Formation of Actin Filament Bundles in the Ring Canals of Developing *Drosophila* Follicles

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Abstract. Growing the intracellular bridges that connect nurse cells with each other and to the developing oocyte is vital for egg development. These ring canals increase from 0.5 μm in diameter at stage 2 to 10 μm in diameter at stage 11. Thin sections cut horizontally as you would cut a bagel, show that there is a layer of circumferentially oriented actin filaments attached to the plasma membrane at the periphery of each canal. By decoration with subfragment 1 of myosin we find actin filaments of mixed polarities in the ring such as found in the "contractile ring" formed during cytokinesis. In vertical sections through the canal the actin filaments appear as dense dots. At stage 2 there are 82 actin filaments in the ring, by stage 6 there are 717 and by stage 10 there are 726. Taking into account the diameter, this indicates that there is 170 μm of actin filaments/canal at stage 2 ($\pi \times 0.5$ μm $\times$ 82), 14,000 μm at stage 9 and ~23,000 μm at stage 11 or one inch of actin filament! The density of actin filaments remains unchanged throughout development. What is particularly striking is that by stages 4–5, the ring of actin filaments has achieved its maximum thickness, even though the diameter has not yet increased significantly. Thereafter, the diameter increases. Throughout development, stages 2–11, the canal length also increases. Although the density (number of actin filaments/μm^2) through a canal remains constant from stage 5 on, the actin filaments appear as a net of interconnected bundles. Further information on this net of bundles comes from studying mutant animals that lack kelch, a protein located in the ring canal that has homology to the actin binding protein, scruin. In this mutant, the actin filaments form normally but individual bundles that comprise the fibers of the net are not bound tightly together. Some bundles enter into the ring canal lumen but do not completely occlude the lumen. All these observations lay the groundwork for our understanding of how a noncontractile ring increases in thickness, diameter, and length during development.

Actin filaments are key elements in the dynamic machinery of motile cells and in the scaffolding that maintains cell shape. However we still have only a rudimentary understanding of how the scaffold is formed in vivo, what the essential non-actin components are, and precisely how the steps in assembly and/or dynamics are regulated.

In the past few years interest in *Drosophila* oogenesis has increased enormously because this well-described and progressive process offers a logical and practical place to understand developmental programming in an organism where genetic strategies followed by molecular analysis have been particularly successful. Cell biologists interested in the formation of actin scaffolds are also interested in *Drosophila* because mutations affecting specific cytoskeletal proteins are available and null mutants lacking these actin binding proteins have a discernible phenotype.

A typical *Drosophila* ovary contains ~16 tubular ovarioles, each representing an independent egg assembly line. Each ovariole is tipped with a distinct region or germarium in which germ line and somatic stem cells become organized into follicles; each follicle leaves the germarium and continues developing as it moves posteriorly within the ovariole. In the germarium each germ line stem cell divides mitotically four times. Cytokinesis is incomplete and accordingly the 16 cells remain connected through cleavage furrows that subsequently develop into ring canals. Among the 16 cells, one develops into the oocyte while the other 15 become nurse cells. The oocyte nucleus enters meiosis and undergoes very little transcription during oogenesis. In contrast the nurse cells become polyploid and synthesize mRNA, proteins, ribosomes, and organelles that are provided to the oocyte by cytoplasmic flow through the ring canals (see Cooley and Theurkauf, 1994). Surrounding this nurse cell-oocyte syncytium is a monolayer of ~1,000 follicle cells of somatic origin. This unit has been termed an egg chamber but corresponds to a follicle in other species.

The ring canals of *Drosophila* have attracted attention

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ever since Warn et al. (1985) showed that the periphery of the ring canal stained with fluorescent phalloidin and thus contained actin filaments. Immunofluorescence studies have shown that at least two other proteins, hts (Yue and Spradling, 1992) and kelch (Xue and Cooley, 1993), are also located in the ring canal (Robinson et al., 1994). Although the ring canals have been examined in thin sections at low resolution, surprisingly no one has described the distribution, polarity, length, number or packing of the actin filaments in the canal in wild-type *Drosophila* or in mutants. Nor has it been determined if the "homogenous material" at the borders of the canal is even filamentous (Mahowald, 1971; Mahowald and Kambysellis, 1980; Yue and Spradling, 1992; Robinson et al., 1994). This lack of information is possibly due to the fact that these egg chambers are difficult to fix. A more likely reason is that thin section analysis before or after detergent extraction, or actin filament decoration with subfragment 1 of myosin, is tedious, labor intensive, and old-fashioned.

The purpose of this paper is to describe the organization of the actin filaments in ring canals in some detail and to suggest how this canal increases in diameter, length and width. A puzzling feature of ring canal actin filaments (recall that the ring canal is derived from the contractile ring) is that this ring does not contract, but in fact does just the opposite, it expands. During an early stage in egg chamber maturation (e.g., stage 2) the ring canal is less than 0.5 μm in diameter, yet at a late stage (e.g., stage 11) it is 10 μm in diameter (Warn et al., 1985). As will be documented below this expansion requires a 134-fold increase in the total length of F-actin as well as an increase in the number of actin filaments.

It is our hope that this detailed description of ring canal development will be useful to other investigators employing this system to understand how actin scaffolds are formed. We also describe the ring canals in *kelch* mutants that fail to express a ring canal protein (Xue and Cooley, 1993) as it helps us to understand the net-like structure of the ring canal in stages 6–11. The kelch protein has homology to the actin binding and crossbridging protein, scruin (Way et al., 1995), found in *Limulus* sperm (Tilney, 1975).

Materials and Methods

Culturing of Drosophila

Wild-type (Canton S) and *kelch* mutant (Xue and Cooley, 1993) flies were obtained from Lynn Cooley (Yale University, New Haven, CT) and cultured using standard methods at 25°C. The kel^{neo} allele was maintained over the *CyO* balancer (Lindsley and Zimm, 1992). kel^{neo}/kel^{neo} adult females were identified by their wild-type (Cy^+/Cy^+) wing phenotype. To obtain ovarioles with follicles at all stages in oogenesis we took newly emerged flies and placed them in new vials for 4 d. 1 d before killing we added a small dollop of yeast paste to stimulate oogenesis. By the time of death the females had enormous abdomens with ovarioles swollen with egg chambers at all stages.

Drosophila Saline

One of the keys to adequate fixation is to dissect out the egg chambers in a *Drosophila* saline that is not hypertonic. Singleton and Woodruff (1994) analyzed *Drosophila* hemolymph and found that the tonicity of the hemolymph is 255 mOsmol/l, lower than that of all existing formulations of *Drosophila* saline. They then designed a saline that had the same osmolarity by freezing point depression as *Drosophila* hemolymph. Physiologically this difference in tonicity is essential in maintaining the egg chambers in a "functioning" state as assayed by (*a*) dye spread from the nurse cells to the oocyte and (*b*) potential differences across the epithelium. We use their glutamate saline solution made up with the following ingredients: 100 mM sodium glutamate, 25 mM KCl, 15 mM $MgCl_2$, 5 mM $CaSO_4$, and 2 mM sodium phosphate buffer at pH 6.9. This solution is slightly hypotonic but it can be made isotonic by the addition of glucose if necessary.

Light Microscope Procedures

After ovarioles and egg chambers are dissected, fixed, and washed, we examine them with a fluorescent microscope using the methods of Peifer et al. (1993). This includes fixation for 15–30 min with freshly made paraformaldehyde in a solution as follows: 5.0 ml of 0.2 M Pipes, 0.4 ml of 25% Triton X-100, 4.0 ml 2.5% paraformaldehyde, 40 μl 250 mM EDTA neutralized to pH 7.0, 0.4 ml 50 mM $MgSO_4$, 160 μl H_2O. The material is then washed in a solution of 50 mM Tris (pH 7.4), 150 mM NaCl and 0.5% Nonidet containing goat serum and BSA, and then incubated with phalloidin conjugated to rhodamine (Sigma Chem. Co., St. Louis, MO). The preparation is then examined with a Zeiss Universal fluorescent microscope (Carl Zeiss, Inc., Thornwood, NY).

Detergent Extraction

After dissection in the glutamate saline solution just described, egg chambers are extracted for 10 min at 4°C in a solution containing 1% Triton X-100, 3 mM $MgCl_2$, and 30 mM Tris (pH 7.5) with agitation on a rotating shaker. After 10 min, the detergent extracted egg chambers are fixed by immersion. In some preparations we add phalloidin to the detergent solution and in the fixative to be sure that the filaments do not break down before fixation.

Decoration with Subfragment 1 of Myosin (S1)

After detergent extraction, the egg chambers are incubated in a solution containing 4 mg/ml S1 in 0.1 M phosphate buffer (pH 6.8) for 20 min at room temperature on a rotating table. The S1 had been prepared many years before and kept frozen in small aliquots in liquid nitrogen at a concentration of 70 mg/ml (Tilney and Tilney, 1994).

After decoration, the specimen is washed in phosphate buffer and fixed at room temperature in 1% glutaraldehyde in 0.1 M phosphate buffer at pH 6.8 containing 2% tannic acid. The preparation is then washed in buffer and postfixed for 45 min at 4°C in 1% OsO_4 in 0.1 M phosphate buffer at pH 6.2 and processed further as mentioned below.

Electron Microscope Procedures

For routine examination of egg chambers before or after detergent extraction they are fixed by immersion in a freshly made solution of 1% glutaraldehyde (from an 8% stock; Electron Microscope Sciences, Fort Washington, PA), 1% OsO_4 and 0.05 M phosphate buffer at pH 6.2. Fixation is carried out for 45 min at 4°C. Because the glutamate in the *Drosophila* saline (small quantities are introduced when the egg chambers are pipetted into fixative) interacts with the fixative after 5 min the egg chambers are put into fresh fixative.

After fixation the egg chambers are washed three times for 5 min each in 4°C water to remove excess phosphate and then stained en bloc overnight in 0.5% uranyl acetate. The specimens are then dehydrated in acetone and embedded in Epon. Thin sections are cut with a diamond knife, stained with uranyl acetate and lead citrate, and examined with a Philips 200 electron microscope. It is essential to examine uncoated grids.

Rationale for Fixation Methods Used

To visualize the actin cytoskeleton one must extract from view as many soluble components as possible because with conventional fixation they obscure the actin cytoskeleton (see Tilney and Tilney 1994). For *Drosophila* egg chambers this turns out to be a particularly thorny problem as each egg chamber is surrounded not only by a layer of follicle cells, but worse, by a thick basement membrane secreted by the follicle cells. Furthermore the nurse cells produce large numbers of soluble macromolecules: ribosomes, tRNAs, mRNAs, and proteins for use later on in development.

The key to fixation of the egg chambers is to use an isotonic or slightly hypotonic saline solution so that the oocyte and nurse cells are not

shrunken before fixation. This seems obvious, but, as already mentioned, most *Drosophila* saline solutions are hypertonic to *Drosophila* hemolymph (Singleton and Woodruff, 1994). We then use a fixative designed to extract the soluble proteins but maintain the cytoskeleton (see Tilney and Tilney, 1994). Briefly, if one uses osmium and glutaraldehyde together, the osmium pokes holes in the plasma membrane that allows small soluble proteins to escape before being cross-linked in place by the fixative. The fixative is made in a low pH phosphate buffer that tends to stabilize the actin filaments to the destructive properties of osmium. To further avoid osmium mediated degradation, fixation is carried out at 4°C for no longer than 45 min (Tilney and Tilney, 1994). More details of the cytoskeleton can be visualized if the egg chamber is detergent extracted before fixation.

Actin Filament Measurements

Thin sections of detergent extracted egg chambers were scanned and photographs of vertical sections (see Fig. 3) through canals were taken at 35,000×. Favorable negatives were enlarged photographically to 200,000–250,000 times. Under a lit magnifier the number of dots or transverse sections of actin filaments was counted. As each dot on the micrograph was counted it was crossed off with a grease pencil so no dots were counted more than once. Some micrographs were counted twice; the difference in actin filament number was less than 1%.

To obtain the density (number of filaments per μm^2) we overlaid micrographs of vertical sections of ring canals with a sheet of mylar and traced the region that was counted. This region was cut out and weighed and compared to a cut out of a μm^2 that had been magnified to the same degree. Because ring canals in stages 6 to 10 consist of separate bundles of filaments, we cut out only that part of the ring canal that contained filaments (e.g., see Fig. 5, the cross section of the ring of a stage 9 egg chamber has 4 portions on the right).

Results

Phalloidin Staining of F-Actin in Ring Canals

Each egg chamber contains a single developing oocyte and 15 nurse cells all surrounded by a layer of follicle cells (Fig. 1). The nurse cells and oocyte are all derived from four consecutive mitotic divisions of a single germ line cell. Cytokinesis is incomplete resulting in all 16 cells being connected together by intracellular bridges that subsequently differentiate into ring canals (Fig. 2). Each oocyte has four ring canals (Figs. 1 and 2) as does one of the nurse cells. Two other nurse cells have three ring canals, four contain two, and the remaining eight have only one(Fig. 2). Because there are four consecutive divisions and time has passed between these divisions, logically the oocyte (labeled *O* in Fig. 2) must have rings of different diameters. This can be seen by carefully examining the oocyte illustrated in Fig. 1, where canals of 4·9, 3·8, 2·9, and 2·6 μm are present. Since the last mitotic division would give rise to the smallest canals and the most recently formed cells must be located at the periphery of the mass of 16 cells, we would predict that the smallest canals would located farthest from the oocyte. This is indeed true (Fig. 1, *arrow*). Thus, the diameters of the ring canals vary in a predictable way that is dependent upon the time elapsed during these mitotic divisions.

Organization of Actin Filaments in Ring Canals

The terminology we use to describe the plane of sections through the ring canals is illustrated in Fig. 3. It is easy to obtain vertical, oblique and grazing sections through canals, but horizontal sections are difficult to get because the egg chambers, even at early stages, are large relative to the thickness of thin sections and the orientation of the canal is not predictable. For example, a stage 4 egg chamber is 40–50 μm in diameter and by stage 9 the egg chamber is 150 × 300 μm while the section thickness is only 0.05 μm.

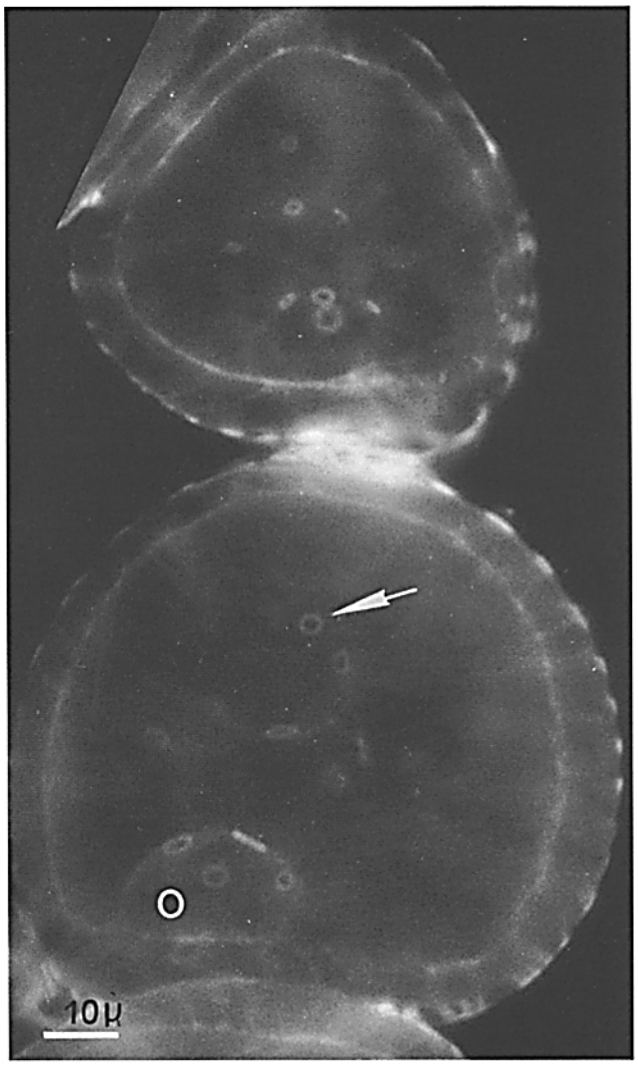


Figure 1. A portion of a single ovariole stained with fluorescent phalloidin illustrating two egg chambers. The oocyte (*O*) of each egg chamber is round and contains four ring canals of different diameters. The arrow points to a ring canal between two nurse cells located furthest from the oocyte. This canal was formed during the last mitotic division of the germ line cell. Its diameter is identical to the smallest of the four ring canals of the oocyte. Ring canals between many of the 15 nurse cells can also be seen.

In Fig. 4 *a* we depict a horizontal section through a ring canal of a stage 9 egg chamber. This canal connects the cytoplasm of a nurse cell with oocyte cytoplasm. These would be located in planes in front and behind this section. Located within the canal are mitochondria, ER, and ribosomes, presumably on their way into the oocyte. At the margins of the canal is a layer of filaments aligned circumferentially around the canal (Fig. 4 *b*). From the phalloidin staining illustrated earlier (Figs. 1 and 4 *a*, *inset*) we can show that these filaments are actin filaments.

In vertical sections through a ring canal (Fig. 4 *c*) the plasma membranes of two adjacent cells can be easily identified even at low magnification because of the layer

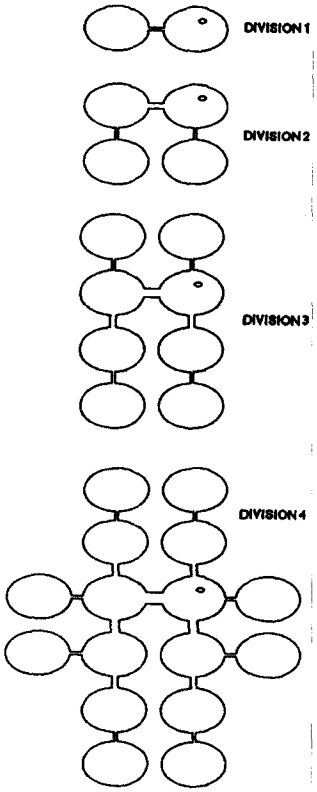


Figure 2. Drawing illustrating the appearance of the ring canals during the four consecutive mitotic divisions of the germline cell in the egg chamber. Each oocyte (*O*) is ultimately connected to four nurse cells. As ring canal diameter increases during the time period that has elapsed during these four divisions the canals will be of different sizes depending upon when division occurred. Significant is the fact that the nurse cells with the smallest canals should be located at the periphery of this 16-cell cluster while the oocyte will have four ring canals, each of a different diameter.

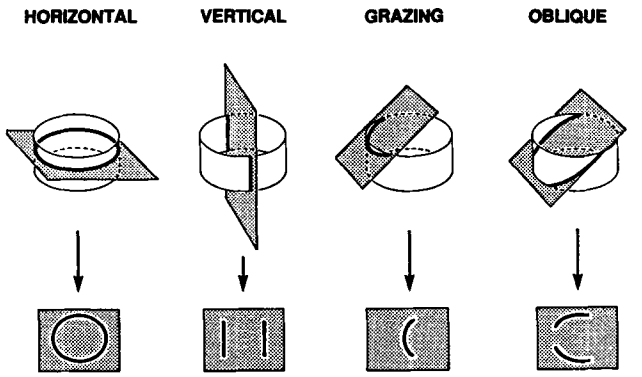


Figure 3. Drawing illustrating four possible section planes that will be made through an individual ring canal, e.g., horizontal, vertical, grazing and oblique. Beneath each is the image of the ring canal visualized in a thin section.

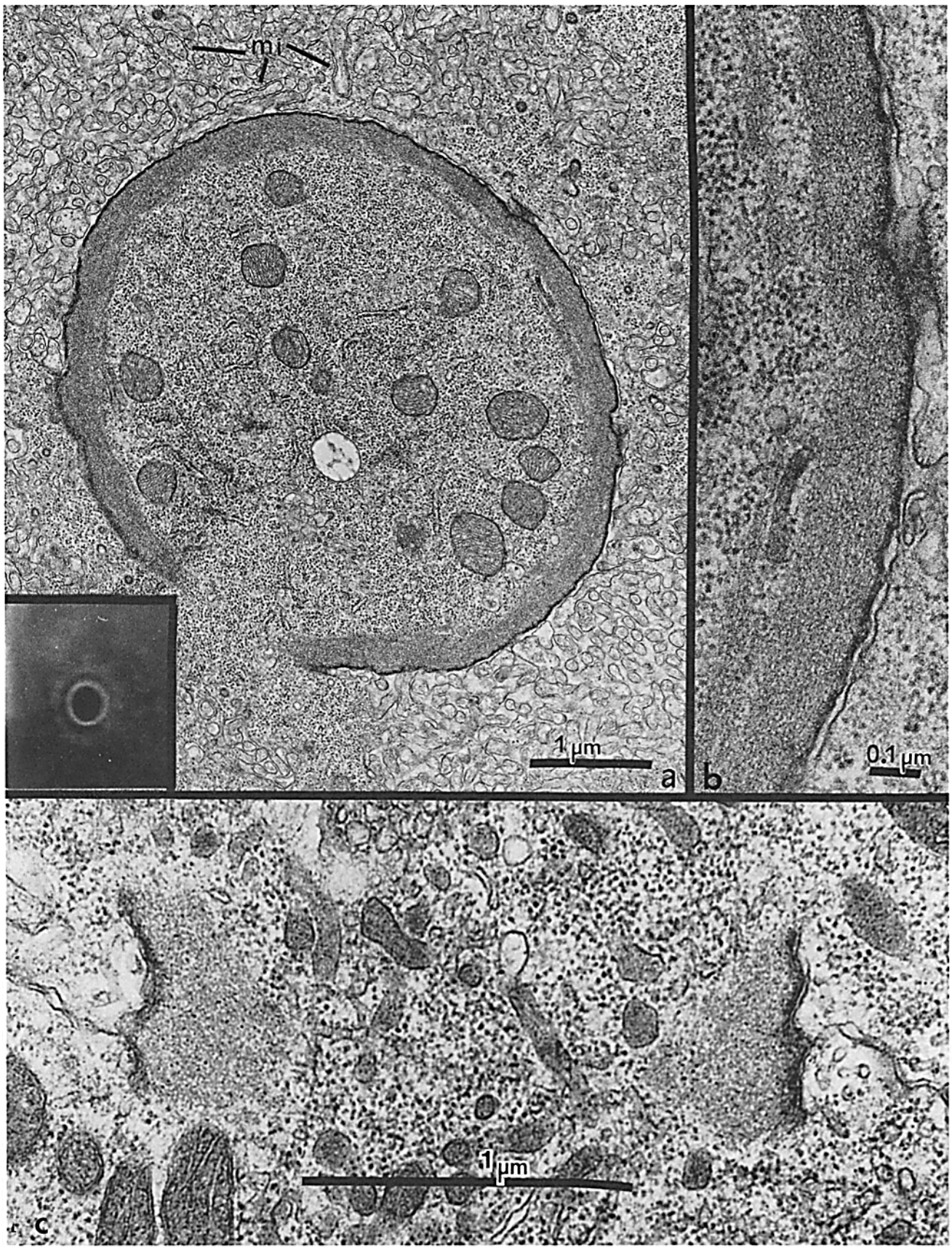
of electron dense material attached to the cytoplasmic surface of the common plasma membrane: inside this is some filamentous material which must be the actin filaments that we visualize by phalloidin staining. Peripheral to the ring canal the oocyte plasma membrane has numerous projections or microvilli extending from the surface of the oocyte membrane (Fig. 4 *a*). The actin filaments in these microvilli account for the diffuse phalloidin staining of the outer ring (Fig. 4 *a*, *inset*).

Careful examination of the ring canals present in stage 6 and older egg chambers reveals that the actin-containing ring is not homogeneous but instead consists of bundles of actin filaments. Similar bundles have recently been described by Riparbelli and Callainí (1995). In spaces between the actin filament bundles we find ribosomes and small vesicles (Fig. 4, *a* and *b*).

From thin sections through egg chambers extracted with Triton the plasma membrane and most of the other cytosolic membranes are solubilized and all that remains are the chromatin, ribosomes, and the cytoskeleton. None of these components diffuse away because they are enclosed by the basement membrane that surrounds the entire egg chamber, a layer secreted by the follicle cells. If collagenase is added either before or during detergent extraction everything disperses as the basement membrane has been solubilized.

The electron-dense material formerly located on the inner surface of the plasma membrane remains attached to the actin filaments in the ring so that the outer margin is still distinct. At the same time the underlying actin filaments are clearly visible because the soluble proteins have diffused away before fixation. Thus, in a horizontal section individual filaments can be resolved; some are several microns in length (data not shown). In vertical sections the actin filaments in the ring appear as dots as they are cut in transverse section (Fig. 5). Vertical sections from stage 6 to stage 10 show clusters of dots or a network of actin filament bundles. In the spaces between the bundles we see ribosomes. Even though the same network pattern exists in non extracted preparations of ring canals of stages 6 to 10, we were concerned that the separations between bundles might be larger in extracted preparations due to the depolymerization of the actin filaments before fixation. This does not seem to be a concern because if we add phalloidin to the detergent solution, which should stabilize the actin filaments so that they do not depolymerize before fixa-

Figure 4. (*a*) A horizontal section through a ring canal of a stage 9 egg chamber. Attached to the cytoplasmic surface of the plasma membrane limiting the canal is a thin layer of electron dense material. Within this material is a layer of actin filaments that encircles the canal. Within the canal proper (which connects the developing oocyte to a nurse cell) are mitochondria, elements of rough ER, a vacuole, and ribosomes. Peripheral to the canal proper are numerous microvilli (*Mi*) that are connected to the plasma membrane of the oocyte (upper part of the sections) and a nurse cell (lower part). We can identify the oocyte microvilli by the coated pits and vesicles used in yolk protein uptake. The insert is a fluorescent micrograph of an individual ring canal in a stage 7 egg chamber stained with fluorescent phalloidin. The fluorescence of the inner ring is due to the actin filaments in the ring canal proper. The outer, more diffuse, fluorescence ring can be ascribed to actin filaments in the microvilli that surround the ring canal. (*b*) Higher magnification of the ring canal illustrated in *a*. The actin filaments in this horizontal section can be seen to be arranged circumferentially around the canal. The electron dense material can be seen to be attached to the cytoplasmic face of the plasma membrane limiting the canal. (*c*) A vertical section through a ring canal. Attached to the cytoplasmic face of the plasma membrane limiting the ring canal is some electron-dense material and within this is at low resolution some amorphous material that at higher resolution appears as electron dense dots or cross sections of actin filaments. This canal connects the cytoplasm of two adjacent cells.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a
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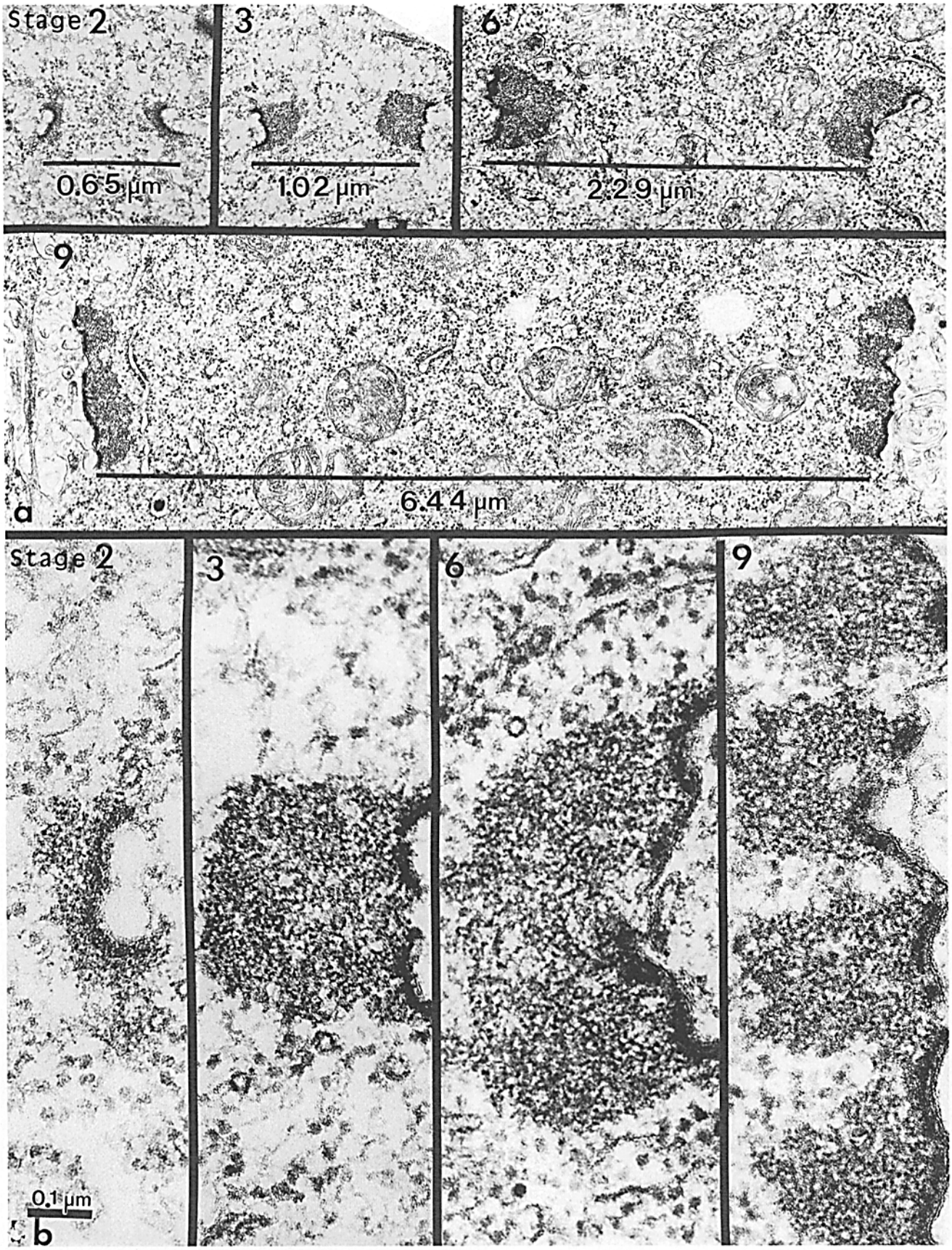
Stage 2
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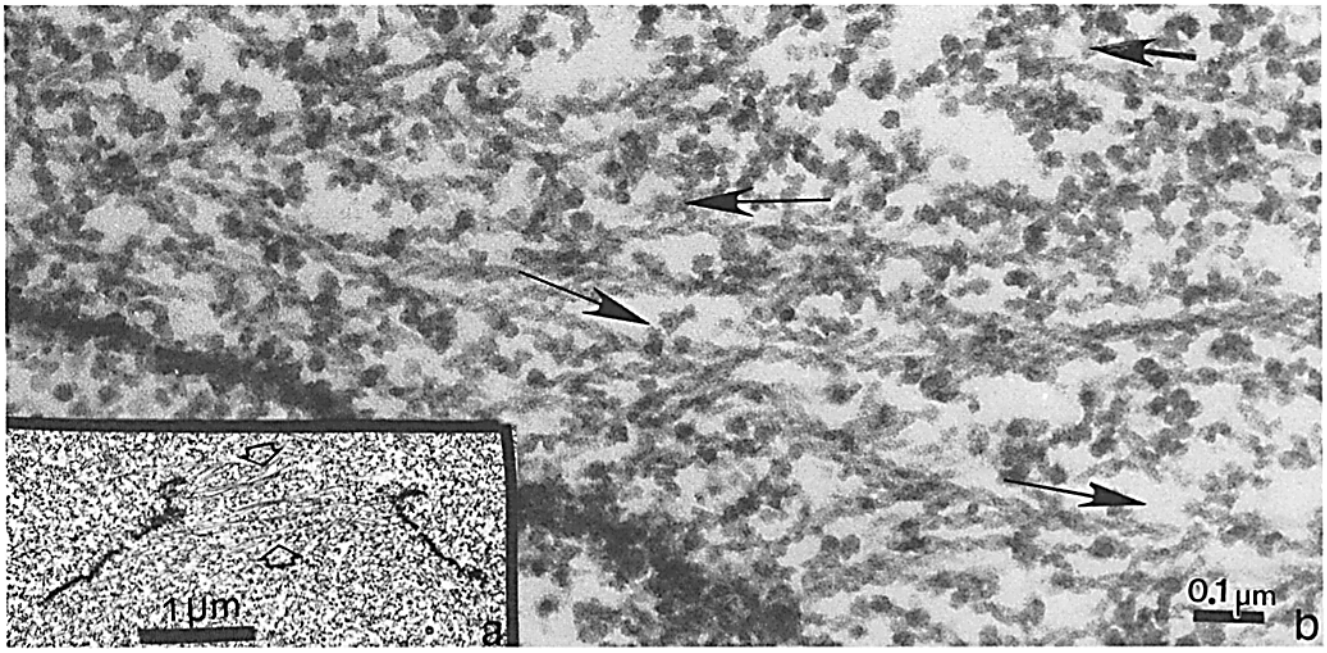

Figure 6. (*a*) Thin section through a portion of an egg chamber of an ovariole that had been detergent extracted for 10 min then incubated in subfragment 1 of myosin before fixation. In this section is a portion of a former ring canal. The electron dense material limiting the canal on its margins is visible. The arrows point to the filaments that will be shown at higher magnification in *b*. (*b*) A portion of the section illustrated in 6 *a* is shown at higher magnification. Near and in some cases extending from the electron dense material that limited the margin of a ring canal are some actin filaments decorated with subfragment 1 of myosin to reveal an arrowhead appearance. Of particular interest is the fact that the filaments illustrated here demonstrate mixed polarities. The polarity of four of these are indicated by the arrows.

tion, the same discontinuities of approximately the same size in the actin ring and the electron dense material are found.

Actin Filaments in the Ring Canals Have Mixed Polarities

Surrounding each ovariole is a thin layer of skeletal muscle that, by contracting, moves the developing egg chambers posteriorly. To visualize the polarity of the actin filaments one can decorate them with subfragment 1 of myosin that gives the filaments an arrowhead appearance. The actin filaments in these muscles decorate beautifully after detergent extraction. Because detergent extraction, which is necessary to allow the S1 to diffuse into the egg chambers, solubilizes formed organelles and membranes, the ring canals do not necessarily remain in their original orientations.

The electron-dense material that was formerly attached to the cytoplasmic surface of the plasma membrane around the ring canals continues to be associated with the actin filaments surrounding the ring canals (Fig. 6 *a*). Inside this dense material is a large population of decorated actin filaments. Two features are obvious; first, individual filaments can be followed for distances of up to 3 μm and second, while individual filaments display unidirectional polarity as judged by the S1 arrowheads, a comparison of neighboring filaments in the same canal reveals some pointing one way, others in the opposite direction (Fig. 6 *b*). Thus, the filaments in the ring canals are of mixed polarities.

Expansion of the Ring Canals during Oogenesis

Besides the obvious increase in ring canal diameter during development, there is also a dramatic increase in thickness and in length of the ring canals (see Fig. 7 for the terminology). We used phalloidin stabilized, extracted preparations because we can count the filament number in vertical sections and at the same time measure the diameter, thickness and length of the canals (Tables I and II and Fig. 5).

Ring canals of stage 2 egg chambers measure 0.5–1.0 μm in diameter by light microscopy (Warn et al., 1985) and 0.65 μm in thin, vertical sections. By stage 11 some have reached their maximum diameter of 10 μm. This change in diameter of ~15-fold is an increase in circumference (πd) of 29.4 μm (10π–0.65π). From these same vertical sections

Figure 5. (*a*) Ring canals of detergent extracted egg chambers of stage 2, stage 3, stage 6, and stage 9. From micrographs such as these we can measure the diameter, thickness, and the length of the ring canals. In stage 2 and 3 egg chambers the actin filament part of the ring is homogeneous, yet by stage 9 the actin containing portion is broken into four discrete bundles on the right hand and 3 on the left. (*b*) Portions of the right side of the ring canals illustrated at higher magnification. The electron dense dots are cross sections of actin filaments.

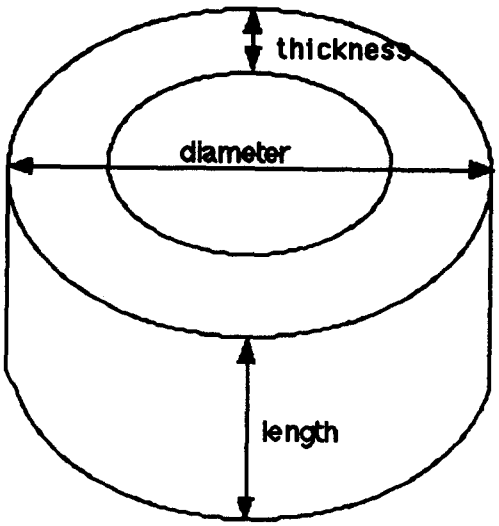

Figure 7. Drawing illustrating the terminology we will use to describe the dimensions of the ring canal.

we can determine the number of actin filaments in each half ring canal by enlarging the requisite micrographs and counting the number of actin filaments seen in cross section (see Table II). In a stage 2 ring canal we count 82 filaments and in a stage 9 ring canal 726.

From the above values we can calculate the total length of F-actin per canal which is the filament number times the circumference. This calculation is interesting because it tells us how much F-actin is added to the ring canal as it increases in diameter. Thus, in a stage 2 egg chamber there is 170 μm of F-actin ($0.65 \times \pi \times 82$) per canal and in a stage 9 ring canal 14,316 μm ($6.28 \times \pi \times 726$). Assuming the same filament number at stage 11 (we have no direct observations on the number), there should be 22,796 μm ($10 \times \pi \times 726$) or a 134-fold increase during development.

How is F-actin assembly increased by 134-fold during ring canal development? Do the ring canals increase in size at a constant rate throughout development or are there periods of rapid expansion followed by periods of slower expansion and how does this relate to increases in the amount of F-actin? To answer these questions we examined specific stages in development and correlated changes in the diameter, width, and length of the ring canals with the amount of F-actin. Specific stages are identified by changes in the morphology of the egg chambers such as the distribution of follicle cells, total dimensions,

Table I. Dimensions of the Ring Canal during Stages in Development

Stage	Thickness	SEM	Length	SEM	Diameter	SEM
2	0.06 (5)	0.01	0.22 (5)	0.01	0.65 (3)	0.18
3	0.24 (4)	0.02	0.33 (5)	0.03	1.02 (2)	0.01
5	0.41 (9)	0.04	0.46 (11)	0.02	1.52 (6)	0.07
6	0.35 (13)	0.02	0.58 (11)	0.03	2.29 (7)	0.17
7	0.26 (8)	0.01	0.90 (2)	0.08	4.62 (5)	0.21
9	0.37		1.87		6.28	

All measurements are in microns. The number in parentheses, number of canals examined.

Table II. Measurement of Actin Filaments in the Ring Canals during Stages in Development

Stage	Filament	Density	Total length
	no./ring	*filament no./μm²*	*μm²*
2	82	3727	170
3	366	4206	1172
6	717	5049	5403
7	768	3918	12,781
9	726	4118	14,316

the appearance of yolk platelets and nurse cell dumping. From information published in the literature (see Spradling, 1993) we first related egg chamber stage, e.g., 2–11, to elapsed time in development as it is the latter that is important here (Fig. 8 *a*). Using this standard curve and information included in Tables I and II we can express the increase in ring canal diameter, thickness and length (Fig. 8, *b–d*) relative to developmental time.

What our measurements show is that the ring canal diameter (Fig. 8 *b*) increases slowly until stage 5; from stage 5 on the diameter increases dramatically. In contrast the thickness of the actin containing portion of the ring increases during stages 2–5, but after stage 5 no further increase in thickness occurs (Fig. 8 *c*). Thus, canal thickness and diameter appear to be increasing at different times during development. The length of the ring increases from stage 2 until stage 10 (Fig. 8 *d*). The growth in the early stages (stages 2–5) is due to an increase in the number of actin filaments. As ring length increases from stage 6 to stage 10 the total number of filaments (as assayed by the number of filaments counted in single cross section through the canal, Tables I and II) remains constant (Table II) but bundles appear with separations between them. Another way of expressing this is to measure the density of the actin filaments as a function of time (Fig. 9 *a*). Here we disregard the spaces between the bundles. For example, in Fig. 5, a vertical section of a stage 9 egg chamber, there are three to four discrete bundles in the ring. The density of actin is calculated from the bundles only, not the space between them. It turns out that the density or number of actin filaments per unit area in a thin section remains constant throughout development (Fig. 9 *a*). After stage 5 increases in canal length must occur by breaks or discontinuities appearing in the F-actin containing rings. Thus, the ring canals of older stages are composed of an interlocking net of filament bundles, a conclusion strengthened by our data on the *kelch* ring canals (see below).

Finally, we plotted the total length of F-actin vs. developmental time (Fig. 9 *c*). What this curve shows is that although the density of the filaments remains constant (Fig. 9 *a*), there is a huge increase in the total amount of actin at later stages in development, e.g., after stage 5. This is due to the striking increase in circumference of the ring canals in these later stages.

We are aware that there are inherent errors in these measurements. First, although we tried to be accurate in assessing the embryonic stages to individual egg chambers, we recognize that until stage 11 each stage is 4–6 h in duration so exact timing is difficult. Second, the diameters of individual ring canals in the same egg chamber vary. These differences in diameter reflect the age of the individual

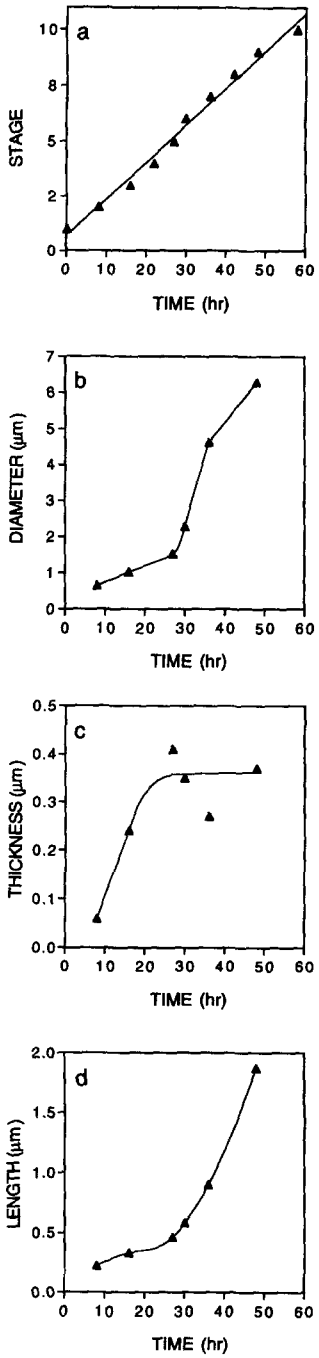

Figure 8. (*a*) Graph of stages in development vs. developmental time in hours (Spradling, 1993); (*b*) the increase of diameter of the ring canals; (*c*) the increase in thickness; and (*d*) increase in length during development. Note that the thickness plateaus at an early stage while the diameter and length increase primarily at later times.

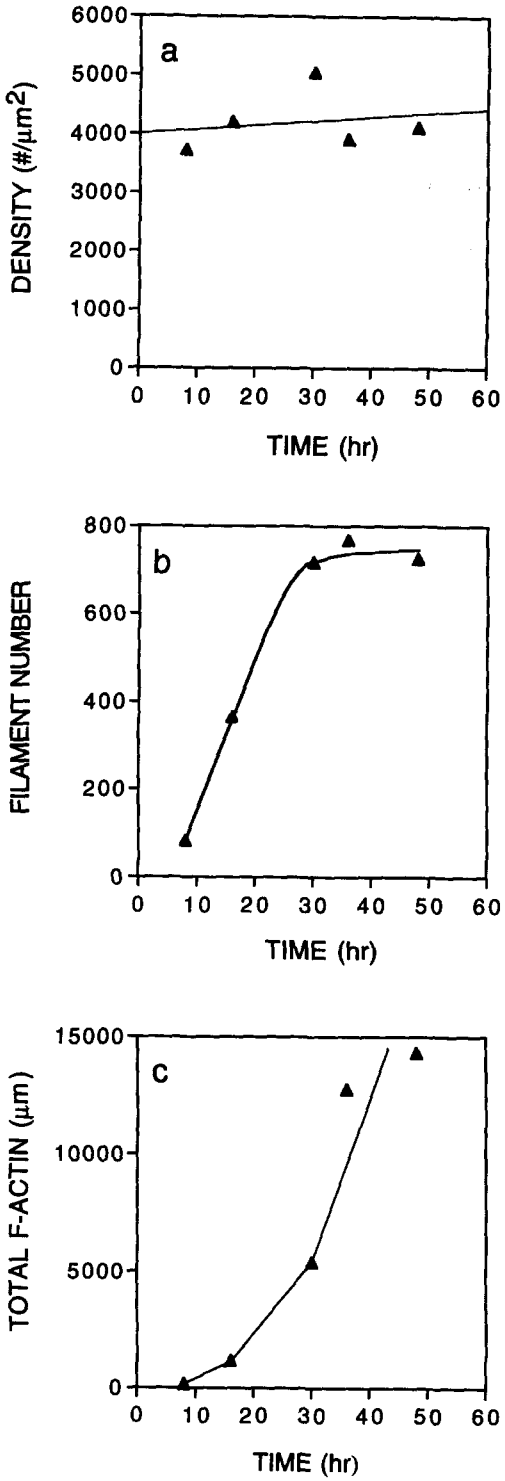

Figure 9. (*a*) Graphs of density of actin filaments vs. developmental time; (*b*) number of actin filaments vs. time; and (*c*) total amount of actin in the ring canal. The density remains constant as does the number of filaments after stage 6, but the total amount of actin continues to increase with time.

ring canals after formation (see Fig. 2). Since the ring canals on the oocyte are positioned at random, when we cut sections through the surface of the oocyte we have no way of knowing if a particular canal is the result of the first or last mitotic division. On the other hand, it is possible that we are measuring the same ring canal in each egg chamber because each egg chamber is positioned in the microtome in the same orientation. Thus, we try to cut a grazing section through the interface between the oocyte and its nearest nurse cells. And finally, there may be differences in the total amount of F-actin in individual ring canals of the same diameter. We counted the number of actin filaments in three vertical sections through 3 different ring canals at stage 10. The values are 677, 734, and 766. If these are representative values, then differences in individual ring canals of the same diameter would result in a relatively small error.

How then does the F-actin in the ring increase? Where are monomers added? How long are individual actin fila-

ments? Why do bundles appear after stage 5 and lie in a net instead of a homogeneous mat? One possibility for the last question is that as the canal increases in diameter entirely new assembly sites are activated on the ring canal membrane. Thus, new bundles of actin would start appearing. Such a hypothesis is strengthened by the data from examining the *kelch* mutant that provides additional information on the net of actin bundles that appears from stage 6 on.

kelch Ring Canals

Cytoplasmic transport from the nurse cells into the oocyte occurs in two phases. The first, or slow phase, begins at stage 5 (Spradling, 1993). A second, or late phase, begins in stage 11 egg chambers when massive dumping of nurse cell cytoplasm into the oocyte occurs during a 30-min period. From the literature the first defect noted in the *kelch* egg chambers is in stage 7–8 egg chambers (Xue and Cooley, 1992) when the oocyte increases in size more slowly than the wild-type even though yolk protein uptake occurs on time. The late phase fails to take place in the *kelch* mutant egg chambers (Xue and Cooley, 1992) and, accordingly, *kelch* females do not lay eggs and are sterile. Antibodies to the kelch protein in wild-type flies localize to the ring canals from stage 3 onwards (Xue and Cooley, 1992; Robinson et al., 1994). From light microscopic observations Robinson et al. (1994) conclude that the *kelch* transport defect is due to occlusion, either partial or complete, of the ring canals by actin filaments.

In thin sections we could find no differences in the actin filament organization comparing wild-type and *kelch* ring canals in thin sections of stage 2 to stage 5 egg chambers. However, *kelch* ring canal diameter and length were greater than those measured in the wild-type from stage 5 to stage 8 (see Table II and Table III). It appears that the *kelch* ring canals are enlarging in diameter and length ahead of schedule. Since we judge stage number in large measure by the size of the egg chamber, it is probable that the *kelch* egg chambers actually may develop more slowly and thus are smaller than wild-type at the same stage. What we notice is that the *kelch* nurse cells tend to degenerate prematurely e.g.. from stage 8 to stage 10. The onset of degeneration is variable and depends on the specific egg chamber and the specific ovariole, some earlier than others. What we mean by degeneration is a vacuolization and loss of density of the cytoplasm and loss of the continuity in the nuclear envelope. In short, degeneration can occur well before the stage when nurse cell dumping should occur.

Changes in the actin filament organization in *kelch* ring canals begin at stage 6 and continue until degeneration sets in. Bundles composed of parallel oriented filaments extending from the dense material associated with the cytoplasmic surface of the plasma membrane in the canal enter into the canal proper (Fig. 10) as if not firmly attached to the dense material. These bundles can join with other bundles and a single bundle of filaments can fray into a number of sub-bundles. The cytoplasm between these fraying and anastomosing strands contains ribosomes, small elements of the rough surfaced ER, and vesicles and vacuoles of varying sizes and density. The behavior of these bundles can be best appreciated by examination of our tracings of serial sections through one canal (Fig. 11).

Table III. Dimensions of kelch Ring Canals during Stages in Development

Stage	Length	SEM	Diameter	SEM
3	0.25 (1)		0.60 (1)	
5	0.60 (4)	0.02	2.58 (3)	0.13
8	0.97 (4)	0.03	4.99 (2)	0.71
9	0.90 (9)	0.48	4.51 (5)	0.48

All measurements are in microns. The number in parentheses, number of canals examined.

By stage 9 to stage 10 measurements of the canal length of *kelch* ring canals is less than that of the wild-type (Table III). This reinforces our reconstructions and emphasizes that what is occurring in this mutant is that the filament bundles, which in the wild type remain at the margins, stray away from the margins into the canal proper. Thus, the canal proper looks like it contains a network of bundles where individual bundles are separated by greater distances than in the wild-type. The canal proper is never completely occluded however; all the canals we have examined have a lumen large enough to pass mitochondria as well as space between the bundles that would allow movement of organelles through the canal. Thus, the failure to produce eggs is not simply due to occlusion or partial occlusion of the ring canals, but is instead somehow related to the degeneration of the nurse cells and oocyte at stages before nurse cell dumping.

Discussion

Organization of Actin Filaments in the Ring Canal

Although it has been assumed that the actin filaments in the ring canals are arranged in a circumferential pattern based upon the arrangement of the actin filaments in the contractile ring of dividing animal cells and in the band inside the zonula adherens in epithelial cells, to our knowledge this is the first detailed report that demonstrates this conclusively in the ring canals of *Drosophila*. From sections of known orientation we find that the actin filaments in the ring canals are circumferentially oriented, aligned parallel to each other, and at all stages packed at approximately the same density although not packed with a high degree of order (e.g., hexagonally packed). By decorating the actin filaments in the ring canals with subfragment 1 of myosin (S1) we can glean two additional facts, namely that the filaments in the ring canal tend to be long, e.g., more that 3 μm in length and are bipolar in orientation.

Ring Canal Increases in Diameter, Thickness, and Length in Two Discrete Phases

During oogenesis there are substantial increases in ring canal diameter (15-fold), circumference (45-fold), thickness (6-fold), and tube length (6-fold) (summarized in Fig. 12). These changes occur in two phases. First the canal increases in thickness from stage 2 to stage 5 during a period when the diameter is essentially unchanged (Fig. 8 *b*). This involves an increase in the number of filaments seen in a transverse section from 80 at stage 2 to ~700 at stage 6. From stage 5 until nurse cell dumping (stage 11) the filament number does not change (Fig. 9 *b*). During the sec-

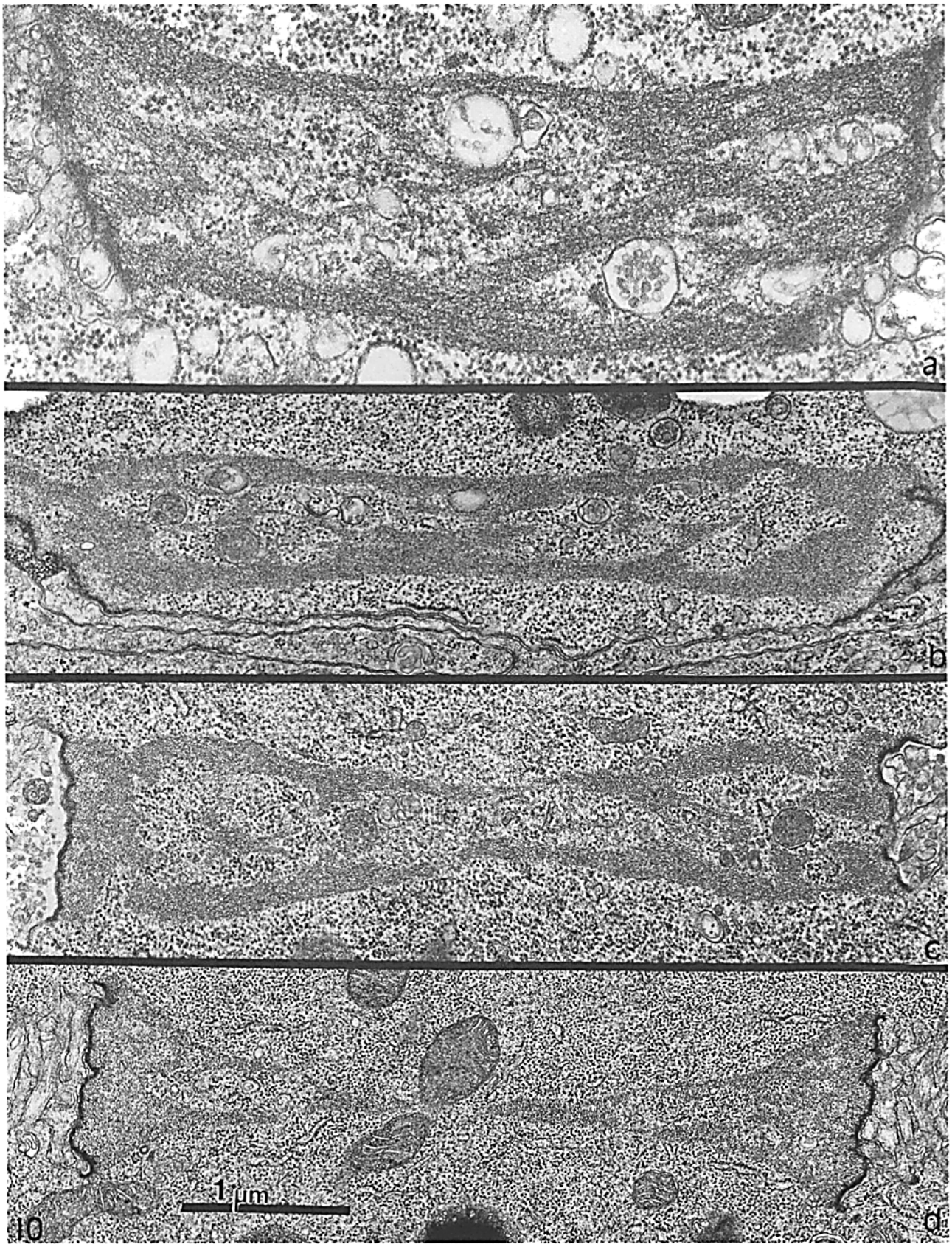

Figure 10. (*a–d*) Thin sections through *kelch* mutant ring canals of a stage 9 egg chamber. Unlike the wild-type ring canals (Figs. 4 and 5), the actin filaments normally located exclusively near the inside electron dense rim of the canal extend as bundles from the rim into the lumen. The filaments within the bundles lie parallel to each other. Adjacent bundles join and separate from each other. In nearly vertical sections a central opening between adjacent cells can be found containing mitochondria.

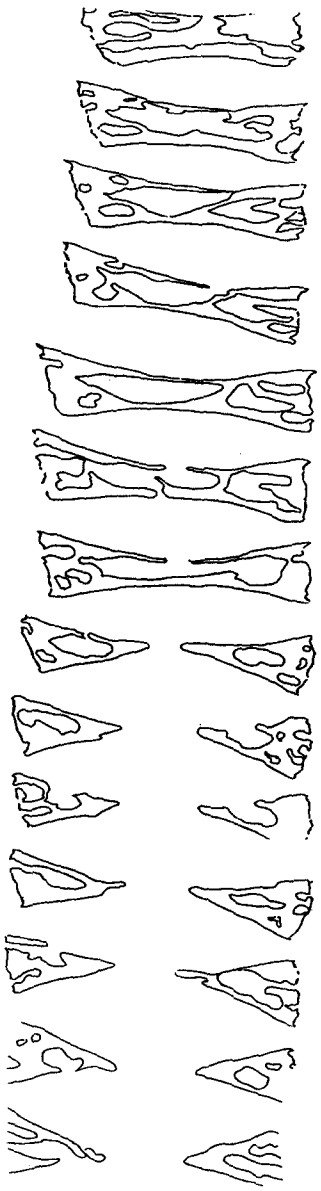

Figure 11. Tracing of serial sections through one ring canal of a stage 9 egg chamber of a *kelch* mutant.

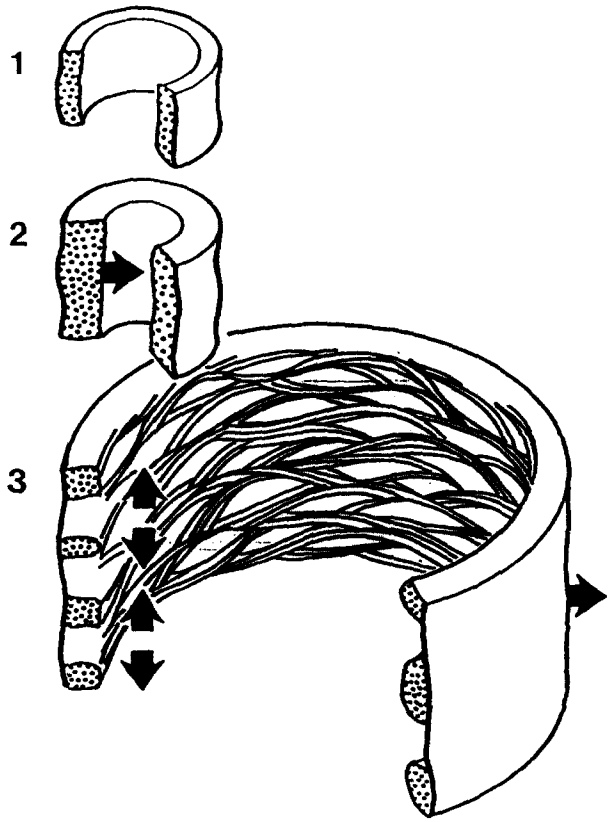

Figure 12. Drawing illustrating the growth of a ring canal from stage 2 (*1*) to stage 4 (*2*) to stage 9 (*3*). See text for details.

ond phase the diameter and accordingly the circumference increases dramatically resulting in a threefold increase in total filament length. This huge increase in the amount of F-actin is accompanied by a conversion of the single actin ring into a ring of anastomosing bundles of filaments (Fig. 12). This 2-phase strategy of ring canal construction makes good sense biologically. Since the function of the actin containing portion of the ring seems to be to stabilize the ring and thus ensure that the canal remains open during development and does not collapse, it must be constructed early in development. This is essential as mRNAs are transported throughout development from the nurse cells to the oocyte (see Cooley and Theurkauf, 1994). Once the wall of the canal has been stabilized, the canal can gradually increase in diameter.

In another actin-containing system, namely the formation of stereocilia in hair cells of the inner ear, there are, as in the formation of the mature ring canal, discrete phases in the formation of stereocilia (see Tilney et al., 1992). These developmental steps are attributable to actin filament nucleation, elongation and crossbridging into bundles.

Expansion of the Ring Canal

Careful analysis of the expansion of the ring canal made us aware of two features that were not recognized or appreciated heretofore. First, from stage 5 onwards the initially homogeneous actin ring is transformed into an anastomosing network of filament bundles. Second, since the density or number of filaments per unit area in our vertical sections through ring canals remains constant throughout development even though the filament number increases in the first phase or forms a network in the second phase, the filaments must be crosslinked to the same degree at all stages. Thus, there must be a constant ratio of crosslinking molecules to F-actin. This is not a trivial point as we estimate that during development there is a 134-fold increase in F-actin such that at stage 10 a single ring canal contains 23 mm ($\pi \times 10\ \mu m \times 726$) or nearly one inch of actin.

Given these facts, the question that immediately comes to mind is how does the ring canal expand. Does the 134-fold increase in F-actin arise exclusively by adding G-actin to the ends of existing filaments or are new filaments assembled, or both? Do filaments slide past one another and if so, how, given that there is no seam in the canal and the filaments are crossbridged together?

One can think of a number of possibilities to explain ring canal expansion. The first is an expanding gel theory. In this model a donut shaped gel expands. The donut and the hole get bigger. This model would predict that the density inside the donut would decrease. Since the density (num-

ber per unit area) of filaments in the actin ring remains constant throughout expansion, this model clearly does not jibe with our results.

A second model is that of sliding filaments past one another to expand the canal. The only known motors that induce sliding of actin filaments are the myosins whose direction of movement is dictated by the polarity of the actin filaments. Since the ring canal is a derivative of a contractile ring used in cytokinesis, it is likely that the barbed ends of the filaments are attached to the membrane. The filaments would then slide past one another by the action of myosin and the ring would contract by necessity because the actin attached to the membrane can only pull the membrane inwards, based on the polarity of actin just like the contractile ring during cytokinesis. Thus, this model is incompatible with expansion.

A third model is that individual filaments in the canal increase in length by the addition of subunits and expansion occurs by the sliding of filaments past one another using cross-links that are made and broken repeatedly. Additional cross-links must be added so that they remain in proportion to the increases in filament length. In this model sliding of the elongating filaments must occur as there is no seam in the canal or a specific end point for all the filaments in the canal. Such a model would lead to extremely long filaments as the circumference of a stage 10 ring is πD or 31.4 μm so that the minimum length of filaments would be at least 30 μm There is very little information on actin filament lengths, but filaments longer than 5–10 μm, the length of filaments in microvilli or stereocilia (see Tilney et al., 1992) are rare. The only documented case is in *Limulus* sperm where 60-μm-long filaments occur, but this is an unusual case as each filament is stabilized by overlapping scruin molecules that inhibit filament or breakdown (Tilney, 1975; Tilney et al., 1981). Thus, although this model is possible, we feel that it is unlikely.

The final model is based on the growth of *Listeria* tails (see Tilney and Tilney, 1994). In this model the ring grows by polymerization of actin at the membrane from new sites. After stage 5 when the thickness of the ring has reached its maximum value, we invision that new filaments are assembled in proportion to increases in the diameter of the ring. This model is consistent with our data with the proviso that bundles of newly assembled actin filaments must slide past other bundles to increase the radius of the ring. What is intriguing about this model is that if the bundles are not tightly associated with each other, they may end up in the lumen of the canal. This may explain our observations on the *kelch* mutant where the bundles of actin filaments enter and partly occlude the lumen of the canal.

What Might Be the Function of the kelch Protein?

The *kelch*-encoded protein has homology with the actin binding protein, scruin, found in *Limulus* sperm (Way et al., 1995). From three-dimensional reconstructions (DeRosier et al., 1982; Bullitt et al., 1988; Schmid et al., 1991, 1994; Owen et al., 1993) it has been determined that a single scruin molecule binds to two adjacent subunits on a single actin filament. By doing so scruin stabilizes the filaments (*a*) so that they do not depolymerize, (*b*) so that the helical symmetry of the filaments is fixed, and (*c*) so that actin binding proteins such as myosin will not interact with the filaments. Furthermore, the outer surfaces of a scruin molecule, e.g., the portion not attached to the actin filament or the back of the molecule, can interact with the outer surface or back of another scruin molecule on an adjacent filament. Consequently scruin may act as a cross-linker of adjacent filaments albeit in a different way than conventional crosslinks such as fimbrin or fascin.

Unlike scruin, which contains two large nearly identical domains on either end of the molecule and many tiny repeated domains, the kelch protein has only one large domain with a series of repeated domains nearly identical to the tiny domains of scruin. Thus, it is likely that kelch can only bind to one subunit in an actin filament rather than two. Whether kelch protein can dimerize has not been established nor is it known whether the backs of kelch proteins located on two adjacent actin filaments can interact and lead to adjacent filament association. Along these lines, we should mention that the NH_2-terminal region of kelch is related to domains responsible for protein–protein interactions among a large class of transcription factors (Bardwell and Treisman, 1994). The BTB domain (also known as the POZ domain) is a NH_2-terminal feature found in transcription factors encoded by the *Drosophila bric-a-brac, tramtrack,* and *Broad-Complex* genes (Harrison and Travers, 1990; DiBello et al., 1991; Zollman et al., 1994). How this domain relates to kelch function is unclear.

From the literature we know that the kelch proteins can be found in the ring canals of stage 2 to stage 11 egg chambers by antibody staining (Robinson et al., 1994). However, no discernible phenotype is seen in *kelch* mutants until stage 7 or stage 8 at which point the oocyte appears slightly smaller in size and grows at a slower rate. Later the characteristic nurse cell dumping in stage 11 egg chambers fails to occur; Robinson et al. (1994) conclude that this is due to canal occlusion or partial occlusion by individual actin filaments, a process that began at stage 7 or 8 as the canals appear somewhat thicker. With the greater resolution achieved with the electron microscope on thin sections and from serial sections of *kelch* mutants we now know that the canals remain open albeit with tendrils of actin filament bundles extending into the canal. The existing opening could allow mitochondria and other large organelles to be transferred to the egg. We found here that the nurse cells in *kelch* mutants begin to degenerate prematurely, well before the nurse cell dumping phase. This degeneration includes loss of the nuclear envelope and vacuolization of the cytoplasm. We know from the literature that if *Drosophila* are in a hostile environment for larval growth, e.g., low humidity, lack of nutrients, or seemingly idiosyncratic factors (see Spradling, 1993) eggs, even mature eggs, are not laid, but resorbed. Thus, factors that slow development will inhibit the eggs from precociously entering the oviduct. We think that may explain why *kelch* females do not lay eggs, the eggs having degenerated before ovulation. The question then becomes: what is the exact function of the kelch protein and why does its absence slow development? One possibility, mentioned in the preceding section is that the kelch protein may act to cross-link adjacent bundles of filaments to each other. As new bundles form and extend from the plasma membrane in-

creasing the diameter of the ring, the kelch protein will then hold these bundles together forming a compact net of peripherally oriented bundles that together form a reasonably compact ring. If the kelch protein is absent, the bundles are no longer compact but enter into the canal and slow transport. Why the nurse cells degenerate prematurely is still not clear, but perhaps it is due to merely a slowing of development. If the lifetime of the nurse cells is under tight temporal control then premature nurse cell degeneration would result in an immature egg that would be resorbed.

We would like to thank L. Cooley and her lab for showing us how to dissect out the egg chambers and for sending us wild type and *kelch* mutants. Thanks go to B. Golder for the drawing in Fig. 4, D. Rugh for Fig. 12 and to M. Sunshine for maintaining the flies. Special thanks go to P. Connelly who, with incredible patience and persistence, cut thin sections through the egg chambers, constantly reorienting the block to obtain the necessary orientations to visualize the ring canals in horizontal and transverse sections. Thanks also go to D. DeRosier for reading a draft of this paper and providing some helpful suggestions and proposing models of how expansion of the ring canal might occur.

Supported by National Institutes of Health grant no. GM-52857 (L.G. Tilney) and American Cancer Society grant no. DB-89A (G.M. Guild).

Received for publication 13 July 1995 and in revised form 16 November 1995.

References

Bardwell, V.J., and R. Treisman. 1994. The POZ domain: a conserved protein-protein interaction domain. *Genes & Dev.* 8:1664–1677.

Bullitt, E.S.A., D.J. DeRosier, L.M. Coluccio, and L.G. Tilney. 1988. Three dimensional reconstruction of an actin bundle. *J. Cell Biol.* 107:597–611.

Cooley, L., E. Verheyen, and K. Ayers. 1992. Chickadee encodes a profilin required for intercellular cytoplasm transport during *Drosophila* oogenesis. *Cell.* 69:173–184.

Cooley, L., and W.E. Theurkauf. 1994. Cytoskeletal functions during *Drosophila* oogenesis. *Science (Wash. DC).* 266:590–596.

DeRosier, D.J., L.G. Tilney, E.M. Bonder, and P. Frankl. 1982. A change in twist of actin provides the force for the extension of the acrosomal process in *Limulus* sperm: the false discharge reaction. *J. Cell Biol.* 93:324–337.

DiBello, P., D. Withers, C.A. Bayer, J.W. Fristrom, and G.M. Guild. 1991. The *Drosophila Broad-Complex* encodes a family of related, zinc finger-containing proteins. *Genetics.* 129:385–397.

Harrison, S.D., and A.A. Travers. 1990. The *tramtrack* gene encodes a *Drosophila* finger protein that interacts with the *ftz* transcriptional regulatory region and shows a novel embryonic expression pattern. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:207–216.

King, R.C. 1970. Ovarian Development in *Drosophila melanogaster*. Academic Press, NY. 227 pp.

Lin, H., L. Yue, and A.C. Spradling. 1994. The *Drosophila* furome, a germline-specific organelle, contains membrane skeletal proteins and functions in cyst formation. *Development (Camb.).* 120:947–956.

Lindsley, D.L., and G.G. Zimm. 1992. The Genome of *Drosophila melanogaster*. Academic Press, San Diego, CA.

Mahowald, A.P. 1971. The formation of ring canals by cell furrows in *Drosophila. Z. Zellforsch.* 118:162–167.

Mahowald, A.P., and M.P. Kambysellis. 1980. Oogenesis. *In* The Genetics and Biology of Drosophila. Vol. 2. M. Ashburner and T.R.F. Wright, editors. Academic Press, London. 141–224.

Owen, C., and D.J. DeRosier. 1993. A 13-Å map of the actin-scruin filament from the *Limulus* acrosomal process. *J. Cell Biol.* 123:337–344.

Pfeifer, M., S. Orsulic, D. Sweeton, and E. Wieschaus. 1993. A role for the *Drosophila* segment polarity gene *armadillo* in cell adhesion and cytoskeletal integrity during oogenesis. *Development (Camb.).* 118:1191–1207.

Riparbelli, M.G., and G. Callaini. 1995. Cytoskeleton of the Drosophila egg chamber: New observations on microfilament distribution during oocyte growth. *Cell Motil. Cytoskeleton.* 31:298–306.

Robinson, D.N., K. Cant, and L. Cooley. 1994. Morphogenesis of *Drosophila* ovarian ring canals. *Development (Camb.).* 120:2015–2025.

Schmid, M.F., J.M. Agris, J. Jakana, P. Matsudaira, and W. Chiu. 1994. Three-dimensional structure of a single filament in the *Limulus* acrosomal bundle: scruin binds to homologous helix-loop-beta motifs in actin. *J. Cell Biol.* 124: 341–350.

Schmid, M.F., P. Matsudaira, T.W. Jeng, J. Jakana, E. Towns-Andrews, J. Bordas, and W. Chiu. 1991. Crystallographic analysis of acrosomal bundle from *Limulus* sperm. *J. Mol. Biol.* 221:711–725.

Satterwhite, L.L., and T.D. Pollard. 1992. Cytokinesis. *Curr. Opin. Cell Biol.* 4: 43–45.

Singleton, K., and R.I. Woodruff. 1994. The osmolarity of adult *Drosophila* hemolymph and its effect on oocyte-nurse cell electrical polarity. *Dev. Biol.* 161:154–167.

Spradling, A.C. 1993. Development genetics of oogenesis in the development of *Drosophila melanogaster* I. Cold Spring Harbor Press, Cold Spring Harbor, NY. 1–70.

Tilney, L.G. 1975. Actin filaments in the acrosomal reaction of *Limulus* sperm. *J. Cell Biol.* 64:289-310.

Tilney, L.G., E.M. Border, and D.J. DeRosier. 1981. Actin filaments elongate from their membrane-associated ends. *J. Cell Biol.* 90:485–494.

Tilney, L.G., D.J. DeRosier, and M.S. Tilney. 1992. Actin filaments, stereocilia, and hair cells: how cells count and measure. *Annu. Rev. Cell Biol.* 8:257–274.

Tilney, L.G., and M.S. Tilney. 1993. The wily ways of a parasite: induction of actin assembly by *Listeria. Trends Microbiol.* 1:25–31.

Tilney, L.G., and M.S. Tilney. 1994. Methods to visualize actin polymerization associated with bacterial invasion. *Methods Enzymol.* 236:476–481.

Warn, R.M., H.O. Gutzeit, L. Smith, and A. Warn. 1985. F-actin rings are associated with the ring canals of the *Drosophila* egg chamber. *Exp. Cell Res.* 157:355–363.

Way, M., M. Sanders, C. Garcia, J. Sakai, and P. Matsudaira. 1995. Sequence and domain organization of scruin, an actin-cross linking protein in the acrosomal process of *Limulus* sperm. *J. Cell Biol.* 128:51–60.

Xue, F., and L. Cooley. 1993. Kelch encodes a component of intercellular bridges in *Drosophila* egg chambers. *Cell.* 72:681–693.

Yue, L., and A.C. Spradling. 1992. Hu-li tai shao, a gene required for ring canal formation during *Drosophila* oogenesis, encodes a homolog of adducin. *Genes & Dev.* 6:2443–2454.

Zollman, S., D. Godt, G.G. Prive, J.L. Couderc, and F.A. Laski. 1994. The BTB domain, found primarily in zinc finger proteins, defines an evolutionarily conserved family that includes several developmentally regulated genes in *Drosophila. Proc. Nat. Acad. Sci. USA.* 91:10717–10721.