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Coregulation of a Neighboring EST with RHOGAP

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COREGULATION OF A NEIGHBORING EST WITH *RHOGAP*

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COREGULATION OF A NEIGHBORING EST WITH *RHOGAP*

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COREGULATION OF A NEIGHBORING EST WITH *RHOGAP*

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It has been reported that the *crossveinless-c* locus of *Drosophila melanogaster* corresponds the *RhoGAP88C* gene. This determination was based on three criteria: proximity in genetic map position, complementation tests, and similarity of phenotype between RhoGAP88C RNAi constructs and the phenotype of hypomorphic *cv-c*^{1} mutant homozygotes, both of which eliminate posterior crossveins (Denholm et al. 2005). There are several genes that map to this region of chromosome 3, including both *RhoGAP88C*, and a poorly annotated PDZ-domain containing gene corresponding to Expressed Sequence Tag (EST) Clot 13975 (approximately 60 kb upstream from the *RhoGAP88C* transcriptional start). A P-element insertion *P[PZ]l(3)06951* is located immediately adjacent to an EST Clot 13975, but the homozygous lethal phenotype of the insertion appears to be unrelated to this EST. The data presented by Denholm et al. (2005) showing that *l(3)06951* fails to complement *cv-c*^{1} is relatively weak; only about 35% of *l(3)06951/cv-c*^{1} heterozygotes have a broken crossvein phenotype. This is in spite of the fact that *l(3)06951* is homozygous lethal, and *cv-c*^{1} homozygotes have a completely penetrant broken/missing crossvein phenotype. I have mobilized *l(3)06951* and recovered lethal insertion mutants 500 bp away from the original insertion that complement *l(3)06951* and fail to complement *cv-c*^{1} with a completely penetrant posterior crossveinless phenotype. I hypothesize based, on our data and that of Denholm

et al. (2005), that *P[PZ]l(3)06951* and *RhoGAP88C* are allelic with one another and distinct from the *cv-c* locus. I have used Real-Time PCR to show that mutations that fail to complement *cv-c* and mutations that fail to complement RhoGAP88C result in lower levels of both RhoGAP and Clot 13975 transcripts. This suggests that the mutations in the region may be affecting transcription levels of multiple mRNAs and may be causing some of the difficulty in assigning mutations to specific genetic loci.

INTRODUCTION

Defects of the posterior crossvein in *Drosophila* have been extensively studied. In 1923, Timofeeff-Ressovsky discovered a gene that caused defects in the posterior crossvein of *Drosophila funebris* (Milkman, 1964). Since this discovery there have been many works published on the formation of crossveins in *Drosophila*. Roughly a dozen spontaneous mutations mapping to all of the major *Drosophila* chromosomes have been recovered that produce broken crossvein or crossveinless phenotypes (Milkman, 1964; Marcus, 2001). Most of these mutations remain uncharacterized at the molecular level and represent an excellent opportunity to dissect the developmental genetics of a simple morphological structure.

Introduction to wing development. Insect wings are only two cell layers thick (Marcus, 2001) and the veins in the wings of *Drosophila* are made up of tubes of thick chitin derived from the remains of the densely packed cells in the wing surfaces (Mohler and Swedberg, 1964). Crossveins form primarily in one wing surface or the other but can pass from one surface of the wing to the other. In insects, crossveins serve as a supporting structure of the wing to aid in flight (Marcus, 2001) and, in some species of moths, crossveins in the wings serve as the organizational center of eyespots (Monterio et al. 2006). Crossvein development occurs in three stages. In the first stage, the position and number of the crossveins are determined along the proximal-distal axis and may involve signaling processes that include Cdc-42 (Agnès et al. 1999), Jun-N-Terminal Kinase (JNK), and as Hsp90 (Rutherford and Lindquist, 1998). Cdc-42 is a G-protein

that is involved in the regulation of the cell cycle and JNK is involved in several processes, such as apoptosis, cell differentiation and proliferation, and other cell cycle processes (Oltmanns et al. 2003). The *Cdc-42* locus was originally discovered in yeast during cell division and has been shown to encode a member of the rho family of small GTPases (Ziman et al. 1991). In *Drosophila melanogaster*, *Cdc-42* is required for many actin-dependent cellular processes as well as important developmental processes, such as cellular proliferation, differentiation, morphogenetic movements and cell signaling events (Genova et al. 2000). Martin-Blanco et al. (1998) and Agnès et al. (1999) have suggested that the *Cdc-42* locus may promote the production of the ligand decapentaplegic (*dpp*). Hsp90 is a heat shock protein that acts as a chaperone for cells undergoing stress during increase heat and has roles in signaling, protein folding and tumor suppression (Rutherford and Lindquist, 1998).

In the second stage of the crossvein formation there is cell-cell signaling between the dorsal and ventral wing surfaces (Garcia-Bellido, 1977) to initiate the crossvein formation, which involves the transforming growth factor-β (TGF-β) signaling pathway (Conley et al. 2000), which includes the ligand *dpp*. The TGF-β pathway is involved in several cellular processes, including cell growth, differentiation and apoptosis. The third stage in the development involves localized signaling through the Egfr-MapK pathway and Notch signaling in the ventral surface of the wing to refine/condense the vein structure (Sturtevant and Bier, 1995; Yu et al. 1996; de Celis et al. 1997; Huppert et al. 1997; Marcus, 2001).

Developmental studies. An effective way to study the development of a particular phenotype is to study mutants that alter that phenotype. In a famous series of experiments, Waddington (1953) discovered that crossvein formation was sensitive to high temperature, paving the way for more developmental studies into the formation of crossveins.

Genetic variation or the effects of specific environmental stimuli at critical stages of development can produce similar phenotypes. From these observations, it was then suggested that the selection for the ability to adaptively modify a specific character would ultimately lead to a genetic mechanism to produce the induced character (Bateman, 1957). How is this possible? In 1896, Baldwin, Osborn and Lloyd Morgan proposed that the selection for an inducible phenotype in a population allowed the population to survive until the time when the specific mutation could occur to produce that phenotype genetically (Gause, 1947). Waddington (1953) suggested an alternative: that by selecting for genotypes capable of producing environmentally induced phenotypes, one also often simultaneously selects for genotypes that produce those phenotypes constitutively (that is, regardless of environmental conditions). This process Waddington had described was given the term "genetic assimilation of an acquired character" (Bateman, 1957).

There have been several studies of mutations that have reduced or completely removed the crossveins. Some of these mutations include *crossveinless* (*cv*), *crossveinless-2* (*cv-2*) and *crossveinless-c* (*cv-c*). The *cv* gene has been characterized at the molecular level and has been shown to be a paralog of *twisted gastrulation* (*tsg-2*)

(Ross et al. 2001; Marcus, 2003), which is required to specify the dorsal-most structures in embryos and which also inhibits two TGF-β homologs in *Drosophila, Decapentaplegic* (*Dpp*) and *Glass bottom boat* (*Gbb*). Previous studies have shown that *crossveinless/tsg-2* is required on the dorsal wing surface to induce cells capable of producing crossveins on the ventral wing surface (Garcia-Bellido, 1977).

Crossvein development. In wild-type *Drosophila* strains, during pupal development a central vesicle forms in the wing discs at 21 hours around where the posterior crossveins (PCV) form. The lumen of the central vesicle is what forms the PCV. In crossveinless-like mutants, when the pupa is 22-24 hours old the longitudinal veins (LVs) are present, but the lumen is eliminated from the central vesicle when the two layers of the wing come together. When this occurs, the developing PCV space is destroyed, and cellular processes continue as in other non-vein areas (Mohler and Swedberg, 1964).

The crossveinless-2 gene product appears to promote the TGF-β signal through the ligands dpp/gbb. The ligand dpp and the TGF-β signaling pathway is believed to play a roll in vein formation in the wings of *Drosophila*. In the wing discs of the developing flies, *dpp* is expressed between the LVs 3 and 4 (Figure 1) and is involved in setting up the longitudinal axis for the wings. The dpp signals seem to be mediated by a type I receptor, Thickveins (Tkv) since cells that lack this gene product fail to form veins. Other factors have been shown to interact with TGF-β -like signals to cause a reduction in the formation of veins. For example, the Sog gene encodes for a Chordin-like

molecule that has been shown to inhibit TGF-β -like signals by binding to ligands to prevent them from activating the receptors (Figure 2). Others, such as tolkin mutants (which is related to the Tolloid proteases) block the formation of CV and the tips of the LVs (Figure 1). These phenotypes that have been observed are similar to the crossveinless class of mutations seen in *Drosophila* (Conley et al. 2000).

Introduction to *crossveinless-c^{1}*. The *crossveinless-c^{1}* (*cv-c^{1}*) mutant in *Drosophila melanogaster* was produced by a spontaneous mutation that is not associated with a visible chromosomal lesion (Lindsley and Grell, 1944) and has been identified by Denholm et al. (2005) as being a mutation in RhoGAP, which also interacts with Cdc-42. I suspect that there is another gene that may carry the *cv-c^{1}* lesion instead of RhoGAP as suggested by Denholm et al. (2005).

MATERIALS AND METHODS

Fly strains

Drosophila strains used in experiments: Oregon-R; *P[PZ]l(3)06951/TM6 Sb; Sb^1 Δ2-3/TM6; DF(3R)Exel6267/TM6B, Tb^1* and *cv-c^1* were obtained from the Drosophila Stock Center, at the University of Indiana; cv-c^2, *st e/TM6 Hu*; cv-c^3, *st e/TM6 Hu*; cv-c^4, *st e/TM6 Hu*; cv-c^5, *st e/TM6 Hu*; cv-c^6, *st e/TM6 Hu*; cv-c^7, *st e/TM6 Hu*; cv-c^8, *st e/TM6 Hu*; cv-c^{M62}/*TM3, Sb*; cv-c^{C524}/*TM3, Sb*; cv-c^{J17}/*TM3, Sb* were obtained from Dr. Barry Denholm, Dr. Rob Roy and Dr. Helen Skaer. All stocks were grown on instant *Drosophila* medium (Carolina Biological) at 25°C.

Mutagenesis Screen

P-element transposon mutagenesis. A transposon is what is commonly known as a jumping gene because of its ability to move from one location to another in the genome (McClintock, 1950). Transposon mutagenesis was used because of its ability to select for a precise excision of the initial P-element transposon followed by a reinsertion into the *cv-c* locus. This method is useful since the sequence of the transposon is known and can be used as a starting point for sequencing the surrounding DNA. This is often referred to as a sequence tagged mutation. To generate new mutations in *cv-c,* the P-element stock used was *P[PZ]l(3)06951/TM6 Sb* which was crossed to *Sb^1 Δ2-3/TM6* which carries a source of transposase, an enzyme that can bind to double-stranded DNA to allow it to be incorporated into the genomic DNA (Robertson et al. 1988; Cooley et al. 1988). The male progeny from this cross with the genotype *P[PZ]l(3)06951/Sb^1 Δ2-3* was crossed

back to virgin females of the parental transposon stock. Males were selected for this cross because there is no recombination in male *Drosophila*. The males that have undergone the precise excision and become viable over the original P-element were crossed to female virgins of *cv-c*^{1} *sbd*^{2}. Individual males with the genotype *P[PZ]l(3)06951/cv-c*^{1} *sbd*^{2} were crossed to virgin females of genotype *TM3/TM6*. The male and virgin female progeny from the same vial were crossed to each other in order to establish the mutant line (Figure 3).

Complementation Tests

cv-c^{1} flies were crossed to *P[PZ]l(3)06951/TM6 Sb* and to *P[PZ]37.6.1/TM6*; adult F1 flies were harvested and stored them in 90% ethanol. Individual wings were removed with forceps and mounted on slides with Euparol mountant. Slides were photographed using an Olympus BH-2 microscope with a 2 Megapixel JVC Digital Camera KY-F75U with Synchroscopy Auto-Montage software.

Inverse PCR and Sequencing

Once the mutant stocks were created, inverse PCR and sequencing of the flanking regions were performed. Genomic DNA was prepared from adult flies carrying the P-element insertion using the Qiagen DNeasy kit. The genomic DNA was digested with Sau3A, MspI, and HinPI restriction enzymes and then circularized with T4 DNA ligase. The sequences flanking the P-element inserts were amplified by inverse PCR using the protocol from the Berkeley *Drosophila* Genome Project, 2003. The PCR primers used match the sequences of the P-element. Forward primers Plac4 or Pry2 and reverse

primers Plac1 or Pry1 were used (Table 1). Plac4 and Plac1 primers were paired together and Pry2 and Pry1 primers were paired together. Successful Plac4 and Plac1 amplifications were sequenced (10 μl reactions) using primers Splac2 and Sp1 and successful Pry2 and Pry1 amplifications were sequenced using the primers Sp3 and Spep1 (Table 1) (Berkley *Drosophila* Genome Project, 2003). The dye-set used was the ABI BigDye® Terminator v3.1 Sequencing Juice (Applied Biosystems, Inc. Fosters City, CA). For each sequencing reaction, an isopropanol precipitation was used to clean up the reaction for use on an ABI 3130 DNA analyzer (Applied Biosystems, Inc. Fosters City, CA). The P-element flanking sequences, obtained by trimming off the transposon from the inverse PCR sequence, were compared to the *Drosophila* genomic scaffold sequence by a BLAST search in FlyBase (Adams et al. 2000), to determine the locations of the insertion sites.

RNA Preparation

mRNA was prepared from third instar larvae using a Qiagen RNeasy kit. Before Real-Time PCR could be performed, reverse transcription PCR (RT-PCR) needed to be performed. Protocol used for RT-PCR was Protoscript® First Strand cDNA Synthesis Kit by New England BioLabs®, Inc. A four-step dilution (concentrations: 400 μL, 80 μL, 40 μL, and 4 μL) of each sample with two replicates of Real-Time PCR for each of the four primer sets was performed on cDNA samples for Oregon-R (wild-type); *cv-c^1/cv-c^1; P[PZ]l(3)06951/TM6 Sb*; *DF(3R)Exel6267/TM6B, Tb^1* (a deficiency that removes the entire region); the point mutants created by Denholm et al. (2005); and the new transposon mutants that I generated, using primers for Clot 13975 and RhoGAP

(Table 2) as well as the housekeeping gene, *elongation factor 1-α (EF1α)*. The *EF1α* gene has been shown to be a useful reference gene for the accurate normalization in real-time PCR studies (Infante et al. 2008). The Clot 13975 and *RhoGAP* primers were designed using VectorNTI with the sequences known for both regions. Primers were then tested using a standard PCR protocol and gel electrophoresis. Two primers sets for the Clot 13975 and only one primer set for the *RhoGAP* sequences worked.

The conditions for Clot 13975 and RhoGAP PCR amplifications were 95°C for 5 minutes; 35 cycles at 95°C for 1 minute, 51°C (Clot 13975) or 56°C (RhoGAP) for 1 minute, 72°C for 1 minute; 72°C for 5 minutes and 4°C hold. The PCR conditions for the EF1α primers are 95°C for 5 minutes; 40 cycles at 95°C for 1 minute, 50°C for 1 minute, 72°C for 1 minute 30 seconds; 72°C for 10 minutes and 4°C hold. For the Clot 1 and RhoGAP primers, 0.5 μL Mg^{2+} had to be added to the Real-Time PCR reaction. For the Clot 2 primer, 1.0 μL Mg^{2+} had to be added to the Real-Time PCR reaction. The dye used for visualization was 25 μL of iQ™ SYBR® Green Supermix at a concentration of 2X (Bio-Rad Laboratories, 2000) for use with the Bio-Rad iCycler Thermal Cycler.

Functional Tests of RhoGAP and Clot 13975

To try to rescue the *cv-c* phenotype, UAS constructs carrying full length *RhoGAP* and *Clot 13975* transcripts are used. Flies carrying UAS-RhoGAP88C construct on Chromosome III was obtained from Dr. Barry Denholm's lab. A genetic crossing scheme was devised to bring $cv\text{-}c^{1}$ and *RhoGAP88C* into the same *Drosophila* stock. Males with homozygous genotype $cv\text{-}c^{1}\ sbd^{2}$ were crossed to females $ap^{xa}/CyO;$

l(3)/TM3Sb. Males from this cross with the genotype *+/CyO; cv-c^{1} sbd^{2}/TM3Sb* or *+/CyO; cv-c^{1} sbd^{2}/l(3)* were then crossed to virgin females from the same cross with genotype *+/ap^{xa}; cv-c^{1} sbd^{2}/TM3Sb*. Males and virgin females from this cross with the genotype *ap^{xa}/CyO; cv-c^{1} sbd^{2}/cv-c^{1} sbd^{2}* were used to make a stock (Figure 4).

To obtain the full-length transcript of the Clot 13975 gene, cDNA of Oregon-R flies were used. PCR was performed using primers for ClotF1 and cDNA cloning primer (Tables 2 and 3) with the same PCR conditions described in previous section for Clot 13975 primers. The amplified products from these PCR reactions were then used as the DNA template for a series of nested PCR reactions using the primers ClotF2 and 3' RACE PCR primers (Tables 2 and 3). These PCR products were then used for TOPO® TA Cloning® Kit by Invitrogen to clone the PCR products into a vector. JM109 competent cells were transformed using the newly cloned vector using Heat-shock transformation. The cloned vector was added to the competent cells and placed on ice for five minutes then transferred to 42°C for 30 seconds to heat-shock the competent cells and placed them back on ice. The newly transformed competent cells were then grown on LB plates with X-gal; colonies were picked off and grown in liquid cultures (Seidman et al. 1997). Plasmid preps were made using UltraClean™ 6 Minute Mini Plasmid Prep Kit by MoBio Laboratories, Inc. The new plasmid preps were then sequenced using M13 forward and reverse primers (Table 3). The sequencing protocol was the same as described above in the section *Inverse PCR and Sequencing.*

RESULTS

Mutagenesis Screen

After crossing the transposon stock *P[PZ]l(3)06951/TM6 Sb* to the stock Sb^1 *Δ2-3/TM6* that carries transposase, I was able to recover three lethal transposon insertions that complemented *P[PZ]l(3)06951* and produced a posterior crossveinless phenotype over $cv\text{-}c^1$. Sequencing of inverse PCR products showed that all three lethal insertions share the same new insertion site, which was not in the RhoGAP coding sequence (Figure 5). Performing the complementation test in the crossing scheme for mutagenesis insured that the transposon was in *cv-c*. When I performed a complementation test, I was able to recover 3 mutations, *P[PZ]5.2.6, P[PZ]7.2.4,* and *P[PZ]37.6.1,* that complemented *l(3)06951*, which Denholm et al (2005) suggested was a mutation in RhoGAP, but failed to complement $cv\text{-}c^1$. This suggests that *l(3)06951* and *cv-c* are different genes.

Complementation Tests

The wing phenotypes observed in *P[PZ]l(3)069551/*$cv\text{-}c^1$ F1 heterozygotes were somewhat different from those previously described by Denholm et al. (2005) for the same genotype. Denholm et al. (2005) reported the complete absence of posterior crossveins in this genotype, while my observations of the same genotype suggest that roughly 35% of individuals have broken posterior crossveins, but these crossveins were never completely absent (Figure 6). *P[PZ]5.2.6/* $cv\text{-}c^1$, *P[PZ]7.2.4/*$cv\text{-}c^1$ and *P[PZ]37.6.1/*$cv\text{-}c^1$ F1 heterozygotes show a complete loss of posterior crossveins, indicating that there is no complementation between the new insertion mutants and $cv\text{-}c^1$.

Inverse PCR

Sequencing of the inverse PCR products for the flanking sequences surrounding *P[PZ]5.2.6/TM6*, *P[PZ]7.2.4/TM6* and *P[PZ]37.6.1/TM6* insertions reveals that all three newly recovered transposon insertions are in the same location and immediately adjacent to a DNA sequence that apparently encodes a PDZ domain. PDZ domains help to anchor transmembrane proteins to the cytoskeleton and hold cell-cell signaling complexes together (Doyle et al. 1996). This PDZ domain-containing sequence appears in multiple *Drosophila* EST libraries, is apparently expressed, and is sometimes referred to as EST Clot 13975. Clot 13975 is approximately 60 kb upstream of the transcriptional start site of RhoGAP88C and may be an alternative gene product involved in producing crossveinless phenotypes.

Real-Time

I performed a four-step dilution of cDNA from wild-type and mutant *RhoGAP* and Clot 13975 samples with two replicates of each sample for real-time PCR analysis. The data gathered was normalized with respect to EF1α PCR amplifications prepared from the same cDNA samples (Infante et al. 2008). After normalization, the data shows that all mutants, both Denholm et al (2005) point mutants (assigned to RhoGAP) and the transposon insertion mutants created in our lab (assigned to Clot 13975), have lower mRNA transcripts for both Clot 13975 and RhoGAP. This information suggests that there is coregulation of the two genes (Tables 4, 5 and 6; Figure 7).

Functional tests of RhoGAP and Clot 13975

The crosses to put UAS-RhoGAP into a *cv-c*^{1} background are ongoing as well as efforts to complete the sequencing of the Clot 13975 cDNA. Once the sequencing of the cDNA for the Clot 13975 is complete, the cDNA will then be cloned into the pUAST construct obtained from Andrea Brand's lab and injected into *ap*^{xa}*/CyO; cv-c*^{1}*sbd*^{2}*/cv-c*^{1}*sbd*^{2} flies. These transformed flies and those from the crosses shown in Figure 4 will be crossed to heat-shocked *(hs)-Gal4* driver lines to finish the functional tests.

DISCUSSION

Is the *cv-c* mutation located in *RhoGAP*? Previous reports stated that *P[PZ]l(3)06951/cv-c*^{1} lacked posterior crossveins (Denholm et al. 2005). When I repeated the crosses necessary to produce this genotype, it occasionally (~35%) produced broken crossveins but the crossveins were never completely absent (Figure 6). The new insertion mutants that I generated, when placed over *cv-c*^{1} (e.g. *P[PZ]37.6.1/cv-c*^{1}) show a completely absent posterior crossvein indicating that there is no complementation between the two alleles. These phenotypes suggest that *cv-c*^{1} may not be a mutation in RhoGAP as previously reported (Denholm et al. 2005) but may be a mutation in another gene. The lesion that produced the original *cv-c*^{1} mutation has never been characterized at the molecular level, and so the transcript that it actually affects is still unknown. By performing Real-time PCR I was hoping to determine which transcript was being reduced in expression by the various mutations and found that both transcripts were being reduced in most of the mutations. This data suggests that there is coregulation of *RhoGAP* and *Clot 13975* since both transcripts are being reduced by our transposon mutants and the point mutants created by Denholm et al (2005).

In eukaryotes, transcriptional regulation usually operates at the level of individual genes and the transcription of any one gene is dependent on a promoter sequence located upstream of the transcriptional start site. The activity of these promoters can be modified by the binding of transcription factors to specific DNA sequences known as regulatory modules (Harding et al. 1989). These can either increase or decrease the amount of

transcription for a specific gene, depending on the activities of the bound transcription factors. In *Drosophila*, it was found that over 20% of genes that are subject to regulation are clustered into coregulated groups made up of 10-30 genes (Spellman and Rubin, 2002). In addition, roughly 29% of unannotated transcription fragments, with characteristics similar to *Clot 13975* are coregulated with one or more known gene (Manak et al. 2006).

The microarray experiments performed by Spellman and Rubin (2002) do not show the mechanism by which the observed similarities of expression of adjacent genes. Spellman and Rubin (2002) believe their findings are consistent with the regulation being at the level of chromatin structure. These researchers came to this conclusion for two reasons; first, the regions they observed with similarities in expression were very large, containing about 15 genes, each with their own core promoter; and second, one or two genes in a group show high levels of differential expression. Spellman and Rubin (2002) explained how the chromatin could effect coregulation of genes. They suggested that in a region with multiple genes, the chromatin was then opened to allow for one of the genes to be transcribed, this could then increase the accessibility of promoter and enhancers for the other genes in the region. This could cause for an increase in transcription of multiple genes (Spellman and Rubin, 2002). Both Denholm et al. (2005) and I made mutations that are unlikely to have disrupted the chromatin structure of a large section of chromosome. Many of the mutants made by Denholm et al. (2005) are point mutations and the mutants made in my lab have an inserted transposon sequence of about 3 kb that

likely would not have grossly disrupted chromatin structure. Yet, these mutations result in down-regulation of both transcripts.

Manak et al. (2006) suggest two additional hypotheses for why an unannotated transcribed sequence fragment might be coregulated with a known gene. One hypothesis is that they are independent transcripts that have similar intensities and time of expression with known genes, perhaps because they are affected by shared enhancer elements. Alternatively, a second hypothesis proposed by Manak et al. (2006) is that the unannotated transcript is coordinately regulated with the known gene because it represents a previously unidentified portion of that gene. My results show that the two transposon insertion locations (*P[PZ]l(3)06951* and the three mutants that I created, all of which are inserts at the same site) are homozygous lethal, but complement each other. This suggests that there are two genes, however, this is not a fool-proof explanation. Intragenic complementation is when two mutations in the same gene complement each other, however, this type of complementation is unusual. There may be other evidence to support an intragenic complementation hypothesis, however, by the map positions of the *RhoGAP* and *Clot 13975* transcripts. *RhoGAP* and the EST containing the PDZ domain for *Clot 13975* are shown to be oriented in the same direction on the same strand of DNA, this increases the likelihood that these are the same transcript. Although, when ESTs are captured, they are usually captured at the 3' end of a transcript. The 3' end of the Clot 13975 is at the 5' end of the RhoGAP transcript.

I therefore conclude that it is highly likely that there are two separate transcripts, one for *rhoGAP* and one for *cv-c*^{1}. The complementation test data support this contention, but the fact that mutations in this region have a tendency to affect the expression of both transcripts, makes it more difficult to assign phenotypes to particular coding sequences. With the functional tests that I am performing currently, I hope to be able to definitively answer the question of where this *cv-c*^{1} mutation occurs.

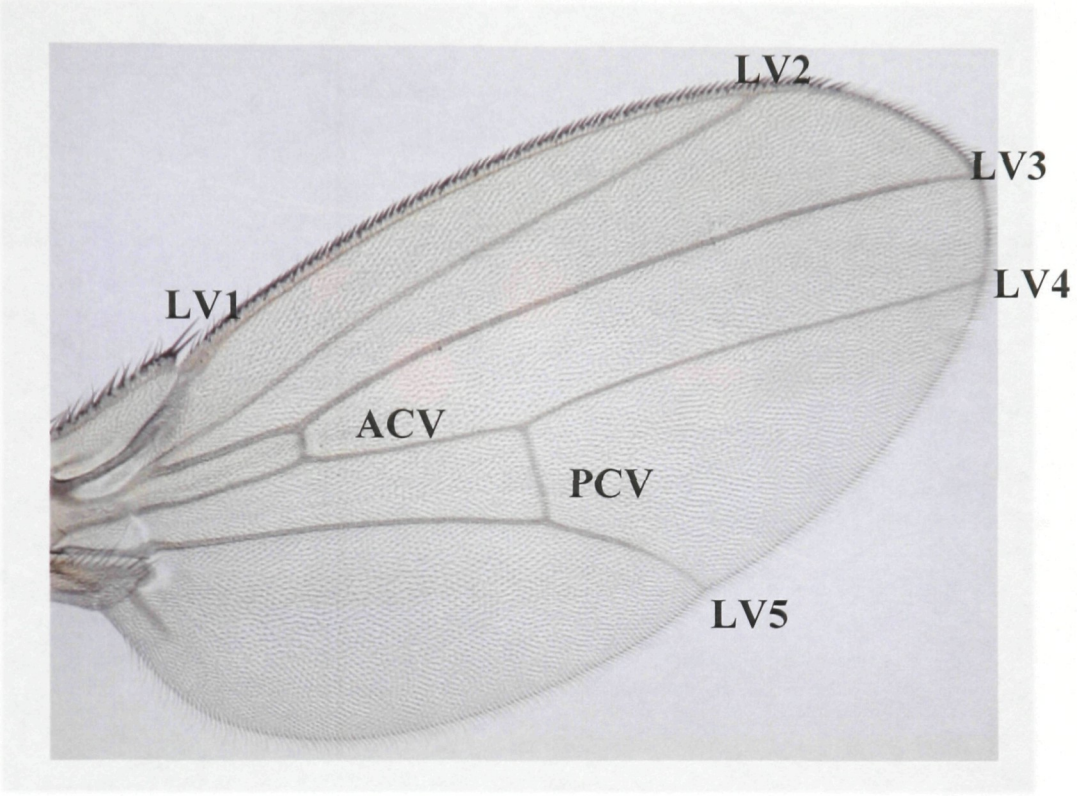


Figure 1. Wild-type wing showing the longitudinal veins (LV), Anterior crossvein (ACV), and Posterior crossvein (PCV).

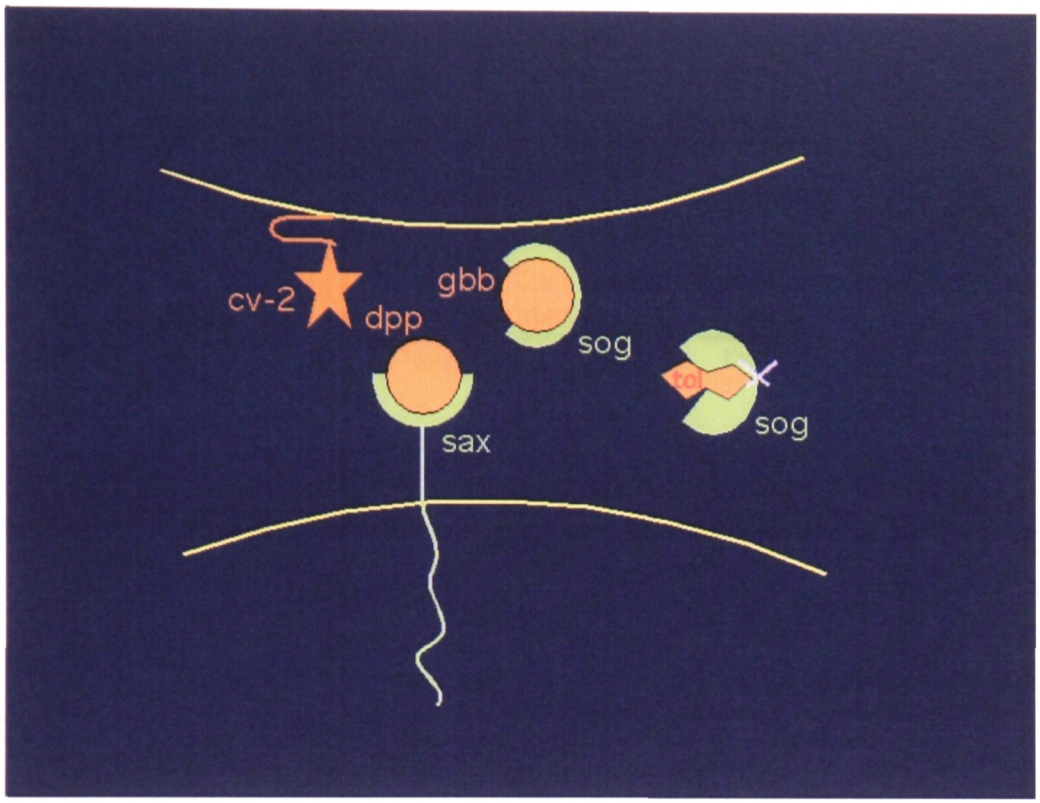


Figure 2. When *cv-2* is activated, this causes the release of the ligands *Glass bottom boat* (*gbb*) and *Decapentaplegic* (*dpp*). These ligands will then bind to Sax receptors, to promote the formation of crossveins. *Sog* gene products will bind and sequester ligands *gbb* and *dpp* to prevent them from activating receptors. *Tolkin* (*tol*) protease can also bind and cleave Sog, inhibiting its activity and facilitating signaling of *gbb* and *dpp*. *cv-2* can be mutated to prevent the release of *gbb* or *dpp*.

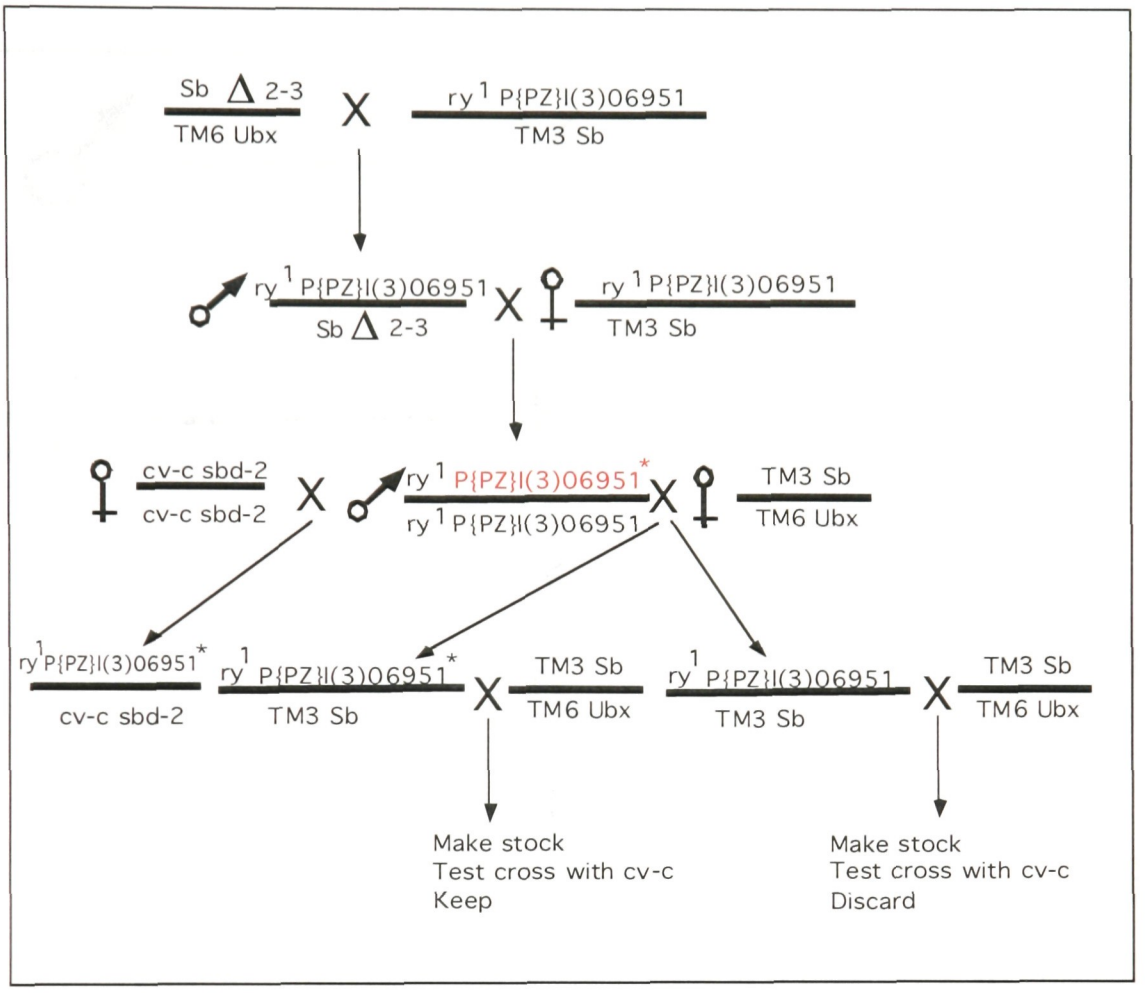


Figure 3. Transposon mutagenesis screen for insertions that complement *P[PZ]l(3)06951*, but fail to complement *cv-c*^{1}. I recovered 3 such insertions *P[PZ]37.6.1, P[PZ]7.2.4, P[PZ]5.2.6* each of which are homozygous lethal, viable over *P[PZ]l(3)06951,* and produce a crossveinless phenotype over *cv-c*^{1}.

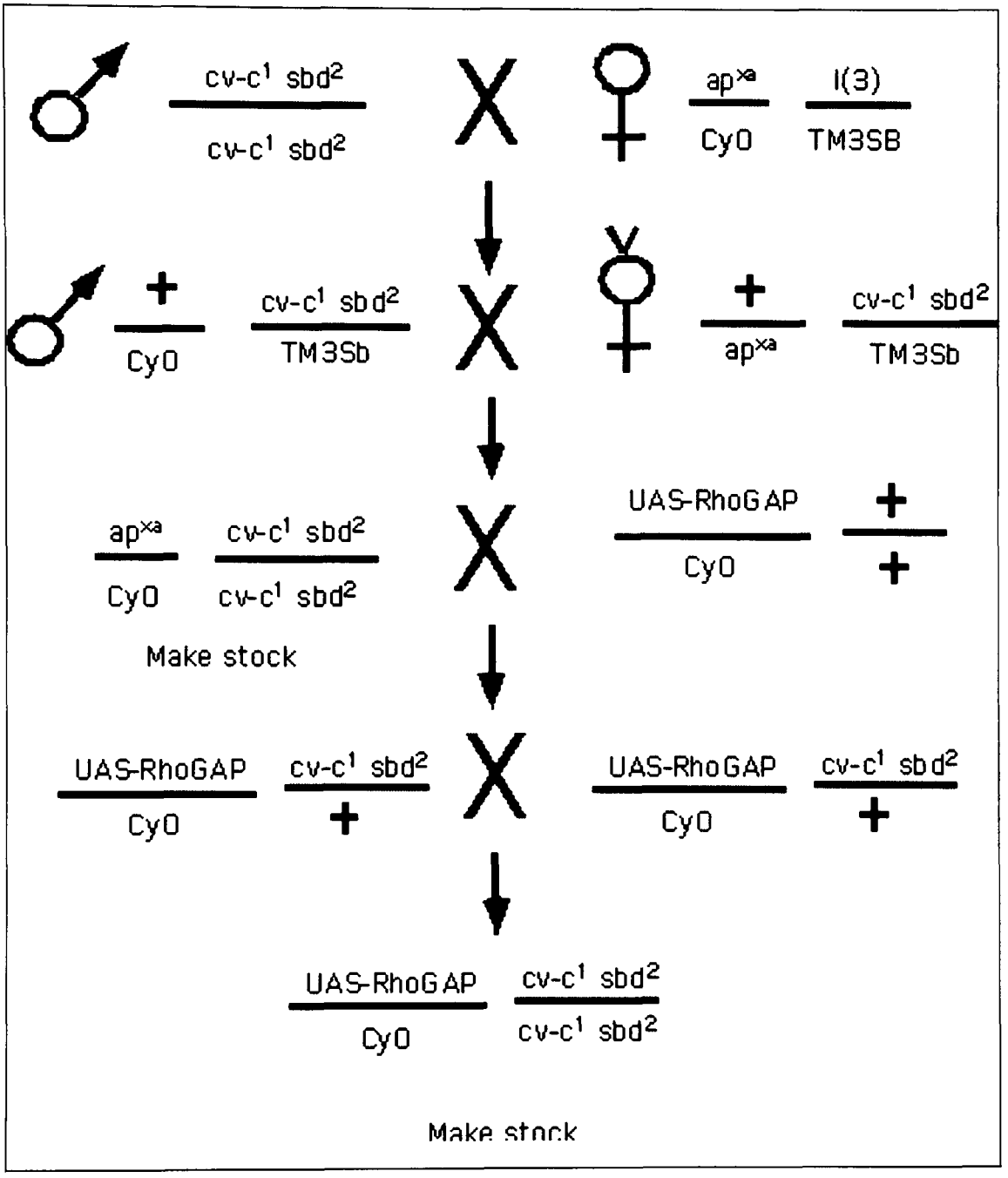


Figure 4. Crossing scheme in preparation for functional tests of *RhoGAP* and *Clot 13975*.

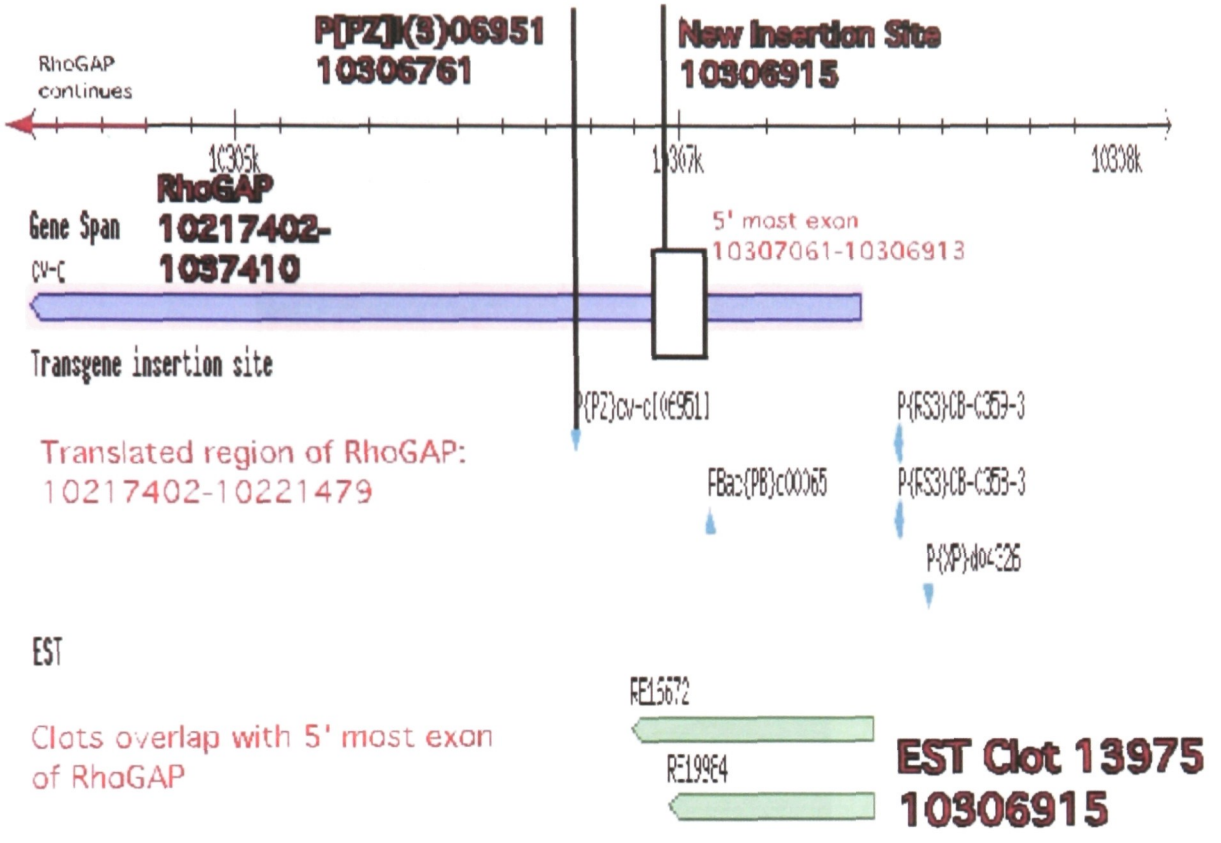


Figure 5. Inverse PCR of transposon insertions indicates that the new mutations are immediately adjacent to EST sequences (in green) that compose *Clot13975* and are outside of the translated region of *rhoGAP* (translated region: 10217402-10221479). Clot13975 consists of a fragment of apparent coding sequence that contains a PDZ domain. I am trying to characterize the rest of the transcript. I suspect that this transcript represents the gene product of $cv\text{-}c^1$. The lesion that causes the $cv\text{-}c^1$ mutation remains undiscovered. P-element transposons indicated by the blue arrows. In FlyBase, the RhoGAP gene is called cv-c.

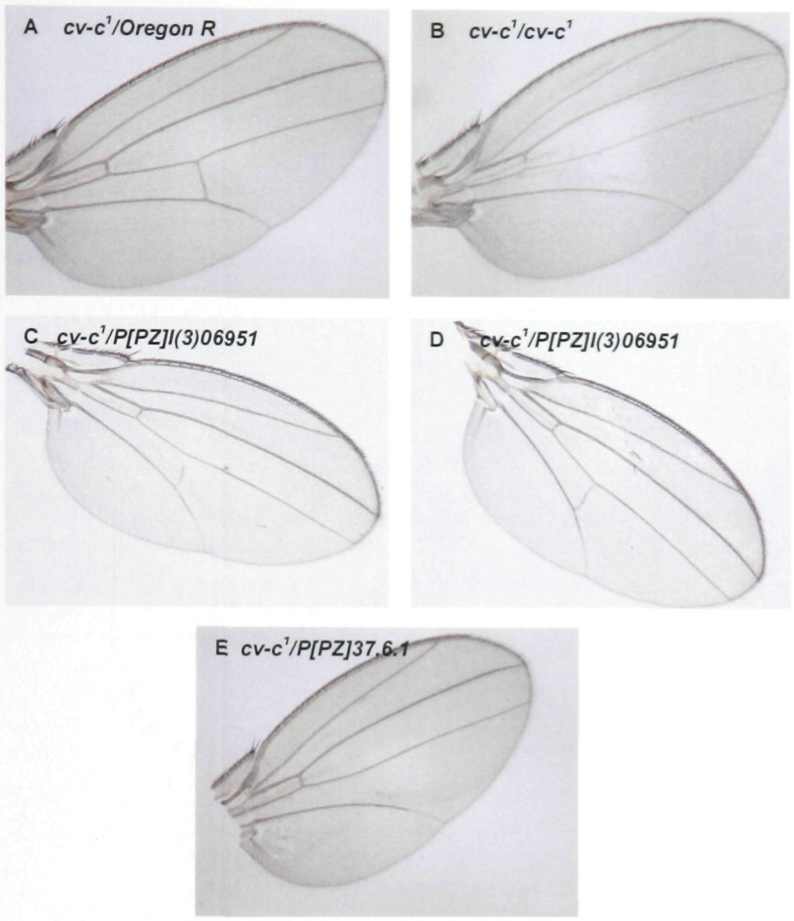
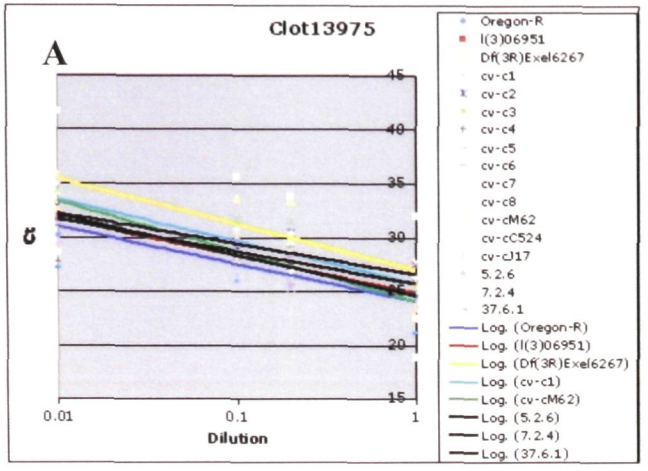


Figure 6. Adult wing phenotypes of (A) Wild-type and (B) *cv-c*^{1} homozygotes and heterozygotes. (C) *cv-c*^{1}*/P[PZ]l(3)06951* produces a partially penetrant broken posterior crossvein phenotype in 35% of the progeny with (D) 65% of the progeny have wild-type crossveins. (E) It produces a completely penetrant missing posterior crossvein phenotype over *P[PZ]37.6.1* and similar insertion mutants from the screen.



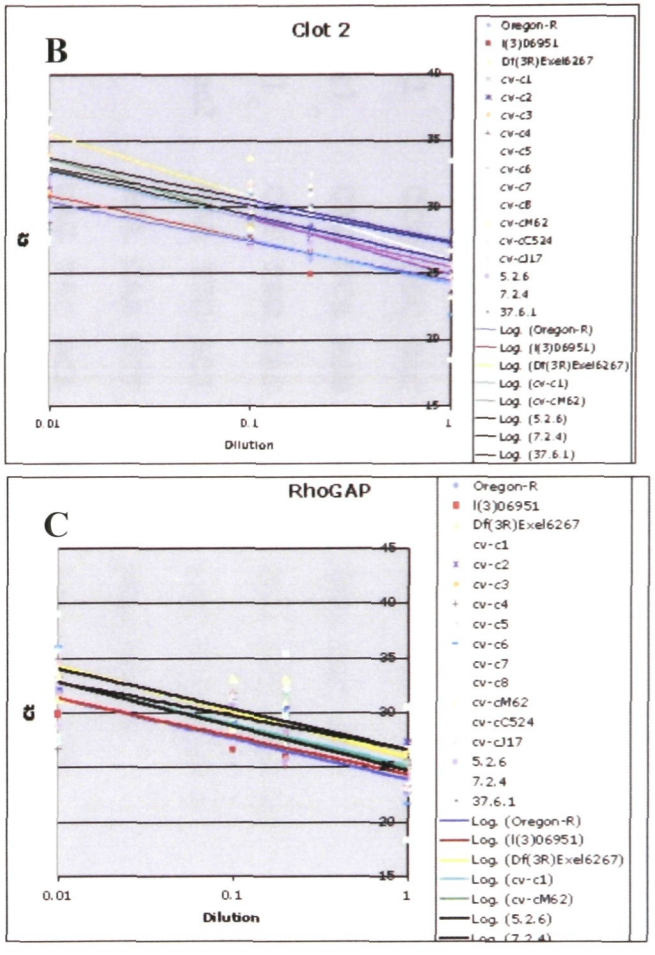


Figure 7. Plot of log-transformed real-time PCR data obtained using primers for portions of Clot13975 and rhoGAP, standardized by comparison with the constitutively expressed transcription factor EF1-alpha. These plots show Oregon-R wild-type levels of gene expression in blue. All of the transposon insertion alleles recovered in our screen show a reduction in both levels of Clot 13975 expression and rhoGAP expression. This is shown by a positive vertical displacement of the Ct values in the plots above. Similarly, all of the alleles in rhoGAP recovered by Denholm et al. (2005) show a reduction in the expression of both gene products and a positive vertical displacement of the Ct values.

Table 1. Inverse PCR primer sequences

Primer	Sequence
Plac4	ACT GTG CGT TAG GTC CTG TTC ATT GTT
Pry2	CCT GCC GAC GGG ACC ACC TTA TGT TAT T
Plac1	CAC CCA AGG CTC TGC TCC CAC AAT
Pry1	CCT TAG CAT GTC CGT GGG GTT TGA AT
Splac2	GAA TTC ACT GGC CGT CGT TTT ACA A
Sp1	ACA CAA CCT TTC CTC TCA ACA A
Sp3	GAG TAC GCA AAG CTT TAA CTA TGT
Spep1	GAC ACT CAG AAT ACT ATT C

Table 2. PCR primers for Clot 13975, RhoGAP, and EF1α (Monteiro and Pierce, 2001)

Primers	Sequence
ClotF1	AGT TGG GTT TCC ACC TCG AC
ClotR1	CCG CGC TAC ATT TTC GTA TT
ClotF2	TTA TTG GCA GTT CTC AGG TT
ClotR2	TTT AAT GGA GAG TGC CTT TT
RhoGAPF1	GCC GTG CAC GGT TGT CGA TTC T
RhoGAPR1	TCT GCA ATC TTG CGT TCG CG
ef44(f)	GCY GAR CGY GAR CGT GGT ATY AC
ef51(r)	CAT GTT GTC GCC GTG CCA AC

Table 3. Cloning primer sequences used to obtain the Clot 13975 sequence.

Primers	Sequence
3' RACE PCR	GGC CAC GCG TCG ACT AGT AC
cDNA Cloning Primer	GGC CAC GCG TCG ACT AGT ACT TTT TTT TTT TTT TTT TV
M13 forward (-20)	GTA AAA CGA CGG CCA GT
M13 forward (-41)	CGC CAG GGT TTT CCC AGT CAC
M13 reverse (-27)	CAG GAA ACA GCT ATG AC
M13 reverse (-48)	AGC GGA TAA CAA TTT CAC ACA GG

Table 4. Normalized Clot 1 Larvae of Ct values from Real-Time PCR. The numbers in this table show the mean values and (standard deviation) of the Ct values for gene expression for the replicates for each sample. The Ct value shows the fractional cycle at which the fluorescence intensity for the sample equals the threshold fluorescence. These values are inversely related to the abundance of the specific transcript (Clot 13975 or RhoGAP) in the sample. When these values are converted to linear form, the changes in gene expression can then be estimated (Infante et al. 2008).

Clot 1 Larvae	Dilution	Oregon-R	l(3)06951	Df(3R)Exel62	cv-c^{1}	cv-c^{2}	cv-c^{3}	cv-c^{4}	cv-c^{5}	cv-c^{6}	cv-c^{7}
	1	24.0(0.7)	25.0(1.1)	26.3(1.6)	25.0(0.5)	25.8(2.4)	24.5(1.6)	24.7(0.1)	23.6(1.3)	24.1(3.1)	25.4(3.1)
	0.2	26.3(0.1)	27.0(0.4)	30.7(3.7)	28.9(3.6)	29.0(2.1)	27.6(1.2)	27.4(2.5)	25.4(1.1)	27.9(0.8)	28.4(2.8)
	0.1	27.9(0.3)	28.4(0.9)	31.7(2.7)	30.8(1.2)	29.3(0.2)	29.2(1.0)	29.0(1.1)	27.8(1.8)	27.7(2.5)	30.2(0.8)
	0.01	30.9(0.9)	32.8(0.5)	35.0(0.9)	32.9(2.2)	32.1(1.2)	31.9(1.0)	30.4(4.0)	31.7(5.9)	31.2(5.6)	26.9(2.4)
		cv-c^{8}	cv-c^{M62}	cv-c^{C524}	cv-c^{117}	5.3.6	7.2.4	37.6.1			
	1	25.5(8.9)	23.5(1.2)	23.8(0.7)	24.1(0.8)	24.4(0.2)	25.8(2.8)	25.5(1.4)			
	0.2	32.0(2.6)	28.3(1.7)	27.3(2.8)	26.5(0.8)	26.9(1.9)	29.4(1.1)	27.9(1.0)			
	0.1	32.3(4.8)	28.9(1.3)	26.6(0.4)	28.9(1.7)	29.3(0.4)	29.7(0.9)	28.8(0.3)			
	0.01	35.1(9.3)	33.0(0.6)	30.7(1.4)	32.4(4.2)	31.7(0.6)	31.7(0.8)	31.5(0.1)			

Table 5. Normalized Clot 2 Larvae of Ct values from Real-Time PCR. The numbers in this table show the mean values and (standard deviation) of the Ct values for gene expression for the replicates for each sample. The Ct value shows the fractional cycle at which the fluorescence intensity for the sample equals the threshold fluorescence. These values are inversely related to the abundance of the specific transcript (Clot 13975 or RhoGAP) in the sample. When these values are converted to linear form, the changes in gene expression can then be estimated (Infante et al. 2008).

Clot 2 Larvae	Dilution	Oregon-R	l(3)06951	Df(3R)Exel62	cv-c^{1}	cv-c^{2}	cv-c^{3}	cv-c^{4}	cv-c^{5}	cv-c^{6}	cv-c^{7}
	1	24.5(0.1)	24.7(0)	25.4(1.8)	24.6(1.6)	25.2(2.7)	25.0(0.6)	25.1(0.1)	25.4(0.6)	23.8(2.8)	25.2(0.6)
	0.2	26.1(0.1)	25.8(1.1)	29.7(2.7)	28.7(1.8)	28.1(0.5)	29.2(0.6)	28.9(3.4)	27.0(0.7)	29.3(1.0)	30.0(2.1)
	0.1	27.9(0.8)	27.7(19.6)	31.7(2.9)	30.0(1.3)	28.3(1.3)	30.0(2.0)	28.7(2.0)	28.1(0.8)	29.0(1.6)	30.7(0.7)
	0.01	30.2(0.6)	31.1(0.3)	35.0(2.9)	32.0(1.3)	32.1(1.0)	32.6(2.1)	31.7(4.1)	32.3(3.3)	32.6(6.0)	31.8(5.8)
		cv-c^{8}	cv-c^{M62}	cv-c^{C524}	cv-c^{J17}	5.3.6	7.2.4	37.6.1			
	1	25.9(10.6)	24.4(1.7)	24.4(1.7)	24.8(1.3)	25.7(0.2)	26.2(0.8)	27.3(1.2)			
	0.2	29.5(20.9)	28.7(1.8)	28.7(1.8)	27.6(1.9)	28.4(1.5)	31.1(2.1)	29.4(1.0)			
	0.1	27.4(19.4)	29.4(1.3)	29.4(1.3)	26.9(0.9)	30.0(0.1)	31.0(1.3)	30.0(1.3)			
	0.01	27.3(19.3)	33.4(0.4)	33.4(0.4)	30.8(2.0)	32.5(0.5)	33.0(0.4)	33.0(0.8)			

Table 6. Normalized RhoGAP Larvae of Ct values from Real-Time PCR. The numbers in this table show the mean values and (standard deviation) of the Ct values for gene expression for the replicates for each sample. The Ct value shows the fractional cycle at which the fluorescence intensity for the sample equals the threshold fluorescence. These values are inversely related to the abundance of the specific transcript (Clot 13975 or RhoGAP) in the sample. When these values are converted to linear form, the changes in gene expression can then be estimated (Infante et al. 2008).

RhoGAP Larvae	Dilution	Oregon-R	l(3)06951	Df(3R)Exel62	cv-c^{1}	cv-c^{2}	cv-c^{3}	cv-c^{4}	cv-c^{5}	cv-c^{6}
	1	23.5(1.3)	24.2(1.6)	25.0(2.3)	23.7(1.0)	25.1(3.0)	24.6(1.1)	24.4(0.8)	24.5(1.3)	23.7(2.9)
	0.2	26.8(0.6)	26.8(1.3)	29.2(5.3)	29.1(3.5)	28.2(1.7)	29.0(2.1)	27.5(3.4)	27.1(1.7)	28.7(2.3)
	0.1	28.0(0.3)	28.0(2.1)	31.7(1.8)	30.1(0.3)	29.4(0.7)	29.3(1.2)	29.7(2.9)	27.9(3.9)	28.8(1.2)
	0.01	31.0(1.5)	31.2(1.9)	33.3(0.2)	31.7(0.1)	32.3(0.9)	31.4(0.1)	30.8(5.9)	31.9(7.0)	31.8(6.0)
		cv-c^{7}	cv-c^{8}	cv-c^{M62}	cv-c^{C524}	cv-c^{J17}	5.3.6	7.2.4	37.6.1	
	1	23.8(0.5)	24.4(8.6)	24.0(1.2)	23.9(0.1)	23.7(0.8)	24.3(0.4)	25.4(1.1)	26.2(1.6)	
	0.2	29.5(3.7)	32.5(4.1)	28.9(2.6)	27.2(2.5)	26.7(1.8)	27.1(2.1)	30.7(2.5)	29.0(2.1)	
	0.1	30.4(0.8)	29.0(20.5)	29.2(1.1)	26.7(0.8)	29.7(2.6)	30.2(0.6)	30.7(0.8)	30.1(1.5)	
	0.01	31.5(8.2)	33.4(8.0)	32.2(1.4)	30.8(2.5)	31.7(4.0)	32.2(0.6)	33.3(1.1)	32.5(0.4)	

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