

Drosophila Kelch Is an Oligomeric Ring Canal Actin Organizer

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Abstract. *Drosophila* kelch has four protein domains, two of which are found in kelch-family proteins and in numerous nonkelch proteins. In *Drosophila*, kelch is required to maintain ring canal organization during oogenesis. We have performed a structure–function analysis to study the function of *Drosophila* kelch. The amino-terminal region (NTR) regulates the timing of kelch localization to the ring canals. Without the NTR, the protein localizes precociously and destabilizes the

ring canals and the germ cell membranes, leading to dominant sterility. The amino half of the protein including the BTB domain mediates dimerization. Oligomerization through the amino half of kelch might allow cross-linking of ring canal actin filaments, organizing the inner rim cytoskeleton. The kelch repeat domain is necessary and sufficient for ring canal localization and likely mediates an additional interaction, possibly with actin.

IN *Drosophila*, kelch is required in the ovary for production of viable eggs. kelch mutants are female sterile as a result of defective cytoplasm transport throughout oogenesis. Kelch is a structural component of the ring canals that provides the intercellular conduits through which cytoplasm is transported from the nurse cells to the oocyte in an egg chamber (for review see Robinson and Cooley, 1996). kelch mutant ring canals have actin filaments that are disorganized and extend into the lumen of the ring canal (Robinson et al., 1994; Tilney et al., 1996). This disorganization apparently causes partial obstruction of the ring canal lumen so that cytoplasm transport is impaired.

Ring canal assembly is initiated with the arrest of mitotic cleavage furrows followed by the addition of several proteins including a protein that immunoreacts with anti-phosphotyrosine antibodies (PY protein), a product of the *hu-li tai shao* (*hts*) locus (Yue and Spradling, 1992) called the *hts* ring canal protein (*hts*-RC; Robinson et al., 1994), actin filaments, and kelch. Kelch is the last of the known proteins to be localized to the ring canal complex (Robinson et al., 1994), and it does not arrive on all ring canals until after the maximum number of actin filaments has been recruited to the ring canal (Tilney et al., 1996). From the time kelch has reached all ring canals until the end of oogenesis, the ring canals expand from a diameter of 3–4 to 10 μ m. In kelch mutants, the ring canals have a normal morphology until the time when kelch would normally reach all of the ring canals. After this time, the ring canals

become disorganized. These data suggest that kelch is required to maintain the organization of the actin filaments during the expansion of the ring canal rather than being required for assembly (Robinson et al., 1994; Tilney et al., 1996).

The *Drosophila* kelch gene produces a single transcript separated into two open reading frames (ORFs) by a UGA stop codon. ORF1 only and full length (ORF1 plus ORF2) kelch proteins are made (Xue and Cooley, 1993; Robinson and Cooley, 1997b). The ORF1 product is a member of a family of kelch-related proteins that includes several Pox virus ORFs (Koonin et al., 1992; Senkevich et al., 1993), mammalian calicin (von Bülow et al., 1995), and *Caenorhabditis elegans* spe26 (Varkey et al., 1995). Currently, the protein databases contain four kelch family proteins from *C. elegans* and at least five mammalian kelch family proteins. Interestingly, the *Drosophila* kelch ORF1 contains ~110 amino acids (the NTR; see Fig. 1 A) at the amino terminus not found in other kelch-related proteins. The *Drosophila* kelch ORF2 domain encodes a protein with no significant homology to known proteins and so far is specific to *Drosophila*. Although the two kelch protein (ORF1 and full length) motif is conserved in several *Drosophila* species, the ORF1 protein is sufficient for kelch function (Robinson and Cooley, 1997b).

Kelch ORF1 contains two conserved domains found in other kelch proteins as well as in nonkelch proteins (Fig. 1 A). The first of these, the BTB or POZ domain, is a 120-amino acid motif that is found immediately after the amino-terminal region (NTR)¹ in kelch. This domain is also found in several zinc finger-containing transcription factors (Harrison and Travers, 1990; Chardin et al., 1991; DiBello et al., 1991; Zollman et al., 1994) and it has been

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1. Abbreviations used in this paper: KREP, kelch repeat domain; NTR, amino-terminal region; ORF, open reading frame.

shown to mediate dimerization in vitro (Bardwell and Treisman, 1994; Chen et al., 1995). A second domain consists of six 50-amino acid repeats known as kelch repeats (see Fig. 1 A; Xue and Cooley, 1993). Kelch repeats are found in several nonkelch proteins including a recently characterized protein in *Physarum polycephalum* called actin-fragmin kinase (Eichinger et al., 1996). The kelch repeat sequence is predicted to fold into a superbarrel or β -flower structure (Bork and Doolittle, 1994), similar to the repeat sequences in a family of bacterial, fungal, and influenza virus enzymes such as neuraminidase, galactose oxidase, and the sialidases (Varghese et al., 1983; Ito et al., 1994).

Suggestions of the function of the kelch repeat domain come from *Limulus* proteins called scruin (Tilney, 1975). The *Limulus* genome contains at least three scruin genes, two of which encode α - and β -scruins (Way et al., 1995a,b). Each scruin consists of two sets of kelch repeats, one in the amino-terminal half and a second at the carboxy-terminal half of the protein. α -scruin was originally identified as an actin filament cross-linking protein found in the acrosomal actin bundle of the *Limulus* sperm (Tilney, 1975). Subsequent work has suggested that each kelch repeat domain in α -scruin forms an actin-binding site (Bullitt et al., 1988; Owen and DeRosier, 1993; Schmid et al., 1994; Way et al., 1995b). *C. elegans* spe26 is required for a normal actin cytoskeleton during spermatogenesis (Varkey et al., 1995), consistent with the repeat motif providing an interaction with actin. β -scruin, on the other hand, is found in the acrosomal vesicle and may not associate with actin (Way et al., 1995a). Mammalian calicin localizes to the calyx, a large nonfilamentous cytoskeletal structure that associates with the sperm head nucleus (von Bülow et al., 1995). The calyx does not contain much, if any, filamentous actin. Consequently, it is possible that the kelch repeat domain can mediate a diversity of functions in different kelch family proteins. However, an attractive hypothesis for *Drosophila* kelch function is that it associates with the ring canal actin, either directly or indirectly, through the kelch repeats, and cross-links the actin filaments by dimerizing through the BTB domain.

To assign functions to the domains of *Drosophila* kelch, we expressed epitope-tagged kelch ORF1 domains in the ovary and examined their ability to rescue fertility and ring canal morphology of *kelch* mutants. Their subcellular distribution in wild type and *kelch* mutant backgrounds was also determined. These experiments uncovered a dominant-negative kelch construct whose behavior suggests that the NTR is required to regulate the timing of kelch localization to the ring canals but is not required otherwise for kelch function. The BTB domain and the 147 amino acids (IVR) intervening between the BTB and kelch repeat domain (collectively called BTBIVR) is required for kelch complex formation. The kelch repeat domain (KREP) is necessary and sufficient for ring canal localization. These data support a model in which kelch ORF1 functions as an oligomeric ring canal actin organizer.

Materials and Methods

Fly Strains

w¹¹¹⁸ (Lindsley and Zimm, 1992) flies were used as wild type in these ex-

periments. The hypomorphic *kelch* mutant, *kel^{neo}*, was described by Xue and Cooley (1993) and Robinson and Cooley (1997b). The molecular null *kelch* mutant, *kel^{DEL}*, was isolated by Schüpbach and Wieschaus (1991) and characterized in Robinson and Cooley (1997b). Transgenic flies expressing untagged kelch ORF1 protein, an alanine-substituted (Alanine), serine-substituted (Serine), and UGA-deleted (Δ UGA) full length kelch proteins were described by Robinson and Cooley (1997b). Fly stocks were maintained under standard conditions.

Construct Generation

To express epitope-tagged domains in the fly ovary, we prepared a myc epitope tagging cassette (KNmyc; Robinson and Cooley, 1997b) that begins with an HpaI site that is found 81 bp upstream of the first ATG in the kelch 5'UTR and includes the first 19 amino acids of kelch that include two potential start methionines. The myc 9E10 epitope (AEEQKLISEEDLN; Evan et al., 1985) was placed after kelch residue 19. The following polylinker in the following reading frame was placed after the myc epitope:

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EcoRI      NdeI      XhoI      BglII      Stop      NotI
GAA TTC CAT ATG CTC GAG GCA GAT CTG TAA GCGGCCGC

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The cassette was subcloned into pCOG, a germline expression vector (Robinson and Cooley, 1997b). All truncations of kelch were made by preparing the appropriate cDNA fragments with back-to-back *EcoRI* and *NdeI* sites at the 5' end of the fragments and a *BglII* or *BamHI* sites at the 3' end of the cDNA fragment. We found that this combination of sites in this reading frame allowed us to move the cDNA fragments easily between pCOG and expression vectors, which allows us to express the proteins in *Drosophila* Schneider cells, bacteria, or *Pichia*.

The cDNA fragments were prepared by PCR amplification and subcloned. The subcloned fragments were analyzed by dideoxynucleotide sequencing to make sure no unwanted mutations had been introduced. The following kelch domains were prepared. The amino acid numbers correspond to the sequence in Xue and Cooley (1993): ORF1: S17 - M688; ORF1-R: S17 - P401; BTB IVR: Q120 - P401; BTB: Q120 - L252; IVR: D253 - P401; IVR KREP: D253 - M688; KREP: M400 - M688; Δ NORF1: Q120 - M688.

Generation of Transgenic Animals

Transformation plasmids were microinjected along with the Δ 2-3 transposase helper plasmid into *w¹¹¹⁸* syncytial blastoderm embryos according to standard techniques. Transgenic flies were identified by the complementation of the *white* eye phenotype by the *white* mini-gene in pCOG. P element insertions were maintained in a wild-type background as well as in *kel^{neo}* and *kel^{DEL}* backgrounds.

Western Analysis

Western analysis of *Drosophila* ovarian extracts was performed as described in Robinson and Cooley (1997b). For anti-kelch monoclonal antibodies, we used anti-kelch 1B (Xue and Cooley, 1993). For anti-hts-RC, we used anti-hts 655 4B (Robinson et al., 1994). For anti-myc staining, we used anti-myc 9E10 (Evan et al., 1985). For anti-KREP, we used mouse polyclonal sera #5-6 (Xue and Cooley, 1992).

Immunolocalization and Confocal Imaging

Immunocytochemical analysis of whole ovaries was performed as described in Robinson and Cooley (1997b). For actin visualization, ovaries were stained with rhodamine-conjugated phalloidin (Molecular Probes, Inc., Eugene, OR). For antibody staining, ovaries were immunostained with hts-RC antibody hybridoma supernatant, anti-hts-RC 655 4B (1:1 dilution; Robinson et al., 1994), kelch hybridoma supernatant, anti-kelch 1B (1:1 dilution; Xue and Cooley, 1993), or anti-myc 9E10 hybridoma supernatant (1:1 dilution; Evan et al., 1985).

Nuclear Staining

Nuclear staining was performed as described in Robinson et al. (1997). We dissected and fixed ovaries from 10–20 females per genotype as described for immunocytochemistry. During the third from final wash in PBT (1 \times PBS, 0.3% Triton-X 100, 0.5% BSA), DAPI (Molecular Probes, Inc.) was added to the PBT at 1 μ g/ml. The specimens were washed two more times and were examined and photographed using a microscope (Axiophot; Zeiss, Inc., Thornwood, NY) with a 40 \times objective (1.3 NA).

Expression, Purification, and Analysis of Recombinant BTB and BTBIVR Proteins

To prepare His-tagged BTB (his:BTB) and BTBIVR (his:BTBIVR) proteins, the respective cDNA fragments were cloned into pET 14b (Novagen, Madison, WI). Proteins were expressed using the HMS174:DE3::pLys S bacterial strain (Novagen). The bacteria were lysed using a French press in a medium salt buffer. The proteins were extensively purified by affinity chromatography using Ni²⁺/NTA agarose resin (Qiagen, Chatsworth, CA).

The molecular weight of his:BTB was determined by SDS-polyacrylamide gel electrophoresis and by dynamic light scattering on a Dynapro-801 instrument. His:BTB and his:BTBIVR proteins were also analyzed by gel filtration on a superose 12 column using an FPLC (Pharmacia Fine Chemicals, Piscataway, NJ) system. For gel filtration, retention times were compared to those of bovine serum albumin (65 kD), ovalbumin (43 kD), carbonic anhydrase (29 kD), and lactalbumin (19.3 kD).

Results

Expression of myc Epitope-Tagged Domains

To investigate the mechanism by which kelch organizes the ring canal actin cytoskeleton, myc epitope-tagged domains were expressed in the ovary using the pCOG vector.

A panel of myc epitope-tagged, truncated kelch domain constructs was engineered, and transgenic lines were established (Fig. 1 B). Western analysis of the lines using anti-kelch 1B antibodies (Xue and Cooley, 1993) and anti-kelch repeat polyclonal sera showed the expression of proteins of the expected size (Fig. 2). Myc:ORF1, myc:ΔNORF1, myc:ORF1-R, myc:BTBIVR, and myc:BTB produced proteins that could be recognized by anti-kelch 1B, which recognizes the BTB domain. Myc:KREP is not recognized by anti-kelch 1B but is recognized by polyclonal sera generated against the repeat region of kelch (Xue, 1992). For some of the transgenes, we had no difficulty isolating lines expressing high levels of protein. However, for some of the transgenes encoding truncated proteins, we generated over 20 inserts to obtain a single useful line (Fig. 1 B). We attribute the difficulty of isolating high expressing lines of some of the proteins to instability of the truncated proteins. We tested the subcellular localization of each transgenically produced protein in the presence or absence of the endogenous kelch proteins using either wild-type, *kel^{meo}* (expresses a small amount of endogenous kelch), or the *kel^{DE1}* (expresses no detectable kelch) mu-

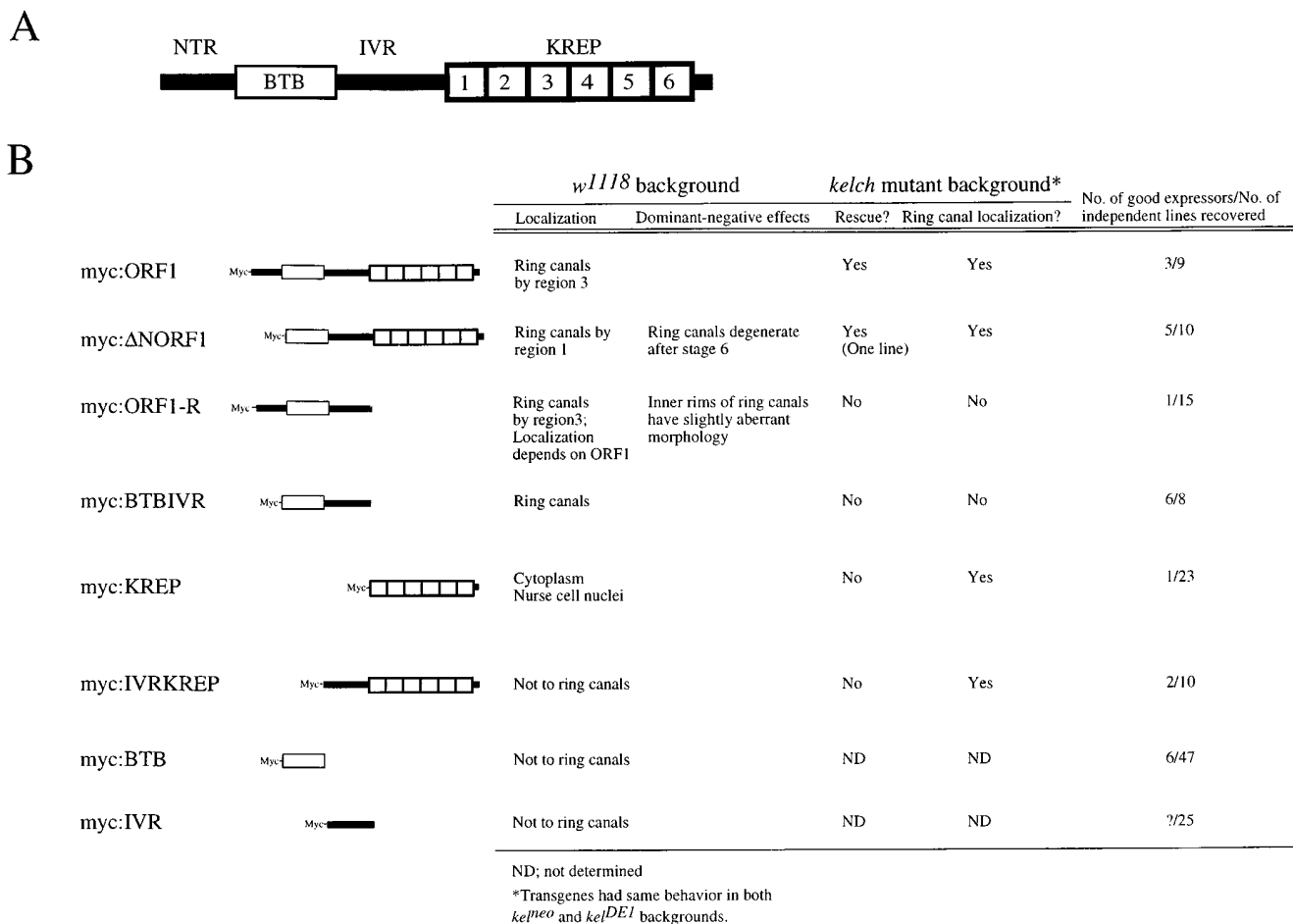


Figure 1. Summary of kelch domain structure and the transgenes studied. (A) Diagram of domain structure of kelch ORF1. NTR includes the amino-terminal of ~110 amino acids, and the BTB domain includes the next 120 amino acids. The IVR is the 147-amino acid intervening region between the BTB and the six 50-amino acid kelch repeat domain (KREP). (B) Amino-terminally myc epitope-tagged domains expressed in the ovary using the pCOG vector. The number of independent lines generated and the number of lines that showed a high level of expression are listed. The subcellular localization of each protein in various genetic backgrounds is provided.

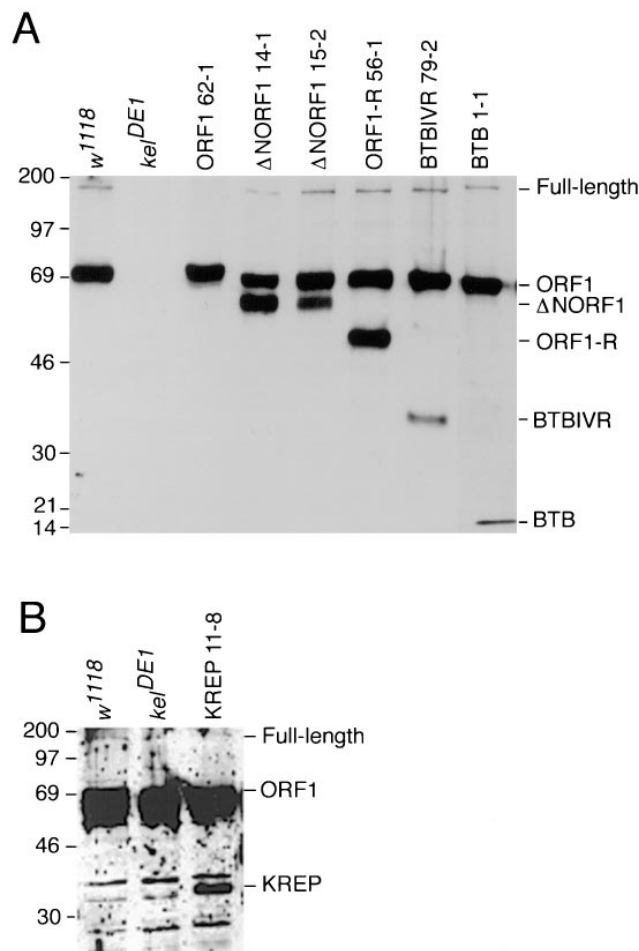


Figure 2. Expression of myc epitope-tagged domains verified by Western analysis. (A) The endogenous 76-kD ORF1 and 160-kD full length kelch proteins are detected in wild-type (*w¹¹¹⁸*) egg chambers; both proteins are absent in *kel^{DE1}* egg chambers. An ~80-kD myc:ORF1 product is detected in *kel^{DE1}* egg chambers. A 65-kD myc:ΔNORF1 (two representative lines, 14-1 and 15-2, are shown), a 47-kD myc:ORF1-R, a 35-kD myc:BTBIVR, and a 17-kD myc:BTB protein are detected in wild-type egg chambers by anti-kelch 1B antibodies. (B) A 33-kD myc:KREP product is detected in one transgenic line with unpurified anti-KREP polyclonal antibodies. Endogenous kelch proteins are also detected, although the ORF1 product is significantly obscured by a prominent group of nonkelch proteins with which the polyclonal antibodies cross-react. These cross-reacting proteins are also detected in *kel^{DE1}* extracts. Only the expected 33-kD KREP protein is unique to the myc:KREP line.

tant backgrounds (Robinson and Cooley, 1997b). We also tested whether the truncated proteins could rescue the *kelch* mutant defects or whether the proteins had any dominant-negative activity (summarized in Fig. 1 B; presented below).

Deletion of the NTR Region

Removal of the NTR region (myc:ΔNORF1) produced a protein that had severe dominant-negative effects on egg chamber morphology and ring canal stability. As a positive control, myc:ORF1 rescued all *kelch* mutant defects, and the protein could be seen on ring canals in a wild-type or

kelch mutant background (Fig. 3, A and B; Robinson and Cooley, 1997b). In addition, no deleterious effects were observed when the myc:ORF1 protein was expressed in a wild-type background. In contrast, we examined carefully several independent lines expressing myc:ΔNORF1 in a wild-type background, all of which showed a dramatic loss of nurse cell plasma membrane integrity (Fig. 3, C and D). This dominant-negative effect began around stage 6 of oogenesis (not shown). The defect was proportional to the level of expression of the myc:ΔNORF1 transgene product; lines expressing lower levels of protein had less dramatic effects, and very low expressing lines had no dominant-negative effects (not shown). Ring canals became difficult to detect after stage 6, and those that did persist were often thin with a larger diameter than normal or had a much reduced diameter. Of those that persisted, myc:ΔNORF1 and actin were seen in the ring canal (Fig. 3, E and F). The loss in membrane stability caused multinucleate nurse cells, and nurse cell nuclei frequently moved into the oocyte. The number of nuclei transported into the oocyte ranged from 0 to 15 with an average of ~4-7 nuclei per oocyte depending on the line (Fig. 3, G-I).

The NTR domain controls timing of kelch localization to ring canals. In wild-type egg chambers and in *kel^{DE1}* egg chambers expressing myc:ORF1, kelch protein began to localize to ring canals in stage 1 egg chambers, but kelch was not readily visible on all ring canals until around stage 4 (Fig. 4, A and B). However, myc:ΔNORF1 began to localize to ring canals much earlier beginning in region 1 in the germarium (Fig. 4 C). This is very early in ring canal development at a time before hts-RC and actin begin to form the ring canal inner rim (Fig. 4 D; Robinson et al., 1994). Localization of myc:ΔNORF1 to ring canals in region 1 occurred in wild-type and *kelch* mutant ovaries. The amount of protein detected on ring canals at this stage was proportional to the level of expression of the transgene (not shown).

One myc:ΔNORF1 line, called myc:ΔNORF1 15-2, had a less dramatic dominant-negative effect and was capable of rescuing the ring canal morphology and fertility of *kel^{DE1}* mutants. This was surprising since the other lines expressing myc:ΔNORF1 at high levels had very similar dominant-negative effects on ring canal and plasma membrane stability in *kel^{neo}* and *kel^{DE1}* backgrounds. In egg chambers carrying myc:ΔNORF1 15-2 in a wild-type background, many of the plasma membranes remained intact, and egg chamber morphology was frequently quite normal (Fig. 5, A and B). The egg chambers did have some plasma membrane disruption as indicated by the number (around 4) of nurse cell nuclei observed in the oocyte (Fig. 3 I). Myc:ΔNORF1 protein localization to ring canals (Fig. 5 A) began earlier than normal at germarial region 2b (Fig. 5, C and D) but did not accumulate as much or as rapidly as in the higher expressing lines. This line rescued the fertility of *kel^{DE1}* mutants and rescued ring canal morphology (Fig. 5, E and F). We did not observe inappropriate transport of nurse cell nuclei into the oocytes in egg chambers from *kel^{DE1}* flies carrying myc:ΔNORF1 15-2 (not shown).

KREP Is Necessary and Sufficient for Ring Canal Localization

To examine the requirements for ring canal localization,

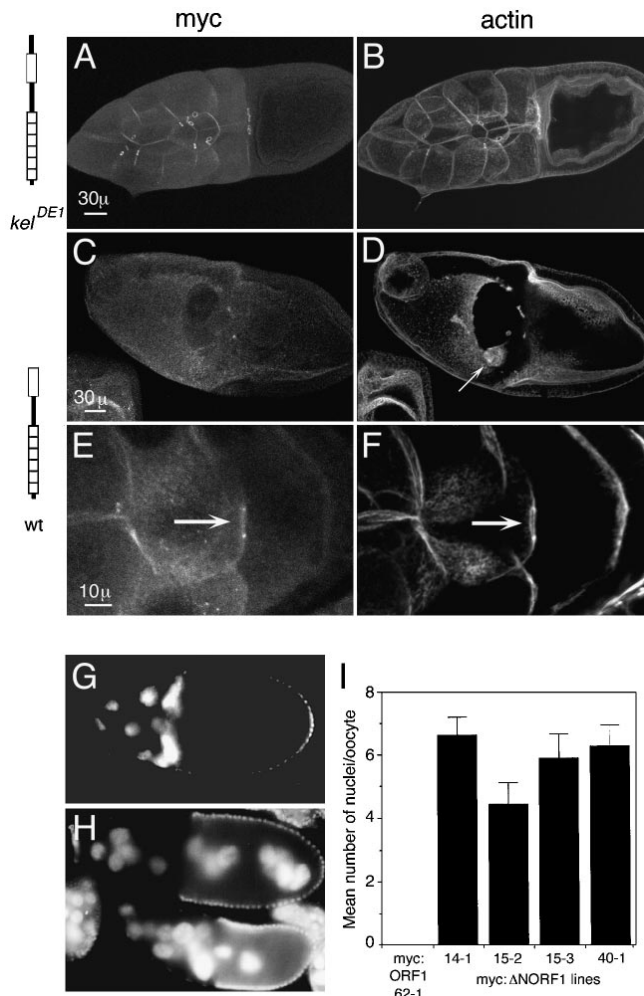


Figure 3. Dominant-negative effect caused by *myc:ΔNORF1*. (A) Myc:ORF1 localizes normally to ring canals. Some protein is also observed localizing to the cortical cytoskeleton. (B) The ring canals and nurse cell plasma membranes in *kel^{DE1}* egg chambers that express *myc:ORF1* are intact and normal, as seen by visualizing filamentous actin. (C–F) Wild-type egg chambers expressing *myc:ΔNORF1*. (C) In wild-type stage 10 egg chambers that express *myc:ΔNORF1*, the nurse cell plasma membranes are severely disrupted and in this case missing. No ring canals are observed by staining for *myc:ΔNORF1*. (D) Actin staining of the same egg chamber as in C verified that the nurse cell membranes are completely disrupted. The border cells (arrow) have migrated toward the oocyte but are mislocalized, probably because there is no place for them to dock. (E) An occasional ring canal persists in this stage 9 egg chamber, and *myc:ΔNORF1* (arrow) can be seen localizing to it. (F) Actin is also localized to the same thin ring canal. Cortical actin is also seen on some of the nurse cell membranes. (A, C, and E) Anti-myc staining to detect the transgene product. (B, D, and F) Rhodamine-conjugated phalloidin staining to detect filamentous actin. (G) A wild-type egg chamber expressing *myc:ORF1* has normal positioning of the nuclei in the nurse cells. (H) Wild-type egg chambers expressing *myc:ΔNORF1* have nuclei transported into the oocyte. (G and H) Nuclei are detected by DAPI staining. (I) Comparison of the number of nuclei transported into the oocytes in four independent wild-type lines that express *myc:ΔNORF1*. No nuclei were observed transported into the oocytes in wild-type egg chambers that express *myc:ORF1* (line 62-1). 17–30 stage 10 or older egg chambers from 5–10 females of each line were counted for the averages. Histograms represent the means, and the error bars represent the standard error of the mean. The phenotype was observed in several additional trials although they were not quantitated. Bars: (A and C) 30 μm; (E) 10 μm.

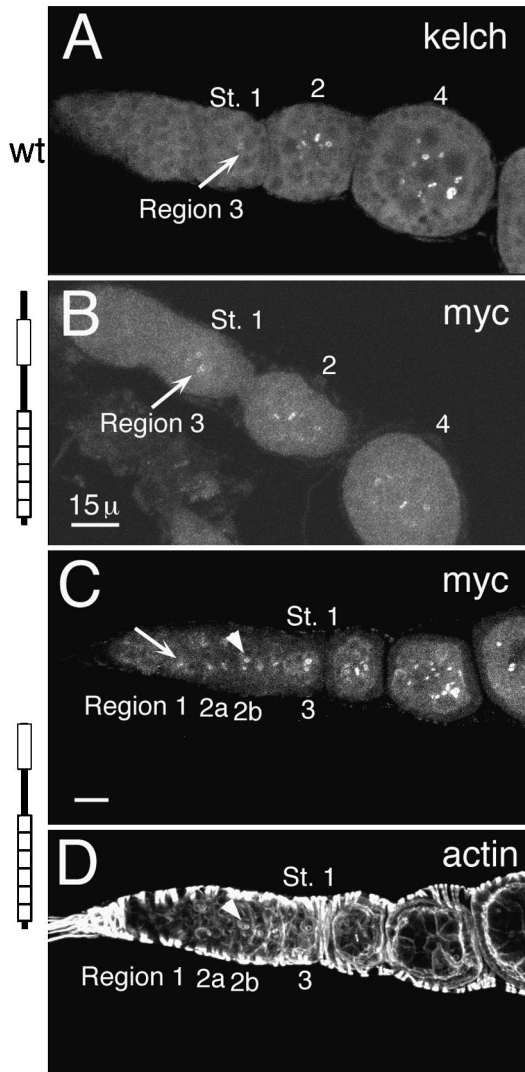


Figure 4. *Myc:ΔNORF1* localizes to ring canals earlier than normal. (A) Endogenous kelch localized to ring canals (arrow) beginning in region 3/stage 1. It takes until stage 4 for it to be detected on all of the ring canals in the egg chamber. This ovariole was stained with anti-kelch 1B antibodies. (B) Myc:ORF1 began to localize to ring canals (arrow) also in region 3/stage 1 egg chambers. It also took a few stages to reach all of the ring canals in the egg chamber. (C) Myc:ΔNORF1 began to localize to region 1 ring canals (arrow). The ring canals identified by the arrowhead correspond to those identified by the arrowhead in D. B and C were stained with anti-myc 9E10 antibodies to recognize the transgene products. (D) The same germarium as in C stained with rhodamine-conjugated phalloidin reveals the accumulation of the robust inner rim of actin filaments. The inner rim of actin filaments begin to accumulate by region 2a but are not really obvious until region 2b (arrowhead). The germaria in B–D are *kel^{DE1}* mutants expressing the respective transgene. Bars, 15 μm.

represent the standard error of the mean. The phenotype was observed in several additional trials although they were not quantitated. Bars: (A and C) 30 μm; (E) 10 μm.

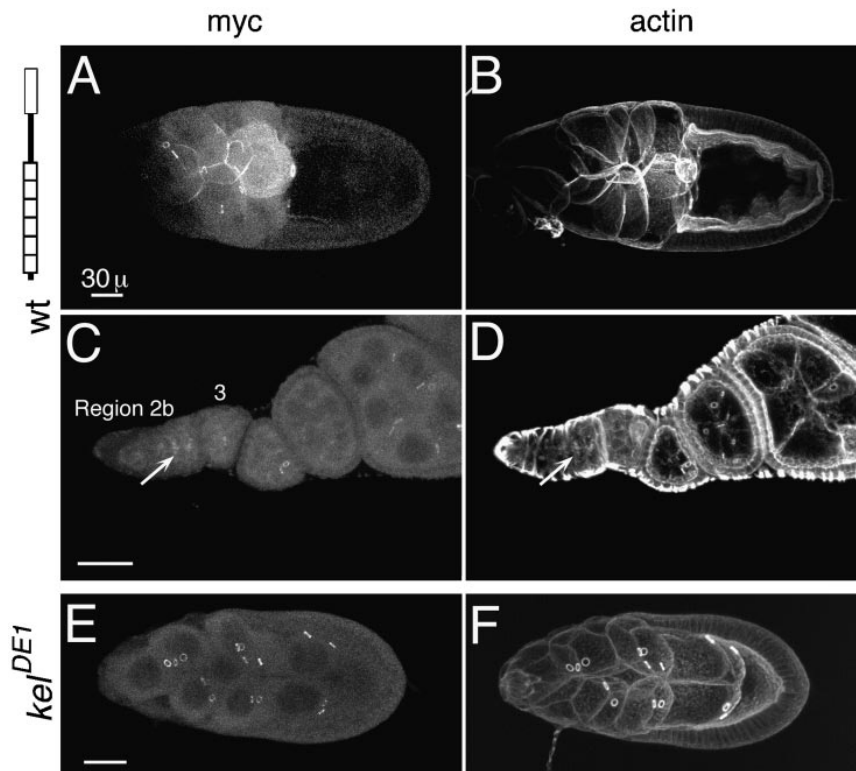


Figure 5. Myc: Δ NORF1 expressed at a low level can rescue *kel*^{DE1} mutants. (A) Myc: Δ NORF1 15-2 expresses myc: Δ NORF1 at a lower level and localizes to ring canals of a wild-type stage 10 egg chamber. (B) The same egg chamber as in A stained for actin shows intact nurse cell membranes and many intact ring canals. (C) The same line myc: Δ NORF1 15-2 in a wild-type background expressed myc: Δ NORF1 that localized to region 2b ring canals (arrow), which is earlier than normal, but the protein was not as abundant. (D) Actin staining of the same germarium as in C also showed actin localization to ring canals (arrow) at the same time as myc: Δ NORF1. (E) Myc: Δ NORF1 15-2 in a stage 9 *kel*^{DE1} egg chamber rescued the ring canals and egg chamber morphology. Myc: Δ NORF1 protein localized to ring canals normally. (F) Actin staining of the same egg chamber in E revealed intact nurse cell membranes and ring canals with a robust, well organized actin inner rim. A, C, and E were stained with anti-myc 9E10 antibodies. Bars, 30 μ m.

we expressed ORF1 minus the kelch repeat domain (myc:ORF1-R) and ORF1-R minus the NTR (myc:BTBIVR) in the ovary. Each of these proteins localized to ring canals in wild-type flies but failed to localize in *kel*^{meo} or *kel*^{DE1} flies (Fig. 6, B–E). ORF1-R also localized to ring canals in *kel*^{DE1} flies when nonepitope-tagged ORF1-only protein or full length kelch proteins, generated by substituting an alanine (alanine full length) or serine (serine full length) residue for the UGA stop codon or by deleting the stop codon (Δ UGA), were provided transgenically (Table I). This indicates that ORF1-R and BTBIVR proteins require a KREP-containing kelch protein for ring canal localization. To test whether KREP is sufficient for localization, we expressed the kelch repeat domain (myc:KREP). We did not detect localization of myc:KREP to ring canals in wild-type egg chambers, but in *kel*^{meo} and *kel*^{DE1} egg chambers, it localized to ring canals (Fig. 6, F and G). This indicates that KREP is sufficient for ring canal localization. A myc:IVRKREP protein also localized to ring canals only in *kelch* mutants (not shown). Finally, we asked whether ORF1-R could interact with the KREP domain or if it interacted with the amino half of kelch ORF1. We generated *kel*^{DE1} flies that expressed myc:ORF1-R and myc:KREP. Anti-myc antibodies gave ring canal staining as expected for myc:KREP. However, antibodies to the BTB domain (anti-kelch 1B) failed to stain ring canals, showing that myc:ORF1-R did not localize to ring canals (Table I). This indicates that ORF1-R interacts with the amino half of an intact kelch ORF1 protein to localize to ring canals.

In wild-type egg chambers expressing myc:ORF1-R, the myc:ORF1-R protein localized to slightly aberrant ring canals characterized by separations in the inner rim. This was most easily observed by anti-myc staining to detect the myc:ORF1-R protein. The actin and anti-hts-RC staining

did not show dramatic defects in the inner rim structure, only occasional separations in the inner rim (not shown; Fig. 1 B).

BTBIVR Is Required for Kelch Function

We examined the morphology of the ring canals from *kel*^{DE1} mutant egg chambers expressing each of the truncated kelch proteins that localize to ring canals to see what domains are required to organize the actin cytoskeleton. Wild-type ring canals have actin-rich inner rims that define an open lumen through which cytoplasm is transported during oogenesis (Fig. 7 A). The *kelch* mutant ring canals have disorganized actin inner rims that partially occlude the lumen (Fig. 7 B). Myc:ORF1 localized to and rescued the morphology of *kelch* mutant ring canals (Fig. 7, C and D). In *kelch* mutants expressing a rescuing dose of myc: Δ NORF1 (from myc: Δ NORF1 15-2), myc: Δ NORF1 localized to and rescued the mutant ring canal morphology (Fig. 7, E and F). This suggests that the NTR region of kelch is not required for kelch's ring canal organizational activity. The BTBIVR region was required since myc:KREP localized to ring canals but was not capable of rescuing the *kelch* mutant ring canal morphology (Fig. 7, G and H).

Both BTB and IVR Regions Are Required for BTBIVR Localization

Since myc:BTBIVR localized to ring canals in wild-type egg chambers, it was possible that either the BTB or the IVR domains were required for this activity. We examined 47 independent lines that carry a myc:BTB transgene (Fig. 1 B). Several of the lines expressed myc:BTB as determined by Western analysis. However, none of the 47 lines

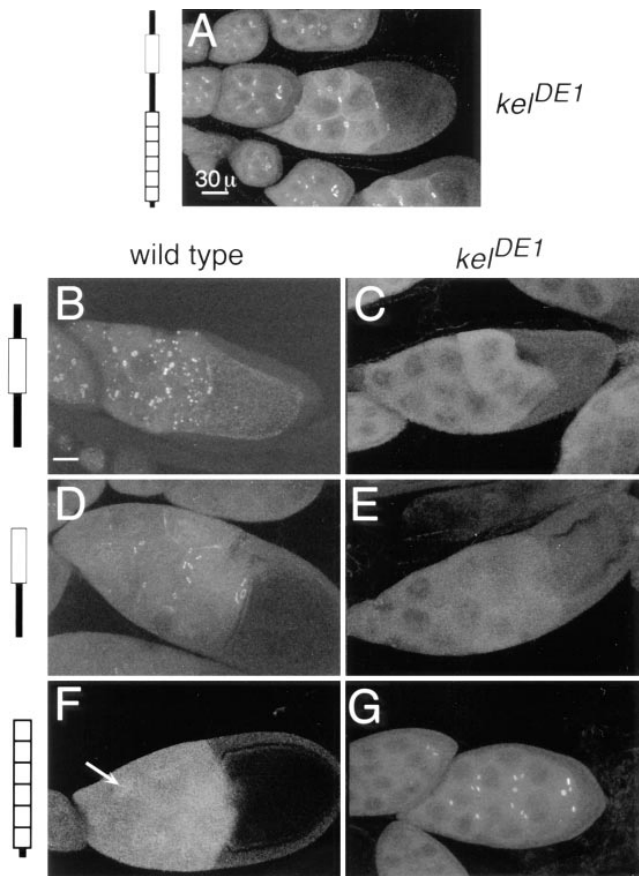


Figure 6. The BTBIVR region depends on an intact ORF1 for ring canal localization. The KREP domain is necessary and sufficient for ring canal localization. Myc:ORF1 localizes to and rescues *kel*^{DE1} ring canals (A). Myc:ORF1-R localized to ring canals in wild-type egg chambers (B) but not to ring canals in *kel*^{DE1} egg chambers (C). In wild-type egg chambers (B), myc:ORF1-R also formed punctate structures in the cytoplasm of the nurse cells. These structures contain no detectable PY protein, hts-RC, or actin (not shown) and appeared to be dependent on the dosage of myc:ORF1-R. Similar structures were seldomly observed in *kel*^{DE1} egg chambers (C). Myc:BTBIVR localized to ring canals in wild-type egg chambers (D) but not to ring canals in *kel*^{DE1} egg chambers (E). Myc:KREP was not detected on ring canals in wild-type egg chambers (F) but did localize to ring canals in *kel*^{DE1} egg chambers (G). Some nuclear localization (F, arrow) of KREP was observed in wild-type egg chambers. All egg chambers were stained with anti-myc 9E10 antibodies. Bars: (A) 30 μm; (B–G) 30 μm.

showed ring canal localization of myc:BTB (not shown). We generated 25 independent lines carrying a myc:IVR transgene and did not detect any localization of myc:IVR to ring canals in any of the 25 lines (Fig. 1 B). It was more difficult to demonstrate expression of myc:IVR by Western immunoblotting because we do not have an antibody directly against the domain, and we find that relatively high expression is required to detect the myc epitope by Western analysis using the anti-myc 9E10 antibodies (Evan et al., 1985). Some lines did have low level nuclear staining that was above background, suggesting that the transgene was expressed (not shown).

Table I. Myc:ORF1-R Localization Summary

Genetic background	myc:ORF1-R
Wild type	Ring canals
<i>kel</i> ^{neo}	Cytoplasm
<i>kel</i> ^{DE1}	Cytoplasm
ORF1 alone	Ring canals
Alanine full length	Ring canals
ΔUGA full length	Ring canals
Serine full length*	Ring canals
KREP	Cytoplasm

*Serine full length protein was tested in a *kel*^{neo}/*kel*^{DE1} background; all other transgene products were tested in a *kel*^{DE1}/*kel*^{DE1} background.

BTB Domain Is Sufficient for Dimerization In Vitro

The localization of myc:BTBIVR to ring canals in an ORF1-dependent manner is consistent with BTBIVR dimerizing with ORF1 through the BTB domain. This interpretation is consistent with the findings that the BTB domain (also called POZ domain) from several zinc finger-containing transcription factors dimerizes in vitro (Bardwell and Treisman, 1994; Chen et al., 1995). To test whether the kelch BTB domain can mediate dimerization, we expressed histidine-tagged BTB (his:BTB) and BTBIVR (his:BTBIVR) domains in *Escherichia coli* and purified the recombinant proteins by nickel (Ni²⁺) chromatography. Both proteins were expressed and could be extensively purified in one step using this recombinant expression system (Fig. 8).

We tested his:BTB for dimerization using dynamic light scattering. The predicted and the apparent (determined by SDS-PAGE) molecular masses of the his:BTB domain are each ~17 kD. Dynamic light scattering indicated that the protein sample was monodisperse with a mean translational diffusion coefficient of 792.5. This gave a Stokes radius of 2.9 nm and an estimated molecular weight of 41 kD. This is consistent with the protein forming dimers in solution. We examined the protein by gel filtration chromatography and compared its retention time to protein standards. Most of the protein had a relative molecular weight of 43 kD, which is again consistent with the protein existing as dimers. A small population of protein (<15%) had an apparent molecular weight comparable to tetramers.

His:BTBIVR was more difficult to analyze since it had a tendency to form aggregates and could not be concentrated >0.4 mg/ml. By gel filtration chromatography, his:BTBIVR separated into three populations. One population eluted from the column with a retention time consistent with monomers, a second population eluted as dimers, and a third population eluted in the void volume of the column consistent with either extremely large molecular weight forms (>500 kD) or aggregates.

Discussion

A Simple Model for Kelch Function

kelch mutant ring canals are highly disorganized and have additional actin filaments that extend into the canal partially obstructing cytoplasm transport (Robinson et al., 1994; Tilney et al., 1996). Although *Drosophila* kelch ORF1 is a member of a large family of kelch proteins, the

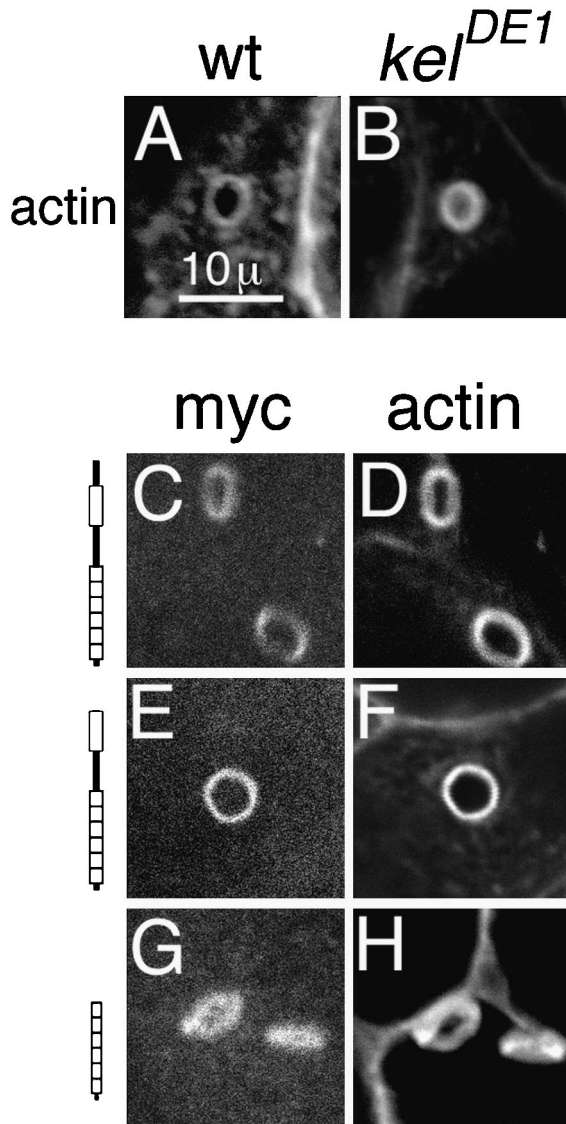


Figure 7. The BTBIVR region is required for kelch's ring canal organizer activity. Actin filaments in wild-type ring canals (A) are neatly organized in the ring canal inner rim, while actin filaments in *kel*^{DE1} ring canals (B) are disorganized and extend into the lumen of the ring canal. Myc:ORF1 localized to *kel*^{DE1} mutant ring canals (C) and organized the inner rim actin (D). Myc:ΔNORF1 produced from the myc:ΔNORF1 15-2 line also localized to *kel*^{DE1} mutant ring canals (E) and organized the actin filaments in the inner rim (F). Myc:KREP also localized to *kel*^{DE1} mutant ring canals (G); however, it was unable to rescue ring canal morphology (H).

biochemical functions for this family have not yet been discerned. However, two domains in kelch are also found in diverse nonkelch proteins. By comparison to the nonkelch proteins, a simple model for kelch function is that there are three interaction domains (Fig. 9). First, kelch might bind to ring canal actin filaments through KREP. Second, it dimerizes through the BTB domain, thereby crosslinking the actin filaments in the ring canal into the well organized inner rim. Finally, since kelch localizes to the ring canals, we would hypothesize that there is a third interaction of kelch that allows it to bind to the ring canal

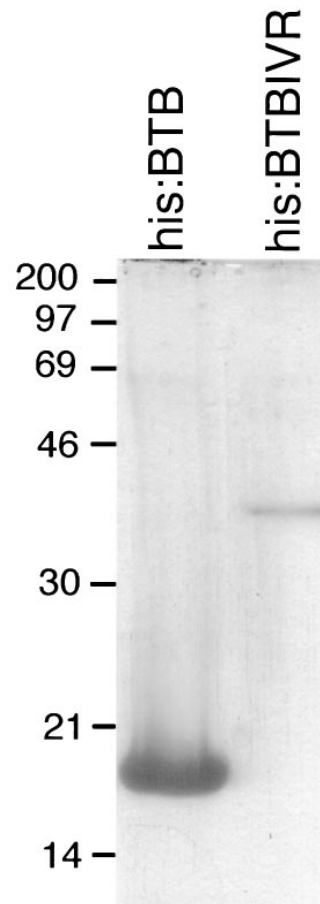
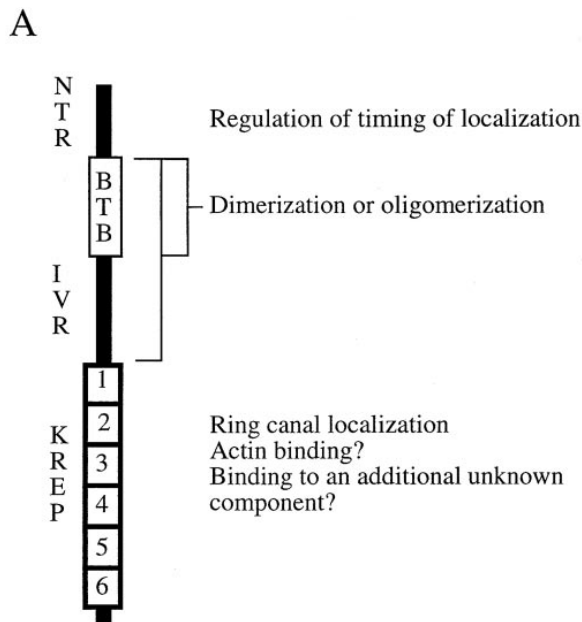


Figure 8. Purified his:BTB and his:BTBIVR proteins. The extensively purified proteins were analyzed by SDS-polyacrylamide gel electrophoresis followed by Coomassie brilliant blue staining. His:BTB had an apparent molecular mass of ~17 kD, and the his:BTBIVR protein had an apparent molecular mass of ~35 kD.

actin specifically. Here, we have performed a structure-function analysis to test various aspects of this model.

NTR Function

Because the NTR region is not present in other kelch family members, we tested whether the NTR is required for kelch function. The myc:ΔNORF1 15-2 line that expresses moderate amounts of protein was able to rescue *kelch* mutant sterility, restoring ring canal morphology. This indicates that the NTR domain is not strictly required for kelch's ring canal organizational activity. However, in highly expressing lines, myc:ΔNORF1 had a strong dominant-negative effect on ring canal stability, indicating that it has a very important function. There was a dramatic loss of plasma membrane stability in stage 6 and older egg chambers. Although it is possible that ΔNORF1 disrupts plasma membrane directly, we believe membrane instability is due to a specific defect in ring canal assembly. First, myc:ΔNORF1 is specifically localized to ring canals in early stages of oogenesis. It is not obviously localized to the cortical cytoskeleton until the time when the ring canals begin to break down. We have also detected some cortical localization of myc:ORF1 that does not result in a breakdown in plasma membrane integrity. Second, myc:ΔNORF1 shows earlier than normal ring canal localization correlating with myc:ΔNORF1's dominant-negative effect being due to a defect in ring canal assembly. Since myc:ΔNORF1 localized to region 1 ring canals, we speculate that the protein disrupts initial assembly of the ring canals



so that they are destabilized later. Supporting this idea, *myc:ΔNORF1*'s localization to region 1 ring canals is even earlier than *hts-RC* and the robust inner rim of actin that normally begins to accumulate by region (Robinson et al., 1994). These results suggest that *kelch* function is fairly tightly controlled and is not required, and in fact is not desirable, until after the initial assembly of the ring canal.

The NTR is apparently required to regulate the localization of *kelch* to ring canals. Since the *ovarian tumor* gene promoter in the pCOG vector provides germline expression beginning in the stem cell, one possible explanation for *myc:ΔNORF1*'s early localization is that it was transcribed earlier than normal. However, *myc:ORF1* expressed using the same pCOG vector had a wild-type time course for localization to ring canals. A second possible explanation is that there might be negative regulators of translation in the NTR region allowing *myc:ΔNORF1* to be translated earlier than normal. However, in high expressing lines of other *kelch* proteins that contain the NTR domain, we could detect the expression of unlocalized proteins in the cytosol in region 2 egg chambers (Robinson, D.N., and L. Cooley, unpublished observations). Therefore, the best explanation for *myc:ΔNORF1*'s early localization is that the NTR regulates the timing of protein localization. The NTR might accomplish this by interacting with an unknown protein that sequesters *kelch* in the cytosol until the time to localize. Alternatively, intramolecular interactions between the NTR and another domain in *kelch* could cause the protein to fold in such a way that it cannot bind to ring canals until the protein is activated. There are no known proteins with domains that have a high sequence homology to the NTR. The NTR is rich in asparagines, glutamines, and histidines having two stretches of polyglutamines including one six residues long and a second eight residues long. These stretches might mediate some of the interactions of the NTR. For example in the human protein, huntingtin, which has a long polyglutamine stretch, the polyglutamine tract is involved in protein-protein interactions (Li et al., 1995; Burke et al., 1996).

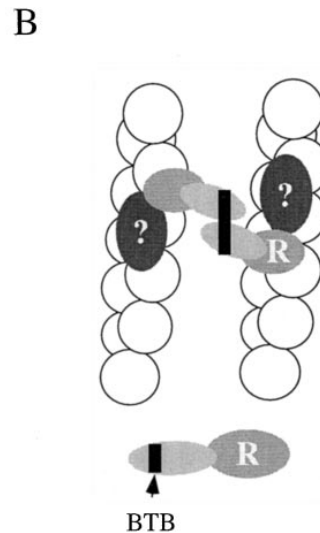


Figure 9. Kelch is an oligomeric ring canal actin organizer. (A) Summary of domain functions. (B) A proposed model for how *kelch* organizes the ring canal actin filaments. Through the KREP domain (R), *kelch* localizes to the ring canal possibly by binding to an unknown ring canal component (?). It likely binds to another component (possibly the actin filaments) also through the KREP domain. The protein forms cross-links through the BTBIVR region of the protein.

In addition to *myc:ΔNORF1*, a *cheerio* hypomorphic mutant (*cher²*) allows early assembly of the ring canals followed by ring canal degeneration (Robinson et al., 1997). This mutant allele also displays loss of nurse cell membrane integrity resulting in aberrant transport of nurse cell nuclei into the oocyte. However, the mechanisms of the defects in *cher²* compared to *myc:ΔNORF1* are different since in *cher²* egg chambers, *kelch* does not localize earlier than normal. Finally, *myc:ΔNORF1* has a much more dramatic and penetrant effect on ring canal and plasma membrane integrity than the *cher²* mutant has.

BTBIVR Mediates Oligomerization

The BTBIVR region was the minimal unit that localized to ring canals in an ORF1-dependent manner. Since ORF1-R failed to localize to ring canals that contained only KREP, the BTBIVR region must interact with the amino half of *kelch*. One model suggests that the BTBIVR region mediates oligomerization. Consistent with this, there are subtle defects in the ring canal inner rims of wild-type egg chambers expressing *myc:ORF1-R*, perhaps because some nonproductive complexes form. However, the *in vivo* experiments cannot distinguish between direct and indirect interactions. *In vitro*, purified recombinant *kelch* BTB and BTBIVR domains are capable of mediating dimerization. The fact that the BTB domain was not sufficient to bind ring canals *in vivo* might indicate that *kelch* BTB-mediated dimerization is a relatively low affinity interaction, and additional residues in the IVR region are required to form a high affinity interaction domain that can promote dimerization *in vivo*.

BTB (also called POZ) domain oligomerization has been analyzed *in vitro* for some of the transcription factors that have this domain. In gel shift assays, the proteins produced a shift expected for dimerization (Bardwell and Treisman, 1994; Chen et al., 1995). The amino-terminal 50 amino acids in the *bric à brac* (*bàb*) BTB domain were sufficient to mediate dimerization (Chen et al., 1995). How-

ever, in another zinc finger protein called ZID, the first 69 amino acids of its BTB domain were insufficient to dimerize (Bardwell and Treisman, 1994), indicating that there are differences in the way different BTB domains interact. Furthermore, some BTB domains have different specificities. For example, the tramtrack (ttk; Harrison and Travers, 1990) BTB domain formed homodimers or heterodimers with the GAGA transcription factor BTB domain (Soeller et al., 1993), while the ZID BTB domain did not interact with the ttk BTB domain (Bardwell and Treisman, 1994). The BTB domain is predicted to be largely α -helical, and α -helical wheel modeling reveals a hydrophobic face that could mediate the dimerization interaction (Chen et al., 1995). Two highly conserved, charged residues (corresponding to *Drosophila* kelch residues: D163 and R171; Xue and Cooley, 1993) map to the hydrophobic face. Mutations that changed the charge of these residues in the b α b BTB domain disrupted in vitro dimerization, while mixed populations with compensatory mutations in trans restored the interaction, suggesting that these residues are involved in electrostatic interactions (Chen et al., 1995). Clearly there are differences in the way this interaction domain functions in different proteins. Comparative structural studies are needed to elucidate the basis of BTB domain interaction.

In vivo, some BTB fusion proteins behave in a dominant-negative manner. Three alleles of *Drosophila pipsqueak* are caused by inappropriate splicing of the transcript that when translated produces a truncated BTB fusion protein. The truncated protein appears to be worse than having no protein at all (Horowitz and Berg, 1995) and can act dominant negatively if overexpressed from a transgene (Horowitz and Berg, 1996). In a rare translocation variant in humans that leads to acute promyelocytic leukemia, a BTB domain-containing zinc finger protein PLZF is fused to the retinoic acid receptor α (RAR α). The PLZF-RAR α acts dominant negatively by disrupting normal RAR α function in retinoic acid-sensitive myeloid cells (Chen et al., 1993, 1994). The BTB domain fusions might sequester proteins in nonproductive complexes or mediate other aberrant interactions contributing to their pathogenesis.

KREP Domain Function

The KREP domain is both necessary and sufficient for localization to ring canals. From sequence comparisons with α -scruin, which has two KREP domains (Way et al., 1995b) that each bind actin (Tilney, 1975; Bullitt et al., 1988; Owen and DeRosier, 1993; Schmid et al., 1994), we speculate that the kelch KREP domain might bind to ring canal actin filaments. Preliminary data indicates that the KREP domain can bind to actin filaments in vitro (Robinson, D.N., and L. Cooley, unpublished results). Since the KREP domain specifically binds to ring canals, we propose that there is at least one additional interaction with an unknown factor that promotes specificity for ring canals. Without the specificity interaction, it is unlikely that KREP would preferentially bind ring canal actin filaments over other actin in the nurse cells since ring canals contain a very small proportion of the actin in the cell. In addition, because myc: Δ NORF1 can localize to ring canals as early as region 1, the unknown factor is present on ring canals

before the addition of hts-RC and the actin filaments that form the inner rim. Since *cheerio* is required for kelch to localize to ring canals (Robinson et al., 1997), it could encode the factor that allows KREP to bind to ring canals.

Another example of a protein in which a kelch repeat domain might mediate two interactions is the *Physarum polycephalum* actin-fragmin kinase (AFK). In this protein, the 35-kD amino half of the protein is a novel protein kinase domain, and the 35-kD carboxy half consists primarily of six kelch repeats. This protein specifically phosphorylates actin in the actin-fragmin complex (Eichinger et al., 1996). Perhaps the AFK KREP domain recognizes sites on both actin and fragmin, giving it its specificity so that the kinase domain can phosphorylate its actin substrate.

Kelch's Ring Canal Organization Activity

Our data suggest that kelch has at least three activities: ring canal localization, dimerization, and ring canal organization. We have separated the ring canal organizational activity from the ring canal localization and dimerization functions using nonrescuing, full length kelch proteins. Previously, we expressed full length kelch proteins by creating alanine-substituted (alanine full length), serine-substituted (serine full length), and UGA-deleted (Δ UGA full length), full length kelch transgenes (Robinson and Cooley, 1997b). The alanine full length kelch was partially functional and was able to rescue the hypomorphic *kel^{neo}* allele but not the molecular null *kel^{DEI}* allele. Serine and Δ UGA full length kelch proteins could not rescue either *kelch* allele. All three of the mutant full length proteins were able to localize to ring canals, indicating that the ring canal specificity interaction is intact. Here, we observed each of the full length kelch proteins interacted with myc:ORF1-R allowing myc:ORF1-R to localize to ring canals. This indicates that the full length kelch proteins are capable of dimerizing normally. Consequently, the full length proteins are defective in a third interaction required for ring canal organization. Perhaps the 84-kD ORF2 domain in the full length protein sterically disrupts the proposed ability of the KREP domain to interact with actin, and the degree of steric hindrance depends on the residue substituted at the stop codon.

Ring Canal Growth

It has been speculated that ring canals increase their diameter from 3–4 μ m (stage 4) to 10 μ m (stage 11) by sliding the actin filaments with respect to one another (Robinson et al., 1994; Tilney et al., 1996). The density of actin filaments and the filament number per cross-sectional area are relatively constant (\sim 720) during these stages of ring canal development (Tilney et al., 1996). This implies that existing actin filaments must increase their length or additional actin filaments are gradually added during expansion to maintain the filament density. During actin filament sliding, cross-linking proteins could break and reform their associations with the actin filaments to facilitate expansion while maintaining ring canal organization. Since the concentration of actin in the ring canal inner rim is so high (millimolar range; Robinson and Cooley, 1997a), low affinity interactions with K_d 's in the μ M range can easily promote the reversibility of these interactions. Kelch is a

good candidate for doing just this, since in *kelch* mutants the ring canals become disorganized during these stages (Robinson et al., 1994; Tilney et al., 1996). The interactions that allow KREP to localize and BTBIVR to dimerize might be relatively high affinity, while the putative actin interaction site might form the hypothesized low affinity interaction site that maintains ring canal organization during growth.

Conclusion

These in vivo studies of kelch function support and extend our model for kelch activity. First, the dominant-negative protein that resulted from the deletion of the NTR was unexpected and points out how important temporal control and order of addition during ring canal assembly are. Identification of proteins that interact with the NTR should provide great insight into the events that regulate the ring canal cytoskeleton assembly. Second, our in vivo and in vitro data support a model in which the BTB domain mediates dimerization. However, in vivo, the BTB domain is not sufficient and requires sequences in the IVR region. Certainly, more complex interactions can be envisioned to explain the data, such as having an additional factor that promotes binding between the BTBIVR domains. In addition, since there are differences in the requirements for BTB domain dimerization from different proteins, further mutagenesis and structural studies are needed to elucidate the molecular basis of this interaction. Third, there are two remaining functions that can be assigned to kelch: ring canal localization and ring canal organization. Our results verify that the KREP domain provides ring canal localization and suggest that it might mediate the ring canal organizational function. Discovery of the binding partner that promotes ring canal localization may identify new ring canal components and will facilitate many new studies on how proteins are added to the ring canals. Ring canal organizational activity likely involves a direct or indirect interaction with the ring canal actin filaments. Experiments are in progress to test direct interactions with actin. Comparative functional studies on the KREP domain would be particularly informative since this motif is wide spread and there is considerable sequence divergence in many kelch-related proteins.

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