Filamin Is Required for Ring Canal Assembly and Actin Organization during *Drosophila* Oogenesis

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Abstract. The remodeling of the actin cytoskeleton is essential for cell migration, cell division, and cell morphogenesis. Actin-binding proteins play a pivotal role in reorganizing the actin cytoskeleton in response to signals exchanged between cells. In consequence, actin-binding proteins are increasingly a focus of investigations into effectors of cell signaling and the coordination of cellular behaviors within developmental processes. One of the first actin-binding proteins identified was filamin, or actin-binding protein 280 (ABP280). Filamin is required for cell migration (Cunningham et al., 1992), and mutations in human α-filamin (*FLN1*; Fox et al., 1998) are responsible for impaired migration of cerebral neurons and give rise to periventricular heterotopia, a disorder that leads to epilepsy and vascular disorders, as well as embryonic lethality. We report the identification and characterization of a mutation in *Drosophila filamin*, the homologue of human α-filamin. During oogenesis, filamin is concentrated in the ring canal structures that fortify arrested cleavage furrows and establish cytoplasmic bridges between cells of the germline. The major structural features common to other filamins are conserved in *Drosophila* filamin. Mutations in *Drosophila filamin* disrupt actin filament organization and compromise membrane integrity during oocyte development, resulting in female sterility. The genetic and molecular characterization of *Drosophila filamin* provides the first genetic model system for the analysis of filamin function and regulation during development.

Key words: filamin • ABP280 • *Drosophila* • actin • ring canals

THE regulation of actin microfilament assembly and organization is essential for intracellular transport, cell movement, changes in cell shape, and the establishment of cell polarity. Necessary intermediates in the determination of actin organization and function during these complex cellular events are the proteins that bind actin. Proteins at the cell membrane that bind actin help to link the cytoskeletal elements of neighboring cells, thereby permitting the coordination of cell behaviors, as well as establishing the linkages between the cytoskeleton and cell surface receptors that mediate responses to cell-cell and cell-matrix signaling. An increasing body of evidence indicates that as regulators of actin organization, actin-binding proteins are the targets of a complex set of inputs from signaling pathways (Schmidt and Hall, 1998). Multidisciplinary approaches in a variety of experimental systems are providing new information on the regulation of individual actin-binding proteins and their role in cell behavior and tissue morphogenesis (Sutherland and Witke, 1999).

In *Drosophila*, oocyte development has proved to be an excellent system in which to study the regulation of actin and actin-binding proteins (Robinson and Cooley, 1997). Oogenesis begins in the anterior compartment of the ovariole, the germarium. A cystoblast divides four times with incomplete cytokinesis to give rise to a cyst of 16 germline cells interconnected by cytoplasmic bridges, or ring canals. One cell will become the oocyte, while the remaining 15 cells differentiate as polyploid nurse cells that synthesize and transport cytoplasmic components through the ring canals to the developing oocyte. Shortly after its formation, the germline cyst is encapsulated with a layer of somatic follicle cells and the resultant egg chamber buds off from the germarium. As the released egg chamber proceeds down the ovariole, a complex set of cellular interactions continues to support the morphogenesis of the egg chamber and the development of the oocyte (Spradling,

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1993; Schupbach and Roth, 1994; Deng and Bownes, 1998). Genetic, cytological, and pharmacological studies have implicated the actin cytoskeleton in multiple aspects of this morphogenetic process, including cytoplasmic transport, the localization of determinants, cell migrations, cell shape changes, and intercellular adhesions.

For cytoplasmic transport within the germline cyst, several distinct actin arrays and associated functions have been characterized (Cooley and Theurkauf, 1994). In one capacity, actin filaments are a major structural component of the ring canals that act as the conduit for cytoplasmic transfer between cells. The initial intercellular bridges established between nurse cells and the oocyte are fortified as ring canal structures in a highly orchestrated process (Robinson and Cooley, 1997). Stabilization of the future ring canal may initiate with the persistence of the actin-binding protein, anillin, in the arrested mitotic cleavage furrow. Anillin associates preferentially with actin filaments in contractile domains and is proposed to stabilize actin filaments within the furrows (Field and Alberts, 1995). As the ring canals grow several additional polypeptides are recruited. An unidentified protein(s) containing phosphotyrosine is associated with the outer rim of the ring canal immediately below the cell membrane and is required for ring canal expansion (Dodson et al., 1998; Roulier et al., 1998). The *hu-li tai shao* (*hts*) gene product and actin microfilaments are also recruited to the inner rim of ring canals (Yue and Spradling, 1992). Kelch, a protein with homology to the actin cross-linking protein, scruin, is the last known component to accumulate in ring canals (Xue and Cooley, 1993; Robinson et al., 1994), and is proposed to bundle and organize actin into the compact ring (Robinson et al., 1994; Tilney et al., 1996). Recent work suggests that kelch functions to hold actin bundles together during the expansion of the ring canals (Tilney et al., 1996). Moreover, expansion is regulated by a parallel pathway that includes the kinase products of the *Tec29* and *Src64* genes (Roulier et al., 1998). The exact physical and functional relationships between the known components of the ring canal, as well as additional components required for membrane attachment of the ring canal, remain to be determined.

The transport of cytoplasmic constituents through ring canals requires additional actin functions. A phase of slow transport during early stages appears to depend on both actin filaments and microtubules (Theurkauf et al., 1992; Bohrmann and Biber, 1994). Subsequently, a rapid phase of transport, or dumping, of nurse cell cytoplasm to the oocyte at stage 10b depends on two distinct cytoplasmic actin networks within the nurse cell complement. A subcortical actin network, together with cytoplasmic myosin, provides the contractile force in nurse cells that drives the rapid dumping of cytoplasm into the oocyte (Gutzeit, 1986; Wheatley et al., 1995; Edwards and Kiehart, 1996). A second network of cytoplasmic actin filaments is assembled before dumping and extends from nurse cell plasma membranes in a radial array to cage the nurse cell nuclei (Gutzeit, 1986; Riparbelli and Callaini, 1995; Guild et al., 1997). The isolation of female sterile mutations has identified three genes, *chickadee*, *quail*, and *singed*, that encode actin-binding proteins that are required for the assembly of the cytoplasmic actin filaments in nurse cells (Cooley et al., 1992; Cant et al., 1994; Mahajan-Miklos and Cooley, 1994; Verheyen and Cooley, 1994).

In this work, we report the characterization of a female sterile mutation that identifies the *Drosophila* homologue of human *ABP280*. Actin-binding protein 280 (ABP280)[1], or nonmuscle filamin, was identified as an ABP by its ability to cross-link and precipitate purified muscle actin (Hartwig and Stossel, 1975; Wang et al., 1975; Stossel and Hartwig, 1976). The association of filamin with actin can induce the orthogonal branching of actin filaments, or serve to bundle parallel actin filaments (Hartwig and Stossel, 1975; Hartwig et al., 1980; Niederman et al., 1983; Gorlin et al., 1990). Filamin binds to a variety of cell membrane proteins and intracellular ligands involved in signal transduction, suggesting its role as a downstream effector in the remodeling of the actin cytoskeleton (Matsudaira, 1994; Glogauer et al., 1998; Loo et al., 1998; Ott et al., 1998; Xu et al., 1998; Zhang et al., 1998; Ohta et al., 1999). Our analysis of the *Drosophila* filamin gene provides evidence for its function in cytoplasmic transport, membrane integrity, and cellular adhesion during *Drosophila* oogenesis.

Materials and Methods

Fly Stocks

The fly stock that contains the marked third chromosome *mwh jv ca*, and the balancer stock *TM6B, h Hu D e ca*, were provided by L. Crosby (Harvard University). The stock Gl^1 *Sb/TM3 Ser* p^p *e* was provided by Dr. Douglas Kankel (Yale University). All third chromosome deficiencies were obtained from the Bloomington *Drosophila* Stock Center (Indiana University). *Df(3R)C4/*p^p*Dp, Sb (89E; 90A)* is a third chromosome deficiency that removes the *sko* locus. The *ru h st* p^p *ss* e^s stock was obtained from the Bowling Green Stock Center (Bowling Green State University), and was recently isogenized in this laboratory for a lethal-free third chromosome. The *P* element insertion line *EP(3)3715* was obtained from the Berkeley *Drosophila* Genome Project. Oregon R flies were used in all cases for wild-type controls. Flies were raised on standard yeast-cornmeal-agar medium at 25°C.

EMS Mutagenesis

EMS mutagenesis was carried out as described previously (Gepner et al., 1996). Male flies of the genotype *mwh jv ca* were starved for ~1.5 h, fed with 25 mM EMS in 1% sucrose overnight, and mass mated with Gl^1 *Sb/TM3 Ser* p^p *e* virgins. F_1 progeny of the genotype *mwh jv ca/*Gl^1 *Sb* were originally screened for modification (enhancement or suppression) of the rough eye phenotype caused by the dominant Gl^1 mutation. One of the third chromosome suppressors of the Gl^1 rough eye phenotype exhibited female sterility when homozygous. Genetic mapping was conducted by meiotic recombination with a third chromosome containing multiple genetic markers *ru h st* p^p *ss* e^s. The results revealed that two distinct mutations were separately responsible for the suppression of Gl^1 and the female sterility. A recombinant chromosome that carried only the female sterile mutation, designated *shi kong* (*sko*), was analyzed in the studies described here.

Generation of Deletions by P Element Excision

The *P* element in the stock *w; EP(3)3715/TM6B, Tb* contains a marker that changes the eye color from white to orange. Excision events were scored by loss of the eye color marker. *P* transposase was introduced by crossing *w; EP(3)3715/TM6B, Tb* flies with *Df(3)/TM3, Sb, Δ2-3*. Individual progeny males of genotype *EP(3)3715/TM3, Sb, Δ2-3* were then mated with virgin *w; sko/TM6B, D* females. Single white-eyed males of genotype *ΔP/TM6B, D* were mated again with *w; sko/TM6B, D* virgins. Female

1. *Abbreviations used in this paper:* ABP, actin-binding protein; EST, expressed sequence tag.

progeny of genotype *ΔP/sko* were tested for sterility. Stocks were established for lines that failed to complement *sko*, using sibling flies of the genotype *ΔP/TM6B, D*.

Isolation of Drosophila filamin cDNAs

Actin-associated proteins were isolated by affinity chromatography, separated by SDS-PAGE, and used as antigens for antibody generation in mice as previously published (Miller et al., 1989, 1991). Mouse antibody no. 4 described in (Miller et al., 1989) was used to screen a Lambda Zap expression library (Hay et al., 1988) constructed from *Drosophila* ovary poly(A)$^+$ RNA. (Antibody no. 4 recognizes two bands on a Western blot, corresponding to SDS band numbers 4 and 5 shown in Miller et al. [1989], and contains a mixture of antisera from two different mice injected with two different antigens.) The library was screened as described (Huynh et al., 1985) with minor modifications. The screen produced two unrelated cDNA clones, one of which is 3.2 kb in length and encodes the *Drosophila* homologue of ABP280 or nonmuscle filamin (Gorlin et al., 1990; Cunningham et al., 1992) used in this work.

The 7.5-kb cDNA clone GH12209 was obtained from the Berkeley *Drosophila* Genome Project via Research Genetics.

DNA and RNA Analysis

Sequence was obtained using T7, SP6, and custom primers, with an ABI377 sequencer. The entire sequence was manually proofread. Portions were read on only one strand, but all base calls were unambiguous. The nucleotide and protein sequence was analyzed using the UWGCG programs and the MacVector Sequence Analysis Software package (Oxford Molecular Group).

Genomic DNA for Southern blots was prepared from adults as previously described (Rasmusson et al., 1994). 5 μg of DNA were digested with restriction enzymes, fractioned on a 1% agarose gel, and transferred to Zeta-Probe nylon membrane (BioRad Laboratories) by standard methods. Total RNA used for Northern blot experiments was isolated as described previously (Rasmusson et al., 1994). RNA was fractionated on 0.75% agarose formaldehyde gels and transferred to Zeta-Probe membrane. DNA probes were labeled with [^{32}P]dATP (Amersham) using random hexamer primers (Amersham Pharmacia Biotech) according to methods described by Vogelstein and Gillespie (1979). Prehybridization and hybridization of DNA and RNA blots were carried out using standard methods.

Genomic clones were isolated by screening a cosmid library made from *iso-1* fly DNA (Tamkun et al., 1992) with a fragment derived from the 3.2-kb filamin cDNA. Two overlapping cosmid clones were used to characterize the transcription unit of the *filamin* gene. DNA labeling and hybridization methods for library screening were those used for Southern and Northern blots.

Generation of Antibodies Specific for Filamin

A filamin-6xHis fusion protein was generated using PCR and the 3.2-kb filamin cDNA as a template. The two primers used to amplify a cDNA fragment that encodes the last 90 residues in the filamin protein were primer 1: 5′-GCGCGCGTCGACGCCAGCAAGGTGGTGTCCAA-3′ and primer 2: 5′-CGCGCGAAGCTTCTTGCATACACGTACACATC-3′.

Vent DNA polymerase (NEB) was used for the PCR reactions. The 6xHis-tagged cDNA was cloned into the vector PQE9 (Qiaexpress vectors; Qiagen) using a HindIII and a SalI site added during the PCR reaction. The 6xHis fusion protein was produced in *E. coli* and purified on a Ni-NTA resin column according to the manufacturer's instructions (Qiagen). The filamin antiserum, 4-3D, was raised in rabbits against the purified bacterial fusion protein, and was blot affinity-purified against the His-tagged fusion protein.

Immunofluorescence and Electron Microscopy

Ovaries were dissected, fixed, and processed for immunocytochemistry as described in McGrail et al. (1995). For staining with propidium iodide or Oligreen (Molecular Probes, Inc.), ovaries were first treated with 20 μg/ml RNase in PBS for 1.5 h at 37°C followed by a one hour wash in PBT (PBS supplemented with 0.1% Triton X-100). Ovaries were incubated in 0.1 μg/ml propidium iodide or Oligreen for 30 min at room temperature. To visualize F-actin, ovaries were stained with phalloidin (Sigma Chemical Co.) diluted 1:1,000 for 30 min at room temperature. The anti-phosphotyrosine monoclonal antibody PY20 was purchased from ICN Biochemical Inc. and used in 1:10 dilution. The anti-anillin antibody is a rabbit polyclonal antibody (Field and Alberts, 1995) and was used at a final concentration of 5 μg/ml. Both anti-kelch and anti-hts are monoclonal antibodies kindly provided by Lynn Cooley (Yale University) and were used at a dilution of 1:10. The anti-hts antibody used here recognizes only the isoform found in the ring canals (Robinson et al., 1994). For immunocytochemistry the anti-filamin (4-3D) antiserum was used at a dilution of 1:1. The secondary antibodies used in the immunofluorescence experiments were either FITC-conjugated or Texas red–conjugated goat anti–rabbit (or mouse). In all cases, the secondary antibodies were preabsorbed against embryos and were diluted 1:100. Images were collected by using an MRC-1024 confocal imaging system (BioRad Laboratories). Details on the procedures for fixation, embedding, sectioning, and staining for electron microscopy are published in Tilney et al. (1996).

Results

Cellular Morphology and Actin Organization Are Defective in sko Mutant Egg Chambers

A female sterile mutation, *shi kong* (*sko*; "out of control" in Chinese), was generated by standard EMS mutagenesis. Homozygous *sko* mutant flies are viable, but females do not lay eggs. Mutant ovaries show an apparent arrest in oocyte growth at stage 10b and no mature egg chambers are observed. Further inspection of *sko* mutant egg chambers reveals several defects in cellular morphology and membrane integrity. In situ staining of DNA reveals the disruption of the normal spatial organization of germline nuclei in *sko* mutant egg chambers, and in late stages, entire nurse cells and/or nurse cell nuclei are sometimes seen to protrude into the posterior compartment normally occupied only by the growing oocyte (Fig. 1 b). Although follicle cells migrate to form a columnar epithelium surrounding the oocyte in *sko* mutants, their shape is frequently more elongated than in wild-type egg chambers. The subsequent centripetal movement of follicle cells along the anterior margin of the oocyte is also abnormal in many mutant egg chambers.

Our analysis of *sko* egg chambers stained with rhodamine-labeled phalloidin reveals an abnormal organization of actin filaments. In early egg chambers (stages 1–6), the level of phalloidin staining in ring canals is frequently reduced, and where visible forms a more diffuse pattern than in wild-type, suggesting that actin microfilaments are less tightly organized within the *sko* mutant ring canals (Fig. 1, c and d). In late stage 10 egg chambers, actin is no longer detected in organized ring canal structures, although some aggregated clusters of short actin filaments may represent the remnants of collapsed ring canals (Fig. 1, f and h). The disruption of actin organization in *sko* egg chambers is not restricted to the microfilaments within ring canals. In early egg chambers, the phalloidin staining pattern suggests that the subcortical network of actin filaments is anomalous along the nurse cell plasma membranes (Fig. 1 d). In late stage 10 egg chambers, the radial arrays of cytoplasmic actin microfilaments that extend from nurse cell membranes toward the centrally located nuclei are also disrupted. Instead, the cytoplasmic actin filaments observed are short, disorganized, and often clumped in small aggregates (Fig. 1 h).

sko Is Required for the Growth of Ring Canals

Because actin organization is defective in *sko* mutant ring

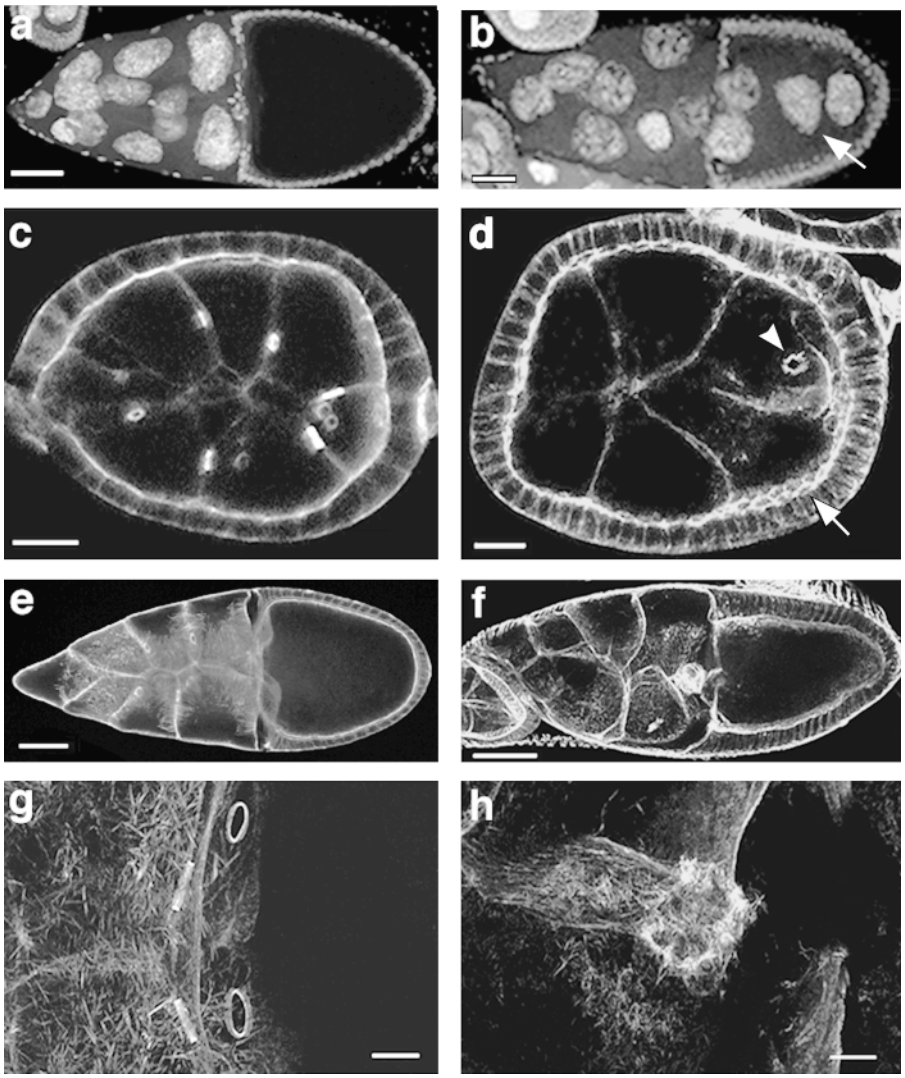

Figure 1. Abnormal cell morphology and disruption of actin organization in *sko* mutant egg chambers. Wild-type (left column) and *sko* (right column) egg chambers are compared and imaged by confocal microscopy. Anterior is to the left. (a and b) Stage 10 egg chambers stained with the fluorescent DNA dye propidium iodide. In the *sko* mutant egg chamber, polyploid nurse cell nuclei are seen in the oocyte compartment (arrow). (c–h) Egg chambers stained with rhodamine-labeled phalloidin to visualize actin filaments. (c and d) Defects of actin organization in ring canal structures (arrowhead) and in the subcortical network (arrow) are evident in stage 3 mutant egg chambers. (e and f) In stage 10 egg chambers, the distinct ring canals in wild-type are absent in the mutant. The radial arrays of filaments that extend between the nurse cell membranes and nuclei are disrupted in the mutant. An altered morphology of nurse cells is apparent. (g and h) High magnification images show the boundary region between the nurse cell and oocyte compartments of stage 10 egg chambers. Ring canals and the cytoplasmic actin network are clearly visualized in wild-type but are defective in *sko*. Bars: (a, b, e, and f) 50 μm; (c, d, g, and h) 10 μm.

canals, we have examined other known ring canal components to further characterize the role of the *sko* gene product. Using an antibody against anillin, we compared patterns of anillin localization in wild-type and *sko* ovaries (Fig. 2, a and b). In wild-type egg chambers, anillin localization at ring canals is observed early in region 1 of the germarium and is easily detected through stage 6, with diminished levels still visible through stage 10. In *sko* ovaries, anillin is present in the stabilized cleavage furrow and persists at ring canals in a pattern similar to wild-type. Moreover, *sko* egg chambers contain the typical 16 cells, including 15 nurse cells and an oocyte. These results indicate that cytokinesis and initial ring canal formation are not defective. However, in contrast to wild-type, the anillin rings in *sko* mutant egg chambers appear reduced in size.

Similarly, the recruitment of the phosphotyrosine protein(s) to ring canals in the *sko* mutant egg chambers appears normal during early stages. Beyond stage 3, while we can detect the phosphotyrosine antigen in ring structures, we also frequently detect small cytoplasmic foci, or particles, some of which are also recognized by rhodamine-phalloidin labeling (Fig. 2, c and d). These foci may represent disrupted or collapsed ring canals. Hts protein is also present in the early ring canals of *sko* egg chambers, but by stage 4, the localization of hts is often abnormal and only a portion of the ring is labeled. The disruption of hts becomes progressively worse in later stage egg chambers (Fig. 2 f). We did detect actin colocalized with hts protein in some ring canals; however, in most cases the ring canals were devoid of actin filaments. We did not observe concentration of kelch protein in *sko* mutant ring canals at any stage of oogenesis. Our results indicate that the *sko* gene product is not required for the initial recruitment of components of the outer rim of the ring canal. Rather, *sko* is required for proper assembly of the inner rim and/or its stability and growth during development.

Thin Section Electron Microscopy of sko Nurse Cells

Thin sections through wild-type and *sko* ring canals at stage 3 and 4 (Fig. 3, a–d) are consistent with what we have described by light microscopy and expand our resolution of the ring canal structures. As noted in Tilney et al. (1996), in vertical section the plasma membrane lining the inner margin of each side of the ring canal appears as a

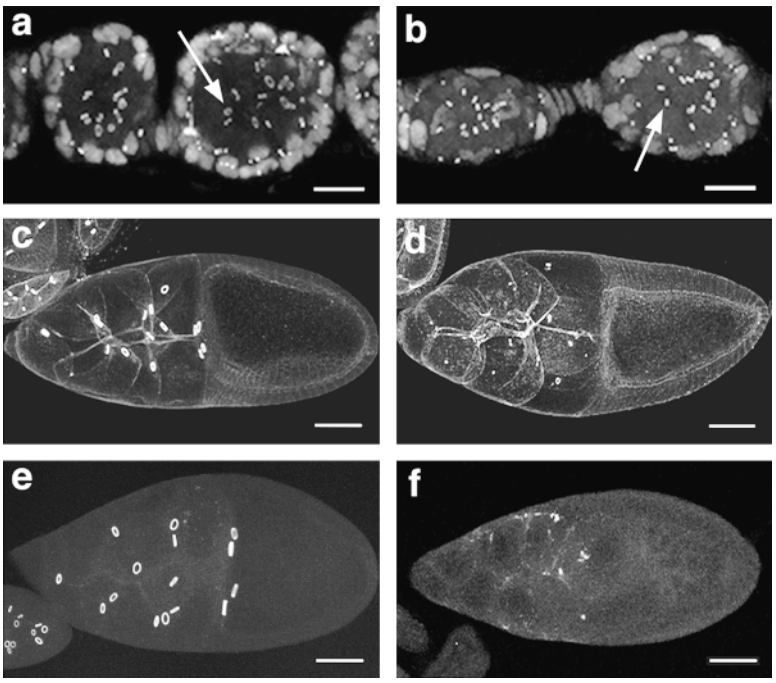

Figure 2. Disruption of inner ring canal components in *sko* ovaries. Anti-anillin staining of wild-type (a) and *sko* (b) egg chambers shows anillin localization to the ring canal outer rim persists in the mutant (arrow). The distinct inner ring canal structures detected at stage 10 by anti-phosphotyrosine (c) and anti-hts (e) are deteriorated in the *sko* mutant egg chambers (d and f, respectively). Small aggregates recognized by these antibodies may represent remnants of ring canals. Bars: (a and b) 10 μm; (c–f) 50 μm.

T-shaped junction limiting adjacent nurse cells (Fig. 3, a and b). Attached to these T-shaped extensions of plasma membrane in both wild-type and *sko* ring canals is a layer of dense material. Inside this material in the wild-type ring canal is a thick layer of actin filaments that is organized circumferentially as a purse string (Fig. 3, a and b; Tilney et al., 1996). This layer of filaments is missing altogether (Fig. 3, c and d) in the *sko* mutants or is reduced to a tiny fraction of the number of filaments seen at this same stage in wild-type. Note that in the *sko* mutants, the T-shaped extensions are formed with their associated dense material, even though actin filaments are sparse or missing altogether. Since anillin is present through stage 6 in *sko* mutants, it seems reasonable that anillin may be one (possibly major) component of this dense material lining the cytoplasmic surface of the canal.

In thin sections through stages 5 to 6 *sko* egg chambers, we also observe a number of changes that occur in the association of the plasma membranes that separate adjacent nurse cell cytoplasms. We see breaks or disruptions in the plasma membrane that allow the cytoplasm of adjacent cells to merge in these regions (Fig. 3, e and f). At these break points the plasma membrane is vesiculated. We also find that many of the ring canals are altered in stages 5 and 6: the normally T-shaped membrane junction appears folded back upon itself (Fig. 3 e). Furthermore, the ring canal as depicted in Fig. 3 e and as seen in other micrographs is frequently incomplete. In these sections we only find half a ring canal; the other half is missing, a situation that we interpret as the breakdown of the ring canal. Overall, the number of actin filaments lining the inner aspect of the ring in stage 2 chambers is initially sparse, and later can completely disappear before membrane disruption and ring canal loss.

Transport within sko Mutant Egg Chambers

To address the impact of *sko* on intercellular transport and cytoplasmic localization we examined the localization of RNAs and proteins known to be required for the proper development of the oocyte (Fig. 4). In previous work, we showed that cytoplasmic dynein accumulates within the presumptive oocyte, beginning very early in the germarium and continuing through later stages (Li et al., 1994). This pattern of dynein accumulation is also observed in *sko* mutant egg chambers, suggesting that the early slow phase of cytoplasmic transport from the nurse cell compartment to the oocyte before stage 9 is not disrupted. By stages 9 to 10 in wild-type egg chambers, dynein becomes concentrated within the oocyte at the posterior pole. In *sko* egg chambers, this posterior concentration of dynein in the oocyte also appears qualitatively normal. Similar to dynein, we find that the posterior accumulation of *staufen* protein and *oskar* mRNA in the oocyte does not depend on *sko* function. Furthermore, despite the distorted margin at the anterior of the oocyte, we still observe the typical anterior concentrations of both *bicoid* and *gurken* mRNAs in *sko* egg chambers.

Identification of the Drosophila filamin Gene in the Region of the sko Mutation

Meiotic recombination experiments showed that *sko* is located on the right arm of the third chromosome in the genetic interval between the recessive markers *st* and *e.* Deletion mapping studies further defined the position of the *sko* mutation to the region between 89E7 and 90A.

During the characterization of the *sko* phenotype, we localized the *Drosophila* homologue of the human actin-binding protein α-filamin to the 89F polytene region using in situ hybridization with a fly *filamin* cDNA. This 3.2-kb cDNA clone, which encodes a portion of *Drosophila* filamin, was previously isolated from an ovary expression library using a polyclonal antiserum raised against partially purified fly actin-binding protein (Miller et al., 1989; Field and Alberts, 1995). The cDNA represents the 3′ end of the

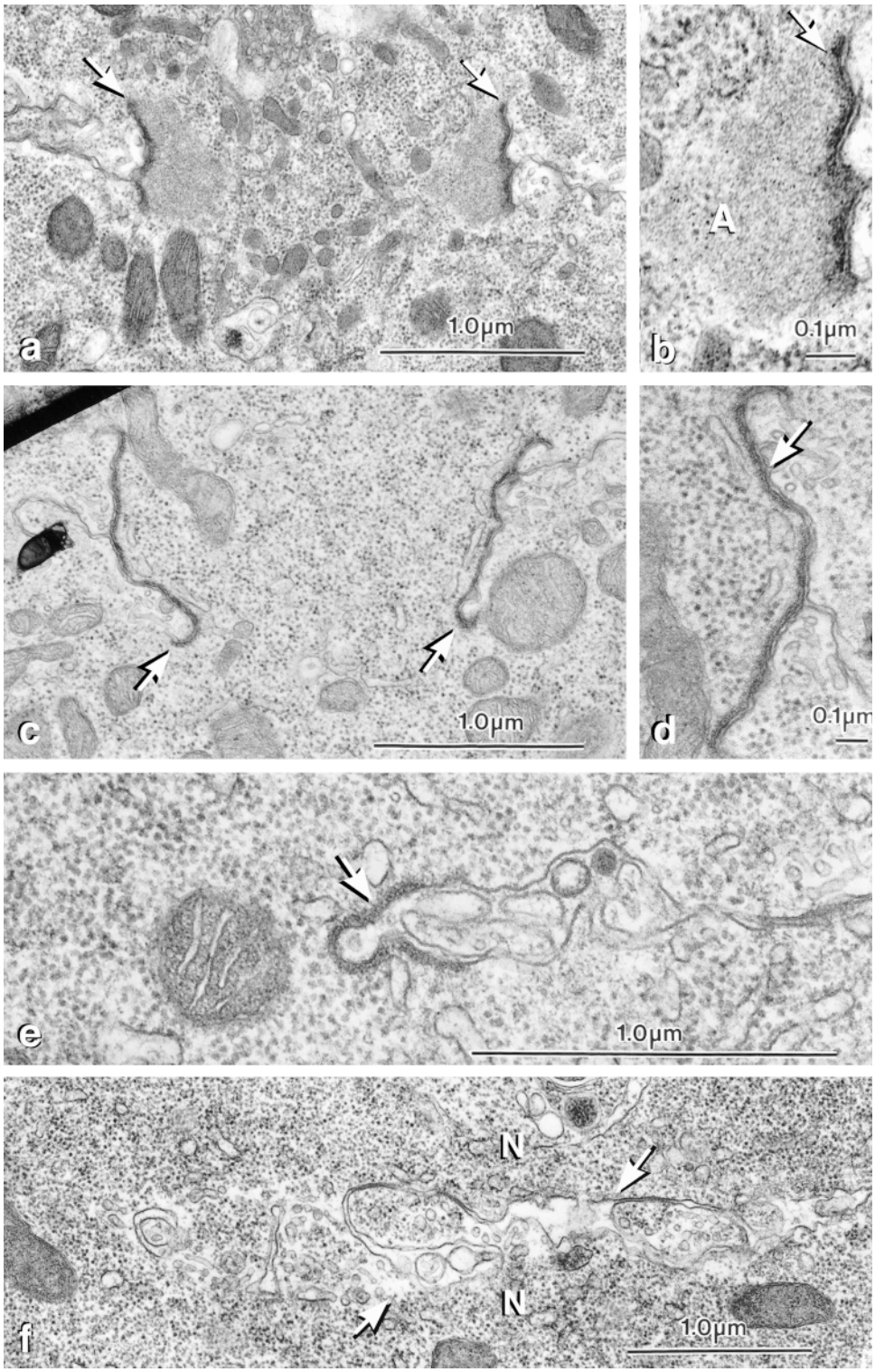

Figure 3. Panels a through d show thin sections through ring canals between nurse cells of stage 4 egg chambers. (a and b) Wild-type: Attached to the cytoplasmic side of the plasma membrane limiting the ring canal is some electron dense material (arrow). Inside this is a fuzzy layer of filamentous material that is composed of a bundle of circumferentially arranged actin filaments, A. See Tilney et al., 1996 for details. (b) Higher magnification of a portion of a. (c and d) *sko/sko* mutant. The plasma membrane of the ring canal is coated on its cytoplasmic surface with the same dense material (arrow) seen in wild-type, but no actin filaments are attached to it. (e and f) Thin sections through nurse cells of *sko/sko* mutant stage 5 egg chambers. (e) By this stage the ring canals are beginning to break down. Although the dense material remains attached to the plasma membrane, the shape of the canal margin has collapsed. Note, the other half of this ring canal was not located in the section and was presumably disrupted. (f) A thin section through the cytoplasm of two nurse cells (N) where the plasma membrane limiting the interface between the cells has vesiculated and is breaking down (arrows).

filamin transcript, including a 513-bp 3′ UTR with a polyadenylation tail. It predicts a polypeptide that is homologous (51% identity) to the COOH-terminal domains of human α-filamin. Genomic Southern blot analysis indicates that this *filamin* cDNA is derived from a single copy gene in *Drosophila* (Fig. 5 a). However, on RNA blots the probe detects two transcripts, 3 and 7.5 kb, which are differentially expressed (Fig. 5 b). The 7.5-kb transcript is expressed in ovaries and embryos, while the 3-kb transcript is expressed in embryos, but not ovaries.

We subsequently identified and sequenced an expressed sequence tag (EST) cDNA clone (data are available from GenBank/EMBL/DDBJ under accession number GH12209), derived from adult head mRNA, that potentially encodes the full-length *Drosophila* filamin protein. The 7,536-bp cDNA contains the original 3′ cDNA sequence and in-

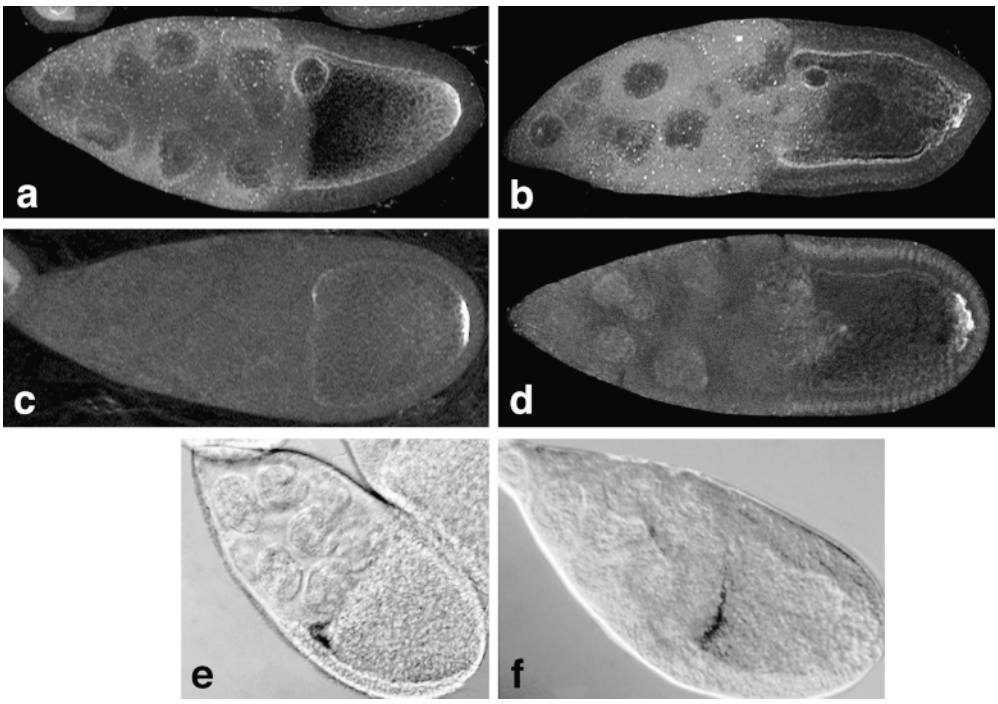

Figure 4. Some intercellular transport functions are still intact in *sko* mutant ovaries. As late as stage 10, cytoplasmic dynein (a and b) and staufen (c and d) proteins show proper localization at the oocyte posterior in both wild-type (left) and mutant (right). At a similar stage, *gurken* mRNA is correctly located at the anterior margin of the oocyte (e, wild-type; f, *sko*).

cludes a polyadenylation tail. An uninterrupted open reading frame begins from the first nucleotide of the cDNA; however, the first ATG codon is located 401 bp downstream. Although the flanking sequence of this ATG differs from the consensus sequence for translation initiation in *Drosophila* (Cavener, 1987), conceptual translation from this initial ATG codon predicts a 2343 residue polypeptide with a deduced mass (~250 kD) and NH_2-terminal sequence that are highly similar to human filamins (Fig. 6) (Takafuta et al., 1998). Amino acid sequence comparisons indicate that the *Drosophila* filamin is ~50% identical and ~60% similar to human and chicken filamins along the entire length of the protein. Although we have not unequivocally defined the 5′ end of the transcript, the data suggest that the fly EST cDNA clone represents a full-length copy of the identified 7.5-kb *filamin* transcript.

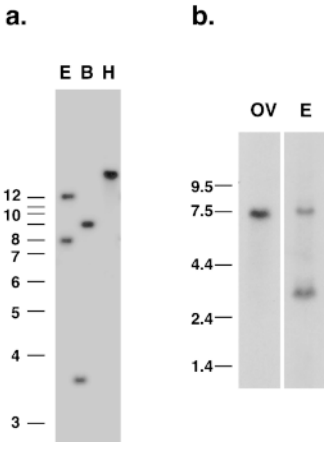

Figure 5. DNA and RNA blot analyses show that two transcripts are expressed by the identified single copy *Drosophila filamin* gene. (a) Genomic DNA was digested with restriction enzymes EcoRI (E), BamHI (B), or HindIII (H), and probed with a 0.7-kb cDNA fragment derived from the 3′ portion of the *filamin* cDNA (bp 4,271–4,972). Two bands detected in lanes E and B are due to restriction sites present in an intron in the corresponding genomic DNA. (b) Total RNA isolated from ovaries (OV) and 8–24-h-old embryos (E) was blotted and hybridized with the cDNA probe used in the Southern analysis. A transcript of ~7.5 kb is recognized in both tissues, and a second transcript of ~3 kb is present in embryos. Size in kb is indicated on the left side of each panel.

To characterize the genomic region of the *Drosophila filamin* gene, we used the filamin cDNA to isolate genomic clones from a cosmid library. A restriction map of the genomic region that contains the *filamin* gene is depicted in Fig. 7. Restriction fragments from across the region were subcloned, partially sequenced, and used in RNA blot experiments to define the *filamin* transcription unit. Both the 3- and 7.5-kb transcripts are detected with a genomic clone that contains the 3′ end of the gene (Fig. 7, probe a), while only the 7.5-kb transcript is recognized by the more 5′ genomic probes b and c. Probe c contains the 5′ end of the EST cDNA sequence. The genomic probe d fails to detect either the 7.5- or the 3-kb transcript, but does identify a neighboring transcript ~4 kb upstream. These results allow us to define the *Drosophila filamin* gene within a ~30-kb genomic region. However, our experiments have not determined the complete intron/exon organization of the gene. Since the 3-kb transcript is detected only by probes derived from the 3′ end of the *filamin* transcription unit, it may be expressed from an internal promotor, or as an alternatively spliced product that eliminates 5′ exons. Further sequence analysis of the 5′ end of this transcript will be required to distinguish between these possibilities. Significantly, the 3-kb transcript lacks the 5′ sequence that encodes the highly conserved actin-binding domain; it may encode a filamin isoform that provides a regulatory function.

Inspection of the 89F polytene region also identified the mutant *cheerio*, which exhibits defects in ring canal assembly similar to the *sko* mutation (Robinson et al., 1997). The analysis of mutant egg chambers from *cheerio* mu-

a.

```
                1                                                   50
      fly                                    MEA -ERDLAEDAQ WKKIQQNTFT
human-a    MSSSHSRAGQ SAAGAAPGGG VDTRDAE.P. T.K......P ..........
human-b                                   .PV T.K......P ..........
human-g                                   .PS T.K......P ..........
chicken              MS TQARGQGGAR AADPDGE.P. T.K......P ..R.......

               51                                                  100
      fly    RWANEHLKTI DRSINNLETD LSDGLRLIAL IEVLSQKRM- PKYNKRPTFR
human-a      ..C.....CV SKR.A..Q.. .......... L......K.H R.H.Q.....
human-b      ..C.....CV NKR.G..Q.. .......... L........Y R..HQ.....
human-g      ..C.....CV GKRLTD.QR. .......... L........Y R.FHP..N..
chicken      ..C....RCV NKR.G..QH. .......... L........Y R..HQ.....

               101                                                 150
      fly    SQKLENVSVA LKFLQDEGIK IVNIDSSDIV DCKLKLILGL IWTLILHYSI
human-a      QMQ....... .E..DR.S.. L.S...KA.. .GN....... ..........
human-b      QMQ....... .E..DR.S.. L.S...KA.. .GN....... V.........
human-g      QM........ .E..ER.H.. L.S...KA.. .GN....... ..........
chicken      QMQ....... .E.WSGRASS WLS...KA.. .GN....... ........L.

               151                                                 200
      fly    SMPMWDGEDD KQLNGSGHTP KQRLLNWIHA KIPDLPINNF TNDWTTGKAV
human-a      ......E.E. E--EAKKQ.. .....G..QN ...Q...T.. SR..QS.R.L
human-b      ...V.ED.G. D--DAKKQ.. .....G..QN ...Y...T.. NQN.QD...L
human-g      .....ED... E--DARKQ.. .....G..QN .V.Q...T.. NR..QD...L
chicken      ...V.ED.G. D--DAKKQ.. .....G..QN ...Y...T.. NQN.QD...L

               201                                                 250
      fly    GALVDACAPG LCPDWELWDP KDAVQNASEA MGLADDWLNV RQLIKPEELV
human-a      .....S.... .....DS..A SKP.T..R.. .QQ.....GI P.V.T.....
human-b      .....S.... ......S... QKP.D..R.. .QQ.....G. P.V.T....I
human-g      .....N.... ......A... NQP.E.SR.. .QQ.....G. P.V.A...I.
chicken      .....S.... ......T... SKP.D..R.. .QQ.....G. P..LP...II

               251
      fly    NPNVDEQSMM TYLSQYPNSK LKTGAPLRP
human-a      D.....H.V. .....F.KA. ..P......
human-b      H.D...H.V. .....F.KA. ..P....K.
human-g      D.....H.V. .....F.KA. ..P...V.S
chicken      H.D...H.V. ...YTF.KA. ..P....K.
```

b.

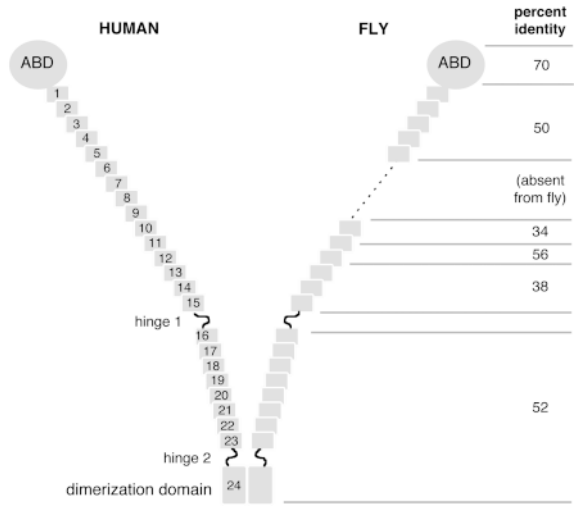

Figure 6. (a) Amino acid alignment of the actin binding domains in *Drosophila* and vertebrate filamins. The *Drosophila* filamin sequence is on top ("fly," accession number GH12209). In human, three filamin isoforms, ABP280 (or α-filamin), β-filamin, and γ-filamin, have been reported and are encoded by separate genes located at different chromosome positions (Takafuta et al., 1998). Sequence alignment shows that the deduced NH_2 terminus of fly filamin matches very well with the human β- and γ-filamins. The start codon of the third human isoform has not been defined, but if the human ABP280 polypeptide initiates at the second ATG, the resultant NH_2 terminus matches those of the other human and fly filamins. Human-a represents human ABP280 (α-filamin) encoded by the gene on the X chromosome (accession number 113001); human-b indicates β-filamin on the third chromosome (accession number 328597); human-g represents γ-filamin derived from chromosome 7 (accession number 4218955) and chicken, chicken filamin (accession number 1079404). Residues identical to the fly sequence are denoted by dots. (b) Schematic of a filamin dimer, comparing the predicted human and *Drosophila* monomers. The drawing is based on previous biochemical and electron microscopy characterizations of human ABP280 structure (Gorlin et al., 1990). Filamin is a homodimer with an elongated subunit structure (160 nm). The self-association of partner subunits occurs at the COOH-terminal end of the molecule within repeat 24, while the actin binding domain (ABD) lies at the opposite free end of each monomeric subunit. The 24 repeats extend from the ABD to the COOH terminus and are interrupted by two hinge regions (hinge 1 and 2). Sequence alignment predicts that repeats 6–9 are missing from the *Drosophila* filamin. The percentage of amino acid conservation between the fly and human sequences is shown at the right.

tants showed that the *cheerio* gene product is required for the incorporation of inner ring canal components and the growth of the ring canal, but the product of the *cheerio* gene is not known. To test whether *cheerio* and *sko* are mutations within the same gene, we conducted a complementation test between *cher*^{1} and *sko*. We report that the two mutations fail to complement one another; *sko/cher*^{1} females are sterile and exhibit defects in ring canal assembly and egg chamber morphology.

Filamin Polypeptides Are Not Detected in sko Mutants

To investigate whether the *sko* mutation represents an allele of *Drosophila filamin*, we looked at the expression of filamin protein(s) in wild-type and *sko* mutant flies. A polyclonal antiserum, 4-3D, was raised against a bacterial fusion protein expressing the COOH-terminal 90 residues of filamin. Extracts from whole flies and tissues of *sko* and *cher*^{1} mutants, as well as from wild-type flies, were analyzed by immunoblotting. As shown in Fig. 8 a, in wild-type ovary extracts the antibody predominantly reacts with a large ~250-kD polypeptide. The size of this polypeptide correlates well with the single 7.5-kb *filamin* transcript expressed in ovary RNA, and is comparable in size to human α-filamin. Significantly, the 250-kD filamin polypeptide is not detected in ovaries derived from sterile *sko*/*sko* females, nor in homozygous *cher*^{1} ovaries. A less abundant polypeptide of ~140 kD is detected by the 4-3D antiserum in wild-type ovary extracts, and is not eliminated in the *sko* or *cheerio* mutant backgrounds. These results demonstrate that the *sko* and *cher*^{1} mutations disrupt the expression of a 250-kD filamin product in ovaries. The loss of the filamin polypeptide coincides with the disruption of actin organization and female sterility.

In embryo and adult extracts, the major polypeptide detected by the 4-3D filamin antiserum migrates at ~97 kD. A product of this mobility could be encoded by the smaller 3-kb filamin transcript, and like the transcript, the 97-kD polypeptide is found in embryos and adults, but not ovaries. The 250-kD polypeptide is also present in embryos and adults, but at relatively lower levels compared with ovary extracts. Both the 250- and 97-kD polypeptides are absent from immunoblots of adult extracts prepared from the *sko/sko* mutant background. By comparison, in *cher*^{1}/*cher*^{1} adult extracts the 250-kD polypeptide is missing, but the 97-kD polypeptide is retained. These results provide further evidence that the *sko* mutation identifies the *filamin* gene. Our data do not eliminate the possibility that the *sko* mutation encodes a truncated polypeptide that is not detected by the filamin antibody. The ~140-kD protein detected in ovaries is also detected in adult extracts, but again is not eliminated by the *sko* or *cher*^{1} mutations.

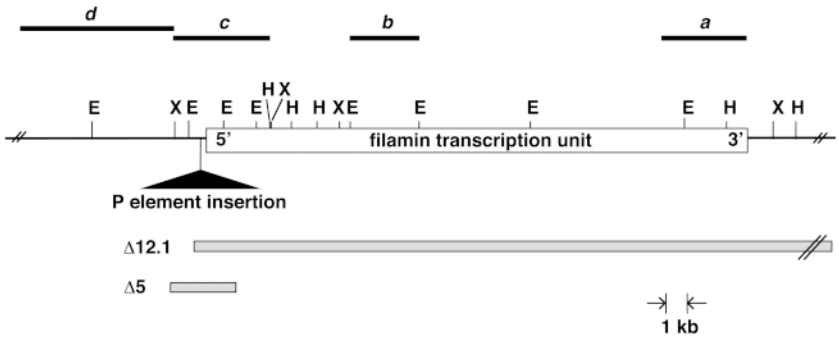

Figure 7. Restriction map of the genomic region of the *Drosophila filamin* gene. Dark lines a–d represent the positions of genomic DNA fragments used as probes described in the text. The *filamin* transcription unit spanning ~30 kb is indicated by an open box. The triangle points to the position of the P-element insertion in the fly stock *EP(3)3715*. Open boxes indicate regions deleted in two deficiencies (*Δ12.1*, *Δ5*) generated by imprecise excisions of the P-element. The 3′ breakpoint of the deficiency *Δ12.1* extends an undetermined distance beyond the *filamin* transcription unit.

Currently, we can not eliminate the possibility that the 140-kD species is a bona fide product of the *filamin* gene. Alternatively, it may represent a polypeptide that cross-reacts with the filamin antiserum. Regardless, our results suggest that the *sko* and *cher*[1] mutations alter the expression of the 250- and 97-kD filamin polypeptides.

The *sko* phenotype predicts that the 250-kD filamin actin-binding protein might function in ovarian ring canal growth. As shown in Fig. 8 b, the 4-3D filamin antiserum decorates the ring canals in wild-type germline cysts. Filamin is concentrated on ring canals at early stages (regions 2–3 in germarium) and persists through late stages (stage 10b). In the *sko* mutant egg chambers, filamin is not detected in ring canal structures. This result is consistent with a model in which filamin acts to tether actin microfilaments within the ring canal to the cell membrane. Moreover, the loss of filamin staining and actin organization in ring canals of *sko* egg chambers supports the interpretation that *sko* encodes filamin.

sko Is a Mutation in the filamin Gene

To confirm that the *sko* phenotype is caused by a mutation in the *filamin* gene, we have generated deletions in the gene by imprecise excision of a P-element inserted 5′ to the *filamin* gene. The P-element insertion line *EP(3)3715* was identified from collections in the *Drosophila* Genome Project. Plasmid rescue and sequence analysis of the genomic sequence flanking the insertion showed that the P-element is inserted 78 bp 5′ to the first nucleotide of the EST cDNA sequence (Fig. 7). We determined that the P-insertion itself does not disrupt *sko* function. However, we took advantage of its position to generate deletions in the *filamin* gene, by mobilizing the P-element insert in the presence of *Δ2-3* P transposase. P-excision lines (*Δ*) were collected and scored for fertility in transheterozygous combination with the *sko* mutation (*sko/Δ*). Two excision lines, *EPSΔ12.1* and *EPSΔ5*, fail to complement the *sko* mutation, resulting in female sterility. Genomic DNA blot analysis confirmed that both lines contain deletions in the *filamin* gene. The deletion line *EPSΔ12.1* removes the entire *filamin* transcription unit. The *EPSΔ5* excision line contains a small deletion at the 5′ end of the transcription unit. The positions of the deletions generated by the P-element excisions are shown in Fig. 7.

As a further verification that the disruption of *filamin* function accounts for the *sko* female sterile phenotype, we examined egg chambers in ovaries derived from *sko/EPSΔ5* and *sko/EPSΔ12.1* heterozygotes. The defects observed are similar to those found in *sko/sko* ovaries. Most characteristically, actin organization in egg chambers is disrupted and ring canal growth is reduced. In late stage egg chambers from *sko/EPSΔ5* and *sko/EPSΔ12.1* females, we see few intact ring canals. Our results show that

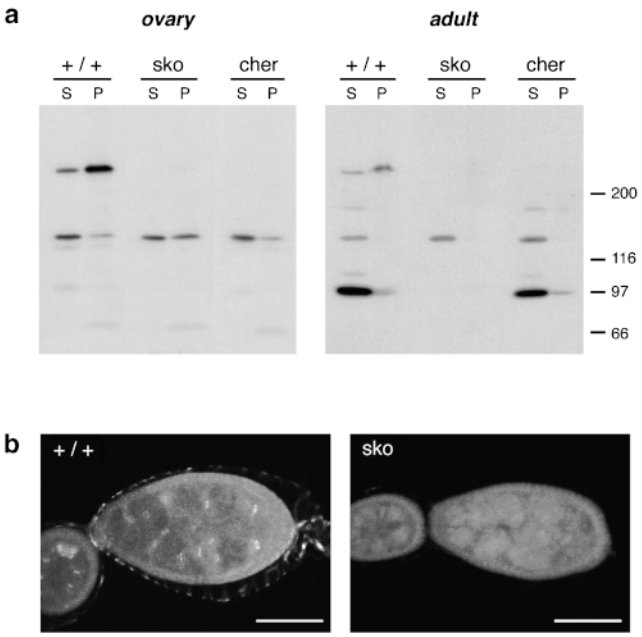

Figure 8. Expression of filamin protein in ovaries and adults. (a) Western blots of protein extracts from wild-type and mutant ovaries and whole flies, probed with anti-filamin antibody 4-3D. In ovaries (left), the ~250 kD protein seen in wild-type (+/+) extracts is not detected in the *sko* or *cheerio*[1] mutants. Extracts of whole male adults (right panel) show a 97-kD protein in addition to the 250-kD species in wild-type flies. Neither polypeptide is detected in *sko* mutant extracts; in *cheerio*[1] mutants, the 97-kD species is detected but the 250-kD species is not. Identical results were obtained using whole female adults (not shown). S, supernatant; P, pellet; equal total protein is loaded in each lane. (b) Localization of filamin in ring canals. Egg chambers from wild-type and *sko* animals were probed with antibody 4-3D and visualized by confocal microscopy. In the stage 3 egg chambers shown, filamin is seen in ring canal structures in wild-type (left panel) but not in the *sko* mutant (right panel). Bars, 50 μm.

deletions that disrupt the *filamin* transcription unit are allelic to the *sko* mutation. We conclude that female sterility and disruption of actin organization in the *sko* mutant egg chambers results from the loss of *filamin* function.

Discussion

Our results provide a characterization of the structure and function of *Drosophila* filamin. We present four lines of evidence that together demonstrate that the female sterile *sko* mutation represents a lesion within the *filamin* gene. First, in situ hybridization of a *filamin* cDNA to polytene chromosomes positions the *filamin* gene in a genomic region that was shown by deletion mapping experiments to contain the *sko* mutation. Second, antibodies that were raised against the COOH-terminal domain of the filamin protein detect two major filamin polypeptides. Both these polypeptides are lost in the *sko* mutants. The 250-kD filamin polypeptide is also eliminated by the $cher^{1}$ mutation, which is allelic to *sko*. Moreover, the antibody detects filamin within ring canal structures of wild-type, but not *sko* mutant egg chambers. Third, deletions (Δ) in the *filamin* gene were generated by P-element excision. These mutations fail to complement the *sko* mutation and exhibit female sterility, similar to females homozygous for the *sko* mutation. Finally, the disruption of actin organization is similar in egg chambers derived from either *sko/sko* or *sko/*Δ females. Thus P-element excisions known to disrupt the *filamin* gene are allelic to the *sko* mutation and, as would be expected, produce a similar phenotype. Given these results, we refer to the *sko* mutation as the $filamin^{sko}$ allele. Previous structural and functional information on the properties of filamins, together with the comparative cytology of mutant and wild-type egg chambers, suggest several functions for *Drosophila* filamin in oogenesis.

Filamin Structure and Function

Filamins are a family of actin cross-linking proteins that have been identified in multiple tissues and cell types from several organisms (Hartwig, 1994). Our analysis of the *Drosophila filamin* sequence predicts a polypeptide that shares the main structural elements present in other filamins, as defined by the extensive characterization of human α-filamin (Fig. 6). Overall there is ~50% amino acid identity with the predicted human (α, β, γ; Takafuta et al., 1998), and chicken filamin sequences. The characteristic signatures of an actin-binding domain are present within an NH_2-terminal region that is ~70% identical to the corresponding region in the human α-filamin sequence (Gorlin et al., 1990). The human sequence predicts an elongated central rod domain comprising 24 repeats of ~96 amino acids each, interrupted in two locations by insertions of 20–40 residues that are thought to provide flexible hinge regions within the rod-like backbone. A series of repeats also constitute the central domain of the fly polypeptide, and sequence alignments indicate that the relative positions of both hinge regions are also conserved. However, a striking distinction is the deletion of repeats 6–9 in the rod domain of the fly filamin. The remaining repeats show 34–52% identity with the corresponding human domains.

In addition to promoting dimerization between filamin monomers, the COOH terminus of human filamin interacts with a number of membrane proteins, including presenilin (Zhang et al., 1998), β1 and β2 integrin (Sharma et al., 1995; Glogauer et al., 1998; Loo et al., 1998; Pfaff et al., 1998), and tissue factor (Ott et al., 1998). These interactions are thought to regulate the linkage between the actin cytoskeleton and the plasma membrane. Evidence for conservation of these functions in the COOH-terminal domain of *Drosophila* filamin was recently provided by a two-hybrid screen for proteins that interact with the toll receptor (Edwards et al., 1997). The toll transmembrane receptor binds the extracellular ligand, Spatzle, to trigger a signaling pathway that includes the downstream cytoplasmic effectors tube, pelle, cactus, and dorsal. Clones encoding the COOH terminus of filamin, plus a variable number of repeats, were identified by their interaction with toll. The COOH-terminal filamin fragment was judged to be competent to dimerize, since coexpression of both the LexA DNA binding and activation domains as separate fusions to *filamin* resulted in elevated reporter expression. How the actin cytoskeleton functions in the toll pathway, and how the dimerization of filamin and its interaction with toll are regulated, will be important to investigate further.

How Might Filamin Function in Ring Canals?

Our results identify filamin as a new component of ring canals. Antibodies raised against a filamin fusion protein detect filamin within the ring canal and mutations in the *filamin* gene disrupt ring canal structure and function. Filamin can provide several functions missing from the previously known complement of ring canal proteins. Most significantly, filamin can cross-link antiparallel actin filaments (Gorlin et al., 1990; Hock et al., 1990), as well as link such cross-linked filaments to the plasma membrane (Fox, 1985; Okita et al., 1985; Cantiello et al., 1991; Glogauer et al., 1998). A simple model for filamin function is that the COOH-terminal tail of filamin anchors the dimer to the plasma membrane in the outer rim of the ring canal. The distal, NH_2-terminal actin-binding sites would act to recruit and/or stabilize actin filaments in the ring canals. This model is consistent with the early arrival of filamin at the ring canal, and with the loss of filamin and actin from the ring canal in the $filamin^{sko}$ mutant background. We have shown that in the presence of the *sko* mutation, the 4-3D antiserum does not detect filamin in situ in ring canals or on immunoblots of ovary extracts. Since this filamin antiserum is directed against the COOH terminus, which participates in membrane association, our results suggest that if a filamin product is encoded by the *sko* mutant, it lacks the COOH terminus and therefore cannot attach to the membrane or recruit actin filaments to the ring canals.

Filamin is required for the maturation of the ring canal and the normal assembly of the inner rim components. Cytokinesis, stabilization of arrested cleavage furrows, and the initial construction of ring canals proceed normally in the *sko* mutant as judged by proper cell numbers in *sko* egg chambers and the early staining pattern of anillin and at least one phosphotyrosine protein. These outer rim

components are thought to fortify the initial intercellular bridges and are present before the assembly of the inner rim components including hts, actin filaments, and kelch protein (Robinson et al., 1994; Field and Alberts, 1995). Only partial staining of the hts protein and actin filaments were obtained in *sko* mutants even at early stages. In addition, no accumulation of kelch protein in ring canal structures was detected. Despite the disruption of inner rim assembly, anillin organization in the outer rim of ring canals was apparent up until stages 5–6. However, the rings were reduced in size and later disintegrated or collapsed. This disruption may result from the inability of the ring canal to fully expand.

How does filamin act to promote ring canal expansion? Before expansion, overlapping actin filaments of opposite polarity extend circumferentially around the ring perimeter with no apparent seam. The sliding of actin filaments of opposite polarities is proposed to account for expansion of the ring canal (Tilney et al., 1996). One explanation for the defect in ring canal growth associated with *filamin* mutations is that actin filaments fail to be recruited to the ring canal or are present in insufficient numbers to support continued sliding during expansion. This interpretation is consistent with partial growth at early stages and the disruption or collapse of ring canals at later stages. During ring canal expansion, the homogeneous monolayer of parallel actin filaments is transformed into a branching network of overlapping actin filament bundles (Tilney et al., 1996). The known property of filamins to cross-link actin filaments at high angles may also serve to establish the spacing between this network of actin bundles and act to stabilize the growth of the ring canal.

Mutations in three other genes, *Tec29*, *Src64*, and *cheerio*, affect the growth of ring canals. The tyrosine kinases Tec29 and Src64 have recently been shown to be essential for ring canal growth during oogenesis (Dodson et al., 1998; Roulier et al., 1998). Mutations in either of these genes result in defective transfer of cytoplasm from the nurse cells to the oocytes and eliminate the staining of ring canals by phosphotyrosine antibodies. Furthermore, antibodies to Tec29 show that the encoded kinase is localized to the ring canal, suggesting that Tec29 protein could provide at least one of the major phosphotyrosine antigens present in ring canals. Src64 protein does not itself accumulate in ring canals, but is required for the accumulation of Tec29. Although expansion of the ring canal is blocked in the Tec29 mutant, the incorporation of actin, hts, and kelch appears normal and suggests a parallel pathway that regulates ring expansion (Roulier et al., 1998). Filamin is a phosphoprotein and its stability (Zhang et al., 1988; Chen and Stracher, 1989) and association with actin may be mediated by its state of phosphorylation (Zhuang et al., 1984; Ohta and Hartwig, 1995). In this regard, it will be of interest to test whether filamin is a substrate of the *Tec29* kinase. A third gene, *cheerio*, was previously identified by a female sterile mutation that exhibits defects in the assembly and the expansion of ring canals (Robinson et al., 1997). The phenotypes of mutations in *cheerio* are similar to the *sko* mutation and our complementation analysis has shown the both are alleles of the *filamin* gene. Based on preliminary examinations, it has been reported that the *cher*^{1} mutation does not eliminate the localization of Tec29 (Roulier et al., 1998). This result is consistent with the proposed model that *Tec29* and *Src64* act in an independent pathway that regulates the growth, but not the assembly of ring canal components (Cooley, 1998; Roulier et al., 1998).

Filamin Function in Cell Adhesion and Membrane Integrity

Defects in the morphology of cells within the nurse cell compartment, as well as the disruption of nurse cell membrane integrity, suggest that filamin function may not be specific to ring canals. Most notably, the protrusion of whole nurse cells or nurse cell nuclei into the oocyte compartment suggests that filamin acts in sites of intercellular adhesion where the cytoskeletons of adjacent cells are linked together. Electron microscopy substantiates the compromised integrity of membranes and indicates that this defect precedes the later disruption of the ring canal structures. While ring canal structures can be observed at stages when breaks in nurse cell membranes are already apparent, the inner rim of such ring canals is often devoid of actin filaments. Our observations suggest that filamin is required for cross-linking actin filaments to the membrane throughout the cortical cytoplasm of nurse cells, as well as in ring canal structures. In this regard, the role of cortical actin filaments in cell adhesion and the maintenance of cell shape and membrane integrity in the *Drosophila* egg chamber has been previously demonstrated (Peifer et al., 1993; Edwards and Kiehart, 1996; Murphy and Montell, 1996). For example, mutations in *armadillo*, a fly homologue of β-catenin, disrupt all three types of actin filaments and exhibit disruptions in cell arrangements and membrane integrity within egg chambers (Peifer et al., 1993). The rapid phase of cytoplasmic transport that results in a rapid increase in oocyte size in stages 10–11 depends on the contraction of the cortical actin network and presumably its linkage to the nurse cell membrane (Gutzeit, 1990; Edwards and Kiehart, 1996). The disruption of the membrane linkage and/or organization of the cortical actin network may also contribute to the arrested growth of oocytes in *sko* egg chambers.

Our interpretation that filamin function is required outside the ring canal is consistent with the functions described for filamins in other cell types. Filamin is known to increase the rigidity of actin networks in vitro, and in vivo filamin increases the elastic modulus of the cell cortex (Brotschi et al., 1978; Hartwig and Kwiatkowski, 1991; Cunningham et al., 1992). Moreover, filamin cross-linking of cortical actin to integrin receptors can mediate the protective responses of cells subjected to mechanical stress (Glogauer et al., 1998). Cells deficient for *filamin* show an increase in permeability of the calcium channel and elevated cell death (Glogauer et al., 1998). Interestingly, mutations in the *Drosophila dcp-1 caspase*, a critical component of the apoptotic pathway, disrupt actin organization, block cytoplasmic transfer, and partially inhibit nurse cell death (McCall and Steller, 1998). Perhaps the regulation of filamin cross-links mediates changes in the mechanical strength of the nurse cell membranes during contraction of the cortical actin network and the subsequent onset of nurse cell death.

In addition to its role in mechanoprotection, the re-

quirement for filamin function in cell movement is well established. Tumor cell lines derived from human malignant melanomas that lack filamin (ABP280) show impaired locomotion (Cunningham et al., 1992; Cunningham, 1995). These cells exhibit extensive blebbing of the cell membrane, but fail to spread and extend lamellae. Moreover, there is evidence that filamin participates in the formation of filopodia in migrating cells as a downstream target of Cdc42-activated RalA. Activated RalA may act to elevate the local concentration of filamin and direct the formation of actin bundles in filopodial extensions (Ohta et al., 1999). The developmental significance for filamin function in cell movement was recently revealed in a study that shows loss-of-function mutations in human *filamin* prevent migration of cerebral cortical neurons (Fox et al., 1998). These mutations give rise to the human disorder periventricular heterotopia (PH). The X-linked PH, or *filamin*, mutations lead to epilepsy and vascular disorders in females and embryonic lethality in males. While the *filamin*^{sko} mutation is not recessive lethal, our studies do not rule out additional functions for filamin during embryogenesis or in adult animals. Indeed, we do find that filamin transcripts and protein products are expressed in embryos and adults. It will be important in future work to establish the functional significance of this expression.

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