Genetic analysis of grinder formation in *Caenorhabditis elegans*: Regulation by RAB-6.2 and its GTPase activating protein EAT-17

APPROVED BY SUPERVISORY COMMITTEE

Leon Avery, Ph.D.

Rueyling Lin, Ph.D.

Rama Ranganathan, M.D., Ph.D.

Dean Smith, M.D., Ph.D.

To my husband and family.

Genetic analysis of grinder formation in *Caenorhabditis elegans*: Regulation by RAB-6.2 and its GTPase activating protein EAT-17

by

SARAH STRAUD ANSELMO

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Genetic analysis of grinder formation in *Caenorhabditis elegans*: Regulation by RAB-6.2 and its GTPase activating protein EAT-17

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Sarah Straud Anselmo, Ph.D.

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Supervising Professor: Leon Avery, Ph.D.

The *C. elegans* grinder is an intricately designed, macromolecular structure located in the terminal bulb of the pharynx. It acts as the teeth of *C. elegans*, crushing bacteria before they are passed to the intestine. The grinder is a specialized cuticular structure and is shed and rebuilt at each larval molt.

While we have a fairly decent view regarding the mechanics of the grinder, we know surprisingly little about its composition or how it is formed. The nematode grinder

has been studied for over 100 years, but no one has yet described the molecular events controlling grinder formation and/or grinder function.

To understand how the grinder is formed, I have focused on cloning and characterizing *eat-17*. *eat-17* mutants have rudimentary, malformed grinders: the grinder plates are smaller than normal, disorganized in structure, and often improperly arranged. I found that *eat-17* encodes an ~825 amino acid Rab GTPase activating protein with a series of C-terminal coiled-coil domains. Its closest human homolog is Evi5, a putative oncogene whose function is not currently understood.

Rabs are key regulators of vesicle transport, and cycle between active, GTP bound and inactive, GDP bound states. GTPase activating proteins (GAPs) catalyze the hydrolysis of GTP, allowing Rabs to be extracted from membranes and recycled for additional rounds of signaling. I found that the GAP activity of EAT-17 is important for its function: 21% (16/76) of mutants expressing a wild-type version of *eat-17* are rescued for defects in grinder formation, while only 2.9% (3/113) of worms expressing catalytically inactive versions of EAT-17 are rescued. I performed RNAi against the 27 putative Rabs in the *C. elegans* genome and found that *rab-6.2* RNAi causes grinder defects similar to those seen in *eat-17* mutants. GFP reporters show that both EAT-17 and RAB-6.2 are expressed in terminal bulb muscle, the site of grinder secretion. By yeast two-hybrid, I have demonstrated a direct interaction between RAB-6.2 and EAT-17. These data suggest that EAT-17 and RAB-6.2 work together in regulating grinder formation. Genetic interaction studies suggest that RAB-6.1 may play a role in a similar cellular process.

Publications

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List of Abbreviations

aa	amino acid
AD	activation domain
Ala/A	alanine
AMV	avian myeloblastosis virus
Arg/R	arginine
Asp/D	aspartic acid
3-AT	3-amino-1,2,4- triazole
BDM	2,3-butanedione monoxime
BLAST	basic local alignment search tool
C	cytosine
CC	coiled-coil domain
cDNA	complementary DNA
CGC	*Caenorhabditis* Genetics Center
C-term.	carboxy terminus
DBD	DNA binding domain
DIC	differential interference contrast
DNA	deoxyribonucleic acid
ds	double-stranded
eat/Eat	eating defective
EM	electron microscopy
EPG	electropharyngeogram
ER	endoplasmic reticulum
EST	expressed sequence tag
Evi-5	ecotropic viral integration 5
F1	daughter of P_o (parent)
F2	granddaughter of P_o (parent)
F3	great-granddaughter of P_o (parent)
GAL	galactose
GAP	GTPase activating protein
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GTP	guanosine triphosphate
HIS	histadine
5-HT	5-hydroxytryptophan
KAN^R	kanomycin resistant
kb	kilobase
L1	larval stage 1
L2	larval stage 2
L3	larval stage 3
L4	larval stage 4
let	lethal
lin	lineage defective
Lys/K	lysine

μL	microliter
Muv	multiple vulvae
ng	nanogram
N-term.	amino terminus
ORF	open reading frame
PCR	polymerase chain reaction
pm	pharyngeal muscle
Rab	Ras-like from brain
RFP	red fluorescent protein
RNA	ribonucleic acid
RNAi	RNA interference
rol/Rol	roller
ROS	rod outer segment
RT-PCR	reverse transcriptase-polymerase chain reaction
SL1	splice leader 1
SL2	splice leader 2
SNP	single nucleotide polymorphism
sup/Sup	suppressor of *unc-93*
T	thymidine
TB	terminal bulb
TBC	Tre-2, Bup2p, Cdc16p
TGN	trans Golgi network
UAS	upstream activating sequence
unc/Unc	uncoordinated
UTR	untranslated region
UV	ultraviolet
WT	wild type
Y2H	yeast two-hybrid

CHAPTER 1

Introduction

1.1 An introduction to Caenorhabditis elegans and its life cycle

C. elegans is a soil dwelling nematode that has three things on its mind: finding food/eating, defecating, and giving birth. *C. elegans* was first chosen by Sydney Brenner as a model organism in 1965, due to its optical transparency, invariant cell lineage, small size, and rapid generation cycle (Brenner, 1974; Wood, 1987; Horvitz and Sulston, 1990).

The two sexes of *C. elegans* are males (XO) and self-fertilizing hermaphrodites (XX). An ordinary wild-type hermaphrodite produces about 300 progeny in her lifetime. These progeny are laid as multicellular embryos and hatch outside of the mother's body (Riddle et al., 1997). The time required for embryos to reach adulthood is approximately three days. The time required for a worm to develop, hatch, produce progeny, and die is a short two to three weeks (Riddle et al., 1997).

Similar to other nematode species, *C. elegans* progresses through four larval molts termed L1-L4 (Bird and Bird, 1991). Each molt is marked by a period of lethargus where worms reduce their rates of locomotion and feeding (Singh and Sulston, 1978). During each molt, the outer covering of the worm (the cuticle) is shed and a new one is made. As in other animals, this process occurs to accommodate the increasing size of the worm.

In arthropods, the exoskeleton is made of chitin, a strong, inflexible material; therefore, molting is strictly required for growth. The *C. elegans* cuticle, unlike that of arthropods, must remain flexible to allow movement to take place. This flexibility allows for increases in body size between molts and after the final molt (Byerly et al., 1976).

The importance of molting in nematodes may have to do with adaptability more than anything else. For most nematode species, the protein composition of the cuticle changes with each molt. Parasitic nematodes use this to their advantage and are capable of resisting the defense mechanisms of their host organisms (Bird, 1959).

In nature, *C. elegans* primarily feeds on soil bacteria. We attempt to simulate this environment in the laboratory by feeding worms the intestinal bacterium E. *coli*. Worms spend a significant amount of time searching for food and eating it when they find it. In fact, the only time a worm does not feed is either during a molt or when food is unavailable (Avery and Horvitz, 1990). If food is lacking for a prolonged period of time, worms enter an alternative larval stage marked by increases in fat storage and decreases in metabolic rate. Worms can remain this way for months, and exit this stage when food becomes available again (Riddle and Albert, 1997).

1.2 The Caenorhabditis elegans feeding apparatus: the pharynx

The *C. elegans* pharynx is a neuromuscular organ composed of 20 neurons of 14 anatomical types, 5 gland cells, 9 marginal cells, 9 epithelial cells, and 34 electrically coupled muscle cells of 8 types (Albertson and Thomson, 1976). It is essentially a self-contained system within the worm, due to its separation from the surrounding fluid by a thick extracellular basement membrane. The pharynx is arranged in triradial symmetry, with three muscle sectors surrounding a central lumen (see Figure 1.1A and B).

The muscle cells of the pharynx are, in actuality, polarized myoepithelial cells and thus have two distinct membrane surfaces (Avery and Thomas, 1997). The basal

membrane of the pharynx is in close contact with neurons that signal the muscle to contract and relax. The apical surfaces of the muscle cells secrete a protective cuticle lining and three specialized cuticular structures- the metastomal flaps, the sieve, and the grinder. These structures face the pharyngeal lumen.

The primary function of the pharynx is to pump food into the intestine of the worm. Food is sucked into the pharynx as a result of pharyngeal contraction. Upon relaxation, the pharynx shuts, trapping bacteria while expelling liquid (Chitwood and Chitwood, 1950). Food is passed from the front end of the pharynx to its distal end through the peristaltic action of the isthmus.

How food is trapped has been of considerable interest. The rate of pharyngeal relaxation is a key regulator of pharyngeal trapping, based on genetic and laser ablation studies (Avery, 1993b; Robatzek et al., 2001). However, pharyngeal relaxation occurs so fast that trapping cannot be observed directly using video recordings. Computer simulations of food transport reveal that proper relaxation timing can explain how food is trapped when assumptions consistent with the principles of fluid flow dynamics are made (Avery and Shtonda, 2003). However, it seems likely that structural components are important for this process as well. The sieve, a cuticular structure made of finger-like projections, is situated at the precise location in the pharynx where food is concentrated before it is swallowed. How this structure contributes to feeding is still unknown (discussed further in Chapter 5).

Within the terminal bulb is a ridged, cuticular structure called the grinder (Figure 1.1C and D). The grinder is secreted from the apical surfaces of terminal bulb muscles pm6 and pm7 with every molt (Albertson and Thomson, 1976). The grinder is composed

of three separate plates that contact one another when the pharynx is at rest. With each muscle contraction, the plates rotate against one another, crushing bacteria that come between them (Avery and Thomas, 1997). Improper grinding leads to the swallowing of whole bacteria, which proliferate in the intestines and make the worms very sick. Preventing the proliferation of bacteria in the intestines of older worms extends lifespan, suggesting that the processes of bacterial proliferation in the gut are reminiscent of bacterial infections that occur in humans (Garigan et al., 2002).

1.3 Grinder composition and formation

The earliest studies of the nematode grinder date back to the mid-late 1800s. At that time, the exact role of the grinder was surprisingly controversial. Some thought the grinder was solely responsible for crushing bacteria (Rauther, 1930). Others thought the grinder's main role was to prevent intestinal materials from entering the pharynx (Bastian, 1865). Still others thought the grinder performed dual roles. Throughout the years, the grinder has been called "bulb-flaps" (Osche, 1959), "bulb-teeth", (Schneider, 1866), "teeth apparatus" (Butschli, 1873), "valve apparatus", "valve flaps", and "bulbar valve" (Doncaster, 1962), denoting these two different views. The grinder has also been called "chitin plates (teeth apparatus)", suggesting its chemical composition (Orley, 1886).

Several observations suggest that the *C. elegans* grinder, at least in part, is constructed from chitin. Calcofluor, a dye used to stain the chitinous cell walls of yeast, stains the three specialized cuticular structures of the pharynx, including the grinder

(Figure 1.2). The *C. elegans* chitin synthase gene F48A11.1 is expressed in the m4 muscle cells (metacorpus) and the gland cells of the pharynx. It is also differentially regulated during each molt (Veronico et al., 2001). The three g1 gland cells, located in the anterior half of the terminal bulb, extend long processes to the buccal cavity and the back of the metacorpus, locations that house the flaps and the sieve, respectively. The two g2 gland cells, located in the posterior half of the terminal bulb, send processes that end just in front of the grinder. In addition, small transport vesicles are seen traveling anteriorly through the g1 processes at the end of each molt (Singh and Sulston, 1978). While these observations are suggestive, it is still not known whether the gland cells play a role in chitin secretion and/or grinder formation.

One inconsistency with this argument is that gland cell activity is concomitant with cuticle shedding rather than cuticle secretion. In plant-parasitic nematodes, the esophageal gland cells are known to secrete enzymes that break down cellular structures, not form them (Bird, 1967). In a single preliminary experiment, laser ablation of the gland cells in C. *elegans* did not cause obvious defects in cuticle secretion, molting, or growth (L. Avery, personal communication). Whether the *C. elegans* gland cells are important for grinder secretion and/or degradation remains to be elucidated.

As stated previously, the cuticle lining of the pharynx (including the grinder) is continuous with the outer body cuticle. This suggests that, in part, these structures are composed of the same building materials. Several lines of evidence demonstrate that collagens are the primary building blocks of the body cuticle. First, many collagen genes are expressed in hypodermal cells, which secrete the body cuticle. Second, many collagen genes are activated just prior to the molt, the time at which cuticle secretion

occurs. Third, the majority of isolated cuticle proteins are sensitive to collagenases (Emmons, 1987). Finally, mutations in collagen genes cause defects in body morphology due to defects in the body cuticle (Emmons, 1987; Thein et al., 2003).

Current estimates suggest that over 150 cuticle collagen genes are present in the C. *elegans* genome, a much higher number than found in vertebrates (Johnstone, 2000; Myllyharju and Kivirikko, 2004). So far, no one has clearly demonstrated that cuticle collagen genes are expressed in the pharynx (J. Kramer, personal communication). Antibodies raised against adult cuticle proteins do not appear to stain the grinder (my personal observations). Further studies are required to determine the exact chemical/protein composition of the grinder.

1.4 Building the C. elegans grinder

Worms progress through four larval stages that are punctuated by molts. Prior to each molt, a new cuticle is secreted under the old. This is followed by a period of lethargus during which time the old cuticle is shed. Like the body cuticle, the grinder is shed and rebuilt at each molt (Wood, 1987).

Most of what we know regarding cuticle secretion has been studied with respect to the hypodermis. The processes governing grinder secretion have not been described in detail. Contrary to grinder secretion, a fair amount is known about how the grinder is shed at each molt (Singh and Sulston, 1978). Just before ecdysis (time at which the body cuticle is shed), the pharynx begins to contract spasmodically, the cuticular lining of the pharynx breaks, and the posterior components of this structure are swallowed. Shortly

after, the old cuticle inflates around the tip of the head, and the nematode pulls back from it repeatedly. This detaches the rest of the cuticle lining, which is expelled through the mouth of the worm. If this process goes awry, the worm is left with a plug of cuticle in its mouth, which prevents it from feeding. At late stages of the molt, just prior to ecdysis, small vesicles (similar to those present in the g1 gland cells) are present in the pm6 and pm7 muscle cells (my personal observations). Their role in grinder formation or breakdown is currently unknown.

Strangely, nematodes such as Pristionchus and Aduncospiculum do not have grinders (B. Shtonda, personal communication). How worms deal with this situation is unknown. Perhaps the intestine secretes digestive enzymes that chemically, rather than mechanically, break down bacteria.

1.5 Rabs: Small GTPases that regulate vesicular trafficking events

Rabs comprise the largest family within the Ras superfamily of small G proteins. Rabs were initially identified in yeast and termed Ypt for their role in "yeast protein trafficking." So far, 11 Ypt/Rabs have been identified in yeast, 60 in mammals, 27 in *C. elegans*, and 57 in *Arabidopsis thaliana* (Martinez and Goud, 1998; Segev, 2001; Rutherford and Moore, 2002).

Rabs are responsible for regulating protein transport between different organelles in the cell. To do this, Rabs cycle between active, GTP-bound and inactive, GDP-bound states. In the active state, Rabs are attached to membranes through their prenylated C-terminal ends. For most Rabs, a consensus CXC motif is geranylgeranylated on both

cysteines by geranylgeranyl transferase II (Beranger et al., 1994b). This post-translational modification is essential for membrane localization (Beranger et al., 1994b; Beranger et al., 1994a).

In general, Rabs regulate trafficking events between specific intracellular compartments (Segev, 2001) (see Figure 1.3). However, at least one Ypt/Rab, Ypt1p, is capable of acting in more than one transport pathway (Jedd et al., 1995). Rabs perform multiple roles in individual pathways as well (see below). Rab families are very well conserved across species; i.e., Rabs that perform particular functions in yeast often play the same roles in mammals.

Initially, Rabs were thought only to be important in the regulation of vesicle docking at target membranes (see Figure 1.4). However, further studies have shown that Rabs are involved in a number of cellular processes including the formation of vesicles at donor membranes, vesicle motility, and direct involvement in the regulation of SNARE function (Segev, 2001; Hammer and Wu, 2002). There is also some evidence that Rabs directly interact with their cargo, thereby providing a way for cargo to regulate its own transport (Smythe, 2002; Spang, 2004).

In their GTP-bound states, Rabs interact with effector proteins. Most Rabs are capable of interacting with many effectors, which allows both specificity and versatility within the signaling pathway (Pfeffer, 2001). Rab activity is regulated through the actions of guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). Rabs are intrinsically capable of undergoing exchange and hydrolysis. However, GEFs and GAPs stimulate these activities by several orders of magnitude.

This type of regulation is necessary; otherwise, Rabs could not effectively perform their physiological roles.

1.6 Rab GTPase activating proteins: Regulators of GTP hydrolysis

Rab GTPase activating proteins terminate Rab signaling by catalyzing their intrinsic GTPase activities (Takai et al., 2001) (see Figure 1.5). Rab GAPs function via an arginine finger mechanism, similar to those for Ras and Rho (Donovan et al., 2002; Bernards, 2003). The arginine finger mechanism was first elucidated by studying the crystal structure of Ras-GDP-GAP in the presence of aluminum fluoride, which forms a stable transition state mimic (Ahmadian et al., 1999). The crystal structure of Gyp1p, a GTPase activating protein for Ypt1p, later showed that Ypt/Rabs act via a similar mechanism (Rak et al., 2003). Mutating the catalytic arginine residues of the yeast Ypt-GAPs, Gyp1p and Gyp7p, severely cripples their function (Albert et al., 1999). This is also the case for the Ypt-GAP, Gyp6p (Strom et al., 1993). All data obtained so far are consistent with the arginine finger hypothesis.

RabGAPs share a conserved 200 amino acid RabGAP/TBC domain first identified in yeast GTPase activating proteins and the cell cycle/spindle checkpoint proteins Bub2p, Tre2p, and *Schizosaccharomyces pombe* Cdc16p. This domain is both necessary and sufficient for GAP catalytic activity *in vitro*. There has been some debate as to the specificity of GAP function. In yeast promiscuity seems inevitable: there are only five known GAPs that act on 11 different Rabs. One of these, Gyp2p, stimulates the GTPase activities of both Ypt6p and Sec4p. Gyp3p, another yeast GAP, stimulates the

GTPase activities of 5/11 known Ypt proteins (Albert and Gallwitz, 1999). The *C. elegans* genome encodes approximately equal numbers of Rabs and GAPs, perhaps indicating that promiscuity is less common in this organism (Bernards, 2002). Unlike GAPs, GEFs and Rab effectors show no marked similarity to one another, although many mammalian homologs of yeast GEFs and effectors are present (Segev, 2001).

For proper signaling to occur, Rabs must interact with their effectors during certain periods of time and GAPs at other periods. How this regulation occurs is unclear, but controlling the local cellular concentrations of these components likely plays a role.

1.7 The role of Rab6 in eukaryotic organisms

Rab6 homologs have been identified in a number of organisms, including yeast, mammals, plants, and protists (Quevillon et al., 2003; Stedman et al., 2003) (see Figure 1.6). In general, Rab6 is localized to Golgi membranes and regulates transport into, out of, and between the different Golgi compartments (see Table 1.1 for details).

In yeast, the Rab6 homolog Ypt6p regulates retrograde trafficking between early endosomes and the trans Golgi network, retrograde trafficking between the Golgi compartments, and possibly Golgi-to-ER transport (Luo and Gallwitz, 2003). Other data suggest that Ypt6p acts at an early step in anteriograde Golgi trafficking (Li and Warner, 1996). In mammals, Rab6A regulates a COPI-independent retrograde transport pathway between the Golgi and ER (White et al., 1999). Rab6A also regulates transport between the individual Golgi compartments (Mayer et al., 1996b). Rab6A', which differs from Rab6A by only three amino acids, seems however to function in the early endosome-to-

TGN transport pathway (Mallard et al., 2002). Rab6B likely acts in a tissue-specific manner in the brain (Opdam et al., 2000). Little is known regarding the function of Rab6C. In *Arabidopsis thaliana*, between two and five Rab6 homolog are thought to exist (Rutherford and Moore, 2002). One of these, RabH1b, can complement the temperature-sensitive phenotype of *ypt-6* mutants, similar to its mammalian counterparts (Bednarek et al., 1994). Rab6 homologs have also been identified in the parasites *Toxoplasma gondii* and *Plasmodium falciparum* (Quevillon et al, 2003; Stedman et al, 2003; (Chattopadhyay et al., 2000). In *C. elegans*, two different genes encode two Rab6 homologs, RAB-6.1 and RAB-6.2. For RAB-6.1, at least two splice forms are present (my unpublished data).

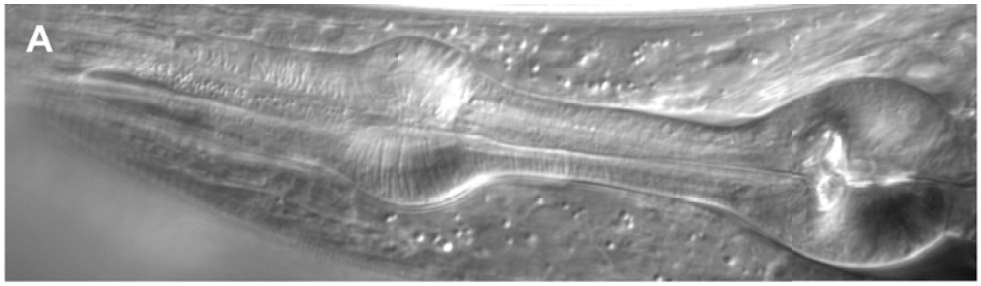
The primary role of Rab6 in vesicle trafficking still remains controversial. As delineated above, Rab6 seems to act in a number of pathways in various organisms. The fact that Rab6 acts in both the exocytic and endocytic pathways is also unusual.

To determine the molecular function of Rab6, several groups have set out to identify its interacting partners. Its interaction with Rabkinesin6, a molecular motor used to transport vesicles along microtubules, suggests that Rab6 is involved in regulating vesicle motility. Recent data suggest that Rab6 is also important for recruiting a family of coiled-coil proteins, termed the Golgins, to the Golgi apparatus (Barr, 1999; Barr and Short, 2003). Most likely, this class of proteins is important for tethering protein complexes to Golgi membranes. Rab6 most likely insures proper membrane recognition.

Currently no mammalian GEFs for Rab6 have been identified. Only one mammalian GAP, GAPCenA, is known. This protein is localized to centrosomes and is thought to regulate organelle dynamics during the cell cycle (Cuif et al., 1999). In yeast,

the protein complex Ric1p-Rgp1p catalyzes guanine nucleotide exchange on Ypt6p (Siniossoglou et al., 2000). Ric1p is also important for the localization of membrane proteins to the trans Golgi network (Bensen et al., 2001), suggesting a dual role as regulator and effector. Gyp6p catalyzes the hydrolysis of Ypt6p-GTP (Strom et al., 1993).

Figure 1.1. Structure of the C. *elegans* pharynx. (A) Nomarski DIC image of a wild-type pharynx. Lateral view. Anterior is to the left. **(B) Schematic depiction of the pharynx.** The pharynx is composed of three parts: the corpus, the isthmus, and the terminal bulb. Food enters the pharynx upon contraction of the corpus. Food is trapped during relaxation, while water is spit out. Food is transported through the isthmus by peristalsis. In the terminal bulb, food is crushed by the grinder, a cuticular specialization of the pharynx. **(C) Three dimensional view of the rhabditoid terminal bulb, showing the arrangement of grinder plates.** The grinder is composed of three plates, arranged as shown. There are two subventral plates and one dorsal plate (marked by arrows). The edges of the grinder plates are highly refractive by DIC optics. The plates contain a series of ridges made of cuticle material. Adapted from Furst von Lieven (2003). **(D) Electron micrograph showing a cross section of the grinder.** Adapted from Albertson and Thomson (1976).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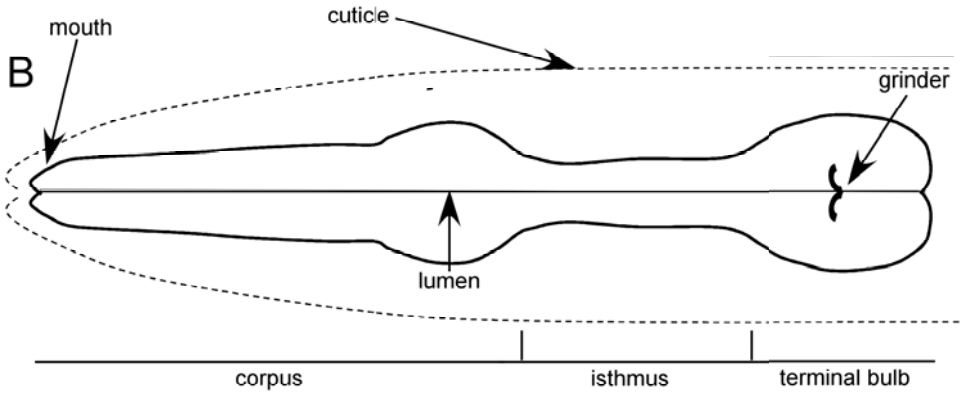
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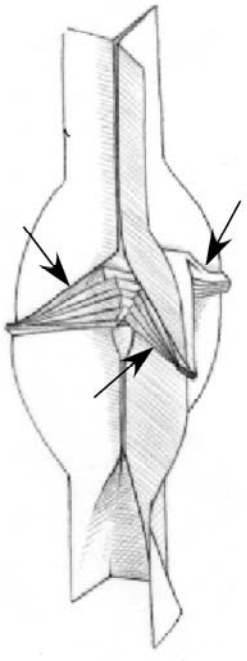

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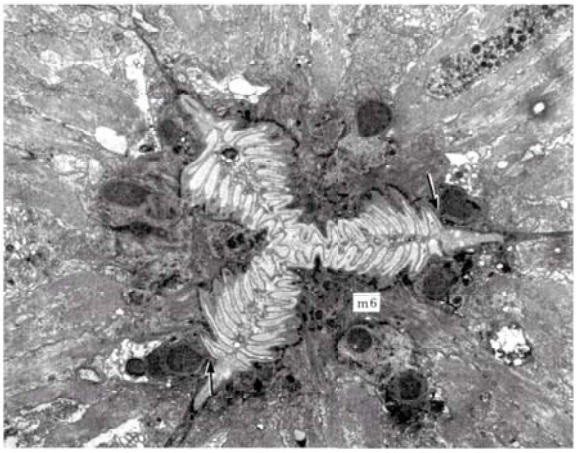
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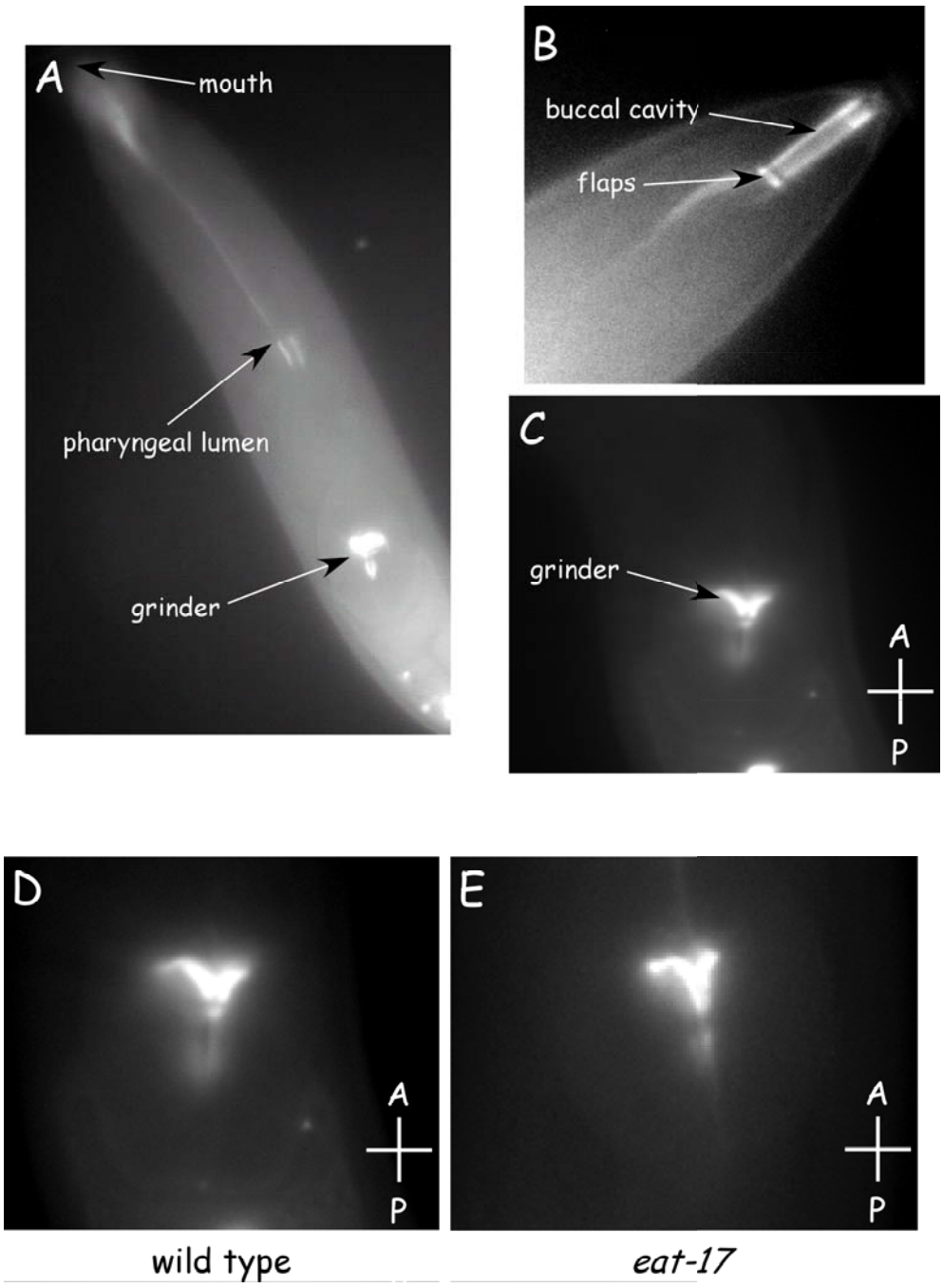


Figure 1.2. Calcofluor staining of the pharynx. Calcofluor stains structures composed of chitin. **(A-C)** Staining of wild-type worms. **(A)** Calcofluor stains three regions of the pharynx, including the grinder. **(B)** Staining of the buccal cavity and flaps, structures located in the head of the worm. Anterior is to the upper right. **(C)** Staining of the grinder. **(D-E)** Staining of wild type and *eat-17* grinders. No marked difference in staining is observed. (This suggests that defects in *eat-17* are not caused by defects in chitin trafficking.)

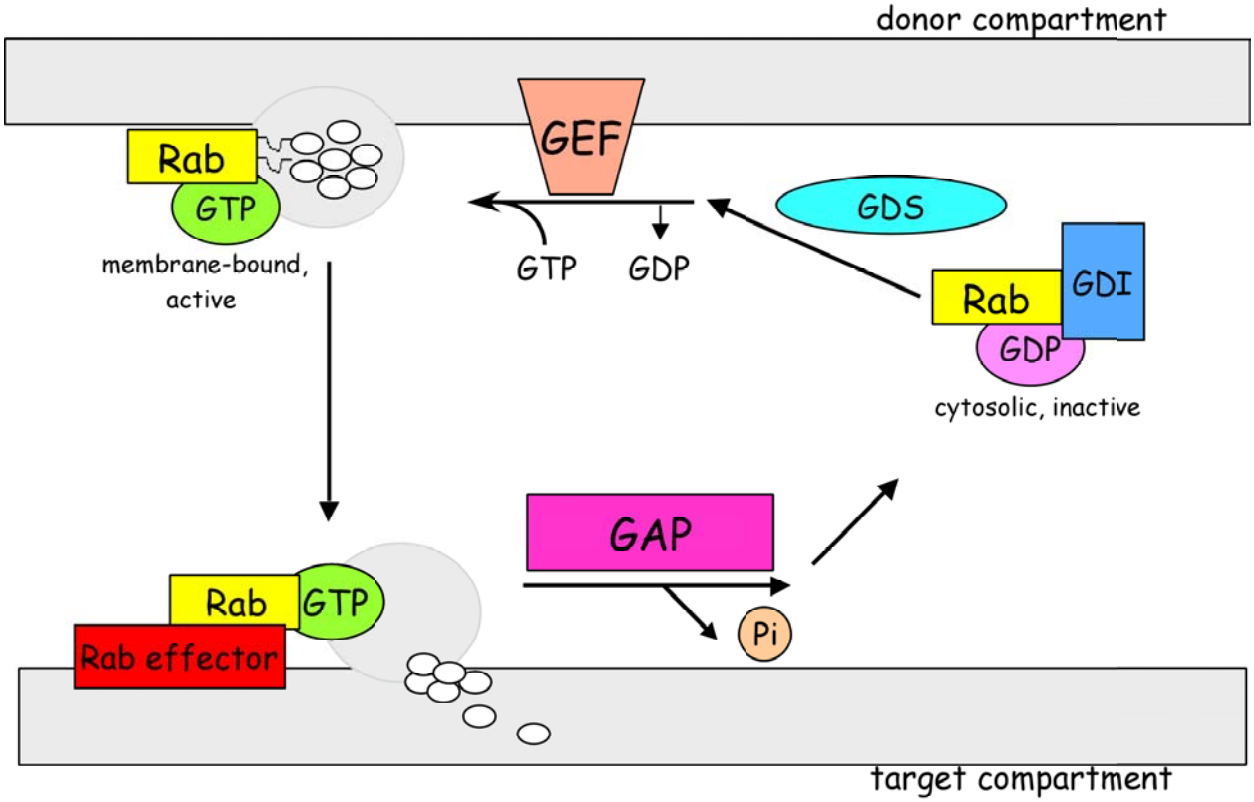


Figure 1.3. The Rab cycle. Rabs exist in either the GTP-bound ON or GDP-bound OFF state. Rabs are membrane bound through two geranylgeranyl groups, which are added to two cysteine residues found at the C-terminus of the protein. These lipid modifications are shown as squiggly lines in the figure. When Rabs are GTP bound, they have a greater affinity for their effectors, which play a number of roles in vesicle transport. Shown here is one such role. The Rab effector (shown in red) acts to tether the cargo-filled vesicle (vesicle shown in gray and cargo shown in white) to the target membrane. Through the action of SNARE proteins, the vesicle docks and fuses with the target membrane, releasing its cargo. At the end of the cycle, GTPase activating proteins (GAPs) (shown in pink) catalyze the hydrolysis of GTP, giving rise to the GDP-bound Rab. In the GDP-bound form, Rabs are extracted from the target membrane and held in the cytosol through their interactions with guanine nucleotide dissociation inhibitors (GDI) (shown in blue). GDIs act to shield the geranylgeranyl lipid groups from the external environment. Rabs are cycled back to the donor compartment by the action of GDS proteins (GDI dissociation stimulator) (shown in cyan). Rabs can again interact with membranes, which places them in proximity with guanine nucleotide exchange factors (GEFs) (shown in light orange). GEFs catalyze the exchange of GDP for GTP, which is in higher concentrations in the cell. Thus begins a new round of signaling.

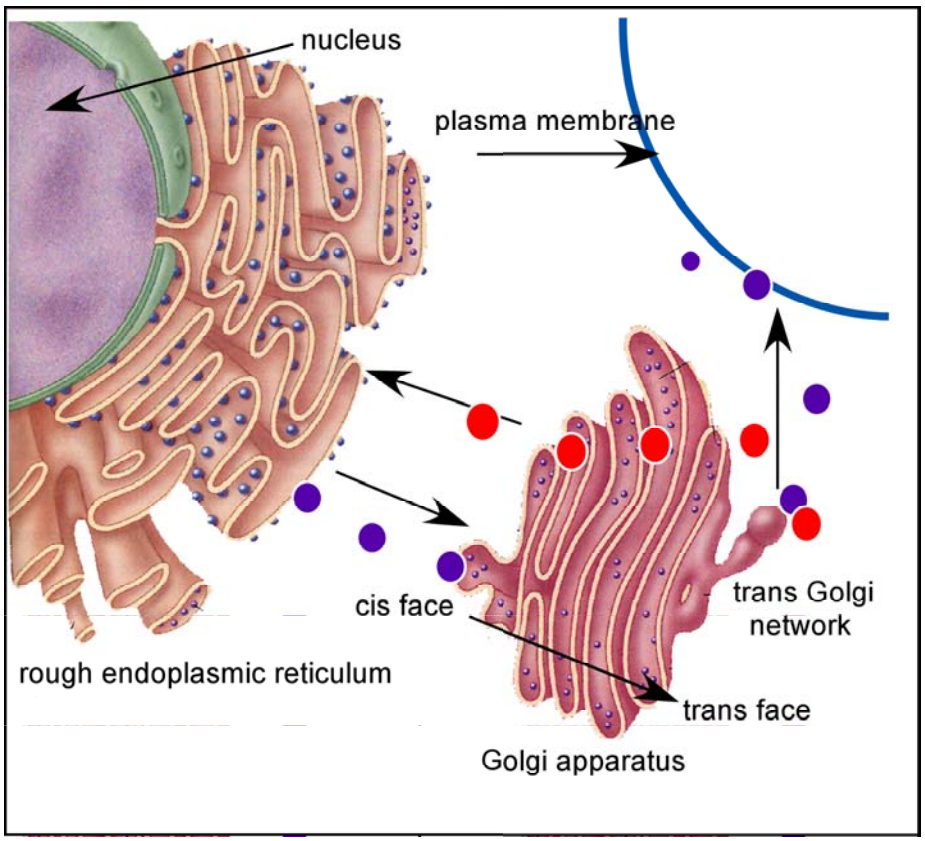


Figure 1.4. Vesicle transport in eukaryotic cells. Anteriograde transport shown in purple. Retrograde transport shown in red. Nascent proteins synthesized by ribosomes on the rough endoplasmic reticulum are transported to the cis face of the Golgi apparatus in transport vesicles. These fuse with the Golgi cisternae and proteins that are released traffic through the Golgi for post-translational modification and folding. Modified proteins destined for secretion enter the cis, medial, and trans Golgi network (TGN), consecutively. Transport vesicles bud from the TGN and make their way to the plasma membrane for exocytosis. Rab proteins are important at each stage of transport, with one or more Rabs acting at specific sites in the cell. Resident proteins that have been lost during transport are returned to their proper compartments (i.e., ER or Golgi) by retrograde trafficking. Two different types of vesicle coats are important for these processes: COPI coats are present on vesicles undergoing retrograde transport and COPII vesicles are generally present on vesicles undergoing anteriograde transport. Image adapted from Wikipedia, the Free Encyclopedia.

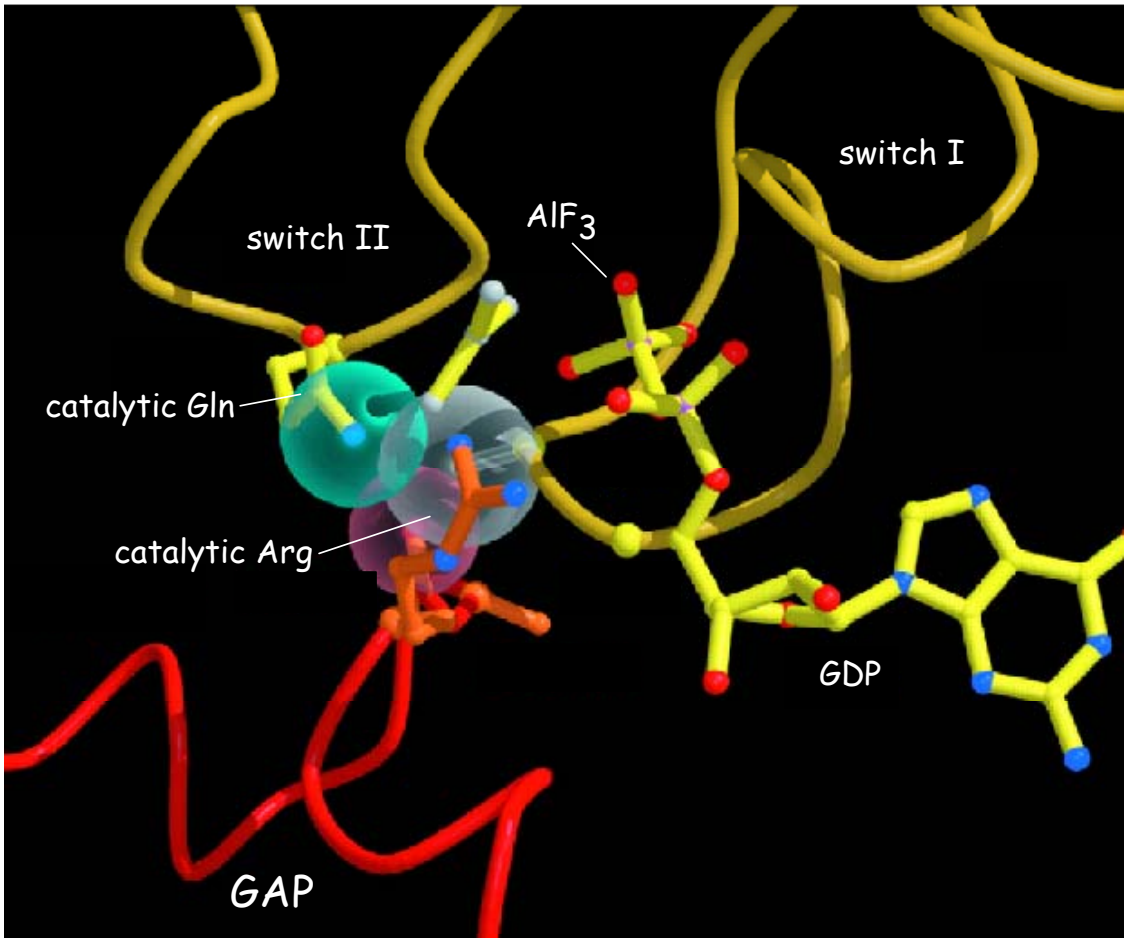


Figure 1.5. Mechanism of GTP hydrolysis. GTPase activating proteins contribute a catalytic arginine residue to the GTPase active site, which stabilizes negative charges accumulating in the transition state. A critical glutamine residue in the switch II region of the small G protein (Ras/Rho/Rab) also contributes to the stability of the transition state. Depicted in the figure is a schematic of the Ras-GDP-GAP-AlF_3 interaction, which is a stable transition state mimic. Adapted from (Scheffzek et al., 1997).

Figure 1.6. Rab6 alignments. C. *elegans* RAB-6.1 and RAB-6.2 are 81% identical (88% similar) at the amino acid level. Mammalian Rab6A shares 77% identity (87% similarity) to RAB-6.1 and 83% identity (94% similarity) to RAB-6.2. The Switch I and Switch II regions (underlined) undergo large conformational changes depending on nucleotide (GDP or GTP) occupancy. Geranylgeranyl lipid attachment sites are shown in purple. Glutamine residues important for GTPase activity are shown in yellow. The threonine residues (shown in blue) are important for nucleotide exchange.

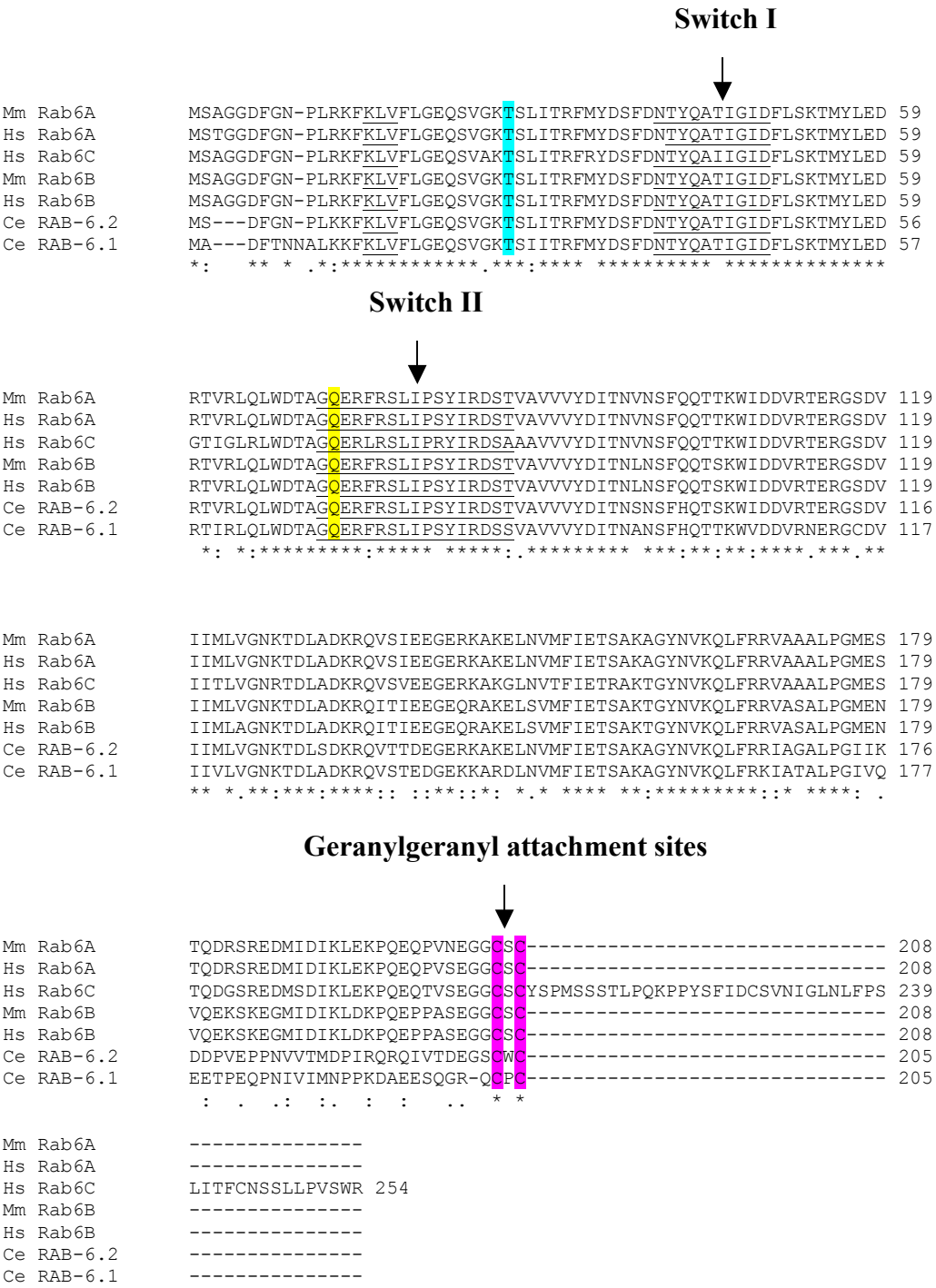

Switch I
Switch II
Geranylgeranyl attachment sites
Mm Rab6A MSAGGDFGN-PLRKFKLVFLGEQSVGKTSLITRFMYDSFDNTYQATIGIDFLSKTMYLED 59
Hs Rab6A MSTGGDFGN-PLRKFKLVFLGEQSVGKTSLITRFMYDSFDNTYQATIGIDFLSKTMYLED 59
Hs Rab6C MSAGGDFGN-PLRKFKLVFLGEQSVAKTSLITRFRYDSFDNTYQAIIGIDFLSKTMYLED 59
Mm Rab6B MSAGGDFGN-PLRKFKLVFLGEQSVGKTSLITRFMYDSFDNTYQATIGIDFLSKTMYLED 59
Hs Rab6B MSAGGDFGN-PLRKFKLVFLGEQSVGKTSLITRFMYDSFDNTYQATIGIDFLSKTMYLED 59
Ce RAB-6.2 MS---DFGN-PLKKFKLVFLGEQSVGKTSLITRFMYDSFDNTYQATIGIDFLSKTMYLED 56
Ce RAB-6.1 MA---DFTNNALKKFKLVFLGEQSVGKTSIITRFMYDSFDNTYQATIGIDFLSKTMYLED 57
Mm Rab6A RTVRLQLWDTAGQERFRSLIPSYIRDSTVAVVVYDITNVNSFQQTTKWIDDVRTERGSDV 119
Hs Rab6A RTVRLQLWDTAGQERFRSLIPSYIRDSTVAVVVYDITNVNSFQQTTKWIDDVRTERGSDV 119
Hs Rab6C GTIGLRLWDTAGQERLRSLIPRYIRDSAAAVVVYDITNVNSFQQTTKWIDDVRTERGSDV 119
Mm Rab6B RTVRLQLWDTAGQERFRSLIPSYIRDSTVAVVVYDITNLNSFQQTSKWIDDVRTERGSDV 119
Hs Rab6B RTVRLQLWDTAGQERFRSLIPSYIRDSTVAVVVYDITNLNSFQQTSKWIDDVRTERGSDV 119
Ce RAB-6.2 RTVRLQLWDTAGQERFRSLIPSYIRDSTVAVVVYDITNSNSFHQTSKWIDDVRTERGSDV 116
Ce RAB-6.1 RTIRLQLWDTAGQERFRSLIPSYIRDSSVAVVVYDITNANSFHQTTKWVDDVRNERGCDV 117
Mm Rab6A IIMLVGNKTDLADKRQVSIEEGERKAKELNVMFIETSAKAGYNVKQLFRRVAAALPGMES 179
Hs Rab6A IIMLVGNKTDLADKRQVSIEEGERKAKELNVMFIETSAKAGYNVKQLFRRVAAALPGMES 179
Hs Rab6C IITLVGNRTDLADKRQVSVEEGERKAKGLNVTFIETRAKTGYNVKQLFRRVAAALPGMES 179
Mm Rab6B IIMLVGNKTDLADKRQITIEEGEQRAKELSVMFIETSAKTGYNVKQLFRRVASALPGMEN 179
Hs Rab6B IIMLAGNKTDLADKRQITIEEGEQRAKELSVMFIETSAKTGYNVKQLFRRVASALPGMEN 179
Ce RAB-6.2 IIMLVGNKTDLSDKRQVTTDEGERKAKELNVMFIETSAKAGYNVKQLFRRIAGALPGIIK 176
Ce RAB-6.1 IIVLVGNKTDLADKRQVSTEDGEKKARDLNVMFIETSAKAGYNVKQLFRKIATALPGIVQ 177
Mm Rab6A TQDRSREDMIDIKLEKPQEQPVNEGGCSC---------- 208
Hs Rab6A TQDRSREDMIDIKLEKPQEQPVSEGGCSC---------- 208
Hs Rab6C TQDGSREDMSDIKLEKPQEQTVSEGGCSCYSPMSSSTLPQKPPYSFIDCSVNIGLNLFPS 239
Mm Rab6B VQEKSKEGMIDIKLDKPQEPPASEGGCSC---------- 208
Hs Rab6B VQEKSKEGMIDIKLDKPQEPPASEGGCSC---------- 208
Ce RAB-6.2 DDPVEPPNVVTMDPIRQRQIVTDEGSCWC---------- 205
Ce RAB-6.1 EETPEQPNIVIMNPPKDAEESQGR-QCPC---------- 205
Mm Rab6A ----------
Hs Rab6A ----------
Hs Rab6C LITFCNSSLLPVSWR 254
Mm Rab6B ----------
Hs Rab6B ----------
Ce RAB-6.2 ----------
Ce RAB-6.1 ----------

Table 1.1. Overview of published Ypt6p/Rab6 results

Rab isoform(s)	Interacting partner	Role	Reference
Ypt6p Rab6A Rab6A` Rab6B	Smg1p/TMF/ARA160	Rab6 recruits Smg1p to Golgi membranes; contributes to Golgi organization; Smg1 belongs to a family of coiled-coil proteins localized to the Golgi apparatus (Golgins)	(Fridmann-Sirkis et al., 2004)
T. gondii Rab6		Rab6 regulates retrograde transport from post-Golgi secretory granules to the parasite Golgi	(Stedman et al., 2003)
Rab6	Dynactin	Dynactin is required for the activity of dynein in intracellular motility processes; Rab6 functions as a specificity or tethering factor that recruits dynactin to Golgi membranes	(Short et al., 2002)
Rab6A Rab6A` Rab6B	Rab6IP2A, Rab6IP2B	Rab6 recruits Rab6IP2 to Golgi membranes; retrograde endosome-to-TGN transport	(Monier et al., 2002)
Rab6A`		Retrograde transport between early/recycling endosomes and TGN	(Mallard et al., 2002)
Rab6	NSF	The-N terminal domain of NSF binds the C-terminal domain of Rab6; isolated from a rat lung cDNA library	(Han et al., 2000)
Rab6	Rab6-KIFL (kinesin)	Membrane trafficking and cytokinesis	(Hill et al., 2000)
Rab6B		Cell-type specific role in retrograde membrane trafficking at the level of the Golgi complex; Rab6B is expressed in brain and neuroblastoma cell line SK-N-SH	(Opdam et al., 2000)
Rab6		Rab6 regulates a retrograde Golgi-to-ER transport pathway; examined using fluorescent markers in live cells	(White et al., 1999)
Rab6	GAPCenA	GAPCenA (`GAP and centrosome-associated') catalyzes hydrolysis of Rab6-GTP; may be involved in coordination of microtubule and Golgi dynamics during the cell cycle	(Cuif et al., 1999)

Rab isoform(s)	Interacting partner	Role	Reference
Drab6		Transports rhodopsin to membranes in Drosophila photoreceptors	(Shetty et al., 1998)
Rab6	Rabkinesin-6	Rabkinesin-6, a molecular motor, plays a role in Golgi dynamics; suggests that Rab6 regulates vesicle transport/motility in a microtubule-dependent manner	(Echard et al., 1998)
Rab6		Overexpression of Rab6(WT) and Rab6(Q72L) in HeLa cells induces the redistribution of trans-Golgi proteins into the ER; Rab-6(Q72L) effects are microtubule-dependent; overexpression of Rab6(T27N) reduces rates of Golgi trafficking	(Martinez et al., 1997)
Ypt6p		Inhibits both early Golgi function and ribosome biosynthesis in yeast; Ypt6 is not essential for viability	(Li and Warner, 1996)
Rab6		Important for transport between cis and medial Golgi cisternae in a reconstituted system; probably tethers Golgi-derived vesicles to their target membranes	(Mayer et al., 1996a)
Rab6	Mouse GDIβ	Identified in a Y2H screen of a mouse brain cDNA library; interacts with WT Rab6 and Rab5 but not GTP-bound Rab6; can remove several Rab proteins from membranes	(Janoueix-Lerosey et al., 1995)
Rab6		Overexpression of Rab6(WT) and Rab6(Q72L) in mouse L cells and human HeLa cells reduces transport between cis/medial and late Golgi compartments, without affecting transport between the ER and cis/medial Golgi or from the TGN to the plasma membrane; overexpression of both Rab6(Q72L) and Rab6(T27N) alter the morphology of the Golgi apparatus and TGN; supports a role in retrograde transport	(Martinez et al., 1994)

Rab isoform(s)	Interacting partner	Role	Reference
Rab6		Rab6 transports rhodopsin from the trans-Golgi to the site of rod outer segment disk formation; may participate in some aspects of ROS disk morphogenesis	(Deretic and Papermaster, 1993)
Ypt6p		Thought to act in endosome-to-Golgi transport, in intra-Golgi retrograde transport, and possibly in Golgi-to-ER trafficking; *ypt-6* mutants also display defects in protein sorting	(Luo and Gallwitz, 2003)
Ypt6p	Vps51p	Ypt6p recruits Vps51p to Golgi membranes	(Siniossoglou and Pelham, 2002)
Ypt6p	Gyp6p	Gyp6p is a GTPase activating protein for Ypt6p; the Gyp6p(R155A) mutation abolishes GAP activity; however, a physical interaction between Gyp6p(R155A) and Ypt6p can be demonstrated	(Will and Gallwitz, 2001)
Ypt6p	Ric1p-Rgp1p	The Ric1p-Rgp1p complex stimulates guanine nucleotide exchange on Ypt6p; required for efficient fusion of endosome-derived vesicles with the Golgi	(Siniossoglou et al., 2000)
Ypt6p	Sys3p	Ypt6p and Sys3p display a genetic interaction in yeast; mutating at both loci results in the accumulation of 40-50 nm vesicles and exacerbates defects in vacuolar protein missorting	(Tsukada and Gallwitz, 1996; Tsukada et al., 1999)
Ypt6p	Gyp6p	First demonstration of a GTPase activating protein for a Rab/Ypt GTPase; Gyp6p shows specificity for Ypt6p	(Strom et al., 1993)

CHAPTER 2

Cloning the *eat-17* gene

Results

2.1 Isolation of eat-17

eat-17 was isolated in a genetic screen for feeding defective mutants, in the hopes of determining the genetic basis for pharyngeal pumping behaviors. *eat-17* mutants are pale in color and have defects in transporting bacteria to the intestine, a phenotype termed "slippery pharynx". *eat-17* mutants also have defects in terminal bulb contraction timing (Avery, 1993a). In addition, *eat-17* mutants have improperly formed grinders (my personal observations). To understand the role of *eat-17* in feeding and grinder formation, I have cloned and characterized this gene.

2.2 eat-17 mapping

Prior to my work, *eat-17* was mapped to the right arm of Chromosome X, between *lin-15* and *sup-10* (Avery, 1993a). I used a three point mapping strategy in combination with a SNP mapping strategy to refine this region (see Methods for details). Using this strategy, I placed *eat-17* between *pkP6096* and *snp-F38E9.1*, reducing the region of interest to 47 kb (Table 2.1, Figure 2.1).

eat-17 also mapped to the left of *ser-1*, to the left of *hda-4*, and to the right of T27A8.2 (Table 2.2). These results are consistent with the SNP mapping data.

2.3 Cosmid rescue

To confirm the mapping results, I tested whether co-injection of T24D11 and F01G12, two overlapping cosmids spanning this region, could rescue the growth defects of *eat-17* mutants. Nine independent lines were isolated and the phenotypes of transgenic worms were examined (4 lines had high transmission frequencies; 5 had transmission frequencies that were very low). For 2/9 (22%) of these, between 50-100% of the transgenic progeny were rescued for defects in both growth and starved appearance. All non-transgenic worms were slow growing and starved.

When I injected either T24D11 or F01G12 alone, none of the F1 transgenic worms were rescued for these defects. I was never able to isolate stable F2 lines for either of these, suggesting that aberrant expression may cause lethality.

2.4 Cloning the eat-17 gene

I examined the genomic region where *eat-17* mapped, looking for predicted genes in this interval. Three genes were identified. F01G12.1 was found to encode a putative copper transporter, not a likely candidate for *eat-17*. T24D11.1 was found to encode a putative Rab GTPase activating protein, implicating it in the process of vesicle trafficking. F01G12.6 was found to encode a protein with several coiled-coil domains. Interestingly, the amino acid sequences of both T24D11.1 and F01G12.6 were strikingly similar to that of the human protein Evi5. Since T24D11.1 and F01G12.6 show similar transcriptional directionality, I tested whether this region comprised a single gene. Using

primers within the third predicted exon of T24D11.1 and within the fifth predicted exon of F01G12.6, I was able to isolate a single transcript by RT-PCR. This result confirms that T24D11.1 and F01G12.6 are parts of the same gene. Because of its identity and the fact that cosmids F01G12 and T24D11 break in this genomic region, this gene became a strong candidate for *eat-17*.

To determine whether a mutation was generated in the *ad707* mutant allele, I sequenced through the predicted *eat-17* coding region. I found a C to T base pair transition at nucleotide 607 in exon 5, creating a stop codon at amino acid 203. This mutation would generate a protein with a truncated RabGAP domain, likely rendering it non-functional. This supported the assertion that T24D11.1/F01G12.6 was *eat-17*.

Next I tested whether RNAi against this gene would phenocopy the *eat-17* genetic mutant. I used the *rrf-3* RNAi hypersensitive mutant (Simmer et al., 2002) because RNAi seems to work poorly in the *C. elegans* pharynx (Timmons, 2003). F1 progeny from injected mothers exhibited a strikingly similar phenotype to the genetic mutant (images of mutants compiled in Figure 3.3). These data confirmed that I had isolated the correct gene. (An overview of the mapping data is shown in Figure 2.1.)

2.5 eat-17 gene structure and splice forms

To determine the gene structure of *eat-17*, I first tested whether I could isolate full-length transcripts with the same intron/exon structure predicted in WormBase (www.wormbase.org). This could never be done. Next I tested individual intron/exon boundaries. Doing this, I found that most of the gene had been predicted correctly. The

first exon, however, was not correct. To identify the 5' end of the transcript, I used SL1 trans-splicing analysis. In *C. elegans*, approximately 60% of all transcripts are either SL1 or SL2 trans-spliced (Blumenthal, 1995; Conrad et al., 1995). My results show that the first exon of *eat-17* is located approximately 4 kb upstream of exon 2. I also found that this transcript contains an ~40 bp 5' UTR. To confirm this splicing pattern, I performed RT-PCR using a primer targeting the newly identified first exon and one targeting the last predicted exon of *eat-17*. I was able to amplify a single 2.5 kb fragment, the predicted size of the full-length transcript (Figure 2.2).

To determine the exact splicing pattern(s) of *eat-17*, I cloned the amplified PCR fragments into vectors and sequenced the inserts. About half of the cDNAs had intron/exon boundaries consistent with WormBase/GeneFinder predictions (Figure 2.3). I named this splice form *eat-17a*. For the rest, an alternative splice acceptor site at the exon3/4 boundary was used. I named this splice form *eat-17a'*. For both of these, exon 5 of T24D11.1 was truncated by 180 nucleotides and spliced directly to exon 2 of F01G12.6 (WormBase terminology). Neither splice form contained the predicted first exon of F01G12.6, termed exon 5B from this point on. The proteins encoded by *eat-17a* and *eat-17a'* differ by only 3 amino acids (Figure 2.3).

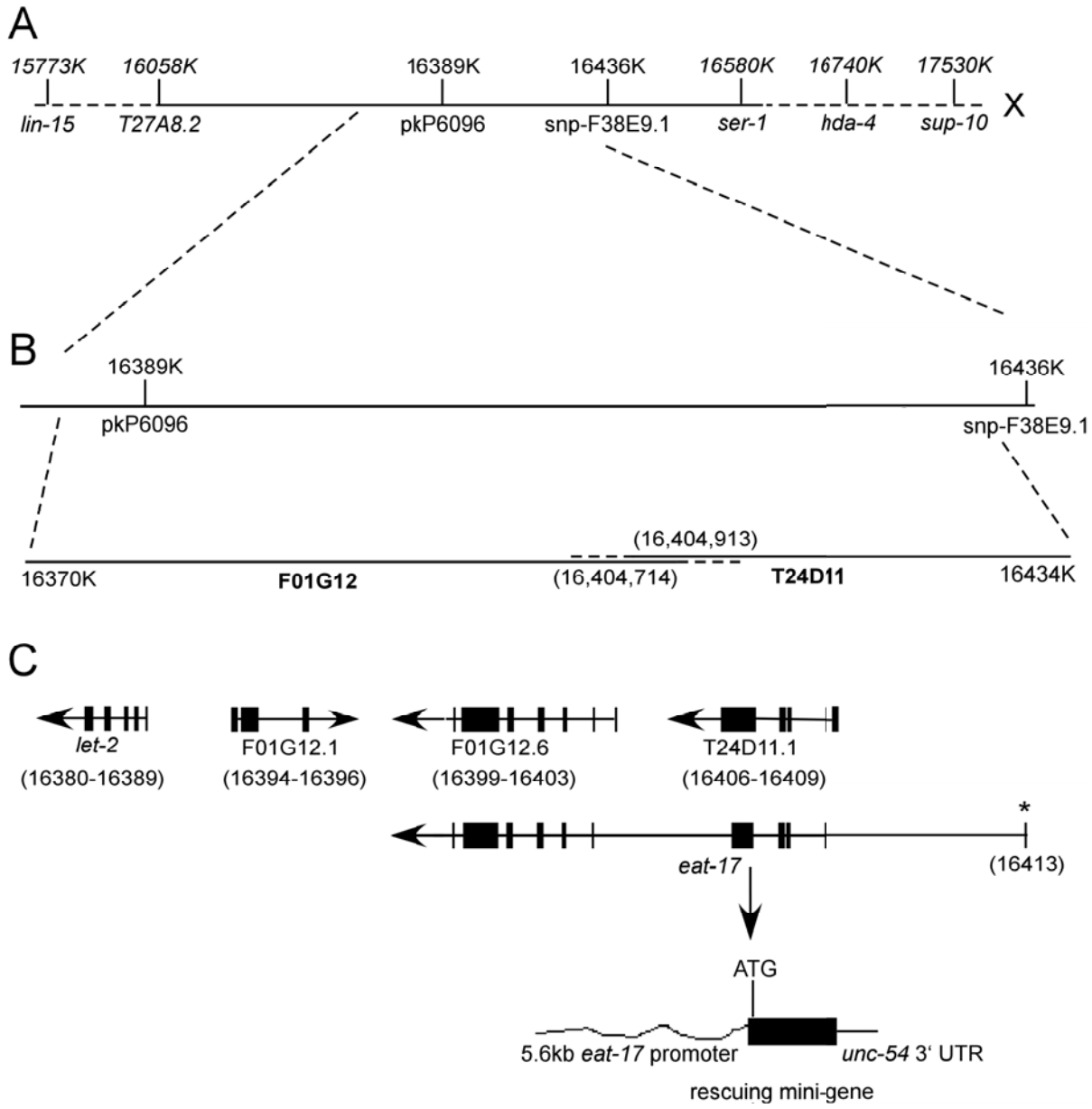
When I designed a set of primers to amplify sequences between exon 1 and exon 5B, I was able to isolate a single non-abundant transcript. I named this transcript *eat-17b*. GeneFinder predictions suggested a splicing pattern that would create a stop codon at the junction between exon 5 of T24D11.1 (termed exon 5A from this point on) and exon 5B. This transcript would encode a truncated protein lacking most of the C-terminus. It

seems most likely that the splice forms encoding full-length proteins are the functional ones. Rescue data are consistent with this assertion.

2.6 eat-17 rescue experiments

As a final test to show that I had isolated the correct gene, I fused *eat-17* cDNAs to predicted *eat-17* regulatory sequences using overlap extension PCR. Because I generated these constructs by PCR, I assumed that each of the major splice forms (*eat-17A* and *eat-17A'*) would be represented in the final product pool. I injected these fragments into *eat-17* mutants and isolated two independent lines that were capable of rescuing the *eat-17* mutant defects (images shown in Figure 3.3). I should note that for both lines most of the transgenic progeny arrested at an early stage of embryogenesis, indicating that overexpression of *eat-17* causes lethality. When I examined transgenic adults from these lines, all were rescued for defects in grinder structure. All together, my data show that the T24D11.1/F01G12.6 gene is *eat-17*.

Figure 2.1. Overview of *eat-17* cloning data. (A) Mapping experiments. Previous to my work, *eat-17* was mapped to the right arm of Chromosome X, between *lin-15* and *sup-10* (Avery, 1993a). To further map *eat-17*, I used a three point mapping strategy to isolate recombinants between these two markers and took advantage of single nucleotide polymorphisms in this region. My results indicate that *eat-17* lies between SNPs *pkP6096* and *snp-F38E9.1*. **(B) Cosmid rescue experiments.** Co-injection of T24D11 and F01G12 rescued growth and grinder defects of *eat-17* mutants, confirming the mapping data. Neither injection of T24D11 nor F01G12 alone was able to rescue these defects. **(C) Single gene rescue experiments.** A 2.5 kb *eat-17* cDNA driven by the native *eat-17* promoter (mini-gene) rescued *eat-17* mutant defects in 21% of transgenic progeny examined. *Bona fide first exon identified by SL1 trans-splicing experiments. Gene structure determined by RT-PCR.


A
15773K
16058K
16389K
16436K
16580K
16740K
17530K
lin-15
T27A8.2
pkP6096
snp-F38E9.1
ser-1
hda-4
sup-10
X
B
16389K
16436K
pkP6096
snp-F38E9.1
(16,404,913)
16370K
F01G12
(16,404,714)
T24D11
16434K
C
let-2
(16380-16389)
F01G12.1
(16394-16396)
F01G12.6
(16399-16403)
T24D11.1
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*
eat-17
(16413)
ATG
5.6kb eat-17 promoter
unc-54 3‘ UTR
rescuing mini-gene

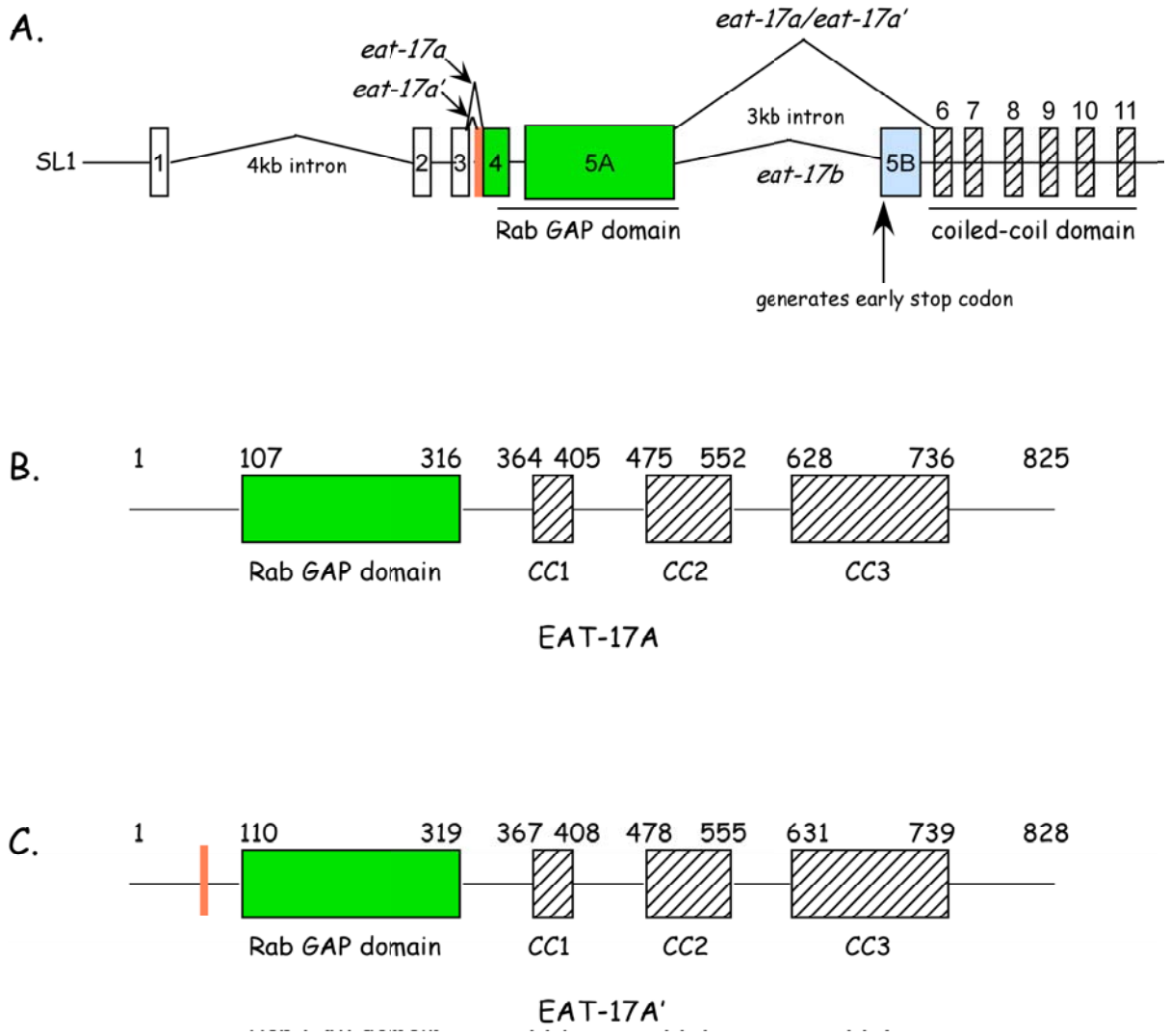

Figure 2.2. (A) *eat-17* gene structure. *eat-17* encodes two major transcripts that differ by nine nucleotides. The alternative splice site is designated by a small orange box. A minor pool of transcripts has an additional exon 5B. A stop codon is generated at the exon 5A/5B junction. **(B) EAT-17A protein structure.** EAT-17A contains several known domains, including a Rab GAP domain between amino acids 107-316 and three coiled-coil domains between amino acids 364-405, 475-552, and 628-736, respectively. **(C) EAT-17A' protein structure.** EAT-17A' contains a three amino acid insert at position 79, which is located prior to the start of the Rab GAP domain. The *ad707* mutation generates a stop codon at amino acid 203 (amino acid 206 for EAT-17A'). This truncates EAT-17 near the middle of the Rab GAP domain, suggesting that the *ad707* mutant is null.

Figure 2.3. EAT-17 nucleotide/amino acid sequences. Shown are the nucleotide/amino acid sequences of EAT-17A and EAT-17A'. The nine nucleotide/three amino acid YFQ insert of EAT-17A' is shown in bold.

```
   1 atggcagccactgcagcgctacgtcggagttcggattacggatcc
     M  A  A  T  A  A  L  R  R  S  S  D  Y  G  S       15
  46 gcagacaccgagtgtggtgagccgtgcgagacaggagccccagta
     A  D  T  E  C  G  E  P  C  E  T  G  A  P  V       30
  91 tcgctaaatgaagtcgatttgcttgccaagatggagcagctgaac
     S  L  N  E  V  D  L  L  A  K  M  E  Q  L  N       45
 136 aaatcaaatgaggaagactctcgaagtgttgcctccaagaagact
     K  S  N  E  E  D  S  R  S  V  A  S  K  K  T       60
 181 ggttccagtgaaagtcgcaaaggtgctcgtgaacattctcccgaa
     G  S  S  E  S  R  K  G  A  R  E  H  S  P  E       75
226 gaagatgag**tattttcag**gaagacttgtggtccgtgtggggagagcttattctc
  E  D  E  **Y  F  Q**  E  D  L  W  S  V  W  G  E  L  I  L    90
 271 aactgggaaattgaagtcaagaagcgtcccaactacattaaggat
     N  W  E  I  E  V  K  K  R  P  N  Y  I  K  D      105
 316 cttgtgaaacgaggcatcccacagcactttcgaatgattgcatgg
     L  V  K  R  G  I  P  Q  H  F  R  M  I  A  W      120
 361 cagaatttatcgaatgcgtcggtgtctagtgtccacgatttgtac
     Q  N  L  S  N  A  S  V  S  S  V  H  D  L  Y      135
 406 agtgactatatgcggcagtcttcggtttatgagaaggttattcaa
     S  D  Y  M  R  Q  S  S  V  Y  E  K  V  I  Q      150
 451 cgcgacattccccgtacctacccagagctcgacttttcaaagat
     R  D  I  P  R  T  Y  P  E  L  D  F  F  K  D      165
 496 ggcgaacgaggccaatcacttctgttcaacgttatcaaagcctat
     G  E  R  G  Q  S  L  L  F  N  V  I  K  A  Y      180
 541 tcagttcacgacaaggaagttgggtactgtcagggtagtgccttc
     S  V  H  D  K  E  V  G  Y  C  Q  G  S  A  F      195
 586 attgttggtctcttgctcctccagatgcccgaagaagaggcgttt
     I  V  G  L  L  L  L  Q  M  P  E  E  E  A  F      210
 631 gctgtgctcgtcagcctcatggaaaactatcggctccgcgagctc
     A  V  L  V  S  L  M  E  N  Y  R  L  R  E  L      225
 676 tacaagccaacaatgacagacttaggattgtgcatgttccagttg
     Y  K  P  T  M  T  D  L  G  L  C  M  F  Q  L      240
 721 gagtgtctcgtgcaggatcaaatgccagatttgtatactcatttt
     E  C  L  V  Q  D  Q  M  P  D  L  Y  T  H  F      255
 766 aataatatgggattcgacacgtcaatgtatgcgtcttcgtggttt
     N  N  M  G  F  D  T  S  M  Y  A  S  S  W  F      270
 811 ttgacacttttcacaactacaatgcctttggacattgccaataga
     L  T  L  F  T  T  T  M  P  L  D  I  A  N  R      285
 856 attatggattgctttttggtagaaggaatggattttatattttgc
     I  M  D  C  F  L  V  E  G  M  D  F  I  F  C      300
 901 atttccatcgcgattcttcaacaagctcgcatcgagcttctccgt
     I  S  I  A  I  L  Q  Q  A  R  I  E  L  L  R      315
 946 ctcgatatggaaggaatgctcaaatacttccagcgtgaagttcgc
     L  D  M  E  G  M  L  K  Y  F  Q  R  E  V  R      330
```

```
 991 gagcgatatgaatttgacgctgatcttcttttcacggttgccaat
     E  R  Y  E  F  D  A  D  L  L  F  T  V  A  N       345
1036 caagttcaactgaatgctaaaaggatgaagcgtctggaaaaggat
     Q  V  Q  L  N  A  K  R  M  K  R  L  E  K  D       360
1081 tacttgacgaagcgtaccaaggagcaggaggaggccgtcgagctg
     Y  L  T  K  R  T  K  E  Q  E  E  A  V  E  L       375
1126 cggcgacttcgtaccgaaaatcgccttctgcgtcaacgaatcgat
     R  R  L  R  T  E  N  R  L  L  R  Q  R  I  D       390
1171 tatctggaagcggaatcttcggcgctggcggatcgtctagtcaag
     Y  L  E  A  E  S  S  A  L  A  D  R  L  V  K       405
1216 ggacaggtaaatcttgctcaagaagctgagaactacatcaatatt
     G  Q  V  N  L  A  Q  E  A  E  N  Y  I  N  I       420
1261 gcacatgagttgaacaagttgcgcgacatgaactctgatgttcac
     A  H  E  L  N  K  L  R  D  M  N  S  D  V  H       435
1306 cgcaagttggagggcgcctatgagactatcagagagttgtcgagt
     R  K  L  E  G  A  Y  E  T  I  R  E  L  S  S       450
1351 gctcggcgcgacaacattatggatactggaacacaagtggacgac
     A  R  R  D  N  I  M  D  T  G  T  Q  V  D  D       465
1396 acgtcgatgattgagcacattcactcgcttcagcaggagctcatc
     T  S  M  I  E  H  I  H  S  L  Q  Q  E  L  I       480
1441 gaggctcacacgaggcaggcggacagtgagaatacgctcagggac
     E  A  H  T  R  Q  A  D  S  E  N  T  L  R  D       495
1486 gccaagttgagggtctcggaactggaaatggccaacaagcgcctt
     A  K  L  R  V  S  E  L  E  M  A  N  K  R  L       510
1531 ttggagaacgagccatcggaagacgttgcaggacttcaagaggag
     L  E  N  E  P  S  E  D  V  A  G  L  Q  E  E       525
1576 cttatttcagtgaagatgcgtgaagctgagagctcactcgctctg
     L  I  S  V  K  M  R  E  A  E  S  S  L  A  L       540
1621 aaggagatgcgtcagaggcttgccgaacttgagcagcactgggcg
     K  E  M  R  Q  R  L  A  E  L  E  Q  H  W  A       555
1666 aaatatgttcatgtacgggcgtttgatccatcgtctgcatctatt
     K  Y  V  H  V  R  A  F  D  P  S  S  A  S  I       570
1711 gaaaaggaatccacgtcagaggctcacagtacccaacagcagcca
     E  K  E  S  T  S  E  A  H  S  T  Q  Q  Q  P       585
1756 tctccgccactcacatccgctcgtgctcgtcttgccaagatcact
     S  P  P  L  T  S  A  R  A  R  L  A  K  I  T       600
1801 gcctcgcttattggaggatccacagaagaaactgataattgtatt
     A  S  L  I  G  G  S  T  E  E  T  D  N  C  I       615
1846 agtgttcgagaacttgaagatcaactcatgggagtacggatcaag
     S  V  R  E  L  E  D  Q  L  M  G  V  R  I  K       630
1891 gaagctgacacattggccgagctcaaagagatgcgacaaaaggtt
     E  A  D  T  L  A  E  L  K  E  M  R  Q  K  V       645
1936 atggagcttgagactcaaaaccatgtgtgcacgaatcagctcaag
     M  E  L  E  T  Q  N  H  V  C  T  N  Q  L  K       660
1981 cgtcaggacgaggagatgaagcgtgtgcgcgaagattcagaagtg
     R  Q  D  E  E  M  K  R  V  R  E  D  S  E  V       675
2026 ttggtgaagaagaggaaagagttggaggatcagttgaaagatgag
     L  V  K  K  R  K  E  L  E  D  Q  L  K  D  E       690
```

```
2071 aaggagaagttggacaacaaggagagcgagttcaacgagggtcga
     K  E  K  L  D  N  K  E  S  E  F  N  E  G  R       705
2116 atcaacgatcgactcaagtactctgaagccatgcagacgattcag
     I  N  D  R  L  K  Y  S  E  A  M  Q  T  I  Q       720
2161 gaccttcagagcagtatttcacagttggagctcaagaaagcggaa
     D  L  Q  S  S  I  S  Q  L  E  L  K  K  A  E       735
2206 aaatggacacaaaatcagttgagaggcagcagtgtctgtgatctt
     K  W  T  Q  N  Q  L  R  G  S  S  V  C  D  L       750
2251 gatgaggaatcgaattcgcatggctcaatttgctcgaacgtagac
     D  E  E  S  N  S  H  G  S  I  C  S  N  V  D       765
2296 catctttcgctggcatctgacgagatgaatgcactactcgccgac
     H  L  S  L  A  S  D  E  M  N  A  L  L  A  D       780
2341 atgactgtccgtatcccaactcttgacgatttggctgaagaagga
     M  T  V  R  I  P  T  L  D  D  L  A  E  E  G       795
2386 tctgcaactgagactgatgagttgcggcctaaggagctcaacgat
     S  A  T  E  T  D  E  L  R  P  K  E  L  N  D       810
2431 ggaaatgacacgacagattcgggtgtacaactgtcggatagccac
     G  N  D  T  T  D  S  G  V  Q  L  S  D  S  H       825
2476 tag 2478
     *
```

Table 2.1. SNP mapping results. See methods section for mapping strategy. *eat-17* was mapped against polymorphisms distinct for N2 and CB4856 wild strains. Sup non-Muv recombinants were isolated, and these were scored for the presence of the *eat-17* mutant allele and the various SNPs listed in the table. The column furthest to the left lists ID codes for individual worms scored. Intervals refer to the location of the crossover event in each worm. For instance, interval 0-1 means a crossover event occurred between *lin-15* and *snp-F23A7*. Interval 1-2 means a crossover event occurred between *snp-F23A7* and *pkP6169* and so on. Worms numbered 100.2, 3.3.1, and 21.7.1 were the most informative for placing *eat-17* (marked by astericks). In the table, C means the worm picked up the CB4856 SNP at that locus. N means the N2 SNP was found. Ns and Cs that are italicized were not scored by PCR but were derived with reference to surrounding SNPs. Non-italicized were determined in actual experiments. The results show that *eat-17* is located between *pkP6096* and *snp-F38E9.1*.

INTERVAL	0	1	2	3	4	5	6	7	8	9	
ID	***lin-15***	**F23A7**	**pkP6169**	**pkP6093**	**pkP6096**	***eat-17***	**F38E9**	**pkP6170**	***sup-10***	**pkP6171**	**INTERVAL**
5.1.1	C	N	N	N	N	N	N	N	N	N	**0--1**
5.1.2	C	N	N	N	N	N	N	N	N	N	**0--1**
6.1.1	C	N	N	N	N	N	N	N	N	N	**0--1**
5.9.1	C	N	N	N	N	N	N	N	N	N	**0--1**
5.9.2	C	N	N	N	N	N	N	N	N	N	**0--1**
9.1.2	C	N	N	N	N	N	N	N	N	N	**0--1**
11.2.1	C	N	N	N	N	N	N	N	N	N	**0--1**
13.9.1	C	N	N	N	N	N	N	N	N	N	**0--1**
5.13.1	C	N	N	N	N	N	N	N	N	N	**0--1**
9.1.1	C	N	N	N	N	N	N	N	N	N	**0--1**
21.7.2	C	N	N	N	N	N	N	N	N	N	**0--1**
21.7.3	C	N	N	N	N	N	N	N	N	N	**0--1**
13.1	C	N	N	N	N	N	N	N	N	N	**0--1**
4.2.1	C	N/D	N	N	N	N	N	N	N	N	**0--2**
100.1	C	N/D	N	N	N	N	N	N	N	N	**0--2**
7.2.1	C	C	N	N	N	N	N	N	N	N	**1--2**
9.2.1	C	C	N	N	N	N	N	N	N	N	**1--2**
5.2.1	C	C	C	N	N	N	N	N	N	N	**2--3**
3.5.2	C	C	C	C	N	N	N	N	N	N	**3--4**
3.5.1	C	C	C	C	N	N	N	N	N	N	**3--4**
100.2	C	C	C	C	C	N	N	N	N	N	**4--5***
3.3.1	C	C	C	C	C	C	N	N	N	N	**5--6***
21.7.1	C	C	C	C	C	C	N	N	N	N	**5--6***
3.10.1	C	C	C	C	C	C	C	N	N	N	**6--7**
5.6.1	C	C	C	C	C	C	C	N	N	N	**6--7**
9.1.1	C	C	C	C	C	C	C	N	N	N	**6--7**
9.9.1	C	C	C	C	C	C	C	N	N	N	**6--7**
22.6	C	C	C	C	C	C	C	N	N	N	**6--7**
24.10.1	C	C	C	C	C	C	C	N	N	N	**6--7**
21.5.1	C	C	C	C	C	C	C	N	N	N	**6--7**
24.9.1	C	C	C	C	C	C	C	N	N	N	**6--7**
29.1	C	C	C	C	C	C	C	N	N	N	**6--7**
4.2.5	C	C	C	C	C	C	C	N	N	N	**6--7**
11.2.2	C	C	C	C	C	C	C	C	N	N	**7--8**

Result: *lin-15*(13) snp-F23A7(2) pkP6169(1) pkP6093(2) pkP6096(1) *eat-17*(2) snp-F38E9(10) pkP6170(1) *sup-10*

A. *lin-15* (8/13) *eat-17* (2/13) *ser-1* (3/13) *sup-10*

eat-17	*ser-1*	# worms
eat-17	+	8
+	(Δ)	3
eat-17	(Δ)	0
+	+	**2**

B. *lin-15* (2/16) T27A8.2 (2/16) *eat-17* (12/16) *sup-10*

eat-17	T27A8.2	# worms
eat-17	+	2
+	(Δ)	12
eat-17	**(Δ)**	**2**
+	+	0

C. *lin-15* (5/11) *eat-17* (5/11) *hda-4* (1/11) *sup-10*

eat-17	*hda-4*	# worms
eat-17	+	5
+	(Δ)	1
eat-17	(Δ)	0
+	+	**5**

Table 2.2. Deletion mapping results. *eat-17* was mapped against three different deletion mutations: *ser-1*, T27A8.2, and *hda-4*. See methods for mapping strategy. **(A)** For *ser-1*, 2/13 Sup non-Muv recombinant progeny exhibited crossover events between *eat-17* and *ser-1*. Both worms lost the *eat-17* and *ser-1* mutations (shown in bold). These data place *eat-17* to the left of *ser-1*. **(B)** For T27A8.2, two worms exhibiting crossover events between *eat-17* and the deletion were isolated. These worms carried both the *eat-17* and T27A8.2 deletions (shown in bold). These data place *eat-17* to the right of this deletion. **(C)** For *hda-4*, 5 recombinants were isolated. All 5 recombinants lost the *eat-17* and *hda-4* mutant alleles. These data show that *eat-17* lies to the left of the deletion.

Materials and Methods

Worm culture:

Worms were grown at 20°C using standard conditions (Sulston and Hodgkin, 1988) with slight modifications (Avery, 1993a). The wild-type strains were Bristol N2 and Hawaiian CB4856. Other strains include DA773 *unc-93(e1500sd)*; *lin-15(n309) eat-17(ad707) sup-10(n183)*, DA1814 *ser-1(ok345)*, RB758 *hda-4(ok518)*, RB787 T27A8.2*(ok570)*, DA707 *eat-17(ad707)*, NL2099 *rrf-3(pk1426)*, and DA1982 *rrf-3(pk1426)*; *eat-17(ad707)*. CB4856, RB758, RB787, and NL2099 were obtained from the CGC (University of Minnesota).

Worms were fed bacterial strain HB101 with a few exceptions (Boyer and Roulland-Dussioux, 1969). To improve brood sizes of feeding-defective mutants, worms were fed *Comomonas* strain DA1877 (Avery and Shtonda, 2003). Bacterial strain DA837 was used to exacerbate the feeding defects of *eat-17* mutants (Davis et al., 1995). HB101 and DA837 are E. *coli* strains. HB101 is easier for worms to eat than DA837.

SNP mapping:

CB4856 males were crossed to DA773 hermaphrodites. *unc-93*; *lin-15 eat-17 sup-10*/CB4856(+) worms were isolated in the F2 generation; these were identified by their ability to produce 25% Muv non-Unc self-progeny and the near-absence of non-Muv non-Unc self-progeny. Sup non-Muv recombinants were isolated in the F3 generation. These were scored for the *eat-17* feeding defect, and their progeny were analyzed by PCR/restriction digestion for SNPs located between *lin-15* and *sup-10*. SNPs examined and primers used for their amplification are shown in Table 2.3.

Information regarding the location of each polymorphism as well as digestion/sequencing information can be found at the following site:

http://genome.wustl.edu/projects/celegans/index.php?snp=1

Mapping against deletion mutants:

A similar strategy was used to map *eat-17* against the following deletions: *ser-1(ok345)*, *hda-4(ok518)*, and T27A8.2*(ok570)*. Instead of scoring for the presence of SNPs, F4 progeny were scored for the presence of each deletion by PCR. Primers used for detecting the deletions are shown in Table 2.4.

Deletion alleles were generated by the *C. elegans* Gene Knockout Consortium (Oklahoma). Worm strains were obtained from the CGC (University of Minnesota).

Cosmid rescue:

Bacteria containing cosmids T24D11 and F01G12 were grown overnight in selective medium (Kan^R). Cosmid DNA was isolated using the Qiagen Plasmid Mini Kit (Valencia, CA) following the manufacturer's recommendations. T24D11 and F01G12 were co-injected into *eat-17(ad707)* mutants at a concentration of 50 ng/µL. Plasmids pPD118.20 *myo-3*::GFP and pRAK3 *rol-6(d)* were used at a concentration of 10 ng/µL as co-injection markers. Cosmids were obtained from Alan Coulson (Wellcome Trust, UK). pPD118.20 was obtained from Andrew Fire (Carnegie Institute, Baltimore, MD). Worm injections were performed using standard techniques (Mello and Fire, 1995). Worms were fed *Comomonas* DA1877 prior to injections. Injected mothers were allowed to recover for several hours on *Comomonas*, then transferred to DA837.

Growth rates of transgenic and non-transgenic progeny were compared to assess rescue. Eggs from transgenic mothers were singled onto plates seeded with DA837 bacteria. Plates were checked every 12 hours to stage the worms. Growth rate is defined as the inverse of the time required for worms to reach adulthood and produce progeny. Worms were scored as adults if they had laid at least one egg. Plates were kept at 20°C for the duration of the experiment except for scoring, when plates were allowed to sit at room temperature (22-23°C).

Analysis of *eat-17* gene structure and identification of splice variants:

To determine the intron/exon structure of *eat-17*, primers were designed to amplify partial and full-length transcripts by RT-PCR. Primer design was initially based on Genefinder predictions shown in WormBase. First strand poly(A)+ cDNA derived from a well-fed population of mixed stage N2 hermaphrodites was used as template in all reactions. Trizol reagent (Invitrogen, Carlsbad, CA) was used to isolate RNA and the First Strand cDNA Synthesis Kit (Roche, Indianapolis, IN) was used to generate cDNA. Manufacturer's instructions were followed in both cases.

Primers used to amplify PCR fragments are shown in Table 2.5 (gene references are based on WormBase predictions). PCR conditions were as follows:

92°C for 1 minute	1 cycle
92°C for 30 seconds	1 cycle
60°C for 30 seconds	1 cycle
68°C for x minutes (1 minute/ 2 kb)	1 cycle
Go to step 2	34 cycles
68°C for 10 minutes	1 cycle
4°C forever	

The resulting products were run on agarose gels (0.8%-2%) to determine band size. All PCR reactions yielded a single product, and all exons except the first appeared to be correct. To assess the number and abundance of different splice variants, I cloned full-length *eat-17* cDNAs into the pGEM-T Easy vector (Promega, Madison, WI) following manufacturer's instructions and sequenced several of the resulting clones from T7 and SP6 promoters (UTSW Sequencing Core, Dallas, TX). Gaps in sequence were covered using a subset of primers listed above. Sequences were aligned and analyzed using Clustal W (EMBL-European Bioinformatics Institute).

SL1 trans-splicing experiment:

To identify the first exon of *eat-17*, I set up PCR reactions using a forward primer recognizing the 22 nt SL1 splice leader sequence (5'- ggtttaattacccaagtttgag -3') and two nested reverse primers recognizing sequences in exon 2 (1050: 5'- tgttcagctgctccatcttg -3'(outside primer)/1051: 5'- cgacttcatttacgcatactg –3'(inside primer)). Products were separated by gel electrophoresis and four rather diffuse bands were isolated and purified (Qiaquick Gel Extraction Kit, Valencia, CA). These were cloned into pGEM-T Easy vectors (Promega, Madison, WI) and sequenced from T7 and SP6 promoters (UTSW Sequencing Core, Dallas, TX). The resulting sequences were BLASTed (blastn) against *C. elegans* ESTs and genomic DNA using WormBase. One set of sequences was most similar to cosmid T24D11. This was further analyzed using Clustal W alignments with the predicted T24D11.1 sequence. Final analysis was performed using the AceDB Genefinder program.

Sequencing:

Genomic DNA was isolated from *eat-17(ad707)* mutants using the Qiagen Dneasy Tissue Kit (Valencia, CA)**.** Predicted exon fragments for T24D11.1 and F01G12.6 were amplified and sequenced using primers shown in Table 2.6.

Products from four independent PCR reactions were mixed prior to sequencing. All sequencing reactions were performed by the UTSW Sequencing Core (Dallas, TX).

Single gene rescue experiments:

To obtain an *eat-17* rescuing fragment, the following products were generated in first round PCR reactions:

Product	**Template**	**Primers**
2.5 kb *eat-17* cDNA	Poly(A)+ cDNA	1076 (5'- *ttgtcaccgccg*atggcagccactgcagcgctac –3')
		1085 (5'- *tagggatgttgaagagtaattggac*ctagtggctatccgacagtt –3')
5.6 kb *eat-17* promoter	N2 genomic DNA	1073 (5'- taggttacggtagttggtacg –3')
		1081 (5'- gtagcgctgcagtggctgc*catcggcggtgacaa* –3')
500 bp *unc-54* 3' UTR	pPD95.75	1080 (5' aactgtcggatagccactaggtccaattactcttcaacatcccta –3')
		MS46 (5'- tttggtatattgggaatgtattctg –3')

PCR conditions were as follows:

Step 1	92°C for 3 minutes	1 cycle
Step 2	92°C for 30 seconds	1 cycle
Step 3	55°C for 30 seconds	1 cycle
Step 4	Ramp at 1.5°/second to 70°C	
Step 5	70°C for x minutes (1 minute/kb)	1 cycle
Step 6	Go to Step 2	29 cycles
Step 7	68°C for 10 minutes	1 cycle
Step 8	4°C forever	

1 μL of each PCR product was transferred to a new reaction mix and these were fused together by overlap extension PCR (Ho et al., 1989). Nested primers used in this case were: 1157 (5' acggtagtgttttatcagtagtg –3') and MS10 (5'- caaacccaaaccttcttccgatc – 3'). PCR products were not gel purified prior to fusion. All products were amplified using the Expand Long Template PCR System (Roche, Indianapolis, IN, Buffer 2). PCR conditions for the fusion reaction were as follows:

Step 1	92°C for 3 minutes	1 cycle
Step 2	92°C for 30 seconds	1 cycle
Step 3	68°C for 6 minutes	1 cycle
Step 4	Go to Step 2	9 cycles
Step 5	92°C for 30 seconds	1 cycle
Step 6	55°C for 30 seconds	1 cycle
	Ramp at 1.5°/second to 70°C	
Step 7	70°C for 10 minutes	1 cycle
Step 8	Go to Step 5	29 cycles
Step 9	68°C for 10 minutes	1 cycle
Step 10	4°C forever	

The resulting 8.6 kb PCR fragment was run on a 0.6% agarose gel and purified using the Qiaquick Gel Extraction Kit (Qiagen, Valencia, CA).

Primers MS46 and MS10 were designed by Mark Steciuk. N2 first strand poly(A)+ cDNA and N2 genomic DNA were prepared as described above. Plasmid

pPD95.75 used in the amplification of *unc-54* 3' UTR was obtained from Andrew Fire (Carnegie Institute, Baltimore, MD). Primers were obtained from IDT (standard desalting unless otherwise noted). The promoter used for this fusion contains sequence directly upstream of the *eat-17* translational start site.

The purified PCR product was injected into *eat-17* mutants at a concentration of 25 ng/μL with pPD118.25 *let-858*::GFP plasmid DNA at a concentration of 18 ng/μL as a co-injection marker. Transgenic animals were identified using the Olympus SZX12 GFP dissecting scope (Olympus Optical Co Ltd, Japan). Expression of this construct caused a significant amount of embryonic lethality. Only transgenic animals that reached adulthood were scored for defects in grinder formation. One stable transgenic line was obtained in which 100% of the adult transgenic animals were rescued for defects in grinder formation (n=8). This line could not be propagated easily, however, due to the large amount of embryonic lethality. Injecting the PCR fragment at lower concentrations (10 ng/μL and 16 ng/μL, respectively) resulted in a significant amount of embryonic lethality, but none of the adult transgenic animals were rescued for defects in grinder formation.

A second set of injections was performed in which the 5.6 kb *eat-17p*::*eat-17* cDNA::*unc-54* 3' UTR construct was injected into *eat-17* mutants at a concentration of 25 ng/μL and the *let-858*::GFP construct was injected at 100 ng/μL. This also resulted in the isolation of a stable line in which 100% of the transgenic progeny were rescued for defects in grinder formation. Like the first line, this line exhibited a significant amount of embryonic lethality and could not be maintained. Rescue of grinder defects always occurred but sometimes the grinder plates were not correctly arranged. Nevertheless, the

differences between transgenic and non-transgenic progeny were significant and could be scored blind. Grinder morphology was observed using the Zeiss Axiophot (Carl Zeiss Instruments Inc, Germany) at 630x or 1000x magnification. Worms were placed on 4% agar pads containing 10mM sodium azide (Sigma, St. Louis, MO) in M9 (Sulston and Hodgkin, 1988) to reduce body movements. All worms observed were gravid adults. In a few cases worms were observed on 4% agar pads containing a drop of M9 supplemented with 20 mM 5-HT (Sigma, St. Louis, MO) and food (either *Comomonas* DA1877 or *E. coli* DA837) to promote pumping.

Table 2.3. SNP mapping primers

SNP	Forward primer	Reverse primer
pkP6169	742: 5'- cctcaggatttaccagtgacac –3'	743: 5'- ttagtcttgcgcccctagag –3'
pkP6093	744: 5'- tagatatcgtggaacccc –3'	745: 5'- cctgggaatccgttttctcc –3'
pkP6096	746: 5'- gattgaacatagctcacagc –3'	747: 5'- tttcgatcgttttggacgcc –3'
pkP6171	748: 5'- cgatgcggtttcctagcttac –3'	749: 5'- attgcccatttcaagccc –3'
pkP6170	785: 5'- cgctgtcacaatctctaaaatg –3'	786: 5'- aaaccctccccactttgttgtc –3'
snp-T21F2	889: 5'- ttgacgcgctcttctactga –3'	890: 5'- ttggggtggttgtttgatct –3'
snp-C06G1	891: 5'- gagcataccgtttggcagat –3'	892: 5'- atggctcagcactctcgaat –3'
snp-F59C12	893: 5'- gaaaaagtggccaaaggtga 3'	894: 5'- aaaaactgacaagcggcaat –3'
snp-T25D1	895: 5'- catttcagaaaccatgaacctg –3'	896: 5'- ttgaagaactactcctctggca –3'
snp-F38E9	897: 5'- ctcacgctgacctctttcct –3'	898: 5'- tttgcatctttggagaattgg 3'
snp-T24D11	899: 5'- gcgggaatgtgcactaaaat –3'	900: 5'- gcgtgtatatttggcagcaa –3'
snp-W09B12	901: 5'- cctcaggatttaccagtgacac –3'	902: 5'- ttagtcttgcgcccctagag –3'
snp-C11G6	903: 5'- tgctggccagactacaaaaa –3'	904: 5'- ctcgaaaagtcctatcagcca –3'
snp-F23A7	905: 5'- tttaaagttcccagctgtgct –3'	906: 5'- gaaagggattctgccacaaa –3'

Table 2.4. Deletion mapping primers

Deletion strain	Primer	Primers outside deletion	Primers inside deletion
DA1814 *ser-1*(ok345)	Forward	5'- aagcatctttgagcgcattt –3'	5'- catagcgagtgtttggagca –3'
	Reverse	5'- aatttcaggggtgtggacat –3'	5'- aatcatttttgaaaccgaccc –3'
RB758 *hda-4(ok518)*	Forward	791: 5'- tcacagctcaccaaagatcg –3'	793: 5'- ttgccaacaggagtaaaggg –3'
	Reverse	792: 5'- gttgttgctgctgcatttgt –3'	794: 5'- ccaatgagtgcctggaattt –3'
RB787 T27A8.2*(ok570)*	Forward	787: 5'- atcgaatacatccgtccagc –3'	789: 5'- ggcaacataccatttccacc –3'
	Reverse	788: 5'- tcttgacccagaaacgaacc –3'	790: 5'- tgacccagaaacgtacccat –3'

Table 2.5. Primers used for gene structure analysis

Gene	Forward primer	Reverse primer	Region amplified
T24D11.1	1035: 5'- atgaatgtcctcccactctttttc –3'	953: 5'-tgcaatcattcgaaagtgctg –3'	5' to the R116/119 codon
T24D11.1	1035: 5'- atgaatgtcctcccactctttttc –3'	955: 5'- acggggaatgtcgcgttgaat –3'	5' to the D152/155 codon
T24D11.1	952: 5'- cagcactttcgaatgattgca –3'	940: 5' ctagtggctatccgacagtt 3'	3' to the R116/119 codon
T24D11.1	954: 5'- attcaacgcgacattccccgt –3'	940: 5' ctagtggctatccgacagtt 3'	3' to the D152/155 codon
T24D11.1	1035: 5'- atgaatgtcctcccactctttttc –3'	1040: 5'- gagacggagaagctcgatg –3'	Exons 1-5
T24D11.1	1036: 5'- aatgtggaatgagagaggcg –3'	1040: 5'- gagacggagaagctcgatg –3'	Exons 1-5
T24D11.1	1037: 5'- gatccgcagacaccgagtg –3'	1040: 5'- gagacggagaagctcgatg –3'	Exons 2-5
T24D11.1	1039: 5'- gaagtgttgcctccaagaag –3'	1040: 5'- gagacggagaagctcgatg –3'	Exons 3-5
T24D11.1	1038: 5'- cgtgtggggagagcttattc –3'	1040: 5'- gagacggagaagctcgatg –3'	Exons 4-5
T24D11.1	1063: 5'- aggcattcgcgttcaaactc –3'	1040: 5'- gagacggagaagctcgatg –3'	Exons 1*-5
T24D11.1/F01G12.6	930: 5'- gaagtcaagaagcgtcccaa –3'	928: 5'- tcgttgaactcgctctcctt –3'	Exon 3 of T24D11.1 to exon 7 of F01G12.6
T24D11.1/F01G12.6	931: 5'- ccgtctcgatatggaaggaa –3'	928: 5'- tcgttgaactcgctctcctt –3'	Exon 5 of T24D11.1 to exon 7 of F01G12.6
T24D11.1/F01G12.6	F01G12for: 5'- gacgtcagcctcatggaaaact -3'	F01G12rev: 5' gccaatgtgtcagcttcctt 3'	Exon 5 of T24D11.1 to exon 5 of F01G12.6

T24D11.1/F01G12.6 (full-length *eat-17* cDNA)	1063: 5'- aggcattcgcgttcaaactc –3'	940: 5'- ctagtggctatccgacagtt -3'	Exon 1* of T24D11.1 to last exon of F01G12.6
T24D11.1/F01G12.6 exon 1**	F01G12for: 5'- gacgtcagcctcatggaaaact -3'	1060: 5' taatacgactcactatagggccttttcgtccaaacaaatcg -3'	Exon 5 of T24D11.1 to exon 1 of F01G12.6

*Exon 1 identified by SL1 trans-splicing experiments

**To determine whether a full-length transcript containing F01G12.6 exon 1 was present in low abundance.

Table 2.6. Primers used for sequencing the *ad707* mutant

Exons amplified	**Forward primer**	**Reverse primer**
	Sequencing primer(s)	
***eat-17*: exons 1-4**	**844 (5' ccacttgtctcattctcaacca –3')**	**847 (5'- tttttggaaaatcgtaaccct –3')**
exon 1 (predicted by Genefinder)	*844 (5' ccacttgtctcattctcaacca –3')*	
exon 2	*845 (5'- tctcttttgggaagttggaa –3')*	
exons 3-4	*846 (5'- aacattgaccaagccgaatc –3')*	
exons 3-4	*847 (5'- tttttggaaaatcgtaaccct –3')*	
***eat-17*: exons 5B-11**	**874 (5'- atgagaacccgcgcccaaa –3')**	**875 (5'- ctagtggctatccgacag –3')**
exon 5B	*874 (5'- atgagaacccgcgcccaaa –3')*	
exon 6	*876 (5'- tccaacacccaacattctca –3')*	
exon 7	*877 (5' atttcagttgccaaaaaccg –3')*	
exon 8	*878 (5'- tggaattagtgggaatgggt –3')*	
exon 9	*879 (5'- tgcaacattcataaaaatgcaa –3')*	
exon 9-10	*875 (5'- ctagtggctatccgacag –3')*	
exon 11	**928 (5'- tcgttgaactcgctctcctt –3')**	**929 (5'- gctctgaaggtcctgaaacg –3')**
	928 (5'- tcgttgaactcgctctcctt –3')	
	929 (5'- gctctgaaggtcctgaaacg –3')	
F01G12.1: exons 1-3	**869 (5'- atggaagtattgaaaagtgtatg –3')**	**873 (5'- tcaatgacaagcatcagtagc –3')**
F01G12.1: exons 1-2	*869 (5'- atggaagtattgaaaagtgtatg –3')*	
F01G12.1: exons 1-2	*870 (5'- ttgccggaaataatcgtttg '3)*	
F01G12.1: exon 3	*873 (5'- tcaatgacaagcatcagtagc –3')*	

CHAPTER 3

Characterizing the EAT-17 protein

Results

3.1 eat-17 encodes a Rab GTPase activating protein with C-terminal coiled-coil domains

As a first step in determining the function of *eat-17*, I performed a BLAST search of all proteins listed in the GenBank database. As expected, Evi5 was the most closely related homolog. Human Evi5 and *C. elegans* EAT-17 share 38% identity (59% similarity). Mouse Evi5 and EAT-17 share 32% identity (54% similarity) (Figure 3.1). Less related, although well conserved, are the human Rab6 GAPs (~30 identity/ 50% similarity).

Rearrangements at the Evi5 locus have been identified in mouse T cell lymphomas and human neuroblastoma cell lines, suggesting that Evi5 is oncogenic in nature (Liao et al., 1997; Roberts et al., 1998). Other than this, little is known about its function. Nothing has been published to date regarding its molecular role.

Although the identity of the mammalian homologs was of little help in determining the function of EAT-17, the N-terminus of the protein did contain a predicted Rab GAP domain. This domain is shared among a number of yeast and human proteins involved in cell cycle regulation. Others have been implicated in protein trafficking in yeast. Because pharyngeal muscle is polarized, with distinct apical and basal membranes, a role for EAT-17 in vesicle trafficking was plausible. In particular, the phenotype of *eat-17* mutants led to the hypothesis that EAT-17 plays a role in proper trafficking of grinder components.

3.2 The GAP activity of EAT-17 is important for its function

As stated in the introduction, Rab GAPs catalyze the hydrolysis of GTP to GDP via an arginine finger mechanism. Therefore, I reasoned that expressing catalytically inactive versions of EAT-17 (R116K and R116A) would fail to rescue defects in *eat-17* mutants if the GAP activity of EAT-17 is important for its function.

When I expressed a wild type version of EAT-17 in mutants, 21% of the transgenic F1 progeny were rescued for defects in grinder formation. In contrast, only 4.1% and 1.6% of transgenic F1s were rescued with the R116K or R116A versions of EAT-17, respectively (Table 3.1). These results show that the catalytic activity of EAT-17 is important for its function, a first step towards determining its molecular role.

3.3 rab-6.2 RNAi phenocopies the eat-17 genetic mutant

To determine which *C. elegans* Rab(s) EAT-17 acts on, I performed RNAi against all 27 putative Rabs in the *C. elegans* genome. I found that none of these was capable of suppressing the *eat-17* mutant phenotype; however, *rab-6.2* clearly phenocopied *eat-17* (Table 3.2; Figure 3.2). The *eat-17* and *rab-6.2 RNAi* mutants showed defects in feeding: worms were small and pale, and at least one additional day was required to reach adulthood (compared to wild type). Defects in grinder formation were strikingly similar. In addition to these defects, *rab-6.2 RNAi* gravid adults had large refractory bodies in their terminal bulbs, similar to ones seen in *eat-17* mutants (Figure 3.3). These structures were never seen in wild type pharynxes. The identity of these structures is still unclear.

Intuitively, one would not expect inhibition of Rabs and their GAPs to phenocopy one another, since GAPs are negative regulators of Rabs. However, studies of other GTPases involved in vesicle trafficking show that Rabs must cycle between their GTP and GDP bound states to regulate secretion appropriately (Walworth et al., 1992; Moskalenko et al., 2002).

RNAi against *rab-6.1*, a close relative of *rab-6.2*, causes no defects in grinder formation. These data, taken at face value, support a one Rab: one GAP hypothesis in *C. elegans*. This will be discussed further in Chapters 4 and 5.

3.4 EAT-17 and RAB-6.2 express in the same tissues

To determine whether EAT-17 and RAB-6.2 function in the same cells, I constructed several GFP reporter fusions. A 5.6 kb *eat-17* promoter fragment driving GFP expresses in a number of cells, including the head hypodermis, the pharynx, the vulva, and the spermatheca. A 2.8 kb *rab-6.2* promoter::GFP fusion expresses in these tissues, as well as the nerve ring. A full-length RAB-6.2::GFP translational fusion expresses in a number of tissues- its most prevalent expression occurs in the head hypodermis and terminal bulb. Because both EAT-17 and RAB-6.2 are expressed in overlapping tissues- particularly the terminal bulb- these data support a role for EAT-17 and RAB-6.2 in the same molecular process (Figure 3.4).

Although EAT-17 and RAB-6.2 are expressed in the terminal bulb, this does not strictly prove that the terminal bulb (or pharyngeal muscle) is their site of action. The *myo-2* promoter is commonly used to express reporters and translational fusions

specifically in pharyngeal muscle. My preliminary data suggest that expressing *eat-17* under this promoter causes severe embryonic arrest, undermining my ability to assay rescue. In the future additional promoter fusions, particularly those that express less strongly that the *myo-2* promoter and specifically in terminal bulb muscle (pm6 and pm7 in particular), should be performed.

Finally, I have not determined the subcellular locations of RAB-6.1, RAB-6.2, and EAT-17. Previous work on Rab6 in yeast and mammalian systems shows that Rab6 is expressed on Golgi membranes and transport vesicles. It is most likely that RAB-6.1 and RAB-6.2 are also expressed at these sites and EAT-17 within this vicinity. Knowing this conclusively would provide strong evidence that RAB-6.1, RAB-6.2, and EAT-17 work together *in vivo*. Preliminary experiments indicate that lethality poses a serious problem in this case as well. To get at these questions, carefully controlled expression levels will be required. Antibody staining would be another good alternative.

3.5 EAT-17 and RAB-6.2 physically interact by yeast two-hybrid

So far I have shown that 1) *eat-17* and *rab-6.2* RNAi worms phenocopy one another, 2) the catalytic activity of EAT-17 is important for its function, and 3) *eat-17* and *rab-6.2* express at the same locations in the worm. All of these data support the idea that EAT-17 is a GAP for RAB-6.2. Although *eat-17* and *rab-6.2* are expressed in the same places at the same time, it is important to show that EAT-17 and RAB-6.2 are capable of physically interacting with one another. To address this question, I took advantage of the yeast two-hybrid system. I fused a series of truncated *eat-17a* cDNAs to

the LexA DNA binding domain and a series of full-length Rab cDNAs to the GAL4 activation domain. If two proteins interact with one another in the yeast two-hybrid system, the DBD and AD come together to activate transcription from the GAL4 UAS (Figure 3.5A) (Fields and Sternglanz, 1994; Janoueix-Lerosey and Goud, 2000; Stevens and Banting, 2000).

My results show that EAT-17 and RAB-6.2 can interact with each other and that this interaction is specific (Figure 3.5B). These data are consistent with the idea that EAT-17 and RAB-6.2 act together in a similar process regulating grinder formation. The fact that the GTP-bound but not the GDP-bound form of RAB-6.2 interacts with EAT-17 points to its role in facilitating GTP hydrolysis (Figure 3.5C). I should note that the activated form of RAB-6.1 also interacts with EAT-17, albeit at reduced levels. This interaction could be physiologically relevant, particularly in the absence of RAB-6.2.

I also found that the GAP domain alone does not interact with RAB-6.2. Sequences C-terminal to the first coiled coil domain, but not CC2 and CC3, are required for this interaction (aa1-460). The importance of this domain is currently unclear.

Figure 3.1. EAT-17/Evi5 sequence alignments. EAT-17 shows greatest sequence similarity to the EVI5/NB4S proteins. These have been implicated in human neuroblastoma and mouse tumorigenesis. EAT-17 contains two types of conserved domains. A putative Rab GAP domain (aa107-316) (shown in yellow) is located in the N-terminal half of the protein. Three predicted coiled-coil domains are located in the C-terminal half of the protein (aa 364-405, aa 475-552, and aa 628-736 for coiled-coil (CC) domains 1, 2, and 3, respectively) (shown in blue). Shown in red is the invariant arginine residue crucial for GAP function. All sequences were obtained through GenBank. Accession numbers are as follows: NM-005665.3 (human Evi5/NB4S), NM-007964.1 (mouse Evi5), NM-167285.1 (Drosophila CG11727-PA), and NM-132488.2 (Drosophila CG11727-PB). Dm=*Drosophila melanogaster*, Hs= *Homo sapien*, and Mm= *Mus musculus*. EAT-17a and EAT-17a' are from *C. elegans*. The functions of the well-conserved Drosophila proteins have not been determined. Residues important for the two-hybrid interaction are shown in green.


Dm CG11727-PA ----------MTLTTTTTASSAE 13
Dm CG11727-PB ----------MTLTTTTTASSAE 13
EAT-17a ----------MAATAALRRSSDY 13
EAT-17a' ----------MAATAALRRSSDY 13
Hs Evi5/NB4S MVTNKMTAAFRNPSGKQVATDKVAEKLSSTLSWVKNTVSHTVSQMASQVASPSTSLHTTS 60
Mm Evi5 MVTTKMTAAFRNPNRRQVATDKVAEKLSSTLSWVKNTVSHTVSQMASQVASPSASLHTTS 60
Dm CG11727-PA SQAKMDVKGGALPGEENLPTSEMDLLAKLEAANKLIESDAKSLNSLHSTHSRKNSDTSQI 73
Dm CG11727-PB SQAKMDVKGGALPGEENLPTSEMDLLAKLEAANKLIESDAKSLNSLHSTHSRKNSDTSQI 73
EAT-17a GSADTECGEPCETG-APVSLNEVDLLAKMEQLNKSNEEDSRSVASKKTGSS--ESRKGAR 70
EAT-17a' GSADTECGEPCETG-APVSLNEVDLLAKMEQLNKSNEEDSRSVASKKTGSS--ESRKGAR 70
Hs Evi5/NB4S SSTTLSTPALSPSSPSQLSPDDLELLAKLEEQNRLLETDSKSLRSVNGSRRNSGSSLVSS 120
Mm Evi5 SSTTLSTPTQSPSSPSKLSPDDLELLAKLEEQNRLIETDSKSLRSVNGSRRNSGSSLVSS 120
Dm CG11727-PA SLTSSGNSVAEEDIWTTWATILNDWEGALKRKNPCVSELVRRGIPHHFRAIVWQQLSGAS 133
Dm CG11727-PB SLTSSGNSVAEEDIWTTWATILNDWEGALKRKNPCVSELVRRGIPHHFRAIVWQQLSGAS 133
EAT-17a EHSPEEDE---EDLWSVWGELILNWEIEVKKRPNYIKDLVKRGIPQHFRMIAWQNLSNAS 127
EAT-17a' EHSPEEDEYFQEDLWSVWGELILNWEIEVKKRPNYIKDLVKRGIPQHFRMIAWQNLSNAS 130
Hs Evi5/NB4S SSASSNLSHLEEDSWILWGRIVNEWEDVRKKKEKQVKELVHKGIPHHFRAIVWQLLCSAQ 180
Mm Evi5 SSASSNLSHLEEDSWILWGRIVNEWDDVRKKKEKQVKELVRKGIPHHFRAIVWQLLCNAQ 180
Dm CG11727-PA DGDKK-QYAEYIKATSACEKVIRRDIARTYPEVEFFKEKDGPGQEALFNVIKAYSLHDRE 192
Dm CG11727-PB DGDKK-QYAEYIKATSACEKVIRRDIARTYPEVEFFKEKDGPGQEALFNVIKAYSLHDRE 192
EAT-17a VSSVHDLYSDYMRQSSVYEKVIQRDIPRTYPELDFFKDGER-GQSLLFNVIKAYSVHDKE 186
EAT-17a' VSSVHDLYSDYMRQSSVYEKVIQRDIPRTYPELDFFKDGER-GQSLLFNVIKAYSVHDKE 189
Hs Evi5/NB4S SMPIKDQYSELLKMTSPCEKLIRRDIARTYPEHNFFKEKDSLGQEVLFNVMKAYSLVDRE 240
Mm Evi5 SMTIKDQYSELLKMTSPCEKLIRRDIARTYPEHNFFKEKDSLGQEVLFNVMKAYSLVDRE 240
Dm CG11727-PA VGYCQGSGFIVGLLLMQMPEEEAFAVLVQIMQQHRMRHMFKPSMSELGLCMYQLENLVQE 252
Dm CG11727-PB VGYCQGSGFIVGLLLMQMPEEEAFAVLVQIMQQHRMRHMFKPSMSELGLCMYQLENLVQE 252
EAT-17a VGYCQGSAFIVGLLLLQMPEEEAFAVLVSLMENYRLRELYKPTMTDLGLCMFQLECLVQD 246
EAT-17a' VGYCQGSAFIVGLLLLQMPEEEAFAVLVSLMENYRLRELYKPTMTDLGLCMFQLECLVQD 249
Hs Evi5/NB4S VGYCQGSAFIVGLLLMQMPEEEAFCVFVKLMQDYRLRELFKPSMAELGLCMYQFECMIQE 300
Mm Evi5 LVTVRAVLSSLDCCCMQMPEEEAFCVFVKLMQDYRLRELFKPSMAELGLCMYQFECMIQE 300
Dm CG11727-PA QIPDMHIHFQQQGFQTTMYASSWFLTLYTTTLNVNLSCRIMDVFLSEGMEFIFKVALALL 312
Dm CG11727-PB QIPDMHIHFQQQGFQTTMYASSWFLTLYTTTLNVNLSCRIMDVFLSEGMEFIFKVALALL 312
EAT-17a QMPDLYTHFNNMGFDTSMYASSWFLTLFTTTMPLDIANRIMDCFLVEGMDFIFCISIAIL 306
EAT-17a' QMPDLYTHFNNMGFDTSMYASSWFLTLFTTTMPLDIANRIMDCFLVEGMDFIFCISIAIL 309
Hs Evi5/NB4S HLPELFVHFQSQSFHTSMYASSWFLTIFLTTFPLPVATRIFDIFMSEGLEIVFRVGLALL 360
Mm Evi5 YLPELFVHFQSQSFHTSMYASSWFLTIFLTTFPLPIATRIFDIFMSEGLEIVFRVGLALL 360
Dm CG11727-PA LTGKDTLLCLDMEAMLKFFQKELPGRVEADVEGFFNLAYSIKLNTKRMKKMEKEYQDLKK 372
Dm CG11727-PB LTGKDTLLCLDMEAMLKFFQKELPGRVEADVEGFFNLAYSIKLNTKRMKKMEKEYQDLKK 372
EAT-17a QQARIELLRLDMEGMLKYFQREVRERYEFDADLLFTVANQVQLNAKRMKRLEKDYLTKRT 366
EAT-17a' QQARIELLRLDMEGMLKYFQREVRERYEFDADLLFTVANQVQLNAKRMKRLEKDYLTKRT 369
Hs Evi5/NB4S QMNQAELMQLDMEGMLQHFQKVIPHQFDGVPDKLIQAAYQVKYNSKKMKKLEKEYTTIKT 420
Mm Evi5 QMNQAELMQLDMEGMLQHFQKVIPHQFDGGPEKLIQSAYQVKYNSKKMKKLEKEYTTIKT 420
Dm CG11727-PA KEQEEMAELRRLRRENCLLKQRNELLEAESAELADRLVRGQVSRAEEEETSYAIQTELMQ 432
Dm CG11727-PB KEQEEMAELRRLRRENCLLKQRNELLEAESAELADRLVRGQVSRAEEEETSYAIQTELMQ 432
EAT-17a KEQEEAVELRRLRTENRLLRQRIDYLEAESSALADRLVKGQVNLAQEAENYINIAHELNK 426
EAT-17a' KEQEEAVELRRLRTENRLLRQRIDYLEAESSALADRLVKGQVNLAQEAENYINIAHELNK 429
Hs Evi5/NB4S KEMEEQVEIKRLRTENRLLKQRIETLEKH----------KCSSNYNEDFVLQLEKELVQ 469
Mm Evi5 KEMEEQGEIKRLRTENRLLKQRIETLEKH----------KCSSTYNEDFVLQLEKELVQ 469
Dm CG11727-PA LRRSYLEVSHQLENANEEVRGLSLRLQENNNSRQSSIDELCMKEEALKQRDEMVSCLLEE 492
Dm CG11727-PB LRRSYLEVSHQLENANEEVRGLSLRLQENNNSRQSSIDELCMKEEALKQRDEMVSCLLEE 492
EAT-17a LRDMNSDVHRKLEGAYETIRELSSARRDNIMDTGTQVDDTSMIEHIHS--------LQQE 478
EAT-17a' LRDMNSDVHRKLEGAYETIRELSSARRDNIMDTGTQVDDTSMIEHIHS--------LQQE 481
Hs NB4S/Evi5 ARLSEAESQCALKEMQDKV--LDIEKRNNSLPDENNIAR----------LQEE 510
Mm Evi5 ARLSEAESQCALKEMQDKV--LDIEKKNNSFPDENNIAR----------LQEE 510

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Dm CG11727-PA      LVKVRQGLAESEDQIRNLKAKVEELEEDKKTLRETTP--------DNSVAHLQDELIASK 544
Dm CG11727-PB      LVKVRQGLAESEDQIRNLKAKVEELEEDKKTLRETTP--------DNSVAHLQDELIASK 544
EAT-17a            LIEAHTRQADSENTLRDAKLRVSELEMANKRLLENEP--------SEDVAGLQEELISVK 530
EAT-17a'           LIEAHTRQADSENTLRDAKLRVSELEMANKRLLENEP--------SEDVAGLQEELISVK 533
Hs NB4S/Evi5       LIAVKLREAEAIMGLKELRQQVKDLEEHWQRHLARTTGRWKDPPKKNAMNELQDELMTIR 570
Mm Evi5            LIAVKLREAEAIMGLKELRQQVRTLEEHWQRHLARTSGRWKDPPKKNAVNELQDELMSIR 570
                   *: .:   *::   ::: : :*  **   :      .        .: :  **:**:: :

Dm CG11727-PA      LREAEASLSLKDLKQRVQELSSQWQRQLAE----------NQRSESERTTNAVDSTP--- 591
Dm CG11727-PB      LREAEASLSLKDLKQRVQELSSQWQRQLAE----------NQRSESERTTNAVDSTP--- 591
EAT-17a            MREAESSLALKEMRQRLAELEQHWAKYVHVRAFDPSSASIEKESTSEAHSTQQQPSPPLT 590
EAT-17a'           MREAESSLALKEMRQRLAELEQHWAKYVHVRAFDPSSASIEKESTSEAHSTQQQPSPPLT 593
Hs NB4S/Evi5       LREAETQAEIREIKQRMMEMETQNQINSNH----------LRRAEQEVISLQEKVQYLSA 620
Mm Evi5            LREAETQAEIREMKQRMMEMETQNQINSNQ----------LRRAEQEVNSLQEKVCSLSV 620
                   :****:.  ::::::*: *:. :                  :.: .*  :   .

Dm CG11727-PA      ------KKLLTNFFD-SSKSSE---HTQKLEEELMTTRIREMETLTELKELRLKVMELET 641
Dm CG11727-PB      ------KKLLTNFFD-SSKSSE---HTQKLEEELMTTRIREMETLTELKELRLKVMELET 641
EAT-17a            SARARLAKITASLIGGSTEETDNCISVRELEDQLMGVRIKEADTLAELKEMRQKVMELET 650
EAT-17a'           SARARLAKITASLIGGSTEETDNCISVRELEDQLMGVRIKEADTLAELKEMRQKVMELET 653
Hs NB4S/Evi5       QN----KGLLTQLSEAKRKQAE---IECKNKEEVMAVRLREADSIAAVAELRQHIAELEI 673
Mm Evi5            KN----KGLLAQLSEAKRRQAE---IECKNKEEVMAVRLREADSIAAVAELQQHIAELKI 673
                          : :.:   . ..::      : ::::* .*::* :::: : *:: :: **:

Dm CG11727-PA      QVQVSTNQLRRQDEEHKKLKEELEMAVTREKDMSNKAREQQHRYSDLESRMKDELMNVKI 701
Dm CG11727-PB      QVQVSTNQLRRQDEEHKKLKEELEMAVTREKDMSNKAREQQHRYSDLESRMKDELMNVKI 701
EAT-17a            QNHVCTNQLKRQDEEMKRVREDSEVLVKKRKELEDQLKDEKEKLDNKESEFNEGRINDRL 710
EAT-17a'           QNHVCTNQLKRQDEEMKRVREDSEVLVKKRKELEDQLKDEKEKLDNKESEFNEGRINDRL 713
Hs NB4S/Evi5       QKEEGKLQGQLN-------KSDSNQYIG---ELKDQIAELNHELRCLKGQKG---FSGQP 720
Mm Evi5            QKEEGKLQGQLN-------RSDSNQYIR---ELKDQIAELTHELRCLKGQRD---FSSRP 720
                   * .  . * : :       :.: :  :    ::.::  :  ..    :..     :. :

Dm CG11727-PA      KFTEQSQTVAELKQEISRLETKNSEMLAEGELR----ANLDDSDKVR------------- 744
Dm CG11727-PB      KFTEQSQTVAELKQEISRLETKNSEMLAEGELR----ANLDDSDKVR------------- 744
EAT-17a            KYSEAMQTIQDLQSSISQLELKKAEKWTQNQLRGSSVCDLDEESNSHGSICSNVDHLSLA 770
EAT-17a'           KYSEAMQTIQDLQSSISQLELKKAEKWTQNQLRGSSVCDLDEESNSHGSICSNVDHLSLA 773
Hs NB4S/Evi5       PFDG-IHIVNHLIGDDESFHSSDEDFIDNSLQETGVGFPLHGKSGSMS------------ 767
Mm Evi5            PFDG-IHIVSHLIGDDELFHSSDEDFIDSSLQESAIGFPLHRKSGPMS------------ 767
                    :    : : .*  . . :. .. :   ..  .      *. ..

Dm CG11727-PA      --DLQDRLADMKAELTALKSRGKFPGAKLRSSSIQSIESTEIDFNDLNMVRRGSTELST- 801
Dm CG11727-PB      --DLQDRLADMKAEYPTP---------------ITSPDTEPWKWIS-------------- 773
EAT-17a            SDEMNALLADMTVRIPTLDDLAE------EGSATETDELRPKELNDGNDTTDSGVQLSDS 824
EAT-17a'           SDEMNALLADMTVRIPTLDDLAE------EGSATETDELRPKELNDGNDTTDSGVQLSDS 827
Hs NB4S/Evi5       ---LDPAVADGSESETEDSVLET----------RESNQVVQKERPPRRRESYSTTV---- 810
Mm Evi5            ---LNPALADGSESEAEDGMLGP----------QESDPEAPQKQPPQR-ESYSTTV---- 809
                      ::  :** .   .                  :      .

Dm CG11727-PA      -
Dm CG1172          -
EAT-17a            H 825
EAT-17a'           H 828
Hs NB4S/Evi5       -
Mm Evi5            -
```

	# rescued	total # examined	% rescue
WT EAT-17	16	76	21%
EAT-17 (R116/119K)	2	49	4.1%
EAT-17 (R116/119A)	1	64	1.6%

Table 3.1. The GAP activity of EAT-17 is important for its function. Wild type and two catalytically inactive versions of EAT-17 (R116/119K and R116/119A) were expressed in *eat-17(ad707)* mutants, and F1 transgenic progeny were assayed for rescue of grinder defects. Results obtained are shown in the table.

Table 3.2. RNAi against C. *elegans* Rabs: *rab-6.2* phenocopies *eat-17*. (A) Double-stranded RNA was injected into *rrf-3* mutants to determine whether RNAi against any of the Rabs could phenocopy *eat-17*. Several mutant phenotypes were observed, but only *rab-6.2* RNAi generated the grinder defects seen in *eat-17* mutants. **(B)** RNAi was performed in the *rrf-3*; *eat-17* background to determine whether RNAi against any of the Rabs could suppress the *eat-17* phenotype. As shown, none could suppress. RNAi against *rab-6.2* enhanced the *eat-17* phenotype.

A.

STRAIN INJECTED	dsRNA INJECTED	**PHENOTYPE**	**PHENOCOPY OF *eat-17***
rrf-3	None	Wild type	
rrf-3	*rab-1*	Embryonic lethal (100%)	
rrf-3	*rab-2*	**Slight growth delay**	NO (no obvious pumping defects)
rrf-3	*rab-3*	Wild type	
rrf-3	*rab-5*	Embryonic lethal (100%)	
rrf-3	*rab-6.1*	Wild type	
rrf-3	*rab-6.2*	**Growth delay**	**YES (defects in grinder formation; swallowing of unground bacteria)**
rrf-3	*rab-7*	Defects are not obvious; however, these worms do not seem to be wild type	
rrf-3	*rab-8*	Slightly long and thin with exaggerated body bends	
rrf-3	*rab-10*	N/D	
rrf-3	*rab-11.1*	Embryonic lethal	
rrf-3	*rab-11.2*	**Did not grow after hatching**	NO (do not pump)
rrf-3	*rab-14*	50% of progeny growth delayed (otherwise wild type); paralyzed rods (5%); slightly Dpy (1%)	
rrf-3	*rab-18*	**Growth delay**; infrequent pumping; as adults, extremely sick and starved; some dead	NO (infrequent pumping)
rrf-3	*rab-19*	Wild type	
rrf-3	*rab-21*	Sick, starved, and strong Dpy	
rrf-3	*rab-27*	Wild type	
rrf-3	*rab-28*	Wild type	
rrf-3	*rab-30*	Wild type	
rrf-3	*rab-33*	Slightly Unc, otherwise wild type	
rrf-3	*rab-35*	Wild type	

STRAIN INJECTED	dsRNA INJECTED	**PHENOTYPE**	**PHENOCOPY of *eat-17***
rrf-3	*rab-37*	Slightly loopy, otherwise wild type?	
rrf-3	*rab-39*	Wild type	
rrf-3	4R79.2	Loopy Uncs, otherwise wild type?	
rrf-3	K02E10.1	N/D	
rrf-3	F11A5.4	Wild type	
rrf-3	F11A5.3	Wild type	
rrf-3	C56E6.2	Loopy Uncs?	

B.

Strain injected	**dsRNA injected**	**PHENOTYPE**	**Suppression of *eat-17* (growth rate measured)**
rrf-3; eat-17	None	None*	
rrf-3; eat-17	*rab-1*	Embryonic lethal (100%)	No
rrf-3; eat-17	*rab-2*	None	No
rrf-3; eat-17	*rab-3*	None	No
rrf-3; eat-17	*rab-5*	Embryonic lethal (100%)	No
rrf-3; eat-17	*rab-6.1*	None	No
rrf-3; eat-17	*rab-6.2*	**Growth delayed**	No
rrf-3; eat-17	*rab-7*	Growth delayed?	No
rrf-3; eat-17	*rab-8*	None	No
rrf-3; eat-17	*rab-10*	None	No
rrf-3; eat-17	*rab-11.1*	Embryonic lethal (~100%)	No
rrf-3; eat-17	*rab-11.2*	Little to no growth after hatching	No
rrf-3; eat-17	*rab-14*	None	No
rrf-3; eat-17	*rab-18*	None	No
rrf-3; eat-17	*rab-19*	N/D	N/D
rrf-3; eat-17	*rab-21*	None	No
rrf-3; eat-17	*rab-27*	None	No
rrf-3; eat-17	*rab-30*	None	No
rrf-3; eat-17	*rab-33*	None	No
rrf-3; eat-17	*rab-35*	None	No
rrf-3; eat-17	*rab-37*	None	No
rrf-3; eat-17	*rab-39*	N/D	N/D
rrf-3; eat-17	4R79.2	None	No

Strain injected	**dsRNA injected**	**PHENOTYPE**	**Suppression of *eat-17* (growth rate measured)**
rrf-3; eat-17	4R79.2	None	No
rrf-3; eat-17	K02E10.1	N/D	N/D
rrf-3; eat-17	F11A5.4	N/D	
rrf-3; eat-17	F11A5.3	None	
rrf-3; eat-17	C56E6.2	None	

*None= no enhancement or suppression of the *rrf-3; eat-17* growth rate; no additional phenotypes (only those seen for *rrf-3* injections).

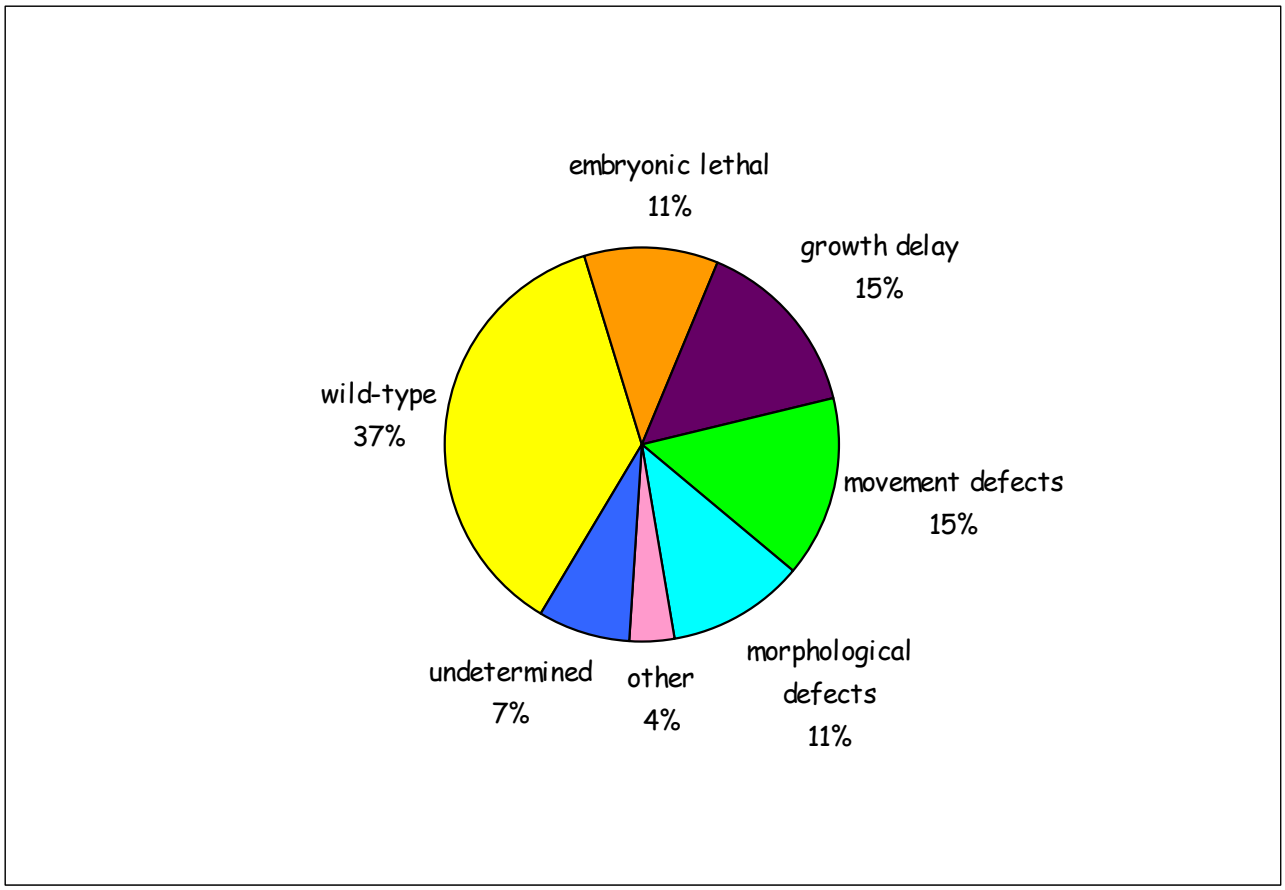
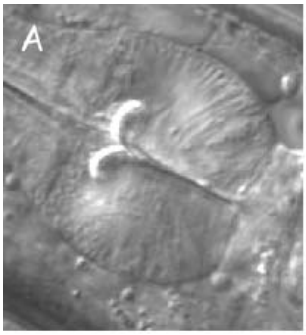
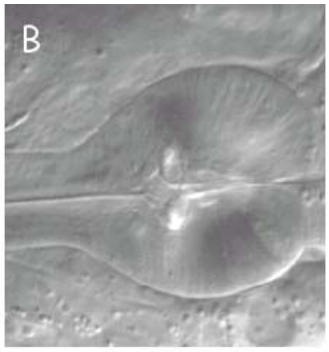


Figure 3.2. Pie diagram of Rab RNAi results. RNAi against *C. elegans* Rabs causes a number of mutant phenotypes. A little over 1/3 have a wild type phenotype, while the rest are evenly distributed between embryonic lethality, delay in growth, and movement and morphological defects. Worms with movement defects are generally uncoordinated. Morphological defects refer to changes in body shape.

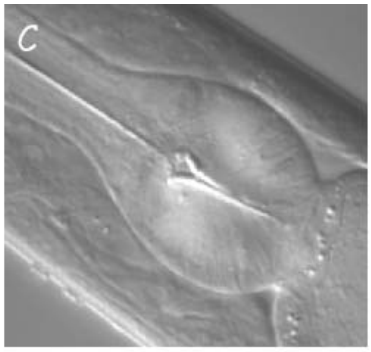
Figure 3.3. Grinder defects of *eat-17* and *rab-6.2 RNAi* pharynxes. Lateral view. Anterior is to the left for all images. **(A-F)** Wild type and mutant pharynxes displaying grinder defects. Images are labeled accordingly. **(G-H)** Rescue of grinder defects. **(G)** Grinder defects are rescued when cosmids F01G12 and T24D11 are co-expressed in *eat-17* mutants. Shown is the pharynx of a transgenic worm carrying F01G12 and T24D11 DNAs as part of an extrachromosomal array. **(H)** Grinder defects are rescued when an *eat-17* mini-gene is expressed in the pharynxes of *eat-17* mutants. **(I-J)** Images of *rrf-3*; *eat-17* and *rrf-3*; *rab-6.2 RNAi* mutant pharynxes. **Insets.** Arrows point to refractory bodies present in the terminal bulbs of both *eat-17* and *rab-6.2 RNAi* mutants.



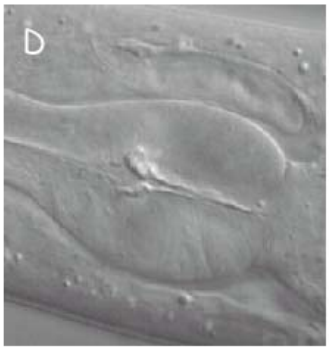
wild type



rrf-3

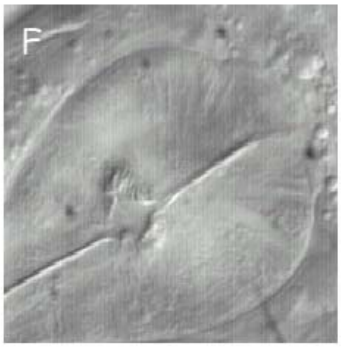


eat-17(ad707)

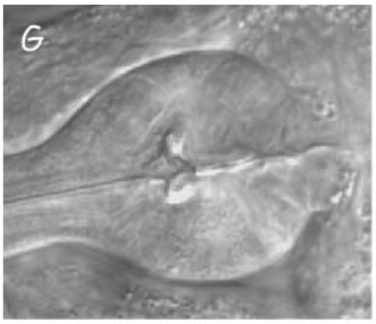


rrf-3; eat-17 RNAi

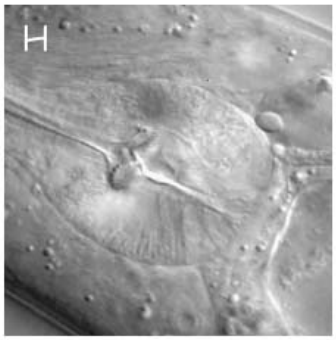
rrf-3; rab-6.1 RNAi



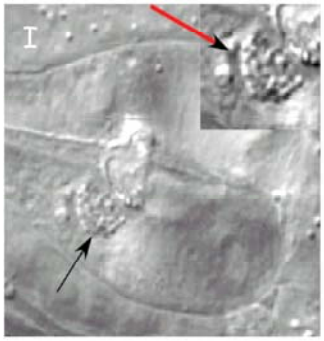
rrf-3; rab-6.2 RNAi



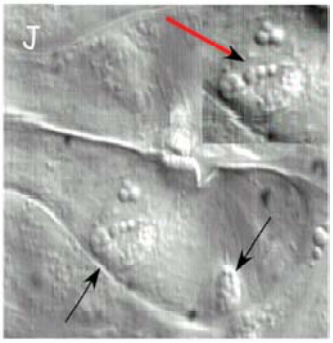
eat-17(ad707); Ex[T24D11+F01G12]



eat-17(ad707); Ex[*eat-17*p::*eat-17* cDNA::*unc-54*]



rrf-3; eat-17(ad707)



rrf-3; rab-6.2 RNAi

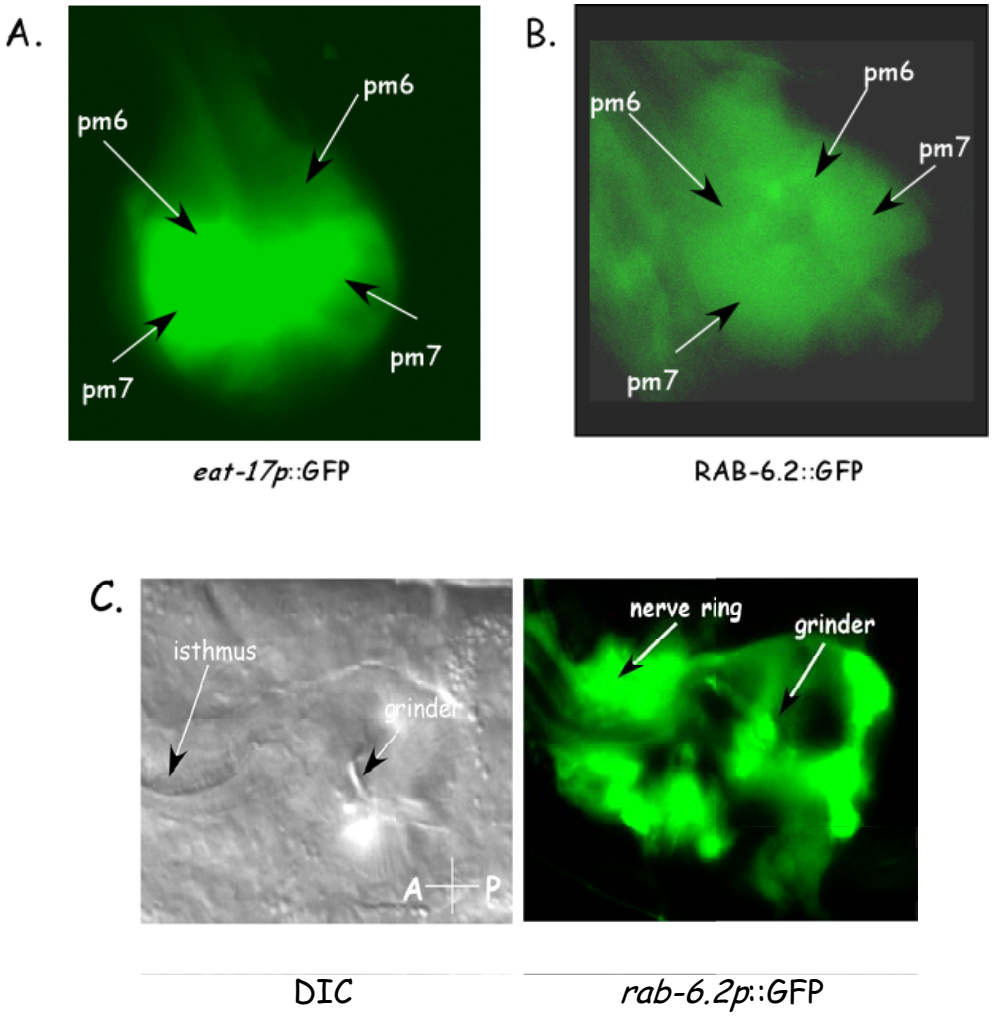
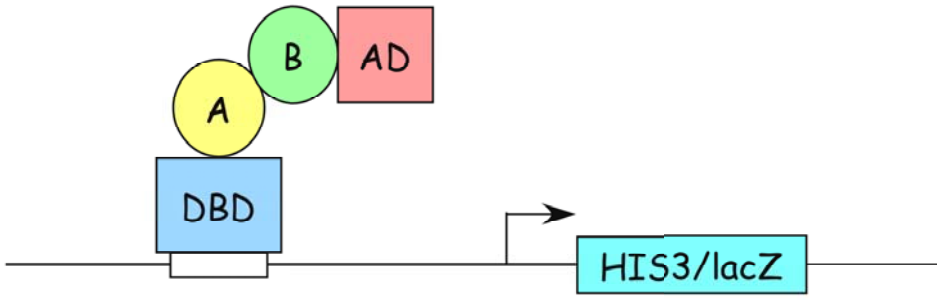


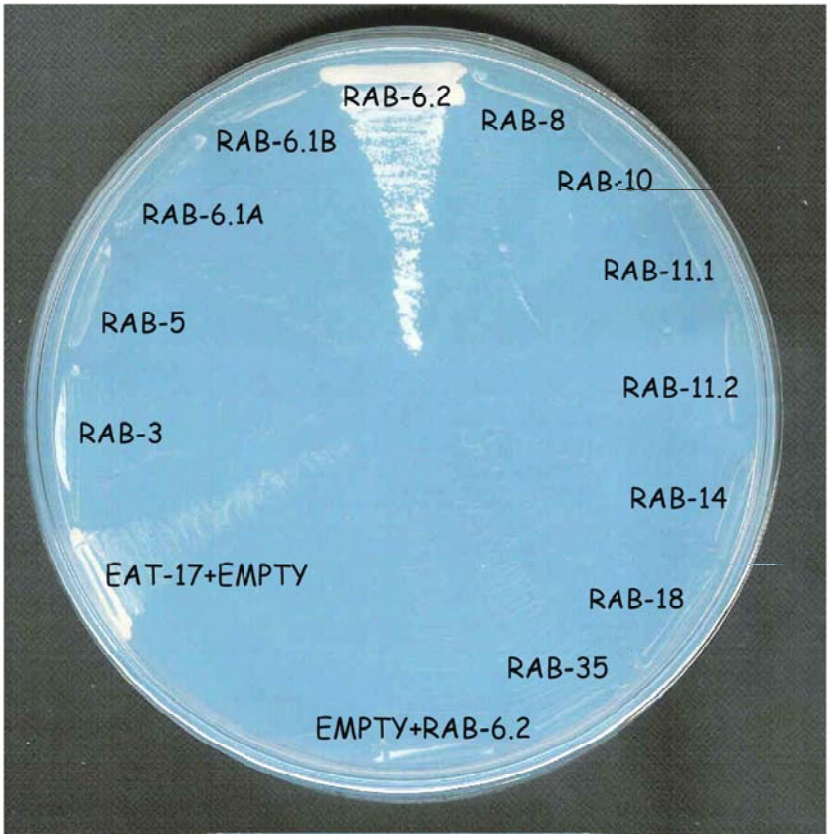
Figure 3.4. Expression patterns of *eat-17* and *rab-6.2*. (A,B) GFP reporter constructs show that EAT-17 and RAB-6.2 are expressed in terminal bulb muscle. **(C)** Expression of GFP driven by the *rab-6.2* promoter. **Left panel.** DIC image. **Right panel.** GFP image. Anterior is to the left in each case.

Figure 3.5. EAT-17 and RAB-6.2 physically interact by yeast two-hybrid. (A) Schematic of the yeast two-hybrid concept. Two proteins of interest, A and B, are fused to the LexA DNA binding domain and the GAL4 activation domain, respectively. If the two proteins physically interact with one another, transcription is activated, producing histadine and/or β-galactosidase. **(B)** EAT-17(aa1-460) and RAB-6.2 show a specific protein-protein interaction by yeast-two hybrid. EAT-17(aa1-316), EAT-17(aa1-364), and EAT-17(aa1-405) do not interact with RAB-6.2, showing that the GAP domain alone is not sufficient for this interaction (not shown). **(C)** RAB-6.2(Q69L) but not RAB-6.2(T27N) interacts with EAT-17(aa1-460). RAB-6.1(Q70L) also shows an interaction, albeit at lower levels.

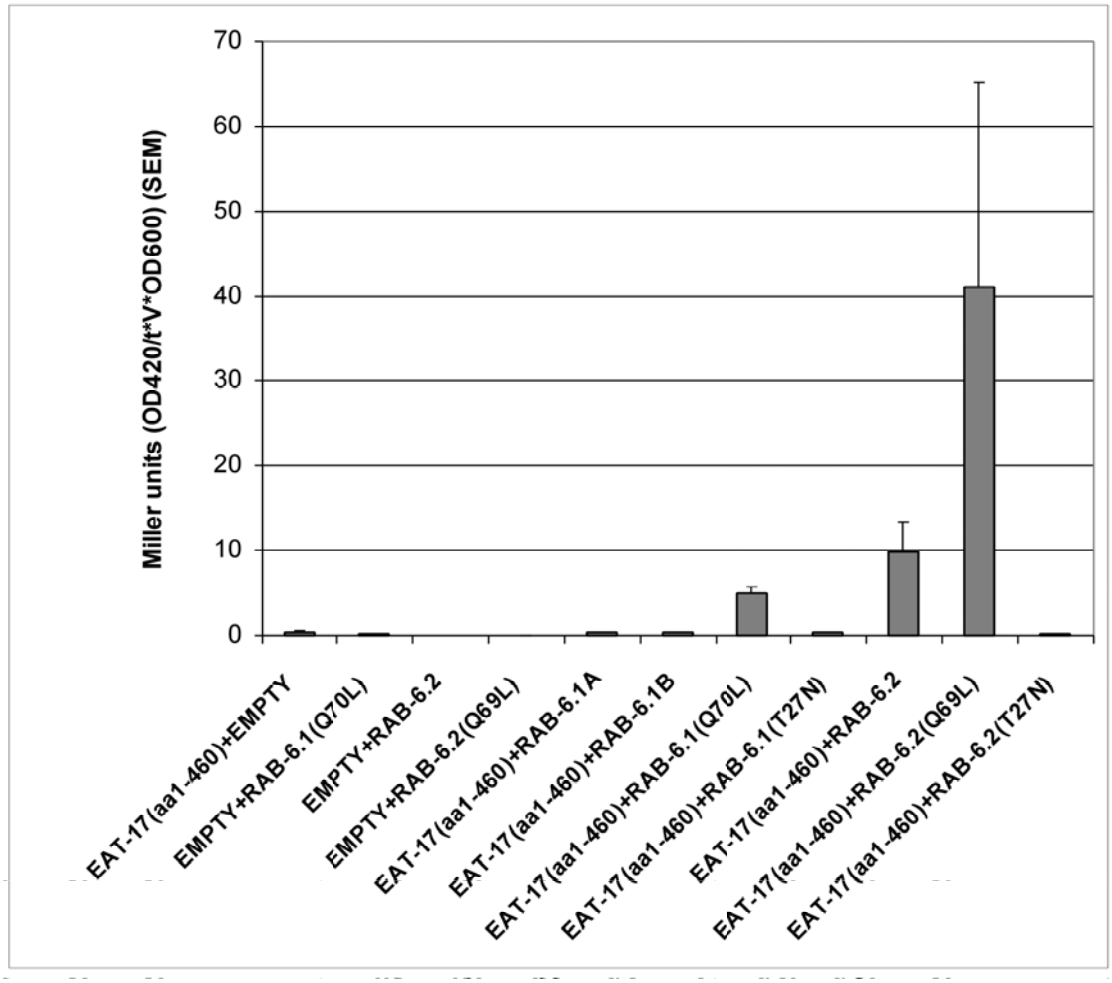
A.


B
AD
A
DBD
HIS3/lacZ

B.


RAB-6.2
RAB-8
RAB-6.1B
RAB-10
RAB-6.1A
RAB-11.1
RAB-5
RAB-11.2
RAB-3
RAB-14
EAT-17+EMPTY
RAB-18
RAB-35
EMPTY+RAB-6.2

C.


Miller units (OD420/t*V*OD600) (SEM)
70
60
50
40
30
20
10
0
EAT-17(aa1-460)+EMPTY
EMPTY+RAB-6.1(Q70L)
EMPTY+RAB-6.2
EMPTY+RAB-6.2(Q69L)
EAT-17(aa1-460)+RAB-6.1A
EAT-17(aa1-460)+RAB-6.1B
EAT-17(aa1-460)+RAB-6.1(Q70L)
EAT-17(aa1-460)+RAB-6.1(T27N)
EAT-17(aa1-460)+RAB-6.2
EAT-17(aa1-460)+RAB-6.2(Q69L)
EAT-17(aa1-460)+RAB-6.2(T27N)

Materials and Methods

Assay of EAT-17 GAP activity:

To assay the GAP activity of EAT-17, rescuing fragments containing the catalytically inactive R116/119K and R116/119A mutations were generated by overlap extension PCR (Ho et al., 1989). In first round PCR reactions, 5' and 3' *eat-17* cDNA fragments were amplified using primers shown in Table 3.3.

Primers 1142-1145 were PAGE purified. Nucleotides shown in bold are residues that were altered. The resulting PCR fragments were purified using the Qiaquick Gel Extraction Kit (Qiagen, Valencia, CA). Each of the products was loaded on a different agarose gel to avoid contamination.

1 μL of each PCR reaction was transferred to a new reaction mix and subjected to a second round of PCR using the overlap extension method. Primers 1076 and 1085 were used in both cases. The PCR protocol is the same as that described previously.

These products were subjected to a third round of PCR to fuse the *eat-17* promoter and *unc-54* 3' UTR to each end. This was done in exactly the same way as for the wild type rescue fragment.

The resulting PCR products were gel purified and injected into *eat-17* worms at a concentration of 25 ng/μL. *let-858*::GFP plasmid DNA was used as a co-injection marker at a concentration of 100 ng/μL. Wild type, R116/119K, and R116/119A injections were performed in parallel. DNA concentrations were determined at the same time using a UV spectrophotometer (an average of six readings).

To score for rescue of grinder defects, F1 gravid adult hermaphrodites were picked arbitrarily to 4% agar pads containing 10 mM sodium azide (Sigma, St. Louis, MO). Defects in grinder formation were recorded and then the presence or absence of GFP expression was determined. In cases where too few transgenic animals were being observed, worms were first picked using the GFP dissecting scope then observed for defects in grinder formation. Images were captured using FLI Grab software (Finger Lakes Instrumentation, Lima, NY).

RNAi experiments:

RNAi was performed as described with minor modifications (Kamath et al., 2003). cDNA sequences of *eat-17* and the 27 predicted *C. elegans* Rabs (M. Nonet, personal communication) were amplified by PCR using primers shown in Table 3.4.

The HiScribe RNAi Transcription Kit (New England Biolabs, Beverly, MA) was used to construct dsRNAs. These were injected at a concentration of ~1μg/μL into the gonads or intestines of young adults. *eat-17* dsRNA was injected into *rrf-3* mutants to test for phenocopy of the genetic mutant. Rab dsRNAs were injected into *rrf-3* mutants to test for an *eat-17* phenocopy and into *rrf-3*; *eat-17* mutants to test for suppression of the Eat phenotype. Worms were grown on *Comamonas* DA1877 prior to injection. Worms were transferred to DA837 immediately, then transferred once more approximately 10-12 hours post-injection. The worms were grown at 16^{o}C to prevent large numbers of dead embryos from being produced (a temperature sensitive phenotype of *rrf-3*). The *rrf-3* strain was obtained from the CGC. I constructed the *rrf-3*; *eat-17*

strain by crossing *rrf-3* males to *eat-17* mutant hermaphrodites. I isolated F2 progeny that were starved in appearance and tested their progeny for the *rrf-3* deletion.

rab-6.1 and *rab-6.2* RNAi results were confirmed in at least two separate experiments.

Expression constructs

***eat-17p*::GFP:**

A 5.6 kb *eat-17* promoter fragment was amplified from N2 genomic DNA using the following primers: 1073: 5'- taggttacggtagttggtacg –3' and 1167: 5'- gaaaagttcttctcctttactcatcggcggtgacaattgg –3'. (In addition to the 5' regulatory sequence, this DNA contains the first six codons of *eat-17*.) GFP was amplified from pPD95.75 plasmid DNA (gift from A. Fire) using primers: 1166: 5'- ccaattgtcaccgccgatgagtaaaggagaagaacttttc –3' and MS46: 5'- tttggtatattgggaatggtattctg –3'. DNAs were fused together by overlap extension PCR using the following nested primers: 1157: 5'- acggtagtgttttatcagtagtg –3' and MS10: 5'- caaacccaaaccttcttccgatc –3'. PCR products were not purified prior to fusion.

The resulting product was purified using the Qiaquick PCR Purification Kit (Qiagen, Valencia, CA). This was injected into wild type N2 adults with pRAK3 *rol-6(d)* as a co-injection marker. Transgenic F2 progeny were placed on 4% agar pads in BDM media (a worm anesthetic) and observed using the Zeiss Axiophot (Carl Zeiss Instruments Inc, Germany) at either 630x or 1000X magnification. Images were taken with FLI Grab software (Finger Lakes Instrumentation, Lima, NY). Further

modifications of the images were performed in Adobe Photoshop. Length of exposure depended on the brightness of the GFP signal.

***rab-6.2p*::GFP:**

A 2.8 kb *rab-6.2* promoter fragment was amplified from N2 genomic DNA using the following primers: 1186: 5'- aatcgcacagcaggcctcc –3' and 1180: 5'- gtgaaaagttcttctcctttactcggattaccaaagtccgacat –3'. (In addition to the 5' regulatory sequence, this DNA fragment contains the first seven codons of *rab-6.2*.)

GFP was amplified from plasmid pPD 95.75 (gift from A. Fire) using the following primers: 1175: 5'- atgtcggactttggtaatccgagtaaaggagaagaacttttcac –3' and MS46: 5'- tttggtatattgggaatggtattctg –3'. Products were fused together by overlap extension PCR using the following primers: 1188: 5'- tttgccgaacggaagagcc –3' and MS10: 5'- caaacccaaaccttcttccgatc –3'. The final product was purified as described above and injected into N2 adults with *rol-6(d)* as a co-injection marker. GFP expression was observed as described above.

RAB-6.2::GFP:

A full-length *rab-6.2* genomic fragment was amplified from N2 genomic DNA using the following primers: 1188: 5'- tttgccgaacggaagagcc –3' and 1182: 5'- cagtgaaaagttcttctcctttactgcaccagcacgatccctc –3'. GFP was amplified from plasmid pPD 95.75 (gift from A. Fire) using the following primers: 1177: 5'- gagggatcgtgctggtgcagtaaaggagaagaacttttcactg –3'and MS46: 5'- tttggtatattgggaatggtattctg –3'. Products were fused together by overlap extension PCR

using the following primers: 1188: 5'- tttgccgaacggaagagcc –3' and MS10: 5'- caaacccaaaccttcttccgatc –3'. The final product contains 1.6 kb of 5' regulatory sequence in addition to the entire coding region of the gene.

This product was purified as previously described and injected into *eat-5* mutant adults at a concentration of 20 ng/μL with an *eat-5* PCR rescuing fragment as a co-injection marker (gift from B. Shtonda). Prior to injection worms were grown on HB101 bacteria. After injection worms were transferred to DA837 and transgenic animals were identified by their ability to grow on this food source (Shtonda, personal communication). GFP images were taken as described.

RAB-6.1::GFP:

A 2 kb *rab-6.1* promoter fragment containing the entire *rab-6.1* coding sequence was amplified from N2 genomic DNA using the following primers: 1185: 5'- tggagaggttatctatcgataac –3' and 1178: 5'- aaggccgtcaatgtccgtgtagtaaaggagaagaacttttcactg –3'.. GFP was amplified from plasmid pPD 95.75 (gift from A. Fire) using the following primers: 1183: 5'- cagtgaaaagttcttctcctttactacacggacattgacggcctt –3' and MS46: 5'- tttggtatattgggaatggtattctg –3'. Products were fused together by overlap extension PCR using the following primers: 1185: 5'- tggagaggttatctatcgataac –3' and MS10: 5'- caaacccaaaccttcttccgatc –3'.

The final product was purified as described above and injected into *eat-5* adults with *eat-5*(+) as a co-injection marker (see above). Unfortunately, expression of this construct in worms caused significant lethality. As a result, stable transgenic lines were not obtained. Expression was observed in a few F1 adult progeny (n=4). While

expression occurred in most tissues, the four worms examined rarely show overlapping expression in these tissues due to mosaicism. All four worms did, however, show strong expression in the head hypodermis (images not shown).

Strain designations for worms expressing *rab-6.2p*::GFP and RAB-6.2::GFP are as follows: DA2035 *adEx2035*[*rab-6.2p*::GFP *rol-6(d)*] and DA2033 *eat-5(ad1402)*; *adEx2033* [RAB-6.2::GFP *unc-122*::RFP *eat-5(+)*].

Yeast two-hybrid experiments

Preparation of first strand cDNA:

RNA was isolated from a well fed, mixed stage wild type N2 population using Trizol Reagent (Invitrogen Life Technologies, Carlsbad, CA). Poly(A)$^+$ first strand cDNA was prepared using the First Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche, Indianapolis, IN).

Preparation of preys:

Inserts for two-hybrid constructs were amplified by PCR using the Expand Long Template PCR Kit (Roche, Indianapolis, IN, Buffer 2). Primers to amplify the preys are shown in Table 3.5.

PCR products were cloned into the pGEM-T Easy vector (Promega, Madison, WI) and sequenced from the T7 and SP6 promoters (UTSW Sequencing Core, Dallas, TX). Constructs with wild type sequence were subcloned into the pACT2 vector (gift from R. Lin), in frame with the GAL4 activation domain.

Preparation of baits:

To construct baits for two-hybrid screening, several truncated versions of *eat-17* were amplified by PCR and cloned into the pGEM-T Easy vector (Promega, Madison, WI). These were sequenced from the T7 and SP6 promoters. Correct sequences were subcloned into the pVJL11 vector (gift from M. Cobb), in frame with the LexA DNA binding domain. Primers used to generate baits are shown in Table 3.6.

PCR conditions for amplifying the two hybrid inserts is as follows:

Step 1	92°C for 3 minutes	1 cycle
Step 2	92°C for 30 seconds	1 cycle
Step 3	55°C for 30 seconds (preys)	1 cycle
	57.5°C for 30 seconds (baits)	1 cycle
Step 4	Ramp at 1.5°/second to 70°C	
Step 5	70°C for 1 minute (preys)	1 cycle
	70°C for x minutes (1 minute/kb) (baits)	1 cycle
Step 6	Go to Step 2	19 cycles
Step 7	68°C for 10 minutes	1 cycle
Step 8	4°C forever	

Mutant versions of the baits were generated using an *in vitro* mutagenesis protocol. Primers containing mutations of interest (shown in bold) are shown in Table 3.7.

Plasmid DNAs containing the wild type *eat-17* truncations (cloned into the pGEM-T Easy vector) were used as templates in PCR reactions. Following PCR DpnI (Invitrogen, Carlsbad, CA) was added to the tubes, and the reactions were incubated at 37°C for 16 hours. The reactions were transformed into DH5α chemically competent cells using standard methods (Sambrook et al., 1989) and grown on LB+AMP plates to select for colonies containing plasmid inserts. Colonies that grew overnight were

cultured in LB+AMP media and DNA was prepped the next day using the Wizard Plus SV Mini-preps DNA Purification Kit (Promega, Madison, WI). The resulting DNAs were sequenced from T7 and SP6 promoters to confirm that the desired mutations had been incorporated (UTSW Sequencing Core, Dallas, TX). Inserts with correct sequence were subcloned into the pVJL11 vector.

PCR conditions for *in vitro* mutagenesis were as follows:

Step 1	92°C for 3 minutes	1 cycle
Step 2	92°C for 30 seconds	1 cycle
Step 3	55°C for 30 seconds	1 cycle
Step 4	Ramp at 1.5°/second to 70°C	9 cycles
Step 5	70°C for x minutes (3 minutes/ kb)	1 cycle
Step 6	Go to Step 2	19 cycles
Step 7	70°C for 10 minutes	1 cycle
Step 8	4°C forever	

PCR reactions were set up as previously described, except that the polymerase was replaced with Pfu DNA polymerase (gift from M. Cobb, UTSW). This protocol is a modification of one described for the QuikChange Site-Directed Mutagenesis Kit manufactured by Stratagene (La Jolla, CA).

Yeast two-hybrid screening:

To test for interactions, combinations of baits and preys were transformed into the yeast strain L40 (Hama et al., 1999). X-gal filter assays (not shown) and quantitative ONPG assays were performed as described (Yeast Protocols Handbook, Clontech). An average of 2-5 colonies were tested in each case. For growth assays, colonies were streaked onto plates lacking histadine and supplemented with 5 mM 3-AT.

Table 3.3. Primers used for generating the R116/119K and R116/119A mutations

PCR product		
(R116/119K)	**Forward primer**	**Reverse primer**
5' fragment	1076 (5'- *ttgtcaccgccg*atggcagccactgcagcgctac –3')	1143 (5'- ttctgccatgcaatcat**ttt**aaagtgctgtgggatgcct –3')
3' fragment	1142 (5'- aggcatcccacagcactt**aaa**atgattgcatggcagaa –3')	1085 (5'- *tagggatgttgaagagtaattggac*ctagtggctatccgacagtt –3')
(R116/119A)		
5' fragment	1076 (5'- *ttgtcaccgccg*atggcagccactgcagcgctac –3')	1145 (5'- ttctgccatgcaatcat**tgc**aaagtgctgtgggatgcct –3')
3' fragment	1144 (5'- aggcatcccacagcactt**gca**atgattgcatggcagaa –3')	1085 (5'- *tagggatgttgaagagtaattggac*ctagtggctatccgacagtt –3')

Table 3.4. Primers used for RNAi experiments

	Forward primer	**Reverse primer**
eat-17	942: 5'- *taatacgactcactatagggc*gacgtcagcctcatggaaaact –3'	943: 5'- *taatacgactcactatagggc*gagccaatgtgtcagcttcctt –3'
rab-1	1088: 5'- *taatacgactcactatagggc*atggcagcaatgaaccctga –3'	1089: 5'- *taatacgactcactatagggc*ttaacaacatccaccgctctt –3'
rab-2	1090: 5'- *taatacgactcactatagggc*atgtcatatgcctacctttca –3'	1091: 5'- *taatacgactcactatagggc*ttaacagcatccagatccacc –3'
rab-3	1092: 5'- *taatacgactcactatagggc*atgaataatcaacaggctgcc –3'	1093: 5'- *taatacgactcactatagggc*ttagcaattgcattgctgttga –3'
rab-5	1094: 5'- *taatacgactcactatagggc*atggccgcccggaacgca –3'	1095: 5'- *taatacgactcactatagggc*ttatttacagcatgaacccttttt –3'
rab-6.1	944: 5'- *taatacgactcactatagggc*atttttctcggcgaacagagt –3'	945: 5'- *taatacgactcactatagggc*gagttgatgaaaagctgcggat –3'
rab-6.2	1096: 5'- *taatacgactcactatagggc*atgtcggactttggtaatccg –3'	1097: 5'- *taatacgactcactatagggc*ttagcaccagcacgatccc –3'
rab-7	1098: 5'- *taatacgactcactatagggc*atgtcgggaaccagaaagaag –3'	1099: 5'- *taatacgactcactatagggc*ttaacaattcgatcccgaattc –3'
rab-8	1100: 5'- *taatacgactcactatagggc*atggcaaaaacttacgactact –3'	1101: 5'- *taatacgactcactatagggc*gttaaagcaaattgcagctccag –3'
rab-10	1102: 5'- *taatacgactcactatagggc*atggctcgccgaccgtatg –3'	1103: 5'- *taatacgactcactatagggc*ctagcagcatcctccactg- 3'
rab-11.1	1104: 5'- *taatacgactcactatagggc*atgggctctcgtgacgatg –3'	1105: 5'- *taatacgactcactatagggc*ttatgggatgcaacactgctt –3'
rab-11.2	1106: 5'- *taatacgactcactatagggc*atgggcaacgaatactactac –3'	1107: 5'- *taatacgactcactatagggc*ttatggaaagcaacactggtt –3'
rab-14	1108: 5'- *taatacgactcactatagggc*atgacggctgctccttacaa –3'	1109: 5'- *taatacgactcactatagggc*ctagcagttgcagtccttct –3'

	Forward primer	**Reverse primer**
rab-18	1110: 5'- *taatacgactcactatagggc*atgtccgacgacagttcaa –3'	1111: 5'- *taatacgactcactatagggc*ctagcatccacacattccgc –3'
rab-19	1112: 5'- *taatacgactcactatagggc*atggacaacgatgatggattt –3'	1113: 5'- *taatacgactcactatagggc*tcaagtgtactgacaacatcg –3'
rab-21	1114: 5'- *taatacgactcactatagggc*atgctcgaaaccaacgtgga –3'	1115: 5'- *taatacgactcactatagggc*tcagcgacagcacttttact –3'
rab-27	1116: 5'- *taatacgactcactatagggc*atgggtgactacgactatctc –3'	1117: 5'- *taatacgactcactatagggc*tcagcaatttgcacaatagga –3'
rab-30	1120: 5'- *taatacgactcactatagggc*atggaggattacaagtatctatt –3'	1121: 5'- *taatacgactcactatagggc*ctaagattgtcgagtacaacag –3'
rab-33	1122: 5'- *taatacgactcactatagggc*atgtcggagcatcatgtgaac –3'	1123: 5'- *taatacgactcactatagggc*tcagcagcagaatccctctt –3'
rab-35	1124: 5'- *taatacgactcactatagggc*atggcgggaactcgggat –3'	1125: 5'- *taatacgactcactatagggc*ttatccacatttgcacttctttt –3'
rab-37	1126: 5'- *taatacgactcactatagggc*atgtttttaaaggttatgctactt –3'	1127: 5'- *taatacgactcactatagggc*tcaattaaacgtgcaacatctg –3'
rab-39	1128: 5'- *taatacgactcactatagggc*atggaaacaaacttcattggtg –3'	1129: 5'- *taatacgactcactatagggc*tcaacatccgcaagctcctg –3'
4R79.2	1130: 5'- *taatacgactcactatagggc*atggaagtagagtcggcga –3'	1131: 5'- *taatacgactcactatagggc*tcaaatacagcaccagctcc –3'
K02E10.1	1132: 5'- *taatacgactcactatagggc*atgaatggaaaaagtattggaaaa –3'	1133: 5'- *taatacgactcactatagggc*tcagacaaaggcgggttcc –3'
F11A5.4	1134: 5'- *taatacgactcactatagggc*atgtcatcagatcatgtgttca –3'	1135: 5'- *taatacgactcactatagggc*ttaacaacatgctttctcctttc –3'
F11A5.3	1136: 5'- *taatacgactcactatagggc*atgtaccctgatcacatgttc –3'	1137: 5'- *taatacgactcactatagggc*ttaacaacatttttttctcttttcc –3'

Table 3.5. Primers used to detect the *rrf-3* deletion

	Forward primer	**Reverse primer**
Inside deletion	569: 5’ gctgcagaagaatcggaaac –3’	570: 5’- cacagcgatggaatgaaatg –3’
Outside deletion	1140: 5’- atgctctcaagccacagaag –3’	1141: 5’- tactcagagaacatagttctc –3’

Table 3.6. Primers used to amplify preys

	Forward primer	**Reverse primer**
rab-1	1088: 5'- *ccatggag*atggcagcaatgaaccctga –3'	1089: 5'- *gagctc*ttaacaacatccaccgctctt –3'
rab-2	1090: 5'- *ccatggag*atgtcatatgcctacctttca –3'	1091: 5'- *gagctc*ttaacagcatccagatccacc –3'
rab-3	1092: 5'- *ccatggag*atgaataatcaacaggctgcc –3'	1093: 5'- *gagctc*ttagcaattgcattgctgttga –3'
rab-5	1094: 5'- *ccatggag*atggccgcccggaacgca –3'	1095: 5'- *gagctc*ttatttacagcatgaacccttt –3'
rab-6.1	944: 5'- *ccatggag*atttttctcggcgaacagagt –3'	945: 5'- *gagctc*gagttgatgaaaagctgcggat –3'
rab-6.2	1096: 5'- *ccatggag*atgtcggactttggtaatccg –3'	1097: 5'- *gagctc*ttagcaccagcacgatccc –3'
rab-7	1098: 5'- *ccatggag*atgtcgggaaccagaaagaag –3'	1099: 5'- *gagctc*ttaacaattcgatcccgaattc –3'
rab-8	1100: 5'- *ccatggag*atggcaaaaacttacgactact –3'	1101: 5'- *gagctc*gttaaagcaaattgcagctccag –3'
rab-10	1102: 5'- *ccatggag*atggctcgccgaccgtatg –3'	1103: 5'- *gagctc*ctagcagcatcctccactg- 3'
rab-11.1	1104: 5'- *ccatggag*atgggctctcgtgacgatg –3'	1105: 5'- *gagctc*ttatgggatgcaacactgctt –3'
rab-11.2	1106: 5'- *ccatggag*atgggcaacgaatactactac –3'	1107: 5'- *gagctc*ttatggaaagcaacactggtt –3'
rab-14	1108: 5'- *ccatggag*atgacggctgctccttacaa –3'	1109: 5'- *gagctc*ctagcagttgcagtccttct –3'
rab-18	1110: 5'- *ccatggag*atgtccgacgacagttcaa –3'	1111: 5'- *gagctc*ctagcatccacacattccgc –3'

rab-19	1112: 5'- *ccatggag*atggacaacgatgatggattt –3'	1113: 5'- *gagctc*tcaagtgtactgacaacatcg –3'
rab-21	1114: 5'- *ccatggag*atgctcgaaaccaacgtgga –3'	1115: 5'- *gagctc*tcagcgacagcacttttact –3'
rab-27	1116: 5'- *ccatggag*atgggtgactacgactatctc –3'	1117: 5'- *gagctc*tcagcaatttgcacaatagga –3'
rab-30	1120: 5'- *ccatggag*atggaggattacaagtatctatt –3'	1121: 5'- *gagctc*ctaagattgtcgagtacaacag –3'
rab-33	1122: 5'- *ccatggag*atgtcggagcatcatgtgaac –3'	1123: 5'- *gagctc*tcagcagcagaatccctctt –3'
rab-35	1124: 5'- *ccatggag*atggcgggaactcgggat –3'	1125: 5'- *gagctc*ttatccacatttgcacttctttt –3'
rab-37	1126: 5'- *ccatggag*atgtttttaaaggttatgctactt –3'	1127: 5'- *gagctc*tcaattaaacgtgcaacatctg –3'
rab-39	1128: 5'- *ccatggag*atggaaacaaacttcattggtg –3'	1129: 5'- *gagctc*tcaacatccgcaagctcctg –3'
4R79.2	1130: 5'- *ccatggag*atggaagtagagtcggcga –3'	1131: 5'- *gagctc*tcaaatacagcaccagctcc –3'
K02E10.1	1132: 5'- *ccatggag*atgaatggaaaaagtattggaaaa –3'	1133: 5'- *gagctc*tcagacaaaggcgggttcc –3'
F11A5.4	1134: 5'- *ccatggag*atgtcatcagatcatgtgttca –3'	1135: 5'- *gagctc*ttaacaacatgctttctcctttc –3'
F11A5.3	1136: 5'- *ccatggag*atgtaccctgatcacatgttc –3'	1137: 5'- *gagctc*ttaacaacatttttttctctttcc –3'
C56E6.2	1138: 5'- *ccatggag*atgcaagtgctccgtcaact –3'	1139: 5'- *gagctc*ctagagcattgaacaacacttt –3'

Table 3.7. Primers used to amplify baits

Bait	Forward primer	Reverse primer
EAT-17 (aa1-316)	1213: 5'- *aagatccatttaaatcg*atggcagccactgcagcgc –3'	1240: 5'- gtcgacctagagacggagaagctcgatg –3'
EAT-17 (aa1-364)	1213: 5'- *aagatccatttaaatcg*atggcagccactgcagcgc –3'	1250: 5'- gtcgacctacttcgtcaagtaatccttttcc –3'
EAT-17 (aa1-405)	1213: 5'- *aagatccatttaaatcg*atggcagccactgcagcgc –3'	1251: 5'- gtcgacctacttgactagacgatccgcc –3'
EAT-17 (aa1-460)	1213: 5'- *aagatccatttaaatcg*atggcagccactgcagcgc –3'	1249: 5'- gtcgacctatccagtatccataatgttgt –3'
EAT-17 (aa1-500)	1213: 5'- *aagatccatttaaatcg*atggcagccactgcagcgc –3'	1241: 5'- gtcgacctagaccctcaacttggcgtcc –3'
EAT-17 (aa500-825)	1243: 5'- aagatccatttaaatcggtctcggaactggaaatggcc –3'	1214: 5'- actgactggtcgacctagtggctatccgacagttg –3'

CHAPTER 4

Genetic interactions between *rab-6.1*, *rab-6.2*, and *eat-17*

Results

4.1 Genetic interactions between rab-6.1, rab-6.2, and eat-17

So far several lines of evidence point to EAT-17 and RAB-6.2 acting together in a pathway that regulates grinder formation. As stated previously, two Rab6 homologs are present in the *C. elegans* genome. RNAi against *rab-6.1* shows no obvious defects in grinder formation.

I was interested in knowing how these three genes interact with one another, so I performed double and triple RNAi experiments in every possible combination (Figure 4.1; Table 4.1). The most interesting finding was that *rab-6.1* and *rab-6.2* display a strong synthetic interaction. RNAi against both of these in the same worm causes a severe larval arrest phenotype in progeny. On closer examination of the worms, I found that all of the arrested larvae had plugs of cuticle blocking the mouth, which could result from a defect in molting. This could either occur at the step of cuticle secretion or at the later step of cuticle shedding. It is plausible that a defect in cuticle secretion actually leads to a defect in cuticle shedding. Because a new cuticle must be deposited before the old one is shed, there must be a mechanism that controls the timing of cuticle shedding. If the cuticle is not formed or gaps in the cuticle are present, one might first expect an increase in molting length to accommodate for decreases in the cuticle secretion rate. It is probable that at some point the cuticle must shed, and this process might go awry if the new cuticle is not present or is incompletely formed. These hypotheses require there to be two signals regulating cuticle shedding, one dependent on cuticle deposition and one

that is independent of this process. I think it is more likely that the *rab-6.1 RNAi*; *rab-6.2 RNAi* phenotype arises from a defect in cuticle secretion. The grinder defects present in *rab-6.2 RNAi* pharynxes support this assertion.

I have observed that many of the *rab-6.1 RNAi*; *rab-6.2 RNAi* larvae arrest at what appears to be the L1 molt. I scored this as an L1 arrest because the gonads of these worms have the characteristic appearance of the late L1 or early L2 stage of development. I looked for alae but could not easily determine if these were present. Because I suspect that double RNAi affects the cuticle, I could not reliably stage worms using this marker. It is interesting that a defect occurs at the head. My GFP reporters suggest that RAB-6.1 and RAB-6.2 are expressed in head hypodermal cells (data not shown).

RNAi against *rab-6.2* in the *eat-17(ad707)* background results in a greatly reduced growth rate. None of the worms reached adulthood in a two-week period. In several of the *eat-17*; *rab-6.2 RNAi* larvae, the cuticles around the head and occasionally the body appeared to be detached (Figure 4.1). The grinders were completely malformed. Based on these results, it appears that *rab-6.2* RNAi actually enhances the *eat-17* phenotype. In the absence of RAB-6.2, it is possible that worms are sensitized to the presence/loss of EAT-17. The growth rate decrease could be due to an increase in molting cycle length; however, this has not been shown definitively.

rab-6.1 RNAi in an *eat-17* genetic background results in a phenotype similar to *eat-17*. This suggests that the only time RAB-6.1 is required is when RAB-6.2 function is compromised.

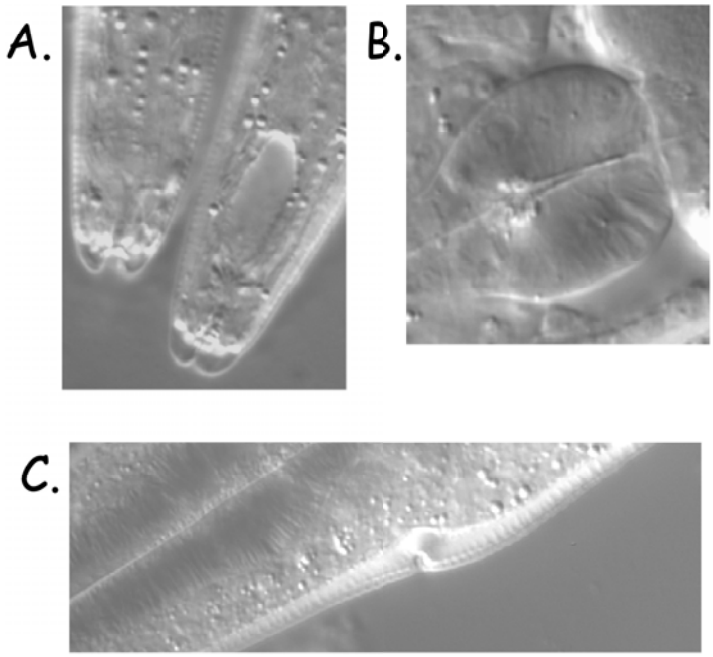
Finally, the *eat-17(ad707)*; *rab-6.1 RNAi*; *rab-6.2 RNAi* triple mutant is not obviously worse that the *rab-6.1 RNAi*; *rab-6.2 RNAi* double mutant, suggesting that EAT-17 acts specifically through RAB-6.1 and/or RAB-6.2. Performing RNAi in the *eat-17* genetic background, however, results in an increase in penetrance from 23% to nearly 100%. The most plausible explanation for this is that RNAi against *rab-6.1* and *rab-6.2* results in partial losses of function. Worms expressing reduced amounts of RAB-6.1 and RAB-6.2 are likely sensitized to the presence/loss of EAT-17. It is unlikely that the increase in penetrance is due to EAT-17 acting through a separate Rab. As stated above, the phenotypes of *eat-17(ad707)*; *rab-6.1 RNAi*; *rab-6.2 RNAi* and *rab-6.1 RNAi*; *rab-6.2 RNAi* worms are similar. Yeast two-hybrid experiments suggest a specific physical interaction between RAB-6.2 (and possibly RAB-6.1) and EAT-17 as well.

My suspicion is that RAB-6.1, RAB-6.2, and EAT-17 are involved in the process of cuticle secretion. RAB-6.1 only seems to be necessary when RAB-6.2 is absent, suggesting that RAB-6.2 is a major player of this pathway, and RAB-6.1 serves as part of a back up system to compensate for the loss of RAB-6.2. When RAB-6.1 and RAB-6.2 are absent, this process is severely compromised, resulting in larval lethality. Because of the cyclic nature of the Rab signaling pathway, the presence of EAT-17 becomes vital when RAB-6.2 function is compromised.

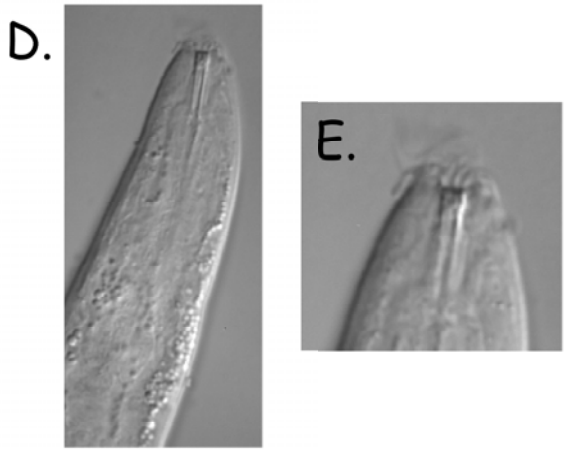
The effects of *eat-17* and *rab-6.2* RNAi seem to be restricted to the grinder. It is possible that the rate of cuticle deposition decreases slightly in these mutants, and we see defects in the grinder because of its intricately complex structure. When both of these are absent, the pathway becomes slightly more deficient, and we begin to see subtle defects outside the grinder. Moreover, it is possible that the decrease in growth rate is

representative of an increase in molting cycle length. When *rab-6.1* and *rab-6.2* are knocked down, the rate of deposition is reduced to an even greater extent, and this is why progeny arrest at the first post-embryonic molt. Currently this is only speculation; several additional experiments are needed to test each of these hypotheses.

Figure 4.1. Synthetic lethal interactions between *rab-6.1*, *rab-6.2*, and *eat-17*. **(A-C). Images of *rrf-3*; *eat-17(ad707)*; *rab-6.2 RNAi* worms.** Worms show defects in molting near the head of the worm (A) and occasionally within the body (C). Defects in grinder formation are also seen (B). Anterior is to the bottom left. **(D-E). Images of *rrf-3*; *rab-6.1 RNAi*; *rab-6.2 RNAi* worms.** Anterior is to the top. Worms arrest at the L1 molt with plugs of cuticle blocking the mouth.



rrf-3; eat-17(ad707); rab-6.2 RNAi



rrf-3; rab-6.1 RNAi; rab-6.2 RNAi

Table 4.1. Synthetic lethal interactions between *rab-6.1*, *rab-6.2*, and *eat-17*

Strain	dsRNA injected	Phenotype	
		Grinder defect	**Larval lethality**
N2	--------	0% (n= 80)	0% (n= 80)
rrf-3	--------	0% (n= 155)	0% (n= 155)
rrf-3	*eat-17*	83.3% (n= 42)	--------
rrf-3	*rab-6.1*	0% (n= 40)	--------
rrf-3	*rab-6.2*	85.2% (n= 54)	--------
rrf-3	*eat-17; rab-6.1*	26% (n= 27)*	--------
rrf-3	*eat-17; rab-6.2*	100% (n= 23)	--------
rrf-3	*rab-6.1; rab-6.2*	68% (n = 25)*/**	23.5% (n= 119)
rrf-3	*eat-17; rab-6.1; rab-6.2*	--------	24.2% (n= 223)
rrf-3; eat-17	--------	100% (n = 32)	0% (n= 183)
rrf-3; eat-17	*rab-6.1*	100% (n= 119)	0% (n= 119)
rrf-3; eat-17	*rab-6.2*	--------	100% (n= 80)***
rrf-3; eat-17	*rab-6.1; rab-6.2*	--------	99.1% (n= 116)

*Decreased penetrance likely due to saturation of the RNAi machinery.
**Percentage calculated by examining a subset of adults.
***Failed to reach adulthood within a two-week period.

Materials and Methods

Described in Chapter 3.

CHAPTER 5

Summary

5.1 Summary

My work has focused on the identification of three components most likely used to regulate secretion in *C. elegans*. RAB-6.2 and EAT-17 act in a pathway necessary for proper grinder formation. RAB-6.1 seems to act redundantly with RAB-6.2.

My findings provide an approach to study the Rab6 pathway in a multicellular organism that is genetically tractable and easily manipulated. Performing suppressor and enhancer screens of *eat-17* and *rab-6.2* should identify other components in the pathway, including regulatory proteins such as GEFs and effectors. GFP fusions to track movement of these proteins should also help determine whether they are differentially regulated during the molt. They will also allow us to determine when and where these proteins act in the cell. Obviously, much work needs to be done to understand the Rab6 vesicle trafficking pathway and the mechanics of grinder formation in C. *elegans*.

One curious finding regards the refractory bodies present in the terminal bulbs of *rab-6.2* RNAi and *eat-17* gravid adults. The identity of these is unknown but it is reasonable to suggest one of two things. First these bodies could represent aberrant Golgi structures. Singh and Sulston (1978) mention the presence of similar structures that arise in hypodermal cells during the molt and have shown by EM that these structures represent synthetically active Golgi. Rab6 siRNA and Rab6-GDP overexpression in mammalian cells cause defects in Golgi structures, resulting from a decrease in the rate of retrograde trafficking. Perhaps something similar occurs in *C. elegans*. It is also possible that the refractory bodies represent vesicles carrying cuticle cargos that have aberrantly

fused to one another at the wrong site in the secretory pathway. Determining the identity of these structures will aid in our understanding of this process as a whole.

What exactly is the problem in *eat-17* and *rab-6.2 RNAi* mutants that causes the grinders to be improperly formed? One explanation is that impairing the Rab6 pathway results in the back up of secretory vesicles in the Golgi. As a result, fewer cuticle proteins reach the apical muscle surfaces, and this results in the formation of small grinders. This model suggests Rab6 plays a role in anteriograde transport.

Another explanation is that Rab6 plays a role in a retrograde pathway that routes membranes and Golgi resident proteins back to their correct compartments. If this pathway is compromised, one expected result would be the improper post-translational modification of proteins trafficking through the secretory pathway. It is known that collagen proteins require a number of modifications before they can form triple helical structures and crosslink to one another extracellularly (Myllyharju, 2003; Page and Winter, 2003; Myllyharju and Kivirikko, 2004). Many ER- and Golgi-resident proteins are important for this process. One might expect that improperly modified collagens secreted at the apical membrane would be unable to form structurally sound grinders. My data are consistent with this hypothesis. As more components of the Rab6 pathway are compromised, I see greater and greater disorganization of the grinder plates: *eat-17=rab-6.2 RNAi<eat-17*; *rab-6.2 RNAi< rab-6.1 RNAi*; *rab-6.2 RNAi = eat-17*; *rab-6.1 RNAi*; *rab-6.2 RNAi*.

Several studies have shown that a balance between anteriograde and retrograde signaling is important for maintaining organelle identity and morphology, in addition to proper signaling (Zerial and McBride, 2001). Several groups have shown that inhibiting

Rab6 function using an RNA interference technique or by over-expressing a GDP-bound version of Rab6 in cells causes defects in the integrity of the Golgi, likely due to a decrease in the rate of retrograde trafficking. Preliminary EM studies of *rab-6.2* and *eat-17* worms show large masses of tubular structures throughout the pharyngeal cells. Whether this is a direct consequence to the loss of gene function or is due to an unrelated phenomenon such as starvation is unknown. Further studies looking at the role of these proteins in vesicle transport are sorely needed.

Finally, my hypothesis throughout this study has been that RAB-6.2 and EAT-17 are important for regulating transport of cuticle proteins to the apical surfaces of pharyngeal muscle. My suspicion is that grinder malformation is due to a defect in collagen trafficking. First, staining grinders with calcofluor reveals no qualitative difference in chitin composition between N2 and *eat-17* grinders. (Having been said, this assay is not quantitative and therefore would not detect subtle changes in chitin composition.) Second, a severely compromised Rab6 pathway results in larval lethality, and specifically worms arrest with plugs of cuticle in their mouths. This is consistent with a defect in molting and most likely indicates that something has gone awry either at the step of cuticle deposition or shedding. While lots of evidence suggests that the body cuticle is composed of collagen, there are no data to support the presence of chitin in the *C. elegans* body cuticle. Obviously, more experiments are required to determine this definitively.

As mentioned earlier, there are several phenotypes of *eat-17* mutants, including a defect in food transport and trapping. My hypothesis is that this defect is caused by a defect in the formation of the sieve. To test this, EM studies must be done.

5.2 Agricultural relevance

Plant parasitic nematodes are a leading cause of crop damage in the United States (Baldwin et al., 2004). Methods for dealing with this problem are currently in development. In nematodes such as *C. elegans* and other species, collagens are regulated at different times and stages during larval development. In parasitic nematodes, the collagen composition of certain cuticles is crucial for enabling the worm to infect its host and infiltrate the organism's tissues. Targeting specific collagens for degradation might aid in alleviating the crop damage problem.

5.3 Human relevance

We are lucky in the United States that human parasitic nematode infections are relatively uncommon. In contrast, nematode infections pose a significant problem in many Third World countries. Nematode infections are the leading cause of river blindness, a disease that affects nearly 20 million of people in West, Central, and East Africa, as well as another million in regions of Central and South America. The nematodes *W. bancrofti* and *Brugia mayali* infect nearly 120 million people worldwide. Individuals infected with these parasites develop elephantitis, "a chronic, often extreme enlargement and hardening of the cutaneous and subcutaneous tissue, especially of the legs and scrotum, resulting from lymphatic obstruction." (The American Heritage Dictionary) Developing methods for the treatment of these infections is important for the overall health of individuals around the world.

5.4 Cosmetic applications

An unusual but potentially interesting application would be to genetically engineer skin cells that exhibit increased collagen trafficking. Collagen treatments are used by many to reduce the signs of aging.

5.5 Medical relevance

EAT-17 is a close homolog of Evi5, a putative human oncogene that has been implicated in stage 4S neuroblastoma. So far, little is known about Evi5 molecularly.

Neuroblastoma is a cancer restricted to young children. The outcome of this disease is largely dependent on the age of the child at diagnosis and the stage of tumor progression. Stage 4S neuroblastoma occurs in very young children, generally under the age of one year. At this stage, the tumor has almost always metastasized to the liver and bones.

Stage 4S neuroblastoma is interesting because of its progression. In some children, tumors disappear without any treatment. Other children develop stage 4 neuroblastoma, which is often fatal even with rigorous treatments. By studying EAT-17, the pathways that control the formation and/or progression of these tumors may begin to be elucidated.

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Vita

Sarah Straud Anselmo was born in Elizabeth, Pennsylvania on October 16, 1975 to John and Carol Straud. In 1993, she graduated eighth in her class from Elizabeth Forward High School in Elizabeth, Pennsylvania. Sarah majored in Biological Sciences and minored in Chemistry at Carnegie Mellon University in Pittsburgh, Pennsylvania, and in 1997 she graduated with University and College honors. As an undergraduate, Sarah conducted research under the supervision by Dr. John Woolford, where she studied the mechanisms of ribosome biogenesis in the yeast *Saccharomyces cerevisiae*. In 1998, Sarah entered the graduate program at the University of Texas Southwestern Medical Center, where she studied the genetics of feeding in *C. elegans*. Her work eventually led to the completion of her thesis in 2004. On April 27, 2002, Sarah married Anthony Anselmo of San Antonio, Texas.

Permanent address:

2245 Ridge Rd.

Boston, PA 15135