The Jaw of the Worm: GTPase-activating Protein EAT-17 Regulates Grinder Formation in *Caenorhabditis elegans*

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ABSTRACT Constitutive transport of cellular materials is essential for cell survival. Although multiple small GTPase Rab proteins are required for the process, few regulators of Rabs are known. Here we report that EAT-17, a novel GTPase-activating protein (GAP), regulates RAB-6.2 function in grinder formation in *Caenorhabditis elegans*. We identified EAT-17 as a novel RabGAP that interacts with RAB-6.2, a protein that presumably regulates vesicle trafficking between Golgi, the endoplasmic reticulum, and plasma membrane to form a functional grinder. EAT-17 has a canonical GAP domain that is critical for its function. RNA interference against 25 confirmed and/or predicted RABs in *C. elegans* shows that RNAi against *rab-6.2* produces a phenotype identical to *eat-17*. A directed yeast two-hybrid screen using EAT-17 as bait and each of the 25 RAB proteins as prey identifies RAB-6.2 as the interacting partner of EAT-17, confirming that RAB-6.2 is a specific substrate of EAT-17. Additionally, deletion mutants of *rab-6.2* show grinder defects identical to those of *eat-17* loss-of-function mutants, and both RAB-6.2 and EAT-17 are expressed in the terminal bulb of the pharynx where the grinder is located. Collectively, these results suggest that EAT-17 is a specific GTPase-activating protein for RAB-6.2. Based on the conserved function of Rab6 in vesicular transport, we propose that EAT-17 regulates the turnover rate of RAB-6.2 activity in cargo trafficking for grinder formation.

CELLS constitutively transport newly synthesized proteins, lipids, and other molecules to their periphery through vesicle trafficking. As the process is conserved from yeast to humans, it is essential for the function and survival of cells. Rab GTPases are small GTP binding proteins that regulate the transport of vesicles between different compartments of the cell (Zerial and McBride 2001; Jordens *et al.* 2005; Grosshans *et al.* 2006). Rab6's are localized to Golgi membranes to mark and target both anterograde cargos from Golgi to post-Golgi compartments (such as the plasma membrane) and retrograde cargos from early/recycling endosomes to Golgi and the endoplasmic reticulum (Jasmin *et al.* 1992; Martinez *et al.* 1994, 1997; Girod *et al.* 1999; Opdam *et al.* 2000; Del Nery *et al.* 2006). For fast turnover, Rabs require guanine nucleotide exchange factors (Rab GEFs) for activation and GTPase-activating proteins (Rab GAPs) to turn off activity (Grosshans *et al.* 2006). While the role of Rab6 in membrane trafficking is well established, very few GAPs or GEFs for Rab6 have been identified; thus their physiological importance is largely unknown. The physiological roles of RabGAPs were addressed only recently in flies (Houalla *et al.* 2010; Uytterhoeven *et al.* 2011) and worms (Chotard *et al.* 2010). In those cases, removing the function of the RabGAPs produced phenotypes almost identical to those of removing the relevant Rabs, showing the fundamental roles of RabGAPs in modulating Rab functions. Currently, the only identified GAP for Rab6 is GAPCenA in humans, which is associated with centrosomes and regulates Golgi dynamics in dividing cells (Cuif *et al.* 1999). Nonetheless, misregulation of GAPs for other small G proteins, such as Ras, directly relates to diseases such as cancer, implicating the essential roles of GAPs in controlling the kinetics of G protein activity (Tanabe *et al.* 2006; Durkin *et al.* 2007; Pamonsinlapatham *et al.* 2009).

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The *Caenorhabditis elegans* grinder is a complex structure required for proper grinding of food (bacteria) before it is passed to the intestine. When food is abundant, worms feed at an average rate of 200 pumps per minute (ppm) based on counts of pharyngeal pumping and grinding motions (Avery and You 2012; Raizen *et al.* 2012). This high frequency of grinding throughout the worm's life suggests transport of grinder components to build a functional grinder is essential for worms' rapid growth and normal development. Considering the location of this structure in the *C. elegans* pharynx, grinder components are likely produced by terminal bulb muscles pm6 and pm7 and trafficked to their apical surfaces (Albertson and Thomson 1976). The exact components of the grinder and molecular mechanisms regulating their transport, however, remain unknown.

Here we report that *C. elegans* gene *eat-17* encodes a Rab GTPase-activating protein (Rab GAP) specific for RAB-6.2, a Rab6 homolog in *C. elegans*. *eat-17* loss-of-function and *rab-6.2* deletion mutants show similar phenotypes, namely disorganized, poorly formed grinders. RNAi against *rab-6.2* produces similar defects. A directed yeast two-hybrid screen using EAT-17 as bait identified RAB-6.2 as its interacting partner. Furthermore, transgenes with mutations in the conserved catalytic arginine residue required for the GAP activity of EAT-17 failed to rescue grinder defects when injected into *eat-17* mutants. Based on these results and the conserved function of Rab6 in vesicle transport, we propose that RAB-6.2 and EAT-17 cooperate in the transport of grinder components to the apical surfaces of the terminal bulb muscles to form a functional grinder.

Materials and Methods

Worm culture

Worms were cultured and handled as described (Sulston and Hodgkin 1988) with the following modifications. First, they were routinely grown on NGMSR plates (Avery 1993). NGMSR differs from NGM in containing 200 pg/ml streptomycin sulfate, 10 pg/ml nystatin, and 2% agar instead of 1.7%. (Streptomycin and nystatin reduce bacterial and fungal contamination. The higher agar concentration delays the burrowing of the worms into the agar.) In addition, all worms were maintained at 20° on *Escherichia coli* HB101 unless indicated otherwise. The wild-type strains are Bristol N2 and Hawaiian CB4856. *eat-17(ad707)* and *rab-6.2(ok2254)* were used throughout this study after being outcrossed twice to N2. Other strains used in this study are DA2120 *adEx2120 [eat-17p*::*GFP rol-6(sd)]*, 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)*, DA1983 *eat-17(ad707)*; *adEx1983 [F01G12 T24D11 rol-6(d)]*, DA1982 *rrf-3(pk1426)*; *eat-17 (ad707)*, DA2035 *adEx2035[rab-6.2p*::*GFP rol-6(d)]*, DA2033 *eat-5(ad1402)*; *adEx2033[RAB-6.2*::*GFP unc-122*::*RFP eat-5 (+)]*, YJ89 *rab-6.2(ok2254)*, MT993 *lin-10(e1439)*; *him-5 (e1467ts)*.

SNP mapping

CB4856 males were crossed to DA773 hermaphrodites. *unc-93*; *lin-15 eat-17 sup-10*/CB4856(+) worms were isolated in the F_2 generation 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 F_3 generation. These were scored for the *eat-17* feeding defect, and their progeny were analyzed by PCR and subsequent restriction digestion for SNPs located between *lin-15* and *sup-10*. SNPs examined and primers used for their amplification are shown in Supporting Information, Table S1.

Deletion mutants of *ser-1(ok345)*, *hda-4(ok518)*, and *T27A8.2(ok570)* were mapped by scoring F_4 progeny for deletion detected by PCR. Primers used for detecting the deletions are shown in Table S2.

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

To determine the intron and exon structure of *eat-17*, primers were designed based on Genefinder predictions shown in WormBase to amplify partial and full-length transcripts by RT–PCR. First-strand poly(A)$^+$ cDNA derived from mixed stage N2 hermaphrodites was used as template in all reactions. Trizol (Invitrogen) was used to isolate RNA and the First-Strand cDNA Synthesis kit (Roche) was used to generate cDNA. Primers used to amplify PCR fragments are shown in Table S3. 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, full-length *eat-17* cDNAs were cloned into the pGEM-T Easy vector (Promega) and sequenced.

SL1 trans-splicing

To identify the first exon of *eat-17*, a forward primer recognizing the 22 nucleotide-SL1-splice leader sequence (5′-ggtttaattacccaagtttgag -3′) and two nested reverse primers recognizing sequences in exon 2 [1050: 5′-tgttcagctgctc catcttg-3′(outside primer)/1051: 5′-cgacttcatttacgcatactg-3′ (inside primer)] were used. Products were purified (Qiaquick Gel Extraction kit), cloned into pGEM-T Easy vectors and sequenced. The resulting sequences were BLASTed (blastn) against *C. elegans* ESTs and genomic DNA using WormBase.

Cosmid rescue

Cosmids used in this study were obtained from Alan Coulson (Wellcome Trust, UK). T24D11 and F01G12 were isolated using the Qiagen Plasmid Mini kit and then co-injected into *eat-17* mutants (50 ng/μl). Plasmids pPD118.20 *myo-3*:: GFP (from Andrew Fire, Stanford University) and pRAK3 *rol-6(d)* were used as co-injection markers at a concentration of 10 ng/μl. Worms were fed *Comamonas* before injection to allow good growth (Avery and Shtonda 2003). Transgenic worms were then fed DA837 to enhance the feeding defect.

Growth rates of transgenic and nontransgenic progeny were compared to assess rescue. Eggs from transgenic mothers

were placed individually onto plates seeded with DA837 and then checked every 12 hr 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.

Generating the eat-17 rescue construct

Rescuing fragments containing the full-length *eat-17* cDNA 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: 5′-*ttgtcaccgccg*atggcagccactg cagcgctac–3′ and 5′-*tagggatgttgaagagtaattgga*cctagtggctatcc gacagtt–3′. The resulting PCR fragments were purified and subjected to a second round of PCR using the overlap extension method to fuse the *eat-17* promoter and *unc-54* 3′-UTR to each end. The primers used to amplify the promoter region are: 5′-taggttacggtagttggtacg–3′ and 5′-gtagcgctgcagtggctgc *catcggcggtgacaa*–3′. The primers used to amplify 500 bp *unc-54* 3′ UTR are 5′-aactgtcggatagccactaggtccaattactcttcaacatcccta–3′ and 5′-tttggtatattgggaatgtattctg–3′. The resulting PCR products were purified and injected into *eat-17* worms (25 ng/μl). The final injected product contained 5.6 kb of sequence located directly upstream of the *eat-17* transcriptional start site. A 2.5-kb full-length *eat-17* cDNA construct was fused to this "promoter region" and the heterologous *unc-54* 3′-UTR was added to promote stability. This construct lacked intron elements, including the large 4-kb first intron. *let-858*::GFP plasmid DNA (18 ng/μl) was used as a co-injection marker.

Two transgenic lines were isolated and examined. F_2 transgenic animals were identified using the SZX12GFP dissecting scope (Olympus). Because many transgenic embryos die, only animals that reached adulthood were scored for the grinder phenotype. For both transgenic lines, 100% of the adult transgenic animals were rescued for defects in grinder formation. Grinder morphology was observed using a Zeiss Axio A2 Imager at either ×630 or ×1000 magnification. Images were acquired using Zeiss Axiovision software.

Assay for EAT-17 GAP activity

To determine the functional importance of the GAP activity of EAT-17, rescuing fragments containing the catalytically inactive R116/119K and R116/119A mutations were generated using the overlap extension method as describe above. Primers used to generate the R116/119K mutation are shown in Table S4. 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. To score for rescue of grinder defects, the F_1 gravid adult progeny of injected mothers were picked to 4% agar pads containing 10 mM sodium azide (Sigma, St. Louis). Defects in grinder formation were recorded and then the presence or absence of GFP expression was determined. Worms were observed using a Zeiss Axio A2 Imager with a ×100 objective lens. Images were acquired using Zeiss Axiovision software.

RNAi

RNAi was performed as described with minor modifications (Kamath and Ahringer 2003). cDNA sequences of *eat-17* and the 25 *C. elegans* Rabs were amplified by PCR using primers shown in Table S5. The HiScribe RNAi Transcription kit (New England Biolabs) was used to generate dsRNAs.

GFP fusions of EAT-17 and RAB-6.2

eat-17p::GFP: A 5.6-kb *eat-17* promoter was amplified from N2 genomic DNA using the following primers: 5′-taggt tacggtagttggtacg-3′ and 5′-gaaaagttcttctcctttactcatcggcggt gacaattgg-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, Stanford University) using primers: 5′-ccaattgtcaccgccgatgagtaaagga gaagaacttttc-3′ and 5′-tttggtatattgggaatggtattctg-3′. DNAs were fused together by overlap extension PCR using the following nested primers: 5′-acggtagtgttttatcagtagtg-3′ and 5′-caaacc caaaccttcttccgatc-3′.

The resulting product was purified and injected into N2 adults with pRAK3 *rol-6(d)* as a co-injection marker. The established transgenic lines were observed using a Zeiss Axio A2 Imager at either ×630 or ×1000 magnification. Images were acquired using Zeiss Axiovision software.

rab-6.2p::GFP: A 2.8-kb *rab-6.2* promoter fragment was amplified from N2 genomic DNA using the following primers: 5′-aatcgcacagcaggcctcc-3′ and 5′-gtgaaaagttcttctcctt tactcggattaccaaagtccgacat-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 pPD95.75 using the following primers: 5′-atgtcggactttggtaatccgagtaaaggagaa gaacttttcac-3′ and 5′-tttggtatattgggaatggtattctg-3′. Products were fused by overlap extension PCR using the following primers: 5′-tttgccgaacggaagagcc-3′ and 5′-caaacccaaaccttcttcc gatc-3′. The final product was purified and injected into N2 adults with *rol-6(d)* as a co-injection marker. GFP expression was observed as described above.

Yeast two-hybrid assay

To generate first-strand cDNA, RNA was isolated from a well-fed, mixed stage N2 population using Trizol. Poly(A)$^+$ first-strand cDNA was prepared using the First-Strand cDNA Synthesis kit for RT–PCR (Roche).

Inserts used for preys were amplified by PCR using the Expand Long Template PCR kit (Roche). Primers to amplify the preys are shown in Table S6. PCR products were cloned into the pGEM-T Easy vector and sequenced. Constructs with wild-type sequence were subcloned into the pACT2 vector (gift from R. Lin, University of Texas Southwestern Medical Center at Dallas), in frame with the GAL4 activation domain.

Six truncated versions of *eat-17* were used as baits after amplification, sequencing and cloning into the pVJL11 vector

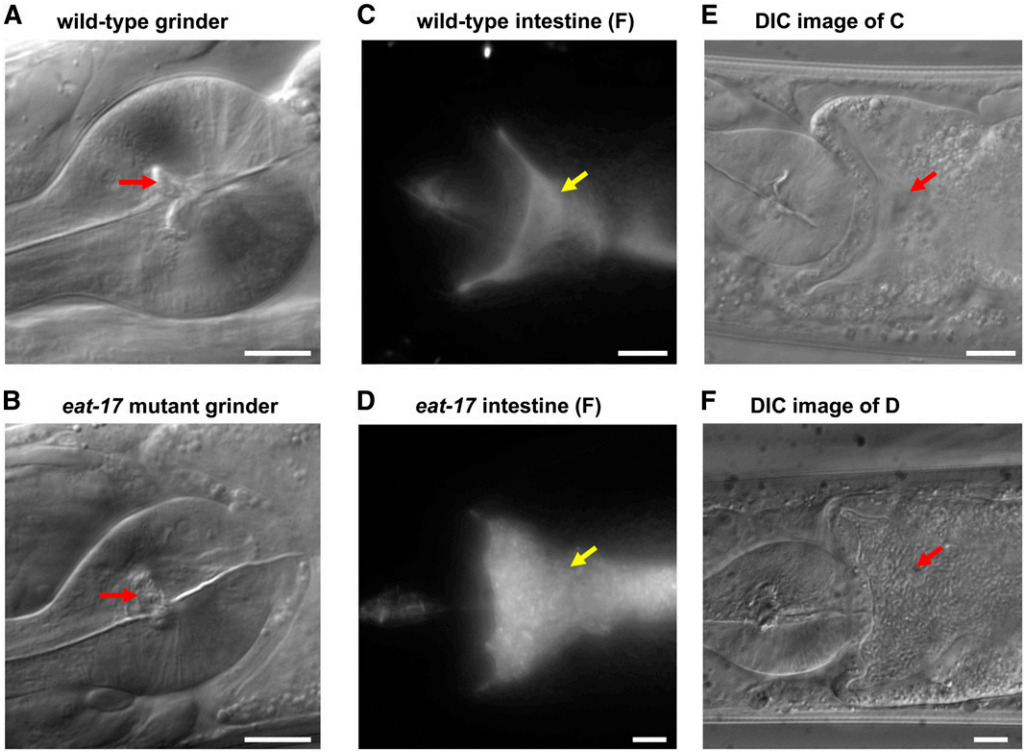

Figure 1 *eat-17* mutants show defects in grinder formation. (A) DIC image of wild-type pharynx. The *C. elegans* grinder is composed of three plates arranged in approximate triradial symmetry. Two fully formed plates are visible in lateral sections (arrow). (B) DIC image of *eat-17(ad707)* mutant pharynx. Most of the material comprising the grinder plates is missing in *eat-17* mutants. (C) The intestine of wild-type worms fed mCherry-expressing *E. coli* shows smooth fluorescence due to fluorescence released from ground bacteria. (D) The intestine of *eat-17* mutants show many unground bacteria indicated by an arrow. (E and F) Corresponding DIC images of wild type (E) and *eat-17* mutant (F), respectively. In F, the arrow indicates DIC image of unground bacteria. For all images, anterior is shown to the left. Bars, 10 μm.

(gift from M. Cobb, University of Texas Southwestern Medical Center at Dallas), in frame with the LexA DNA binding domain. Primers are shown in Table S7. Mutant versions of the baits were generated using an *in vitro* mutagenesis kit (Invitrogen). Primers containing mutations are shown in Table S4.

To test for interactions, combinations of baits and preys were transformed into the yeast strain L40 (Hama *et al.* 1999). X-gal filter assays and quantitative ortho-nitrophenyl-β-D-galactopyranoside (ONPG) assays were performed as described (Yeast Protocols Handbook, Clontech). An average of two colonies was tested for each. For growth assays, colonies were streaked onto plates lacking histidine and supplemented with 5 mM 3-amino-1,2,4-triazole (3-AT). Miller units are calculated from $OD_{420}/t \times V \times OD_{600}$.

Chitin staining

Chitin was stained as described (Ruvkun and Finney 2005). Fluorescein-conjugated chitin-binding probe (P5211S, NEB) was used at a 1:100 dilution.

Grinder function assay

Worms were fed mCherry-expressing HB101 as described (You *et al.* 2008). Images were acquired using Zeiss Axiovision software.

Results

eat-17 mutants are Eat due to defects in grinder formation

eat-17 mutants were isolated from a genetic screen for defects in feeding behavior. They are defective in trapping bacteria and have slightly asynchronous terminal bulb contractions during feeding (Avery 1993). The most striking phenotype, however, is a defect in their grinders; wild-type grinders consist of highly refractile cuticle ridges that can be observed by DIC optics (Figure 1A, arrow). These ridges are completely disorganized in *eat-17* mutants (Figure 1B). In wild-type worms, the grinder is composed of three separate plates that are arranged in approximate triradial symmetry (Albertson and Thomson 1976; von Lieven 2003). These plates are secreted from the apical surfaces of pharyngeal muscle cells pm6 and pm7 in the terminal bulb (Albertson and Thomson 1976). During feeding, the plates rotate against one another, grinding any material that comes between them. Due to defects in the grinder, *eat-17* mutants cannot grind bacteria efficiently. As a result, unground bacteria pass into the intestine (Figure 1, C–F) contributing to the Eat phenotype.

eat-17 encodes a GTPase activating protein with coiled-coil domains at the C terminus

Previous work placed *eat-17* on chromosome X, between *lin-15* and *sup-10* (Avery 1993). We further mapped *eat-17* to a 47-kb region between two SNP markers, *pkP6096* and *snp-F38E9.2* (Figure 2A), using a multipoint SNP mapping strategy (Wicks *et al.* 2001). Three genes were predicted in this interval (Figure 2B): F01G12.1 encodes a putative copper transporter, T24D11.1 (currently designated as *tbc-4*) is predicted to encode a Rab GAP containing a TBC (Tre2/Bub/Cdc16 homology) domain—a canonical GAP domain—and F01G12.6 is predicted to encode a protein with several coiled-coil domains. Interestingly, both T24D11.1 and F01G12.6

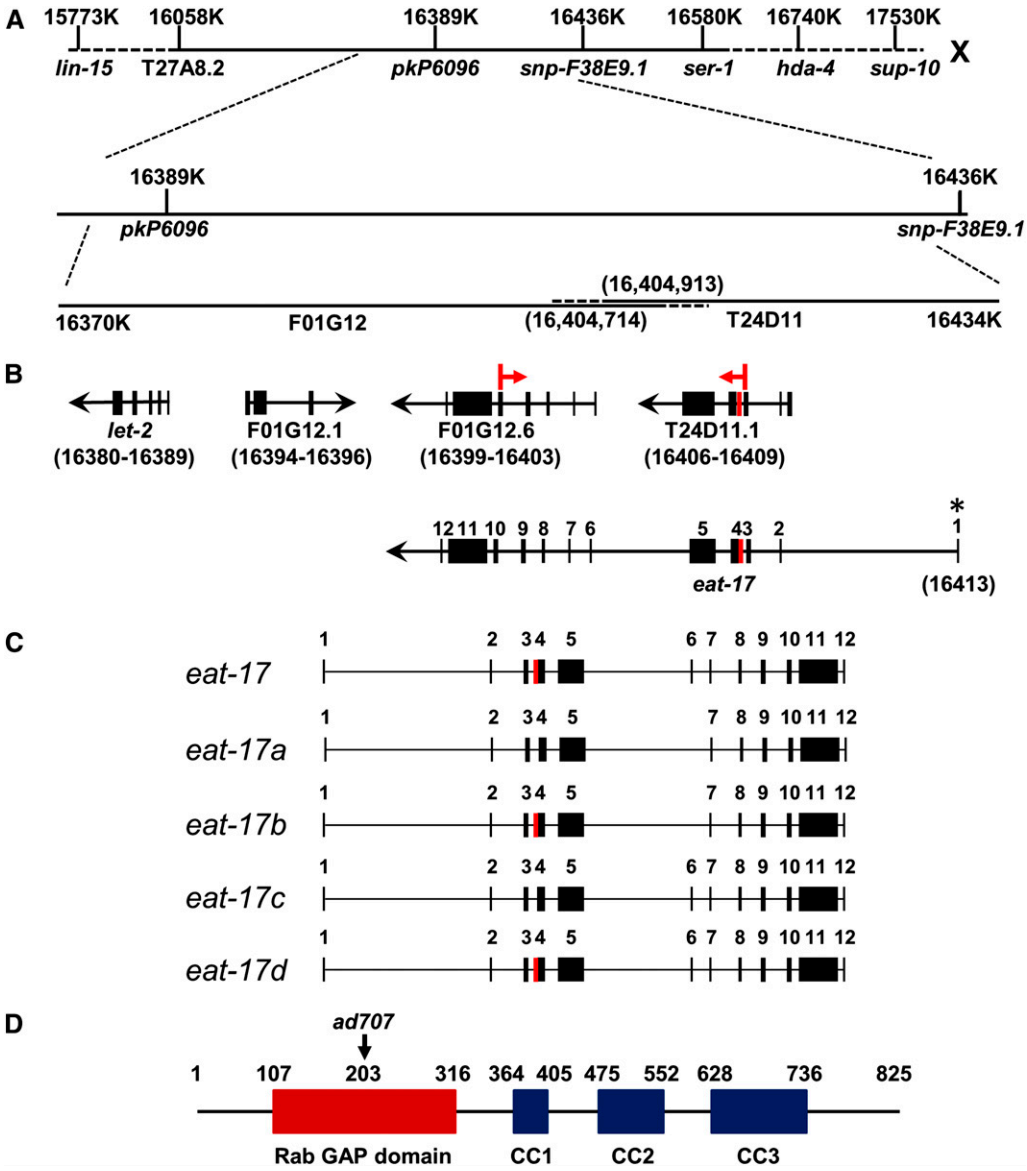

Figure 2 *eat-17* gene structure. (A) Region in *C. elegans* genome where *eat-17* maps. *eat-17* was mapped to 47 kb between two SNP markers *pkP6096* and *snp-F38E9.2* using a multipoint SNP mapping strategy. (B) Genes predicted to be located in the interval and the gene structure of *eat-17*. Red arrows indicate the exons targeted to amplify and validate *eat-17* transcripts. (C) Exon usages of four possible splice forms deduced from SL1 and RT–PCR analysis. The red box indicates exon 4B. The two different splice forms *eat-17a* and *eat-17b* encode two proteins differing by three amino acids. (D) Predicted protein structure of EAT-17 and the location of the mutation in the *ad707* allele. An N-terminal Rab GAP domain is followed by three C-terminal predicted coiled-coil domains. The arrow indicates the position of the mutated amino acid in *ad707*.

share homology with the human protein Evi5 (Figure S1), a Rab11 GAP, whose mutation is associated with neuroblastoma (Liao *et al.* 1997; Dabbeekeh *et al.* 2007). Because T24D11.1 and F01G12.6 are transcribed in the same direction (Figure 2B) and because we were unable to retrieve cDNAs coding individual genes through RT–PCR, we hypothesized that these two predicted genes comprised a single gene. RT–PCR using primers within the third predicted exon of T24D11.1 and the fifth predicted exon of F01G12.6 identified a single transcript (indicated with arrows in Figure 2B), confirming T24D11.1 and F01G12.6 are parts of the same gene. Furthermore, by injecting two cosmids, T24D11 and F01G12, which completely cover this region, we rescued the growth, feeding, and grinder defects of *eat-17* mutants. Injecting either alone did not rescue the phenotype (data not shown).

To determine the gene structure of *eat-17*, we first tried to isolate full-length transcripts with the intron/exon structure predicted in WormBase (www.wormbase.org) but failed. Next we identified individual intron/exon boundaries and found that the first exon was incorrectly predicted. To identify the 5′ end of the transcript, we used SL1 *trans*-splicing analysis. In *C. elegans*, ~60% of all transcripts are either SL1 or SL2 *trans*-spliced at their 5′ ends (Blumenthal 1995; Conrad *et al.* 1995). The results show that the first exon of *eat-17* is located ~4 kb upstream of exon 2 (indicated with * in Figure 2B). We also learned that this transcript contains ~40 bp of 5′-UTR. Using a primer targeting the newly identified first exon and one targeting the last predicted exon of *eat-17*, we were able to amplify a single 2.5-kb fragment. To confirm that we had isolated the full-length transcript, we generated a rescuing transgene in which this 2.5-kb sequence was attached to an ~5.6-kb region of sequence upstream of the predicted *eat-17* transcriptional start site (*eat-17* promoter) at its 5′ end and to a 500-bp *unc-54* 3′-UTR. When this construct was injected into *eat-17* mutants, we found

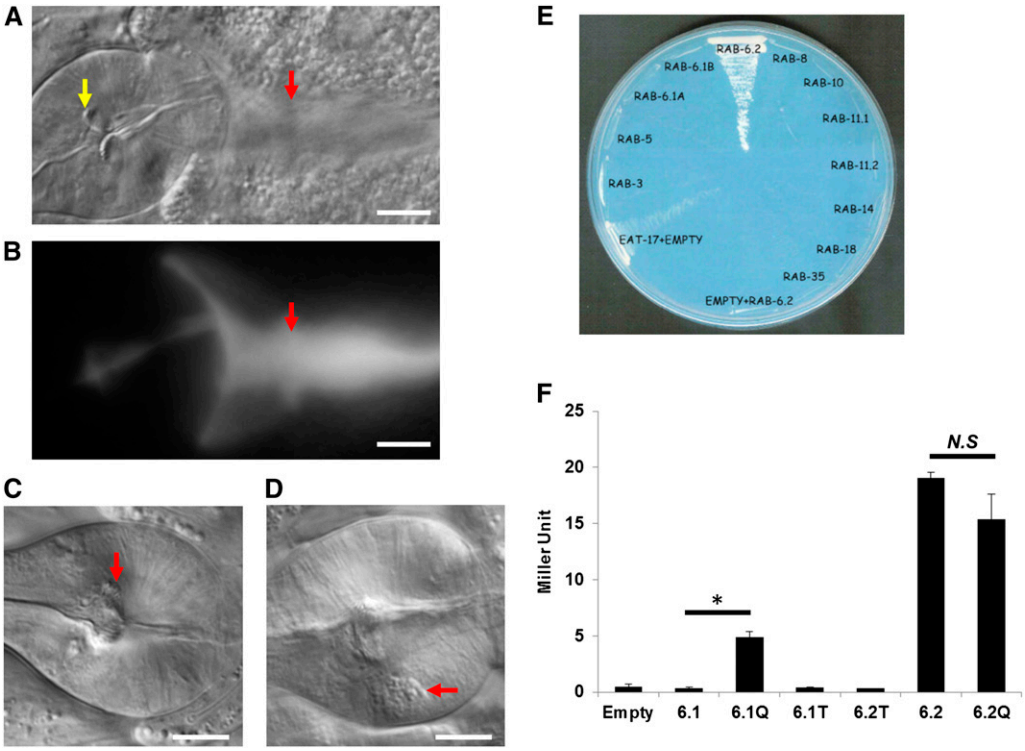

Figure 3 EAT-17 interacts with RAB-6.2. (A) *eat-17* rescue. Expressing a wild-type *eat-17* cDNA construct in mutant worms rescues defects in grinder formation. Grinder plates are of normal size (yellow arrow) and there are no unground bacteria in the intestine (red arrow). (B) Fluorescence image of A. Smooth fluorescence in the intestine indicates the bacteria were ground (red arrow). (C) *rab-6.2(ok2254)* mutants show a grinder defect. Grinder is formed but the size is small. (D) *eat-17(ad707)* grown at 15° containslarge refractile body in the terminal bulb (arrow). (E) Yeast two-hybrid screen using EAT-17 as bait identified RAB-6.2 as the only interacting Rab protein. (F) A constitutively GTP bound form of RAB-6.1 enhances binding of EAT-17 to RAB-6.1. On the other hand, a constitutively GDP bound form of RAB-6.2 (6.2T) abolishes binding of EAT-17 to RAB-6.2. The EAT-17 construct used was a truncated protein (amino acids 1–460) containing only first coiled coil domain. The truncated protein binds to RAB-6.2 very efficiently, suggesting the first coiled-coil domain is sufficient for the binding. Abbreviations are as follows: 6.1, RAB-6.1 full-length wild type 6.1Q, RAB-6.1 with Q70L mutation; 6.1T, RAB-6.1 with T27N mutation; 6.2T, RAB-6.2 with T27N mutation; 6.2, RAB-6.2 full-length wild type 6.2Q, RAB-6.2 with Q69L mutation. $*P < 0.05$; NS, not statistically significant by Student *t*-test. Miller units calculated as $OD_{420}/t \times V \times OD_{600}$. Bars, 10 μm.

that 100% of surviving F_2 transgenic progeny were rescued for defects in their grinders (Figure 3A).

To determine the exact splicing pattern(s) of *eat-17*, we cloned the amplified PCR fragments into vectors and sequenced the inserts (see *Materials and Methods*). We named the possible splice products EAT-17 a, b, c, and d (Figure 2C). EAT-17a has intron/exon boundaries consistent with WormBase/GeneFinder predictions. EAT-17b uses an alternative splice acceptor site at the exon 3/4 boundary (exon 4A, labeled in red, Figure 2, B and C). For both of these, predicted exon 5 of T24D11.1 was truncated by 180 nucleotides and spliced directly to predicted exon 2 of F01G12.6 in WormBase. Neither splice form contained the predicted first exon of F01G12.6. The proteins encoded by EAT-17a and EAT-17b differ by only 3 amino acids. When we designed a set of primers to amplify sequences between exon 1 and exon 6 (corresponding to the predicted first exon of F01G12.6), we could isolate transcripts that included exon 6. We named the predicted transcripts that include exon 6 EAT-17c and EAT-17d (Figure 2C). GeneFinder predictions suggested a splicing pattern that would create a stop codon at the junction between exon 5 and exon 6. This transcript would encode a truncated protein lacking most of the C terminus. It seems most likely that the a and b splice forms encoding full-length proteins are the functional ones.

Analysis of the predicted protein sequence revealed two conserved domains in EAT-17: a Rab GTPase activating (Rab GAP) domain and three coiled-coil domains (CC1–3, Figure 2D). The Rab GAP domain implicates EAT-17 in vesicle trafficking, a role consistent with the grinder defects in *eat-17* mutants. To identify the specific mutation in *eat-17(ad707)*, we sequenced the predicted *eat-17* coding region (based on our RT–PCR results) and found a C-to-T base-pair transition at nucleotide 607 in exon 5 of the *ad707* mutant allele, creating a stop codon at amino acid 203 (Figure 2D). This mutation would generate a protein with a truncated Rab-GAP domain, likely rendering it nonfunctional.

To determine whether EAT-17 contains GAP activity, we mutated the catalytic arginine residue (R116) to either lysine (K) or alanine (A) to abolish the GAP activity and expressed these mutated constructs in *eat-17* mutants (see *Materials and Methods*). A total of 21% of F_1's carrying a wild-type copy of *eat-17* were rescued for defects in grinder formation (Figure 3, A and B), while only 4.1 and 1.6% of mutants expressing either the R116K or R116A mutations were rescued (Table 1). Collectively, these data show that the catalytic activity of EAT-17 is critical for its function and that EAT-17 is a GAP protein.

EAT-17 and RAB-6.2 interact to regulate grinder formation

To identify the substrates of the GAP activity of EAT-17, we performed RNA interference against 25 of the 29 predicted Rab genes in the *C. elegans* genome (Audhya *et al.* 2007)

Table 1 GAP activity is required for full EAT-17 function

	No. rescued	Total no. examined	% rescue
Wild-type *eat-17* construct	16	76	21
R116K	2	49	4.1
R116A	1	64	1.6

eat-17 mutants were injected with DNA constructs encoding either wild-type or one of two mutant versions (R116K or R116A) of the EAT-17 protein (see *Materials and Methods*). F_1 transgenic progeny were blindly assayed for rescue of grinder defects. GFP was used to identify transgenic progeny afterward.

and looked for a grinder phenotype similar to that of *eat-17* mutants. RNAi was performed in worms mutant for *rrf-3*, a hypersensitive background for RNAi (Simmer *et al.* 2002). Among the 25 Rab genes tested, RNAi against *rab-6.2* alone produced a phenotype similar to *eat-17* (Table 2), whereas *rrf-3* mutants show no obvious defects in grinder formation (data not shown). When we examined *rab-6.2* deletion mutants, the defects in the size and organization of the grinder plates were identical to those of *eat-17* mutants (Figures 1B and 3C), confirming our RNAi results.

To examine whether EAT-17 and RAB-6.2 directly bind to each other and whether the binding is specific, we performed directed yeast two-hybrid assays with EAT-17 and each of the 25 RAB proteins. Among all the RABs, only RAB-6.2 interacted with EAT-17 (Figure 3E, data not shown). To examine if the guanylate nucleotide binding status of RAB-6.2 is critical, we introduce two types of mutations into RAB-6.2: (1) glutamine (Q69) to leucine (L) to produce a constitutively GTP bound form and (2) threonine (T27) to asparagine (N) to produce a constitutively GDP bound form (Martinez *et al.* 1994, 1997). The interaction between EAT-17 and RAB-6.2 was completely abolished when the T27N mutation was introduced (Figure 3E, 6.2T), but it was not further enhanced with the Q69L mutation (Figure 3C, 6.2Q), as compared to wild-type RAB-6.2 (Figure 3F, 6.2). This suggests that the reaction reached maximum binding with wild-type RAB-6.2 and that we could not enhance binding any further under the conditions in which we performed the assay.

RAB-6.1, the only other Rab6 in the *C. elegans* genome, shares 81% amino acid sequence identity with RAB-6.2. Because of the high similarity between the two Rab6's, we tested whether EAT-17 could bind to RAB-6.1 as well. Although we did not detect any binding between wild-type RAB-6.1 and EAT-17 in our assay, RAB-6.1(Q70L) (equivalent to Q69L in RAB-6.2) showed enhanced binding to EAT-17, suggesting that EAT-17 binds to RAB-6's through the conserved guanylate nucleotide binding site. Taken together, these data strongly support the hypothesis that RAB-6.2 is a specific substrate of EAT-17 and that EAT-17 promotes hydrolysis of GTP by binding the active (GTP bound) form of RAB-6.

Our studies identified EAT-17 as a GAP protein with three coiled-coil domains. Coiled-coil domains are generally involved in protein–protein interactions, and GAPCenA, a mammalian RabGAP, interacts with Rab6 through a coiled-coil domain (Cuif *et al.* 1999). When we tested several truncated EAT-17 constructs, EAT-17 (aa 1–316), EAT-17 (aa 1–364), and EAT-17 (aa 1–405), they did not interact with RAB-6.2, showing that the GAP domain alone is not sufficient for this interaction (data not shown). A construct of EAT-17 containing the first coiled-coil domain [EAT-17 (aa 1–460)], however, shows significant interaction with RAB-6.2 (Figure 3E). Collectively, our results show that the GAP domain of EAT-17 mediates its catalytic activity and the first coiled-coil domain mediates its interaction with RAB-6.2. Presumably, the coiled-coil domains are important to provide EAT-17 with specificity toward RAB-6.2. Altogether, these data suggest a conserved structural mechanism of interaction between Rab6's and their Rab GAP proteins through the coiled-coil domain (Cuif *et al.* 1999).

Table 2 RNAi of *rab-6.2* phenocopies *eat-17* mutants

dsRNA injected	Phenotype
None	Wild type
rab-1	Embryonic lethal
rab-2	Slight growth delay
rab-3	Wild type
rab-5	Embryonic lethal
rab-6.1	Wild type
rab-6.2	Growth delay with abnormal grinder
rab-7	Wild type
rab-8	Loopy movement, exaggerated body bends
rab-10	ND[a]
rab-11.1	Embryonic lethal
rab-11.2	Arrested as L1s
rab-14	Some show growth delay, Dpy[b]
rab-18	Growth delay, infrequent pumping but normal grinder
rab-19	Wild type
rab-21	Sick, starved, and strong Dpy
rab-27	Wild type
rab-28	Wild type
rab-30	Wild type
rab-33	Slightly Unc[c], otherwise wild type
rab-35	Wild type
rab-37	Loopy movement, otherwise wild type
rab-39	Wild type
4R79.2	Loopy movement, Unc, otherwise wild type
K02E10.1	ND
F11A5.4	Wild type
F11A5.3	Wild type
C56E6.2	Loopy movement, Unc

Among 25 Rabs tested, RNAi of *rab-6.2* shows an identical grinder phenotype to *eat-17*. Full-length dsRNAs for each Rab listed were injected into *rrf-3* RNAi hypersensitive mutants. Worms were grown at 15° to enhance the phenotype.
[a] ND, Not determined.
[b] Dpy, dumpy phenotype, worms are shorter than wild type.
[c] Unc, uncoordinated movement phenotype, worms move abnormally.

EAT-17 and RAB-6.2 are co-expressed in terminal bulb muscle, the site of grinder secretion

To determine whether EAT-17 and RAB-6.2 function together in the terminal bulb, we examined the expression patterns of transgenic lines that carry the promoters of each

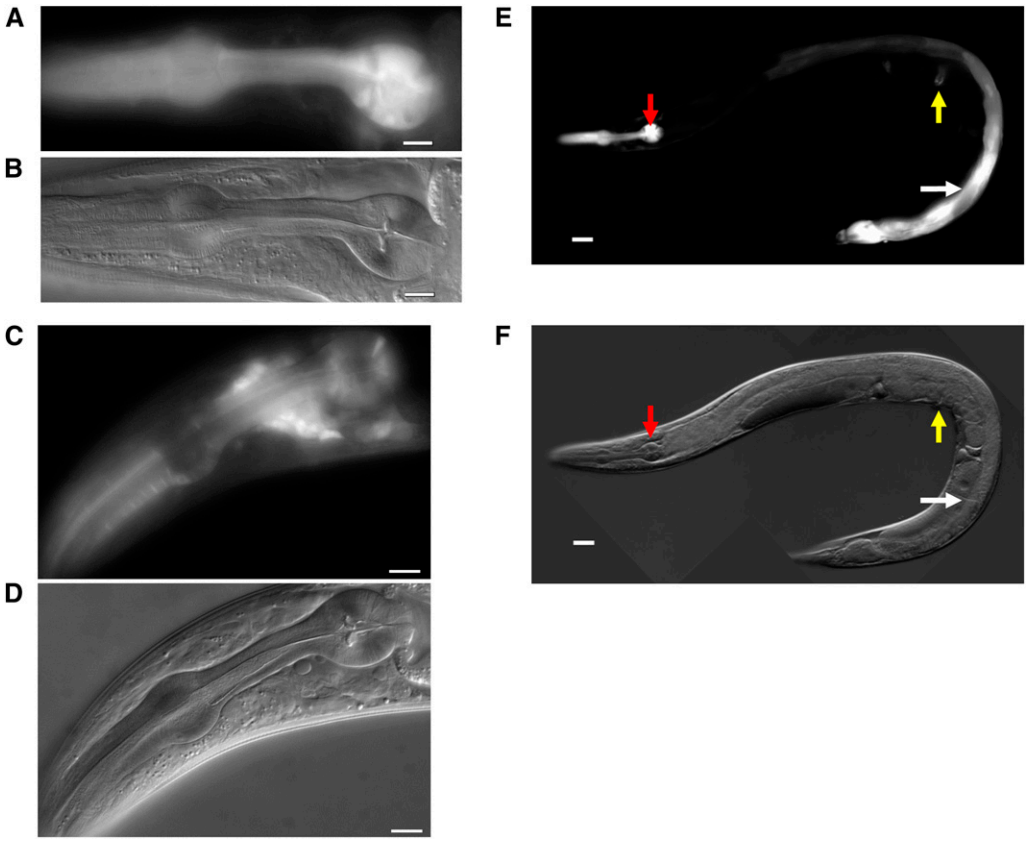

Figure 4 EAT-17 is expressed in pharyngeal muscle, intestine, and vulva. (A and B) Corresponding GFP and DIC images of EAT-17::GFP. (C and D) Corresponding GFP and DIC images of RAB-6.2::GFP. Both EAT-17 and RAB-6.2 are highly expressed in the pharyngeal muscle. RAB-6-2 is also highly expressed in neurons. (E and F) Corresponding GFP and DIC images of EAT-17::GFP in the pharynx (red arrows), intestine (white arrows), and vulva (yellow arrows). For Figure 4, A–D, bars, 10 μm. For Figure 4, E and F, bars, 30 μm.

gene fused with GFP (see *Materials and Methods*). Both EAT-17 and RAB-6.2 are strongly expressed in the terminal bulb muscles that secrete the components of the grinder (Figure 4, A–D, see *Materials and Methods*). Our construct also shows EAT-17 expression outside of the pharynx, such as in intestine and vulva (Figure 4, E and F).

This tissue expression pattern of EAT-17 overlaps largely with that previously reported for RAB-6.2 (Zhang *et al.* 2012), supporting the inference that EAT-17 is a specific GAP to regulate RAB-6.2 activity in those tissues.

Chitin deposition is superficially intact in eat-17 and rab-6.2 mutants

Chitin provides mechanical support to exoskeletons in many invertebrate animals. In yeast, proper chitin deposition is essential and strictly regulated by controlling the trafficking of its synthase, chs3p (Schorr *et al.* 2001). Because chitin is one of the components of the grinder and its synthase is regulated by trafficking, we stained the grinders of *eat-17* and *rab-6.2* mutants for chitin to examine if chitin deposition is misregulated in these mutants. Despite the global disorganization of the grinder, the overall chitin deposition was indistinguishable among *eat-17*, *rab-6.2*, and wild-type (Figure 5, A–F). However, when grown at a low temperature, *eat-17* mutants occasionally have large refractory bodies in the terminal bulbs that secrete the components of the grinder (Figure 3D). Interestingly, at least in appearance, those bodies share similar refractile characteristics with that of grinder. These results may suggest that the materials trafficked by RAB-6.2 from terminal bulb could be the components of the grinder and that the refractile bodies in *eat-17* could be these components, accumulating in the muscle because of the trafficking defect.

Discussion

In this study we mapped and characterized *eat-17*, a new GTPase-activating protein gene in *C. elegans*. Using two different approaches, RNA interference and yeast two hybrid, we showed that EAT-17 interacts with RAB-6.2, a conserved small G protein regulating membrane trafficking from Golgi (Del Nery *et al.* 2006; Girod *et al.* 1999). The fact that both approaches identify only RAB-6.2 as a substrate of EAT-17 among 25 tested Rabs strongly suggests EAT-17 is a specific GAP for RAB-6.2. Both *eat-17* and *rab-6.2* mutants show identical defects in their grinders, the part of the feeding organ that crushes food, and which, therefore, is essential for rapid growth and normal development. A new grinder replaces the old one during every molt, implying that the grinder components needed to be transported during every molt to build a new one. Our observations that a mutation in *rab-6.2*, which functions in cargo trafficking, and a mutation in its GAP protein EAT-17 show their most striking phenotypes in the grinder, suggest that precise regulation of trafficking

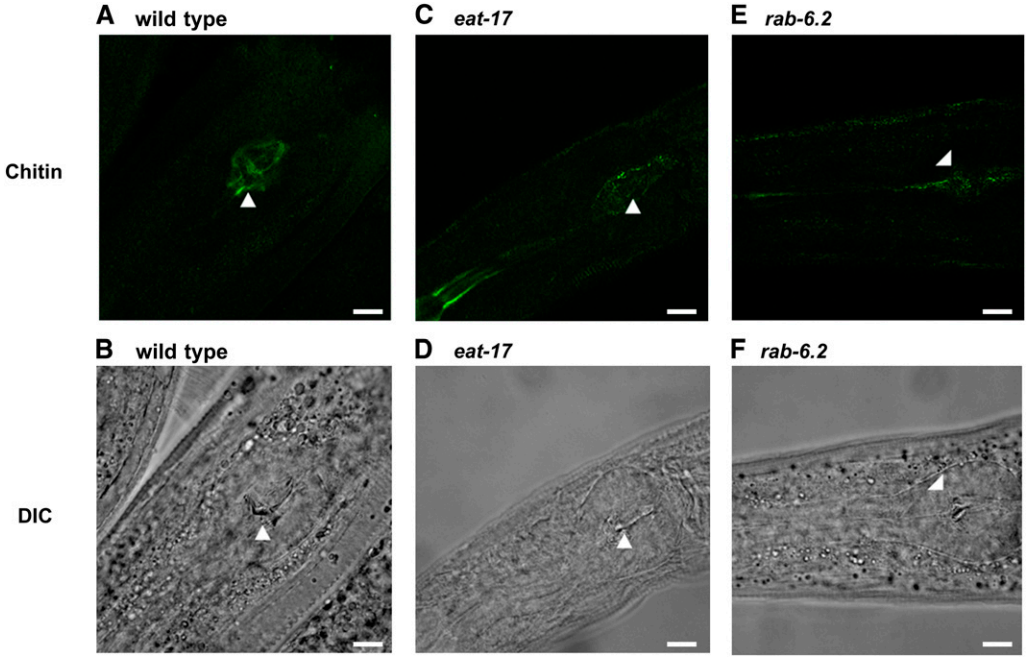

Figure 5 Chitin deposition in the grinders of *eat-17* and *rab-6.2* mutants is normal. (A and B) Chitin staining of grinder from wild type and corresponding DIC image. (C and D) From *eat-17* mutants and corresponding DIC image. (E and F) From *rab-6.2* mutants and corresponding DIC image. Arrowheads indicate the position of the grinder. Bars, 10 μm.

of grinder components is critical to build a functional grinder.

Although we do not know the identities of the cargos in RAB-6.2 targeted vesicles, we found that *eat-17* mutants grown at 15° have refractory bodies in the terminal bulbs. The bodies appear similar (*i.e.*, highly refractile) to the grinder. This suggests that those bodies may be grinder components accumulating in the muscle because of slow trafficking at low temperature. In fact, Chotard *et al.* (2010) showed that the mutant of *tbc-2*, which encodes another RabGAP in *C. elegans*, accumulates enormous RAB-7-positive late endosome in the intestine containing refractile materials. These results strongly suggest that the refractile bodies can be a common phenotype in endosomal trafficking mutants and that EAT-17 regulates endosomal trafficking.

Small G proteins are substrates of GAPs for their fast turnover. The importance of GAPs is demonstrated in many examples such as the oncogene Evi5, which is homologous to EAT-17 (Liao *et al.* 1997; Dabbeekeh *et al.* 2007). Only one other GAP for Rab6 has been identified so far, GAPCenA, which also interacts with Rab6 through a coiled-coil domain. The requirement of the coiled-coil domain for interaction appears to be unique for the Rab6 GAPs. Studies have shown that a class of Rab6 effector, Golgins, interacts with Rab6's through their coiled-coil domains to direct vesicle capture and sorting within the Golgi. It is possible that Rab6's bind to their GAPs and effectors through a similar mechanism mediated by coiled-coil domains (Sinka *et al.* 2008).

Our discovery of EAT-17 and its specificity toward RAB-6.2 strongly suggest that there are specific GAPs yet to be discovered for RAB-6.1, the only other Rab6 in *C. elegans*, sharing 81% amino acid sequence identity with RAB-6.2. *rab-6.1* and *rab-6.2* are partially redundant; *rab-6.1(RNAi)* worms are superficially normal, and *rab-6.2(RNAi)* worms have defects only in the grinder. Knockout of both genes, however, produces gross defects in molting and growth. The fact that EAT-17 does not interact with RAB-6.1 to regulate grinder formation and the similarity of the *eat-17* phenotype to that of the *rab-6.2* single mutant or *rab-6.2(RNAi)* is consistent with the proposed EAT-17 specificity toward RAB-6.2.

Recently Zhang *et al.* (2012) identified LIN-10 as an RAB-6.2 effector to regulate glutamate receptor recycling in *C. elegans* neurons. However, we did not see any grinder defect in *lin-10* mutants (data not shown). In addition, we could not detect EAT-17 expression in neurons in which LIN-10 interacts with RAB-6.2. Although this does not exclude the possibility that LIN-10 functions as an effector for RAB-6.2 and EAT-17 pathway in other places, LIN-10 does not obviously function in grinder formation. This suggests LIN-10 affects RAB-6.2 function probably specifically in neurons, supporting the notion that differential expression of specific GEFs and GAPs in different places and at different times provides spatial and temporal specificity to the G proteins (Segev and Kahn 2012).

That mutations in a Rab and one of its GAPs cause an identical phenotype demonstrates how critical the precise kinetics of the Rab provided by their GAPs are. Furthermore, this implies that we could identify Rab interactors such as their GAPs by looking for mutants exhibiting phenotypes similar to those of Rab mutants. Among 20 TBC GAP domain-containing proteins, only two have been characterized (Mukhopadhyay *et al.* 2007; Chotard *et al.* 2010). Our finding provides an insight that may be useful for discovering more GAPs for these Rabs. In addition, using the Eat phenotype caused by the grinder defect in *eat-17* mutants, we could identify other interactors such as RAB-6.2 effectors

through genetic screens to isolate suppressors or enhancers of *eat-17* mutants.

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The Jaw of the Worm: GTPase-activating Protein EAT-17 Regulates Grinder Formation in *Caenorhabditis elegans*

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Dm CG11727-PA ----------MTLTTTTTASSAE 13
Dm CG11727-PB ----------MTLTTTTTASSAE 13
EAT-17a ----------MAATAALRRSSDY 13
EAT-17b ----------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-17b 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-17b EHSPEEDEYFQEDLWSVWGELILNWEIEVKKRPNYIKDLVKRGIPQHFRMIAWQNLSNAS 130
Hs Evi5/NB4S SSASSNLSHLEEDSWILWGRIVNEWEDVRKKKEKQVKELVHKGIPHHFRAIVWQLLCSAQ 180
Mm Evi5 SSASSNLSHLEEDSWILWGRIVNEWDDVRKKKEKQVKELVRKGIPHHFRAIVWQLLCNAQ 180
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EAT-17b VSSVHDLYSDYMRQSSVYEKVIQRDIPRTYPELDFFKDGER-GQSLLFNVIKAYSVHDKE 189
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Mm Evi5 SMTIKDQYSELLKMTSPCEKLIRRDIARTYPEHNFFKEKDSLGQEVLFNVMKAYSLVDRE 240
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Mm Evi5 QMNQAELMQLDMEGMLQHFQKVIPHQFDGGPEKLIQSAYQVKYNSKKMKKLEKEYTTIKT 420
Dm CG11727-PA KEQEEMAELRRLRRENCLLKQRNELLEAESAELADRLVRGQVSRAEEEETSYAIQTELMQ 432
Dm CG11727-PB KEQEEMAELRRLRRENCLLKQRNELLEAESAELADRLVRGQVSRAEEEETSYAIQTELMQ 432
EAT-17a KEQEEAVELRRLRTENRLLRQRIDYLEAESSALADRLVKGQVNLAQEAENYINIAHELNK 426
EAT-17b 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-17b LRDMNSDVHRKLEGAYETIRELSSARRDNIMDTGTQVDDTSMIEHIHS--------LQQE 481
Hs NB4S/Evi5 ARLSEAESQCALKEMQDKV--LDIEKRNNSLPDENNIAR----------LQEE 510
Mm Evi5 ARLSEAESQCALKEMQDKV--LDIEKKNNSFPDENNIAR----------LQEE 510

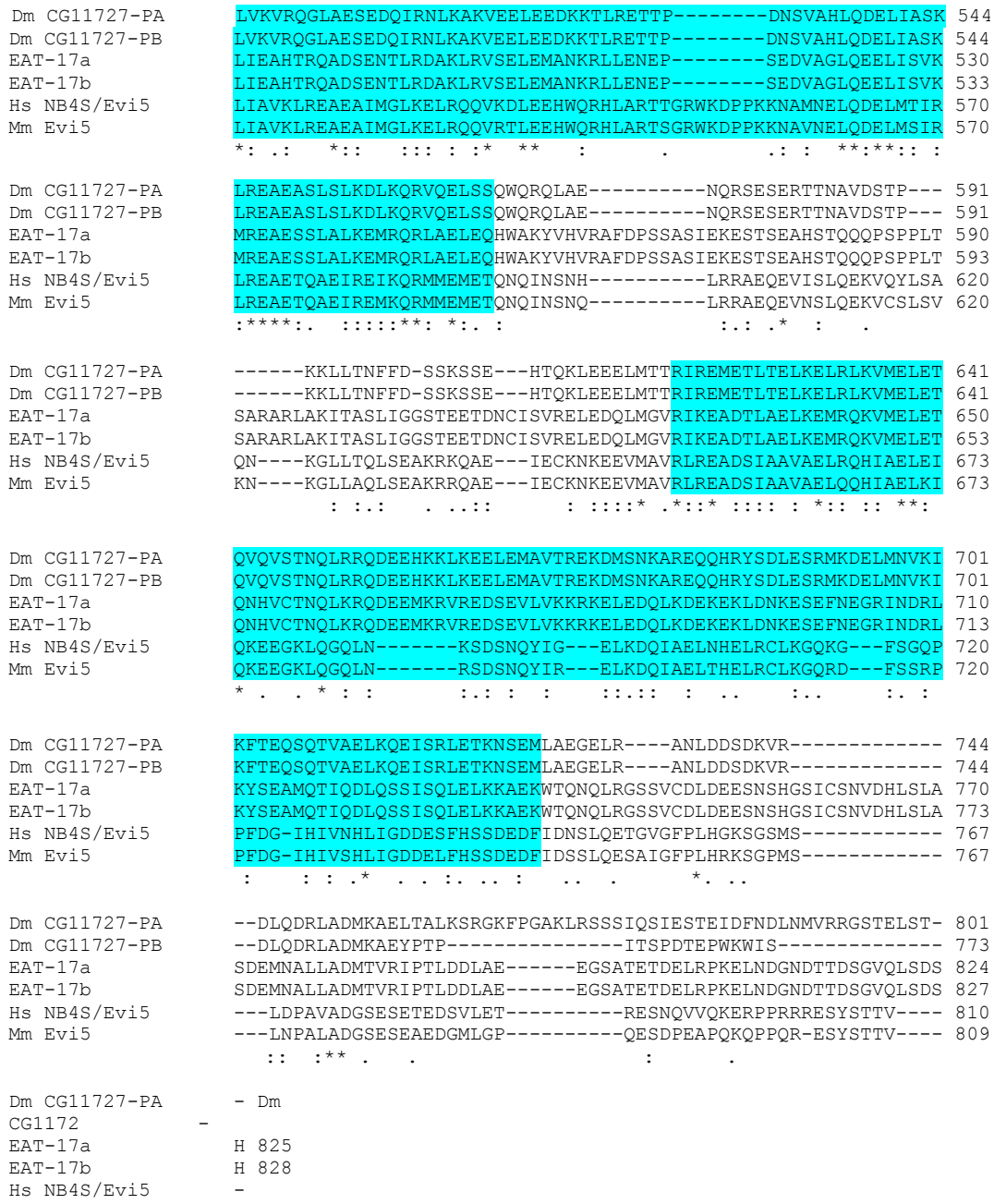

Figure S1 Sequence alignments between EAT-17 and Evi5 homologs

EAT-17 shows greatest sequence similarity to the EVI5/NB4S proteins which 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 and indicated with an arrow 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 sapiens*, and Mm= *Mus musculus*. EAT-17a and EAT-17b are two spliced form of EAT-17 from *C. elegans*.

Table S1 Primers used for SNP mapping

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

Table S2 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 S3 Primers used for gene structure analysis

Gene	Forward Primer (5'- 3')	Reverse Primer (5'- 3')	Amplified Region
T24D11.1	atgaatgtcctcccactctttttc	tgcaatcattcgaaagtgctg	5' to the R116/119 codon
T24D11.1	atgaatgtcctcccactctttttc	acggggaatgtcgcgttgaat	5' to the D152/155 codon
T24D11.1	cagcactttcgaatgattgca	ctagtggctatccgacagtt	3' to the R116/119 codon
T24D11.1	attcaacgcgacattccccgt	ctagtggctatccgacagtt	3' to the D152/155 codon
T24D11.1	atgaatgtcctcccactctttttc	gagacggagaagctcgatg	3' Exons 1-5
T24D11.1	aatgtggaatgagagaggcg	gagacggagaagctcgatg	Exons 1-5
T24D11.1	gatccgcagacaccgagtg	gagacggagaagctcgatg	Exons 2-5
T24D11.1	gaagtgttgcctccaagaag	gagacggagaagctcgatg	Exons 3-5
T24D11.1	cgtgtggggagagcttattc	gagacggagaagctcgatg	Exons 4-5
T24D11.1	aggcattcgcgttcaaactc	gagacggagaagctcgatg	Exons 1*-5
T24D11.1/F01G12.6	gaagtcaagaagcgtcccaa	tcgttgaactcgctctcctt	Exon 3 of T24D11.1 to exon 7 of F01G12.6
T24D11.1/F01G12.6	ccgtctcgatatggaaggaa	tcgttgaactcgctctcctt	Exon 5 of T24D11.1 to exon 7 of F01G12.6
T24D11.1/F01G12.6	gacgtcagcctcatggaaaact	gccaatgtgtcagcttcctt	Exon 5 of T24D11.1 to exon 5 of F01G12.6
T24D11.1/F01G12.6 (*eat-17* cDNA)	aggcattcgcgttcaaactc	ctagtggctatccgacagtt	Exon 1** of T24D11.1 to last exon of F01G12.6
T24D11.1/F01G12.6	gacgtcagcctcatggaaaact	taatacgactcactatagggccttttcgtc caaacaaatcg	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.

Table S4 Primers used for generating the R116/119K and R116/119A mutations

PCR product	Forward primer (5′-3′)	Reverse primer (5′-3′)
R116/119K		
5′ fragment	ttgtcaccgccgatggcagccactgcagcgctac	ttctgccatgcaatcat**ttt**aaagtgctgtgggatgcct
3′ fragment	aggcatcccacagcactttt**aaa**atgattgcatggcagaa	tagggatgttgaagagtaattggacctagtggctatccgacagtt
R116/119A		
5′ fragment	ttgtcaccgccgatggcagccactgcagcgctac	ttctgccatgcaatcat**tgc**aaagtgctgtgggatgcct
3′ fragment	aggcatcccacagcactttt**gca**atgattgcatggcagaa	tagggatgttgaagagtaattggacctagtggctatccgacagtt

Nucleotides shown in bold are residues that were altered.

Table S5 Primers used for RNAi

	Forward Primers (5'-3')	Reverse Primers (5'-3')
eat-17	*taatacgactcactatagggc*gacgtcagcctcatggaaaact	*taatacgactcactatagggc*gagccaatgtgtcagcttcctt
rab-1	*taatacgactcactatagggc*atggcagcaatgaaccctga	*taatacgactcactatagggc*ttaacaacatccaccgctctt
rab-2	*taatacgactcactatagggc*atgtcatatgcctacctttca	*taatacgactcactatagggc*ttaacagcatccagatccacc
rab-3	*taatacgactcactatagggc*atgaataatcaacaggctgcc	*taatacgactcactatagggc*ttagcaattgcattgctgttga
rab-5	*taatacgactcactatagggc*atggccgcccggaacgca	*taatacgactcactatagggc*ttatttacagcatgaaccctttt
rab-6.1	*taatacgactcactatagggc*atttttctcggcgaacagagt	*taatacgactcactatagggc*gagttgatgaaaagctgcggat
rab-6.2	*taatacgactcactatagggc*atgtcggactttggtaatccg	*taatacgactcactatagggc*ttagcaccagcacgatccc
rab-7	*taatacgactcactatagggc*atgtcgggaaccagaaagaag	*taatacgactcactatagggc*ttaacaattcgatcccgaattc
rab-8	*taatacgactcactatagggc*atggcaaaaacttacgactact	*taatacgactcactatagggc*gttaaagcaaattgcagctccag
rab-10	*taatacgactcactatagggc*atggctcgccgaccgtatg	*taatacgactcactatagggc*ctagcagcatcctccactg
rab-11.1	*taatacgactcactatagggc*atgggctctcgtgacgatg	*taatacgactcactatagggc*ttatgggatgcaacactgctt
rab-11.2	*taatacgactcactatagggc*atgggcaacgaatactactac	*taatacgactcactatagggc*ttatggaaagcaacactggtt
rab-14	*taatacgactcactatagggc*atgacggctgctccttacaa	*taatacgactcactatagggc*ctagcagttgcagtccttct
rab-18	*taatacgactcactatagggc*atgtccgacgacagttcaa	*taatacgactcactatagggc*ctagcatccacacattccgc
rab-19	*taatacgactcactatagggc*atggacaacgatgatggattt	*taatacgactcactatagggc*tcaagtgtactgacaacatcg
rab-21	*taatacgactcactatagggc*atgctcgaaaccaacgtgga	*taatacgactcactatagggc*tcagcgacagcacttttttact
rab-27	*taatacgactcactatagggc*atgggtgactacgactatctc	*taatacgactcactatagggc*tcagcaatttgcacaatagga
rab-30	*taatacgactcactatagggc*atggaggattacaagtatctatt	*taatacgactcactatagggc*ctaagattgtcgagtacaacag
rab-33	*taatacgactcactatagggc*atgtcggagcatcatgtgaac	*taatacgactcactatagggc*tcagcagcagaatccctctt
rab-35	*taatacgactcactatagggc*atggcgggaactcgggat	*taatacgactcactatagggc*ttatccacatttgcacttctttt
rab-37	*taatacgactcactatagggc*atgtttttaaaggttatgctactt	*taatacgactcactatagggc*tcaattaaacgtgcaacatctg
rab-39	*taatacgactcactatagggc*atggaaacaaacttcattggtg	*taatacgactcactatagggc*tcaacatccgcaagctcctg
4R79.2	*taatacgactcactatagggc*atggaagtagagtcggcga	*taatacgactcactatagggc*tcaaatacagcaccagctcc
K02E10.1	*taatacgactcactatagggc*atgaatggaaaaagtattggaaaa	*taatacgactcactatagggc*tcagacaaaggcgggttcc
F11A5.4	*taatacgactcactatagggc*atgtcatcagatcatgtgttca	*taatacgactcactatagggc*ttaacaacatgctttctccttttc
F11A5.3	*taatacgactcactatagggc*atgtaccctgatcacatgttc	*taatacgactcactatagggc*ttaacaacatttttttctcttttcc

Table S6 Primers used to amplify preys

Preys	forward primers (5'-3')	reverse primers (5'-3')
rab-1	*ccatggag*atggcagcaatgaaccctga	*gagctc*ttaacaacatccaccgctctt
rab-2	*ccatggag*atgtcatatgcctacctttca	*gagctc*ttaacagcatccagatccacc
rab-3	*ccatggag*atgaataatcaacaggctgcc	*gagctc*ttagcaattgcattgctgttga
rab-5	*ccatggag*atggccgcccggaacgca	*gagctc*ttatttacagcatgaacccttt
rab-6.1	*ccatggag*atttttctcggcgaacagagt	*gagctc*gagttgatgaaaagctgcggat
rab-6.2	*ccatggag*atgtcggactttggtaatccg	*gagctc*ttagcaccagcacgatccc
rab-7	*ccatggag*atgtcgggaaccagaaagaag	*gagctc*ttaacaattcgatcccgaattc
rab-8	*ccatggag*atggcaaaaacttacgactact	*gagctc*gttaaagcaaattgcagctccag
rab-10	*ccatggag*atggctcgccgaccgtatg	*gagctc*ctagcagcatcctccactg
rab-11.1	*ccatggag*atgggctctcgtgacgatg	*gagctc*ttatgggatgcaacactgctt
rab-11.2	*ccatggag*atgggcaacgaatactactac	*gagctc*ttatggaaagcaacactggtt
rab-14	*ccatggag*atgacggctgctccttacaa	*gagctc*ctagcagttgcagtccttct
rab-18	*ccatggag*atgtccgacgacagttcaa	*gagctc*ctagcatccacacattccgc
rab-19	*ccatggag*atggacaacgatgatggattt	*gagctc*tcaagtgtactgacaacatcg
rab-21	*ccatggag*atgctcgaaaccaacgtgga	*gagctc*tcagcgacagcacttttact
rab-27	*ccatggag*atgggtgactacgactatctc	*gagctc*tcagcaatttgcacaatagga
rab-30	*ccatggag*atggaggattacaagtatctatt	*gagctc*ctaagattgtcgagtacaacag
rab-33	*ccatggag*atgtcggagcatcatgtgaac	*gagctc*tcagcagcagaatccctctt
rab-35	*ccatggag*atggcgggaactcgggat	*gagctc*ttatccacatttgcacttctttt
rab-37	*ccatggag*atgtttttaaaggttatgctactt	*gagctc*tcaattaaacgtgcaacatctg
rab-39	*ccatggag*atggaaacaaacttcattggtg	*gagctc*tcaacatccgcaagctcctg
4R79.2	*ccatggag*atggaagtagagtcggcga	*gagctc*tcaaatacagcaccagctcc
K02E10.1	*ccatggag*atgaatggaaaaagtattggaaaa	*gagctc*tcagacaaaggcgggttcc
F11A5.4	*ccatggag*atgtcatcagatcatgtgttca	*gagctc*ttaacaacatgctttctccttttc
F11A5.3	*ccatggag*atgtaccctgatcacatgttc	*gagctc*ttaacaacatttttttctcttttcc
C56E6.2	*ccatggag*atgcaagtgctccgtcaact	*gagctc*ctagagcattgaacaacacttt

Table S7 Primers used to amplify baits

Bait	Forward primer (5′-3′)	Reverse primer (5′-3′)
EAT-17 (aa1-316)	*aagatccatttaaatcg*atggcagccactgcagcgc	gtcgacctagagacggagaagctcgatg
EAT-17 (aa1-364)	*aagatccatttaaatcg*atggcagccactgcagcgc	gtcgacctacttcgtcaagtaatccttttcc
EAT-17 (aa1-405)	*aagatccatttaaatcg*atggcagccactgcagcgc	gtcgacctacttgactagacgatccgcc
EAT-17 (aa1-460)	*aagatccatttaaatcg*atggcagccactgcagcgc	gtcgacctatccagtatccataatgttgt
EAT-17 (aa1-500)	*aagatccatttaaatcg*atggcagccactgcagcgc	gtcgacctagaccctcaacttggcgtcc
EAT-17 (aa500-825)	aagatccatttaaatcggtctcggaactggaaatggcc	actgactggtcgacctagtggctatccgacagttg