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Examination of the function of two kelch proteins generated by stop codon suppression

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SUMMARY

The *Drosophila kelch* gene produces a single transcript with a UGA stop codon separating two open reading frames (ORF1 and ORF2). From the transcript, 76 kDa ORF1 and 160 kDa full-length (ORF1 + ORF2) proteins are made. The expression of these two proteins is regulated in a tissue-specific manner causing the ratio of full-length to ORF1 protein to vary in different tissues. The only detected defect for *kelch* mutants is female sterility, and kelch protein is localized to the ovarian ring canals. *kelch* mutant ring canals are disorganized and have partly occluded lumens, causing a failure to transport cytoplasm. ORF1 and full-length kelch proteins co-sediment with ring canals suggesting that both proteins are found in the ring canals. Transgenetic analysis reveals that ORF1 kelch protein is sufficient to rescue ring canal morphology and fertility. In addition, we have mutated the UGA stop codon to a UAA stop codon and to three sense codons that allow constitutive readthrough. Analysis of these mutants reveals that a full-length kelch protein can partially compensate for the loss of endogenous kelch, but the residue included at the stop codon is critical for function. Finally, these studies suggest that the mechanism of stop codon suppression of kelch is by tRNA suppression.

Key words: ring canal, kelch, stop codon suppression, actin, *Drosophila*

INTRODUCTION

A particularly sensitive and rapid method of regulating gene expression occurs at the level of translation, allowing gene products to be generated without new transcription. This can enable cells to respond rapidly to environmental cues, giving them greater temporal control over their identity during development. Translation can be controlled by regulating translational initiation, by transient masking of mRNAs, or by regulating the polyadenylation of the mRNA (reviewed in Curtis et al., 1995).

Another mechanism of translational control of a gene's expression is through stop codon suppression or readthrough. A number of genes contain stop codons that create multiple open reading frames in the transcript. Cellular genes that have this structure can be found in organisms ranging from bacteria to mammals and in several retroviruses. In bacteria, archaebacteria and eukaryotes, the best characterized mechanism for suppression of an in-frame UGA stop codon is through incorporation of a selenocysteine (reviewed in Böck et al., 1991). Additionally, programmed frameshifting at a UGA stop codon has also been demonstrated for the cellular gene, ornithine decarboxylase antizyme (Matsufuji et al., 1995).

In vertebrates, stop codon suppression by selenocysteine incorporation is necessary to produce active enzymes such as glutathione-peroxidase (Chambers et al., 1986) and type I (Berry et al., 1991a), type II (Davey et al., 1995) and type III (Salvatore et al., 1995) iodothyronine deiodinases. Selenoprotein P is especially interesting because it contains ten in-frame UGAs at which selenocysteines are incorporated (Hill et al., 1991). Selenoprotein P and another protein, selenoprotein W (Vendeland et al., 1995), which contains only one selenocysteine, have unknown functions. The mammalian sperm protein, mitochondrial capsule selenoprotein (MCS), contains three selenocysteines encoded by UGAs and likely provides a structural role in the mitochondrial sheath, perhaps by forming multiple disulfide and diselenide bonds (Karimpour et al., 1992).

Retroviruses such as Moloney murine leukemia virus normally suppress a UAG stop codon to produce the gag-pol fusion protein. UAA and UGA can also be suppressed in this context (Feng et al., 1989b). Suppression of the endogenous UAG or the UAA stop codon results in incorporation of a glutamine, but suppression of UGA allows incorporation of arginine, cysteine or tryptophan in the gag-pol fusion protein. The translation of gag-pol fusion proteins in mouse cells demonstrates that the necessary machinery exists in mammalian cells to suppress all three stop codons, provided that the necessary cis-acting elements are present (Feng et al., 1989a, 1990).

In *Drosophila*, *kelch* (Xue and Cooley, 1993) and *out at first* (*oaf*) (Bergstrom et al., 1995) produce transcripts with a single in-frame UGA stop codon. The protein products of the *oaf* gene have not been characterized (Bergstrom et al., 1995). *kelch* produces a 76 kDa open reading frame 1 (ORF1) product and a 160 kDa full-length (ORF1 + ORF2) product (Fig. 1A). The expression and function of the kelch proteins are the subject of this paper.

In *Drosophila*, *kelch* is required in the ovary for production of viable eggs. *kelch* mutants are female sterile as a result of improper cytoplasm transport throughout oogenesis. Antibodies generated against kelch ORF1 recognize the ORF1 and full-length kelch proteins and immunostain the ring canals in the *Drosophila* egg chamber (Xue and Cooley, 1993). Ring canals provide the intercellular conduits through which cytoplasm is transported from the nurse cells to the oocyte in an egg chamber (reviewed in Robinson and Cooley, 1996). Examination of *kelch* mutant ring canals revealed that the actin filaments in the ring canal 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 the ring canals until the end of oogenesis, the ring canals expand from a diameter of 3-4 μm to 10 μm. 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).

Drosophila kelch ORF1 is a member of a growing family of kelch-related proteins that includes several Pox virus ORFs (Koonin et al., 1992; Senkevich et al., 1993), *Caenorhabditis elegans* spe26 (Varkey et al., 1995) and several mammalian proteins including calicin (von Bülow et al., 1995). Kelch ORF2 has no homology to other proteins in the database. Kelch ORF1 contains two conserved domains found in other kelch proteins as well as in non-kelch proteins. ORF1 contains a 120-amino-acid motif called a BTB or POZ domain that is inset from the amino terminus about 100 amino acids. This domain is also found in several zinc-finger-containing transcription factors (Harrison and Travers, 1990; DiBello et al., 1991; Chardin et al., 1991; Zollman et al., 1994) and it has been 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 (Xue and Cooley, 1993). Kelch repeats are found in several non-kelch proteins including the recently characterized *Physarum polycephalum* actin-fragmin kinase (Eichinger et al., 1996). 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). β-scruin, on the other hand, is found in the acrosomal vesicle and does not appear to associate with actin (Way et al., 1995a). An attractive hypothesis for *Drosophila* kelch function is that it associates with the ring canal actin filaments through the kelch repeats and cross-links them by dimerizing through the BTB domain.

In this work, we have characterized the developmental expression pattern of kelch and found that suppression of the UGA stop codon is regulated. We provide evidence that both ORF1 and full-length kelch proteins associate with the ovarian ring canal complex. Finally, we dissect the functions of ORF1 and full-length kelch proteins and study codon specificity of the stop codon suppression mechanism of *kelch*. The primary conclusion of these experiments is that the ORF1 kelch protein is sufficient for *kelch* function in *Drosophila*.

MATERIALS AND METHODS

Fly strains

w^{1118} (Lindsley and Zimm, 1992) flies were used as wild type in these experiments. kel^{neo} was described by Xue and Cooley (1993). kel^{DE1} and kel^{PL25} were isolated by Schüpbach and Wieschaus (1991). kel^{PL25} produces no detectable kelch protein (not shown). Deficiency strain *Df(2L)TW137*, which uncovers the *kelch* locus, was obtained from the Bloomington *Drosophila* Stock Center at Indiana University. Fly stocks were maintained under standard conditions.

Western analysis

Drosophila ovaries were dissected in *Drosophila* IMADS buffer (Singleton and Woodruff, 1994) and ground in 1× Laemmli's buffer under reducing conditions. Protein concentrations of the ovary extracts were measured using the BioRad Protein Assay. Proteins separated by SDS-polyacrylamide gel electrophoresis were transferred to Hybond-ECL nitrocellulose (Amersham). Following transfer, membranes were blocked in Blotto-Tween (5% powdered milk, 0.2% Tween and PBS) for 2 hours, then incubated in hybridoma cell supernatant (1:10 in Blotto-Tween) for 1 hour to overnight. 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, we used anti-myc 9E10 hybridoma supernatant (Evan et al., 1985). Membranes were washed four times for 10 minutes in 0.2% Tween in PBS at room temperature and incubated for 30 minutes at room temperature in goat anti-mouse IgG secondary antibody conjugated to horseradish-peroxidase (Pierce Chemical Co.) in Blotto-Tween (1:10,000). After four washes, signals were detected using ECL western blotting detection reagents (Amersham) according to manufacturer's specifications.

Ring canal fractionation and sucrose gradient centrifugation

Egg chamber starting material was isolated either by hand dissection or by a mass isolation procedure adapted from Mahowald et al. (1983) and Theurkauf et al. (1992). Flies produced from 1.5 g of embryos were anesthetized using CO_2 and collected. The flies were ground in batches in 200-300 ml of *Drosophila* IMADS buffer (Singleton and Woodruff, 1994). Grinding involved several 2-3 second pulses in a Hamilton Beach blender. The resulting slurry was poured through a series of metal sieves with 500 μm and 250 μm pore sizes (Tyler, Inc.)

to remove fly parts. The filtrate was allowed to settle then the supernatant was decanted. Rounds of settling and decanting eventually yielded as much as a 30 ml bed volume of tissues that were as much as 60-80% egg chambers.

5 ml of egg chambers were ground in a dounce homogenizer in 15 ml of ring canal buffer (RC buffer): 200 mM NaCl, 50 mM Tris pH 7.5, 3 mM $MgCl_2$, 1% Triton X-100, 0.1 mM PMSF (Sigma), 1.35 TIU aprotinin (Sigma), 12 units of rhodamine-conjugated phalloidin (Molecular Probes). The extracts were incubated on ice for 30 minutes. After incubation, they were filtered through Nitex nylon mesh (Tetko, Inc.) with pore sizes of 500 μm, 250 μm, then 10 μm. The filtrate was centrifuged for 15 minutes at 1200 rpm in a Sorvall GLC-2 clinical centrifuge. The supernatant was decanted and the pellet was recovered. The pellet was resuspended in 500 μl of RC buffer.

For sucrose density gradient centrifugation, 55%-65% sucrose gradients were prepared using a Hoefer SG25 gradient maker, a peristaltic pump and a Buchler Auto Densi Flow II gradient collector. Sucrose solutions were prepared in 200 mM NaCl, 50 mM Tris pH 7.5 and 3 mM $MgCl_2$. 12 ml gradients were made and the 500 μl resuspended ring canal pellet was layered onto the gradient. The gradients were centrifuged in a Beckman SW 41 Ti rotor at 20,000 rpm (68,530 ***g***) for 4 hours (6.24×10^{10} ω^2t). Fractions were collected using the Buchler Auto Densi Flow II gradient collector, a peristaltic pump and an LKB fraction collector. 32 12-15-drop fractions were collected. Even-numbered fractions were diluted in RC buffer and TCA precipitated. Pellets were washed three times in acetone and resuspended in 40 μl of 2× Laemmli's buffer. 0.05 N NaOH was added to raise the pH of the samples. 15 μl of each sample were examined for the presence of kelch and hts-RC proteins by western immunoblot detection. Odd fractions were examined by fluorescence microscopy for the presence of ring canals.

Construct generation

pCOG (Fig. 3A) contains the *ovarian tumor* (*otu*) gene promoter that was demonstrated to drive expression of a β-galactosidase reporter gene throughout oogenesis, beginning in the stem cell (Comer et al., 1992; Rodesch et al., 1995). We received an *Mlu*I genomic fragment that contains the *otu* gene from Pam Geyer. An *Eco*RI-*Hpa*I fragment that corresponds to nucleotides 1 to 740 from the *otu* genomic sequence (Steinhauer et al., 1989) was isolated. The *Eco*RI-*Hpa*I fragment contains most of the 548 bp fragment that has been shown to direct expression of *E. coli lacZ* in the *Drosophila* ovary (Comer et al., 1992) and the *Eco*RI-*Hpa*I fragment itself has been shown to drive expression of *lacZ* in the ovary (Rodesch et al., 1995). The *Eco*RI-*Hpa*I fragment was cloned into the *Eco*RI and *Hpa*I sites of pCaSpeR 2 transformation vector (Pirrotta, 1988). The *Eco*RI site was destroyed by a Klenow fill-in reaction. pCOG also contains the *fs(1)K10* 3′ untranslated region (UTR) cloned from the *Not*I-*Pst*I fragment of pGerm8 (Serano et al., 1994). This fragment includes a polyadenylation signal sequence but does not include the oocyte localization signal found in the endogenous *fs(1)K10* 3′UTR. These sequences were built into the pCaSpeR 2:*otu*. A *Bgl*II site that remained in the polylinker was destroyed by a Klenow fill-in reaction and an *Eco*RI site was added using *Eco*RI linkers (New England Biolabs).

To generate pCOG:wtKcD, the *kelch* cDNA clone, NBCD22 (Xue and Cooley, 1993), was isolated from pNB40 (Brown and Kafatos, 1988) by digesting with *Hin*dIII, filling in the overhanging ends and digesting with *Not*I. The resulting NBCD22 fragment includes 44 bp of the Xenopus β-globin 5′UTR, 96 bp of *kelch* 5′UTR, the entire coding region of *kelch*, the *kelch* 3′UTR and polyadenylation sequence. This was ligated into the *Hpa*I and *Not*I sites of pCOG.

To generate pCOG:ORF1, we started with a construct we had made previously, in which the NBCD22 *Hin*dIII-*Not*I fragment had been cloned into pCaSpeR-hs (Pirrotta, 1988) as was described for pCOG:wtKcD. An *Eco*RI site exists just 5′ to the NBCD22 *kelch* cDNA. ORF1 was removed from pCaSpeR-hs:wtKcD by digesting the plasmid with *Eco*RI and *Msc*I. An *Msc*I site is found 43 bp 3′ to the UGA stop codon at position 2343 (Xue and Cooley, 1993) that separates ORF1 from ORF2. This fragment was cloned into pCOG vector that had been prepared by filling in the *Not*I site after digestion and digesting with *Eco*RI.

To generate pCOG:UAA, Alanine, ΔUGA and Serine mutants, the UGA stop codon at position 2343 (Xue and Cooley, 1993) was mutated by site-directed PCR mutagenesis using the gene splicing by overlap extension method (Horton et al., 1989). Appropriate oligonucleotides that complement the sequence flanking the UGA stop codon but include the desired mutations were used. The TGA was changed to TAA for the UAA mutant, changed to GCA for the Alanine mutant, deleted for the ΔUGA mutant, and changed to TCA for the Serine mutant. All PCR fragments were screened for unwanted random PCR mutations and the desired mutations were verified by dideoxynucleotide sequencing. We replaced the UGA-containing wild-type DNA restriction fragment in pCOG:wtKcD with the respective mutant restriction DNA fragment to generate the appropriate mutant transformation plasmid.

To express epitope-tagged ORF-specific products in the fly ovary, we prepared a myc epitope-tagging cassette (KNmyc) that starts at a *Hpa*I site 81 bp upstream in the kelch 5′UTR and includes the first 19 amino acids of kelch containing 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:

*Eco*RI	*Nde*I	*Xho*I	*Bgl*II	Stop	*Not*I
GAA TTC	CAT ATG	CTC GAG	GCA GAT CTG	TAA	GCGGCCGC

The *Hpa*I-*Not*I cassette was subcloned into pCOG. ORF1-and ORF2-only cDNAs were prepared with back-to-back *Eco*RI and *Nde*I sites at the 5′ end of the fragments. A *Bam*HI site was added to the 3′end of the ORF1 cDNA fragment. ORF2 had a *Not*I site in the 3′UTR. ORF1 cDNA for Nmyc-ORF1 encoded residues Ser17 to Met688. ORF2 cDNA for Nmyc-ORF2 encoded Met690 to Asp1476. The amino acid numbers correspond to the sequence in Xue and Cooley (1993).

Generation of transgenic animals

Transformation plasmids were microinjected along with the Δ2-3 transposase helper plasmid into w^{1118} syncytial blastoderm embryos, according to standard techniques. Transgenic flies were identified by the complementation of the *white* eye phenotype by the *white* minigene in pCOG. P element insertions were mapped to respective chromosomes. They are maintained in a wild-type background as well as in kel^{neo} and kel^{DE1} backgrounds.

Immunolocalization and confocal imaging

Whole ovaries were dissected in *Drosophila* IMADS buffer (Singleton and Woodruff, 1994), fixed in 1% formaldehyde saturated with heptane, rinsed three times in PBS and washed three times for 10 minutes in PBT (0.3% Triton X-100 (Sigma), 0.5% BSA (Sigma) in PBS) at room temperature. For actin visualization, ovaries were incubated in 1-2 units of rhodamine-conjugated phalloidin (Molecular Probes) for 2 hours at room temperature. For antibody staining, ovaries were incubated in hts-RC antibody hybridoma supernatant, anti-hts-RC 655 4B (Robinson et al., 1994) (1:1), kelch hybridoma supernatant, anti-kelch 1B (Xue and Cooley, 1993) (1:1), or anti-myc 9E10 hybridoma supernatant (Evan et al., 1985) (1:1). The ovaries were incubated with rocking for 2 hours at room temperature, then overnight at 4°C. After washing four times for 15 minutes in PBT, bound antibodies were detected using a goat anti-mouse IgG secondary antibody conjugated to fluorescein isothiocyanate (Jackson Laboratories). Immunolocalizations were visualized by collecting 1-2 μm optical sections on a laser-scanning confocal microscope (BioRad MRC 600), then optical sections were compiled and

displayed using the CoMOS software package (BioRad). A 25× (0.8 NA), 40× (1.0 NA) or 63× (1.4 NA) objective was used in each case. Images were processed using Adobe Photoshop.

Egg production and embryo survival analysis

Groups of 10-25 virgin females were collected and placed in an embryo collection bottle with approximately 10 wild-type males. The flies were aged for 2-3 days before beginning to collect embryos. The flies were fed small amounts of wet yeast on molasses agar plates that were changed daily. Embryos were collected for approximately 24 hour intervals (exact time periods were recorded) in order to eliminate any effects of daily variations in egg production. The number of embryos was counted. Hourly egg production per female was calculated. Percentage survival to 1st instar larvae was determined by counting the number of unhatched embryos remaining after 48 hours. For statistical analysis, N = one day's collection of embryos. Each N generally represents a group of two to several hundred embryos. All results are listed as the mean±s.e.m.

Metabolic labeling of pupal extracts with ^{75}Se

Approximately 30 embryos were loaded into a new vial containing standard fly media. 150 μCi of isotopic sodium selenite ($Na_2{}^{75}SeO_3$; Amersham) were mixed in a dollup of wet yeast paste and added to the vials. The embryos were allowed to mature to mid-pupal stage. Pupae were collected, rinsed and whole extracts were prepared in Laemmli's sample buffer for SDS-PAGE. The gels were dried on Whatman's 3M paper and exposed to film.

RESULTS

Expression of two proteins

The *kelch* locus produces a single transcript that encodes two ORFs separated by a single UGA stop codon (Fig. 1A). Antibodies generated against the BTB domain of the ORF1 protein recognized the 76 kDa ORF1 and the 160 kDa full-length (ORF1+ORF2) protein species in whole ovary extracts (Xue and Cooley, 1993; Fig. 1B). The ratio of full-length kelch to ORF1 kelch proteins was approximately 1:20. Both of these proteins were disrupted by mutations in the *kelch* locus (Xue and Cooley, 1993).

Characterization of the developmental expression pattern revealed that, in the adult, most of the kelch protein was found in the ovary (Fig. 1B). However, in female adult fly carcasses from which the gonads were removed, both proteins were present but at a ratio of approximately 1:1 (Fig. 1B). A similar ratio and level were seen in the male carcasses and little detectable protein was seen in the male gonad (not shown). This suggested that suppression of the stop codon could be controlled in a tissue-specific manner.

Kelch was expressed throughout development; however, during metamorphosis, the ratio of full-length to ORF1 was again high compared to the adult ovary (Fig. 1B, compare pupae to ovary). Since *kelch* mutations affect the ovary, we suspected that the major source of the increase in full-length protein relative to ORF1 was due to expression in the pupal ovary. If this was the case, the expression should primarily be in female pupae. To test this, third instar larvae were collected and separated into males and females. These were allowed to pupate and synchronized by testing for the formation of air bubbles that form at 4 hours of metamorphosis (Roberts, 1986). Pupae were aged and then protein extracts were prepared. In late third instar larvae (3L), ORF1 and a small amount of full-length protein was observed in both males and females (Fig.

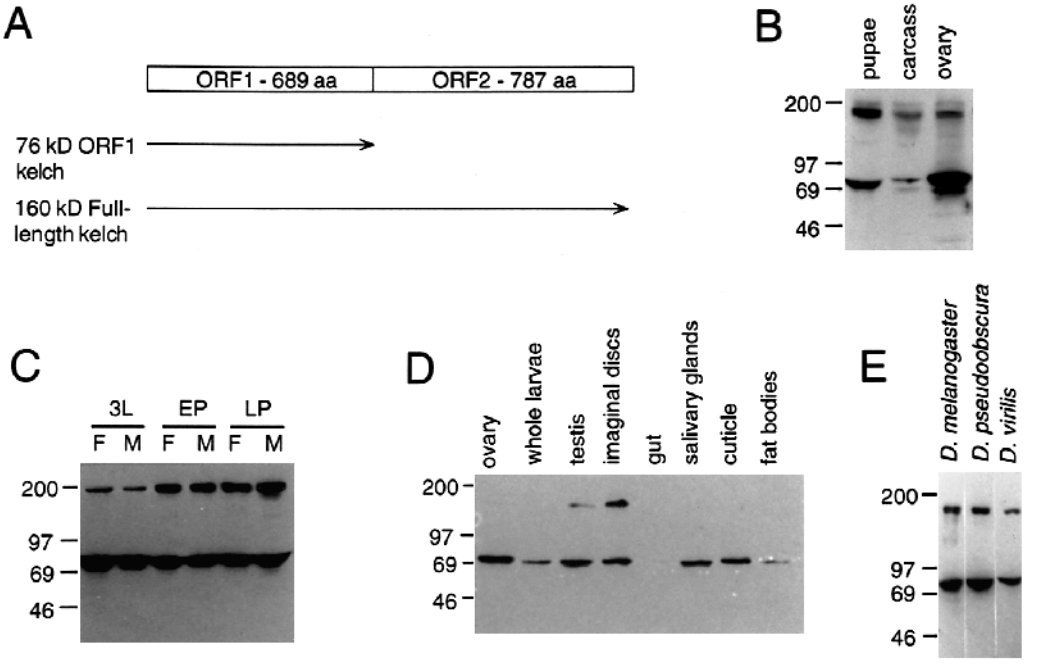

Fig. 1. Two proteins produced from the *kelch* transcript. (A) *kelch* produces a single message that encodes a 76 kDa ORF1 product and a 160 kDa full-length product through stop codon suppression. (B) Western blot of protein extracts from different tissues reveals different ratios of full-length kelch to ORF1 kelch. Pupae, adult female carcasses lacking ovaries, and adult ovary are shown. 40 μg of protein were loaded in each lane. (C) The ratio of full-length to ORF1 protein increases with entry into metamorphosis. Western blot of protein extracts from female (F) and male (M) third instar larvae (3L), female (F) 24-27 hour pupae (EP), male (M) 21-24 hour pupae (EP), and female (F) and male (M) 58-69 hour pupae (LP). One quarter of an animal equivalent was loaded per lane. (D) The ratio of full-length to ORF1 kelch varies in third instar larval tissues. The ratio is increased in larval testis and imaginal discs. The ovary lane was prepared from adult ovary. Whole larvae, testis, imaginal discs (includes accompanying larval brain), gut, salivary gland, cuticle and fat bodies were collected from third instar larvae. 20 μg of protein were loaded in each lane. (E) Two kelch proteins are produced in related *Drosophila* species. Western blot of whole pupae protein extracts using anti-kelch 1B monoclonal antibodies reveals ORF1 and full-length kelch proteins in related *Drosophila* species. Approximately 1/4 pupae equivalent was used for *D. melanogaster*, and 3/8 pupae equivalent for *D. pseudoobscura* and *D. virilis* were used.

1C). In early pupae (EP), full-length kelch increased relative to ORF1 in both males and females. This increase continued into late metamorphosis (LP) in both males and females (Fig. 1C). Since the ratio of full-length protein to ORF1 was elevated in both males and females, pupal ovary-specific expression was ruled out. To determine which tissues were expressing elevated levels of full-length kelch, we dissected late third instar larvae and separated respective tissues. Western analysis of extracts of each tissue type revealed that imaginal discs, the presumptive adult tissue, showed an increase in full-length kelch relative to ORF1 (Fig. 1D). Larval testis also showed a slight increase compared to other tissues. Longer exposures revealed some full-length kelch in adult ovary, whole larvae extracts, salivary gland and cuticle. We performed immunofluorescence experiments to determine the subcellular localization of the kelch proteins in imaginal discs. There was some enrichment of the kelch proteins subcortically in the epithelia of the imaginal discs, although the antibodies had a relatively high background in the absence of highly concentrated localization (not shown).

Since the *kelch* transcript has such an unusual structure, we were curious as to whether the two protein motif was conserved across *Drosophila* species. We prepared whole pupal extracts from several *Drosophila* species (*D. melanogaster*, *D. yacuba*, *D. pseudoobscura*, *D. willistoni* and *D. virilis*) and found that all species tested made both a 76 kDa ORF1 product and a 160 kDa full-length product (Fig. 1E, only *D. melanogaster*, *D. pseudoobscura* and *D. virilis* shown). The kelch antibodies recognized ring canals in *D. virilis* egg chambers (not shown), so it is likely that the function of *kelch* in *Drosophila* species is conserved. We infer from the protein expression pattern that the *kelch* gene structure is likely to be conserved.

Two kelch proteins co-sediment with fractionated ring canal complexes

To determine which of the kelch proteins is found in the ring canal complex, we first tried to make antibodies to the ORF2 protein. After several attempts with three different antigens from the ORF2 protein, no antibodies were obtained that produced a strong, dependable response. Consequently, to determine which of the kelch proteins was found in the ring canal complex we performed fractionation experiments to investigate which would co-sediment with ring canals. The ring canal complexes were separated from either hand-dissected or mass-isolated egg chambers using a non-ionic detergent buffer, and extracts were filtered to remove many of the nurse cell and follicle cell nuclei. The extracts were then centrifuged gently to sediment ring canals and other

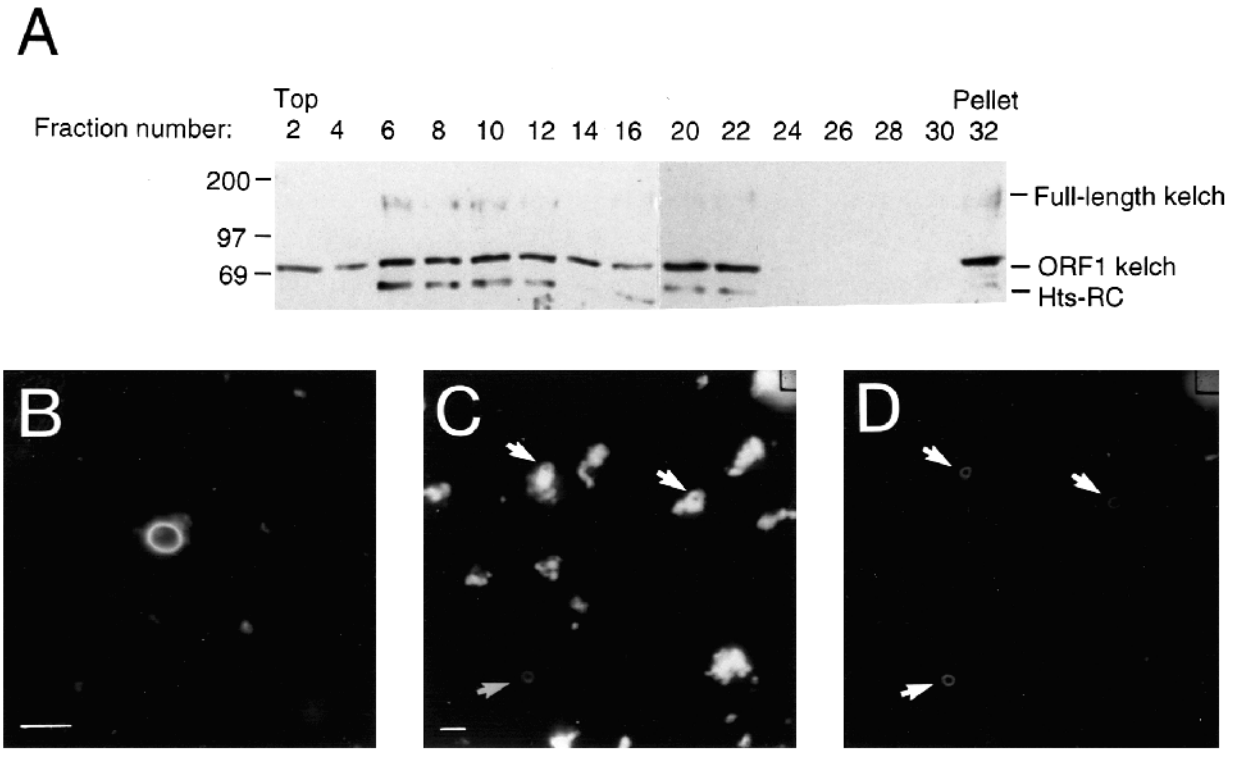

Fig. 2. ORF1 and full-length kelch proteins co-sediment with ring canals. (A) ORF1 and full-length kelch proteins and hts-RC proteins co-fractionate and co-sediment on sucrose density gradients. A cytoskeletal fraction containing ring canals was isolated and sedimented on a 55%-65% sucrose density gradient. Fractions were collected and examined by western analysis using anti-kelch 1B and anti-hts-RC monoclonal antibodies and fluorescence microscopy. Ring canals were observed in fractions 6-22 and in fraction 32. Equivalent amounts of each fraction were loaded in each lane. (B) An example of an intact ring canal extracted from egg chambers. Ring canals were extracted in the presence of rhodamine-conjugated phalloidin, which stabilizes the actin filaments and makes them visible by fluorescence microscopy. Scale bar, 10 μm. (C) Actin-rich ring canals (arrows) are visible among other actin-rich cytoskeletal elements. Ring canals isolated from egg chambers were stabilized and detected by staining with rhodamine-conjugated phalloidin. (D) Hts-RC protein remains associated with the actin ring canals. The same ring canals (arrows) as in C were labeled with anti-hts-RC antibodies and detected with FITC-conjugated secondary antibodies. Scale bar in C and D, 10 μm.

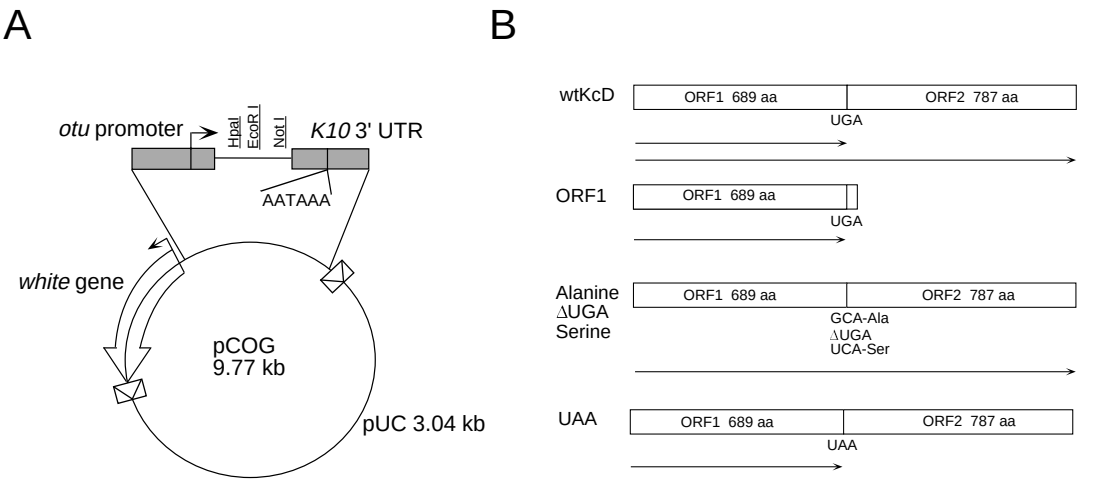

Fig. 3. Transformation experiments to elucidate the roles of two kelch proteins. (A) A new vector, pCOG, provides expression in the *Drosophila* germline. The vector utilizes the *ovarian tumor (otu)* gene promoter and the *fs(1)K10* 3′UTR and transcription termination sequences. The polylinker consists of *Hpa*I, *Eco*RI and *Not*I restriction sites. The vector is built into the pCaSpeR 2 transformation vector, which includes the *white* mini-gene as a selectable marker, a pUC replication origin and an *Ampicillin*^{R} gene. (B) Schematic of six transgenes designed to test the functions of ORF1 and full-length kelch proteins. The wild-type transgene (wtKcD) contains the wild-type *kelch* cDNA with the UGA in-frame stop codon. ORF1 has had ORF2 removed so that only the ORF1 protein is made. Alanine consists of the full-length cDNA except that the UGA has been mutated to a GCA, which encodes alanine. ΔUGA has had the UGA removed so that constitutive readthrough results. Serine has had the UGA mutated to UCA, which encodes serine. UAA has had the UGA mutated to a UAA stop codon. The arrows represent the protein products that were produced from the transgenes (see Fig. 4).

cytoskeletal structures. The pellets were resuspended and fractionated on 55%-65% sucrose gradients. The sucrose gradient fractions were analyzed by immunofluorescence microscopy to look for the presence of ring canals and by western analysis on equivalent amounts of each fraction to look at the presence of known ring canal proteins (Fig. 2A). Some ORF1 kelch was seen at the top of the gradient. In the gradient, hts-RC, ORF1 kelch and full-length kelch always co-sedimented in a broad distribution, probably due to the heterogeneity of ring canal size. In the fractions that contained all three proteins, intact ring canals could also be seen by rhodamine-conjugated phalloidin staining (Fig. 2B,C). The ring canals could be immunostained with anti-hts-RC antibodies (Fig. 2D). These results strongly suggest that both kelch proteins, as well as hts-RC and actin, co-sediment in a complex. They also indicate that the ring canal complex is a discrete unit of cytoskeleton that remains stably associated after Triton X-100 extraction.

Transgenically produced wild-type kelch proteins or ORF1-only kelch rescue fertility and ring canal morphology in a null *kelch* mutant; full-length proteins do not

In order to determine specific roles of kelch ORF1 and full-length proteins, we expressed individual proteins in the germline to test for rescue of the *kelch* mutant phenotype. To do this, we developed a vector, pCOG, that would provide expression of heterologous cDNAs early in the germline (Fig. 3A; see Materials and methods). We made six transgenes (Fig. 3B) and tested for their ability to complement *kelch* mutants. For each transgene, we generated 8-10 independent inserts for analysis. Most of the lines were assayed for expression and rescue of fertility of *kelch* mutants. Ring canal morphology of the two highest expressing lines of each transgene were examined.

We expressed the wild-type kelch proteins (wtKcD), ORF1 and three mutant full-length kelch proteins in the background of a homozygous mutant *kelch* allele, *kel*^{DE1}, which does not produce any detectable amount of kelch proteins (Fig. 4A). In this molecular null background, the wtKcD transgene produced both ORF1 and full-length proteins while the ORF1 transgene only produced the expected ORF1 product (Fig. 4A). To generate full-length proteins (Fig. 3B), we mutated the TGA to a GCA, which encodes alanine (Alanine); deleted the TGA (ΔUGA); or mutated the TGA to a TCA, which encodes a serine (Serine). Each of these mutations caused constitutive readthrough so that only the full-length protein was made (Fig. 4A). Only the wtKcD and ORF1 transgenes were capable of rescuing the female sterility of the molecular null *kel*^{DE1} mutant; Alanine, ΔUGA and Serine did not rescue the female sterility of this allele (Fig. 4A).

Since only the wtKcD and ORF1 transgenes were capable of rescuing the female sterility of *kel*^{DE1}, the full-length proteins may not have been able to localize to the ring canals in the absence of ORF1. We used anti-kelch antibodies to immunostain egg chambers that were homozygous for *kel*^{DE1} but were expressing one of the transgenes. In wild-type egg chambers, the antibodies recognized ring canals (Fig. 5A), whereas no staining was seen in egg chambers homozygous for the *kel*^{DE1} allele (Fig. 5B). Ring canal staining was seen in *kel*^{DE1} egg chambers that also contained the products of the wtKcD, ORF1, Alanine, ΔUGA or Serine transgenes (Fig. 5C,D,E,F and G, respectively). Thus, differences in rescue by the transgenes were not due to differing abilities of the respective transgene products to localize to ring canals.

We examined how effective the transgenic proteins were at correcting ring canal morphology. Egg chambers were stained with rhodamine-conjugated phalloidin or anti-hts-RC antibodies and examined by confocal microscopy. In wild-type flies, ring canals were neatly organized, forming tight bands of staining that defined an open lumen (Fig. 6A,B). In *kel*^{DE1} egg chambers, the ring canal rims were highly disorganized with a reduced lumen (Fig. 6C,D). Ring canals from flies rescued with wtKcD (Fig. 6E,F) or ORF1 (Fig. 6G,H) had normal morphology. Some ring canals that contained Alanine (Fig. 6I,J) and ΔUGA (Fig. 6K,L) had lumens intermediate in size

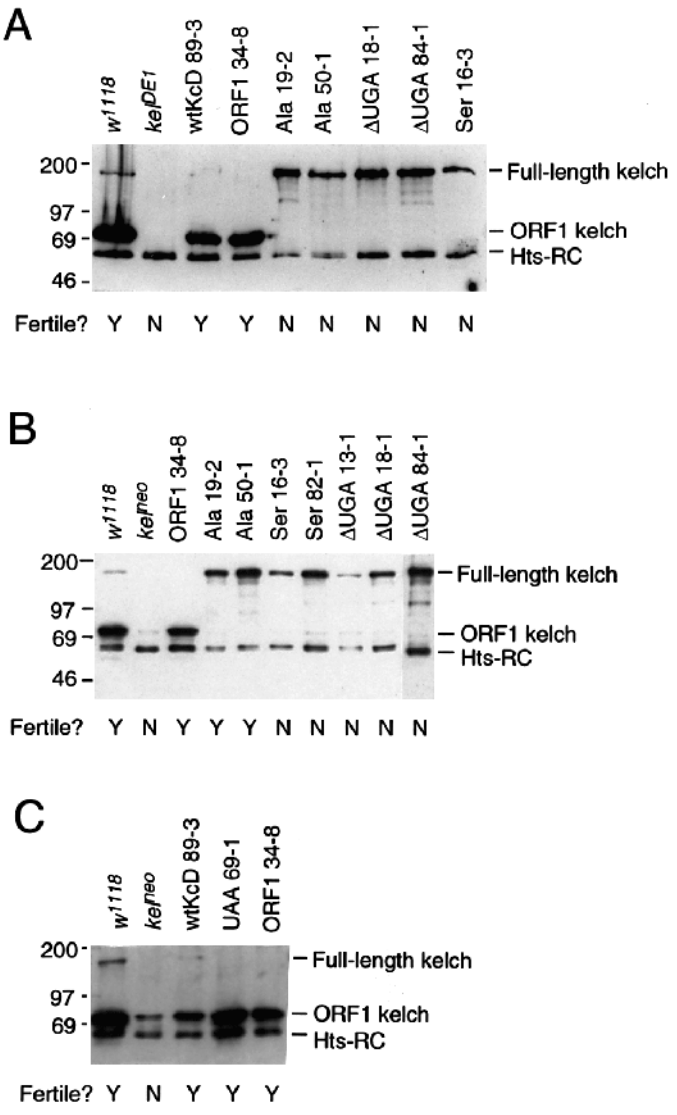

Fig. 4. Expression of transgene products in *kel*^{DE1} and *kel*^{neo} backgrounds. (A) wtKcD and ORF1 could rescue the fertility of the *kel*^{DE1} allele, but Alanine (Ala), ΔUGA and Serine (Ser) could not. The endogenous *kelch* gene (*w*^{1118}) produced ORF1 and full-length protein, as expected. *kel*^{DE1} produced no detectable full-length or ORF1 kelch proteins. A representative wtKcD line produced the expected ORF1 and full-length proteins. ORF1 produced the expected ORF1 product. Each full-length transgene also produced the expected full-length product. Numbers indicate transgenic lines with independent insertion sites for each transgene. Each of these are representative lines. Fertility: Y, yes; N, no. The western blot was performed using anti-kelch 1B monoclonal antibodies to detect ORF1 and full-length kelch and anti-hts-RC monoclonal antibodies to detect hts as a loading control. The same antibodies were used for western blots and total ovary extracts were used in each case in A, B and C. 40 μg of protein were loaded per lane. (B) The ORF1 and Alanine lines rescued the fertility of the *kel*^{neo} allele. Serine and ΔUGA proteins could not. Western blots indicate that each transgene made the appropriate size product. Some ORF1 protein was detected in each line due to the *kel*^{neo} allele. Fertility: Y, yes; N, no. 40 μg of protein were loaded per lane. (C) UAA is not suppressed. Both ORF1 and full-length kelch proteins were expressed from the wtKcD transgene. No detectable full-length protein was made by the UAA transgene. ORF1 made the expected ORF1 product. All three transgenes were capable of restoring fertility to the *kel*^{neo} background. Fertility: Y, yes; N, no. 50 μg of protein was loaded per lane.

Table 1. Morphology distribution of ring canals from *kel*^{DE1} flies containing a particular transgenically produced kelch protein

Genotype	Wild type (%)	Intermediate (%)	Kelch-like (%)	*n*
Stages 4-7				
w^{1118}	100	0	0	60
kel^{DE1}	0	0	100	73
wtKcD	59	13	28	112
ORF1	40	25	35	144
Alanine	0	8.1	92	99
ΔUGA	0	0	100	78
Serine	0	0	100	62
Stages 8-10				
w^{1118}	100	0	0	67
kel^{DE1}	0	0	100	74
wtKcD	87	7.9	5.1	215
ORF1	69	24	7.2	250
Alanine	3.7	23	74	191
ΔUGA	0	2.1	98	190
Serine	0	0	100	145

n, number of ring canals examined.

between wild type and *kel*^{DE1}, while ring canals that contained Serine (Fig. 6M,N) were largely kelch-like.

We quantitated the prevalence of ring canals of wild-type, intermediate and kelch-like morphologies with each transgene in the *kel*^{DE1} background (Table 1). Ring canals were examined from two classes of egg chambers: stages 4-7 and 8-10. In stages 4-7, roughly half of wtKcD ring canals and ORF1 ring canals were rescued to a wild-type morphology, whereas most ring canals from Alanine, ΔUGA and Serine transgenes were not. By later oogenesis, 87% of wtKcD ring canals and 69% of ORF1 ring canals were restored to wild type. Only 5-7% of the ring canals from these two transgenes were not rescued at all. In stages 8-10, only a few ring canals from Alanine transgenes were wild type while 74% of Alanine ring canals were kelch-like. 98% of ΔUGA ring canals and 100% of Serine ring canals were kelch-like. Therefore, wtKcD and ORF1 kelch protein products were able to rescue ring canal inner rims, while the full-length kelch proteins were unable to rescue ring canal morphology of the molecular null *kel*^{DE1} allele.

ORF1 and Alanine rescue fertility and ring canal morphology of a hypomorphic *kelch* mutant

Since homozygotes for the *kel*^{neo} allele have a small amount of detectable ORF1 protein (Fig. 4B), we tested whether any of the full-length transgene products would provide more rescuing activity in this background. ORF1, Alanine, Serine and ΔUGA transgenes were expressed in the *kel*^{neo} background and each transgene produced the expected size product (Fig. 4B). Surprisingly, the two highest expressing Alanine lines (Ala 19-2 and Ala 50-1; presented here) were capable of rescuing the female sterility of *kel*^{neo}, while none of the ΔUGA and Serine lines was able to rescue the female sterility of *kel*^{neo}.

Ring canals were examined from *kel*^{neo} flies containing each transgene. In mid- and late-stage wild-type egg chambers, ring

canals formed a well organized band of hts-RC protein that defined a clear lumen (Fig. 7A,G). In *kel*^{neo} egg chambers, mid- and late-stage ring canals were disorganized and had a reduced lumen (Fig. 7B,H). ORF1 rescued mid-and late-stage ring canals, restoring an open lumen (Fig. 7C,I). Alanine, ΔUGA and Serine transgenes did not rescue ring canal morphology in mid-stage ring canals (Fig. 7D,E,F), but Alanine did restore ring canal organization by late oogenesis (Fig. 7J). ΔUGA and Serine did not restore ring canal organization as well in late oogenesis (Fig. 7K,L).

We quantitated the distribution of wild-type, intermediate and kelch-like morphologies of ring canals found in *kel*^{neo} egg chambers containing the transgenes (Table 2). ORF1 rescued about one third of the ring canals in early stages to wild type, while one third were still kelch-like. All of the ring canals containing Alanine, ΔUGA or Serine transgenes were kelch-like in mid-oogenesis. By late oogenesis, ORF1 rescued 92% of the ring canals to a wild-type morphology. 47% of Alanine ring canals had a wild-type morphology while none of the Serine- or ΔUGA-containing ring canals were rescued to wild type. The higher percentage of *kel*^{neo} ring canals rescued by Alanine-substituted full-length kelch appeared to be sufficient for the rescue of fertility in these lines.

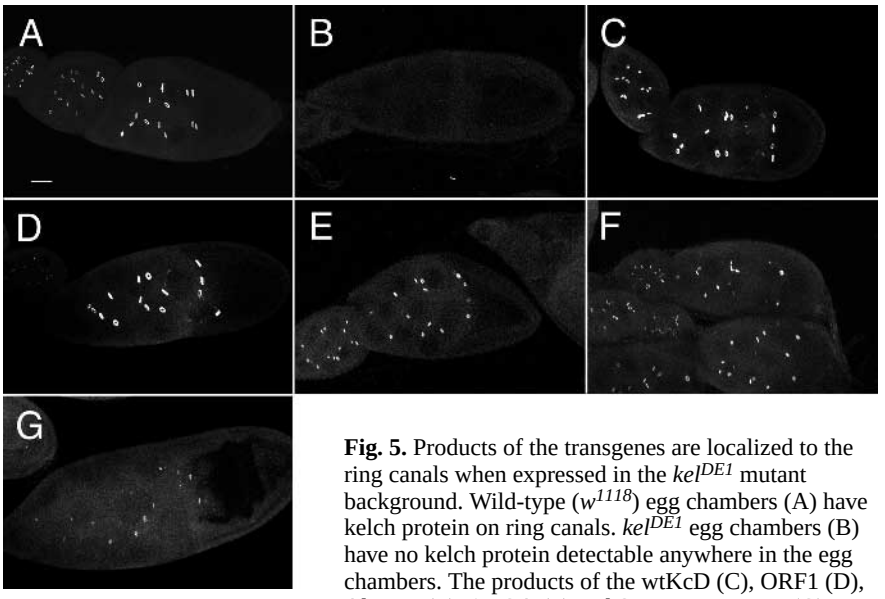

Fig. 5. Products of the transgenes are localized to the ring canals when expressed in the *kel*^{DE1} mutant background. Wild-type (*w*^{1118}) egg chambers (A) have kelch protein on ring canals. *kel*^{DE1} egg chambers (B) have no kelch protein detectable anywhere in the egg chambers. The products of the wtKcD (C), ORF1 (D), Alanine (E), ΔUGA (F) and Serine transgenes (G) are localized to ring canals. Egg chambers in A-G were immunostained with anti-kelch 1B antibodies. Scale bar in A, 30 μm and applies to A-G.

Ring canal localization determinants reside in kelch ORF1

Since transgenic ORF1 and full-length kelch proteins localized to ring canals, it was clear that ring canal localization determinants reside in kelch ORF1. However, it was also possible that ORF2 contributes ring canal binding sites that are sufficient for ring canal localization. To test this, we prepared trans-

Table 2. Morphology distribution of ring canals from *kel*^{neo} flies containing a particular transgenically produced kelch protein

Genotype	Wild type (%)	Intermediate (%)	Kelch-like (%)	*n*
Stages 4-7				
w^{1118}	100	0	0	105
kel^{neo}	0	0	100	60
ORF1	35	31	34	126
Alanine	0	0	100	50
ΔUGA	0	0	100	50
Serine	0	0	100	50
Stages 8-10				
w^{1118}	100	0	0	105
kel^{neo}	0	0	100	135
ORF1	92	8	0	88
Alanine	47	52	1	91
ΔUGA	0	46	54	65
Serine	0	18	82	38

n, number of ring canals examined.

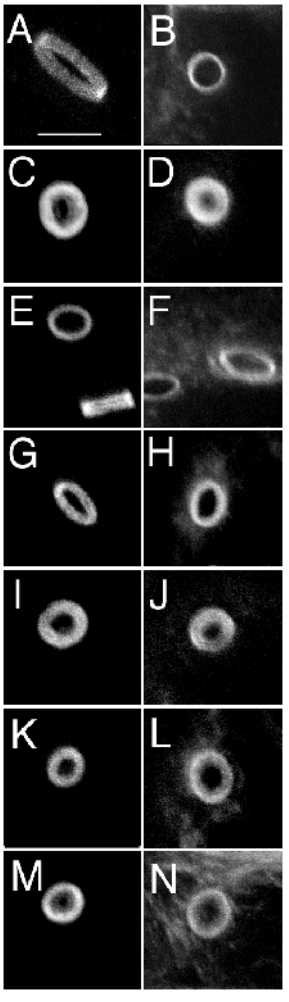

Fig. 6. wtKcD and ORF1 rescue the morphology of *kel*^{DE1} ring canals; full-length proteins do not. Stage 10 wild-type (*w*^{1118}) ring canals (A and B) have a clearly defined lumen. Hts-RC and actin are organized into a ring. Stage 10 *kel*^{DE1} ring canals (C and D) are severely disorganized. Hts-RC and actin extend into the lumen, partially occluding it. wtKcD (E and F) and ORF1 (G and H) rescue the morphology of stage 10 ring canals. The hts-RC and actin are properly organized. Alanine (I and J), ΔUGA (K and L) and Serine transgenes (M and N) do not rescue ring canals in stage 10 egg chambers. Hts-RC and actin are disorganized. A, C, E, G, I, K and M were immunostained with anti-hts-RC antibodies. B, D, F, H, J, L and N were stained with rhodamine-conjugated phalloidin to identify actin filaments. Scale bar in A, 10 μm and applies to A-N.

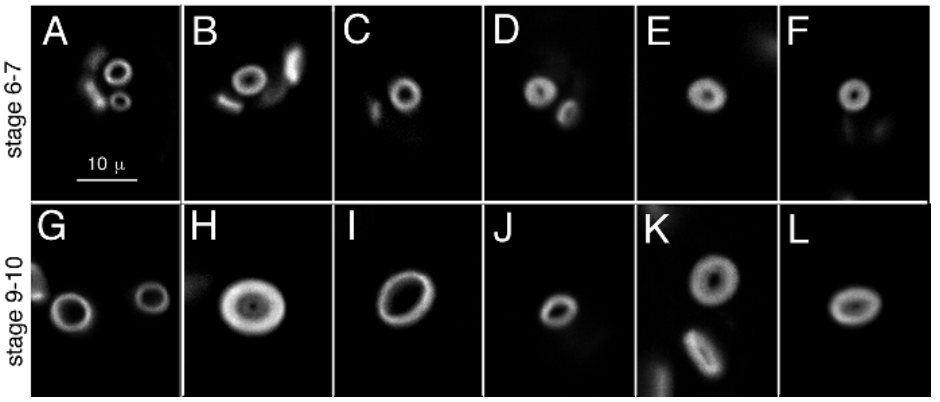

Fig. 7. ORF1 and Alanine transgenes rescue the ring canal morphology of *kel*^{neo} mutants; ΔUGA and Serine do not. Wild-type (*w*^{1118}) ring canals (A and G) have a clearly defined lumen in mid-and late-stage ring canals. *kel*^{neo} ring canals (B and H) are disorganized in both mid-and late-stage ring canals. ORF1 (C and D) rescues ring canal morphology. Alanine (D), ΔUGA (E) and Serine (F) do not rescue ring canal morphology in mid-stage ring canals. Alanine (J) rescues the morphology of some stage 10 ring canals. ΔUGA (K) and Serine (L) do not rescue ring canal morphology of late-stage ring canals. A, B, C, D, E and F are stage 6-7 ring canals. G, H, I, J, K and L are stage 9-10 ring canals. All ring canals in A-L were immunostained with anti-hts-RC antibodies. Scale bar in A, 10 μm and applies to all A-L.

genic animals that express myc-tagged ORF1-only (Nmyc-ORF1) and ORF2-only (Nmyc-ORF2) proteins using pCOG (Fig. 8). In wild-type, *kel*^{neo} and *kel*^{DE1} backgrounds, Nmyc-ORF1 localized to ring canals whereas Nmyc-ORF2 did not. Nmyc-ORF1 was capable of rescuing fertility and ring canal morphology of *kel*^{neo} and *kel*^{DE1} mutants, while Nmyc-ORF2 did not rescue any *kelch* mutant defects (not shown).

ORF1 is sufficient for egg production and maternal function

Since ORF1 was very effective at restoring fertility and ring canal morphology of the null *kelch* mutant, we tested these females for their ability to produce eggs that could hatch at normal rates. ORF1-containing lines produced eggs at nearly wild-type rates. Wild-type flies produced 1.37±0.146 (n=13) eggs per hour per female while P[w^+, ORF1 34-8], *kel*^{DE1}*/Df(2L)TW137* produced 0.924±0.199 (n=4) eggs per female per hour, which was slightly less (Student's t test: $0.01<P<0.05$). P[w^+, ORF1 34-8], *kel*^{neo}*/kel*^{PL25} females produced 1.49±0.498 (n=3) eggs per hour, which was not significantly different from wild type (Student's t test: $P>0.20$). The ORF1 transgene did significantly increase egg production compared to *kelch* mutants. *kel*^{DE1}*/Df(2L)TW137* females produced 0.037±0.034 (n=4) eggs per hour, which was significantly less (Student's t-test: $0.0005<P<0.005$) than the hourly egg production of P[w^+, ORF1 34-8], *kel*^{DE1}*/Df(2L)TW137* females. Due to the variability in egg production of flies over several days, we conclude that there was little significant difference in egg production between wild-type flies and mutant flies carrying an ORF1-only transgene.

Embryos laid by ORF1-containing mutant females survive at wild-type levels. 94.5±0.82 % (n=13) of embryos laid by wild-type mothers survived to hatching while 91.5±1.4 % (n=4) of embryos laid by P[w^+, ORF1 34-8], *kel*^{DE1}*/Df(2L)TW137* mothers survived. Statistically, the difference in these two genotypes is significant (Student's t-test: $0.025<P<0.05$). 97.6±0.704 % (N=3) of embryos from P[w^+, ORF1 34-8], *kel*^{neo}*/kel*^{PL25} mothers also survived to 1st instar larvae. In this background, ORF1 produced slightly better than the endogenous wild-type gene (Student's t-test: $0.005<P<0.01$). Each of these survives dramatically better than embryos produced from *kel*^{DE1}*/Df(2L)TW137* females, of

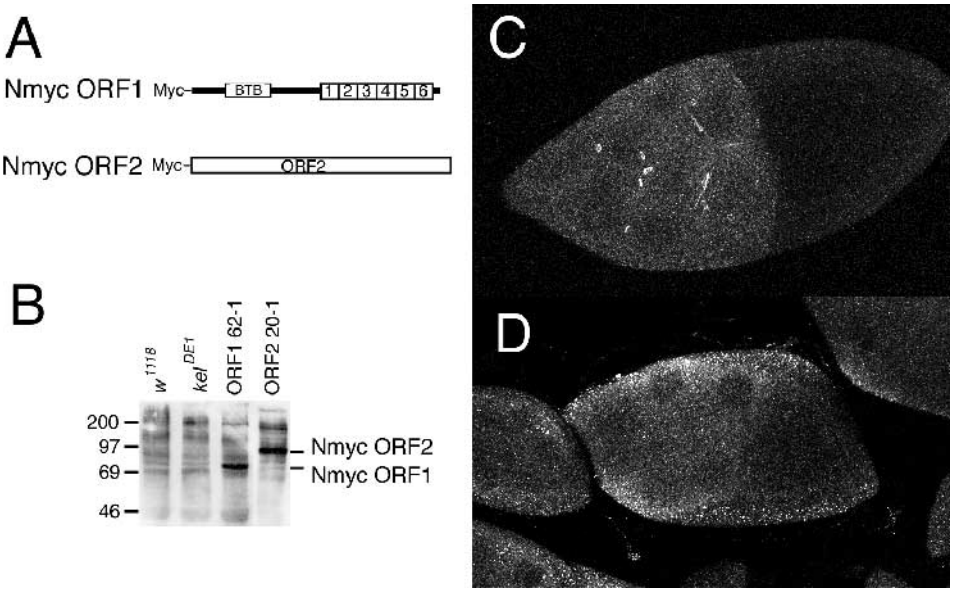

Fig. 8. All kelch ring canal localization determinants reside in ORF1. (A) A schematic diagram of amino-terminally myc epitope-tagged ORF1 and ORF2 transgenes. The BTB and kelch repeats (numbered 1-6) are shown in myc-ORF1. (B) Nmyc-ORF1 (approx. 80 kDa) and Nmyc-ORF2 (approx. 95 kDa) transgene products are detected in whole ovary extracts by western analysis using anti-myc 9E10 antibodies. (C) The Nmyc-ORF1 protein localizes to ring canals in a wild-type background. (D) Nmyc-ORF2 product does not localize to wild-type ring canals. The transgene products in C and D are detected using anti-myc 9E10 antibodies.

which 0% hatch. Since each group of females was mated to wild-type males for these experiments, these data suggest that there is no maternal requirement for full-length kelch protein. Previously we have seen no reduction in viability due to absence of zygotic kelch. Together, these data suggest that there is no genetic requirement for full-length kelch protein.

UAA is not suppressed

To test whether stop codon suppression is UGA-dependent, we generated a mutant (UAA) in which the TGA was changed to a TAA stop codon. Each of eight lines expressing this UAA transgene produced kelch ORF1 but no detectable amount of full-length kelch (Fig. 4C), suggesting that stop codon suppression is UGA-dependent. The UAA transgene, like wtKcD and ORF1, was able to rescue the fertility of *kel^{neo}* mutants (Fig. 4C).

Since the most likely mechanism of suppression of a UGA codon is incorporation of selenocysteine by a suppressor tRNA and since the kelch stop codon suppression mechanism appeared to be UGA dependent, we attempted to metabolically label full-length kelch by feeding isotopic selenium to larvae. In whole pupal extracts we were able to detect incorporation of isotopic selenium in three proteins with molecular masses of 68, 42 and 25 kDa (not shown). However, we were unable to show incorporation of isotopic selenium in immunoprecipitated full-length kelch, probably because the protein is not abundant enough for detection of a single labeled amino acid.

DISCUSSION

Two kelch proteins in the ring canal complex

The *kelch* gene produces two proteins from one message by stop codon suppression. Two lines of evidence indicate that both kelch proteins associate with the ovarian ring canal complex. First, both proteins co-sediment with the hts-RC protein and ring canals in sucrose density gradients. Second, when we expressed only a full-length kelch protein that contains either an Alanine, Serine or no amino acid (ΔUGA) at the UGA stop codon, these proteins associated with the ring canals, indicating that the full-length protein is capable of associating with the ring canal complex. Localization of Nmyc-ORF1 to ring canals, together with the failure of Nmyc-ORF2 to localize to ring canals, suggests that the full-length protein localizes by virtue of the ORF1 component of the protein. We are currently using the myc-epitope tagging system to study the structure-function relationships of kelch ORF1.

ORF1 protein is sufficient for *Drosophila* development

Transgenes that produce ORF1 kelch are capable of rescuing all detectable defects from loss of the endogenous *kelch* locus. Ring canal morphology of a molecular null mutant, *kel^{DE1}*, containing ORF1 kelch was rescued in 40% of stage 4-7 ring canals and 69% of stage 8-10 ring canals. The wtKcD transgene appears to perform slightly better in this background, suggesting a possible function for the full-length protein in the maintenance of ring canal morphology. In stages 4-7, wtKcD rescues 59% of the ring canals to wild type and, by stages 8-10, it rescues 87% of the ring canals to wild type. Although these results suggest that wtKcD might work a little better than ORF1, we do not have enough lines of each transgene that express at high enough levels to be able to conclusively demonstrate a difference between these two transgenes.

Perhaps what is really significant is that the percentage of ring canals not improved by either transgene is very similar at each stage of development. In stage 4-7 egg chambers rescued with wtKcD or ORF1, about one third of the ring canals were *kelch*-like. In stage 8-10 egg chambers rescued with the same transgenes, only 5-7% of the ring canals were *kelch*-like. One explanation for this difference is that the transgenes are not expressed at high enough levels early in oogenesis to improve ring canal morphology of all young ring canals. Endogenous wild-type kelch normally begins to accumulate by stage 1 but does not reach all ring canals until stage 4. We observed that each of the transgene products was localized to ring canals by stage 4, although the initial accumulation that begins by stage 1 was occasionally delayed in some ovarioles in the transgenic females (Robinson and Cooley, unpublished results).

The apparent reduction in the percentage of ring canals that are *kelch*-like between stages 4-7 and stages 8-10 suggests that the kelch ORF1 is capable of improving the organization of the ring canal during growth. This raises important questions about how the ring canals grow and how the kelch ORF1 functions in the ring canal. It has been proposed that ring canal growth involves sliding the ring canal actin filaments with respect to one another in order to increase the circumference of the ring canal (Robinson et al., 1994; Tilney et al., 1996). Tilney et al. (1996) demonstrated that the actin filaments in the ring canal are bipolar and extend circumferentially around the ring canal. Additionally, they showed that the maximum number (approximately 720) of actin filaments per cross-sectional area in wild-type egg chambers associate with ring canals by stage 4 when the ring canals are 3-4 μm in diameter. Thus, in order for the ring canals to expand to 10 μm by stage 11, they likely slide with respect to one another while adding additional filaments or while increasing the length of existing actin filaments. If kelch ORF1 is a dimeric actin cross-linker as we have hypothesized, then ORF1 may be able to provide additional cross-links to the filaments during growth, improving the morphology of the ring canal.

ORF1 kelch is also sufficient for *Drosophila* development. We have been able to maintain lines that have no detectable endogenous kelch but contain only the pCOG-ORF1 transgene. Egg production, embryo hatching rates and adult viability are normal in these flies. Although the loss of full-length kelch might result in subtle defects, it does not appear to be required for viability or for general morphogenesis.

Full-length kelch can partially rescue *kelch* mutants

To test the function of the full-length protein, we expressed three mutant transgenes, Alanine, ΔUGA and Serine, that provide expression of full-length kelch proteins. None of the full-length proteins was capable of rescuing female sterility of the molecular null *kel^{DE1}* allele. If ORF1 functions as an actin filament cross-linker during ring canal growth, this activity might be blocked by the presence of the ORF2 portion of the protein. In contrast, in the presence of a small amount of ORF1 protein (*kel^{neo}* background), Alanine, but not Serine or ΔUGA, was capable of rescuing the fertility. However, Alanine's rescue was only partial because even by stages 8-10 only about half of the ring canals had a wild-type morphology. These results

were very interesting for two reasons. First, they suggest that at least some ORF1 protein is required for *kelch* function. Second, the dramatic difference in the rescuing activity of Alanine versus ΔUGA and Serine suggests that the amino acid incorporated in the protein at the junction between ORF1 and ORF2 is important for proper function. Although this residue does not appear to affect the ability of the protein to localize to ring canals, it may confer some important protein folding information. The ΔUGA protein had a little more activity than the Serine full-length protein, suggesting that the serine residue may be specifically deleterious to the folding of the protein. In contrast, it is possible that Alanine is a more permissive residue that minimizes steric hindrance by the 84 kD ORF2 fused to ORF1. Of course, rescue of ring canal morphology and female sterility might not be the correct assays to measure the function of full-length kelch proteins.

Tissue-specific regulation of expression of two proteins

The levels of full-length kelch compared to ORF1 kelch are regulated throughout development. The ratio of full-length to ORF1 reaches a maximum approaching 1:1 during metamorphosis. This increase occurs in a largely tissue-specific manner, suggesting that the most efficient stop codon suppression occurs in the imaginal discs. However, we cannot rule out the possibility that the relative levels of ORF1 compared to full-length kelch are controlled, at least in part, at the level of protein stability.

The increase in the full-length kelch compared to ORF1 in the imaginal tissues suggests that full-length kelch might have an important function in these tissues. However, since mutants in the *kelch* locus only affect egg chamber development, we cannot assign a function to kelch in imaginal discs. The conservation of the two-kelch-protein motif across several *Drosophila* species suggests that both kelch proteins could be providing an important, perhaps redundant, function during metamorphosis.

Implications of stop codon context and our mutants on the mechanism of regulated suppression

Since stop codon context has been demonstrated to be non-random, the context of the UGA in *kelch* is likely to be important. The nucleotide immediately 3′ to the *kelch* UGA is an A. In *Drosophila*, UAA(A/G) is the preferred stop codon for highly expressed genes and UGA(A/G) is generally preferred in most eukaryotes, although less so in *Drosophila* (Brown et al., 1990). In mammals, stop codon context has also been shown to influence the decision to incorporate a selenocysteine or to terminate. A UGA followed by a C or a U was three times more likely to be suppressed than terminated, while a UGA followed by an A or a G was three times more likely to be terminated than suppressed (McCaughan et al., 1995). The UGA A in *kelch* might favor termination, which is what we see in the ovary where the ORF1 product is the predominant form. The UAA codon in the UAA *kelch* mutant transgene may not be decoded by a suppressor tRNA, or we might have introduced a stop codon that simply favors termination so that any product of stop codon suppression is below the level of detection. Cell-type specific factors may allow suppression to be favored over termination, perhaps by blocking the UGA to translation release factors.

The major candidate mechanisms for stop codon suppression in *kelch* are incorporation of selenocysteine or another amino acid by a suppressor tRNA, tRNA hopping, (Weiss et al., 1987; reviewed by Valle and Morch, 1988 and Atkins et al., 1990) or RNA editing. tRNA hopping that preserves the same reading frame has been shown to occur when the takeoff and landing codons are similar (Weiss et al., 1987; Huang et al., 1988; reviewed by Atkins et al., 1990). The *kelch* UGA is flanked 5′ and 3′ by in-frame AUG codons, making such a mechanism an intriguing possibility.

A mechanism of stop codon recoding by RNA editing is formally possible. Recoding has been shown to occur in the transcripts of the three members of the cellular glutamate (AMPA) receptor genes (Sommer et al., 1991). A double-stranded RNA adenosine deaminase changes an adenosine to inosine (Melcher et al., 1996) in a mechanism that requires exon and intron sequences in the pre-spliced RNA (Higuchi et al., 1993). While adenosine deaminase could change the sequence of the UGA stop codon to UGI, encoding a tryptophan, the mechanism would be quite different since a wild-type *kelch* cDNA (wtKcD) expressed in the ovary produces kelch ORF1 and full-length proteins.

Most well-characterized examples of genes that undergo UGA suppression do so by the incorporation of selenocysteine from a suppressor tRNA. Incorporation requires a cis-acting element called a selenocysteine insertion sequence (SECIS), which generally resides 3′ of the UGA in the mRNA, often in the 3′UTR (Berry et al., 1991b; Berry and Larsen, 1993). Suppression requires a special elongation factor (Forchhammer et al., 1989; Yamada, 1995) such as SELB in *E. coli* (Forchhammer et al., 1989), a selenocysteyl-tRNA and GTP, which form a ternary complex that competes with the release factor so that suppression occurs rather than translation termination. The SECIS may bias the competition to favor suppression by interacting with the translation machinery or by blocking the UGA from the ribosome release factor (reviewed in Böck et al., 1991). In *E. coli*, SELB has been shown to bind directly to SECIS, perhaps positioning the tRNA so that it is favored in the competition (Baron et al., 1993). In mammals, specific proteins have now been shown to bind directly to the SECIS (Shen et al., 1995; Hubert et al., 1996).

We favor selenocysteine-incorporation into kelch as the mechanism of stop codon suppression, since the stop codon readthrough mechanism for *kelch* appears to be UGA-specific and the *kelch* transcript has a potential SECIS in the 3′ UTR (Robinson and Cooley, unpublished). To try to test this, we metabolically labeled whole pupae by feeding isotopic selenium to larvae. Although we were unable to demonstrate incorporation into full-length kelch, probably because of the rarity of full-length kelch, we were able to see incorporation of isotopic selenium into three major protein species, suggesting that the selenocysteine-incorporation apparatus is intact in *Drosophila* and making this mechanism a reasonable possibility for full-length kelch.

With this in mind, we substituted a serine residue to try to mimic a selenocysteine. The shape and size of serine is similar but is much less reactive. Interestingly, the selenocysteine in glutathione-peroxidase (Chambers et al., 1986) and in the iodothyronine deiodinases (Berry et al., 1991a) is in the active site of these enzymes. Substitution of leucine at the stop codon of type I deiodinase eliminated the activity of the enzyme

(Berry et al., 1991a, 1994). Substitution of a serine at the stop codon in glutathione-peroxidase abolished enzyme activity while a cysteine-substituted enzyme still had some activity (Rocher et al., 1992). In contrast, by the criteria of rescue of ring canal morphology and female sterility, substitution of alanine in full-length kelch produced a partially active protein while a serine-substituted protein was inactive. Since kelch is most likely providing a structural, not enzymatic, role and an alanine-substituted full-length kelch works, it is apparently not necessary to have a strong nucleophile like selenocysteine at the junction between ORF1 and ORF2.

Conclusion

Stop codon suppression can be a very efficient mechanism of gene regulation. It can be regulated in a tissue-specific manner, allowing the ratio of two protein products to vary. Since we cannot assign a function to full-length kelch, the significance of this level of gene regulation for *Drosophila kelch* remains unclear; however, it is likely to have been conserved throughout the evolution of *Drosophila* species. We have discovered that the ORF1 product of *kelch* is sufficient for *Drosophila* development in a laboratory setting and is capable of rescuing the defect in ring canal morphology and female sterility that results from disruption of the endogenous *kelch* locus. However, the degree to which ORF1 rescues ring canal morphology has only been assessed by confocal microscopy. Ultrastructural analysis using electron microscopy might reveal a more subtle function for full-length kelch. Examination of the function of different mutant full-length kelch proteins suggests further constraints on the amino acid that may be included at the stop codon. Identification of the amino acid residue that is incorporated in the endogenous full-length kelch protein will permit these results to be more fully interpreted. The finding that ORF1 kelch is sufficient for *Drosophila* development has allowed us to concentrate on ORF1 for understanding the biochemical function of kelch in ring canal assembly.

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