region 1, although region 2a is normally depicted behind region 1 (Fig. 2D). The orderly younger-cyst-anterior and older-cyst-posterior arrangement of different stages occurred faithfully only from region 2b onwards.

Correlation of the cyst stages with the formation and localization of the synaptonemal complex

To determine the relationship of cyst stages with the progression of the meiotic cell cycle, we co-stained germlia with antibodies against anillin, Tmod (tropomodulin), and C(3)G (crossover suppressor on 3 of Gowen) (Fig. 7). Tmod was recently identified as a synaptonemal complex (SC) protein and associates with paired chromosomes (Page and Hawley, 2001). Low levels of C(3)G protein were detected first in stage IV-c (Fig. 7A). In a stage IV-f cyst, C(3)G was localized in four of the 16 cystocytes (Fig. 7B), with high levels in the two with four ring canals (white arrows) and low levels in the two with three ring canals (yellow arrows). In stage nona-2b3 cysts, C(3)G was detected only in the pro-oocytes (Fig. 7C, only one was seen in the plane of focus).

Interestingly, the fusome structure revealed by Tmod antibody staining was distinct from the one revealed by α-spectrin antibody staining, especially after stage IV-e. When the α-spectrin-stained fusome became heterogeneous or formed gaps, the Tmod-stained fusome skeletonized (compare Fig. 5F with Fig. 7B). Most of the Tmod-stained fusome structure remained up to stage nona-2b3, allowing for the identification of the mitotic origins of all ring canals even at stage nona-2b3 (compare Fig. 5L with Fig. 7C). However, the morphological change of the Tmod-stained fusome was not as dramatic as the α-spectrin-stained fusome. Therefore, we used α-spectrin immunostaining to determine the age of cysts and used Tmod immunostaining to determine the mitotic origins of ring canals for late 16-cell stages.

**Determination of mitotic origins and the sizes of contractile rings and ring canals**

To follow the progress of IC, we developed a method to accurately measure the sizes of contractile rings and ring canals (Fig. 8A). We dissected ovaries from 10 to 20 females for each genotype and immunostained with anti-anillin and anti-α-spectrin antibodies. We then generated confocal Z-stacks of 30 randomly chosen germaria and analyzed germline cysts contained in these germaria.

To determine the mitotic origins of each contractile ring or ring canal, we first determined the number of cells in a cyst based on the proximate number of ring canals and the morphology of the fusome in the cyst. Next, we identified all the contractile rings and ring canals and their mitotic origins according to the morphology of their associated fusome and the ring canal distribution in the cyst. For example, with a 16-cell cyst, we first identified the M1 ring, which is located in the center of the cyst. From one of the two M1-linked pro-oocytes, we identified its associated M2, M3, and M4 rings according to the following: from the center of the cyst progressing outward along the fusome, there is no ring canal beyond each M4 ring, one M4 beyond each M3, and three ring canals (one M3 and two M4s) beyond each M2. Then we did the same for the other pro-oocyte.

To determine the size of each contractile ring or ring canal, we examined several consecutive focal planes containing a specific ring to identify the plane with the maximum diameter measurement of the ring and measured the ring size as shown in the boxed area in Fig. 8A. The diameter of a ring canal or contractile ring is determined according to their shapes and sizes.

### Table 1

<table>
<thead>
<tr>
<th>Stages</th>
<th>Fusome in the nascent ring (s)</th>
<th>Fusome between rings</th>
<th>Anillin location</th>
<th>Germarium region</th>
<th>Applicable to cyst</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No fusome</td>
<td>Plug</td>
<td>Connecting</td>
<td>Bud</td>
<td>Mature</td>
</tr>
<tr>
<td>a</td>
<td>All</td>
<td>None</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>b</td>
<td>≥1</td>
<td>None</td>
<td></td>
<td></td>
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<tr>
<td>g</td>
<td>≥1</td>
<td>m</td>
<td>w to vv</td>
<td></td>
<td></td>
</tr>
<tr>
<td>nona-2a1</td>
<td>≥1</td>
<td>m</td>
<td>nd</td>
<td></td>
<td></td>
</tr>
<tr>
<td>nona-2a2</td>
<td>≥1</td>
<td>Mostly</td>
<td>nd</td>
<td></td>
<td></td>
</tr>
<tr>
<td>nona-2b1</td>
<td>≥1</td>
<td>Almost all</td>
<td>m</td>
<td></td>
<td>2b</td>
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The prefixes were not included, i.e., stage a can be I-a, II-a, III-a, or IV-a. For example, according to our classification, stage III-c is an 8-cell cyst with at least one fusome plug fusing with the pre-existing fusome. Abbreviations: ≥1, at least one; ≥1, more than one; m, medium; na, not all; nd, not detectable; s, strong; vs, very strong; vv, very weak; w, weak.
inner and the outer edges vary depending on the contrast and brightness settings of images, while the midpoint remains constant.

Colocalization of Pav-GFP with anillin

To ensure that the ring canal measurements truly reflect the size of ring canals and not simply the localization of anillin, we analyzed ring canals of flies carrying green fluorescent protein (GFP) tagged-Pav (Pav-GFP). Live-cell imaging demonstrated that the ring canals revealed by Pav-GFP are similar to anillin immunostained ring canals (Fig. 9A). To determine the relative distribution of Pav-GFP and anillin, we immunostained the ovaries of pav-GFP flies with anti-anillin and anti-α-spectrin antibodies and examined these ovaries with widefield microscopy followed by deconvolution. To our satisfaction, Pav-GFP and anillin were colocalized in the ring canals in the germaria (Figs. 9B–J), although anillin disappeared from older ring canals while Pav-GFP remained in the ring canals throughout the oogenesis.

Incomplete cytokinesis (IC) in wild type D. melanogaster ovaries

Effect of the orientations of rings on the measurements of their diameters

Ideally, one would like to measure the diameter of rings with an o- shape. However, this orientation is very rare; we found 9 out of a sample size of 211 (4%; Fig. 8B). To determine the effect of the “presentation” of the rings on the ring size measurements, we compared the diameters of stage IV-c M4 rings in different orientations (Fig. 8B). Interestingly, the measured diameters from the rings with different shapes are similar; their size differences are within the detection limit of 0.1 μm of confocal microscope. Therefore, we

![Fig. 6. Germaria are highly dynamic. Shown are numbers of germline stem cells, cystoblast, and cysts at various stages in 30 germaria. Note that the frequencies of finding different staged cysts vary greatly.](image)

![Fig. 7. Correlation of the localization of the meiotic marker C(3)G and cyst stages. (A) Low level of C(3)G protein was detected first in stage IV-c. (B) C(3)G was localized in four of the 16 cells at stage IV-f, with high levels in the two cells with four ring canals (white arrows) and low levels in the two with three ring canals (yellow arrow, only one can be seen in the plane of focus). (C) C(3)G was detected only in the two pro-oocytes (only one can be seen in the plane of focus) at stage nona-2b3. Germaria were immunostained with C(3)G (turquoise, column 1), anillin (green, column 2), and Tmod (red, column 3) antibodies. All images are projections of several confocal planes and have the same magnification. Dashed lines outline the cysts of the indicated stages. Anterior is on the left. Scale bar: 2 μm.](image)
contractile rings were different for each mitotic division. On average, not yet been reported, we found that the maximum constrictions of comparison. Surprisingly, in region 1, where ring canal sizes have varied greatly; therefore, the a stages were excluded from the At stage a, contractile rings were actively constricting and ring sizes canals from stages I-a to nona-2b4 (Fig. 10A, Supplementary Table 1). Different contractile ring constriction end points for different mitotic divisions

To determine if nascent ring canals are formed with the same dimensions, we compared the sizes of M4 ring canals with a fusome plug, a connecting fusome, a fusome bud, or a mature fusome at stage IV-c (Fig. 8C). No significant ring canal size differences were observed. This suggests that maximum constriction has been reached at or before the formation of the fusome plug. In other words, only when the constriction is arrested, the fusome plug starts to form inside the cleavage furrow.

A similar comparison of the sizes of M4 rings at stage IV-c between various germaria indicated that the ring canal sizes at the same stage were very similar among different individuals (Fig. 8D). Therefore, we pooled the measurements of ring canals at the same stages from various germaria together to generate a time course of cyst formation (Fig. 10).

Effect of fusome amounts on contractile ring and ring canal diameters

Effect of fusome amounts on contractile ring and ring canal diameters To determine when a contractile ring reaches its maximum constriction, we compared the sizes of M4 ring canals with a fusome plug, a fusome bud, or a mature fusome at stage IV-c (Fig. 8C). No significant ring canal size differences were observed. This suggests that maximum constriction has been reached at or before the formation of the fusome plug. In other words, only when the constriction is arrested, the fusome plug starts to form inside the cleavage furrow.

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Different contractile ring constriction end points for different mitotic divisions

To determine if nascent ring canals are formed with the same diameter for all four mitotic divisions, we measured the sizes of ring canals from stages I-a to nona-2b4 (Fig. 10A, Supplementary Table 1). At stage a, contractile rings were actively constricting and ring sizes varied greatly; therefore, the a stages were excluded from the comparison. Surprisingly, in region 1, where ring canal sizes have not yet been reported, we found that the maximum constrictions of contractile rings were different for each mitotic division. On average, nascent M1 was 1.46 ± 0.12 μm, M2 1.20 ± 0.16 μm, M3 1.01 ± 0.10 μm, and M4 0.79 ± 0.12 μm (Supplementary Table 2-1, stages n-b to n-e, n equals to I, II, III, and IV for M1, M2, M3, and M4, respectively). Therefore, at the point of maximum constriction the largest contractile rings were found following the 1st mitotic division of cystoblasts, while the smallest were found following the 4th mitotic division (Fig. 10A, M1: cornflower blue, top; M2: dark magenta, second top; M3: yellow green, second bottom; or M4: orange, bottom).

To determine whether the size differences of ring canals from different mitotic divisions are statistically significant, we pooled ring canal measurements from the same mitotic origins during different time frames. We compared the average sizes of the M1s, M2s, M3s, and M4s immediately following their formation (Fig. 10B, left) throughout the four mitotic divisions (Fig. 10B, middle) and during the fourth mitotic cycle (Fig. 10B, right) (Supplementary Tables 2-1 and 2-2). Regardless the method of pooling, the M1s were significantly larger than the M2s, which were larger than the M3s, which were larger than the M4s (P values < 0.005, Supplementary Tables 2-1 and 2-2).

Ring canals do not grow throughout the subsequent mitotic divisions

Fig. 10A also shows that the sizes of ring canals, regardless of their origin of mitotic divisions, did not change significantly throughout the subsequent mitotic divisions. This became more obvious when we compared ring canals from the pooled measurements (Fig. 10C, Supplementary Tables 3-1 and 3-2). No increase in diameters was detected for any of the ring canals. In fact, a slight decrease was observed. This indicates that the variance in ring sizes between pooled the measurements together for all rings at the same stage, regardless of their orientations.

Effect of fusome amounts on contractile ring and ring canal diameters

Effect of fusome amounts on contractile ring and ring canal diameters To determine when a contractile ring reaches its maximum constriction, we compared the sizes of M4 ring canals with a fusome plug, a connecting fusome, a fusome bud, or a mature fusome at stage IV-c (Fig. 8C). No significant ring canal size differences were observed. This suggests that maximum constriction has been reached at or before the formation of the fusome plug. In other words, only when the constriction is arrested, the fusome plug starts to form inside the cleavage furrow.

A similar comparison of the sizes of M4 rings at stage IV-c between various germaria indicated that the ring canal sizes at the same stage were very similar among different individuals (Fig. 8D). Therefore, we pooled the measurements of ring canals at the same stages from various germaria together to generate a time course of cyst formation (Fig. 10).

Different contractile ring constriction end points for different mitotic divisions

To determine if nascent ring canals are formed with the same diameter for all four mitotic divisions, we measured the sizes of ring canals from stages I-a to nona-2b4 (Fig. 10A, Supplementary Table 1). At stage a, contractile rings were actively constricting and ring sizes varied greatly; therefore, the a stages were excluded from the comparison. Surprisingly, in region 1, where ring canal sizes have not yet been reported, we found that the maximum constrictions of contractile rings were different for each mitotic division. On average, nascent M1 was 1.46 ± 0.12 μm, M2 1.20 ± 0.16 μm, M3 1.01 ± 0.10 μm, and M4 0.79 ± 0.12 μm (Supplementary Table 2-1, stages n-b to n-e, n equals to I, II, III, and IV for M1, M2, M3, and M4, respectively). Therefore, at the point of maximum constriction the largest contractile rings were found following the 1st mitotic division of cystoblasts, while the smallest were found following the 4th mitotic division (Fig. 10A, M1: cornflower blue, top; M2: dark magenta, second top; M3: yellow green, second bottom; or M4: orange, bottom).

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Ring canals do not grow throughout the subsequent mitotic divisions

Fig. 10A also shows that the sizes of ring canals, regardless of their origin of mitotic divisions, did not change significantly throughout the subsequent mitotic divisions. This became more obvious when we compared ring canals from the pooled measurements (Fig. 10C, Supplementary Tables 3-1 and 3-2). No increase in diameters was detected for any of the ring canals. In fact, a slight decrease was observed. This indicates that the variance in ring sizes between
different mitotic divisions in early region 2a is not due to ring canal growth. Instead, it is due to greater constriction of the contractile ring for each subsequent round of mitotic division.

Ring canals grow in a sequential manner after completion of all mitotic divisions

From nona-2a1 onward, ring canals grew in size. For instance, the M1 rings expanded from 1.25 μm at stage nona-2a1 to 1.95 μm at stage nona-2b4, similar to the results reported by Mahowald and Strassheim (Mahowald and Strassheim, 1970). Interestingly, the rings from different mitotic divisions did not start growing at the same time (Figs. 10A and 11, Supplementary Tables 4-1 and 4-2). Significant increase in the diameters of M1 rings was observed at stage nona-2b1, M2 at stage nona-2b3, and M3 at stage nona-2b4. No significant increase was observed for M4 rings up to stage nona-2b4. This suggests that some modification(s) has to occur before a ring canal is competent to increase in size.

Discussion

Cyst formation and incomplete cytokinesis

The earliest observations of ring canals were reported over a century ago (George, 1865; McGregor, 1899; Sertoli, 1877; v. Ebner, 1888). Cyst formation has remained mainly a research topic for transmission electron microscopy (TEM) due to the small size of the ring canals. In order to view an entire cyst with this approach, serial thin section TEM and 3-dimensional reconstruction must be employed. Thus, it is both costly and laborious to follow IC in detail using TEM.

We have used multi-color co-immunostaining and confocal microscopy to study how the contractile rings are prevented from closing as germline cysts form. We have found that (1) D. melanogaster female germline cyst formation can be divided into 29 stages (the first 20 stages are the four mitotic divisions, 5 stages for each division; the last stage is the stage 1 egg chamber); (2) the contractile ring/ring canal sizes can be accurately measured; (3) the ring canal sizes at the same stage are very similar among different flies of the same genotype; (4) different mitotic divisions have distinct constriction end points; (5) there is no ring canal growth throughout the four mitotic divisions; and (6) from stage nona-2a1 onward ring canals grow in an orderly manner according to their age. Our results open a new door for elucidating the molecular mechanism of IC and thus germline cyst formation.

Staging of incomplete cytokinesis

Both the level and localization of anillin and α-spectrin change with the cell cycle and the age of a cyst. Co-immunostaining of these two markers allows us to stage IC during D. melanogaster oogenesis.

Fig. 9. Colocalization of Pav and anillin during IC. (A) A single-plane confocal image of a live pav-GFP germarium. The germarium was dissected and cultured in Grace’s medium supplemented with fetal bovine serum and insulin. Note that the Pav-GFP marked contractile rings, ring canals and nuclei, similar to anillin. (B–J) Pav and anillin are colocalized in contractile rings and ring canals during IC. Panels B to J are deconvoluted widefield fluorescent images of a pav-GFP germarium immunostained with anillin antibody (green: green fluorescence of Pav-GFP fusion protein, red: anillin immunostaining). Panels D, G, and J are overlays in which where Pav-GFP and anillin colocalize appears yellow. Panels E to J are examples of an oval-shaped ring canal (E–G) and a colon-shaped ring canal (H–J). Anterior is to the left in panels A to D. Scale bars: 10 μm in panels A to D, 1 μm in panels E to J.
This detailed, molecular-based staging is necessary to follow IC throughout the four mitotic divisions and to determine precisely when and where ring canal growth is initiated. Moreover, it allows us to identify rings, between different germaria, that are at the same stage of development. Thus, we are able to accurately compare ring sizes between different individuals with the same or different genotypes. This paves the way for the identification of genes specifically required for IC. Our novel staging method provides language and landmarks that allow us to describe IC and to chart this previously unexplored area.

Ring shapes, ring orientations, and ring sizes

Ring canals have been perceived as cylinders based on their appearance in TEM (Figs. 12A and B). A cross-section of a cylinder is O-shaped, while a longitudinal section looks like a pair of bars (Fig. 12B). An oblique section appears oval- or C-shaped, depending on the position of the section. In our confocal images, the rings may appear colon-shaped or bar-shaped but never resemble a pair of bars, suggesting that the rings are donut-shaped, not cylindrical (Fig. 1D).

Why does a ring that is donut-shaped appear cylindrical in TEM? One explanation is that external pressure squeezes the ring, causing a reduction in its thickness but an increase in its height (Fig. 12C). This may happen during dehydration or infiltration when preparing TEM samples, leading to unequal shrinkage or deformation of the donut-like rings. Alternatively, structural proteins that comprise the rings may resist forces strongly in one plane, but weakly in another. The cylindrical shape seen via TEM may therefore be a result of forces applied during sample preparation distorting the rings. It is conceivable that our fixation procedure could distort the shape of ring canals, though the live-cell imaging using Pav-GFP argues against this.

Variability of contractile ring and ring canal sizes between flies

No significant differences among the rings from different germaria from various flies were observed, indicating that the sizes of ring canals from different flies at the same stage are similar. Therefore, we pooled the measurements from different flies together in our analysis. We have performed the measurements with three different strains of flies. Similar results were obtained with all three. This suggests that it is possible to identify genes that, when mutated, will interfere with the constriction of the contractile rings during IC and that this mechanism may be conserved in both oogenesis and spermatogenesis across various organisms (this will be described in a different paper).

Differential constriction for different mitotic divisions

The most striking data from our ring measurements are that the ring canals from different mitotic divisions differ in diameter from the...
outset; the ring canals are progressively smaller for each successive mitotic division. This may result from distinct cell volumes for cells of different mitotic generations. Koch and King (1966) observed that in germainium region 1 and region 2, single cells (germline stem cells and cystoblasts) were the largest, and the cystocytes of the 16-cell cysts were the smallest. They suggested that the cystoblast and the cells derived from it did not double their birth size before entering the next round of mitotic division and thus each generation of cells was smaller than the preceding generation. It is possible that a larger cell forms a thicker mitotic spindle and generates a larger opening when its contractile ring stops constricting.

Initiation of ring canal growth

The most surprising observation of our ring measurements is that the ring canals do not increase in size throughout all four mitotic divisions. The ring canal size differences seen in early region 2a are not caused by ring canal growth but by differential maximum constriction of contractile rings for each round of mitotic division, which is contrary to what was commonly believed. Ring canal growth does not initiate until stage nona-2a1. Therefore, IC and ring canal growth are two separate processes, temporally and spatially.

When all the mitotic divisions are completed, ring canals begin to expand. Interestingly, ring canal growth occurs in an ordered, sequential fashion; the M1 ring begins to expand first, followed by M2 and then M3 and lastly M4. We observed that the recruitment of ring canal marker Ovhts-RC also occurs in the same order (unpublished observation).

One mechanism to explain differences in ring canal size was proposed by Koch and King (1969). They compared the ring canals in one 16-cell cyst in region 2b to another cyst, which was in region 3 and had completed the 4th mitotic division about 24 h earlier. They found that the width and the thickness of ring canals increase from M4 → M3 → M2 → M1. In addition, the canals from the 2nd, 3rd, and 4th division in the older cluster resembled those formed at the 1st, 2nd, and 3rd divisions in the younger group. They concluded that as time passed the canal rim increased in thickness and diameter, and a coating of material built up on its inner circumference. A seemingly reasonable extension, which was commonly believed, was that ring canals grew as soon as they were formed. An M1 ring canal was larger than an M4 ring canal because it had more time to grow. Our observations demonstrate that this extension is incorrect; ring canals do not increase in size until late region 2a. Nonetheless, when the ring canals grow, they do so in an orderly manner, which is consistent with and explains the observation of Kock and King.

The ordered, sequential growth of ring canals and recruitment of Ovhts-RC suggests that ring canal growth is a regulated process. It is tempting to speculate that the fusome mediates these ordered processes since each ring canal is specifically associated with a unique branch of the fusome network. If the fusome provides the trigger for the ring canals to grow, then mutations that disrupt fusome formation and the synchronicity of mitotic divisions should cause mis-regulation of Ovhts-RC recruitment. Unfortunately, all known mutations that disrupt fusome formation also interfere with mitotic division and cell fate determination, making it difficult to identify cytokinesis-specific effects.

One caveat for the fusome hypothesis is that germline cysts from different organisms do not all contain fusomal material. It is not clear whether the bridge-partitioning complex (BPC), which is generated...
during each successive germ cell division in mammals (Dym and Fawcett, 1971; Weber and Russell, 1987), functions as a fusome analog. BPC is transient in that it is formed during the prophase/prometaphase transition and disappears in late telophase (Miething, 2003; Weber and Russell, 1987), while the fusome persists throughout the four mitotic divisions. BPC serves as an essential barrier to temporarily prevent intraclonal exchange of nuclear material when a nuclear membrane is lacking and thus maintains genetic integrity of male germ cells during synchronous divisions (Miething, 1995). In flies, the fusome may perform a similar barrier function, though the fusome is often characterized as a highway for the transport of materials, such as centrioles and mitochondria (Cox and Spradling, 2003; Mahowald and Strassheim, 1970).

Alternatively, certain gradual modification(s) may occur prior to ring canal morphology change and accumulation of Ovhts-RC. The levels of this modification could act as a signal to identify the age of the ring canals. One candidate modifier is a phosphotyrosine containing protein(s). Phosphotyrosine epitope(s) was observed to localize to ring canals after the third mitotic division (Robinson et al., 1994). It will be interesting to determine whether this epitope differentiates the mitotic origins of ring canals.

Conclusions

Our results indicate that D. melanogaster oogenesis is an ideal system for studying incomplete cytokinesis. We have defined necessary terminology and have established a method to describe and follow the progression of IC. The ring measurements demonstrate that M1, M2, M3, and M4 ring canals are initially formed with progressively smaller diameters and that ring canals do not increase in size until stage nona-2a1. These discoveries have built a foundation for solving the mystery of IC and germ line cyst formation.

Experimental procedures

Fly strains and fly husbandry

Fly stocks were maintained on standard corn meal-molasses food. Newly eclosed flies were fed with wet yeast daily for optimal oogenesis for 2 to 3 days and then dissected. Three different strains of flies were used for ring canal measurements: Oregon R, w1118, and hsDMYPT;+;DMYPT03802/TM3 (or DMYPT03802 FRT/TM3). Similar results were obtained regardless of the strain used. Figs. 2–8 were generated using hsDMYPT;+;DMYPT03802/TM3 (or DMYPT03802 FRT/TM3), which were generated by crossing hsDMYPT;+;DMYPT03802/TM3 with w;+;DMYPT03802 FRT/TM3. DMYPT03802 is a P-element insertion mutation of DMYPT, the myosin binding subunit of myosin phosphatase (Tan et al., 2003). Figs. 10 and 11 were generated with Oregon R. Fig. 9 was generated with Pav-GFP, which was a gift from David Glover (University of Cambridge) (Minestrini et al., 2002). Ovaries were cultured for live-cell imaging as described in Prasad and Montell (2007).

Immunocytochemistry

Fixation and immunocytochemistry of ovaries was carried out as described by Lynn Cooley (http://info.med.yale.edu/cooley/Protocol%20Ovaries.html) with minor modifications. Briefly, we (a) dissected the ovaries in EBR (NaCl 130 mM, KCl 4.7 mM, CaCl2 1.9 mM, HEPES (pH6.9) 10 mM); (b) fixed ovaries in 200 μl devitellinizing buffer (1 volume of buffer B, which is 100 mM KH2PO4/K2HPO4 (<25:20) pH 6.8, 450 mM KCl, 150 mM NaCl, and 20 mM MgCl2.6H2O, 1 volume of 36% formaldehyde, 4 volume of water) and 600-μl heptane for 5 min; (c) removed solution and rinsed ovaries with PBS (g/l) (20 NaCl, 0.5 KCl, 0.5 KH2PO4, and 2.78 Na2HPO4.2H2O) three times; (d) washed
with PBS with 0.1% Triton X-100 three times (10 min each); (e) blocked ovaries in PBST (PBS with 0.3% Triton X-100) and 1 μg/μl of BSA (1 to 10 dilution of NEB’s 100× BSA) or normal goat serum (5%) for 20 min; (f) incubated the ovaries in 50 to 100 μl of primary antibody mix in PBS with 0.1% Triton X-100 and 0.1% NaN3 at room temperature for 2 h or at 4 °C overnight, rinsed with PBST three times, then washed four times (10 min each); (g) incubated in the secondary antibody mix at room temperature for 1 h, rinsed with PBST once, and then washed with PBST three times (15 min each); (h) after rinsing with PBS for 2 times, the ovaries were teased apart and mounted with ProlongGold mounting media (Invitrogen), cured for 1 h, sealed with colorless nail polish, kept at room temperature overnight, and then stored at 4 °C.

The following primary antibodies and dilutions were used: rabbit anti-anillin, 1 μg/ml (gift of C. Field; Field and Alberts, 1995), mouse anti-C(3)G 1:500 (gift of S. Hawley; Page and Hawley, 2001), mouse anti-α-spectrin 1:100 [3A9 supernatant, Developmental Studies Hybridoma Bank (DSHB)], mouse anti-Ovh-ts-RC 1:50 (DSHB, supernatant; Robinson et al., 1994), and guinea pig anti-tropomodulin 1:1000 (gift of H. Bellen; Lighthouse et al., 2008). Secondary antibodies (Invitrogen) were as follows: Alexa488 donkey anti-rabbit IgG, Alexa546 goat anti-mouse or guinea pig (highly cross-adsorbed), and Alexa647 goat anti-guinea pig or mouse (highly cross-adsorbed) 1:500.

Images in Fig. 9 were captured with Olympus BX61 (60× objective, image scaling: 0.12 μm, 0.50 μm), followed by deconvolution with Slidebook v4.0.2.2, and processed with Zeiss LSM Image Examiner. All other images were captured on a Zeiss LSM 510 META (×63, C-Apachromat objective, zoom 3×, Z step size 0.5 μm) and processed with Zeiss LSM Image Examiner and Microsoft PowerPoint.

Measurement of contractile ring and ring canal sizes

Ovaries from 10 to 20 females for each genotype or treatment were dissected and immunostained with antibodies of anillin and α-spectrin. Confocal Z-stacks were generated for 30 such stained germaria. To analyze the data with Zeiss LSM Image Examiner, we identified a germline cyst and determined its stage based on its location in the gerarium, its fusome morphology, and the intensity and distribution of its anillin staining. Once the plane containing the maximum diameter of a contractile ring or ring canal is determined, we recorded the ring size and the coordinates of the center of the ring. These coordinates are the identification number for that ring. In addition to the size of the ring, we also recorded the shape of the ring canal, its association with the fusome, the morphology of the fusome, and the intensity of anillin nuclear staining.

Statistical analysis

All tests for significant differences used a parametric ANOVA with Duncan post hoc tests when warranted.

Acknowledgments

We are grateful to E. Fernandez and T. Phillips for confocal assistance, D. Cornelison for letting us use her Olympus microscope, C. Field, S. Hawley, H. Bellen, and DSHB for antibodies, D. Glover for fly stocks, T. Zars, B. McClure, C. Foote, K. Cone, K. Bennett, J. Bai, R. Binari, and D. Sezier for constructive discussions, T. Zars for helping with statistics, M. Chen, K. Powers, C. Shi, and W. Wu for lab assistance, and unknown reviewers for critical readings and constructive suggestions of the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.ydbio.2009.10.018.