C. elegans tubby regulates life span and fat storage by two independent mechanisms

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Summary

In *C. elegans*, similar to in mammals, mutations in the *tubby* homolog, *tub-1*, promote increased fat deposition. Here, we show that mutation in *tub-1* also leads to life span extension dependent on *daf-16/FOXO*. Interestingly, function of *tub-1* in fat storage is independent of *daf-16*. A yeast two-hybrid screen identified a novel TUB-1 interaction partner (RBG-3); a RabGTPase-activating protein. Both TUB-1 and RBG-3 localize to overlapping neurons. Importantly, RNAi of *rbg-3* decreases fat deposition in *tub-1* mutants but does not affect life span. We demonstrate that TUB-1 is expressed in ciliated neurons and undergoes both dendritic and ciliary transport. Additionally, *tub-1* mutants are chemotaxis defective. Thus, *tub-1* may regulate fat storage either by modulating transport, sensing, or responding to signals in ciliated neurons. Taken together, we define a role for *tub-1* in regulation of life span and show that *tub-1* regulates life span and fat storage by two independent mechanisms.

Introduction

Adult onset obesity is a growing problem worldwide since it is a leading cause of type II diabetes, heart disease, and hypertension. One of the few single-gene mutations that cause obesity in mammals is in the gene *tubby*. Mutations in *tubby* result in adult onset obesity, insulin resistance, infertility, as well as progressive sensorineural degeneration of retinal and cochlear hair cells by apoptosis (Carroll et al., 2004). The TUBBY protein is highly expressed in the cochlea, retina, in CNS neurons of hypothalamus, hippocampus, and throughout the brain, suggesting that it is involved in controlling satiety and/or regulating feeding behavior (Carroll et al., 2004). Several studies have suggested possible functions of TUBBY, including (1) it may function as a transcription factor downstream of a G protein signaling pathway (Boggon et al., 1999; Santagata et al., 2001); (2) it may act as an adaptor molecule that integrates several pathways and is phosphorylated by the insulin receptor (Kapeller et al., 1999); and (3) it may regulate vesicle transport (Ikeda et al., 2002b).

TUBBY homologs have been identified in many organisms across the plant and animal kingdom (Carroll et al., 2004; Ikeda et al., 2002a; Lai et al., 2004). In *Caenorhabditis elegans* (*C. elegans*), deletion of the *tubby* ortholog, *tub-1*, leads to accumulation of triglycerides (TG), the major form of stored fat (Ashrafi et al., 2003). This indicates that the function of TUBBY is conserved in evolution. *C. elegans* is a convenient and experimentally tractable organism that provides a powerful tool to investigate the mechanisms of *tub-1* function. Here, we define a novel role for *tub-1* in life span regulation through the well-characterized insulin-like signaling pathway. However, the increased fat storage in *tub-1* mutants is independent of this pathway. By performing a yeast two-hybrid screen with TUB-1, we identify a novel RabGAP protein (RBG-3) and show that TUB-1 and RBG-3 are expressed in the same set of neurons. Importantly, we find that RBG-3 functions in *tub-1*-dependent fat storage, but has no effect on life span. We also show that TUB-1 undergoes dendritic as well as ciliary transport in the neurons, suggesting a function either in neuronal transport or as a cargo in transport. Alternatively, TUB-1 mobility is part of the signal transduction cascade. Additionally, *tub-1* mutant animals are defective in chemotaxis. In summary, *tub-1* regulates life span and fat storage through independent downstream mechanisms.

Results and discussion

Mutation in *tub-1* leads to life span extension through the insulin/IGF-1 pathway

In *C. elegans*, mutations in several genes, such as *daf-2* and *tph-1*, cause an extension in life span and also result in an increased fat content in the intestine (Ashrafi et al., 2003; Kimura et al., 1997; Sze et al., 2000). It has been speculated that these mutants shift from fat-metabolizing to fat-storing adults (Ashrafi et al., 2003). Additionally, mutations in these genes result in an increase in TG content (Ashrafi et al., 2003). We asked whether *tub-1* mutants that have a higher TG content also show a change in life span. We performed life span analysis at 25°C on two null alleles of *tub-1*: *tub-1(nr2004)* and *tub-1(nr2044)* (Liu et al., 1999; Figure 1A). The *tub-1(nr2004)* has a 3.2 kb deletion that encompasses the entire *tub-1* coding region, while the *tub-1(nr2044)* has a 1.8 kb deletion that eliminates the entire coding region but the first exon. We find that both mutations in *tub-1* extend life span from 14.0 ± 0.1 days in wild-type to 16.8 ± 0.2 days ($p < 0.0001$) in *tub-1(nr2004)* and 17.4 ± 0.2 days ($p < 0.0001$) in *tub-1(nr2044)*. Therefore, in addition to increased fat storage, mutations in *tub-1* lead to a 20% extension of life span. Since both alleles of *tub-1* extend life span to a similar extent and are presumed null alleles (Liu et al., 1999), only *tub-1(nr2004)* was characterized further. The

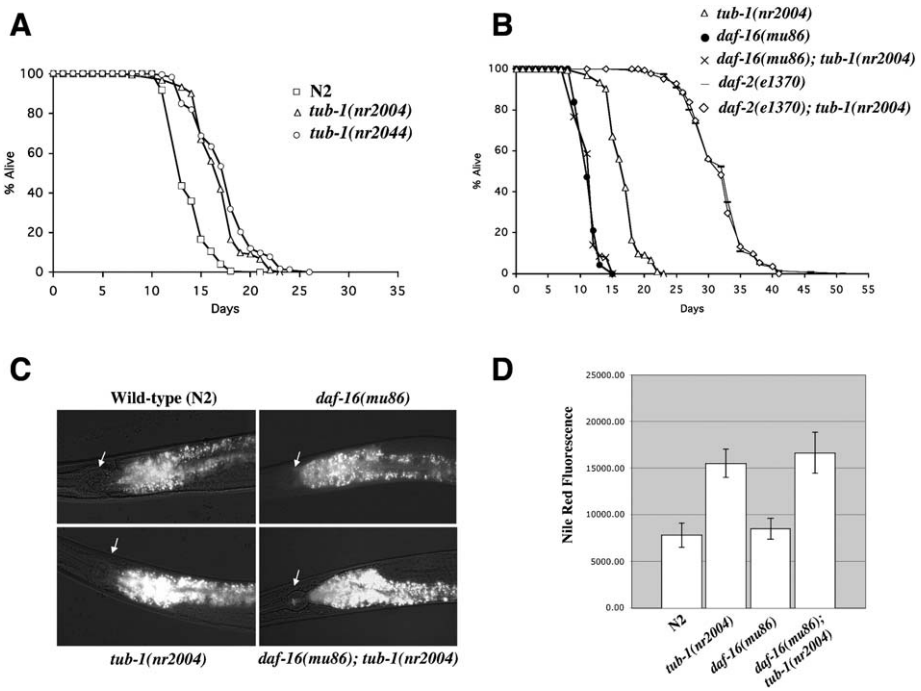


Figure 1. *tub-1* regulates life span and fat by independent pathways

A) Life span analysis of two mutant alleles of *tub-1*. All life span data are presented as mean life span ± standard error (total number of animals scored). Wild-type (N2): 14.0 ± 0.1 (193); *tub-1(nr2004)*: 16.8 ± 0.2 (167); *tub-1(nr2044)*: 17.4 ± 0.2 (164).

B) Life span analysis of *tub-1(nr2004)* in combination with *daf-16(mu86)* and *daf-2(e1370)*. *tub-1(nr2004)*: 16.8 ± 0.2 (167); *daf-16(mu86)*: 11.4 ± 0.12 (142); *daf-16(mu86); tub-1(nr2004)*: 11.4 ± 0.14 (150); *daf-2 (e1370)*: 31.8 ± 0.4 (155); *tub-1(nr2004); daf-2 (e1370)*: 31.7 ± 0.3 (166).

C) Nile red staining of wild-type (N2), *tub-1(nr2004)*, *daf-16(mu86)*, and *daf-16(mu86); tub-1(nr2004)*; the pharynx is indicated with an arrow.

D) Quantification of the Nile red staining of fat in wild-type (N2), *tub-1(nr2004)*, *daf-16(mu86)*, and *daf-16(mu86); tub-1(nr2004)*. Error bars indicate standard error.

life span and the fat phenotypes of *tub-1* mutants were also observed with *tub-1* RNAi (data not shown).

Life span extension and increased fat storage in mutants of the insulin-like signaling pathway depend on a functional copy of *daf-16*, the ortholog of the mammalian transcription factor FOXO3a (Ashrafi et al., 2003; Lin et al., 1997; Ogg et al., 1997). *daf-16* is also required for life span extension brought about by mutations in the reproductive signaling pathway (Hsin and Kenyon, 1999) as well as the JNK pathway (Oh et al., 2005). However, mutations in several genes in *C. elegans* such as *clk-1* and *eat-2* show an extended life span independent of the insulin-like signaling pathway (Lakowski and Hekimi, 1996; Lakowski and Hekimi, 1998). To test whether life span extension in *tub-1* mutants is dependent on *daf-16*, we crossed *tub-1(nr2004)* to *daf-16(mu86)* and analyzed the life span of the double mutant (Figure 1B). We found that life span extension of *tub-1(nr2004)* is completely suppressed by a mutation in *daf-16.* Life span is as follows: *tub-1(nr2004)* mutants 16.8 ± 0.2 days was reduced to 11.4 ± 0.1 days in *daf-16(mu86); tub-1(nr2004)* which is similar to the life span of *daf-16(mu86*) mutants alone (11.4 ± 0.1 days). Thus, life span extension in *tub-1* mutant is dependent on *daf-16*, and genetically, *daf-16* acts downstream of *tub-1*.

Next, we tested whether the increased life span in *tub-1* mutant requires input from the insulin-like receptor, *daf-2*, an upstream component of the insulin-like signaling pathway. If two genes function in the same genetic pathway, combining mutations of the two genes can result in a life span similar to that of one of the single mutants. We constructed a *tub-1(nr2004); daf-2(e1370)* double mutant and analyzed the life span of this strain at 25°C (Figure 1B). Mutation in *tub-1* does not further extend the long life span of *daf-2(e1370)*, indicating that *tub-1* may function in the same pathway as *daf-2*. Life spans are as follows: *daf-2 (e1370)*, 31.8 ± 0.4 days; *tub-1(nr2004)*, 16.8 ± 0.2 days; and the double mutant *tub-1(nr2004); daf-2(e1370)*, 31.7 ± 0.3 days. This is in agreement with the observation in mammalian systems where in response to insulin and IGF, TUBBY is phosphorylated by the insulin receptor in vitro (Kapeller et al., 1999). In summary, we position *tub-1* downstream of the insulin-like receptor (*daf-2*) but upstream of *daf-16* for life span regulation (see Figure 5). However, it is also formally possible that *tub-1* acts upstream of *daf-2*.

tub-1 regulates fat independent of *daf-16*

Mutation in *daf-2* results in life span extension as well as increased fat storage (Kimura et al., 1997; Ashrafi et al., 2003). Thus far, it has been suggested that these two processes are coupled because both phenotypes in *daf-2* mutants are dependent on *daf-16* (Ashrafi et al., 2003; Ogg et al., 1997). We examined whether a similar coupling between fat storage and life span regulation occurs in *tub-1* mutants by comparing fat staining of *daf-16(mu86); tub-1(nr2004)* double mutants and *tub-1(nr2004)* single mutants using the vital dye Nile red. Surprisingly, *daf-16(mu86)* does not affect the fat storage phenotype of *tub-1* mutants (i.e., the increased fat storage in *tub-1* mutants is not suppressed by a mutation in *daf-16*; Figures 1C and 1D). This observation suggests that *tub-1* controls fat metabolism independent of *daf-16* and that the increased life span observed in *tub-1* mutants is not simply due to the increased fat storage. Moreover, it reemphasizes the notion that multiple pathways are involved in fat storage regulation. DAF-16 regulates transcription of multiple downstream target genes that are involved in both life span as well as fat storage regulation (Lee et al., 2003; Murphy et al., 2003). It is the output of several major pathways including the *daf-2* signaling pathway, germ line pathway, as well as the JNK pathway (Kenyon, 2005;

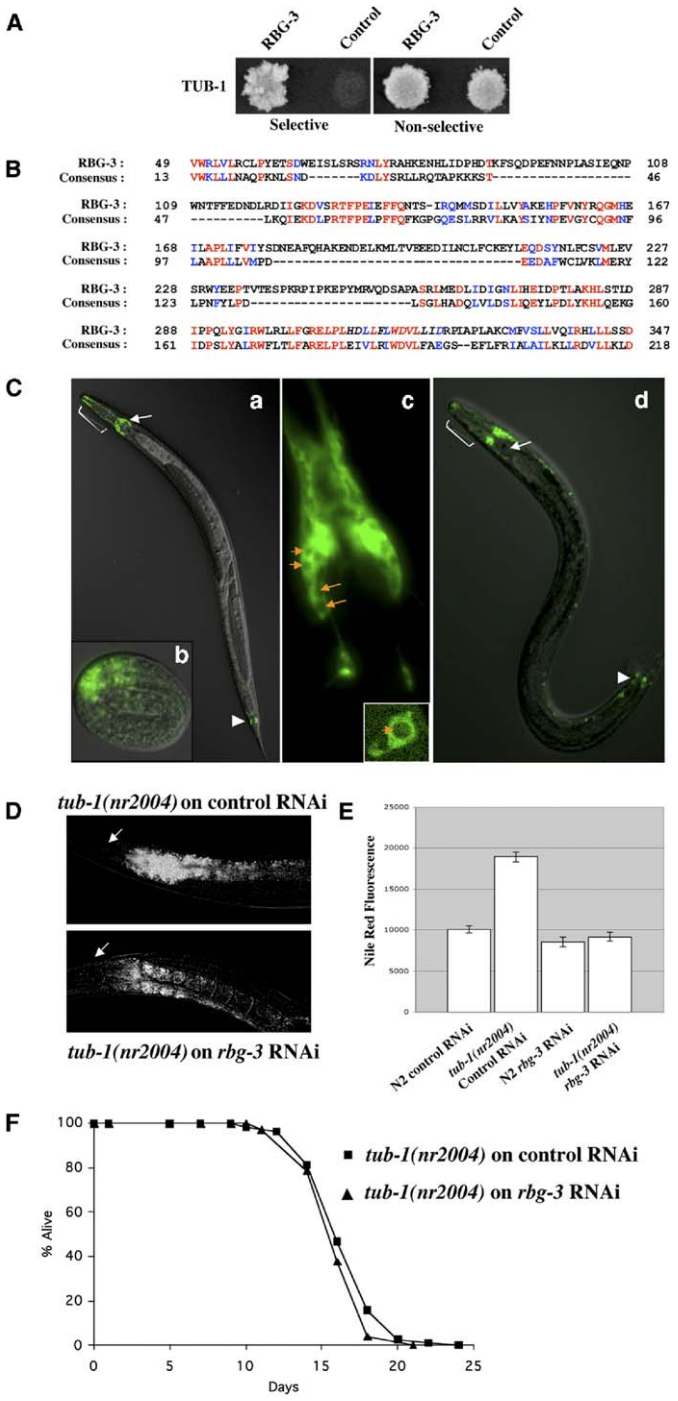


Figure 2. *tub-1* interacts with *rbg-3*, a novel RabGAP

A) Yeast two-hybrid interaction of TUB-1 and RBG-3. Selective plates contain 40 mM 3AT.

B) Alignment of TUB-1 with the consensus TBC domain (Smart00164.10 TBC) sequence commonly found in Tre-2, BUB2p, Cdc6p, Gyp6 and Gyp7.

C) Neuron-specific expression of TUB-1::GFP, position of nerve ring and phasmid neurons indicated by arrow and arrowhead, respectively (**a**); several of the neurons send processes to the tip of the head or tail, as indicated by brackets; expression of TUB-1::GFP in larvae inside the eggshell (**b**); predominant cytoplasmic localization of TUB-1::GFP, the position of the nucleus indicated with colored arrows (inset showing a magnified cell body with *tub-1::gfp* excluded from nucleus [**c**]); expression pattern of RBG-3::GFP, position of nerve ring and phasmid neurons indicated by arrow and arrowhead, respectively (**d**).

D) Nile red staining of *tub-1(nr2004)* and wild-type (N2) animals on vector control RNAi or *rbg-3* RNAi, pharynx indicated with an arrow.

E) Quantification of Nile red fluorescence of *tub-1(nr2004)* and wild-type (N2) animals on vector control RNAi or *rbg-3* RNAi. Error bars indicate standard error.

F) Life span analysis of *tub-1(nr2004)* on vector control RNAi or *rbg-3* RNAi. All life span data is presented as mean life span ± standard error (total number of animals scored). *tub-1(nr2004)* on vector control RNAi: 16.8 ± 0.2 (107); *tub-1(nr2004)* on *rbg-3* RNAi: 16.4 ± 0.2 (98).

Oh et al., 2005; Wang et al., 2005). These pathways may regulate DAF-16 by independent posttranscriptional modifications (Brunet et al., 1999; Brunet et al., 2004; Oh et al., 2005) which in turn regulate different subsets of target genes. TUB-1 signal transduction may modify DAF-16 to transcribe genes that are involved mainly in life span regulation, and for fat storage, TUB-1 may employ an independent signal transduction pathway. It is also possible that TUB-1 indirectly regulates several of the DAF-16 target genes that control fat metabolism through yet-unknown transcription factors at a level downstream of DAF-16. Thus, the regulation of fat storage genes downstream of DAF-16 and TUB-1 may overlap.

TUB-1 interacts with a novel RabGTPase-activating protein to regulate fat storage

To investigate the mechanism of TUB-1 function, we performed a yeast two-hybrid screen (Walhout and Vidal, 2001) to identify proteins that can interact with TUB-1. Using full-length TUB-1 as bait and a *C. elegans* cDNA library as prey (Walhout et al., 2000), a putative TUB-1 interactor (B0393.2) was found (Figure 2A). B0393.2 is a novel, predicted TBC domain-containing RabGTPase-activating protein (RabGAP). We name this protein RBG-3. RBG-3 is similar to Ypt/Rab-specific GTPase-activating protein Gyp6 (http://ws120.wormbase.org). The TBC domain, the catalytic domain of RBG-3, is similar to that found in Tre-2, BUB2p, Cdc6p, Gyp6, and Gyp7, and is located at the N terminus between amino acids 49 and 387 (Albert and Gallwitz, 1999; Marchler-Bauer et al., 2003; Figure 2B). YPT/Rab GTPases and GAPs are key regulators of vesicular transport in eukaryotic cells (Segev, 2001).

TUB-1 and RBG-3 are exclusively expressed in neurons

Next we asked if TUB-1 and RBG-3 are coexpressed by creating GFP fusions and examining the tissue-specific expression patterns. For *tub-1*, we fused 2.8 kb of the promoter region and the entire coding region in frame to *gfp* and injected the construct along with the coinjection marker *rol-6(su1066)* into gonads of wild-type worms. We obtained multiple stable transgenic lines and examined GFP fluorescence at different stages of development. To determine if *tub-1::gfp* was functional, we injected *tub-1::gfp* into *tub-1(nr2004)* mutant animals and found that the increased fat storage phenotype was rescued (see Figure S1 in the Supplemental Data available with this article online). GFP expression was found in several neurons, including the ciliated amphid neurons (ASI, ADL, ASK, AWB, ASH, ASJ) in the head region and ciliated phasmid neurons (PHA and PHB) in the tail, as judged by colocalization of GFP with DiI (Hedgecock et al., 1985; Figures 2Ca, 2Cc, and S2) as well as in PDE (dopaminergic neurons located on the lateral side of the posterior body) and several labial neurons in the head. Several of these neurons have processes leading to the

tip of the head or tail (Figure 2Ca). The GFP fluorescence was also observed in form of small aggregates (0.3 μm to 0.9 μm) along the neuronal processes. Expression in neurons was visible early in developing young larvae enclosed in the egg (Figure 2Cb). Surprisingly, we found that TUB-1::GFP is localized mostly to the cytoplasm with very little signal in the nucleus (Figure 2Cc). This is in contrast to results from mammalian cell culture where *tubby* is primarily found in the nucleus (Boggon et al., 1999; He et al., 2000). The cytoplasmic localization suggests that either *C. elegans* TUB-1 only transiently functions in the nucleus or a very small proportion in the nucleus is sufficient for the function or it is required only in the cytoplasm.

We then created an RBG-3 translational fusion with GFP using ~1.9 kb promoter region and the entire *rbg-3* coding region, fused in-frame to *gfp*, and injected the construct along with the coinjection marker *rol-6(su1066)* into wild-type worms and examined the expression of RBG-3::GFP fusion protein. We found that RBG-3::GFP is exclusively expressed in the amphid neurons in the head as well as the phasmid neurons (Figure 2Cd). The expression is mostly cytoplasmic and correlates with the expression pattern of TUB-1::GFP. Thus, TUB-1 and RBG-3 can interact and are expressed in similar sets of neurons, raising the possibility that they regulate similar processes.

RBG-3 regulates fat storage and not life span

Since *tub-1* functions to regulate life span and fat storage through two independent pathways, we examined whether *rbg-3* functions in either or both of these processes. We cloned the *rbg-3* open reading frame into an RNAi feeding vector (Fire et al., 1998) and exposed *tub-1(nr2004)* mutants or wild-type animals to *rbg-3* RNAi to examine the effect on fat storage by Nile red staining. *rbg-3* RNAi dramatically decreases the fat storage of *tub-1* mutants while causing slight reduction in wild-type fat storage (Figures 2D and 2E). Therefore, RBG-3 can interact with TUB-1, expresses in similar neurons as TUB-1, and suppresses increased fat storage phenotype of *tub-1* mutant, qualifying it as a novel downstream target of TUB-1.

Interestingly, life span of *tub-1* mutants is not significantly affected by *rbg-3* RNAi. Life span of *tub-1*(*nr2004*) mutants on *rbg-3* (RNAi) is 16.8 ± 0.2 days which is similar to the life span when a vector control was used (16.4 ± 0.2 days, Figure 2F). Thus, *tub-1* depends on *daf-16* for life span extension and not for fat storage, while it depends on the *rbg-3* for fat storage, but not for life span (Figures 5A and 5B). Taken together, we have defined two separate pathways downstream of *tub-1*, one affecting life span through the transcription factor *daf-16* and the other affecting fat storage through *rbg-3*, a novel RabGAP.

TUB-1 undergoes transport in ciliated neurons

RabGAPs enhance the intrinsic GTPase activity of Rab family proteins converting them to the inactive GDP bound form (Bernards, 2003). RabGTPases regulate vesicular transport of both exo- and endocytic pathways (Bernards, 2003; Olkkonen and Stenmark, 1997; Stenmark and Olkkonen, 2001). Therefore, we asked if TUB-1 is involved in neuronal transport. Indeed, a similar connection has been suggested in mammals where a genetic modifier of *tubby* hearing loss phenotype in mice was found to be a protein involved in vesicle trafficking (Ikeda et al., 2002b). We examined the TUB-1::GFP in the ciliated neurons by time-lapse photography and found that TUB-1::GFP particles transport along the dendrites of the ciliated neurons as well as undergo movement in the ciliary axoneme and processes in retro- as well as anterograde direction (Figure 3; Movies S1–S3). Therefore, the cytoplasmic location of TUB-1 and interaction with a RabGAP combined with movement of the protein in the ciliated neurons support the hypothesis that TUB-1 is involved in neuronal trafficking or functions as a cargo in transport in *C. elegans*.

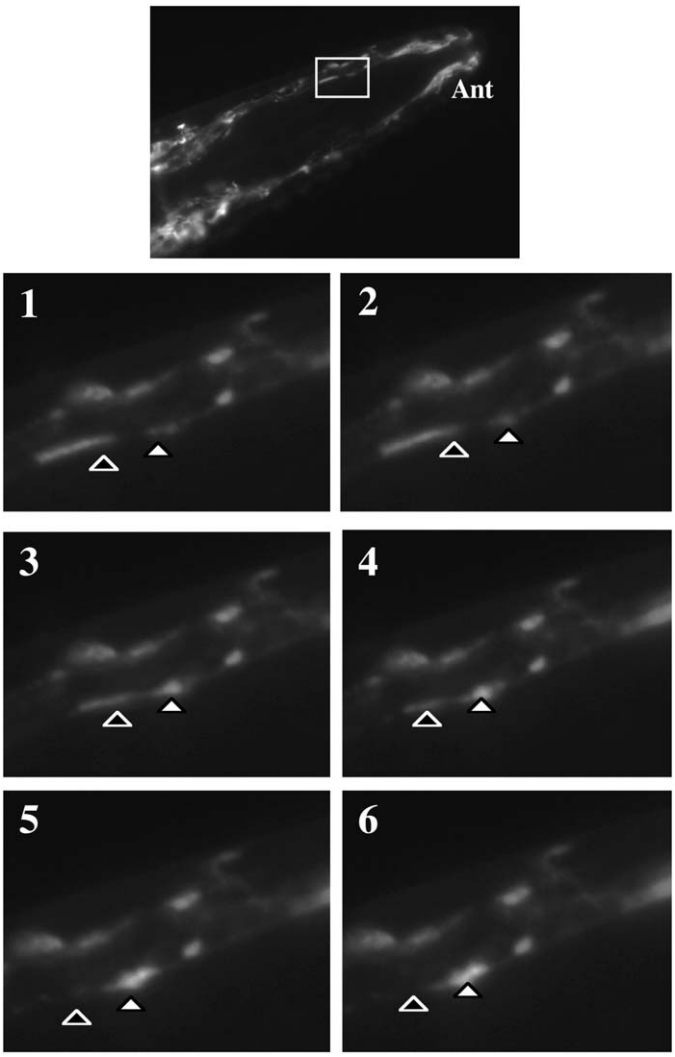


Figure 3. TUB-1::GFP particle movement in neurons

Region of neuron enclosed with dashed line is magnified digitally in panels 1–6. Progressive anterograde transport is shown in panels 1–6, with arrows marking reference points. Anterior is toward right. The progress was captured over a period of 6 s. The rate of progression of the TUB-1::GFP particles is ~1.4 μm/s.

***tub-1(nr2004)* are defective in chemotaxis**

In *C. elegans*, ciliated neurons are the location for sensory receptors and other signaling molecules responsible for detecting and transmitting external stimuli that control chemotaxis (Scholey, 2003). Through intraflagellar and dendritic transport, flagellar precursors as well as sensory molecules are delivered to the ciliary axonemes (Koushika and Nonet, 2000; Pazour and Rosenbaum, 2002; Scholey, 2003; Snell et al., 2004). Loss

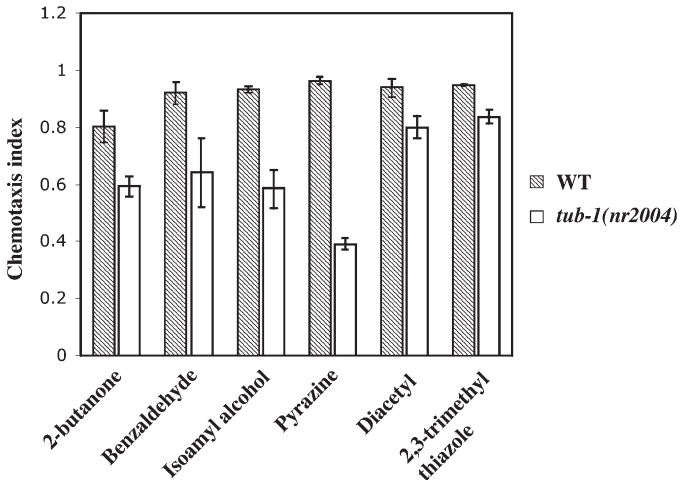
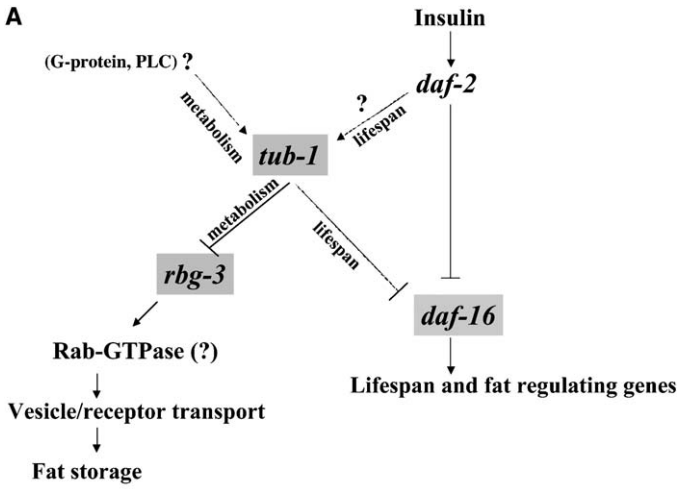


Figure 4. *tub-1* mutant animals are defective in chemotaxis

Responses of wild-type animals are indicated by hatched bars while those of the *tub-1(nr2004)* mutant animals are indicated by open bars. Assays were standard population chemotaxis assays on round plates. Error bars indicate the standard error. Dilution of the odorants were as follows: 1:500 butanol, 1:200 benzaldehyde, 1:100 isoamyl alcohol, 10 mg/ml pyrazine, 1:1000 diacetyl, and 1:1000 2,4,5-trimethylthiazole.



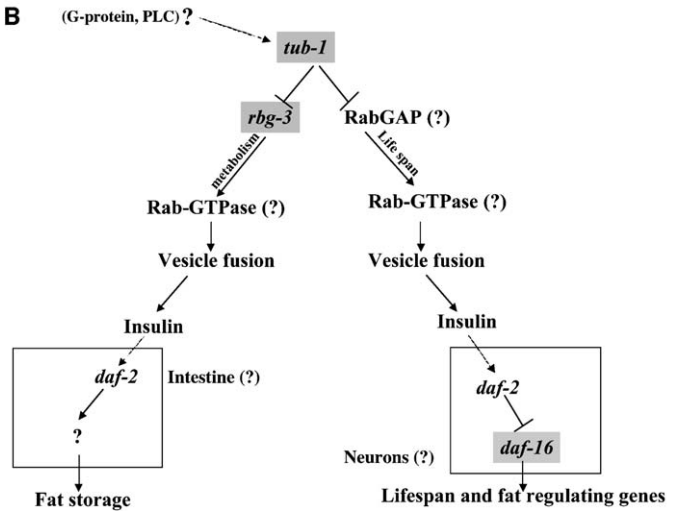


Figure 5. Models for TUB-1 regulation of life span and fat storage by independent pathways

A) For life span regulation, TUB-1 couples to the insulin-like receptor (*daf-2*) and depends on *daf-16*. For regulation of metabolism and fat storage, TUB-1 interacts with *rbg-3*, a RabGAP, and possibly controls neuronal transport.
B) Cell nonautonomous regulation of fat and life span by TUB-1.

of neuronal transport leads to severely defective ciliary neurons characterized by defective dye filling and defective chemosensing (Cole et al., 1998), increased fat storage (Blacque et al., 2004), as well as extension of life span (Apfeld and Kenyon, 1999). Here we found that *tub-1(nr2004)* mutants show an extended life span and increased fat storage but dye-fill normally (indicating that the chemosensory apparatus is intact and open to the environment). Interestingly, when we tested *tub-1(nr2004)* for chemosensory defects, we found that the *tub-1* mutants are defective in sensing various chemoattractants including isoamyl alcohol, benzaldehyde, and butanone that are sensed by the AWC neurons, as well as pyrazine and 2,4,5-trimethylthiazole that are sensed by the AWA neurons (Figure 4). Thus, although the *tub-1(nr2004)* does not have an apparent defect in dye filling, they are defective in AWC and AWA neuron-mediated chemosensation. As chemosensing is the main form of sensory perception in *C. elegans*, this defect may be compared to the retinal degeneration in mutant *tubby* mice (Carroll et al., 2004). This observation also strengthens our hypothesis that TUB-1 is involved in neuronal transport, although it is unclear whether it is the cargo or the regulator of cargo transport.

The neuroendocrine system is an important determinant of life span (Apfeld and Kenyon, 1998; Kenyon, 2005; Wolkow et al., 2000). Here, we show that TUB-1 is exclusively neuronal and controls both life span and fat metabolism systemically in *C. elegans*. We identify a novel component to the neuronal signal for *tub-1* fat metabolism, *rbg-3*, a novel RabGAP. Importantly, alterations in the small GTPases, including Rabs and Arfs, have increasingly been implicated in human disease including Bardet-Biedl syndrome (BBS) where affected individuals are also obese and have retinal degeneration (Fan et al., 2004; Stein et al., 2003). Thus, Rabs and their regulator proteins like RabGAPs may provide unique therapeutic targets to regulate obesity.

Our results indicate that TUB-1 regulates both life span as well as fat metabolism through two independent pathways. Unlike the insulin-like signaling pathway mutants (e.g., *daf-2*), the life span phenotype can be uncoupled from the increased fat storage phenotype in *tub-1* mutants. This indicates that increased life span is not the result of increased fat storage. For life span regulation, *tub-1* couples with the insulin-like signaling pathway and requires *daf-16*. However, fat metabolism is controlled through direct regulation of *rbg-3*, a RabGAP that ultimately controls neuronal transport of yet-unknown molecules. Our data suggests a model (Figure 5A) where sensory signals governing fat storage/development decision are sensed by the insulin-like signaling pathway or other unknown pathways.

These signals are processed through the insulin-like pathway as well as the *tub-1* pathway, however, in different ways. The insulin-like pathway processes the signal by directly controlling gene expression through *daf-16*, while the *tub-1* pathway processes it through modulating transport of important molecules in the ciliated neurons. An alternative interpretation is that TUB-1 functions in processing and release of different insulin-like ligands in neurons upstream of the insulin-like signaling pathway (Figure 5B). In such a case, TUB-1 would regulate more than one RabGAP, each involved in release of a specific insulin-like ligand. It is possible that one specific insulin-like peptide signals cell nonautonomously to the insulin receptor in the target tissue to regulate life span, as has been shown in *Drosophila* (Wessells et al., 2004). For regulating fat, TUB-1 may employ RBG-3 to release an insulin-like peptide (specific for fat, not life span) that signal cells nonautonomously to an insulin-like signaling pathway in a different target tissue. Indeed, in *Drosophila*, it has been found that the insulin-like ligand *dilp-2* affects aging (Hwangbo et al., 2004; Wang et al., 2005), while *dilp-3* and *dilp-5* affect the nutritional status (Ikeya et al., 2002). Together, our data suggest a model where *tub-1* regulates both life span and fat storage through independent downstream mechanisms.

Experimental procedures

Strains

All strains were maintained and handled as described in Brenner (1974) and Sulston and Hodgkin (1988). All strains were maintained at 15°C.

Strain construction

The *tub-1(nr2004)* has a 3.2 kb deletion that encompasses the entire *tub-1* coding region while the *tub-1(nr2044)* has a 1.8 kb deletion that eliminates the entire coding region but the first exon. The *tub-1(nr2004)* allele is backcrossed 15 times while the *tub-1(nr2044)* allele is backcrossed 3 times. The breakpoint sequences for *tub-1(nr2004)* are CCCATAGCAGAGAACTCC and GTTCGATTTTCCTTG, while those of *tub-1(nr2044)* are TTCAGCA TTGTA and CGGTTGCTCCCT.

For *daf-16(mu86); tub-1(nr2004)*, *tub-1(nr2004)* males were obtained by heat shock and mated to *daf-16(mu86)* hermaphrodites at 20°C. Three days later, ten putative F1 cross progeny were transferred from the mating plate to individual plates and allowed to have progeny at 20°C. Single worm PCR was performed on the F1 parents to determine whether they are heterozygous for the *tub-1(nr2004)* allele using primers 5′-GTGCGGTCTTCC CAAGTTGA-3′, 5′-ATTGTGTTTGATTTCACGCTTTC -3′, and 5′-CGCTAA CAATCTCGGTTGCTC-3′. From the confirmed heterozygous plates, a total of 40 F2 worms were transferred to fresh plates and allowed to have eggs. The F2 parents were then lysed in lysis buffer and the lysate split into two tubes. One half was scored for the presence of a homozygous mutant allele of *tub-1(nr2004)* using primers as above. The other half was used to determine the presence of a mutant *daf-16(mu86)* allele using primers 5′-CAATGAGCAATGTGGACAGC-3′ and 5′-CCGTCTGGTCGTTGTCT TTT-3′. The double mutant strain was rechecked in the next generation using the above primers.

For the *tub-1(nr2004); daf-2(e1370); tub-1(nr2004)*, males were mated to *daf-2(e1370)* hermaphrodites at 15°C. Six days later, ten putative F1 cross progeny were transferred from the mating plate to individual plates. They were allowed to have progeny at 15°C and were then shifted to 25°C. From the plates that segregated dauer and wild-type worms, 20 dauers were transferred to separate plates and shifted to 15°C. Once the dauer recovered and laid eggs, the adults were tested by PCR to determine the presence of a homozygous *tub-1(nr2004)* allele. The presence of a homozygous mutant *daf-2(e1370)* allele in the strain was then reconfirmed by shifting to 25°C and checked for formation of 100% dauers.

Life span analysis

Life span assays were performed at 25°C. Adult hermaphrodites from each strain were transferred to fresh nematode growth medium (NGM) plates at 15°C and allowed to undergo one full generation to ensure the worms were well fed and had not gone through dauer. L4s or young adults were then transferred to new NGM plates containing 0.1 mg/ml of 5′flourodeoxyuridine (FUDR) to prevent the growth of progeny (Gandhi et al., 1980; Mitchell et al., 1979). Animals were tapped every 2–3 days and scored as dead when they did not respond to the platinum wire pick. Life span is defined as the number of days that worms are alive after they were transferred to FUDR plate (day 1). All the life span assays were repeated at least three times. Data shown is a sum of all of the experiments.

Yeast two-hybrid analysis

The ORF encoding TUB-1 was retrieved from the ORFeome library (Reboul et al., 2003) and cloned into $pDEST_{DB}$ via a Gateway LR reaction as described (Walhout et al., 2000). The resulting DB-TUB-1-containing plasmid was then transformed into the yeast strain MaV203. Transformants were selected on Sc-Leu media. To screen TUB-1 versus the worm AD-cDNA library (Walhout et al., 2000), 30 μg library DNA was used. The transformation reaction was plated directly onto Sc-Leu, -His, -Trp plates with 20 mM 3AT. Approximately 1.1×10^6 colonies were screened. Potential positives were picked after 6–8 days of incubation at 30°C, spotted both onto an Sc-Leu, -His, -Trp "masterplate" as well as an Sc-Leu, -His, -Trp + 3AT plate for reanalysis of potential positives. The latter plate was incubated for 4–5 days at 30°C, while the masterplate was replica-plated after two days to a YEPD plate containing a nitrocellulose filter for the β-Gal assay. To sequence ORFs corresponding to colonies positive for both the *HIS3* and *lacZ* reporter, yeast colony PCRs were performed as described (Walhout et al., 2000). For sequencing purposes, PCR products were purified using a Qiagen PCR purification kit (Qiagen). Sequencing and BLAST comparisons to identify the sequence encoding a potential interactor were performed as described previously (Walhout et al., 2000).

Preparation of transgenic worms and GFP fluorescence microscopy

The GFP-tagged constructs were prepared according to Hobert (2002). For the *tub-1::gfp* construct, 2.8 kb promoter region upstream of start codon as well as the entire 2.8 kb coding region was amplified using a forward primer (A; 5′-TCCGTCCATCGTATCGTCAAGCC-3′) and a reverse primer (B; 5′-AGTCGACCTGCAGGCATGCAAGCTTTCACATGCAAGTTTTCCGTGG-3′) that is tailed with sequence from the coding region of *gfp*. The *gfp* coding and *unc-54* 3′UTR region was amplified from plasmid pPD95.75 using primers 5′-AGCTTGCATGCCTGCAGGTCGACT-3′ (C) and 5′-AAGGGCC CGTACGGCCGACTAGTAGG-3′ (D). The two pieces were fused by another round of PCR using nested primers from the promoter region (A*; 5′-TTT ATGTCGATGCTGAGAGATCG-3′) and *unc-54* UTR (D*; 5′-GGAAACAGTTA TGTTTGGTATATTGGG-3′). For the *rbg-3::gfp* constuct, the forward primer (A) 5′-CGTCACCAACTACGGTATGTGTGG-3′ and the reverse primer (B) 5′-AGTCGACCTGCAGGCATGCAAGCTCAATGGAGGGCGTTTTGTT GCC-3′ was used to amplify the 1.9 kb promoter and 2.5 kb coding region. For nested 5′ (A*), primer 5′-CGAGAAATGCGGAGAAATTGCG-3′ was used. The resulting PCR products were purified using PCR purification kit (Qiagen) and injected at 50 ng/μl into the gonad of worms along with pRF4, (*rol-6(su1066)* plasmid) as a coinjection marker (100 ng/μl) to generate stable extrachromosomal transgenic lines (Mello et al., 1991).

The transgenic worms were mounted in 10 mM levamisole and visualized using Zeiss Axioscope 2+ microscope. Live images as well as still photos were taken using the OpenLab.3.1.7 software.

rbg-3 RNAi

A portion of the coding region of *rbg-3* was amplified from total cDNA of N2 using primers 5′-CAAGTATTCGGCAAATGATGTCAG-3′ and 5′-GAT GACTGCTTCCAGCAACCGTG-3′ and cloned into pL4440. The construct was transformed into *E. coli* HT115 strain carrying the DE3 lysogen. The RNAi feeding bacteria was prepared according to Conte and Mello (2003). Briefly, a single colony was inoculated in 2 ml Terrific Broth (TB) containing 50 μg/ml of ampicillin and tetracycline and grown overnight at 37°C. The culture was used to seed 1L TB containing 50 μg/ml of ampicillin and incubated with shaking a 37°C for 8 to 16 hr. The bacteria were allowed to settle overnight at the bottom of the flask at 4°C, and the supernatant aspirated

carefully. The bacteria were resuspended in the remaining TB and centrifuged. The pellet was washed with 5 volumes of 1× M9 and resuspended in 1× M9. NGM plates containing ampicillin and IPTG were seeded with about 100–150 μl bacteria and dried overnight. L4 worms were placed onto the plates and allowed to go through one generation before scoring of phenotype. Plates seeded with HT115 transformed with the empty vector were used as controls.

Nile red staining of stored fat

Nile red staining of stored fat was performed according to Ashrafi et al. (2003). Briefly, Nile red powder (Molecular Probes) was dissolved to a concentration of 0.5 mg/ml in acetone and stored at –20°C. The stock was diluted 1:250 and overlayed on plates seeded with OP50 or RNAi bacteria and allowed to dry. Two gravid adults or L1s were placed on the plates and the staining was observed in young adult worms prior to starvation under a fluorescence microscope (Zeiss Axioscope 2+ microscope) using a rhodamine filter (emission 560–590 nm). Images were captured and processed using the OpenLab.3.1.7 software (Improvision Inc.) under identical settings. For quantitation, similar areas of the worms were selected. The total fluorescence was calculated as the product of mean fluorescence and the area selected.

Chemotaxis assays

Chemotaxis assays were performed according to Bargmann and colleagues (Bargmann et al., 1993; Bargmann and Horvitz, 1991). Unseeded NGM plates were used for the assay. All the assayed chemicals were diluted in ethanol. Shortly before the assay, 1μl of volatile odorant (1:500 butanol, 1:200 benzaldehyde, 1:100 isoamyl alcohol, 10 mg/ml pyrazine, 1:1000 diacetyl, and 1:000 2,4,5-trimethylthiazole) was spotted slightly off-center on the assay plate. As a control, 1 μl ethanol was applied approximately 4 cm from the attractant. One microliter of 1% sodium azide was applied on both the spots to anesthesize animals that move to either spot. Around 100–150 worms were washed two times in 1× M9 buffer and placed equidistant from either spot. After 30 min, the plates were chilled at 4°C, and the number of worms within 2 cm of each spot was counted. Chemotaxis index was calculated as (number of worms at attractant – number of worms at control)/total number of worms assayed.

Supplemental data

Supplemental Data include two figures and three movies and can be found with this article online at http://www.cellmetabolism.org/cgi/content/full/2/1/35/DC1/.

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