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Genetic Interactions Between The Guanine Nucleotide Exchange Factor Gefmeso And Gtpase Signaling Components In The Drosophila Wing Reveal Microenvironment Dependent Variation Within Gtpase Signaling N

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GENETIC INTERACTIONS BETWEEN THE GUANINE NUCLEOTIDE EXCHANGE FACTOR GEFMESO AND GTPASE SIGNALING COMPONENTS IN THE DROSOPHILA WING REVEAL MICROENVIRONMENT-DEPENDENT VARIATION WITHIN GTPASE SIGNALING NETWORKS

by

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B.S. University of Central Florida, 2008

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ABSTRACT

The Ras superfamily of GTPases are important regulators of morphogenesis involved in control of cytoskeletal dynamics, intracellular trafficking, apical-basal polarity and cell migration. Mis-regulation of GTPase signaling interferes with development and is linked to pathogenesis. Traditionally, GTPase signaling has been depicted as a series of independent linear pathways. However, recently it has become apparent that multiple GTPases can interact to regulate a single cellular process, functioning in poorly understood networks of cross talk between pathways during development. Jim Fristrom (unpublished data) identified a mutation (18-5) that interacts with components of the GTPases Rho1, Rala, and Cdc42 signaling in multiple developmental contexts. Genetic analysis, physical mapping studies, and sequencing of the mutant allele have indicated that the gene was an allele of GEFmeso (CG30115), which encodes guanine nucleotide exchange factor. To show that 18-5 is an allele of GEFmeso, I demonstrated that a GEFmeso transgene could functionally rescue developmental defects associated with the 18-5 mutation. I also investigated cross talk and network variation in signaling interactions between GEFmeso and other GTPase signaling components in the Drosophila wing. My data provide evidence for microenvironment-dependent variation in GTPase signaling networks in specific domains of the wing, and reveal intercellular variation in GTPase signaling within an otherwise uniform epithelium.

ACKNOWLEDGMENTS

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CHAPTER ONE: INTRODUCTION

GTPase signaling and Principles of Regulation

The Ras superfamily of small GTPases are ubiquitous, highly conserved proteins regulating proliferation, cell migration, morphogenesis and differentiation across many species. GTPases accomplish these tasks by acting as key components linking extracellular signals to intracellular responses (Wennerberg et al, 2005; Hancock, 2003; Lindquist, 2006). The Rho (Ras Homology) family of GTPases are a subfamily of the Ras superfamily, and the most well studied members in vertebrates are RhoA, Rac1 and Cdc42, regulating stress fiber, lamellipodia and filopodia formation, respectively (Wennerberg et al, 2005; Hancock, 2003). Most members of the superfamily are cycled between active (GTP bound) and inactive (GDP bound) states by guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). Guanine dissociation factors (GDIs) stabilize and sequester GTPases in an inactive state (Wennerberg et al, 2005; Boulter et al, 2010). The active GTP-bound GTPase interacts with downstream effector proteins to trigger signaling cascades that elicit context-dependent cellular responses (Figure 1). Mis-regulation of the signaling pathways controlled by these GTPases is associated with many forms of pathogenesis.
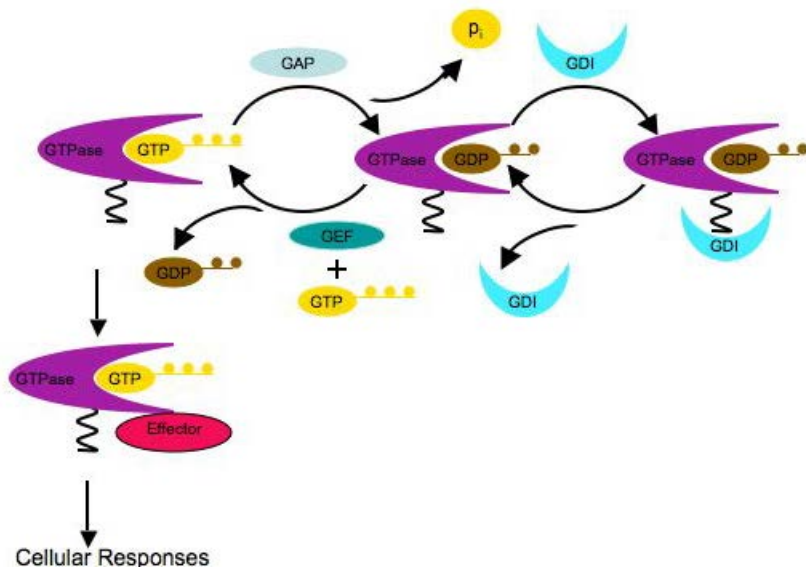
In the active GTP-bound state, GTPases interact with effectors to trigger cellular responses.

Figure 1: Cycling of GTPases between active and inactive states is regulated by GEFs, GAPs and GDIs

Epithelial Development and the Requirement for the Rho family of GTPases

Epithelia create selectively permeable barriers, forming boundaries between body compartments and structures. During developmental processes, epithelial tissues undergo extensive remodeling during developmental processes. Establishment of cell polarity, directed cell migration, cell rearrangement and tissue morphogenesis are important developmental events.

Polarization of cells within epithelia is often observed during organogenesis. For example, the establishment of apical-basal polarity is a critical process required to properly form the lining of the gastrointestinal tract, and assembly of apical tight

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junctions required in intestinal epithelia is a ROCK-dependent process (Walsh et al 2001). In Drosophila, each individual cell of the wing become polarized across a plane of the epithelium, forming a single distal-pointing hair per cell (Van Aelst and Symons, 2002; Yan et al, 2009). Aberrantly activated Rho1(Drosophila RhoA) or Cdc42 in the wing affects planar polarity, with phenotypes depending on the severity of the mutation such as shortened wing hairs, multiple wing hairs, or no wing hairs (Baron et al, 2000; Winter et al, 2001; Yan et al, 2009).

Directed cell migration is required for development and for physiological processes. Cells often must migrate either as individual cells or as part of an epithelial sheet to a new position. During Drosophila embryonic development, epithelial sheets migrate dorsally in a Rho GTPase-dependent process, meeting in the midline and enclosing the animal (Harden, 2002).

Morphogenesis requires a precisely coordinated series of cell behaviors including cell migration, cell rearrangements, and cell shape changes contributing to the final adult form. For example, in *Drosophila* the final shape of the adult leg is dependent on cell shape changes driven by Rho1-dependent contraction of the actin-myosin belt (vonkalm et al, 1994; Halsell et al, 2000; Bayer et al, 2003; Condic et al, 1991). Rho GTPases are also required during many aspects of neuronal development, such as myelination and neuronal migration (Luo, 2002; Park and Feltri, 2011).

GTPase Signaling and Pathology

The Ras family of GTPases are frequently found to have hyperactive mutations in cancers (Pruitt and Der, 2001; Tidyman and Rauen, 2009). However, of the Rho family of GTPases, RhoH is the only protein with a known mutation in human cancers. Frequently, the expression and activation levels are the main cause of a disease phenotype (Boettner and Van Aelst, 2002; Fritz et al, 2002). Studies examining RhoA and Rac1 expression in cancer patients show upregulation in hepatocellular carcinomas and breast cancers when compared to healthy tissues (Fukui et al, 2006; Schnelzer et al, 2000; Jordan et al, 1999). In addition, malignant breast tissues have increased immunohistochemical staining for Rac1 when compared to non-malignant tissue (Schnelzer et al, 2000; Jordan et al, 1999). Several other GTPases (RhoA, Rac1 and Cdc42) have elevated expression in tissues from patients with testicular cancer, and the expression level positively correlates with more advanced stages (Kamai et al, 2004; Kamai et al, 2002). Similar correlations are observed when comparing RhoA protein levels in malignant and non-malignant breast tissue (Fritz et al, 2002).

GTPase Signaling Exhibits Cross Talk and is Organized as a Network

Traditionally, GTPase signaling was depicted as a linear chain of events where growth factors stimulate a GTPase leading to effector activation, and in turn inducing downstream effects such as stress fiber formation, focal adhesion, or membrane ruffling (Ridley and Hall, 1992; Burridge and Wennerberg, 2004). However, in the past five to ten years it has become apparent that there is considerable cross talk among GTPase

signaling pathways which can involve GTPase regulators, GTPases and their effector molecules (Pertz, 2010; Burridge and Wennerberg, 2004).

Cross talk between GTPases is used to control the spatial distribution of GTPase regulators. For example, Rap1 localizes the Vav2 GEF to pseudopodia in Hela cells during cell spreading where it activates Rac1 (Arthur et al, 2004). In MDCK and Hela cells Rac1 localizes RhoGAPp190B to membrane ruffles where Rac1 promotes GAP activity and inhibits RhoA (Bustos et al, 2008).

Post-translational modifications to one GTPase can also influence activation of another GTPase. For example, in HEK293, Hela, and vascular smooth muscle cells**,** phosphorylation of RhoA displaces Rac1 from RhoGDI, leading to activation of Rac1 (Boulter et al, 2010; Rolli-Derkinderen et al, 2010).

The effector of one GTPase can also regulate the activity of an effector of another GTPase. For example, upon Ras stimulation in COS-1 cells and MEFs, Raf-1 (Ras effector) binds to and inhibits Rok-α (RhoA effector) (Ehrenreiter et al, 2005; Niault et al 2009). In human melanoma A7 cells and HEK293 cells, ROCK (RhoA effector) phosphorylates and activates FilGAP (negative regulator of Rac), resulting in membrane blebbing (Ohta et al, 2006).   In an added layer of complexity, it is apparent that some

regulators have the capacity to act as activators and repressors. For example, the Abr and Bcr proteins each contain activating Dbl and inactivating GAP domains, and can both positively and negatively regulate Rac and Cdc42 (Chuang et al, 1995).

The evidence for crosstalk between GTPase signaling cascades has been well established; and crosstalk appears to be so extensive that the term 'network' may be a more accurate way to describe GTPase signaling pathway organization. Currently, FRET and other techniques are being used to investigate GTPase organization within subcellular compartments, however little is known about how these networks operate and vary *in vivo*. Investigations that study GTPase signaling networks in a range of developmental contexts are needed to provide a more accurate picture of the organization of GTPase networks within and between tissues and their contributions to pathogenesis.

<u>GEFmeso: A Developmental Integrator of Multiple GTPase Signals in Multiple Tissues</u>

Our laboratory previously reported a genetic screen for mutations that affect the Rho1 (*Drosophila* RhoA) signaling pathway leading to activation of zipper (nonmuscle myosin II heavy chain) during leg and wing imaginal disc morphogenesis (Bayer et al, 2003). One allele identified in the screen, designated 18-5, was of special interest because it interacted with Rho1 and potentially encoded a novel member of the Rho1 signaling pathway. 18-5 homozygote adult escapers are rare and exhibit severe leg and wing malformations and an ectopic cross vein wing phenotype. Subsequent work in our lab

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has shown that the 18-5 allele also interacts with Rho1 to control embryonic head involution and dorsal closure (Smallwood and von Kalm, unpublished observations).

Genetic mapping experiments localized the 18-5 allele to a region of chromosome two, containing a putative guanine nucleotide exchange factor encoded by gene CG30115 (Fox, 2006). Blanke and Jackle (2006) characterized CG30115, which they named GEFmeso after identifying it in a yeast-two hybrid screen for proteins that bind to the DRala GTPase. The GEFmeso gene encodes two protein products, designated long and short, which differ in the N-terminal region (Figure 2). The GEFmeso short transcript is observed only in 0-3hr embryos. GEFmeso long expression is first detected in 3 hr embryos and is thereafter continuously observed into adulthood (Blanke and Jackle, 2006; Flybase).
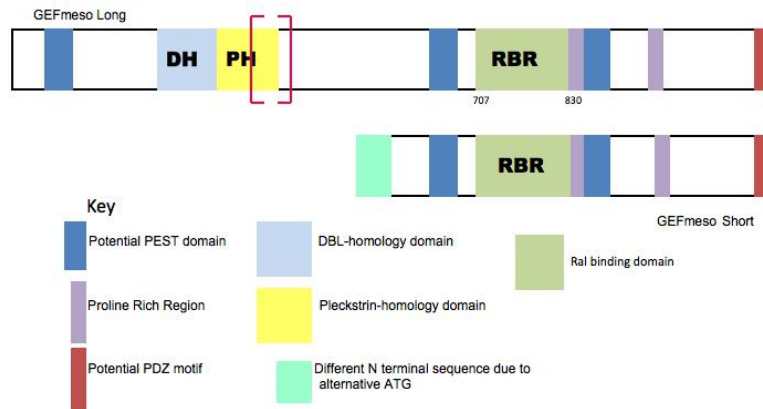
Red brackets indicate region of GEFmeso deleted by the 18-5 mutation. Figure adapted from Blanke and Jackle, 2006

Figure 2: Protein Domain Structure of GEFmeso Long and Short Proteins

In *in* vitro binding assays, GEFmeso binds to GTP-bound DRala and nucleotide-depleted Cdc42, but not to Rac, Mtl (a Drosophila Rac ortholog) or Rho1 (Blanke and Jackle, 2006). In addition, RNAi against GEFmeso in the wing results in an ectopic cross vein phenotype similar to the 18-5 homozygous mutant phenotype (Figure 3). Sequencing of the CG30115/GEFmeso gene in 18-5 homozygotes revealed a 502-nucleotide deletion within the gene. The deletion removes the last 52 amino acids of the pleckstrin homology domain and creates a frame shift that leads to premature termination of translation and loss of the Ral and PDZ binding motifs in the GEFmeso long protein (Morgan, Smallwood and von Kalm, unpublished observations; Figure 2). The GEFmeso short transcript is not affected by the 18-5 deletion.

Wing vein phenotypes of an 18-5 homozygote (left image) and GEFmeso RNAi targeted throughout the wing. Arrows indicate ectopic cross veins

Figure 3 The 18-5 Homozygote Phenotype is Comparable to Phenotypes Observed in GEFmeso RNAi Animals

Collectively, the data suggest that the 18-5 mutation is an allele of GEFmeso and that GEFmeso is an integrator of multiple signals coordinating GTPase activity during development. GEFmeso interacts with multiple GTPases in biochemical (DRala and Cdc42; Blake and Jackle, 2006) and genetic contexts (Rho1 and Cdc42; Fox, 2006; Bayer

et al, 2003). Moreover, the interactions occur in multiple cell types throughout

embryonic and adult development, indicating that GEFmeso may be a global signal

regulator/integrator for several GTPases. Therefore, GEFmeso and its interacting

partners are an ideal starting point to compare GTPase networks in similar developmental

contexts.

 I hypothesize that GEFmeso is a global regulator of GTPase network activity during

Drosophila development. To address this hypothesis, I examined the role of GEFmeso in

a GTPase network in the developing wing, which is a simple epithelium consisting of

vein and intervein cells. Specifically, I identified interactions between GEFmeso and

GTPases and their regulators in developing wing veins. Wing vein development is well

suited for these studies because it is a GTPase-dependent process with easily scored

mutant phenotypes. In addition, the wing is dispensable to survival allowing for the

identification of interactions that might be lethal in another developmental context.

Here, using transgenic rescue, I confirm that the 18-5 mutation is an allele of GEFmeso.

I then explore the organization of GTPase networks in microdomains of the developing

wing. Finally, I examine interactions between GEFmeso and GTPase signaling

components in wing microdomains. I conclude that GTPase networks differ between

microdomains of the wing epithelium. This is the first study to my knowledge

documenting and comparing GTPase signaling networks within a tissue *in vivo*.

CHAPTER TWO: METHODS

Drosophila Stocks

The Drosophila 18-5 mutant used in this study is described in Bayer et al, 2003. All mutants were obtained from the Bloomington Drosophila Stock Center at Indiana University (Bloomington, IN). All RNAi lines were either obtained from the Transgenic RNAi Project (TRiP) at Harvard Medical School or from the Vienna Drosophila RNAi Center (Dietzel et al, 2007; Ni et al, 2008). Stocks were maintained either at 18°C or 25°C on standard cornmeal/sugar/yeast media.

Lethal Phase Analysis of 18-5 Mutant Homozygotes

Embryonic lethality was determined by collecting 0- to 2-hr embryos from 18-5/CyO,P[Dfd-EYFP} stocks. Animals were aged for 13 hours and homozygotes selected based on absence of fluorescence. Animals were monitored beginning at 24 hours after egg laying up to 72 hours after egg laying and dead embryos were counted. Embryonic lethality was calculated as (number of dead mutant embryos/total number of mutant embryos) x 100. The experiment was repeated twice for a total of 259 embryos scored.

Larval lethality was determined by collecting non-fluorescing first instar animals from 18-5/CyO,P{Dfd-EYFP} stocks. Larvae were allowed to develop at 25°C at a density of 50 animals per vial and were removed from vials as they pupariated. Larval lethality was calculated as (number of mutant larvae-number of pupae)/number of mutant larvae x 100.

The experiment had three separate collection replicates for a total of 48 vials and 2,400 larvae.

To calculate pupal lethality, larvae from the larval lethal phase analysis were allowed to develop to pupariation and adult eclosers were counted. Pupal lethality was calculated as (number of pupae-number of adult eclosers)/number of pupae x 100. The experiment had three replicates for a total of 1,366 pupae. For the stage of pupal lethality analysis, a random sample of 1,284 animals were chosen and observed for stage of arrested development.

<u>Construction of the hs-GEFmeso Transgenic Construct</u>

Full length GEFmeso was amplified from a cDNA (LP15490) (DGRC, EST collection) using primer pair (GEFmeso short reverse and GEFmeso long fwd2) listed in Table 1 and JumpStart Taq (Sigma, catalogue no. D4184).

Table 1 Primers Used to Clone GEFmeso and Test GEFmeso Transgenes

Primer Name	Primer Sequence (5'-3')
GEFmeso Short Reverse	ACTAGTTCTAGAGGCTCGTCTGTGCATCACTA
GEFmeso Long Forward 2	AGATCTGCGGCCGCGGGCGAAAACTAACCCTACC
GEFmeso.L.RT.F	TCACTGTTCTGGGAGAGTTGC
GEFmeso.L.RT.R	CCAGCTGSTAGACCAGCTCCT

PCR parameters were 94°C for 3 min and 30 cycles of (1 min 94°C, 1 min 55C, 1 min 72°C). The amplification remained at 20°C until it was stored at 4°C Amplicons were visualized on a 0.8% low EEO agarose gel in 1xTAE buffer and PCR purified using a Quaquick PCR purification Kit (Qiagen Catalogue no. 28104). Amplicons were cloned into the vector pCaSpeR-hs (Thummel and Pirrotta, 1992) following digestion with the restriction enzymes XbaI/NotI and separated on an 0.8% SeaPlaque GTG agarose gel in 1xTAE buffer. Products were gel purified as described above using a Qiaquick Gel Extraction Kit 9Qiagen, Catalogue No. 28704) and ligated using a Rapid DNA ligation kit (Roche, Catalogue no. 11635379001). The ligation mixture was transformed into Max efficiency DH5α cells (Invitrogen) according to the manufacturer's specifications with one exception. 50µl cells were mixed with the ligation mixture instead of 100µl cells. The Transgene was injected into w[1118] embryos and established as stably transformed lines (11 lines total). Genetic tests chromosome balancers were used to determine which chromosome the transgene inserted in. An insertion on chromosome three, line 40A3 was used for the rescue experiments. Activity of the transgene was verified by induction at 37°C in larvae for 60 minutes with no recovery time, and GEFmeso expression was measured RT-PCR and comparison to GEFmeso expression in non-induced transgenic animals. The primer pair (GEFmeso.L.RT.F/GEFmeso.L.RT.R) used for RT-PCR is listed in Table 1.

Genetic Analysis of GTPase Interactions in the Wing

To test for the requirement of various GTPases during wing vein formation, ten virgin females carrying the transgene(s) Bx>Gal4 driver or Bx>Gal4; UAS>Dcr2 were mated to five males carrying a RNAi responder construct. Cultures were incubated at 18 $^{\circ}$C, 25 $^{\circ}$C, ot 29 $^{\circ}$C, depending on the strength of the phenotypes observed. For 25 $^{\circ}$C cultures, animals were allowed to lay eggs for five days in vials, and were then turned over onto fresh food for a further five days. Progeny were collected for 19 days; beginning the day the parents were added to the vial. All 18 $^{\circ}$C and 29 $^{\circ}$C matings were incubated at 25 $^{\circ}$C for three days, then the parents were removed and the vials were moved to 18 $^{\circ}$C and 29 $^{\circ}$C respectively. Progeny were collected for 16 days for 29 $^{\circ}$C experiments and 38 days for 18 $^{\circ}$C experiments; beginning the day the parents were added to the vial. Animals were collected, and stored in fly preservative (3:1 70% ethanol: glycerol). Wings were dissected from the animals, washed twice in 100% glycerol and mounted in 100% glycerol. Preparations were heated to 50-55 $^{\circ}$C for several hours, scored and photographed using an Olympus BX-FLA microsocope and Axiovision software. Animals carrying multiple transgenic constructs for co-expression analysis were constructed using appropriate balancers to combine two constructs within the same genotype. These animals were mated to Bx>Gal4 virgins and treated as described above. Progeny were collected and mounted as described above.

To investigate the effect of over-expression of wild type GEFmeso in the wing, we made a UAS>GEFmeso transgenic construct. UAS>GEFmeso was made as described for the

hs-GEFmeso construct except the cDNA was ligated into the pUAST vector (Brande and

Perrimon, 1993).

CHAPTER THREE: RESULTS

We have previously reported the isolation of a mutant allele, designated 18-5, which interacts genetically with the Rho1 and Sb-sbd loci to control leg and wing imaginal disc morphogenesis (Bayer et al, 2003). Three lines of evidence suggest that 18-5 is an allele of GEFmeso (CG30115; Blanke and Jackle). First, genetic mapping experiments place lethality associated with the 18-5 mutation in a region containing *GEFmeso* (Fox, 2006). Second, rare 18-5 homozygous adults have wing defects similar to those observed following RNAi knockdown of *GEFmeso* in the wing (Fox, 2006; Blanke and Jackle, 2006). Third, sequencing of the *GEFmeso* locus in 18-5 homozygous animals reveals a deletion that impairs GEFmeso function (Morgan, Smallwood, and von Kalm, unpublished).

In order to investigate the relationship between the 18-5 allele and *GEFmeso,* I performed a functional rescue with a GEFmeso transgene. Prior to conducting rescue experiments I conducted a lethal phase analysis of 18-5 mutant homozygotes to better understand the developmental requirements for the 18-5 gene product and to establish an appropriate period for transgene expression.

Lethal Phase Analysis of the 18-5 allele

Embryonic, larval, and pupal survival data for 18-5 homozygotes are shown in Table 2. Approximately one-quarter of 18-5 homozygous embryos fail to hatch, and slightly more

than one-half of homozygous larvae fail to survive to pupariation. Of animals that enter

metamorphosis, only 2% eclose as adults successfully, indicating a major requirement for

the 18-5 gene product during adult development.

Table 2 18-5 Embryonic, Larval and Pupal Lethal Phase Analysis for 18-5 Homozygotes

Genotype	Embryonic Lethality	Larval Lethality	Pupal Lethality
18-5/18-5	26.6 +/- 3.0	57.4 +/- 1.3	98.0 +/- 0.3

Embryonic viability was calculated as the percentage of homozygous embryos that failed to hatch as larvae (see methods for details). Larval viability was calculated as the percentage of larvae that failed to survive to pupariation. Pupal viability was calculated as the percentage of adults that eclosed of animals that were able to form pupal cases. Values shown are percentages +/- SEM from two (embryonic) or three (larval and pupal) replicates.

To further characterize the requirement for the 18-5 gene product during adult

development I determined the stage of pupal lethality in mutant animals (Table 3). As

shown in Table 3, lethality occurs throughout the prepupal and pupal stages, but is

focused in the early and later stages of adult development. Interestingly, almost a quarter

of animals die as late pupae during eclosion. Of animals that survived to initiate

eclosion, nearly all animals showed a partial eclosion phenotype (Tables 3, 4).

Dissection of late stage pupa from their pupal cases revealed that 76.4% (n=127) exhibit

a wing defect where at least one wing everts in an anterior rather than posterior direction

(Figure 4.B,D). Some animals that are able to eclose lose their wings during the eclosion

process. Visual examination of dissected late stage pupae also revealed a low frequency

of leg extension defects (data not shown).

Table 3 Lethal Phase Analysis of the Pupal Phase in 18-5 Homozygotes

Phase	% of N
Died as Elongated/Prepupae	10.2
Died as Prepupae and Young Pupae	27.6
Died as Mid Pupae (unpigmented)	25.7
Died as Late Pupae (pigmented)	10.4
Died during eclosion	24.2
Animals Eclosed	1.9

Percentages represent animals that died during pupal development or eclosed as adults (Animals eclosed). N=1366. Numbers calculated from three replicates.

Table 4 Analyses of 18-5 Homozygotes that Die at Eclosion

Eclosion Status	% of N
Partially eclosed	92.4
Eclosed	7.6

Percentages represent pupae that initiated eclosion and either eclosed or failed to complete eclosion (partially eclosed). N=357. Numbers calculated from three replicates.
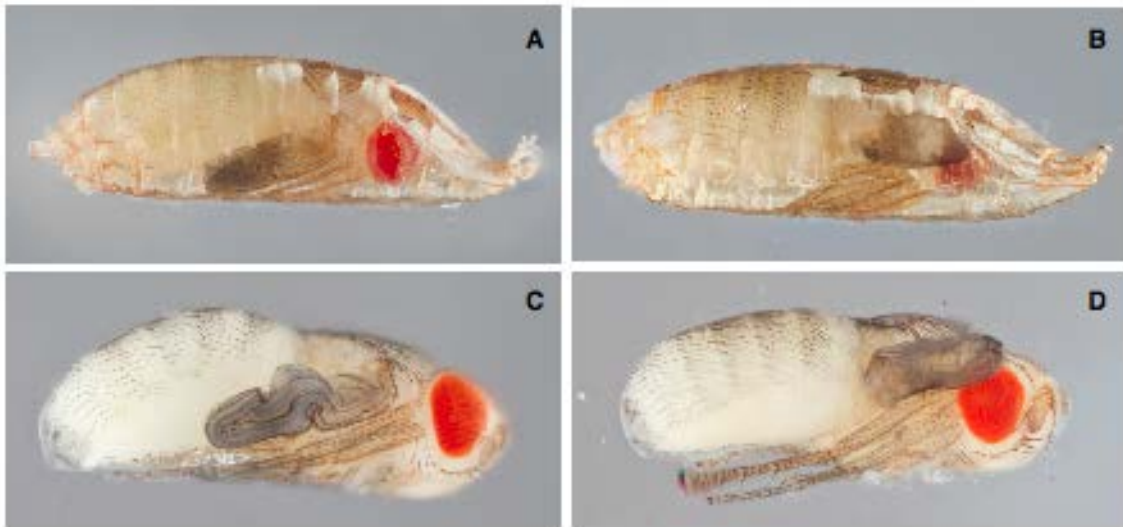
A) OreR pupa. B) 18-5/18-5 pupa. C) Dissected OreR pupa. D) Dissected 18-5/18-5
pupa with an anteriorally everted wing

**Figure 4 GEFmeso transgene rescues the anteriorally everted wing phenotype
associated with 18-5**

Functional Rescue of 18-5 Mutant Homozygotes with a Wild-Type GEFmeso Transgene

Genetic mapping experiments indicate that lethality associated with the 18-5 mutation

maps to a short interval on chromosome two that includes the guanine nucleotide

exchange factor, GEFmeso (Fox and Smallwood, unpublished data). Furthermore, 18-5

homozygotes have ectopic wing veins that mirror phenotypes observed in animals where

GEFmeso RNAi is expressed in the wing (Blanke and Jackle, 2006). To confirm if 18-5

is a mutation in GEFmeso, I asked if a heat-inducible GEFmeso transgene could rescue

mutant phenotypes associated with 18-5 homozygotes. I generated transgenic animals

with a wildtype copy of GEFmeso under control of a heat shock-inducible promoter,

induced expression in an 18-5 homozygous genetic background and assessed changes in

viability, leg malformation and wing eversion orientation. GEFmeso is expressed

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throughout Drosophila development, however, expression peaks during late embryogenesis and again beginning at the onset of morphogenesis, continuing for two days afterward (Blanke and Jackle, 2006; Graveley et al, 2011). Strong expression of GEFmeso during early adult development is consistent with the observation that a majority of animals show an anteriorally-everted wing likely due to morphogenetic defects during the first 12 hours of the pupal period. The expression data and lethal phase analysis indicate that there is a heightened requirement for GEFmeso expression during the early pupal stages. Therefore, I induced the GEFmeso transgene twice during this phase of development (37°C, 60 min each). The first induction occurred in late larvae within a few hours of pupariation. The second induction of GEFmeso expression occurred 12 hours later. This regimen increased adult viability 15-fold compared to untreated animals lacking the GEFmeso transgene (Table 5). In contrast, a single induction of GEFmeso delivered only to late larvae, increased adult viability 10-fold (Table 5) compared to untreated animals lacking a transgene. Heat treatment alone in the absence of a transgene did not rescue 18-5 lethality and reduced viability compared to untreated controls (Table 5).

Expression of the GEFmeso transgene also suppressed the frequency of the wing eversion and leg malformation phenotypes of 18-5 homozygotes. Expression of the GEFmeso transgene in late larvae and then twelve hours later reduced the frequency of the anterior everted wing phenotype from 75% to 5.5% (Table 6). Weaker rescue of the everted wing phenotype was observed if the induction of the GEFmeso transgene was limited to late

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larvae. In addition, expression of the GEFmeso transgene rescued leg malformation associated with homozygous 18-5 mutant animals from 77% to 28% (Table 7). Collectively, these data indicate the 18-5 mutation is an allele of GEFmeso. Henceforth, I will refer to 18-5 as GEFmeso.

Table 5 A GEFmeso Transgene Rescues Lethality Associated with 18-5 Mutant Homozygotes

Genotype	Treatment	% Viability	N
18-5/18-5 ; +/+	Untreated	1.9+/-0.3	1,366
18-5/18-5 ; t/+	Untreated	4.6 +/- 1.8	554
18-5/18-5 ; +/+	Late larvae + 12hrs (PP)	0.0 +/- 0.0	76
18-5/18-5 ; t/+	Late larvae +12hrs (PP)	29.4 +/- 6.6	234
18-5/18-5 ; t/+	Late larvae	20.5+/-1.9	156

Animals with and without transgene (indicated by t) were either treated at $37^{o}C$ for 60 minutes for the indicated protocol or left untreated and survival to adulthood was determined.

Table 6 A GEFmeso Transgene Rescues the Anterior Everted Wing Phenotype Associated with 18-5 Mutant Homozygotes

Genotype	Treatment	Anterior Everted Wing Phenotype (%)	N
18-5/18-5 ; t/+	Untreated	73.3	206
18-5/18-5	Untreated	76.4	127
18-5/18-5 ; t/+	Late larvae + 12hrs	7.5	108
18-5/18-5 ; t/+	Late Larvae	38.2	64

Animals with and without transgene (indicated by t) were either treated at $37^{o}C$ for 60 minutes at the indicated times or left untreated and the frequency of animals with at least one anteriorally-everted wing was determined

Table 7 A GEFmeso Transgene Rescues Leg Malformation Associated with 18-5 Mutant Homozygotes

	WT	Mild	Severe
Control	23.0% (26)	15.3% (26)	61.5% (26)
1X as Late Larvae	50.0% (32)	6.2% (32)	43.7% (32)
Late Larvae +12hr	72.2% (72)	9.7% (72)	18.0% (72)

Animals were treated at 37°C for 60min at the indicated time points. Control animals were not treated and maintained at 25°C. The numbers shown indicate the percentage of animals with malformed legs, with the total number of animals of the indicated genotype shown in parentheses. Experiments represent three replicates.

The Requirements for Rho GTPases During Wing Vein Formation Vary in Different Microenvironments of the Wing

GTPases are master regulators of most cell behaviors and a variety of tools have been developed for *in vivo* and *in vitro* investigations of their functions. Drosophila geneticists utilize a range of tools (mutants, RNAi, over-expression, dominant negative and constitutively active constructs) to investigate the roles of GTPases in various aspects of development. Tools allowing gene expression manipulation and cell imaging techniques provide evidence that cell processes coordinating tissue development (cell polarization, morphodynamics and migration) are GTPase-dependent. More recently, insights brought to the field by biochemical methods such as fluorescent tagging, imaging, and binding assays have shown GTPases may function antagonistically, synergistically or independent of one another to regulate cell behaviors. These biochemical approaches demonstrate that GTPase signaling programs are variable at the cellular level (Pertz, 2010). However, the question of whether intercellular GTPase signaling networks variation occurs within a single tissue is still unknown.
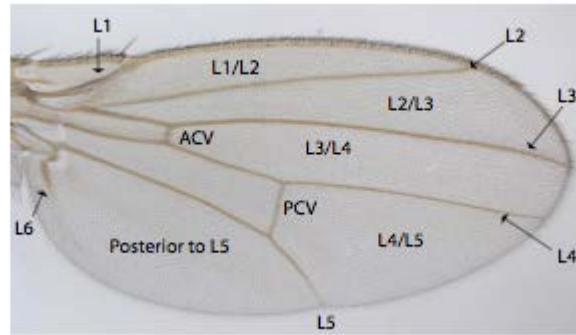
In the adult, a wild type wing has six longitudinal veins (L1-L6) separated by intervein tissue. Positioned along the proximal-distal axis are the anterior and posterior cross veins (ACV and PCV).

Figure 5 Topography of the Adult Wing

In order to investigate intercellular variability of GTPase networks, I have focused on a single developmental context, the *Drosophila* wing and specifically the formation of the two predominate tissues, vein and intervein (Figure 5). Since GTPase signals are intricately modulated, I opted to use an RNAi loss-of-function approach to investigate relationships between GTPases during wing vein development. I also used over-expression constructs, but only when there was an RNAi phenotype. Gain-of-function constitutively active and dominant negative mutants and transgenic constructs were excluded from the analysis because they have the potential to alter multiple GTPase signaling pathways, potentially confounding interpretation. To focus this investigation, I limited the analysis to regions of the wing requiring GEFmeso function.

GEFmeso is Required for Wing Vein Morphogenesis

GEFmeso is expressed ubiquitously in the wing imaginal disc in third instar larvae
(Blanke and Jackle, 2006). Furthermore, disruption of expression or proper function
results in two distinct wing phenotypes. First, GEFmeso[18-5] interacts genetically with
members of the Rho1 signaling pathway resulting in a 'crumpled' malformed wing
phenotype (Bayer et al, 2003). Second, perturbing GEFmeso function or expression
alters the wing vein pattern (Blanke and Jackle, 2006).

Animals homozygous for GEFmeso[18-5] have ectopic anterior cross veins and ectopic vein
material between longitudinal veins (L) two and three (Iketani, Fox and von Kalm,
unpublished observations). RNAi against GEFmeso targeted to the wing using the Bx-
Gal4 driver also results in ectopic cross veins adjacent to the anterior cross vein and/or
between L2 and L3 (Figure 3). These results indicate that GEFmeso is required to
suppress vein formation in the intervein region proximal and distal to the anterior cross
vein. Ectopic veins observed in 18-5 homozygotes and GEFmeso RNAi animals
indicates GEFmeso suppresses vein formation and in the intervein region between L2 and
L3. Consistent with these observations, over-expression of GEFmeso throughout the
entire wing using the Bx-Gal4 driver results in a loss of anterior cross vein phenotype.

Over-expression of GEFmeso also results in a loss of posterior cross vein material and the appearance of ectopic vein material surrounding the distal regions of L3 and L4. In contrast, GEFmeso RNAi expressed in the wing or GEFmeso[18-5] mutants exhibit neither phenotype. There are two interpretations of these observations. First, GEFmeso is able to suppress posterior cross vein formation and maintain intervein identity in the distal regions of L3 and L4, but may not be required in these microdomains. Alternatively, these phenotypes could be an artifact caused by over-expression of a protein that is not endogenous in these regions of the wing.

Requirements for GTPase signaling in Microdomains of the Wing

To investigate variation in networks requiring GEFmeso, I first identified GTPases required for wing vein development using an RNAi approach and assessed the impact on various microdomains in the wing (cross veins, longitudinal veins and intervein regions). I tested twelve genes by expressing RNAi transgenes in the wing and examined twelve microdomains (ACV, PCV, L2-L5 and intervein regions L1/L2, L2/L3, L3/L4, posterior to L5). I used the Beadex-Gal4 (Bx-Gal4) driver to express transgenes in the wing. This driver is located on the X chromosome and therefore the transgene has stronger expression in males than heterozygous females. After expressing the RNAi transgenes, I catalogued vein abnormalities as either ectopic tissue or loss of wild type tissue for each microdomain. The results from the RNAi studies revealed that the genes fit into one of three categories: 1) the gene is not required for vein development, 2) the gene is required for vein morphogenesis in a limited number of microdomains and is dispensable in

others, or 3) the gene is required globally for vein development (Table 8). In the following sections, I discuss each gene tested individually. I begin by discussing genes that have global effects on wing vein development and then discuss genes with more limited phenotypes.

Table 8 Genes Tested in this Study and the Number of Microdomains Affected

Gene	Number of Microdomains Affected
GEFmeso	3
Ras85D	6
Egfr	6
Rho1	3
Mtl	3
Ras64B	2
RhoGAPp190	2
RhoGEF2	3
Rac1	2
Rala	3
Cdc42	2
Roughened	0

A) w[1118] control, B) Bx-G4>UAS-Egfr.RNAi 18°C, C) Bx-G4>UAS-Ras85D.RNAi 18°C

Figure 6 Egfr and Ras85D are Global Regulators of Wing Vein Formation

Ras85D and Egfr are Global Regulators of Wing Vein Formation

Of the twelve genes tested, two signaling components (Egfr and Ras85D) behave as global regulators throughout the wing epithelium (Figure 6). The criterion to be classified, as a global regulator is to disrupt proper vein formation in at least half of the microdomains examined after RNAi expression. The role of Egfr as a global regulator of of wing vein development has been well established and was confirmed in this study (Blair, 2007; Appendix). In addition, this study revealed a second and previously unreported global regulator, Ras85D, which is required for vein formation in many regions of the wing (Figure 6C; Table 9).
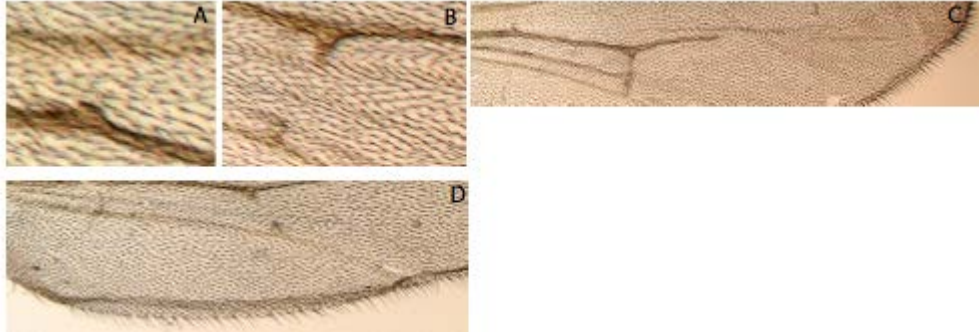
All animals are Bx-G4>UAS-Ras85D.RNAi at 25°C. A) Anterior cross vein, B) Posterior cross vein, C) Longitudinal vein four, and D) Longitudinal vein five

Figure 7 Ras85D RNAi Wing Phenotypes

Animals expressing Ras85D RNAi in the wing had several phenotypes. First, vein material was lost from the distal and/or the center portions of the vein. Second, while L2 was typically unaffected, L5 was severely affected with loss of all material except the proximal section. Third, the anterior and posterior cross veins were either partially or completely absent (Table 9 and Figure 7 A, B). Collectively, these results indicate that Ras85D is required for formation of L3, L4, and L5, and both cross veins. If Ras85D is a global regulator of wing vein formation we predict that over-expression will lead to ectopic vein material throughout the wing. As expected, over-expression of Ras85D in the wing results in ectopic wing vein material in L2-L5 and the intervein region between L4 and L5 (Appendix A).

Table 9 Microenvironments that Require Ras85D

Genotype	Sex	Micro-envronment	Wing Phenotype (%)		n	T (°C)
			Loss	Ectopic		
Bx-G4>UAS-Ras85D.RNAi	M	ACV	100	-	50	25
Bx-G4>UAS-Ras85D.RNAi	F	ACV	97.4	-	39	25
Bx-G4>UAS-Ras85D.RNAi	M	ACV	96.7	-	61	18
Bx-G4>UAS-Ras85D.RNAi	F	ACV	62.5	-	56	18
Bx-G4>UAS-Ras85D.RNAi	M	PCV	97.8	-	50	25
Bx-G4 >UAS-Ras85D.RNAi	F	PCV	97.4	-	39	25
Bx-G4 >UAS-Ras85D.RNAi	M	PCV	96.7	-	61	18
Bx-G4>UAS-Ras85D.RNAi	F	PCV	82.1	-	56	18
Bx-G4>UAS-Ras85D.RNAi	M	L4	100	-	50	25
Bx-G4>UAS-Ras85D.RNAi	F	L4	89.7	-	39	25
Bx-G4>UAS-Ras85D.RNAi	M	L4	21.3	-	61	18
Bx-G4>UAS-Ras85D.RNAi	F	L4	10.7	-	56	18
Bx-G4>UAS-Ras85D.RNAi	M	L5	100	-	50	25
Bx-G4>UAS-Ras85D.RNAi	F	L5	97.4	-	39	25
Bx-G4>UAS-Ras85D.RNAi	M	L5	1.6	-	59	18
Bx-G4>UAS-Ras85D.RNAi	F	L5	23.2	-	59	18

In contrast to Ras85D, wings expressing Egfr RNAi had a complex phenotype involving both loss of and ectopic vein material across the wing epithelium. For example, loss of both anterior and posterior cross veins and loss of vein material from L4 and L5 was observed, indicating a requirement for Egfr to promote vein formation in these regions. In addition, ectopic cross veins, and spots of vein material in the L2/L3 intervein region were observed (Figure 8C and Table 10). In contrast to Egfr RNAi, Ras85D RNAi has a uniform loss of vein material phenotype across the entire wing epithelium
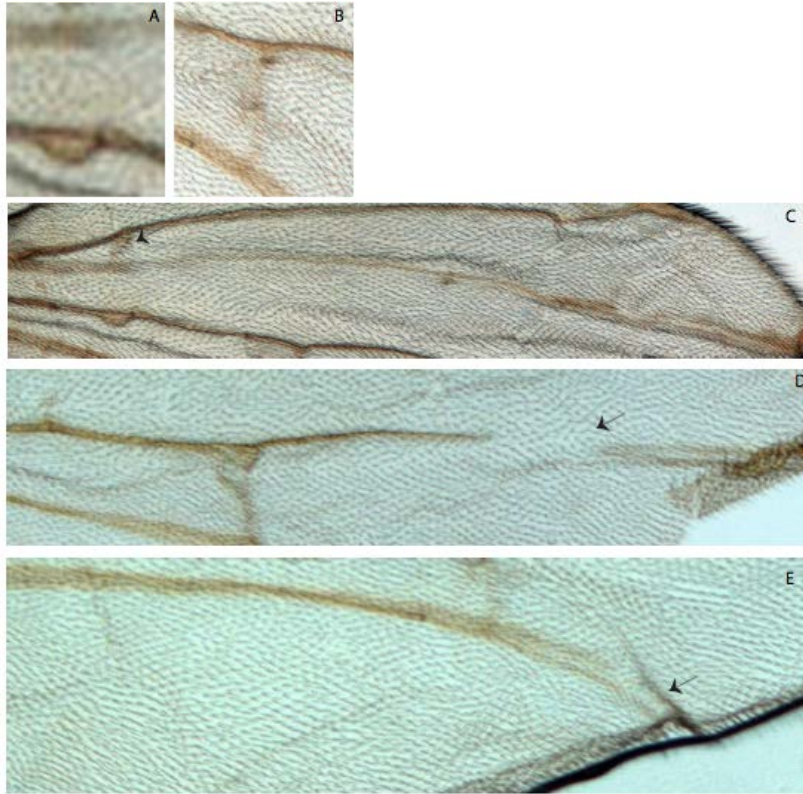
All animals are Bx-G4>UAS-Egfr.RNAi 18°C
Loss of the anterior cross vein phenotype, B) Loss of the posterior cross vein phenotype,
C) Arrowhead is pointing to ectopic material in the L2/L3 Intervein region, D)
Arrowhead is pointing to a loss of longitudinal vein four phenotype and E) Arrowhead is
pointing to an incomplete longitudinal vein five phenotype

Figure 8 Microdomains that Require Egfr

Table 10 Microenvironments that Require Egfr

Genotype	Sex	Micro-environment	Wing Phenotype (%) Loss	Ectopic	n	T (°C)
Bx-G4>UAS-Egfr.RNAi	F	ACV	100	-	47	25
Bx-G4>UAS-Egfr.RNAi	M	ACV	NS	NS	NS	25
Bx-G4>UAS-Egfr.RNAi	F	ACV	100	-	47	18
Bx-G4>UAS-Egfr.RNAi	M	ACV	100	-	22	18
Bx-G4>UAS-Egfr.RNAi	F	PCV	97.3	-	38	25
Bx-G4>UAS-Egfr.RNAi	M	PCV	NS	NS	NS	25
Bx-G4>UAS-Egfr.RNAi	M	PCV	45.5	-	22	18
Bx-G4>UAS-Egfr.RNAi	F	PCV	82.9	-	47	18
Bx-G4>UAS-Egfr.RNAi	F	L2/L3	-	29.7	47	18
Bx-G4>UAS-Egfr.RNAi	M	L2/L3	0	0	22	18
Bx-G4>UAS-Egfr.RNAi	F	L4	86.8	-	38	25
Bx-G4>UAS-Egfr.RNAi	M	L4	NS	NS	NS	25
Bx-G4>UAS-Egfr.RNAi	M	L4	100	-	22	18
Bx-G4>UAS-Egfr.RNAi	F	L4	17.0	-	47	18
Bx-G4>UAS-Egfr.RNAi	F	L5	92.1	-	38	25
Bx-G4>UAS-Egfr.RNAi	M	L5	NS	NS	NS	25
Bx-G4>UAS-Egfr.RNAi	M	L5	100	-	22	18
Bx-G4>UAS-Egfr.RNAi	F	L5	36.1	-	47	18

NS indicates wings were too malformed to be scored.

<u>Rho1 is Required for Anterior Cross Vein Formation and Suppresses Formation of the</u>

<u>Posterior Cross Vein and Vein material in the L2/L3 Intervein Region</u>

The role of Rho1 in wing vein development has not been well established and our results indicate a more limited role in wing vein formation. Like Egfr, Rho1 can promote or suppress vein development in different regions of the wing.

Table 11 Microenvironments that Require Rho1

Genotype	Sex	Micro-environment	Wing Phenotype (%) Loss	Ectopic	n	T (^{o}C)
Bx-G4>UAS-Rho1. RNAi	M	ACV	41.2	-	38	25
Bx-G4>UAS-Rho1. RNAi	F	ACV	53.7	-	95	25
Bx-G4>UAS-Rho1. RNAi	M	ACV	39.1	-	46	18
Bx-G4>UAS-Rho1. RNAi	F	ACV	54.3	-	57	18
Bx-G4>UAS-Rho1.RNAi	M	PCV	20.6	50.0	38	25
Bx-G4>UAS-Rho1.RNAi	F	PCV	13.7	66.3	95	25
Bx-G4>UAS-Rho1.RNAi	M	PCV	10.8	26.2	46	18
Bx-G4>UAS-Rho1.RNAi	F	PCV	8.7	28.0	57	18
Bx-G4>UAS-Rho1.RNAi	M	L2/L3	-	14.7	38	25
Bx-G4>UAS-Rho1.RNAi	F	L2/L3	-	13.7	95	25
Bx-G4>UAS-Rho1.RNAi	M	L2/L3	-	21.7	46	18
Bx-G4>UAS-Rho1.RNAi	F	L2/L3	-	10.5	57	18

At 25°C, Rho1 RNAi resulted in an incomplete loss of the anterior cross vein as well as a blurred boundary between intervein and vein material. At 18°C, the phenotype was similar with a faint to complete or incomplete loss of the ACV. We conclude that Rho1 is required for anterior cross vein formation. In contrast, Rho1 has a complex phenotype in the PCV with some animals exhibiting PCV loss and others with ectopic vein material (Table 11; Figure 9 A, B). In addition, reduced Rho1 expression resulted in ectopic vein spots along the proximal-distal axis in the L2/L3 intervein region (Figure 9 C and Table 11).
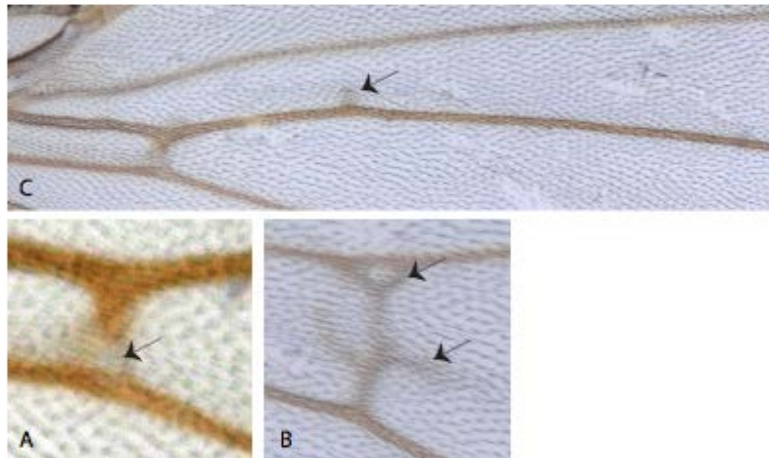
A) Anterior cross vein (Bx-G4>UAS-Rho1.RNAi 25°C), B) Posterior cross vein (Bx-G4>UAS-Rho1.RNAi 25°C) and C) Intervein L2/L3 (Bx-G4>UAS-Rho1.RNAi 18°C),

Figure 9 Microdomains that Require Rho1

<u>GEFmeso and Mtl Interact in the Anterior Cross Vein and longitudinal Vein Five</u>

Mtl is one of three Rac orthologs found in Drosophila (Lindquist, 2006; Van Aelst and Symons, 2002; Hakeda-Suzuki et al, 2002). Reduced Mtl expression caused mild incomplete loss of the PCV indicating a requirement for vein formation in this region in

the wing (Figure 10 A and Table 12). In contrast, RNAi against Mtl resulted in ectopic

vein material around L4 and L5 (Figure 10 B, C and Table 12). RNAi against Mtl

affected male wings but did not exhibit phenotypes in females, indicating a stronger

knockdown is needed to affect vein pattern.
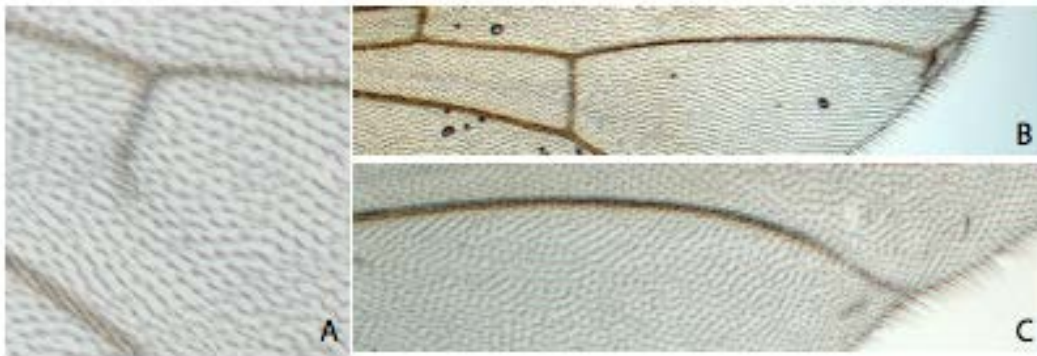
A) An incomplete PCV phenotype (Bx-G4>UAS-Mtl.RNAi 25°C), B) Ectopic vein
material extending from L5 (Bx-G4>UAS-Mtl.RNAi 29°C), and C) Short, ectopic
vein material in the intervein region posterior to L5 (Bx-G4>UAS-Mtl.RNAi 29°C)

Figure 10 Microdomains that Require Mtl

Table 12 Microenvironments that Require Mtl

Genotype	Sex	Micro-environment	Wing Phenotype (%) Loss	Wing Phenotype (%) Ectopic	n	T (^{o}C)
Bx-G4>UAS-Mtl.RNAi	M	PCV	18.3	-	60	25
Bx-G4>UAS-Mtl.RNAi	M	PCV	17.5	-	57	29
Bx-G4>UAS-Mtl.RNAi	M	L4	-	38.5	57	29
Bx-G4>UAS-Mtl.RNAi	M	L5	-	26.6	60	25
Bx-G4>UAS-Mtl.RNAi	M	L5	-	59.6	57	29

Rac1 is Required to Define the Boundaries of Longitudinal Veins Four and Five

Rac1 is required for morphogenesis, however a role in wing vein development has not been documented (Eaton et al, 1995). RNAi Rac1 caused ectopic veins to form around L4 and L5 (Table 15 and Figure 12). At a higher temperature (stronger RNAi expression) the L5 phenotype became variable with both loss and gain of material observed (Table 15). Again, only males had phenotypes after Rac1 RNAi expression indicating a high level of RNAi expression is needed to produce a phenotype.

Table 13 Microenvironments that Require Rac1

| | | | Wing Phenotype (%) | | | |
Genotype	Sex	Micro-environment	Loss	Ectopic	n	T (^oC)
Bx-G4>UAS-Rac1.RNAi	M	L4	0	40	45	29
Bx-G4>UAS-Rac1.RNAi	M	L5	0	14.4	47	25
Bx-G4>UAS-Rac1.RNAi	M	L5	15.7	53.3	45	29
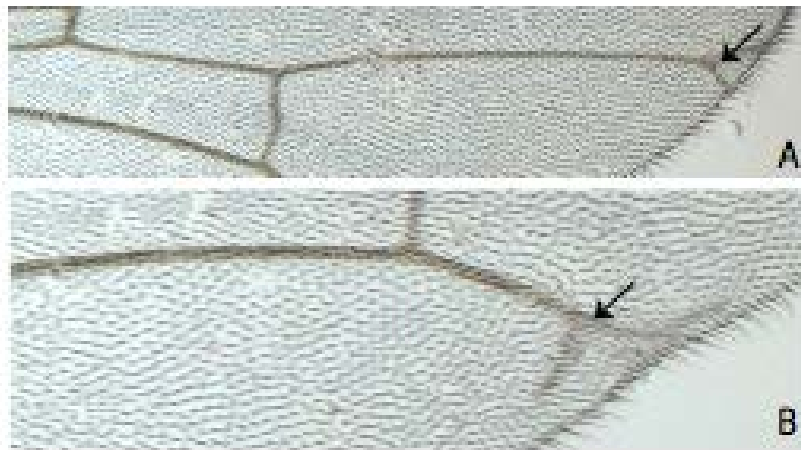
Both wings are Bx-G4>UAS-Rac1 RNAi at 25^oC. A) Ectopic extension from L4 is indicated by the arrowhead and B) Ectopic extension from L5 is indicated by the arrowhead

Figure 11 Microenvironments that Require Rac1

Ras64B is Required to Refine The Distal Ends of Longitudinal Veins Four and Five

The role of Ras64B on wing vein formation is unknown, but previous work indicates Ras64B may specify vein formation. Expression of activated Ras64B in the wing results in ectopic veins in intervein region L2/L3, ectopic extensions from the PCV and a thickened L3 (de Celis, 1997). RNAi against Ras64B results in ectopic vein extensions from the distal end of L4 and L5 (Table 13). No other defects were observed, raising the possibility that the previously reported observations involving expression of an activated Ras64B in the wing may not play a normal role there. Only males had phenotypes, which indicates that a strong knockdown is required to have an effect on the wing vein pattern.

Table 14 Microenvironments that Require Ras64B and RhoGAPp190

Genotype	Sex	Micro-environment	Loss	Ectopic	n	T (^{o}C)
			Wing Phenotype (%)			
Bx-G4>UAS-Ras64B.RNAi	M	L4	-	33.8	59	29
Bx-G4>UAS-Ras64B.RNAi	M	L5	-	52.5%	59	29
Bx-G4, UAS-Dcr2>UAS-RhoGAPp190.RNAi	M	ACV	62.2	-	56	25

RhoGAPp190 is Active in the Anterior Cross Vein

Currently, the role of RhoGAPp190 during wing vein formation is unknown. I expressed RNAi against RhoGAPp190 in the wing to determine if this GTPase regulator is required

for vein patterning. UAS-Dicer2 was included and required to amplify the RNAi

response to a biologically significant level. Only one region of the wing was affected.

RNAi against RhoGAPp190 caused the ACV to become faint, albeit still visible (Table

13). In contrast, over-expression of RhGAPp190 leads to a widespread ectopic vien

phenotype (C. Bayer, unpublished data) suggesting that RhoGAPp190 may act

redundantly in many regions of the wing.

RhoGEF2 Suppresses Vein Formation in the Intervein Regions, the Posterior Cross Vein and Longitudinal Vein Four

Linking role for RhoGEF2 in wing vein development has not been previously reported.

RNAi against RhoGEF2 resulted in ectopic vein material around the PCV, L4, and in the

L2/L3 intervein region (Figure 11 and Table 14).

Both images are from Bx-G4>UAS-Dcr2; UAS-RhoGEF2.RNAi animals at 25°C
A) Ectopic vein material in the L2/L3 Intervein intervein region and B) Ectopic extension from the PCV

Figure 12 Microdomains Requiring RhoGEF2

Table 15 Microenvironments that Require RhoGEF2

Genotype	Sex	Micro-environment	Wing Phenotype (%)		n	T (^{o}C)
			Loss	Ectopic		
Bx-G4;UAS-Dcr2>UAS-RhoGEF2.RNAi	M	PCV	0	19.6	56	25
Bx-G4;UAS-Dcr2>UAS-RhoGEF2.RNAi	F	PCV	0	24.6	57	25
Bx-G4;UAS-Dcr2>UAS-RhoGEF2.RNAi	M	L2/L3	0	28.5	56	25
Bx-G4;UAS-Dcr2>UAS-RhoGEF2.RNAi	M	L4	0	23.2	56	25

<u>Rala is Required to Form the Anterior and Posterior Cross Veins</u>

The effect of Rala RNAi was limited to the ACV and PCV where a strong loss of vein phenotype was observed (Figure 13 and Table 16).
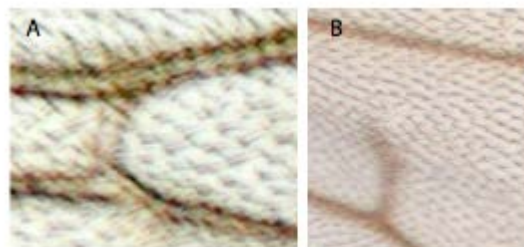
A) Faint loss of ACV phenotype (Bx-G4>UAS-Rala.RNAi at 25°C) and B) Incomplete loss of PCV phenotype (Bx-G4>UAS-Rala.RNAi at 18°C)

Figure 13 Microdomains Requiring Rala

Table 16 Microenvironments that Require Rala

Genotype	Sex	Micro-environment	Wing Phenotype (%)		n	T (^{o}C)
			Loss	Ectopic		
Bx-G4>UAS-Rala.RNAi	F	ACV	94.2	-	87	25
Bx-G4>UAS-Rala.RNAi	M	ACV	89.4	-	19	25
Bx-G4>UAS-Rala.RNAi	M	ACV	46.8	-	47	18
Bx-G4>UAS-Rala.RNAi	M	PCV	94.7	-	19	25
Bx-G4>UAS-Rala.RNAi	F	PCV	96.5	-	15	25
Bx-G4>UAS-Rala.RNAi	M	PCV	91.4	-	59	18
Bx-G4>UAS-Rala.RNAi	F	PCV	28.8	-	47	18

Interactions Between GEFmeso and Other GTPase Signaling Components in the Wing

The single gene analysis indicated specific roles for GTPase signaling components in the wing. I next asked if genetic interactions between gene pairs are observed in different regions of the wing. To narrow the focus, I examined interactions between GEFmeso and other GTPase signaling genes. These experiments were performed by simultaneous RNAi against the GTPase signaling gene and GEFmeso or by RNAi in combination with heterozygosity for the GEFmeso[18-5] mutation (i.e. 18-5/+).

Reduced GEFmeso Expression Suppresses the Rala RNAi Phenotype in the Anterior and

Posterior Cross Veins

Blake and Jackle (2006) showed in vitro that GEFmeso binds to GTP-bound Rala in a region distinct from the DHPH domain. I have shown that Rala and GEFmeso are required for the ACV development, albeit with opposite activities (loss of ACV with RNAi Rala and ectopic ACV with RNAi GEFmeso). In addition, over-expression of GEFmeso (but not RNAi knockdown which has no phenotype) leads to loss of the PCV, a phenotype also seen with RNAi Rala. To investigate *in vivo* interactions between GEFmeso and Rala I tested for interactions between GEFmeso and Rala in the anterior and posterior cross veins by co-expressing Rala RNAi with GEFmeso RNAi or in a GEFmeso[18-5] heterozygous background. In both instances, the penetrance of the Rala RNAi phenotype was suppressed. For example, in the ACV 94% of animals show a loss of ACV phenotype following Rala RNAi, whereas only 5% of animals have this phenotype in a Rala RNAi GEFmeso RNAi animals. Suppression of the Rala phenotype was consistently stronger in the ACV than the PCV (Tables 17 and 18). In addition, the amount of ectopic vein material in the ACV of double RNAi animals increased which is a phenotype associated with RNAi GEFmeso but not RNAi Rala. These results indicate an antagonistic relationship between GEFmeso and Rala. A genetic interaction is not unexpected since *in vitro* experiments show physical interactions between GEFmeso and Rala (Blanke and Jackle, 2006).

Table 17 Interactions Between Rala and GEFmeso in the Anterior Cross Vein

Genotype	Sex	Micro-environment	Wing Phenotype (%)		n	T (^{o}C)
			Loss	Ectopic		
18-5/+	F	ACV	1.7	-	58	25
Bx-G4>UAS-GEFmeso.RNAi	F	ACV	-	4.6	107	25
Bx-G4>UAS-Rala.RNAi	F	ACV	94.2	-	88	25
Bx-G4>UAS-Rala.RNAi; 18-5/+	F	ACV	49	-	53	25
Bx-G4> UAS-Rala.RNAi; UAS-GEFmeso.RNAi	F	ACV	5.5	11.1	36	25

Table 18 Interactions between GEFmeso and Rala in the Posterior Cross Vein

Genotype	Sex	Micro-environment	Wing Phenotype (%)		n	T (^{o}C)
			Loss	Ectopic		
18-5/+	F	PCV	0	-	58	25
Bx-G4>UAS-GEFmeso.RNAi	F	PCV	0	-	107	25
Bx-G4>UAS-Rala.RNAi	F	PCV	96.5	-	88	25
Bx-G4>UAS-Rala.RNAi; 18-5/+	F	PCV	71.6	-	53	25
Bx-G4> UAS-Rala.RNAi; UAS-GEFmeso.RNAi	F	PCV	75	-	36	25

Reduced GEFmeso Expression Enhances the Rac1 Anterior Cross Vein Phenotype

I also tested for interactions between GEFmeso and Rac1. A small percentage (3%) of Rac1 RNAi animals have an anterior cross vein defect. In contrast, GEFmeso[18-5] and GEFmeso RNAi animals have ectopic ACV phenotypes. Rac1 RNAi expressed in a GEFmeso[18-5] heterozygous background enhanced the Rac1 loss of ACV phenotype 7-fold (Table 19). A similar pattern, but weaker interaction occurred in the PCV (Table 19). Overall, the data indicate an interaction between Rac1 and GEFmeso in the ACV.

Table 19 GEFmeso and Rac1 interact in the Anterior Cross Vein

| Genotype | Sex | Wing Phenotype (%) | | N | T (^{o}C) |
		ACV Loss	PCV Loss		
18-5/+	M	0	0	60	25
Bx-G4>UAS-Rac1.RNAi	M	3.1	14.7	95	25
Bx-G4>UAS-Rac1.RNAi; 18-5/+	M	22.8	24.5	57	25

There were several microdomains where either GEFmeso or Rac1 was required for vein development, but there was no genetic interaction in these microdomains. For example, RNAi Rac1 results in ectopic tissue extending from L4, L5 and in the intervein region posterior to L5. Simultaneously removing a copy of 18-5 did not affect these phenotypes (Table 20). Thus the interaction between Rac1 and GEFmeso is context dependent and varies in different microdomains in the wing.

Table 20 Rac1 and GEFmeso Do Not Interact Genetically in Longitudinal Veins Four and Five or Posterior to Longitudinal Vein Five

Genotype	Sex	L4	L5	Posterior to L5	n	T (^{o}C)
			Ectopic Veins(%)			
18-5/+	M	0	0	0	60	25
Bx-G4>UAS-Rac1.RNAi	M	9.5	13.4	29.8	95	25
Bx-G4> UAS-Rac1.RNAi; 18-5/+	M	0	19.2	26.3	57	25

Reduced GEFmeso Expression Enhances Mtl RNAi Phenotypes in the Anterior Cross Vein, the Posterior Cross Vein, and Longitudinal Vein Five

I tested for interactions between GEFmeso and Mtl *in vivo*. Mtl RNAi animals do not have an ACV phenotype, but do have a loss of PCV phenotype. Expression of Mtl RNAi in a GEFmeso[18-5] heterozygous mutant enhances the loss of cross vein phenotype in both veins (Table 21). Mtl RNAi results in ectopic tissue surrounding longitudinal vein five. The two major phenotypes are extensions from longitudinal vein five or ectopic tissue directly posterior to L5 (compare Figures. 10.C and 12.B to observe differences in the phenotype). Mtl RNAi results in 32% of animals with ectopic material in or around L5. In contrast, expression of Mtl RNAi in a GEFmeso[18-5] heterozygous background increased the penetrance of this phenotype to 70% (Table 21). Thus, Mtl and GEFmeso appear to interact genetically in the ACV, PCV in the region around L5.

Table 21 Mtl and GEFmeso Interact in the Posterior Cross Vein, Longitudinal Vein Five and in the Intervein Region Posterior to Longitudinal Vein Five

		Loss of Veins (%)		Ectopic Veins (%)			
Genotype	Sex	ACV	PCV	L5	Posterior to L5	n	T ($^{\circ}$C)
18-5/+	M	0	0	0	0	60	25
Bx-G4> UAS-Mtl.RNAi	M	8.3	18.3	26.6	5.7	60	25
Bx-G4> UAS-Mtl.RNAi; 18-5/+	M	20.3	37.2	14	56	59	25

Reduced GEFmeso Expression Enhances the Ras85D RNAi Loss of Longitudinal Vein

Phenotypes

Since Ras85D is a global regulator of wing development, we tested for an interaction

with GEFmeso. RNAi Ras85D animals exhibited loss of ACV, PCV, L2, L3, L4, and L5

vein material. The strongest phenotypes were in the ACV, PCV and L5 (Appendix A).

The weakest phenotype was in L2. Reduced GEFmeso expression (either GEFmeso[18-5]

or GEFmeso RNAi) did not modify the Ras85D RNAi phenotypes in the ACV or PCV

(Appendix C). This may indicate that GEFmeso and Ras85D do not interact in the cross

vein regions of the wings. Alternatively, the penetrance and severity of the Ras85D

phenotype may be too high to detect a synergistic interaction.

In contrast, simultaneous reduction of GEFmeso (GEFmeso[18-5] or GEFmeso RNAi) and

Ras85D enhanced the Ras85D loss of vein phenotype (Table 22). I found enhancement

of the Ras85D RNAi phenotype was most profound in regions of the wing where Ras85D

has the weakest phenotypes. For example, in females, where there is weaker expression

of Ras85D RNAi, removing a copy of GEFmeso increases the loss of L2 vein phenotype

by 4-fold whereas in males, where Ras85D RNAi is stronger, the loss of vein phenotype

increased only 2-fold (Table 22 and Appendix C). In a second example, L2 had the

weakest phenotype RNAi Ras85D and after removing a copy of GEFmeso[18-5], the

phenotype increased 4-fold. In L5, where RNAi Ras85D had the most severe phenotype,

removing a copy of GEFmeso[18-5] did not change the phenotype (Table 22). These results

indicate a global, synergistic relationship between GEFmeso and Ras85D in the

longitudinal veins.

Table 22 GEFmeso Enhances the Ras85D Phenotype in the Longitudinal Veins

Genotype	Sex	L2	L3	L4	L5	N	T (°C)
		Loss of Vein Phenotype (%)					
Bx-G4>UAS-Ras8D.RNAi;	F	60	93.3	100	93.3	30	25
18-5/+	M	66.6	94.4	100	94.4	18	
Bx-G4>UAS-GEFmeso	F	47.5	100	100	100	40	25
RNAi; UAS-Ras85D.RNAi							
	M	66.6	94.4	100	94.4	18	25
Bx-G4>UAS-Ras85D.RNAi	F	12.8	76.9	89.7	97.5	58	25
	M	29.7	100	100	100	60	
18-5/+	M	0	0	0	0	58	25
	F	0	0	0	0	60	
Bx-G4>GEFmeso.RNAi	F	0	0	0	0	107	25

In summary, I tested for interactions between GEFmeso and four genes in the wing using an RNAi-loss of function approach. I found distinct differences in the interaction profiles between GEFmeso and other GTPase genes across microenvironments in the wing (Table 23). This genetic data provides evidence that GTPase signaling networks exhibit intercellular variation within an otherwise uniform epithelium.

Table 23 Interactions between GEFmeso and GTPases Across the Wing

Gene 1	Rala	Rac1	Mtl	Ras85D
ACV	A	WI	I	NI, NI
PCV	A	WI	I	NI
L2	NI	NI	NI	S
L3	NI	NI	NI	S
L4	NI	NI	NI	S
L5/Posterior to L5	NI	NI	I	WS
L1/L2	NI	NI	NI	NI
L2/L3	NI	NI	NI	NI
L3/L4	NI	NI	NI	NI
L4/L5	NI	NI	NI	NI

Antagonistic (A): One gene suppresses the phenotype of the other
Synergistic (S): One gene enhances the phenotype of the other
I: Interaction between two genes that cannot be characterized as antagonistic or synergistic
NI: No interaction
NI: GEFmeso has a phenotype that is not modified by Gene 2
NI: Gene 2 has a phenotype that is not modified by GEFmeso
W: Weak interaction (interaction, synergistic, or antagonistic)

CHAPTER FOUR: DISCUSSION

There is mounting evidence that the concept of GTPase signaling as a series of linear or branched pathways connected by 'cross talk' is too simplistic. A more accurate reflection of cellular events may be one in which GTPases act within the context of networks with multiple nodes and points of connectivity. In this network model, some nodes are more important for network function than others (i.e. have non-redundant functions), and mutations affecting nodes that are critical for network function are more likely to disrupt the network (i.e. have phenotypes) than less essential nodes (Costanza et al, 2010). While variation between GTPase networks has been explored at a sub-cellular level, such variation is unknown in a tissue. At the subcellular level in migrating MEFs, RhoA activation is localized within the first two micrometers from the membrane at sites of protrusion, whereas Rac1 and Cdc42 are activated at and beyond two micrometers from the membrane (Pertz, 2010; Kiyokawa et al, 2011; Machacek et al, 2009). Several other recent studies examine subcellular GTPase signaling patterns (Machacek et al, 2009; Aoki et al 2004; Itoh et al, 2002; Kurakowa et al, 2009; Pertz, 2010; Pertz et al, 2006), but none have attempted to examine intercellular GTPase network signaling in similar developmental contexts *in vivo*. Thus, comparing signaling networks between similar cells is the next step to understanding the developmental roles of GTPases.

GEFmeso is a Guanine Nucleotide Exchange Factor that Interacts with GTPases in Multiple Developmental Contexts

Previous studies have established that the signaling program of a GTPase, and its interacting partners are not only cell-type dependent but also are tightly regulated spatially and temporally. Although *in vitro* assays give insight into intermolecular and intramolecular interactions on a protein domain level, identifying and exploring the variation in GTPase interactions *in vivo* is important to validate their signaling activities. To do this, we investigated a mutant, 18-5, that interacts genetically with Rho1 during leg morphogenesis and embryogenesis (Halsell et al, 2000; Maggie, 1999; Strutt, 1997). Here I provide evidence demonstrating that 18-5 is a mutation in the guanine nucleotide exchange factor GEFmeso. I show that a GEFmeso transgene can functionally rescue mutant phenotypes observed in 18-5 homozygous animals. The phenotypes rescued were lethality, an anteriorly everted wing phenotype, and leg malformation, indicating GEFmeso is vital for embryonic and adult development. The role of the GEFmeso short transcript remains to be investigated. The short transcript lacks the DHPH domains, which would confer GEF activity, but does retain the Ral GTPase binding region and other protein binding motifs (Schmidt and Hall, 2002; Blanke and Jackle, 2006). I did not attempt to perform the 18-5 rescue with the short transcript, but it would be interesting to determine if it has functions that overlap with the long transcript. However, the long and short transcripts are expressed at distinct, non-overlapping time points, and therefore it is unlikely that GEFmeso short could function in place of the long transcript.

The GEFmeso long protein is active in multiple tissues (wing, embryonic tissue, and leg) and has genetic and/or physical interactions with several GTPases. GEFmeso binds

physically to GTP-bound Rala and nucleotide depleted Cdc42 *in vitro*, suggesting

GEFmeso is an exchange factor for Cdc42 and an effector of Rala. However, *in vitro*

binding studies reveal GEFmeso does not bind to Rho1 (Blanke and Jackle, 2006).

Therefore it is likely that GEFmeso communicates with Rho1 indirectly. Previously it

was unknown if GEFmeso interacts with Rala *in vivo*. However, I have shown an

antagonistic relationship between GEFmeso and Rala in the anterior cross vein regions of

the wing. The *in vivo* relationship between GEFmeso and Cdc42 remains to be

determined.

GEFmeso shares DH sequence similarity with Dbl and Dbl's Big Sister (Dbs), both of

which are Rho1 and Cdc42 exchange factors (Blanke and Jackle, 2006), suggesting

GEFmeso may be an activator of Rho1 and Cdc42. In DBS, the PH is required for the

DH domain to bind the GTPase (Schmidt and Hall, 2002; Rossman et al, 2002). The PH

domain has traditionally been thought to mediate binding to the phosphoinositides of the

membrane, but more recent studies have shown the PH domain also increases the

catalytic activity of the DH domain and in some cases may interact directly with the

GTPase (Baumeister et al, 2006). Sequencing analysis of the GEFmeso[18-5] mutation

reveals a deletion that retains the DH domain, but loses approximately half of the PH

domain resulting in a frameshift and premature truncation of the protein. The

consequences of the GEFmeso[18-5] deletion are likely reduced efficiency of the catalytic

domain and reduced ability to localize to the plasma membrane.

GTPases have microenvironment-dependent interactions

GEFmeso interacts genetically and biochemically with multiple GTPases in a variety of developmental contexts. Thus GEFmeso is a good starting point to examine potential variation in the intracellular structure of GTPase networks. To address these questions, I first surveyed the requirement for a subset of GTPases and a few regulators in different microdomains in the wing. I identified a new global regulator of vein fate, Ras85D, by expressing RNAi against Ras85D through out the wing. RNAi against Ras85D caused loss of vein tissue from all veins in the wing. This result was not surprising since loss of function Ras mutants result in loss of veins and Ras signaling can be detected in developing wing veins (Blair, 2007; Baonza and Garcia-Belido, 1999; Ciapponi et al 1999). As expected, RNAi against Egfr (a second global designator of vein fate) disrupted most microdomains of the wing. The phenotypes of RNAi animals was more complex than that of RNAi Ras85D. Egfr suppressed vein material in intervein region L2/L3 but promoted vein formation in the ACV, PCV and longitudinal veins 3-5. This result is consistent with reports that Egfr signaling switches on and off in the veins at different time points in development (Martin-Blanco et al, 1999) and an RNAi approach could result in opposite phenotypes depending on the temporal expression pattern of the Gal4 driver. Ras85D is downstream of Egfr and both are required for wing vein development (Sotillos and Campuzano, 2000; Marcoux and Vuori, 2005; Munoz-Descalzo et al, 2007). The role of GEFmeso in the Egfr-Ras signaling pathway remains to be investigated. Although, we did not investigate the interaction between GEFmeso and Egfr, I show that GEFmeso interacts with Ras in four wing microdomains. One interesting avenue to explore would be the interaction between Ras85D and Egfr in the

L2/L3 intervein region. This intervein region was the only region where ectopic veins were observed in an Egfr RNAi animal. Egfr acts upstream of Ras, and therefore the contrasting phenotype is unexpected.

I asked if proteins required for wing vein development exhibit consistent behavior across the wing epithelium. In general we found that proteins behaved consistently across the wing, however some proteins showed microenvironment variation in behavior. Examples are Rho1 and Mtl which suppressed vein development in some microenvironments and promoted veins in others. Rac1 and Mtl both promoted vein formation in the cross veins but suppressed veins around longitudinal vein five. It is worth mentioning that the Rac1 RNAi phenotype in the ACV had low penetrance, however it did interact with GEFmeso[18-5] resulting in a substantial loss of ACV tissue. Furthermore, both Mtl and Rac1 showed similar interactions with GEFmeso. Phenotypes for both proteins were enhanced in the anterior and posterior cross veins when GEFmeso was simultaneously reduced. However, Rac1 did not interact genetically with GEFmeso in the longitudinal vein five microdomain while Mtl did.   GEFmeso failed to interact with Rac1 in the L2/L3 intervein region or in the longitudinal veins four and five.

I also showed that GEFmeso interacts with Rala and Ras85D in a microenvironment-dependent manner. The interaction between GEFmeso and Rala is antagonistic in the

cross veins, however, I failed to detect any genetic interaction in the intervein region between L2/L3, a microenvironment where GEFmeso suppresses vein formation.

Ras85D and GEFmeso Interact Globally in the Wing

Finally, we show that GEFmeso interacts globally with Ras85D in the wing and the interactions are microenvironment dependent. Both proteins are required for anterior cross vein formation but there was no interaction between them in this region of the wing. It is possible that a synergistic interaction could not be detected due to the high penetrance of the Ras85D phenotype. However, an antagonistic relationship between Ras85D and GEFmeso can be ruled out since suppression of the RNAi Ras85D phenotype was not observed. Alternatively, Ras85D and GEFmeso may operate in non-interacting networks in the anterior cross vein. The interaction between GEFmeso and Ras in the longitudinal veins was an unexpected result because animals expressing RNAi against GEFmeso, or GEFmeso[18-5] homozygotes do not have longitudinal vein defects. However, reduced GEFmeso signaling consistently enhanced the Ras85D loss of vein phenotype. The interaction is weak in longitudinal vein five; the longitudinal vein where the Ras85D phenotype is strongest. One possible explanation for the observations is that GEFmeso activity is redundant in this region of the wing and a phenotype is only observed when additional genes (Ras85D) are also knocked down. In summary, the interaction data for GEFmeso and Rala, Rac1, Mtl and Ras85D provide evidence for microenvironment-dependent variation in GTPase signaling networks in specific domains of the wing, and reveal intercellular variation in GTPase signaling within an otherwise uniform epithelium.

Summary

I characterized the genetic interactions of GEFmeso, a gene that interacts with multiple GTPases, within a single tissue. I have shown that despite structural uniformity across the wing, the signaling programs utilized vary in different microenvironment. I have also shown that GTPase signaling pathways can have opposite phenotypic effects depending on microenvironment. Thus I have demonstrated that the ways GTPase networks are constructed and interconnected are not uniform across an epithelium. This work adds to existing studies that have demonstrated subcellular variation in GTPase networks and points to the complexity of understanding protein network organization at a subcellular and intercellular level. Ultimately, this work has relevance to human pathology such as cancer, where signaling networks unique to the cancer and localized to a specific niche in the tumor (i.e. stem cell niche), may be targeted with minimum toxicity to cells.

APPENDIX A: RESULTS OF SINGLE RNAI EXPRESSION DATA

Genotype/T(^{o}C)/ Stocks Used	Sex	N	WT	Defect	ACV	PCV	L2	L3	L4	L5	L1/L2	L2/L3	L3/L4	L4/L5	Posterior to L5
Bx-G4>UAS-Ras64B.dsRNA/25/ BL8860/BL29318	F	99	88	Loss	0	<1	1	1	1	4	0	0	0	0	0
				Ect.	0	4	0	3	0	0	0	0	0	0	0
	M	100	23	Loss	27	14	0	0	0	17	0	0	0	1	0
				Ect.	0	0	0	1	24	15	0	10	1	3	11
Bx-G4>UAS-Ras64B.dsRNA/29/ BL8860/BL29318	F	60	95	Loss	0	0	0	0	0	0	0	0	0	0	0
				Ect.	0	0	0	1.6	1.6	1.6	0	0	0	0	0
	M	59	20	Loss	6.7	20.3	0	0	0	0	0	0	0	0	0
				Ect.	0	0	5	3.3	33.8	52.5	0	3.3	0	1.6	13.5
Bx-G4>UAS-RhoGAPp190. RNAi/25/BL 8860, BL 6430	F	137	99	Loss	0	0	0	0	0	0	0	0	0	0	0
				Ect.	0	1	0	0	0	0	0	0	0	0	0
	M	114	94	Loss	7	0	0	0	0	0	0	0	0	0	0
				Ect.	0	0	0	0	0	0	0	0	0	0	0
Bx-G4>UAS-RhoGAPp190. RNAi/29/BL 8860, BL 6430	F	60	96.6	Loss	0	1.6	0	0	0	0	0	0	0	0	0
				Ect.	0	0	0	0	0	0	0	0	0	0	0
	M	55	46.5	Loss	46.5	0	0	0	10.3	0	0	0	0	0	0
				Ect.	0	0	0	0	0	0	0	0	0	0	0
Bx-G4>UAS-Dcr2;UAS-p190.TRiP/25/BL 25706/BL 31070	F	57	91.3	Loss	3.8	3.8	0	0	0	0	0	0	0	0	0
				Ect.	0	0	0	0	0	0	0	0	1.7	0	0
	M	61	6.5	Loss	62.2	21	0	0	1.6	1.6	0	0	0	0	0
				Ect.	0	0	1.6	0	6.5	0	0	1.6	0	0	1.6
Bx-G4>UAS-Ras85D.TRiP/18/ BL 8860, BL 29319	F	56	10.7	Loss	62.5	82.1	0	17.8	10.7	23.2	0	0	0	0	0
				Ect.	0	0	0	0	0	0	0	0	0	0	0
	M	61	0	Loss	96.7	96.7	4.9	37.7	21.3	1.6	0	0	0	0	0
				Ect.	0	0	0	0	0	0	0	0	0	0	0

Numbers represent percents of total wings scored that had the indicated defect. N is the sample of wings scored for defects

Genotype/T(^{o}C)/ Stocks Used	Sex	N	WT	Defect	ACV	PCV	L2	L3	L4	L5	L1/L2	L2/ L3	L3/ L4	L4/ L5	Posterior to L5
Bx-G4>UAS-Ras85D.TRiP/25/ BL 8860, BL 29319	F	39	0	Loss	97.4	97.4	12.8	76.9	89.7	97.4	0	0	0	0	0
				Ect.	0	0	0	0	0	0	0	0	0	0	0
	M	50	0	Loss	100	97.8	29.7	100	100	100	0	0	0	0	0
				Ect.	0	0	0	0	0	0	0	0	0	0	0
Bx-G4>UAS-Dcr2;UAS-RhoGEF2.TRiP/25/BL 25706, BL 31239	F	57	49.1	Loss	0	0	0	0	0	0	0	0	0	0	0
				Ect.	0	24.5	0	3.5	14	0	0	5.2	0	0	0
	M	56	25	Loss	0	3.5	0	0	0	0	0	0	0	0	0
				Ect.	0	19.6	1.7	8.9	23..2	5.3	1.7	28.5	0	1.7	8.9
Bx-G4>UAS-RhoGEF2.TRiP/25/ BL8860, HMS01118	F	58	17.2	Loss	0	10.3	0	0	0	0	0	0	0	0	0
				Ect.	0	55.1	0	1.7	27.5	0	0	3.4	0	1.7	0
	M	56	5.3	Loss	3.5	33.9		1.7	0	1.7	0	0	0	0	0
				Ect.	1.7	32.1	1.7	5.3	53.5	37.5	0	12.5	0	3.5	16
Bx-G4>UAS-Rac1.TRiP/25/ BL 8860, BL 28985	F	104	96	Loss	0	<1	0	0	0	0	0	0	0	0	0
				Ect.	0	<1	0	0	<1	0	0	0	0	0	0
	M	95	65.2	Loss	3.1	14.7	0	0	0	0	0	0	0	0	0
				Ect.	0	0	0	0	9.5	13.4	0	0	0	0	29.8
Bx-G4>UAS-Rac1.TRiP/29/ BL 8860, BL 28985	F	50	72	Loss	0	0	6	2	14	0	0	0	0	0	0
				Ect.	6	10	0	0	4	4	0	0	0	0	0
	M	45	13.3	Loss	6.6	6.6	2.2	0	2.2	15.5	0	0	0	0	24.5
				Ect.	2.2	0	2.2	0	40	53.5	0	0	0	2.2	4.4
Bx-G4>UAS-Rho1.dsRNA/25/ BL 8860, BL 9910	F	95	8.4	Loss	53.7	13.7	0	0	0	0	0	0	0	0	0
				Ect.	1.1	66.3	0	1.5	0	0	0	13.7	0	1.1	0
	M	38	11.8	Loss	41.2	20.6	0	1.5	1.5	4.4	0	0	0	0	0
				Ect.	2.9	50	1.5	10.3	16.2	0	0	14.7	2.9	7.4	0

Numbers represent percents of total wings scored that had the indicated defect. N is the sample of wings scored for defects

Genotype/T(^{o}C)/ Stocks Used	Sex	N	WT	Defect	ACV	PCV	L2	L3	L4	L5	L1/L2	L2/ L3	L3/ L4	L4/ L5	Posterior to L5
Bx-G4>UAS-Rho1.dsRNA/18/ BL 8860, BL 9910	F	57	26.3	Loss	54.3	8.7	0	0	0	5.2	0	0	0	0	0
				Ect.	0	28	3.5	3.5	5.2	0	5.2	10.5	0	0	0
	M	46	23.9	Loss	39.1	10.8	0	0	0	0	0	0	0	0	0
				Ect.	0	26.2	6.5	2.1	6.5	4.3	0	21.7	0	0	0
Bx-G4>UAS-Mtl.TRiP/25/ BL8860/ BL 28622	F	60	80	Loss	0	10	0	0	0	1.6	0	0	0	0	0
				Ect.	0	1.6	0	0	5	0	0	0	0	0	0
	M	60	48.3	Loss	8.3	18.3	0	0	0	01.6	0	0	0	0	0
				Ect.	0	0	0	0	10	26.6	0	0	0	0	0
Bx-G4>UAS-Mtl.TRiP/29/ BL8860/ BL 28622	F	43	93	Loss	0	0	0	0	0	0	0	0	0	0	0
				Ect.	0	4.6	0	0	2.3	0	0	0	0	2.3	0
	M	57	26.3	Loss	3.5	17.5	0	0	0	1.7	0	0	0	0	0
				Ect.	0	0	0	0	38.5	59.6	0	0	0	0	1.7
Bx-G4>UAS-Cdc42.TRiP/18/ BL8860/ BL 28021	F	10	0	Loss	50	50	0	0	0	0	0	0	0	0	0
				Ect.	40	0	0	0	40	0	0	40	0	0	0
	M	NS		Loss											
				Ect.											
Bx-G4>UAS-GEFmeso Long/18/BL8860, Line 75B2	F	53	1.8	Loss	69.8	88.6	0	0	0	0	0	0	0	0	0
				Ect.	0	3.7	0	0	9.4	3.7	0	0	0	0	0
	M	42	0	Loss	100	100	0	0	0	14.2	0	0	0	0	0
				Ect.	0	0	28.5	23.8	45.2	26.1	0	2.3	0	0	0
18-5/18-5/ 25	F	13	53.8	Loss	0	0	0	0	0	0	0	0	0	0	0
				Ect.	23	15.3	0	0	0	0	0	30.7	0	0	0
	M	25	36	Loss	4	0	0	0	0	0	0	0	0	0	0
				Ect.	28	24	0	0	0	0	0	40	0	0	0

Numbers represent percents of total wings scored that had the indicated defect. N is the sample of wings scored for defects
NS: Animals were too malformed and phenotype could not be scored

Genotype/T(^{o}C)/ Stocks Used	Sex	N	WT	Defect	ACV	PCV	L2	L3	L4	L5	L1/L2	L2/ L3	L3/ L4	L4/ L5	Posterior to L5
Bx-G4>UAS-CG30115.RNAi/ 25/ BL 8860, v39952 (I)	F	107	95.3	Loss	0	0	0	0	0	0	0	0	0	0	0
				Ect.	4.6	0	0	0	0	0	0	0	0	0	0
	M	NA		Loss											
				Ect.											
Bx-G4>UAS-G30115.RNAi/ 29/ BL 8860, v39952 (I)	F	59	67.7	Loss	0	1.6	0	0	0	0	0	0	0	0	0
				Ect.	28.8	1.6	0	0	1.6	1.6	0	0	0	0	0
	M	NA		Loss											
				Ect.											
Bx-G4>UAS-Dcr2; UAS-CG30115.RNAi/ 29/ BL 25706, v39952 (I)	F	59	27.1	Loss	0	0	0	0	0	0	0	0	0	0	0
				Ect.	67.7	1.6	1.6	6.7	0	0	0	0	0	0	0
	M	NA		Loss											
				Ect.											
Bx-G4>UAS-Dcr2; UAS-CG30115.RNAi/ 25/ BL 25706, v39952 Line I2	F	60	41.6	Loss	0	1.6	0	0	0	0	0	0	0	0	0
				Ect.	30	28.3	0	0	1.6	0	0	10	3.3	0	0
	M	60	40	Loss	0	1.6	0	0	0	0	0	0	0	0	0
				Ect.	41.6	10	0	1.6	5	1.6	0	6.6	0	0	0
Bx-G4>UAS-CG30115.RNAi/ 29/ BL 8860, v39952 Line I2	F	58	63.7	Loss	3.4	1.7	0	0	0	0	0	0	0	0	0
				Ect.	20.6	6.8	0	0	3.4	0	0	1.7	0	0	0
	M	59	6.7	Loss	1.6	0	0	0	0	1.6	0	0	0	0	0
				Ect.	22	15.2	1.6	0	71.1	16.9	0	1.6	0	5	54.2
Bx-G4>UAS-Rala.RNAi/25/BL 8860, Bl 29580	F	95	0	Loss	94.2	96.5	ND	ND	ND	ND	ND	ND	ND	ND	ND
				Ect.	0	0	ND	ND	ND	ND	ND	ND	ND	ND	ND
	M	99	0	Loss	89.4	94.7	ND	ND	ND	ND	ND	ND	ND	ND	ND
				Ect.	0	0	ND	ND	ND	ND	ND	ND	ND	ND	ND

Numbers represent percents of total wings scored that had the indicated defect. N is the sample of wings scored for defects
NA: Insertion on X. Males are not the relevant genotype. ND: Not determined

Genotype/T(^{o}C)/ Stocks Used	Sex	N	WT	Defect	ACV	PCV	L2	L3	L4	L5	L1/L2	L2/L3	L3/L4	L4/L5	Posterior to L5
Bx-G4>UAS-Rala.RNAi/18/BL 8860, BL 29580	F	59	67.7	Loss	0	28.8	0	0	0	0	0	0	0	0	0
				Ect.	0	1.6	0	1.6	3.2	0	0	0	0	0	0
	M	47	4.2	Loss	46.8	91.4	0	0	0	0	0	0	0	0	0
				Ect.	0	0	0	0	0	0	0	0	0	0	0
Bx-G4>UAS-Egfr.RNAi/25/BL 8860, BL 25781	F	38	0	Loss	100	97.3	0	100	86.6	92.1	0	0	0	0	0
				Ect.	0	0	0	0	2.6	0	2.6	0	0	2.6	0
	M	NS		Loss											
				Ect.											
Bx-G4>UAS-Egfr.RNAi/18/BL 8860, BL 25781	F	47	0	Loss	100	82.9	0	82.7	17	36.1	0	0	0	0	0
				Ect.	0	6.3	0	4.2	8.5	0	12.7	29.7	0	0	0
	M	42	0	Loss	100	45.5	13.6	100	100	100	0	0	0	0	0
				Ect.	0	4.5	0	0	0	0	0	0	0	4.5	0
Bx-G4>UAs-Roughened.RNAi/25/ BL8860, BL 29434	F	60	98.3	Loss	0	0	0	0	0	0	0	0	0	0	0
				Ect.	0	0	0	0	0	0	0	1.6	0	0	0
	M	58	68.9	Loss	3.4	1.7	0	0	0	0	0	0	0	0	0
				Ect.	0	0	1.7	1.7	5.3	5.1	0	0	0	1.7	12.7

Numbers represent percents of total wings scored that had the indicated defect. N is the sample of wings scored for defects

APPENDIX B: RESULTS FOR SINGLE OVER-EXPRESSION RESULTS AND CONTROLS

Genotype/T(^{o}C)/Stocks Used	Sex	N	WT	Defect	ACV	PCV	L2	L3	L4	L5	L1/L2	L2/L3	L3/L4	L4/L5	Posterior to L5
w[1118]/25	F	50	100	Loss	0	0	0	0	0	0	0	0	0	0	0
				Ect.	0	0	0	0	0	0	0	0	0	0	0
	M	49	93.8	Loss	6.1	2	0	0	0	0	0	0	0	0	0
				Ect.	0	0	0	0	0	0	0	0	0	0	0
w[1118]; 18-5/+/29	F	60	70	Loss	8.3	1.6	0	0	0	0	0	0	0	0	0
				Ect.	5	0	0	0	0	0	11.6	1.6	1.6	0	6.6
	M	60	88.3	Loss	5	0	0	0	0	0	0	0	0	0	0
				Ect.	5	0	0	0	0	0	0	0	0	0	1.6
w[1118]/18	F	58	98.2	Loss	1.7	0	0	0	0	0	0	0	0	0	0
				Ect.	0	0	0	0	0	0	0	0	0	0	0
	M	60	96.6	Loss	0	0	0	0	0	0	0	0	0	0	0
				Ect.	0	0	0	0	0	0	0	3.3	0	0	0
Bx-G4>UAS-RhoGAPp190/18	F	59	96.6	Loss	0	1.60	0	0	0	0	0	0	0	0	0
				Ect.	0	0	0	0	0	0	0	0	0	0	0
	M	58	45.6	Loss	46.5	0	0	0	10.3	0	0	0	0	0	0
				Ect.	0	0	0	0	0	0	0	0	0	0	0
W[1118] ;18-5/+/25	F	61	98.2	Loss	1.7	0	0	0	0	0	0	0	0	0	0
				Ect.	0	0	0	0	0	0	0	0	0	0	0
	M	60	96.6	Loss	0	0	0	0	0	0	0	0	0	0	0
				Ect.	0	0	0	0	0	0	0	3.3	0	0	0
Bx-G4>UAS-GEFmeso Long/25/ BL 8860, Line 75B2	F	20	0	Loss	100	90	0	0	0	0	0	0	0	0	0
				Ect.	0	0	0	ND	ND	ND	ND	ND	ND	ND	ND
	M	18	0	Loss	100	94.4	0	0	5.5	33.3	0	0	0	0	0
				Ect.	0	0	5.5	0	5.5	16.6	0	5.5	5.5	0	5.5

APPENDIX C: RESULTS FOR CO-EXPRESSION WING VEIN ANALYSIS

Genotype/T(^{o}C)	Sex	N	WT	Defect	ACV	PCV	L2	L3	L4	L5	L1/L2	L2/L3	L3/L4	L4/L5	Posterior to L5
Bx-G4>UAS-Rala.RNAi; 18-5/25	F	53	13.2	Loss	49	71.6	0	0	0	38	0	0	0	0	0
				Ect.	0	0	0	0	0	0	0	0	0	0	0
	M	ND		Loss											
				Ect.											
Bx-G4>UAS-Rala.RNAi; 18-5/18	F	70	87.1	Loss	1.4	2.8	0	0	0	0	0	0	0	0	0
				Ect.		2.8	0	0	0	2.8	0	0	0	0	0
	M	ND		Loss											
				Ect.											
Bx-G4>UAS-Rala.RNAi ; UAS-CG30115 RNAi (I)/18	F	60	96.6	Loss	0	0	0	0	0	0	0	0	0	0	0
				Ect.	0	17.8	0	0	1.7	0	5.3	5.3	0	0	0
	M	NA		Loss											
				Ect.											
Bx-G4>UAS-Rala.RNAi ; UAS-CG30115 RNAi (I)/25	F	36	0	Loss	5.5	75	11.1	2.7	5.5	50	0	0	0	0	0
				Ect.	11.1	0	0	0	5.5	0	0	8.3	0	0	0
	M	NA		Loss											
				Ect.											
Bx-G4>UAS-Rac1.RNAi; 18-5/+/25	F	48	72.9	Loss	0	0	0	0	0	0	0	2	0	0	0
				Ect.	0	0	0	0	0	0	0	0	0	0	0
	M	57	26.3	Loss	22.8	24.5	0	0	0	0	0	0	0	0	0
				Ect.	0	0	0	0	0	0	0	0	0	7	26.3
Bx-G4>UAS-Mtl.RNAi; 18-5/+/25	F	53-	96	Loss	0	1.9	0	0	0	0	0	0	0	0	0
				Ect.	0	3.9	0	0	0	0	0	0	0	0	0
	M	59-	12.2	Loss	20.3	37.2	0	0	0	1.7	0	0	0	0	0
				Ect.	0	3.3	0	0	6.7	27.1	0	0	0	8	33.8

Genotype/T(^{o}C)/ Stocks Used	Sex	N	WT	Defect	ACV	PCV	L2	L3	L4	L5	L1/L2	L2/ L3	L3/ L4	L4/ L5	Poster ior to L5
Bx-UAS-Ras85D.RNAi ; 18-5/+/25	F	30	0	Loss	86.6	86.6	60	93.3	100	93.3	0	0	0	0	0
				Ect.	0	0	0	0	0	0	0	0	0	0	0
	M	18	0	Loss	100	100	66.6	94.4	100	94.4	0	0	0	0	0
				Ect.	0	0	0	0	0	0	0	0	0	0	0
Bx-UAS-Ras85D.RNAi ; 18-5/+/18	F	49	8.1	Loss	71.4	81.6	2	40.3	42.8	32.6	2	0	0	0	0
				Ect.	0	0	0	0	0	0	0	0	0	0	0
	M	42	0	Loss	100	97.6	0	73.8	14.2	28.5	0	0	0	0	0
				Ect.	0	0	0	0	0	0	0	0	0	0	0
Bx-G4>UAS-Ras85D.RNAi; UAS-CG30115.RNAi (I)/ 25	F	40	0	Loss	95	97.5	47.5	100	100	100	0	0	0	0	0
				Ect.	0	0	0	0	0	0	0	0	0	0	0
	M	NA		Loss											
				Ect.											

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