Drosophila Kelch regulates actin organization via Src64-dependent tyrosine phosphorylation

Reed J. Kelso,1 Andrew M. Hudson,2 and Lynn Cooley1,2

1Department of Cell Biology and 2Department of Genetics, Yale University School of Medicine, New Haven, CT 06520

The Drosophila kelch gene encodes a member of a protein superfamily defined by the presence of kelch repeats. In Drosophila, Kelch is required to maintain actin organization in ovarian ring canals. We set out to study the actin cross-linking activity of Kelch and how Kelch function is regulated. Biochemical studies using purified, recombinant Kelch protein showed that full-length Kelch bundles actin filaments, and kelch repeat 5 contains the actin binding site. Two-dimensional electrophoresis demonstrated that Kelch is tyrosine phosphorylated in a src64-dependent pathway. Site-directed mutagenesis determined that tyrosine residue 627 is phosphorylated. A Kelch mutant with tyrosine 627 changed to alanine (KelY627A) rescued the actin disorganization phenotype of kelch mutant ring canals, but failed to produce wild-type ring canals. Electron microscopy demonstrated that phosphorylation of Kelch is critical for the proper morphogenesis of actin during ring canal growth, and presence of the non-phosphorylatable KelY627A protein phenocopied src64 ring canals. KelY627A protein in ring canals also dramatically reduced the rate of actin monomer exchange. The phenotypes caused by src64 mutants and KelY627A expression suggest that a major function of Src64 signaling in the ring canal is the negative regulation of actin cross-linking by Kelch.

Introduction

Although much is known about the key elements in actin filament formation and regulation in vitro (Pollard et al., 2000), our understanding of temporal and spatial regulation of actin dynamics in vivo is far from complete. Observations of actin dynamics at the leading edges of migrating cells have begun to describe the in vivo kinetics of actin assembly during cell migration (for review see Condeelis, 2001; Pantaloni et al., 2001). Dicyostelium and the budding yeast Saccharomyces cerevisiae have both proved to be valuable in the in vivo characterization of components involved in regulation of the actin cytoskeleton (for review see Noegel and Schleicher, 2000; Pryunte and Bretscher, 2000). We are able to combine microscopic studies of large actin structures with the expansive palate of genetic tools available in Drosophila to examine the regulation of F-actin in the ovarian ring canal.

In Drosophila melanogaster, 15 syncytial nurse cells and 1 oocyte are enveloped by a monolayer of somatic follicle cells and constitutes an egg chamber, the structural and functional unit of the Drosophila ovary (for review see Spradling, 1993). A ring canal is a gateway through which mRNAs, proteins, and nutrients flow from nurse cells into the oocyte during the entire course of oogenesis. Ring canals are derived from arrested mitotic cleavage furrows that are modified by the addition of several proteins. These include abundant F-actin (Koch and King, 1969), at least one protein that is recognized by antiphosphotyrosine antibodies (PY protein), a mucin-like glycoprotein (Kramerova and Kramerov, 1999), the Hts ring canal protein (HtsRC)* (Yue and Spradling, 1992; Robinson et al., 1994), ABP280/filamin (Li et al., 1999; Sokol and Cooley, 1999), Tec29 and Src64 tyrosine kinases (Dodson et al., 1998; Roulier et al., 1998), and Kelch (Xue and Cooley, 1993; Robinson and Cooley, 1997a).

As nurse cell cytoplasm transport proceeds, the diameter of ring canals grows from <1 µm to 10–12 µm. This represents the addition of over one inch of filamentous actin during a period in which the filament density remains constant (Tilney et al., 1996). Near the end of oogenesis, the ring canal actin transforms from a single continuous bundle into several interwoven actin cables (Tilney et al., 1996). Ring canal expansion probably involves the nucleation of

*Abbreviations used in this paper: 2D, two-dimensional; BTB, broad complex, tramtrack, and bric-à-brac; FRAP, fluorescence recovery after photobleaching; GFP, green fluorescent protein; HtsRC, Hts ring canal protein; KREP, kelch repeat; POZ, poxvirus and zinc finger; SFK, Src family kinase.
new actin filaments and an increase in actin filament length, coupled with filament reorganization that requires the establishment of reversible actin cross-links.

Previous work has shown that the Kelch protein is required for ring canal morphogenesis (Xue and Cooley, 1993; Tilney et al., 1996; Robinson and Cooley, 1997a). Ring canal actin in kelch mutant egg chambers is severely disorganized and partially occludes the lumen. This leads to a defect in cytoplasm transport and the production of small, sterile eggs (Xue and Cooley, 1993). Kelch is a multidomain protein (Fig. 1 A) and a member of a superfamily of proteins defined, in part, by the presence of six 50-amino acid kelch repeats (KREPs). Based on sequence similarity to galactose oxidase, the KREP domain is predicted to fold into a six-bladed β-propeller (Bork and Doolittle, 1994; Adams et al., 2000). In Limulus the KREP domain is present in at least three scrin proteins, each of which contains two KREPs (Way et al., 1995). The KREP domains of α-scrin each form an F-actin binding domain that allows α-scrin to act as an actin filament–cross-linking protein (Tilney, 1975; Bullitt et al., 1988; Sanders et al., 1996; Sun et al., 1997). Another KREP protein, Mayven, is found in human brain extracts and tightly colocalizes with F-actin in cultured human U373-MG astrocytoma/glioblastoma cells (Soltysik-Espanola et al., 1999). The second conserved domain in Kelch is the BTB/POZ ( broad complex, tramtrack, and bric-à-brac; also known as the poxvirus and zinc finger domain) dimerization domain (Ahmad et al., 1998). The molecular makeup of the Kelch protein and the morphology of the kelch mutant ring canals suggest that Kelch could organize actin filaments by acting as a dimeric cross-linking protein (Robinson and Cooley, 1997a).
cross-links in ring canals. The nonphosphorylatable mutant also caused a reduction in actin monomer turnover kinetics. This suggests that reversible cross-links are required to allow dynamic actin monomer turnover and maintain overall ring canal morphology. These observations suggest that a major cytoskeletal target of Src64 signaling at the ring canal is the actin–cross-linking protein Kelch.

Results

Kelch is phosphorylated and interacts with actin in a Src64-dependent manner

Based on motif searches (Hofmann et al., 1999), we determined that there were two predicted tyrosine phosphorylation sites in Kelch. The first was in the BTB dimerization domain at residue 132 (Fig. 1A), and the second was within the fifth KREP at residue 627 (Fig. 1A and B, shaded). The fifth KREP in α-scruin has been shown to bind F-actin (Sun et al., 1997). We performed site-directed mutagenesis to change the tyrosines to alanines, which are incapable of being phosphorylated. Transgenes encoding these constructs were designated P[kelY132A] and P[kelY627A]. At least three transgenic lines for each mutant were examined in a kelch mutant background (see Materials and methods for a detailed description of the genotypes tested).

To examine Kelch phosphorylation in the Drosophila ovary, we performed 2D electrophoresis of ovary lysates from several genetic backgrounds. When wild-type ovary lysates were treated with phosphatase inhibitors, a tyrosine-phosphorylated protein that comigrated with one of two Kelch isoforms was detected (Fig. 2, A, C, and H). Antibodies to phosphoserine and phosphothreonine showed no immunoreactivity comigrating with Kelch (unpublished data). Comparison to pH standards showed that the shift observed between the two Kelch isoforms was equivalent to the addition of a single phosphate. In the absence of phosphatase inhibitors, Kelch migrated as a single spot with no corresponding phosphotyrosine staining (Fig. 2B and D). Egg chambers dissected from src64/H9004 flies did not contain the phosphorylated form of Kelch (Fig. 2E). In the presence of phosphatase inhibitors, Kelch protein from kelDE1;P[kelY132A]/H11001 ovaries continued to be tyrosine phosphorylated (Fig. 2F). However, Kelch tyrosine phosphorylation in kelDE1;P[kelY627A]/+ ovaries was absent (Fig. 2G). To characterize the effects of phosphorylation on the ability of Kelch to bind actin, we performed an actin overlay experiment. Total ovary lysates from wild-type ovaries were separated using 2D electrophoresis, and the blots were incubated with F-actin and then actin antibodies to detect bound actin. Only nonphosphorylated Kelch (KelY627A) bound actin (Fig. 2I). To verify that actin binding was due to Kelch, we tested ovary lysates from a kelch mutant and determined that there was no longer actin binding present in the area where Kelch protein would have focused (unpublished data).
Kelch binds and cross-links F-actin via the fifth KREP

We tested the ability of purified recombinant Kelch protein to interact with F-actin. In preliminary experiments, the majority of Kelch sedimented in the presence of F-actin and remained in the supernatant in its absence (Fig. 3 A and unpublished data). Saturation binding was determined by incubating purified Kelch with phalloidin-stabilized F-actin in increasing ratios, followed by 100,000 g centrifugation (Fig. 3 A). As the Kelch/F-actin ratio increased, more Kelch remained in the supernatant. The stoichiometry of Kelch binding to actin was estimated using a saturation curve in which the ratio of Kelch to actin in the pellet was plotted against increasing concentrations of Kelch (unpublished data). From this curve, the molar ratio of Kelch to F-actin was calculated as 1:4.

To determine if Kelch could cross-link F-actin, low speed sedimentation at 16,000 g was performed. When a saturating concentration of Kelch was added, most of the F-actin pellet (unpublished data), suggesting that the actin was being cross-linked into bundles. Negative staining of F-actin, sedimented with or without Kelch, was performed to further determine the nature of the interaction. The actin-alone sample contained mainly single filaments (Fig. 3 B). In contrast, pellets from mixtures of Kelch and actin contained mostly loose bundles of actin filaments (Fig. 3 C). These observations suggested that full-length Kelch acted to bundle ring canal F-actin.

We mapped the F-actin binding domain to a single KREP by expressing and purifying each individual repeat and then performing high speed centrifugation in the presence of F-actin. KREP 1–4 and 6 failed to cosediment with F-actin (Fig. 3 D, repeat 4, lanes 1 and 2). KREP five was capable of binding F-actin in a saturable manner (Fig. 3 D, lanes 3–12). To address the effect of phosphorylation, we introduced aspartate or glutamate residues at position 627 to mimic phosphorylation (Jordan and Karess, 1997; Waites et al., 2001). Both substitutions disrupted the ability of KREP five to bind F-actin, resulting in >50% of each mutant repeat remaining in the supernatant after high speed centrifugation (Fig. 3 E, lanes 7 and 11). To demonstrate that the substitution made in the KelY627A protein would not affect actin binding, we tested the substitution in vitro. Neither alanine nor phenylalanine substitutions disrupted actin binding (Fig. 3 E, lanes 13–20). These data suggest that F-actin binding by KREP five is likely to be reduced by phosphorylation of the tyrosine residue at position 627.

Characterization of KelY627A ring canal morphology

To study the role of Kelch phosphorylation in vivo, we examined kelch mutant flies expressing KelY627A protein in the germline. Wild-type stage 10A egg chambers stained with the Kel1B monoclonal antibody had a ring canal staining pattern that colocalized with F-actin (Fig. 4 A and A’). Egg chambers from kelch mutants showed a complete absence of Kelch protein staining (Fig. 4 B), and the well-characterized phenotype of ring canal actin disorganization with partial occlusion of the lumen (Tilney et al., 1996; Robinson and Cooley, 1997a) (Fig. 4 B’). The expression of one copy of P[kelY627A] in kelch mutants resulted in restoration of
Kelch localization to ring canals and a rescue of F-actin organization (Fig. 4, C and C'), showing that the KelY627A protein had F-actin binding and cross-linking activity in vivo. The phenotype of KelY627A was not changed in a src64<sup>A17</sup> background (Fig. 4, D and D'). At a higher magnification, horizontal sections of wild-type ring canals were characterized by the appearance of two parallel rims of actin (Fig. 4 E). In kelch mutants, there was the typical collapse of actin into the lumen, almost completely obstructing the ring canal (Fig. 4 F). Comparison of wild type, kel<sup>DE1</sup>; P[kelY627A]/+, and src64<sup>A17</sup> revealed an increase in the concavity of the actin rim in both mutants, causing the appearance of a bicycle rim shape as well as a decrease in the diameter of the ring canal (Fig. 4, compare E, G, and H). These observations suggested that the phenotype in kel<sup>DE1</sup>; P[kelY627A] closely resembled src64<sup>A17</sup>. Additionally, the introduction of two copies of P[kelY627A] into a wild-type background caused a decrease in ring canal diameter (Fig. 4 I). This dominant-negative phenotype indicated that the introduction of “irreversible” cross-links formed by KelY627A perturbed the function of endogenous Kelch.

Using light microscopy, we measured ring canal diameters in five backgrounds: wild type, src64<sup>A17</sup>, kel<sup>DE1</sup>; P[ORF1], kel<sup>DE1</sup>; P[kelY627A], and P[kelY627A]; kel<sup>DE1</sup>; src64<sup>A17</sup> (see Materials and methods for details of each genotype). P[ORF1] is a transgene encoding wild-type Kelch protein that completely rescues the kelch phenotype (Robinson and Cooley, 1997b). The diameters of stage 5 ring canals in all the genotypes examined were similar with only a slight shift toward smaller rings in the three mutants (Fig. 5, stage 5). In contrast, the diameters of stage 10 A wild-type and kel<sup>DE1</sup>; P[ORF1] ring canals were significantly larger than those of kel<sup>DE1</sup>; P[kelY627A], src64<sup>A17</sup>, and P[kelY627A]; kel<sup>DE1</sup>; src64<sup>A17</sup> (Fig. 5, stage 10 A). The distributions of src64<sup>A17</sup>, kel<sup>DE1</sup>; P[kelY627A], and P[kelY627A]; kel<sup>DE1</sup>; src64<sup>A17</sup> diameters were identical, suggesting a common defect in ring canal morphogenesis.

Electron microscopy of ring canals during oogenesis
To better characterize the phenotype caused by KelY627A protein expression, we performed thin section electron microscopy on wild-type and mutant egg chambers. Horizontal sections cut through a ring canal reveal the two sides of the ring canal (diagramed in Fig. 6 A). Stage 6 ring canals from wild type, kel<sup>DE1</sup>; P[kelY627A], and src64<sup>A17</sup> had roughly similar morphologies (Fig. 6, compare B–D); the plasma membranes forming the outer ring canal rim were perpendicular to the lumen with a continuous actin cytoskeleton making up the inner rim. However, late stage ring canals had distinct differences. In wild type, the continuous F-actin layer of ring canals transformed into separate actin cables (Fig. 6 E, arrows). This was accompanied by a remodeling of the electron-dense material located on the outer rim (Fig. 6 E, arrowheads) so that electron-dense material remained in apposition to the actin filaments. In both kel<sup>DE1</sup>; P[kelY627A] and src64<sup>A17</sup> mutants, the ring canal F-actin remained continuous with no separation into cables (Fig. 6, F and G). The electron-dense material of the outer rim also remained continuous, suggesting that the actin segregation shares a pathway with outer rim restructuring. Finally, there was a dramatic distortion of ring canal morphology resulting in a pointed rather than flat shape (Fig. 6, F and G). We concluded that the failure to resolve the F-actin rim into cables and the subsequent distortion of the underlying membrane caused the “bicycle rim” shape seen by light microscopy.

Using the thin section images, we measured several ring canal diameters (Fig. 7 A) in wild type and kel<sup>DE1</sup>; P[kelY627A]. By stage 6, kel<sup>DE1</sup>; P[kelY627A] inner rim actin was 25–35% thicker than wild-type actin (Fig. 7 B). This significant difference (P < 0.01) in thickness remained roughly constant throughout ring canal growth. Although there was a significant decrease in kel<sup>DE1</sup>; P[kelY627A] ring canal diameter (Fig. 7 C), it was less than what was observed by light microscopy (Fig. 5, stage 10 A). This discrepancy is probably due to the inherent difficulty of using serial thin sections to define the diameter of a circular ring canal. Wild-type and mutant ring canal lengths, measured by tracing the length of the outer rim, remained similar during ring canal growth (Fig. 7 D). Filament number per section (Fig. 7 A) increased by 50% in kel<sup>DE1</sup>; P[kelY627A] by stage 10 A (Fig. 7 E). The density of actin filaments (Fig. 7 A) in wild type and kel<sup>DE1</sup>; P[kelY627A] was identical (Fig. 7 F). Therefore, although there were comparable densities of actin filaments in wild type and kel<sup>DE1</sup>; P[kelY627A], the failure to form actin cables in kel<sup>DE1</sup>; P[kelY627A] led to a significant increase in the total amount of actin present in each section.
Figure 6. Thin section electron micrographs of ring canals. (A) A schematic illustrating the components visualized in a vertical section of a ring canal (see inset). The inner rim is rich in F-actin (dots), whereas the electron-dense material forming the outer rim is continuous with the plasma membrane. The lumen of the ring canal rim is large enough for mitochondria to pass through. (B) Wild-type stage 6 ring canals have a tightly organized band of actin evenly distributed across the length of the outer rim. (C) kelDE1; P[kelY627A]/+ mutant ring canals have a slightly thicker actin band at stage 6 (see Fig. 7 for quantitation). (D) src64Δ17 mutant ring canals also have thicker actin at stage 6. (E) Wild-type stage 10A ring canals have separated actin cables (arrows). This separation is mirrored in the rearrangement of the electron-dense outer rim (arrowheads). (F) kelDE1; P[kelY627A]/+ ring canals do not exhibit the same segregation of actin into cables. Moreover there is no fragmentation of the outer rim, and it instead folds into a pointed shape. (G) src64Δ17 ring canals phenocopy kelDE1; P[kelY627A]/+ at stage 10A. Bars, 1 μm.
Actin monomer exchange decreases in KelY627A

To further characterize the phenotype caused by KelY627A protein, we performed the fluorescence recovery after photobleaching (FRAP) assay using actin tagged with green fluorescent protein (GFP–actin). The expression of GFP–actin was driven by a germline-specific promoter to label ring canals throughout all stages of development. FRAP was measured in stage 10A egg chambers from transgenic flies expressing wild-type Kelch (ORF1) and KelY627A. 18  P[UAS-GFP–Actin]/P[ORF1], 21 P[UAS-GFP–Actin]/P[kelY627A], and 15 P[UAS-GFP–Actin];src64417 ring canals were analyzed. Three representative experiments are shown (Fig. 8, A–C). After bleaching, ring canals in P[UAS-GFP–Actin]/P[ORF1] recovered fluorescence with a $t_{1/2}$ of 65 s (Fig. 8 D). In contrast, fluorescence in P[UAS-GFP–Actin]/P[kelY627A] ring canals recovered with a $t_{1/2}$ of 227 s (Fig. 8 D). P[UAS-GFP–Actin];src64417 had a $t_{1/2}$ of 244 s. These data indicate that in ring canals with nonphosphorylatable Kelch or reduced Src64, actin monomers took >3.5 times as long to exchange as in control ring canals.

Discussion

We have shown that Drosophila Kelch protein is an actin filament–cross-linking protein essential for the organization of actin in ovarian ring canals. F-actin binding by Kelch is negatively regulated by phosphorylation of the tyrosine residue at position 627, which is within the fifth KREP. Because KREP five contains the actin binding site, the addition of a phosphate is likely to sterically interfere with actin filament binding as has been found for other actin-binding proteins, villin (Zhai et al., 2001) and α-actinin (Izaguirre et al.,...
2001). Phosphorylation of Kelch tyrosine 627 depends directly or indirectly on the Src64 kinase, and the expression of a nonphosphorylatable Kelch protein phenocopies src64 mutations. Additionally, actin has reduced exchange rates in the ring canal as measured by FRAP using GFP–actin. These data suggest that Kelch may be the primary, if not only, cytoskeletal target of Src64-dependent kinase activity in ring canals, and the regulated cross-linking of Kelch plays a role in the remodeling of actin during ring canal growth.

**Regulation of ring canal expansion**
The dynamics of actin filaments in ring canals have been elegantly described at the ultrastructural level (Riparbelli and Callaini, 1995; Tilney et al., 1996). Ring canals are built at the positions of arrested cleavage furrows that form during the mitotic divisions of germline cells. The mechanism of cleavage furrow arrest is likely to be conserved among animal species because incomplete cytokinesis occurs during the proliferation of germline cells in many animals (for review see Robinson and Cooley, 1996). In *Drosophila*, once egg chambers are fully assembled, ring canal growth happens in two phases. First, the thickness of the actin rim increases to ~0.3 μm as the diameter of the ring grows slowly to 2 μm. Subsequently, the thickness of the actin rim and the density of actin filaments remain constant while the rate of ring canal expansion increases. The net increase of actin within ring canals overall is 134-fold (Tilney et al., 1996). During the rapid phase of ring canal growth, actin filaments must be polymerized, probably at the plasma membrane, to expand the ring canal rim, and disassembled at the cytoplasmic face to maintain the lumen. Our analysis of the Kelch protein shows that precise regulation of actin filament cross-linking by phosphorylation is critical during rapid ring canal growth.

The behavior of ring canals that contain KelY627A provides significant insight into Kelch function. The absence of Kelch phosphorylation leads to ring canals that accumulate more actin filaments than normal, possibly due to a slowing in the rate of actin depolymerization relative to the rate of polymerization. After about stage 8 of oogenesis, the failure to resolve the continuous sheet of actin filaments into discreet cables may be another consequence of inhibiting depolymerization. The presence of more "permanent" Kelch cross-links may reduce the accessibility of the filament network to depolymerizing factors. In vitro experiments have demonstrated that actin–cross-linking proteins alone are capable of inhibiting the rate of pyrenyl F-actin depolymerization (Cano et al., 1992). Another possible explanation for these phenotypes is that because Kelch cross-links are no longer easily reversible, filament reorientation or sliding is restricted during ring canal growth.

The FRAP experiments provide additional insight into the actin dynamics at the ring canal. First, ring canal actin is highly dynamic. The rate of actin monomer turnover that we found in wild-type ring canals is comparable to the kinetics of actin turnover found in the leading edge of motile goldfish epithelial keratocytes (Theriot and Mitchison, 1991). This would be consistent with a population of actin that is constantly undergoing a rapid cycle of polymerization and depolymerization. Second, the presence of nonregulated Kelch clearly results in a dramatic reduction in the dynamics of actin. This supports the model that mutant Kelch protein reduces accessibility to other actin-binding proteins, in this case proteins involved with polymerization or depolymerization. We propose that this effect could be due to bound Kelch acting as a stabilizing protein much in the same way that tropomyosin protects F-actin from actin depolymerizing factor/cofilin (for review see Pollard et al., 2000).

Studies in our lab involving the actin polymerization factor Arp2/3 have demonstrated that ring canal stability and growth is dependent on the presence of a functional Arp2/3 complex (Hudson and Cooley, 2002). The effects of mutations in Arp2/3 complex subunits are progressively more severe as egg chambers develop, and by stage 6, ring canals begin to collapse. In *kelch* null mutants, the actin filaments are initially well organized, begin to show signs of disorganization around stage 4, and are completely disorganized starting at stage 6 (Tilney et al., 1996; Robinson and Cooley, 1997a). Interestingly, thin section electron micrographs of *kel*P[kelY627A]+ show signs of actin filament disruption beginning at stage 6. The coincidence of *kelch* and Arp2/3 complex mutant phenotypes with the onset of rapid ring canal expansion and the presence of highly dynamic actin suggest a model where ring canal growth is powered by de novo actin polymerization accompanied by regulated cross-links. Therefore, ring canal growth may be mechanistically similar to the movement of plasma membranes at the leading edge of motile cells. Future work on ring canal actin organization should include platinum replica electron microscopy to understand the overall organization of the ring canal actin filament network. This will allow direct comparison to the actin filament networks of lamellipodia in *Xenopus laevis* keratocytes and fibroblasts (Svitkina and Borisy, 1999).

Intriguingly, the accumulation of actin during earlier stages of oogenesis is apparently independent of both Kelch and the Arp2/3 complex. Characterization of other mutants affecting ring canals has revealed genes required for initial stages of ring canal assembly. These include the *cheerio* gene that encodes the actin filament–cross-linking protein ABP280/filamin (Li et al., 1999; Sokol and Cooley, 1999). In *cheerio* mutants, ring canal actin is absent. In addition, HtsRC is required for the early accumulation of actin filaments (Robinson et al., 1994); however, it has not been determined whether HtsRC interacts directly with F-actin or affects actin polymerization. Therefore, additional research is needed to elucidate the mechanism of early ring canal biogenesis.

The regulation of Kelch–actin cross-links could be accomplished by Src64 directly phosphorylating Kelch. Alternatively, Src64 may activate another protein tyrosine kinase, such as Tec29 (Roulier et al., 1998), which in turn phosphorylates Kelch. However, the shared phenotype seen by electron microscopy of the *src64* and *P[kelY627A]* ring canals is strongly suggestive of Kelch being the major downstream component of a Src64 cascade. Analysis of Kelch phosphorylation in *tec29* mutants is difficult because available *tec29* alleles are lethal. SFKs have been shown to signal rearrangements in the actin cytoskeleton in other contexts. In *Drosophila*, embryonic mutant for *src64* or *tec29* fail to complete epidermal closure at the end of gastrulation. This is, in part, because the leading edge cells contain reduced quantities of F-actin, and the cells only partially elongate and fail to migrate completely.
Kelch family members and actin

It should be noted that not all Kelch family members are actin-binding proteins (for review see Adams et al., 2000). For example, nuclear matrix protein (NRP/B) is a novel nuclear matrix protein that contains a highly conserved KREP domain. NRP/B is specifically expressed in primary neurons and participates in the regulation of neuronal process formation (Kim et al., 1999). A direct interaction with actin by the ectodermal neurotroph-1 protein has been demonstrated by coimmunoprecipitation; however, it does not exclusively colocalize with F-actin in Drosophila, and it is perinuclear in neuronal cell lines (Hernandez et al., 1997). A Kelch family member that interacts with actin is called Mayven. Mayven localizes to the leading edge of the lamellipodia in U373-MG astrocytoma/glioblastoma cells (Soltysik-Espanola et al., 1999). Mayven is also localized with the focal adhesion kinase (Soltysik-Espanola et al., 1999), suggesting it could play a role in actin reorganization at focal adhesion plaques. A role for phosphorylation in the regulation of Mayven has not been reported.

In Limulus, it has been postulated that the Kelch homologue ω-scruin acts as a protein that allows F-actin to rapidly twist and slide during acrosome extension or “true discharge” (Sherman et al., 1999). Biochemical studies performed on ω-scruin (Sun et al., 1997) have shown that the cysteine corresponding to Drosophila Kelch residue 628 lies within the ω-scruin actin binding domain (Fig. 2 B, boxed). Thus, both Kelch and ω-scruin contain an actin binding site within KREP number 5. However, ω-scruin does not have a tyrosine comparable to Kelch residue 627 in the primary sequence; therefore, regulation of ω-scruin cross-linking is likely to be different than that for Kelch. ω-Scruin regulation may target scruin–actin interactions rather than scruin–actin interactions.

Materials and methods

Drosophila stocks and germline transformation

w1118 (Lindsley and Zimm, 1992), src6417 (Guarnieri et al., 1998; obtained from S. Beckendorf, University of California, Berkeley, CA); kef<sup>305</sup> (Xue and Cooley, 1993; Robinson and Cooley, 1997b), and kef<sup>317</sup> (Schüpbach and Wieschaus, 1991) were maintained under standard culture conditions. Mutants for transgenic studies were generated using PCR amplification, sequenced to confirm that there were no PCR-induced errors, and then subcloned into the germline expression vector pCOG (Robinson and Cooley, 1997b). Mutant constructs were microinjected along with the PinPoint Xa Kelch constructs. Soluble Kelch and KREP fragments were obtained by following the manufacturer's instructions (Invitrogen). The constructs were sequenced and subcloned into the PinPoint Xa-3 vector (Promega) using HindIII and Nltt. Escherichia coli DE3 cells were transformed with the PinPoint Xa-3 Kelch constructs. Soluble Kelch and KREP fragments were obtained by following the manufacturer's instructions. Only full-length Kelch constructs were cleaved from the purification tag using Factor Xa and the manufacturer's instructions.

Image acquisition and FRAP

GFP-actin–expressing ovaries were dissected onto a 22-mm × 40-mm glass coverslip as previously described (Theurkauf and Hazelrigg, 1998). FRAP experiments were conducted on a Zeiss 510 scanning laser microscope (Center for Cell Imaging, Yale University, New Haven, CT). Images were captured with either a 63× 1.4 NA objective or a 40× 1.3 NA objective using a pinhole diameter equivalent to one to two times the Airy disk diameter. In each experiment, at least 20 consecutive baseline images were obtained before bleaching. A region of the ring canal was selected, and fluorescence was bleached by scanning the region with high intensity illumination (100% transmittance). After photobleaching, fluorescence of the entire field was collected by the ArKr laser at 1% power every second for at least 4 min after photobleaching. The fluorescence intensity in the photobleached region of the ring canal was normalized to the fluorescence measured in a nonbleached region of the same ring canal, and in the cytoplasm. Wild-type and mutant rate constants of three curves were compared, and a P value of <0.01 was taken as significant. Nonlinear regression and t tests were performed using Microsoft Excel 2000. Images were prepared for publication using Adobe Photoshop.

Protein electrophoresis and Western blot analysis

Ovaries were dissected in the presence or absence of 25 mM sodium vanadate, 10 mM sodium fluoride, and 0.05% hydrogen peroxide in IMADS buffer and incubated on ice for 5 min. 10 ovaries were then solubilized with 25 μl isoelectric focusing sample buffer (8 M urea, 4% [wt/vol] CHAPS, 40 mM Tris, 65 mM DTE, and a trace of bromophenol blue). Samples were focused using Immobiline DryStrips, pH 4–7 linear (Amersham Pharmacia Biotech). The second dimension was performed using standard SDS-PAGE techniques on 8% gels and transferred onto nitrocellulose membranes (Hybond ECL; Amersham Pharmacia Biotech). Membranes were blocked with PBS containing 5% nonfat dry milk and incubated with the following antibodies and dilutions: 1:10 anti-kerb 1B (Xue and Cooley, 1993); 1:10 anti-actin JLA20 (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA); and 1:2,000 anti-phosphorylated myosin PY20 (ICN Biomedicals); and detection of biotin-labeled Kelch proteins was performed using a 1:5,000 dilution of avidin-HRP (Pierce Chemical Co.). Secondary antibodies conjugated with HRP were purchased from
Pierce Chemical Co. Visualization of HRP was performed using an ECL detection kit (Amersham Pharmacia Biotech).

Egg chamber staining
Ovaries were dissected, fixed, and processed as previously described (Robinson and Cooley, 1997a). For filamentous actin staining, egg chambers were incubated in 5 U of rhodamine-conjugated phalloidin (Molecular Probes) per 200 μl of PBSTO (1 × PBS, 0.3% Triton X-100, 0.5% BSA). For antibody staining, ovaries were immunostained with anti-kelch 1A at a 1:1 dilution (Xue and Cooley, 1999), or anti-phosphotyrosine PY20 (ICN Biomedicals) at a 1:500 dilution. Secondary antibodies conjugated with Alexa Fluor 488 were purchased from Molecular Probes. Fluorescence intensity measurements were collected using the ZEISS image analysis software.

F-actin binding assays
Purified rabbit skeletal muscle monomeric actin was purchased from Cytokeleton Inc. Actin concentration was estimated as previously described (Pollard, 1976). The concentration of Kelch and KREPs was determined using a modified Bradford assay (Bio-Rad Laboratories). G-actin was polymerized in A-buffer (10 mM imidazole, pH 7.0, 75 mM KCl, 2.5 mM MgSO4, 1 mM EGTA, 1 mM ATP, 0.1% NaN3). Immediately before use, purified Kelch or KREPs and actin were centrifuged at 100,000 g at 4°C for 1 h to pellet any aggregated protein. Mixtures of F-actin with Kelch or KREPs were incubated for 10 min at 25°C. Low speed and high speed cosedimentation assays were performed as previously described (Matova et al., 1999).

Far Western blot analysis
For binding experiments, total ovary lysates were separated using 2D electrophoresis as described above. Nonreduced SDS gels were incubated at 4°C overnight in PBS containing 5% milk and 0.1% Tween 20, followed by incubation with 10 μg of F-actin (Sigma-Aldrich) for 2 h at 4°C. The blots were washed, and a 1:10 dilution of rabbit anti-actin antibody JLA20 was added for 1 h. After washing, HRP-conjugated anti-rabbit antibody (Pierce Chemical Co.) was added for 1 h. Bands were visualized using ECL reagents.

References
Adams, J., R. Kelso, and L. Cooley. 2000. The kelch repeat superfamily of proteins: propellers of cell function. Trends Cell Biol. 10:17–24.
Ahmad, K.F., C.K. Engel, and G.G. Prive. 1998. Crystal structure of the BTB domain from PLZF. Proc. Natl. Acad. Sci. USA. 95:12123–12128.
Bork, P., and R.F. Doolittle. 1994. Drosophila kelch motif is derived from a common enzyme fold. J. Mol. Biol. 236:1277–1282.
Bourguignon, L.Y., H. Zhu, L. Shao, and Y.W. Chen. 2001. CD44 interaction with c-Src kinase promotes cortactin-mediated cytoskeleton function and actin polymerization in vivo. Trends Cell Biol. 11:288–293.
Condeelis, J. 2001. How is actin polymerization nucleated in vivo? Trends Cell Biol. 11:288–293.
Dodson, G.S., D.J. Guarnieri, and M.A. Simon. 1998. Srcf64 is required for ovarian ring canal morphogenesis during Drosophila oogenesis. Development. 125:2883–2892.
Guarnieri, D.J., G.S. Dodson, and M.A. Simon. 1998. SRC64 regulates the localization of a Tec-family kinase required for Drosophila ring canal growth. Mol. Cell. 1:831–840.
Harris, J.R. 1999. Negative staining of thinly spread biological particulates. In Electron Microscopy Methods and Protocols. Vol. 117. M.A. Nasser Hajibagheri, editor. Humana Press, Totowa, NJ. 13–30.
Hernandez, M.C., P.J. Andres-Barquin, S. Martinez, A. Bulkone, J.L. Rubenstein, and M.A. Israel. 1997. ENC-1: a novel mammalian kelch-related gene specifically expressed in the nervous system encodes an actin-binding protein. J. Neurosci. 17:3058–3051.
Hofmann, K., P. Bucher, L. Falquet, and A. Bairoch. 1999. The PROSITE database, its status in 1999. Nucleic Acids Res. 27:215–219.
Jordan, P., and R. Karess. 1997. Myosin light chain-activating phosphorylation sites are required for oogenesis in Drosophila. J. Cell Biol. 139:1805–1819.
Koch, E.A., R.C. King. 1969. Further studies on the ring canal system of the ovarian ovocytes of Drosophila melangaster. Z. Zellforsch. Mikrosk. Anat. 102:129–152.
Kramerova, I.A., and A.A. Kramerov. 1999. Mucinoprotein is a universal constituent of stable intercellular bridges in Drosophila melanogaster germ line and somatic cells. Dev. Dyn. 216:349–360.
Li, M.G., M. Serr, K. Edwards, S. Ludmann, D. Yamamoto, L.G. Tilney, C.M. Field, and T.S. Hays. 1999. Filamin is required for ring canal assembly and actin organization during Drosophila oogenesis. J. Cell Biol. 146:1061–1074.
Pollard, T. 1976. The role of actin in the temperature-dependent gelation and contraction of actin filament networks. J. Cell Biol. 68:579–601.
Pollard, T.D., L. Blanchon, and R.D. Mullins. 2000. Molecular mechanisms controlling actin filament dynamics in nonmuscle cells. Annu. Rev. Biochem. Biophys. Biostruc. 79:455–565.
Pruyne, D., and A. Bretscher. 2000. Polarization of cell growth in yeasts. J. Cell Sci. 113:571–585.
