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GENETIC ANALYSIS OF THE ACTIN CYTOSKELETON IN THE *DROSOPHILA* OVARY

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ABSTRACT

The *Drosophila* ovary provides a favorable model system in which to study cellular morphogenesis. The development of a mature egg involves a syncytium of 16 germline cells and over 1000 somatically derived follicle cells. Intercellular transport, stable intercellular bridges, cell migrations, cell shape changes, and specific subcellular localization of many embryonic patterning determinants contribute to egg development and require a dynamic cytoskeleton. We discuss many of the recent genetic and cell biological studies that have led to insights into how the actin cytoskeleton is assembled and regulated during the morphogenesis of the *Drosophila* egg.

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INTRODUCTION

Egg development is an attractive model for studying cellular morphogenesis, pattern determination, and syncytial development. In *Drosophila melanogaster*, production of a mature egg requires that over 1000 somatic cells and 16 germline cells coordinate their behavior. Subsets of these cells contribute specialized functions, all directed at the production of a viable, fertilizable egg in which the early patterning of the fly embryo is already established. Female and male germ cells in *Drosophila* and in a wide variety of other animals, including mammals, undergo at least one phase of syncytial development, but the significance of this style of development is poorly understood.

In recent years, the study of oogenesis in *Drosophila* has led to the realization that this is a favorable system in which to explore the genetic regulation of actin. We present recent work that contributes to our understanding of how the cytoskeleton is regulated during oogenesis and how it is involved in morphogenesis of oocytes. We do not attempt to be comprehensive about any particular protein or complexes of proteins. Instead, we concentrate on cases where genetic approaches have been used and highlight how studies on the actin cytoskeleton are beginning to converge with work on regulatory molecules and adhesive complexes in the *Drosophila* ovary.

OVERVIEW OF OOGENESIS

The *Drosophila* ovary is composed of 16 to 18 discrete units called ovarioles in which developing egg chambers are formed and mature (Figure 1; reviewed in Spradling 1993). Each ovariole is composed of two distinct regions: the germarium and the vitellarium (Figure 1*A*). *Drosophila* oogenesis is initiated by the division of a stem cell that resides at the anterior-most tip of region 1 of the germarium. The division produces a daughter stem cell and a daughter cell called a cystoblast. The cystoblast undergoes four mitoses characterized by incomplete cytokinesis, which give rise to a 16 germ-cell syncytium. At the sites of the arrested cleavage furrows, a specialized cytoskeleton is assembled to form the stable intercellular bridges called ring canals. The axis of each mitotic division is oriented approximately perpendicularly to that of the previous division. This produces a cluster of cells in which two cells have four ring canals, two have three, four have two, and eight have one. One of the cells with four ring canals differentiates into the oocyte, while the remaining 15

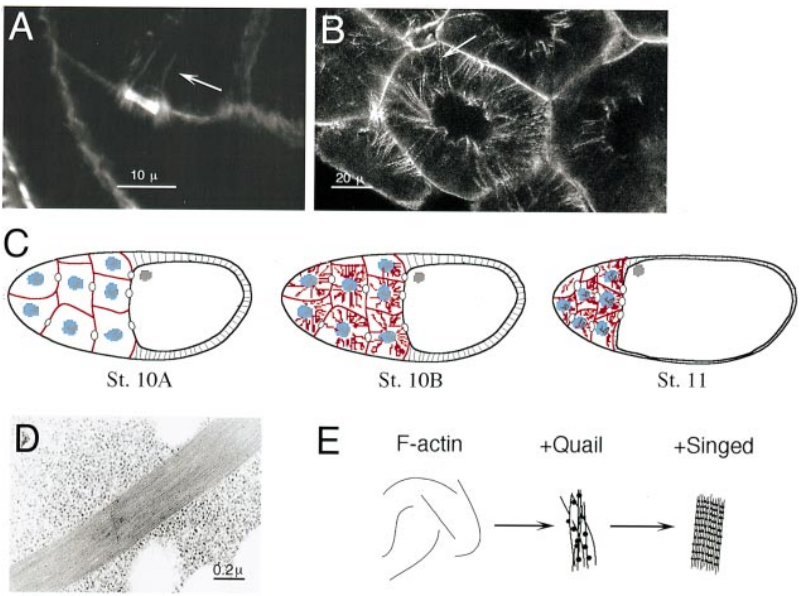

Figure 2 Cytoplasmic actin bundles required during directed and rapid phases of transport. *(A)* An actin filament basket *(arrow)* is present around ring canals in stage 9 egg chambers. These are assembled between stages 7 and 8 and perdure until stage 10. They are seen on both sides of the ring canals that connect nurse cells but only on the nurse cell side of a ring canal that interconnects a nurse cell and the oocyte. *(B)* An elaborate array of cytoplasmic actin bundles extends from the nurse cell plasma membrane to the nucleus in this stage 11 egg chamber. The bundles have a striated appearance *(arrow)* when viewed by rhodamine-phalloidin staining. *(C)* No cytoplasmic bundles are observed in stage 10A egg chambers. In stage 10B, the cytoplasmic actin bundles are assembled in the cytosol of the nurse cells, preceding rapid cytoplasm transport, which occurs in stage 11. *(D)* An electron micrograph of a wild-type nurse cell cytoplasmic actin bundle reveals a densely packed array of actin filaments. *(E)* In a model for wild-type cytoplasmic actin bundle assembly that involves two bundlers, quail brings actin filaments together, packing them into a loose bundle. Singed then orders the filaments into a hexagonally packed bundle.

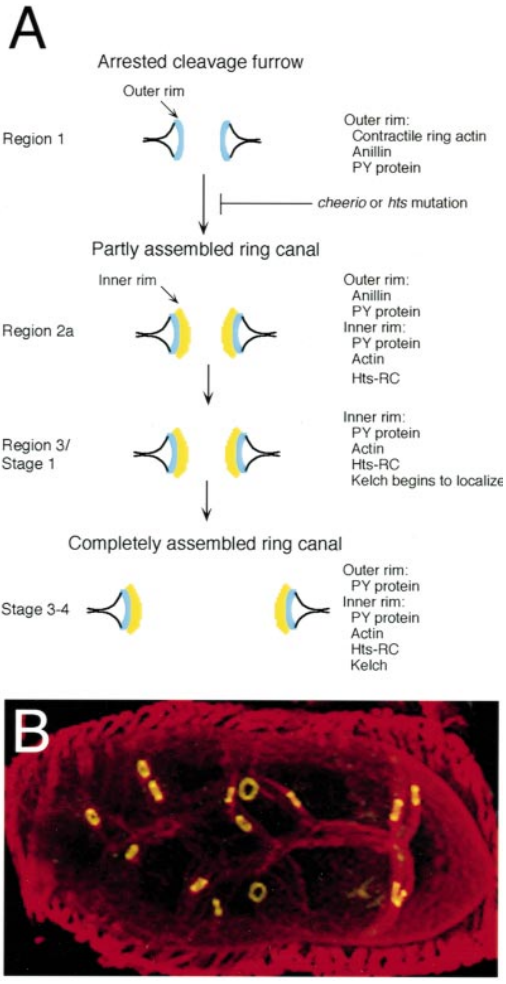

Figure 4 Pathway of ring canal assembly. *(A)* Ring canals are assembled at arrested cleavage furrows by the sequential addition of several proteins. Contractile ring actin and anillin associate with cleavage furrows. The first ring canal-specific protein immunoreacts with anti-phosphotyrosine antibodies and associates with the plasma membrane, forming the outer rim of the ring canal. The inner rim proteins are localized beginning with the hu-li tai shao ring canal-specific (Hts-RC) protein and a robust ring of actin filaments. Kelch is the last known protein to localize to ring canals and requires a few stages of egg chamber development before it is easily detectable on all ring canals. The assembly of the inner rim depends on the products of the *cheerio* and *hu-li tai shao* genes. *(B)* In a stage 9 egg chamber stained with rhodamine-conjugated phalloidin to detect filamentous actin, actin *(red)* is observed in the subcortial cytoskeleton, in the surrounding muscle sheath, and in the ring canals. Anti-kelch antibodies detect kelch *(green)*, which specifically colocalizes with actin in the ring canals *(yellow)*.

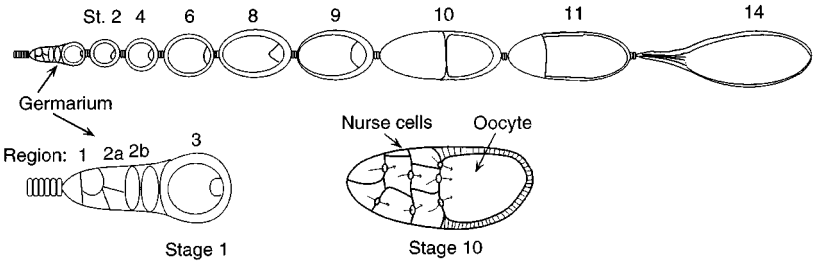

Figure 1 Cartoon of the stages of *Drosophila* oogenesis. The egg chamber is assembled in the germarium and emerges as a stage 2 egg chamber. In the vitellarium, it grows and matures until stage 10 when the nurse cells rapidly transport their cytoplasmic contents into the oocyte. Transport is facilitated by cytoskeletal structures called ring canals that maintain the openings between the germline cells. Finally, by stage 14, the fully developed egg is ready for fertilization. For a detailed review of the process of oogenesis, see Spradling (1993).

become polyploid nurse cells. The fusome, a cytoskeletal structure that is rich in spectrin and adducin and that extends through the ring canals, is required to regulate the cell divisions and may have a role in oocyte determination (Yue & Spradling 1992, Lin et al 1994, Lin & Spradling 1995, de Cuevas et al 1996). The interconnected germ-cell cluster is encapsulated by a layer of somatically derived follicle cells forming the complete egg chamber. Soon after this occurs, the egg chamber pinches off from the germarium and enters the vitellarium as a stage 2 egg chamber where it matures, ultimately producing a stage 14 mature egg.

In addition to the major cellular morphogenic events in the germline cells, several groups of follicle cells undergo a number of morphological changes. After encapsulating the germline cluster, they are columnar in shape. Between each egg chamber in the vitellarium is a small group of cells known as stalk cells. By stage 9, the majority of cells in the columnar epithelia migrate posteriorly until they surround the oocyte. The follicle cells that remain over the nurse cells become squamous and stretch over the nurse cell cluster. Also during stage 9, a small group of follicle cells, called border cells, delaminate from the anterior tip of the egg chamber and migrate through the nurse cell cluster until they reach the oocyte. These cells are required for proper formation of the pore of the micropyle, which provides the sperm entry port. At stage 10A, a group of follicle cells at the anterior of the oocyte migrate centripetally between the oocyte and the nurse cells. The columnar follicle cells over the oocyte synthesize and secrete the vitelline and chorionic membranes, which form the egg shell. The centripetally migrating follicle cells secrete the anterior egg shell structures including the operculum and the dorsal appendages. Genes that disrupt the

behavior of any of these groups of cells can ultimately disrupt the formation of these egg shell structures.

ACTIN CYTOSKELETON IN TRANSPORT

The *Drosophila* oocyte is nearly inactive transcriptionally and therefore relies on other cells for its maturation. Throughout oogenesis, the nurse cells synthesize and deliver cytoplasmic components, including mRNA, protein, and organelles, to the oocyte. The transport of cytoplasm is mediated by the cytoskeleton. For earlier reviews, see Mahajan-Miklos & Cooley (1994a) and Cooley & Theurkauf (1994).

Directed Transport

During stages 7 to 10, the selective transfer of particles through ring canals into the oocyte has been observed using video-enhanced contrast time-lapse microscopy (Bohrmann & Biber 1994). The movement of these particles is sensitive to actin-depolymerizing drugs, suggesting that actin filaments in the vicinity of the ring canals participate in particle transport. Indeed, discrete actin structures resembling baskets can be seen at the ring canals of stage 6 to 10 egg chambers (Figure 2*A*; Riparbelli & Callaini 1995). The filaments of the actin baskets extend from ring canals, roughly in parallel for a short distance, before converging. Interestingly, the baskets are present on nurse cell ring canals but not on the oocyte side of ring canals connecting nurse cells to the oocyte. A myosin motor has not been localized to these structures.

Rapid Transport

The final stage of transport occurs during the rapid contraction of nurse cells in stage 11 when the remaining cytoplasmic contents of nurse cells are entirely transferred to or are "dumped into" the oocyte. This process is sensitive to treatment with cytochalasins and, therefore, is actin dependent (Gutzeit 1986a). Abundant filamentous actin is located subcortically in both the nurse cells and follicle cells, as well as in an extensive array of cytoplasmic bundles in the nurse cells (see below). Therefore, nurse cell regression could be driven by contraction of the follicle cells, the nurse cells, or both. In the nurse cells, either the subcortical or cytoplasmic population of actin filaments could participate in generating the contractile force. However, two pieces of evidence argue against the participation of cytoplasmic actin filaments. First, the cytoplasmic actin filaments are stable during the short incubation of egg chambers in cytochalasin required to halt transport (Gutzeit 1986a). Second, nonmuscle myosin II is located subcortically in nurse cells but does not colocalize with cytoplasmic bundles that extend to the nucleus (Wheatley et al 1995, Edwards & Kiehart 1996).

Recent work on cytoplasmic myosin has shown that nurse cell contraction is a germline-dependent process powered by subcortical actin-myosin complexes in nurse cells. Two different strategies were used to reduce myosin activity, both using mutations in the myosin regulatory light chain (RMLC) gene *spaghetti squash* (*sqh*). Germline clones of a hypomorphic allele of *sqh* result in egg chambers with two types of defects: reduced nurse cell number and failure to transport nurse cell cytoplasm in stage 11 (Wheatley et al 1995). The aberrant nurse cell number phenotype suggests that myosin is required for cytokinesis. The defect in cytoplasm transport shows clearly that nurse cell contraction is a germline-dependent process and rules out a significant contribution by follicle cells. The other strategy used to reduce myosin function was to rescue a severe allele of *sqh* with a *sqh* cDNA clone driven by the heat-inducible hsp70 promoter thereby transiently ceasing induction of the gene (Edwards & Kiehart 1996). Rescued adult females held under nonheat shock conditions became sterile, with a number of problems in egg chamber development, including major defects in follicle cell migration and a failure to transport cytoplasm at stage 11. These results are consistent with the germline clone data and also reveal a critical role for actomyosin activity in somatic epithelial movements.

The identification of cytoplasmic myosin as the motor for cortical nurse cell contraction suggests that the mechanism for triggering nurse cell contraction could involve regulated phosphorylation of the RMLC. Phosphorylation of RMLC is known to activate the vertebrate enzyme and the motor capability of myosin in smooth muscle (reviewed in Sellers 1991). The role of phosphorylation of *Drosophila* muscle RMLC has been examined by creating site-directed mutations of the phosphorylated serines and testing their ability to rescue null mutations in the gene (Tohtong et al 1995). In this case, the phosphorylation sites are not required for muscle development but are required for the stretch activation response in flight muscle. A similar approach might prove useful in analyzing the ovarian function of nonmuscle RMLC encoded by *sqh*. The identification of the *Drosophila* nonmuscle RMLC kinase and genetic analysis of its function might add to our understanding of the final stages of nurse cell cytoplasm transport.

Nurse Cell Cytoplasmic Actin Bundle Assembly

A remarkable change in nurse cell actin distribution occurs during stage 10 as a prelude to contraction during stage 11 (Figure 2*C*). Staining with rhodamine-phalloidin reveals that early in stage 10, filamentous actin is present subcortically and at the ring canals. The subcortical filaments are short and cross the cortical cytoplasm parallel to the nurse cell membrane (Riparbelli & Callaini 1995). By the end of stage 10, an extensive array of actin bundles is arranged radially around each nurse cell nucleus (Figure 2*B–D*). The bundles form gradually,

starting at the plasma membranes and extending to the nuclear membranes where they bend to form a cage around each nucleus (Gutzeit 1986a, Riparbelli & Callaini 1995). At the plasma membrane, actin bundles of neighboring cells intercalate, suggesting that the membrane insertion sites protrude into their neighbors (Riparbelli & Callaini 1995). During stage 11, the actin bundles become less organized, as well as shorter and thicker (Gutzeit 1986a, Riparbelli & Callaini 1995). This suggests that the bundles may collapse as the cytoplasmic volume is decreased during rapid transport.

Ultrastructural analysis of nurse cell actin bundles showed that the majority of the bundles are not uniform. They are interrupted by either small gaps or electron-dense bodies as if the bundles were composed of short segments arranged in tandem. Similarly, rhodamine-phalloidin stained bundles sometimes have a striated appearance (Figure 2*B*; Riparbelli & Callaini 1995). The striated appearance might mean that the nurse cell bundles are constructed in a modular fashion similar to what appears to happen in developing bristles (Tilney et al 1996a). During pupation, bristle cells send out an extension supported by 7–11 columns of actin filament bundles positioned at the plasma membrane. The actin filament bundles reach a final length of 400 μm in the macrochaetes. Analysis of these bundles by both light and electron microscopy showed that the bundles have transverse breaks every 3 μm on average (Tilney et al 1996a). All the bundles in a given bristle have breaks at the same level, suggesting that new modules of actin bundles are initiated synchronously. Careful analysis of growing nurse cell actin bundles is needed to determine how similar their development is to that of bristle actin bundles.

Three genes required for nurse cell actin bundle formation have been identified: *chickadee, quail*, and *singed*. Females carrying mutations in each of these genes are sterile and produce egg chambers in which nurse cell cytoplasmic actin bundles do not form. When the nurse cells contract in these mutant egg chambers, the large polyploid nuclei become lodged in the ring canals, halting the flow of cytoplasm into the oocyte. The eggs produced from these "dumpless" egg chambers are, therefore, much smaller than normal and are not laid by the female. This phenotype indicates that the cytoplasmic actin bundles provide structural support for the nuclei during the rapid flow of cytoplasm.

The *chickadee* gene encodes profilin, a small actin monomer binding protein. Biochemical analysis of profilin from other systems has shown it to have several activities including the apparently contradictory properties of sequestering actin monomers, which inhibits actin polymerization, and stimulating nucleotide exchange on actin monomers, which leads to an increased rate of filament polymerization (Theriot & Mitchison 1993). In nurse cells, where mutations in *chickadee* disrupt actin bundle formation, profilin is most likely required to allow fast rates of actin polymerization. Analysis of a full range

of *chickadee* alleles has revealed that profilin is required in many other stages of fly development, including earlier in germ-cell development (Verheyen & Cooley 1994). Null alleles are lethal at the late embryonic stage. Severe but viable alleles of *chickadee* display defects in germ-cell division in both males and females. Egg chambers are produced with fewer than normal numbers of cells, much as is seen for mutations in *sqh* (Wheatley et al 1995). Presumably, profilin is required to produce the actin that is in the contractile ring of dividing cells.

Both *singed* and *quail* encode actin filament bundling proteins. *Drosophila singed* encodes a homologue of fascin (Paterson & O'Hare 1991, Bryan et al 1993, Cant et al 1994), a monomeric filament bundler originally characterized biochemically from sea urchins (Bryan & Kane 1978). In vitro, fascin produces hexagonally packed actin filament bundles with a 12 nm periodicity caused by the fascin cross-links (Bryan & Kane 1978, Cant et al 1994). In fruit flies, null mutations in *singed* affect only two processes: bristle formation and oogenesis. Interestingly, actin bundles have a 12 nm periodicity in both developing bristles (Tilney et al 1995) and late stage 10 nurse cells (Riparbelli & Callaini 1995). The nurse cell actin filament bundles are absent in *singed* mutations. The small residual actin bundles in the bristle cells of *singed* mutants are reduced, and they are not hexagonally packed (Tilney et al 1995). The bristle develops with many kinks and bends (Cant et al 1994). Apparently, the actin bundles of nurse cells are more sensitive to the loss of fascin than are the bundles in bristle cells.

The *quail* gene encodes a protein with similarity to villin (Mahajan-Miklos & Cooley 1994b). Villins from other systems have been found to bundle actin filaments at calcium concentrations less than 10^{-6} M and sever, cap, and nucleate actin filaments at calcium concentrations greater than 10^{-6} M (Friederich et al 1990 and references therein). The villin-like protein encoded by *quail* is unusual because it is expressed only in the germline; other villins are present in the actin cores of epithelial brush border microvilli.

The presence of two actin filament bundlers in nurse cells is similar to other cell types. Villin and fimbrin are found in the epithelial brush border (Louvard et al 1992; reviewed in Friederich et al 1990); fimbrin (reviewed in Tilney et al 1992), and at least one other bundler are in stereocilia of the ear (Tilney et al 1995). In addition to fascin in the fly bristle, the second bundler is likely to be a product of the *forked* gene (Petersen et al 1994, Tilney et al 1995). The two-protein motif for bundle assembly argues that the two bundlers present in a given cell have nonredundant functions. This notion is supported by the distinct biochemical properties of fascin, fimbrin, and villin. A genetic test for bundling protein redundancy was carried out by over-expressing quail protein in *singed* mutant nurse cells lacking fascin (K Cant, B Knowles, S Mahajan-Miklos, M Heintzelman & L Cooley, unpublished data). Flies were made that

contained a *singed* mutation—therefore no fascin—and four copies of wild-type *quail*, two endogenous genes, and two transgenes. The fertility of these flies was rescued. Actin bundles were present in the nurse cells although they were not as highly organized as in wild type. Therefore, quail protein by itself is capable of forming bundles that provide sufficient support for nurse cell nuclei during rapid cytoplasm flow. This suggests that the two bundling proteins do have some redundant function. Perhaps the presence of two bundling proteins evolved to provide a more efficient method of producing highly ordered actin bundles. One protein may bring the filaments together, and the second may help order them into a nearly crystalline array (Figure 2*E*; Tilney et al 1995).

The domain structure of villins is well understood (see Friederich et al 1990); in contrast, sequence analysis of fascin family members has failed to reveal candidate actin-binding domains or other functional domains. A mutagenesis screen for mutations in *singed* was carried out to identify important amino acid residues (Cant & Cooley 1996). Out of 15 *singed* mutations recovered, only two accumulate singed protein; the other 13 disrupt the protein expression altogether. The new mutations change glycine at position 409 to glutamic acid (this allele is fertile) and serine at position 289 to asparagine (this allele is female sterile). The G409E mutation is within a short region that is highly conserved in the fascin family; this region deserves further testing to determine its function. The S289N mutation is positioned near the middle of the fascin sequence, perhaps affecting the tertiary structure of the protein or the alignment of the two actin-binding sites. An intragenic suppressor mutation of S289N was isolated that changed a serine at position 251 to phenylalanine (Cant & Cooley 1996). This change restored fascin function to near wild-type levels. When the three-dimensional structure of fascin is available, the positions of these three amino acids will be of particular interest.

ACTIN CYTOSKELETON IN OOCYTE PATTERNING

Actin Cytoskeleton Interaction with Microtubules

During stages 8 through 10, prior to final nurse cell cytoplasm transport, a number of molecules that specify the embryonic axes are transported to the oocyte and localized to specific subcellular domains. The primary determinant of anterior identity, *bicoid* mRNA, is localized to the anterior cortex of the oocyte; the posterior determinants *oskar* mRNA and staufen and vasa proteins are localized to the posterior of the oocyte, and *gurken* mRNA becomes localized to the dorsal anterior cortex where it specifies dorsal cell fates (reviewed in Ray & Schüpbach 1996). All these events are dependent on microtubules that are arranged in an anterior-to-posterior concentration gradient in the oocyte during stages 8–10. During stage 10, the oocyte microtubules reorganize into arrays of parallel microtubules adjacent to the oocyte cortex. These microtubules are

required to mediate ooplasmic streaming that occurs during stage 11 and mixes rapidly entering nurse cell cytoplasm (Gutzeit 1986b).

Recent data indicate that the reorganization of microtubules during stage 10 is coordinated with, and possibly regulated by, changes in the actin cytoskeleton. There is a thick peripheral network of actin filaments in the oocyte in early stage 10 oocytes that decreases in thickness throughout stage 10 except at the anterior (Riparbelli & Callaini 1995). The reduction in actin filaments occurs at the same time that microtubules organize into parallel arrays, suggesting that microtubules cannot reorganize and promote ooplasm streaming until the actin network dissipates. Indeed, treatment of stage 8 egg chambers with cytochalasin D causes premature ooplasm streaming accompanied by microtubule reorganization (Manseau et al 1996). A consequence of premature streaming is that embryonic axis determinants such as *oskar* mRNA and staufen protein are not localized, which suggests that ooplasm mixing interferes with the binding of these determinants to a localized anchor in the pole plasm (Manseau et al 1996).

Three mutants have been identified in which premature ooplasm streaming occurs; one, *chickadee*, disrupts multiple actin-dependent processes (Verheyen & Cooley 1994, Manseau et al 1996). The reduction in germline profilin in *chickadee* may impair the oocyte actin cytoskeleton, creating a permissive environment for microtubule arrays. The other two genes that cause premature ooplasm streaming are *cappuccino* (*capu*) and *spire* (Theurkauf 1994, Emmons et al 1995). *capu* encodes a protein with similarity to vertebrate formins encoded by the limb deformity locus (Emmons et al 1995). Regions of similarity, including a proline-rich domain called formin homology 1 (FH1) and formin homology 2 (FH2), are separated by about the same length spacer in capu, formins, and other family members such as yeast Bni1p and *Drosophila* diaphanous (Castrillon & Wasserman 1994). Profilin has been shown to interact with capu protein in a yeast two-hybrid assay (Manseau et al 1996); it likely binds to the proline-rich FH1 domain of capu because profilin is known to bind poly-L-proline (Theriot & Mitchison 1993). Profilin has also been shown to bind yeast Bni1p at the FH1 domain (Evangelista et al 1997). In addition, profilin clones were isolated as suppressors of lethality induced by a truncated Bni1p, which suggests an in vivo interaction exists between Bni1p and profilin in yeast (Evangelista et al 1997). If capu interacts directly with profilin in nurse cells, it is possible that capu affects actin polymerization. In this case, the early ooplasm swirling in *capu* mutants could result from an unstable oocyte actin cytoskeleton. It will be interesting to learn the identity of the *spire* gene product.

Cytoplasmic Tropomyosin II in Localization of Posterior Determinants

Evidence for a role of actin in the localization of posterior determinants in the oocyte is the finding that pole cells do not form in cytoplasmic *tropomyosin II*

(*TmII*) mutants (Erdélyi et al 1995, Tetzlaff et al 1996). Females with mutations in cytoplasmic tropomyosin produce embryos that fail to develop pole cells and therefore mature into sterile adults. The embryos from *TmII* mutant mothers do not have *oskar* mRNA or staufen protein localized at the posterior pole. However, the localization of *nanos* mRNA to the posterior pole is unaffected, indicating that tropomyosin is required for pole cells but not abdominal formation (Tetzlaff et al 1996). The localization of *bicoid* mRNA at the anterior and *gurken* mRNA at the dorsal/anterior corner of the oocyte is not affected in *TmII* mutants, suggesting that the effect is specific to molecules located at the posterior pole. This phenotype is reminiscent of what happens in *capu* and *chickadee* mutants and in egg chambers treated with cytochalasin D to induce early ooplasm streaming (Emmons et al 1995, Manseau et al 1996), which raises the possibility that *TmII* mutants could also be causing premature ooplasm streaming by destabilizing the oocyte actin cytoskeleton. However, the actin cytoskeleton (Erdélyi et al 1995, Tetzlaff et al 1996) and ooplasm streaming (Erdélyi et al 1995) in *TmII* mutants are reported to be normal. Furthermore, the disruption of *oskar* mRNA localization appears to be more severely affected in embryos than in oocytes (Tetzlaff et al 1996). Perhaps tropomyosin is necessary for the anchoring or maintenance of pole cell determinants rather than the well-being of the entire oocyte actin cytoskeleton. This model is supported by the localization of the cytoplasmic *TmII* mRNA to the posterior pole of oocytes and embryos (Tetzlaff et al 1996).

REGULATION OF ACTIN CYTOSKELETON

Rho Family of GTPases

Cell shape changes during morphogenesis require a dynamic actin cytoskeleton. The rho family of small GTPases functions in many of the actin rearrangements seen in cultured cells such as stress fiber and focal contact formation, membrane ruffling, lamellopodia formation, and filopodial extension (Ridley & Hall 1992, Ridley et al 1992). To study the role of these proteins in many developmental systems, investigators have determined the effects of site-directed mutations: constitutive active, GTP-bound mutant forms of small GTPases based on the oncogenic V12 ras proteins (reviewed in Barbacid 1987) and dominant-negative N17 mutant proteins that are expected to remain in the GDP-bound state, allowing the proteins to compete for and titrate out the guanine exchange factor (Schweighoffer et al 1993). The mutant proteins expressed in cells of interest are used to study their effects on the actin cytoskeleton.

In *Drosophila*, the rho family of proteins includes Drac1, RhoL (a rho-like protein), and Dcdc42. Expression of mutant forms of Drac1 and Dcdc42 in *Drosophila* has shown that they are involved in diverse processes such as

myoblast fusion, axonal outgrowth (Luo et al 1994), wing epithelia morphogenesis (Eaton et al 1995, 1996), and embryonic dorsal closure (Harden et al 1995). In the ovary, expression of dominant-negative mutant rac in border cells disrupts their migration (Murphy & Montell 1996). Border cells do not migrate properly, which results in eggs with malformed micropyles. The border cells fail to form the filopodial-like extensions that normally reach between the nurse cells. Using inducible heat shock dominant-negative Drac1, the movement of the border cells can be halted at any point during migration, which indicates that rac function is not required simply to initiate movement. Interestingly, border cells normally have a relatively large amount of filamentous actin and express at least two actin-binding proteins, fascin and profilin, at especially high levels. Mutations in *chickadee* occasionally result in a delay in border cell migration (Verheyen & Cooley 1994). *singed* mutants, however, do not show defects in border cell migration (Cant et al 1994), possibly indicating redundant actin-bundling activities.

Constitutively active and dominant-negative RhoL and Dcdc42, when expressed in the germline cells, cause a variety of defects in the actin cytoskeleton (Murphy & Montell 1996). Dcdc42 mutants cause the subcortical actin in stage 6 and older egg chambers to become discontinuous, and the ring canals are released from the membranes. This often results in cell fusion and the formation of multinucleate cells. Although the effects of the dominant-negative Dcdc42 protein are somewhat less severe than the constitutively active form of Dcdc42, the data indicate that regulated Dcdc42 activity is required for maintaining the nurse cell subcortical cytoskeleton. In addition, dominant-negative RhoL causes some cell fusion characterized by release of ring canals from the membranes. Perturbations of RhoL activity disrupt nurse cell-follicle cell contacts and cause morphogenic defects in the follicle cell layer, including elongation and stacking of follicle cells. In addition to the specific defects seen with each of the mutant Drac1, RhoL, and Dcdc42 proteins in cellular morphogenesis, dominant-negative forms of each of the proteins prevent the formation of the cytoplasmic actin bundles at stage 10B. This results in the characteristic failure to transport cytoplasm seen for other dumpless mutants.

A number of candidate effector proteins for each of the rho family of GTPases have been identified (reviewed in Tapon & Hall 1997). For example, GTP-bound cdc42 and rac small GTPases bind to the serine/threonine $p65^{PAK}$ kinase (PAK), stimulating its kinase activity (Manser et al 1994, Martin et al 1995). The *Drosophila* PAK (DPAK) localizes with sites of dynamic actin structures and focal adhesion proteins (Harden et al 1996). In the *Drosophila* ovary, the downstream effectors of the small GTPases are unknown. However, DPAK might serve as one effector of Dcdc42 and/or Drac1. Determination of its expression and its subcellular localization in the ovary might be informative

regarding its potential role there. In addition, the minimal cdc42 and rac-binding domain in PAK is the 18-amino-acid CRIB motif. The CRIB motif is found in a variety of proteins, some of which contain kinase domains, and others, such as the human MSE55 protein, contain a proline-rich motif. It is possible that some of these proteins serve as linkers between cdc42 or rac and SH3 domain proteins or profilin (Burbelo et al 1995). Finally, in yeast, cdc42 interacts with the formin, Bni1p (Evangelista et al 1997). This raises the possibility that the *Drosophila* formin, capu, mediates some of cdc42's activities, perhaps by linking cdc42 to profilin.

TGF-β

A recent study suggests a role for the TGF-β pathway in controlling cytoplasm transport in the stage 10 egg chamber (Twombly et al 1996). The TGF-β ligand family members include dpp (Padgett et al 1987), 60A (Wharton et al 1991, Doctor et al 1992), and screw (Arora et al 1994). Only the dpp ligand appears to be expressed in the ovary. Dpp activity itself is not required in the germline cells of the ovary (Irish & Gelbart 1987); however, it is expressed in a dynamic pattern in the anterior follicle cells beginning around stage 8 (Twombly et al 1996), placing it in a good location to signal from the soma to the underlying germline. The TGF-β receptors include the type I and type II receptors that heterodimerize upon ligand binding, thus activating the serine-threonine kinase activity of the receptor. There are three type I receptors in *Drosophila* encoded by *saxophone* (*sax*), *thick veins* (*tkv*) (Nellen et al 1994, Penton et al 1994, Xie et al 1994, Brummel et al 1994), and *Atr I* (Wrana et al 1994), all of which are expressed in the germline cells of the ovary. The saxophone and thick veins receptors, along with the type II receptor encoded by the *punt* gene, likely mediate dpp signaling (Childs et al 1993, Ruberte et al 1995, Letsou et al 1995).

A number of genetic experiments have explored the role of TGF-β signaling in egg chamber development (Twombly et al 1996), many of which point to a role in egg shell production. For example, a reduction of dpp expression using a conditional allele led to egg chambers with abnormal anterior egg shell structures. Similarly, *saxophone* mutant flies rescued with a *hs-sax* transgene and held under nonheat shock conditions produced eggs with abnormal dorsal appendages. Each of these genotypes produced some eggs that were shorter than normal, which hints at a role in nurse cell cytoplasm transport.

In the context of actin regulation, experiments with transgenic females that express heat shock–inducible dominant-negative *saxophone* revealed particularly intriguing defects (Twombly et al 1996). In flies carrying this construct, a fraction of the egg chambers arrested and degenerated after stage 9, as was seen for the loss-of-function alleles. Other egg chambers failed to undergo cytoplasm

transport. Visualization of the actin cytoskeleton in these ovaries revealed that cytoplasmic actin bundles failed to assemble. This suggests that sax may transmit the signal triggering the stage 10B egg chamber to build cytoplasmic actin bundles and dump cytoplasm. An attractive hypothesis is that the signal is dpp dependent and originates in the follicle cells overlying the nurse cells.

Because there are multiple type I receptor genes, one may be partially redundant to another, resulting in the incompletely penetrant phenotypes. Thus germline clones that are mutant for multiple type I receptor genes might allow an analysis of more penetrant phenotypes. On the other hand, if heterodimer formation of type I and type II receptors is essential for signaling, germline mutant clones of loss-of-function type II receptor *punt* mutants might allow the examination of complete loss-of-dpp signaling. Germline clones of a hypomorphic alleles of *punt* have been made (Ruberte et al 1995). Although the ovarian phenotype has not been reported, ventralized eggs were produced, consistent with punt's role in embryonic patterning (Ruberte et al 1995). It will be useful to learn the phenotype of germline clones of null alleles of *punt.*

Drosophila schnurri encodes a zinc-finger transcription factor related to the human MBP family of transcription factors and is required to mediate dpp-regulated transcriptional activity during embryonic patterning (Arora et al 1995, Grieder et al 1995, Staehling-Hampton et al 1995). *schnurri* germline clones produce very few eggs, which suggests that *schnurri* is required in oogenesis (Grieder et al 1995). TGF-β signaling has been reported to stimulate cardiac or skeletal muscle actin transcription in certain cell types (Brand et al 1993). Interestingly, relative levels of nonmuscle actin mRNAs more than double between stage 7 and stage 11, possibly in order to create the necessary actin pools required for the dramatic formation of cytoplasmic actin bundles and to provide additional actin for the syncytial embryo (Ruddell & Jacobs-Lorena 1984). Perhaps dpp signaling through schnurri is partly responsible for the upregulation of nonmuscle actin genes and possibly other cytoskeletal proteins in oogenesis.

CELL ADHESION AND THE ACTIN CYTOSKELETON

Armadillo

The highly coordinated behaviors of both the follicle cell epithelium and the underlying germline cells require sturdy cell-cell adhesion. One complex of proteins involved in cell-cell adhesion is the catenin-cadherin complex that includes E- or N-cadherin, β-catenin, α-catenin, and a 120-kDa protein ($p120^{cas}$), which is a src substrate (reviewed in Kemler 1993, Peifer 1995). β-catenin links α-catenin to E-cadherin, and α-catenin links the cadherin complex to the actin cytoskeleton either directly or through α-actinin. E-cadherin can then mediate homotypic cellular adhesive properties (reviewed in Kemler 1993, Peifer 1995).

Genetic analysis of *Drosophila* β-catenin encoded by the *armadillo* locus (Riggleman et al 1989) has revealed that the protein is bifunctional, with roles in the wingless signaling pathway and cell adhesion (reviewed in Peifer 1995). Because wingless signaling is not required during oogenesis in the germline (Baker 1988) and may have only a limited role in the soma very early in the germarium (Forbes et al 1996), the egg chamber is an ideal tissue in which to study the adhesive properties of armadillo. Armadillo is expressed abundantly in follicle cell epithelia and is enriched at the apical-lateral surfaces of the plasma membranes that are juxtaposed to the germline cells. In addition, the polar follicle cells at the anterior and posterior ends show elevated expression of armadillo. Armadillo also is localized somewhat uniformly in a slightly punctate pattern at the plasma membrane of the germline cells (Peifer et al 1993).

Germline clones of *armadillo* hypomorphic and null alleles allowed its function in cell adhesion to be studied (Peifer et al 1993). Embryos produced from germline clones of weak *armadillo* mutants have defects in the formation of adherens junctions and enhanced zygotic defects in wingless signaling (Cox et al 1996). Germline clones of moderate and severe alleles produce egg chambers that are dumpless as a result of failing to assemble the cytoplasmic actin bundles. In the case of null alleles of *armadillo,* a variety of defects are observed in the ovary (Peifer et al 1993). They have defects in the positioning of the oocyte and aberrant cell shape, and ring canals are sometimes released from the membranes. Germline clones of severe alleles also have defects in maintaining plasma membrane integrity, resulting in multinucleate nurse cells similar to those observed in protein kinase A (PKA) mutants (discussed below) and mutant rho family proteins. In addition, a small class of egg chambers produced from clones of the most severe alleles have aberrant numbers of cells consistent with a defect in packaging of the germ-cell clusters by the follicle cells. An egg chamber with too many germline cells and two oocytes is generally flanked by an egg chamber with too few cells and no oocyte.

The functions of armadillo have been mapped to domains within the protein. Armadillo is a modular protein (Figure 3) consisting of an NH_2-terminal domain of 165 amino acids, a central domain of 13 42-amino acid imperfect repeats (arm repeats), and a COOH-terminal domain of approximately 120 amino acids (Riggleman et al 1989). The α-catenin binding site in armadillo has been mapped to a 76-amino acid region in the NH_2-terminal domain (Orsulic & Peifer 1996, Pai et al 1996). The *Drosophila* E-cadherin (*D*E-cadherin) binding site maps to arm repeats 3 through 8 (Orsulic & Peifer 1996, Pai et al 1996).

In the ovary, mutant *armadillo*, which lacks the α-catenin binding site, can localize both to the adherens junctions in the follicle cells and weakly to the nurse cell plasma membranes (Orsulic & Peifer 1996). Deletion of central arm repeats 3 through 6 or simply repeat 8 disrupts *D*E-cadherin binding, resulting in a mutant armadillo protein that does not localize to the plasma membrane

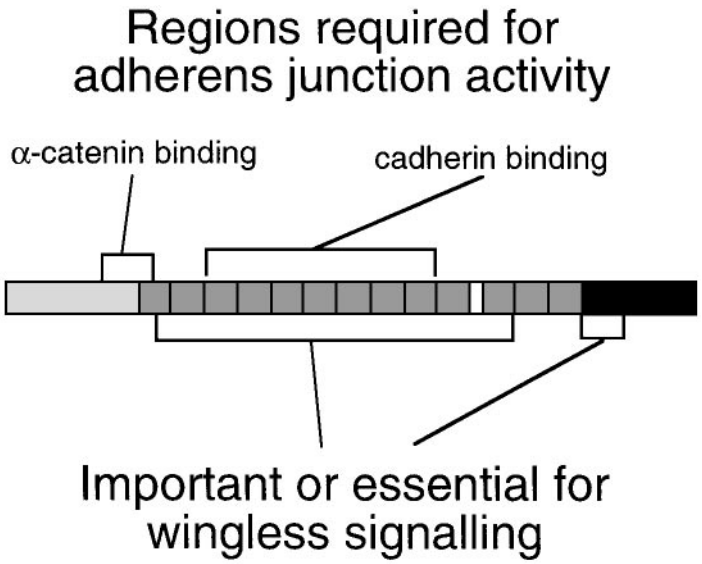

Figure 3 Domain structure and function of armadillo (modified from Orsulic & Peifer 1996). Armadillo consists of an acidic amino terminal domain (*light gray box*), 13 imperfect 42 amino acid repeats (*medium gray boxes*) and a Gly/Pro-rich carboxy-terminal domain (*black box*). The white box indicates an insertion between repeats 10 and 11. The interaction domains are indicated. The cadherin binding site in vertebrate β-catenin has also been shown to bind to fascin (Tao et al 1996). Regions shown to be involved in wingless signaling are also indicated.

in follicle cell adherens junction or to plasma membranes of nurse cells. These mutant armadillo proteins that are defective in *D*E-cadherin or α-catenin binding also do not rescue the cell adhesion defects in mutant germline clones of a null *armadillo* allele (*arm*^{YD35}).

With the discovery that *shotgun* encodes *D*E-cadherin (Uemura et al 1996, Tepass et al 1996, reviewed in Knust & Leptin 1996), further genetic analysis of cell adhesion is now possible. *shotgun* mutations lead to defects in epithelial rearrangements during the embryonic morphogenesis of organs such as the Malpighian tubules and the trachea. In the ovary, *shotgun* germline clones have defects in cell adhesion in which the nurse cells become round (Oda et al 1997). In contrast to the *armadillo* mutants, the overall actin cytoskeleton of the germline cells appears to remain intact, suggesting that *D*E-cadherin and *armadillo* have multiple functions, some of which are independent of one another.

These data suggest that the cadherin-catenin complex can mediate the cell adhesive properties of the germline cells and tether the adhesive complexes to the subcortical actin cytoskeleton. Given that *armadillo* mutants have defects in

the assembly of cytoplasmic actin bundles, it is attractive to speculate that the cadherin-catenin complex also anchors the actin bundles to the plasma membrane. Because loss of *shotgun* does not show defects in cytoplasm actin bundle assembly, it is possible that additional cadherin-like molecules or other proteins with similar functions are expressed in the ovary to help anchor the actin bundles to the membrane. In a recent report, fascin was shown to bind to the central arm repeats of β-catenin; fascin also competes with E-cadherin for binding (Figure 3; Tao et al 1996). This raises the possibility that some armadillo and fascin might interact in cytoplasmic actin bundle assembly independent of armadillo's functions in the cadherin-catenin complex. Finally, it seems intuitive that cell-cell contacts between the nurse cells must be dynamic and capable of reorganization or even release as the nurse cells shrink during cortical contraction that leads to cytoplasm dumping. How are the contacts regulated during dumping? If they are anchoring the actin bundles to the membrane, does the whole cadherin complex dissociate during nurse cell contraction or can reorganization be facilitated by regulating homotypic E-cadherin associations alone? Further examination of this complex in the ovary will help shed light on how cell adhesion can be regulated during cellular morphogenesis.

Protein Kinase A

PKA is the major mediator of cAMP signaling from a variety of plasma membrane receptors whose ligands include neurotransmitters and hormones. In *Drosophila*, PKA has been shown to antagonize the hedgehog signaling pathway (for example, see Blair 1995 and references therein). The major catalytic subunit is encoded by the DC0 gene (Kalderon & Rubin 1988), and mutants in this gene have defects in embryonic morphogenesis that cause larval arrest (Lane & Kalderon 1993). Female sterile alleles have egg chambers with plasma membrane degeneration that causes the release of the ring canals into the cytoplasm (Lane & Kalderon 1993, 1995). This defect is observed after stage 6 and appears to become more severe as the egg chambers get older. Careful examination of the time course of nurse cell membrane degeneration by actin staining showed that the ring canals were released intact from the membranes, allowing the membranes to open and cells to fuse. The cortical cytoskeleton remained associated with the membrane during degeneration so the defect does not appear to affect association of the cortical actin cytoskeleton with the plasma membrane per se. Immunohistochemistry revealed that the catalytic subunit is localized uniformly to the nurse cell cortex consistent with PKA having critical substrates at the nurse cell membrane (Lane & Kalderon 1995). In addition, there was no apparent enrichment near the ring canals, suggesting that the ring canal release phenotype might be the result of general plasma membrane instability rather than from a specific effect on ring canals. The sensitivity of

ring canal association with the membranes may simply highlight the structural significance of the ring canal cytoskeleton and its membrane attachments in maintaining a stable opening in the plasma membrane.

Lane & Kalderon (1995) propose that PKA may directly affect cell adhesion molecules that are critical for maintaining plasma membrane integrity. Armadillo contains a consensus PKA phosphorylation site, making it a candidate target for PKA. As discussed previously, germline clones of *armadillo* mutants display a range of defects including a breakdown in nurse cell plasma membrane integrity. However, there is no failure of armadillo to localize to the plasma membrane in PKA mutants, suggesting that if there is an effect of PKA on armadillo, it may be more subtle than just affecting localization (Lane & Kalderon 1995). The PKA phenotype might also arise from the loss of regulation of other substrates.

RING CANAL ASSEMBLY AND FUNCTION

Ring canals are large, prominent actin structures that form the junctions between the germline cells and provide the conduits through which cytoplasm is transported (reviewed in Robinson & Cooley 1996). These actin structures provide a unique opportunity to study the function of the cytoskeleton and its associated proteins because of their nearly perfect ring shape. Perturbations in either the assembly or growth of the ring canals are revealed in the morphology of the ring canal and are generally visible with a light microscope (Robinson et al 1994). Finally, because of the geometric nature of the ring canal, we can consider many theoretical aspects of assembling such a large complex of proteins.

Assembly of wild-type ring canals occurs through the sequential addition of a number of cytoskeletal proteins to arrested cleavage furrows (Figure 4*A*; Robinson et al 1994). During cytokinesis, the contractile ring contains the cleavage furrow protein anillin (Field & Alberts 1995) and contractile actin filaments (Robinson et al 1997). After the third mitotic division, at least one protein that immunoreacts with anti-phosphotyrosine antibodies localizes to the outer rims of nascent ring canals. After the fourth mitotic division and the movement of the cluster of 16 germline cells into region 2a of the germarium, a product of the *hu-li tai shao* (*hts*) gene, called hts-RC, and additional actin filaments localize subcortically to the arrested cleavage furrow membrane, forming a robust actin-rich inner rim (Warn et al 1985, Robinson et al 1994). Additional phosphotyrosine epitopes accumulate in the inner rim of the ring canal. In region 3 (stage 1 egg chambers), kelch proteins begin to localize to the inner rims of the ring canals (Xue & Cooley 1993, Robinson et al 1994).

Mutations in the *cheerio* (Robinson et al 1997) or *hts* (Yue & Spradling 1992) genes block the accumulation of the inner rim. The phenotypic analysis

of a null allele of *cheerio* suggests that it functions downstream of at least one phosphotyrosine epitope and upstream of *hts* (Robinson et al 1997). Molecular characterization of *hts* has shown it to be a complex gene encoding multiple transcripts (Yue & Spradling 1992). An ovary-specific transcript encodes a protein with homology to vertebrate adducin through the amino half of the protein, and with no known homology through the carboxy half of the protein. The ring canal-specific hts-RC product is derived from the novel carboxy half, probably through processing of a precursor product (Robinson et al 1994). Kelch (Figure 4*B*) is not required for the initial assembly of ring canals but is required throughout the growth and expansion of the ring canal to maintain proper organization of the inner rim (Robinson et al 1994, Tilney et al 1996b).

Ring canals are dynamic structures that grow throughout oogenesis (Robinson et al 1994, Tilney et al 1996b). Upon cleavage furrow arrest, they have diameters that range from 0.5 to 1 μm. By stage 4 when kelch has reached all of the ring canals, the ring canal outer diameter has expanded to 3–4 μm. Finally, by stage 11, when cytoplasm dumping occurs, the ring canal diameter is approximately 10 μm. In stages 4–5, the inner rim has on the order of 720 bipolar actin filaments in a cross-sectional view, and between stages 4 and 11, the number as well as the density of actin filaments in a cross-sectional view remain roughly constant (Tilney et al 1996b). Consequently, a stage 9 ring canal with an outer diameter of 6.28 μm has a total length of 14,323 μm of actin filaments (taken from a specific example in Tilney et al 1996b). From this information, it is possible to make additional calculations[1] to determine the concentration of the actin in the ring canal inner rim. From these calculations, the actin concentration is in the millimolar range (not 6–7 mM as previously stated in Robinson & Cooley 1996), indicating that very high concentrations of proteins might exist in ring canals.

To expand the ring canal, the actin filaments might slide with respect to one another, increasing the diameter (Robinson et al 1994, Tilney et al 1996b). The

[1]From the number of actin filaments per cross-sectional area of a ring canal (Tilney et al 1996b), we can make additional calculations to estimate the concentration of actin in a ring canal. First, because an actin filament consists of a repeating unit of 13 monomers per 36 nm (Egelman 1985), we can determine the moles of actin in a ring canal. For example, considering the same stage 9 ring canal with 14,323 μm of actin, the number of monomers is 14,323 $\times$ 10^3 nm of actin/36 nm per repeating unit $\times$ 13 monomers per repeating unit $=$ 5.2 $\times$ 10^6 actin monomers in the ring canal. The number of moles of actin in this stage 9 ring canal then is 8.589 $\times$ 10^{-18} mol. The volume of the same stage 9 ring canal can be calculated by considering the difference of two cylinders that define the ring canal annulus with a depth of 1.87 μm, an outer diameter of 6.28 μm, and an inner diameter of 5.54 μm. The volume of ring canal annulus as described is 12.84 μm^3, which converts into 1.284 $\times$ 10^{-14} L. The molarity of actin monomers in the ring canal annulus then is 670 μM. Similar calculations, using data from Tilney et al (1996b), from younger ring canals reveal actin concentrations as high as 4 mM in stage 2 ring canals and 2 mM actin in stage 7 ring canals. We conclude there is millimolar actin in the ring canal inner rim.

actin filaments then could be lengthened or additional filaments might be added to the ring canal to maintain the density and filament number during growth. Cross-linking proteins that organize the actin filaments might bind to the ring canals through high-affinity interactions. Because there are high concentrations of actin monomers, low-affinity interactions (on the μM range) between ring canal organizer proteins and ring canal actin can allow the cross-links between actin filaments to be formed, broken, and reformed, maintaining organization of the inner rim during expansion.

Because *kelch* mutant ring canal actin inner rims are disorganized, kelch is one candidate protein that might form dynamic cross-links in order to maintain inner rim organization during ring canal growth. The *kelch* gene produces a single transcript with a UGA stop codon separating two open reading frames (ORFs) that produces two products: ORF1-only and full-length ORF (ORF1 + ORF2) proteins as a result of stop codon suppression (Xue & Cooley 1993, Robinson & Cooley 1997). However, kelch ORF1 is sufficient for *kelch* function (Robinson & Cooley 1997a). Kelch ORF1 has two domains found in other non-kelch proteins including a BTB domain and a domain consisting of six 50-amino-acid kelch repeats. The BTB domain is found in a group of zinc finger transcription factors where it has been shown to mediate dimerization (Harrison & Travers 1990, DiBello et al 1991, Chardin et al 1991, Bardwell & Treisman 1994, Zollman et al 1994, Chen et al 1995). The kelch repeat domain is also found in non-kelch proteins such as *Physarum polycephalum* actin-fragmin kinase (Eichinger et al 1996) and *Limulus* scruins (Way et al 1995a,b). The kelch repeats in one scruin isoform, α-scruin, mediate interactions with the actin filaments in the *Limulus* acrosomal actin bundle (Bullitt et al 1988, Owen & DeRosier 1993, Schmid et al 1994, Way et al 1995b).

A model for kelch activity indicates that it organizes ring canals by cross-linking the actin filaments. It might accomplish this by binding to the actin filaments or another actin-associated protein through the repeat domain and dimerizing through the BTB domain. Structure-function analysis of kelch ORF1 (Robinson & Cooley 1997b) revealed that a truncated ORF1 protein lacking the repeat domain localizes to ring canals, but its localization requires the coexpression of an intact ORF1 protein. In addition, a recombinant BTB domain dimerizes in vitro. These results support a model of kelch dimerization through the BTB domain in vivo. A truncated ORF1 protein consisting of only the kelch repeat domain localizes to ring canals in the absence of other kelch proteins. These experiments show that the repeat domain is necessary and sufficient for ring canal localization. Additional evidence suggests that the kelch repeat domain actually has two interaction domains, one that allows ring canal-specific localization and one that is required for ring canal-organization activity (Robinson & Cooley 1997b). At least one of these proposed interactions for kelch ORF1 might be a low-affinity interaction that allows the actin filament cross-links to

break and reform during growth. Because it is not possible to measure a dissociation constant in vivo, identification of kelch-binding partners will facilitate these kinds of experiments in vitro.

FUTURE PROSPECTS

Genetic analysis of the actin cytoskeleton and interacting proteins in the *Drosophila* ovary has suggested functions for many structural proteins and has identified some candidate regulatory molecules. However, it is not well understood what signals the morphological events during *Drosophila* oogenesis. Although TGF-β signaling might play some role in regulating the actin morphological changes that occur at stage 10, the partial penetrance of many of the phenotypes associated with *saxophone* makes the role of TGF-β signaling uncertain. In addition, many of the binding partners for the structural proteins described remain unknown. The *Drosophila* ovary, with the large size of the germline cells and its amenability to genetic analysis, provides a great opportunity to study the function of the actin cytoskeleton and its associated proteins and promises to support many future studies that will help to elucidate the role of the actin cytoskeleton in cellular morphogenesis.

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