Regulation of Metabolism and Lifespan

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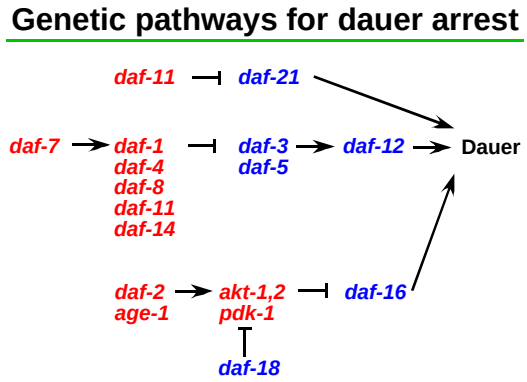
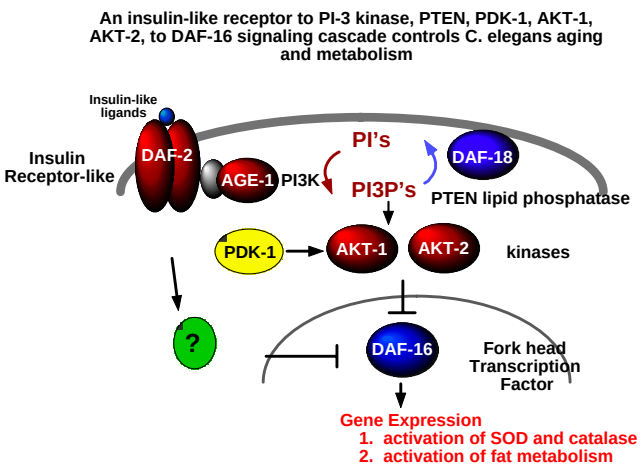
During the last decade, major progress on the genetics of life span has been realized through the study of long-lived mutants identified in the nematode *Caenorhabditis elegans*. The biochemical functions of many of these genes is now known and because they are related to processes (e.g. metabolism, free radical production) implicated in aging of vertebrates, have potential general significance to aging. The clearest example of such a biochemical convergence is the finding that an insulin-like signaling pathway regulates longevity and metabolism in *C. elegans*. *daf-2*, *age-1*, and *pdk-1* mutants that are components of the *C. elegans* insulin signaling pathway live 2x to 3x longer than wild type. This insulin-like signaling pathway is part of a global endocrine system that controls whether the animals grow reproductively or arrest at the dauer diapause stage.

Genes that regulate the function of this neuroendocrine pathway were identified by two general classes of mutants: dauer defective and dauer constitutive mutants. For example, *daf-2* dauer constitutive mutant animals, form dauers in the absence of high pheromone levels. Conversely, *daf-16* dauer defective mutants do not form dauers under normal dauer pheromone induction conditions, and suppress the dauer constitutive phenotype induced by *daf-2* mutations. The *daf* genes constitute multiple parallel signaling pathways that converge to regulate *C. elegans* diapause. The *daf-2/age-1/pdk-1/daf-18/akt-1/akt-2/daf-16* subpathway corresponds an insulin-like signaling pathway, and the *daf-7/daf-1/daf-4/daf-8/daf-14/daf-3* subpathway corresponds to a TGF-betalike neuroendocrine signaling pathway. Even though these pathways conspire to regulate metabolism and dauer arrest, only the *daf-2* insulin like pathway has effects on the longevity of reproductively growing adults.



A large number of *C. elegans* insulin superfamily genes have been found and are expressed in subsets of sensory neurons, as well as the intestine and somatic gonad. Human insulin expressed using the *ins-1* regulatory region antagonizes DAF-2. Since all of the INS proteins are predicted to adopt a similar tertiary structure, they may all bind to DAF-2, the only member of this receptor superfamily in the worm genome. Some INS proteins may be DAF-2 agonists while others may be antagonists. Dauer arrest in *C. elegans* is normally regulated by an as yet uncharacterized dauer pheromone, that is detected by sensory neurons. The dauer pheromone causes down regulation of *daf-7*, the TGFbeta ligand of the pathway that acts in parallel to the *daf-2* pathway. It also regulates the expression of the daf-28 insulin that acts upstream of the daf-2 insulin receptor. The detection of the dauer pheromone depends on ciliated sensory endings; cGMP signaling in those sensory neurons has been implicated, suggesting that the pheromone receptor may be a 7 transmembrane receptor that couples to cGMP phosphodiesterase, as in mammalian odor sensation.

age-1 acts downstream of *daf-2*and encodes the worm ortholog of mammalian phosphatidylinositol 3-kinase (PI 3-kinase) p110 catalytic subunit. Reduction of function mutations in age-1 cause a two fold increase in life span. PI 3-kinases generate a membrane-localized signaling molecule, phosphatidylinositol P3drslt (PIP3), which binds to the pleckstrin homology domain of mammalian Akt/PKB and are required for its activation. There are two Akt/PKB orthologs in *C. elegans*. Simultaneous inhibition of both *akt-1* and *akt-2* activities using RNA interference causes nearly 100% arrest at the dauer stage, whereas inactivation of either gene alone does not. One of the kinases that phosphorylates



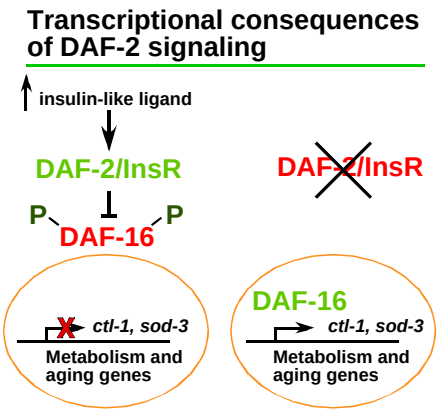
Akt/PKB and is required for its activation is 3-phosphoinositide-dependent kinase-1 (PDK1). A loss of function mutation in *pdk-1* increases *C. elegans* life span almost two fold, similarly to a mutation in *age-1*.

The dauer arrest, fat accumulation, and longevity phenotypes of *daf-2* and *age-1* mutants are suppressed by *daf-18* mutations. *daf-18* encodes the *C. elegans* ortholog of mammalian PTEN lipid phosphatase gene. By genetic epistasis experiments, *daf-18* acts downstream of the AGE-1 PI3K, but upstream of AKT-1 and AKT-2 in this signaling cascade. The DAF-18 lipid phosphatase may normally decrease the level of PIP3 signals, perhaps insulating signals emanating from the DAF-2/AGE-1 signaling complex from other PIP3 signals in the cell, or resolving insulin-like signaling episodes. It is not clear from the genetic analysis whether DAF-18/PTEN activity is regulated during insulin-like or other signaling.

Mutations in *daf-16* also completely suppress the dauer arrest and metabolic shift of animals bearing *daf-2, age-1, pdk-1* mutations, or RNAi inhibited *akt-1* and akt-2 activity. *daf-16* mutations also suppress the increase in longevity caused by decreased *daf-2, age-1,* or *pdk-1* signaling. Thus DAF-16 is active in the absence of these upstream inputs and acts to increase life span. *daf-16* encodes two proteins with forkhead DNA binding domains. The mammalian orthologs to DAF-16 are human FKHR, FKHRL1, and AFX (82). DAF-16 contains four consensus sites for phosphorylation by Akt/PKB and three of these sites are conserved in the human DAF-16 homologs AFX, FKHR, and FKHRL1. Mammalian FKHR, FKHRL1 and AFX activities are regulated by AKT phosphorylation; these transcription factors are nuclearly localized only when insulin like signaling (AKT activity) is low. Consistent with the activity of *C. elegans akt-1* and *akt-2* upstream of *daf-16* to inhibit its activity, mutation of those Akt sites in FKHRL1 cause nuclear localization in the presence of insulin or IGF-I signaling. The nuclear localization of a functional DAF-16/GFP fusion protein is similarly controlled by upstream pathway activity.

The mammalian DAF-16 orthologs have been shown to regulate the expression of target genes such as the metabolic genes PEPCK and glucose 6 phosphatase. DAF-16 binds to this same insulin response sequence in vitro. *C. elegans* DAF-16 may regulate the *C. elegans* homologs of these and other metabolic genes. Our genomic approach to the detection of DAF-16 target genes has identified 115 genes that in Drosophila as well as C. elegans bear DAF-16 binding sites. We have not validated all of these possible target genes but about 30% of those tested so far have been validated both by functional tests as well by expression analysis.

The *C. elegans* insulin pathway regulates the expression of key free radical detoxifying enzymes, consistent with free radical theories of aging. *ctl-1* catalase and *sod-3* Mn superoxide dismutase genes are expressed at higher levels in a *daf-2* mutant than in a *daf-2;daf-16* double mutant. These enzymes convert the toxic superoxide radicals and peroxides to less reactive products. Consistent with this, treatment of *C. elegans* with free radical detoxifying mimetics, cause a 44% increase in life span of wild type and 60% increase in the life span of the above mutants expected to produce more free radicals than normal.



The *C. elegans* insulin-like signaling pathway regulates metabolism as well as free radical protection. These mutants accumulate much larger stores of fat. In addition, the rate of CO2 production, a measure of metabolic rate, is reduced in a *daf-2* mutant to about 30% that of wild type. The fat accumulation, decline in metabolic rate, as well as the longevity increase is fully suppressed by a mutation in *daf-16*. There is also conflicting data on metabolic control by *daf-2* pathway mutants: however because the other studies used a free radical surrogate measure of metabolism rate, and were done under non-optimal growth conditions, they must be viewed with caution.

Drosophila orthologs of the mammalian and nematode insulin receptor, insulin receptor substrate, *age-1* PI3 kinase, AKT/PKB, PTEN, and S6 kinase regulate cell, organ, and total body size. Mutations in *Drosophila* IRS also affects metabolism, causing a dramatic increase in fat storage, like mutations in the *C. elegans* insulin signaling pathway.

Mammals are also likely to use an insulin like pathway to regulate longevity. Life span is dramatically increased in dwarf mice with defects in growth hormone signaling, and decreased IGF-I signaling, as well as in mice with defects in insulin signaling within fat and neurons. Importantly, we have found that it is insulin signaling inthe nervous system that is key for longevity control. Similar results have emerged from an analysis of insulin and IGF-I signaling in the mammalian nervous system.

The increase in longevity associated with decreased DAF-2 signaling is analogous to mammalian longevity increases associated with caloric restriction. The involvement of an insulin signaling pathway in worm aging may be mechanistically related to the longevity increase caused by caloric restriction in mammals. Insulin secretion by the pancreas is regulated by nutritional and autonomic neural inputs, and this endocrine signal of metabolic status is

detected by target tissues to regulate the activities of metabolic enzymes that synthesize or breakdown glucose, amino acids, fat, etc.

Life span of *C. elegans* is also coupled to rate of feeding. Perhaps like caloric restriction, some *eat* mutants which ingest bacteria less efficiently than wild type live up to 50% longer. The increase in life span of the *eat* mutants is not fully suppressed by *daf-16* mutations, suggesting that the caloric restriction pathway engages some pathway besides the *daf-2* insulin like signaling pathway.

The connection between longevity and diapause control may not be parochial to *C. elegans*. Diapause arrest is an essential feature of many vertebrate and invertebrate life cycles, especially in regions with seasonal temperature and humidity extremes. Animals in diapause arrest slow their metabolism and their rates of aging, and can survive for periods for much longer than their reproductive life span.

Mutations in the *C. elegans* coenzyme Q biosynthetic gene *clk-1* cause a modest increase in life span, in addition to a two-fold slowing of the rate of development. But the *clk-1* null mutant uses coenzyme Q from *E.. coli*. Presumably, the level or regulation of the *E. coli* derived coenzyme Q is not normal, leading to the modest increase in life span. Consistent with this finding, RNAi of many mitochrondrial components causes an increase in lifespan.

In addition to their possible roles in longevity control, the insulin signaling genes identified by C. elegans genetics may reveal components of insulin signaling in mammals that are important for the understanding and eventual treatment of diabetes. Diabetes is a common disease that affects the production or response to insulin, causing devastating metabolic dysregulations. The molecular basis of the defective insulin response in the more common adult onset or type II diabetes is unknown. It is clear that it is at least in part a genetic disease: the disease shows autosomal dominant transmission but is likely to be multifactoral based on pedigree analysis. In addition it is clear that both genetically and environmentally induced obesity is a major modulator of diabetes symptoms.

1. Function of DAF-16 and its human ortholog FKHRL1 and the DAF-18 ortholog human PTEN in the *daf-2* insulin-like signaling pathway

daf-16 encodes multiple isoforms that are expressed in distinct tissue types and are probable orthologs of human FKHRL1 (FOXO3), FKHR, and AFX. To prove orthology between *daf-16* and these related genes, we tested the ability of human FKHRL1 to substitute for DAF-16. A *daf-16*-promoter:FKHRL1 fusion gene supplies *daf-16* gene activity to a *daf-16; daf-2*double mutant. Thus human FKHRL1 can function like DAF-16 in mediating *C. elegans* insulin signaling. Mammalian insulin and insulin-like signaling regulate FKHR, FKHRL1, and AFX function by controlling nuclear localization via AKT phosphorylation. Mammalian FKHRL1 is negatively regulated by AKT/PKB kinases. *C. elegans akt-1* and *akt-2* negatively regulate *daf-16*. On DAF-16, there are four sites that conform to the consensus of mammalian AKT phosphorylation. We assayed the phenotype of AKT phosphorylation-defective DAF-16A1-4A mutant in *daf-16(mgDf47)*. This daf-16 mutant missing AKT consensus sites cause dauer arrest in *daf-2(+)* animals, proving that *daf-16* is the major output of insulin signaling in *C. elegans*. FKHR, FKRHL1, and AFX may similarly be the major outputs of mammalian insulin signaling. We as well as others found that DAF-16 nuclear localization is regulated by *daf-2* insulin-like signaling.

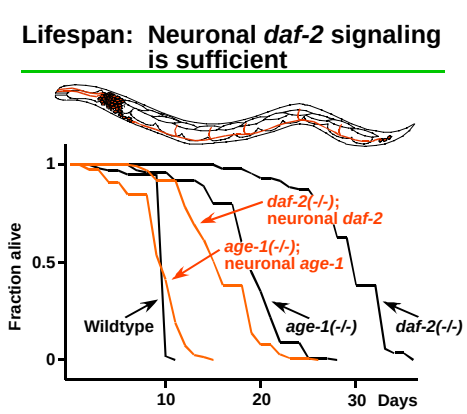
DAF-18 and PTEN share 43% identity within the amino-terminal phosphatase core domain and both proteins display a C2 phospholipid binding domain. We asked whether PTEN could substitute for DAF-18 in *C .elegans*. Mammalian *PTEN* cDNA or *daf-18* cDNA were expressed under the control of 5' regulatory sequences of *daf-18*. *daf-2 (e1370)* single mutants form dauers when raised at the restrictive temperature of 25°C and become long-lived adults when transferred at 25°C from the L4 stage. We identified a null allele of daf-18, mg198 a stop codon upstream of the conserved phosphatase domain, in a genetic screen for suppressors of daf-2 dauer arrest. Double mutant *daf-2 (e1370); daf-18 (mg198)* developed as reproductive adults at 25°C and have a shorter lifespan compared to *daf-2(e1370)* mutants. Expression of mammalian PTEN under the control of the *daf-18* promoter in *daf-2(e1370); daf-18(mg198)* mutants restored the *daf-2(e1370)* dauer constitutive phenotype at 25°C as efficiently as DAF-18.

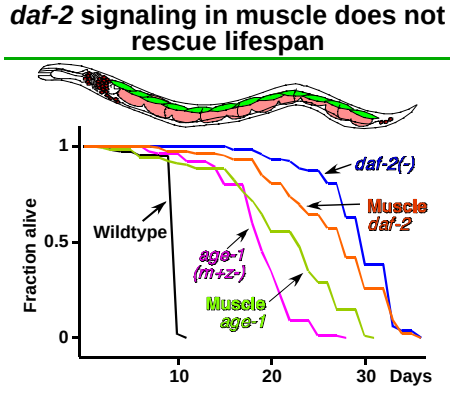
2. *C. elegans* lifespan is regulated by insulin-like signaling in the nervous system

Several components of the *daf-2* pathway, such as *akt-1, pdk-1* and *daf-16*, are widely expressed throughout development, as determined from GFP fusion reporter genes. Studies of *daf-2* genetic mosaic animals showed that animals lacking *daf-2* activity from the entire AB cell lineage, which generates nearly all of the hypodermis and nervous system and half of the pharynx, have significantly extended lifespans (*3*). However, mosaic animals lacking *daf-2* activity from blastomere daughters of AB, AB.a and AB.p which generate about half of the hypodermis, nervous system, and pharynx, show very modestly extended lifespans. These studies showed that *daf-2* can act non-autonomously to regulate lifespan, but did not assign *daf-2* longevity control to particular cell types.

To define the cell type(s) from which the *daf-2* insulin-like signaling pathway functions to control *C. elegans* lifespan, metabolism and development, we restored *daf-2* pathway function to only restricted cell types by using distinct promoters to express *daf-2* or *age-1* cDNAs in either neurons, intestine or muscle cells of a *daf-2* or *age-1* mutant. Because regulation of longevity may require gene activity over the entire life of the animal, the expression of GFP fusions to these promoters was examined in wild type at all stages, including aged adults.

Genotype	Cell type with *age-1* or *daf-2*	Lifespan days ± SD (n)	Intestinal fat level (% of population)			Dauer larvae	
			Low	High	(n)	%	(n)
wild-type	wild-type	10.3 ± 1.9 (402)	83	17	(51)	0	(148)
age-1(-) mutants							
age-1(-) (m+z-)	none	19.5 ± 5.1 (362)	75	25	(20)	0	(>100)
age-1(-) (m-z-)	none	-	5	95	(41)	100	(>100)
Pdpy-30::age-1	all cells	11.6 ± 3.4 (198)	94	6	(75)	0	(1185)
Punc-14::age-1	all neurons	10.5 ± 3.7 (198)	97	3	(35)	0	(749)
Pmec-7::age-1	10 neurons	17.9 ± 6.8 (160)	100	0	(39)	17	(897)
Punc-54::age-1	muscle	21.2 ± 6.7 (201)	95	5	(38)	3	(1400)
Pges-1::age-1	intestine	18.8 ± 5.8 (343)	20	80	(66)	43	(597)
daf-2(-) mutants							
daf-2(-)	none	28.8 ± 4.8 (101)	5	95	(20)*	100	(175)
Pdpy-30::daf-2	all cells	15.0 ± 2.9 (21)	30	70	(18)	27	(161)
Punc-14::daf-2	all neurons	16.8 ± 3.9 (101)	25	75	(11)	5	(173)
Punc-119::daf-2	all neurons	18.3 ± 9.8 (128)	nt	nt	-	62	(513)
Punc-54::daf-2	muscle	27.6 ± 6.3 (98)	0	100	(18)	100	(335)
Pges-1::daf-2	intestine	19.2 ± 3.5 (197)	10	90	(35)	91	(911)





The long lifespan of *daf-2* and *age-1* mutants is rescued by neuronal expression of *daf-2* or *age-1*, respectively, using the pan-neuronal *unc-14* promoter. Neuronally-restricted *age-1* expression fully restored wild-type adult lifespan to an *age-1 (mg44)* null mutant. This rescue is comparable to the positive control, ubiquitous expression of *age-1* from the *dpy-30* promoter in the *age-1* mutant. Neuronally-restricted *daf-2* expression from the *unc-14* promoter also rescues the long lifespan of *daf-2(e1370)* mutants. The long *daf-2(e1370)* lifespan is also rescued when *daf-2* is expressed from the *unc-119* promoter, another neuron-specific promoter. Furthermore, we have now fused age-1 to another pan neuronal promoter ric-19 and find that this fusion gene rescues the lifespan and metabolic defects of an age-1 null mutant. It is important to note that these fusion genes did not cause lifespan to overshoot wild type lifespan, even though gene dosage was not carefully controlled to wild type levels. Thus insulin-

like signaling in the nervous system is necessary for wild type lifespan but is not so dose dependent that increased insulin signaling causes shorter than normal lifespan.

In contrast to neuronal expression of *daf-2* and *age-1*, restoration of *daf-2* pathway activity to muscles from the promoter for muscle myosin, *unc-54*, is not sufficient to rescue the long lifespan of *daf-2* or *age-1* mutants. Similarly, expression of *daf-2* or *age-1* in the intestine, the major site of fat storage, from the *ges-1* promoter does not rescue lifespan as efficiently as neural expression of these genes. Intestinally-restricted *daf-2* expression shows weak rescue of the long lifespan of *daf-2(e1370)*, whereas intestinal *age-1* expression does not rescue the long lifespan of *age-1(mg44)* mutants. The lack of longevity rescue was observed in multiple transgenic lines for both *daf-2* and *age-1*. In addition, the muscle or intestinal *daf-2* and *age-1* transgenes expressed sufficient gene activities to partially rescue dauer arrest phenotypes, showing that the fusion genes were functional.

In indirect immunofluorescence experiments, purified anti DAF-2 antibodies reveal DAF-2 in the cell bodies and processes of neurons, and the nerve ring, the major neuropil of the animal. We raised and affinity-purified rabbit anti-DAF-2 antisera. A pair of cells anterior to the nerve ring also express DAF-2. Positions of the cells are variable, suggesting that they are the XXX cells that also express daf-9 and the eak-3, eak-4, eak-5, eak-6, npc-2, and hsd-1 genes. No such immunofluorescence was observed in the *daf-2(m646)* null mutant, indicating that the antibodies are specific to DAF-2. While the highest level of DAF-2 is present in the nervous system, we observe weak immunofluorescence in other tissues, such as the hypodermis as well. DAF-2 protein levels decreased dramatically in wild type animals cultivated in the absence of food compared to well-fed animals. This suggests that the DAF-2 abundance is coupled to nutritional status or the sensation of food. Food levels regulate dauer arrest. It is possible that food modulates the production of insulin-like DAF-2 ligands, which in turn affect DAF-2 activity, and DAF-2 abundance.

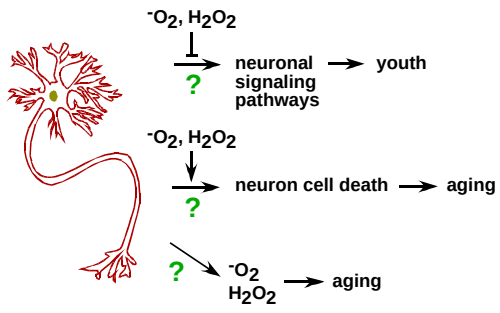
The more potent regulation of longevity by neuronal *daf-2* pathway signaling could represent distinct outputs from some or all neurons, or, simply, that neuronal promoters restore *daf-2* pathway activity to more cells than muscle or intestinal promoters. The adult hermaphrodite nematode contains 302 neuronal cells, 95 body-wall muscle cells and 20 intestinal cells. While neurons constitute the largest number of cells, the total mass of neurons, which are smaller than nematode muscle or intestinal cells, is considerably less than the mass of muscle or intestinal cells. Further analysis of animals with *daf-2* pathway signaling restored to restricted neuronal subtypes (proposed in Aim 2) should elucidate whether *C. elegans* lifespan is controlled by a specific set of neurons or, alternatively, by a quorum of neurons that can be of any neuronal subtype.

The XXX cells and nerve ring express DAF-2

The expression of catalase and Mn-SOD are transcriptionally regulated by DAF-16. Neurons may be particularly sensitive to free radical damage during aging. In fact, over-expression of Cu/Zn superoxide dismutase (SOD) in only motor neurons can extend *Drosophila* lifespan by 48%. It is striking that aging in two different organisms can be controlled from neurons and is correlated with increased free radical protection in those neurons.

Loss of DAF-2 activity from neurons, relieving the negative regulation of DAF-16, induces higher expression levels of these free radical scavenging enzymes, thereby protecting neurons from oxidative damage. By this model, neuronal *daf-2* signaling might regulate an organism's lifespan by controlling the integrity of specific neurons that secrete neuroendocrine signals, some of which may regulate the lifespan of target tissues in the organism. Our results, together with those from *Drosophila*, suggest that oxidative damage to neurons may be a primary determinant of lifespan.

Models for neuronal control of lifespan



Controls and caveats: The interpretation that insulin-like signaling in the nervous system is key is highly dependent on our characterization of the pan neuronal and other neuronal promoters. We have revisited these promoters to carefully document their expression patterns. Two issues are paramount: are the promoters expressed only in the cells where we see GFP and do the promoters continuously express even in older adults? For the unc-14 promoter, it is highly expressed in all neurons throughout the life of the animal, including in aged adults, but it also shows a much weaker haze of expression in many other tissues in older animals. But the late onset promoter

promiscuity is a concern. Other observations support the conclusion that it is expression in the nervous system that is key: First, we have tested a number of additional promoters. The unc-119 promoter is also pan neuronal and has less of a general haze of expression in aged adults. And the pan neuronal ric-19 promoter is expressed at all stages only in the nervous system, without any haze of generalized expression at any stage. These promoters driving age-1 rescue aging as well as the unc-14 promoter. These data support the view that it is neuronal expression rather than weaker extra neuronal expression that rescues aging. But the neuronal specificity is still a quantitative argument: it is the site of brightest GFP but we cannot be sure that the promoter is not active in other cell types as well. Of course, that critique is true of any in situ based gene expression assessment: there could be low level expression that is important. Actually this critique applies to genetic mosaic analysis as well: there could be perdurance of a gene product in a cell lineage that has lost the gene locus itself, akin to maternal loading of gene products. Such a critique is especially germane to an animal like C. elegans with only 10 cell divisions from beginning to end. Second, RNAi of age-1 in the strain bearing the unc-14 pro: age-1 in an age-1(mg44) null mutant background does not induce dauer arrest, suggesting that it is not promoter promiscuity in non neuronal tissues that rescues dauer arrest. We are now testing the age-1 RNAi this strain for lifespan. Third, the Kenyon lab has shown that it is daf-2 or daf-16 action in early adults that is key for regulation of aging, not activity late in life. This suggests that the late onset promoter promiscuity may occur after the longevity regulation action in early adults.

Superficially inconsistent with the view that daf-2 regulation of aging occurs in the nervous system is the finding that RNAi of daf-2 or age-1 works pretty well at increasing lifespan (though not as well as a genetic lesion). This would not be expected if the major focus of daf-2 RNAi was neuronal, given how difficult it is to phenocopy neuronal unc genes for example. However, the fact that RNAi of daf-2 and age-1 does increase lifespan does not rule out the nervous system as the focus of their action. First, increase of lifespan is a weak allele phenotype for daf-2 and age-1: slight decrements in insulin like signaling dramatically increases lifespan. So if RNAi works to decrease daf-2 or age-1 gene activity by a factor of two for example, it may dramatically increase lifespan without affecting dauer arrest. RNAi in neurons may work sufficiently well to induce aging but not dauer phenotypes. Second, it is not clear that all neurons are defective for RNAi. Given that the cell XXX is the focus of daf-2 for dauer arrest (see Progress report C4), one other neural type could be the focus for aging regulation and for some reason RNAi may work in that cell. So we continue to search for that neuron and to continue to triangulate in on which cells are the focus of insulin like regulation of lifespan.

But there is evidence of aging regulation from outside of the nervous system as well. First, genetic mosaic analysis points to weak daf-2 regulation of lifespan from the gut lineages. In addition, our promoter analysis driving daf-2 within the gut shows a weak regulation of lifespan. Second, the ablation of the germ line causes potent increase in lifespan, suggestive of signals from that tissue to also induce senescence. These signals could be insulins, because many are expressed in the germ line and because the somatic gonad has been shown to affect lifespan via the *daf-2* pathway. The lifespan signals from the somatic gonad may act to regulate neuronal *daf-2* pathway activity.

3. A PDK1 homolog transduces AGE-1 PI-3 kinase signals in *C. elegans*

To identify new components regulating dauer formation, we performed a genetic screen for mutants that arrest at the dauer stage constitutively (Daf-c). Two alleles (*sa680* and *sa709*) from this screen are recessive, and fail to complement. In support of the placement of *sa680* in the insulin receptor-like signaling pathway, its dauer arrest phenotype is partially suppressed by a gain of function mutation in the gene *akt-1* that also suppresses the dauer arrest induced by loss of *age-1* PI3K signaling. *sa680* maps to the genetic region is *mg142*, a dominant mutation that was isolated in a screen for suppression of the Daf-c phenotype of an *age-1(mg44)* null mutant. The *mg142* dominant mutation suppresses the *age-1* Daf-c phenotype, while the recessive *sa680* and *sa709* mutants phenocopy the *age-1* Daf-c phenotype, suggesting that *mg142* may activate the same gene that is inactivated by *sa680* and *sa709*. The *C. elegans* homolog of human PDK1 is located in this region. We determined the *pdk-1* DNA sequence in the *sa680, sa709,* and *mg142* strains by PCR amplification and direct sequencing and found mutations in the pdk-1 open reading frame. The Daf-c phenotypes of *pdk-1(sa680)* and *pdk-1(sa709)* were both efficiently rescued by a *pdk-1(+)* transgene, confirming their assignment as alleles of *pdk-1*.

A loss of function mutation in *pdk-1* increases *C. elegans* life span almost two fold, similarly to a mutation in *age-1*. *daf-16(m27)* suppresses the longevity phenotype of *pdk-1(sa680)*. These results show that longevity regulation signals from the DAF-2 and AGE-1 signaling pathway are propagated by PDK-1, via AKT-1 and AKT-2, to the DAF-16 transcriptional outputs. A human PDK1 Ala277Val mutant, that is equivalent to pdk-1(mg142), activates PDK1 kinase activity towards Akt/PKB. The equivalent substitution mutation was constructed in human PDK1 and its kinase activity on human AKT substrate was 2.9 fold increased compared to wild type.

4. Cell nonautonomous role of DAF-9 cytochrome P450 in *C. elegans* larval development and gonadal migration

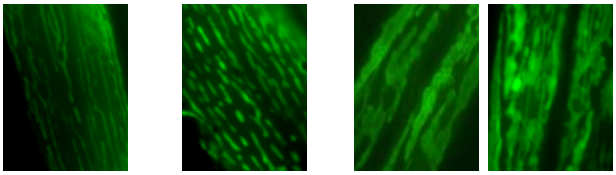
Genetic mosaic analysis showed that the DAF-2 insulin/IGF-I like receptor and the DAF-4 type II TGF-β receptor control reproductive development in a cell nonautonomous manner**.** A secondary signal is thought to be responsible for communication between the peptide hormone responsive tissues, such as the nervous system, and the rest of the body. Genetic analysis suggests that *daf-9* functions downstream of or in parallel to *daf-2* and *daf-7* and upstream of *daf-12*). *daf-9* encodes a cytochrome P450 enzyme whereas *daf-12* encodes a nuclear receptor (35,50). Furthermore, cholesterol withdrawal inhibits recovery from dauer arrest by *daf-9* hypomorphs (50). This argues that *daf-9* may participate in the modification of cholesterol in the biosynthetic pathways to steroid hormones. Because *daf-9* acts upstream of the nuclear receptor gene *daf-12*, DAF-9 may mediate the production of a lipophilic hormone that regulates DAF-12 activity. A rescuing *daf-9*::GFP fusion gene driven by the *daf-9* promoter is expressed in two head cells, XXX L and XXX R at all stages, in the hypodermis from mid-second larval stage (L2) to the fourth larval stage (L4), and in the spermatheca of adult hermaphrodite. Genetic analysis suggests that *daf-9* functions either downstream of or in parallel to *daf-16* and *daf-3* in the dauer pathway (35,50). When a *daf-9*::GFP fusion gene was introduced into *daf-2(e1370)* and *daf-7(e1372)* mutant animals, hypodermal expression of *daf-9*::GFP was absent in dauers but the neuronal *daf-9*::GFP expression persisted. Hypodermial *daf-9*::GFP expression is also down in dauer animals derived from starvation. In a daf-12 null mutant, dramatic reduction of hypodermal expression of *daf-9*::GFP was observed while expression in the head neurons and spermatheca persisted. Hence, DAF-12 activates *daf-9* gene expression in the hypodermis. Furthermore, *daf-9* and *daf-12* appear to form a feedback regulatory loop in which activated DAF-12 is down-regulated by an increase in antagonistic ligand production by DAF-9. We used the collagen gene *dpy-7* promoter to direct hypodermal expression of *daf-9* during larval stages. Restoration of *daf-9* activity in the hypodermis alone was sufficient to prevent dauer arrest of *daf-9(e1406)* mutant animals; these transgenic animals developed into reproductive adults with the same growth rate as animals carrying a *daf-9* transgene driven by its own promoter. We obtained similar results by restoring *daf-9* activity only in neurons under the control of the pan-neuronal *unc-14* promoter. Therefore, hypodermal or pan-neuronal expression of *daf-9* allows rescue of dauer arrest caused by a lack of *daf-9* gene activity. These results suggest that daf-9 p450 activity within a large number of tissues can rescue dauer arrest but that it is normally expressed in the XXX as well as hypodermal cells.

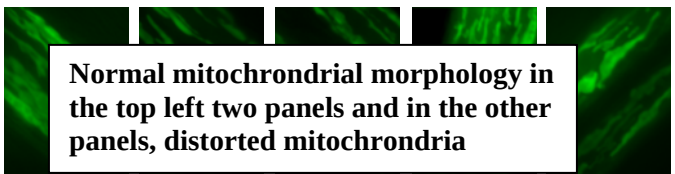
The XXX cells express daf-9

We could not induce dauer arrest by daf-9 RNAi suggesting that the XXX neuron is not sensitive to RNAi. But in some of our enhanced RNAi mutants, daf-9 RNAi does induce dauer arrest and is suppressed by daf-12. These mutants are therefore good strains to use for RNAi screens for the neuroendocrine output from the nervous system that regulates aging downstream of daf-2 signaling.

5. Food and metabolic signaling defects in a *C. elegans* serotonin synthesis mutant

Food level not only modulates *C. elegans* motor outputs, it also affects a metabolism-regulating neurosecretory axis. Food is one of the sensory inputs (a secreted pheromone and growth temperature are others) that control whether wild-type *C. elegans* enter the dauer stage, and store large amounts of fat. We found that 10-15% of *tph-1(mg280)* animals arrest at the dauer stage even in the presence of abundant food, unlike wild type. The dauer arrest phenotype is suppressed by growing *tph-1(mg280)* animals on plates supplied with serotonin. *tph-1 (mg280)* animals accumulate larger stores of fat than wild-type animals. Bacterial food and low temperature may normally up-regulate the production, release or response to serotonin to in turn up-regulate DAF-7 and insulin-like neuroendocrine signals for reproductive development and low fat storage. In the *tph-1* mutant, these neuroendocrine signals are decoupled from such food and temperature inputs. Serotonin signaling has been implicated in the control of mammalian feeding and metabolism. Thus mammalian 5HT levels may be regulated by nutrition and other food sensory cues to in turn regulate feeding behaviors, as well as neuroendocrine signals analogous or homologous to *C. elegans* DAF-7 TGF- eta and insulin-like hormones.





Normal mitochrondrial morphology in the top left two panels and in the other panels, distorted mitochrondria

6. A systematic RNAi screen reveals a critical role for mitochondria in *C. elegans* longevity

To identify in a systematic manner all of the of gene classes important for specifying *C. elegans* lifespan, we utilized a comprehensive set of more than 5,600 genes in an RNAi screen to identify genes whose inactivations

extend longevity. We found that RNAi inactivation of a large number of genes key to mitochondrial function dramatically extends *C. elegans* lifespan. For example, the chromosome I library contains RNAi clones corresponding to 2,663 genes, and we found that RNAi inactivation of 52 (1.8%) caused reproducible lifespan extension. These lifespan regulators encompassed genes of diverse cellular functions: gene expression, protein processing, signal transduction, stress response, energy metabolism and other uncharacterized functions. Intriguingly, 15% represent genes specific for mitochondrial function. Importantly, RNAi of these genes caused an increase in lifespan most comparable to RNAi of insulin pathway genes. It is also worth noting that none of the 5,690 RNAi inactivations extends *C. elegans* longevity more potently than RNAi of the insulin pathway genes.

Several of the mitochondrial lifespan regulators identified in the RNAi screen also exhibited pleiotropic phenotypes, especially when exposed to RNAi earlier in development. The lethality observed for some of the mitochondrial components inactivated by RNAi might represent the phenotype of severe loss of mitochondrial function, whereas lifespan extension might represent a weak loss-of-function phenotype.

These mitochondrial components regulate lifespan independently from the insulin signaling pathway; *daf-16(mgDf47)* null mutant worms, which decouple animal lifespan from the upstream insulin signaling, lived significantly longer when feeding on all but one (F13G3.7) of the RNAi bacteria targeting the mitochondrial components. Thus, most of the mitochondrial components regulate lifespan downstream of *daf-16* or parallel to the insulin-like longevity regulating pathway.

ATP levels were substantially reduced in worms undergoing RNAi against mitochondrial components. The oxygen consumption rate in the different RNAi-treated worms in general correlated well with their ATP content.

Using a muscle-specific GFP fusion gene that targets mitochondria to visualize the mitochondrial morphology in live worms, dramatic defects were observed in mitochondrial structure of worms with RNAi-inactivated mitochondrial components. Distorted mitochondria were observed in worms undergoing RNAi specific for the various electron transport chain subunits. Thus we can use this GFP fusion gene as a reporter for classifying genes whether they affect mitochondrial function or not.

Long-lived *daf-2* mutants are highly tolerant to multiple stress treatments, including paraquat, H_2O_2, and heat shock, and this heightened stress resistance is generally thought to be key to the extended longevity in *daf-2* worms. Interestingly, most of the long-lived worms with compromised mitochondria did not show increased resistance to paraquat. In contrast, worms with reduced *daf-2* signaling were highly resistant to paraquat in this experiment. It is possible that when facing increased ROS production, the long-lived worms with compromised electron transport sense the free radicals and induce defense mechanisms to counteract ROS damage and extend longevity.

This comprehensive RNAi screen provides us the first global view of animal longevity genes. Our results reinforce the idea that energy metabolism is critical for the determination of animal lifespan. The other gene inactivations that increase *C. elegans* longevity but are not annotated to be mitochondrial or metabolic enzymes are likely to reveal pathways that either respond to the metabolic shift caused by decreased energy production, or impact longevity in parallel to energetics.

From our new completed RNAi screen of all 16,857 clones, we have the following list:

~100 RNAi genes that potently affect lifespan: 23 metabolism (11 mito), 11 signaling, 6 neuro (neurotransmitter, 7Tm), 5 transcription, 1 small molecule transport, 2 RNA/protein synthesis, 2 RNA binding, 2 proteases (~insulinase), 1 collagen, 1 chromatin, 58 unknown.

Plus~ 500 other positives that we need to confirm with more tests on various sensitized strains. We are following up the weakly positives as well as the stronger positives because RNAi works poorly in the nervous system. Thus if any of these genes act within neurons they may score more poorly on the phenotypic assay depending on RNAi. Because we will be testing these primary screen candidates on strains with much better RNAi, they may make the transition to the list of stronger candidates.

7. Systematic genome-wide RNAi screen to identify mediators of the extended longevity of *daf-2(e1370)*.

The increase in lifespan of a daf-2 mutant is dependent on the action of various daf-16 downstream genes. We used the 17,000 clone RNAi library to inactivate genes in the long-lived *daf-2* mutant animals [*daf-2 (e1370)*]. This screen identifies genes which when inactivated shorten lifespan and thus normally function to enhance lifespan. This screen will identify more than just the direct targets of daf-16, in fact the entire response cascade.

To screen for genes suppressing the *daf-2* increased longevity phenotype, populations of *daf-2* mutant animals synchronized at the first larval stage (L1) were placed on each of 17,000 E coli each expressing a distinct dsRNA corresponding to one C. elegans gene, and approximately 22 days after reaching adulthood the percentages of live/dead animals were measured. At this time point, 100% of *daf-2* mutant animals exposed to *daf-16* RNAi are dead but animals treated with control RNAi are viable. RNAi clones with dead animals also showing developmental defects

were excluded, as mortality is due to sickness and not premature aging. To date, we have screened through 4,500 RNAi clones and found 1.8% of the clones suppress the enhanced lifespan of *daf-2* mutant animals. Two genes out of 39 identified by microarrays comparisons of daf-2 vs. wild type were also found in our screen: gei-7, encoding a dual enzyme functioning in the glyoxylate cycle, and K07C6.4, encoding a member of the cytochrome P450 family involved in electron transport. This RNAi screen and our conserved DAF-16 target gene informatics-based approach both identified *pnk-1* as a suppressor of daf-2 longevity. p*nk-1* encodes one of two pantothenate kinases in *C. elegans*, the rate-limiting enzymes in coenzyme A synthesis.

Positives from our screen were classified based on how completely the increase in lifespan of *daf-2* mutant animals was suppressed and were assigned into one of twelve categories based on biological function. Six transcription factors were strong suppressors of *daf-2* longevity, including *daf-16* and heat shock factor-1 (*hsf-1*), a transcription factor known to regulate aging. These positives validate the effectiveness of the screen. Genes involved in signal transduction including known and predicted kinases, phosphatases, G-protein coupled receptors, and chemoreceptors are prominently represented. Other classes include genes involved in metabolism, electron transport, RNA synthesis/processing, protein degradation, and proteolysis. However, the largest category of positive clones target genes of unknown function (40%). Secondary screens and functional tests will confirm true positives. Most importantly, we are still in the process of classifying our positives as to whether they also affect wild type lifespan and whether they show signs of premature aging, rather than general sickness. This is a major aim of the grant proposal.

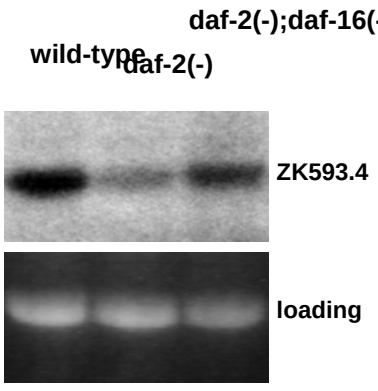
8. Insulin-regulated DAF-16 target genes that control *C. elegans* lifespan and metabolism

The work in this paper was supported by the Ellison Medical Foundation. We will not follow these genes up in detail in this RO1 but will use some of the identified genes as reporters for aging genetic and genomic studies.

Multiple *daf-16* transcriptional targets are likely to mediate the diverse functions of *daf-2*/insulin-like signaling. Because the *daf-2*/insulin receptor to *daf-16*/FOXO pathway regulates both longevity and metabolism in *C. elegans*, *Drosophila*, and mammals, DAF-16/FOXO might control homologous target genes in the different species to mediate the conserved functions. DAF-16 and its mammalian homologs bind to an identical consensus DNA sequence (TTGTTTAC) *in vitro*, and FOXO3 binds to this consensus site in the *MnSod* promoter in mammalian cells, and binding is required for FOXO3 trans-activation of *MnSod*. We identified DAF-16 transcriptional targets by searching for DAF-16 binding sites in the regulatory regions of genes. Given the high expected rate of detecting a DAF-16 binding site by chance alone (3,700 sites expected by chance), the search of such a site upstream of a *C. elegans* gene and upstream of its ortholog in a divergent animal species would enrich for authentic DAF-16 sites.

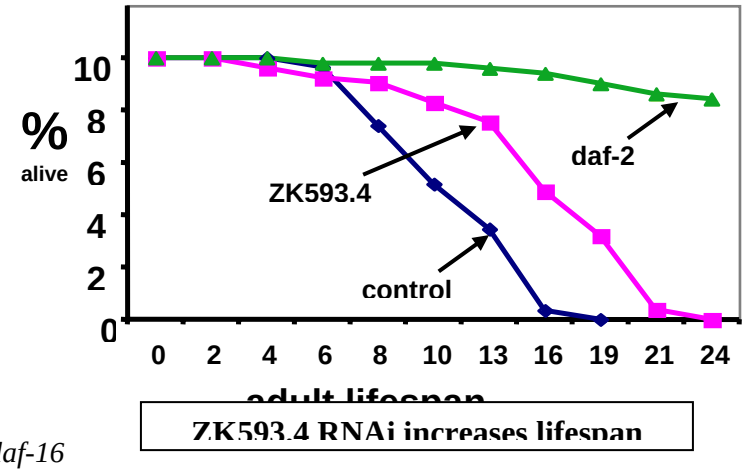
We surveyed 1 kb upstream of the predicted ATG of 17,085 *C. elegans* and 14,148 *Drosophila* genes, and identified 947 *C. elegans* and 1,760 *Drosophila* genes that contain at least one perfect-match to the consensus DAF-16 binding site within the 1 kb promoter region. We then compared these DAF-16 binding site containing worm and fly genes with a list of 3,283 *C. elegans* and *Drosophila* that are orthologous to each other, and identified 17 genes that are orthologous between *Drosophila* and *C. elegans* and bear a DAF-16 binding site within 1 kb of their start codons in both species. One *Drosophila* and one *C. elegans* candidate target gene had more than one DAF-16 binding site within the 1 kb region. We have also searched for DAF-16 binding sites in the intergenic regions upstream of each annotated worm gene to the next upstream gene, and each annotated fly gene up to the next upstream gene, and filtered for DAF-16 sites in both the worm and fly orthologous genes to reveal a total of 115 DAF-16 candidate target genes. Interestingly, the gene PEPCK is prominent on this list. This is the classic insulin regulated gene studied in mammals. It is very satisfying that our informatic search between worms and flies rediscovered this classic insulin-regulated mammalian gene.

We compared the RNA expression level of a random set of these candidates in wild-type, *daf-2(e1370)*, and *daf-2(e1370);daf-16(mgDf47)* animals. We found that 6 of the 17 (~35%) predicted DAF-16 downstream genes were differentially expressed in *daf-2* and *daf-2;daf-16* mutant animals, indicating that their expression was regulated by insulin signaling through *daf-16*. Three of the 6 genes were expressed at 3x to 7x higher levels in a *daf-2* mutant compared to wild type or the *daf-2; daf-16* double mutant. This fraction of genes robustly regulated by the *daf-2* pathway is much higher than that expected to occur by chance; data from a microarray analysis indicates that 1% of the 16,721 *C. elegans* genes tested were regulated by three-fold or more.



The expression of ZK593.4, T21C12.2, and F43G9.5 was downregulated and that of C10G11.5, F52H3.5, and C39F7.5 was upregulated in the *daf-2* mutant in a *daf-16* dependent manner. We used RNAi to reduce each of their expression and assayed whether lifespan, dauer arrest, and fat storage were affected, using wild-type or *rrf-3(pk1426)*, and *daf-2(e1370)* or *age-1(hx546)* strains. We expect RNAi inactivation of the genes downregulated in the *daf-2* mutant to promote *daf-2* mutant phenotypes, including lifespan extension, dauer arrest, and increased fat storage, and RNAi of the genes upregulated in the *daf-2* mutant to suppress the *daf-2* mutant phenotypes.

ZK593.4 expression is regulated by daf-2 and daf-16



ZK593.4 RNAi increases lifespan

A number of the 17 genes tested passed these functional filters. For example, RNAi of ZK593.4 (*rbp-2*) and T21C12.2 (*hpd-1*), genes that are downregulated in the *daf-2* mutant, caused *rrf-3(pk1426)* animals to live considerably longer than those undergoing control RNAi or RNAi of an unrelated gene. *rbp-2* might specifically regulate lifespan, whereas *hpd-1* might have a broader role in *daf-16* regulation of both dauer arrest and longevity. *rbp-2* encodes a homolog of the mammalian RB binding protein 2 (RBP2), which is implicated in gene expression control and chromatin remodeling. *sir-2*, which modulates longevity in yeast and in *C. elegans*, encodes a histone deacetylase, also highlighting a role for chromatin remodeling in longevity control. *rbp-2* might be regulated by DAF-16 to further modify chromatin when *daf-2* signaling is decreased. *hpd-1* encodes the enzyme 4-hydroxyphenylpyruvate dioxygenase involved in the catabolism of phenylalanine and tyrosine to fumarate and acetoacetate. Insulin signaling might regulate the catabolism of tyrosine to coenzyme Q, a key intermediate in electron transport.

9. Regulation of DAF-2 receptor signaling by human insulin and *ins-1*, a member of the unusually large and diverse *C. elegans* insulin gene family

Insulin and its related proteins define a superfamily of secreted proteins that share a structural motif stabilized by a set of stereotypical disulfide bonds. Bombyxin in silk moths, and the neurons that secrete locust and molluscan insulin-related proteins regulate metabolism, implicating insulin-like proteins in metabolic control broadly in animal phylogeny.

To comprehensively identify *C. elegans* insulin superfamily genes, we analyzed the entire *C. elegans* genomic sequence with multiple sequence searching tools. This procedure revealed 37 candidate genes, *ins-1* through *ins-37*. The INS-1 protein is the most closely related to human insulin by primary sequence comparison , structural homology models, and because it has a probable cleaved C peptide. Consequently, we explored its action in the DAF-2 signaling pathway.

The *ins-1* transgene, providing approximately twenty times the normal gene dosage of *ins-1*, causes 43% and 84% of the animals, respectively, to arrest at the dauer stage, suggesting that *ins-1* acts antagonistically to DAF-2.

Human insulin was expressed in the same cells that express *ins-1*, using the 5' flanking region of *ins-1* to drive a human insulin cDNA. This *ins-1* regulatory region drives the expression of GFP in a variety of neural and non-neural tissues. Human insulin expression causes a low level of dauer arrest in wild type at 26°C and strongly enhances dauer arrest in the *daf-2(e1365)* and *daf-7(e1372)* backgrounds at 20°C. Thus human insulin can also engage the worm insulin signaling pathway.

We constructed gene fusions of the upstream promoter and enhancer regions for 14 of the *ins* genes, including *ins-1, ins-18, ins-9*, and *ins-22*, to the coding region of GFP. These *ins* genes are expressed primarily in subsets of sensory neurons throughout most of the life cycle. All *ins* genes tested except *ins-11* are expressed in at least some amphid sensory neurons. *ins-1* and *ins-9*, are expressed in the amphid sensory neurons ASI and ASJ, which regulate dauer arrest. *ins-1* is also expressed in ASH and three other unidentified neurons in the nerve ring, as well as NSM. While *ins-9* is expressed exclusively in amphid sensory neurons, *ins-1* is also expressed in other neurons, intestine, and vulval muscles. Most *ins* genes are also expressed in a small number of other neurons.

Many of the *C. elegans ins* genes are organized into clusters of three to seven genes. The clustering, tandem arrangement, and closer sequence similarity between members of a given cluster suggests that these clusters arose relatively recently by gene duplication.

10. *daf-28* encodes a *C. elegans* insulin superfamily member that is regulated by environmental cues and acts in the DAF-2 signaling pathway

We found that that *daf-28* encodes an insulin-like protein, which when mutated causes dauer arrest and down regulation of DAF-2/IR signaling. A *daf-28::*GFP fusion gene is expressed in ASI and ASJ, two sensory neurons that regulate dauer arrest. *daf-28::*GFP expression in ASI and ASJ is down-regulated under dauer-inducing conditions and in mutants of DAF-11/guanylyl cyclase, a predicted component of the dauer pheromone sensing pathway. Thus *daf-28* expression in sensory neurons is regulated by the environmental cues that normally trigger dauer arrest.

DAF-28 is most related to the other β type INS proteins in *C. elegans*; most of the β insulins including DAF-28 have a predicted F-peptide in proinsulin. Among these *ins* genes, only *ins-4* and *ins-6* at high gene dosage suppress *daf-28(sa191)*. Consistent with this result, INS-4 and INS-6 are most similar to DAF-28 among the INS proteins in *C. elegans*.

daf-28 transcription responds to the DAF-11/guanylyl cyclase signal, which functions in the sensory neurons to transduce dauer pheromone signals to cyclic GMP gated channels to in turn control dauer arrest. *daf-28* expression is age-sensitive. P_{daf-28}::GFP is expressed throughout adulthood, unlike *daf-7* expression, which is limited to larval stages. The observations that P_{daf-28}::GFP expression in head neurons increases from an average of 4 neurons to 8 neurons as animals age, and that gonadal GFP expression is only observed in old animals, suggest that *daf-28* expression is subject to regulation by aging-related factors. In addition, the overall level of P_{daf-28}::GFP expression is elevated in several mutants with sensory neuron defects, which also live longer than wild-type animals (3). This observation suggests that *daf-28* may be regulated by some sensory factors that also regulate longevity. DAF-28 alone may not be a determinant in longevity, but alteration of its expression pattern may reflect the regulation of *ins* genes as animals age. In fact, many *ins* genes have been shown to change expression levels during the aging process (69). It will not be surprising if the higher levels of expression observed with microarray and SAGE analyses are the result of broadened expression patterns such as those we have observed for *daf-28.*

11. Insulin-like Signaling Negatively regulates RNA Interference in *C. elegans*

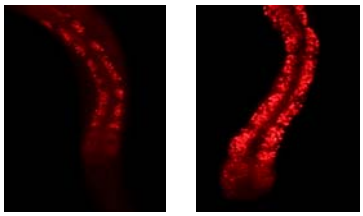
Mutants lacking *age-1* activity show enhanced response to RNAi. *age-1(mg305)* displays a temperature-sensitive enhanced RNAi response at the non-permissive temperature of 25C. For instance, loss-of-function mutation in the *lin-1* ETS transcription factor gene results in multiple vulva structure (Muv) in *C. elegans*. RNAi of *lin-1* causes multiple vulva in 96% of the *age-1(mg305)* mutants at 25 C, but 0% at 20 C. RNAi of *lin-1* has no effect on the wild-type animals at both temperatures. An additional mutation in *daf-16*, the downstream fork-head transcription factor gene in the insulin-like pathway, rescues both the enhanced RNAi and dauer-constitutive phenotypes of *age-1(mg305)*. Mutants of *daf-2*, a gene encoding an insulin/IGF-like receptor in the insulin-like pathway, resemble *age-1* in their enhanced response to RNAi. These data suggest that the insulin-like signaling may play an inhibitory role in RNAi. Northern analysis shows that after RNAi the target mRNA level is significantly decreased in *age-1(mg305)* or in the previously known RNAi enhancer mutant *rrf-3(pk1426)* (103a), whereas no change is observed in wild type. Therefore, the insulin-like pathway affects RNAi by facilitating the suppression of target mRNA.

Three parallel signaling cascades regulate dauer arrest in *C. elegans*: the *daf-2/* insulin-like, *daf-7*/ TGF- -like, and *daf-11/* cyclic GMP pathways (66d). Mutants lacking activity of either pathway arrest as dauers. To decide whether the RNAi-enhanced phenotype is caused by some general dauer-inducing signal shared by all three pathways, we tested the RNAi response of dauer-constitutive mutants in other two pathways, *daf-7/ TGF-* and *daf-11/ guanylate cyclase*. *daf-11(mg295ts)* behaves as wild type to dsRNA. *daf-7(mg1372)* is slightly more sensitive than wild type to the *lin-1* (22% multiple vulval structure) and *hmr-1* (41% embryonic lethal) dsRNAs, but wild-type to the *his-4* (7% embryonic lethal) or *col-183* (not dumpy) dsRNAs (Table 2). Therefore, we conclude that mutations in the insulin-like pathway, but not the cyclic GMP pathway, negatively affect RNAi. Mutants lacking the insulin-like signaling, such as *daf-2* and *age-1*, are more tolerant than wild type to environmental stresses (46a). RNA interference induced by exogenous dsRNA in terms of viral RNA or transposon is also a defensive response to protect genome stability. It is, therefore, intriguing to propose that the two defense systems interact with each other and both are under tight control.

12. Genome-wide RNAi analysis of *C. elegans* fat regulatory genes

Regulation of body fat storage involves signalling between feeding regulatory centers in the brain and sites of fat storage and utilization in the body. We used the vital dye Nile Red to visualize fat storage droplets in living worms. Addition of Nile Red to *E. coli*, the lab diet of *C. elegans*, resulted in uptake and incorporation of the dye into lipid droplets in intestinal cells, the major site of worm fat storage. The increased fat stores in animals with decreased *daf-*

2(e1370) insulin-like signalling were readily detectable in living animals by Nile Red staining. To correlate the differences in body fat visualized by Nile Red with actual fat content, total lipids were extracted from wild type and mutant animals, separated by thin layer chromatography into triacylglyceride and phospholipid components, and quantified by gas chromatography. Consistent with the fat histochemical results, *daf-2(e1370)* animals had 2.5-fold greater total fat content than wild type animals. The increased fat content of these animals was strongly suppressed by the *daf-16(*mg*Df47)* null mutation. Moreover, excess fat in *daf-2(e1370)* animals was largely in the form of storage triacylglycerides, suggesting that in *C. elegans* as in mammals, triacylglycerides are the main fat storage form. Using the Nile Red fat assay, we screened for gene inactivations that affect fat content, fat droplet morphology, and the pattern of fat droplet deposition. Of 16,757 genes tested by RNAi, 1.8% (305 genes) caused reduced fat or a distorted fat deposition pattern, while 0.7% (112 genes) resulted in animals with increased fat or enlarged fat droplet size. RNAi inactivation of another 261 genes caused reduced fat but were also accompanied by larval arrest, embryonic lethality or sterility. This latter group includes known genes with critical roles in fat biosynthesis and metabolism such as acetyl-CoA carboxylase, fatty acid synthase, and the fatty acid desaturase *fat-7*. The diverse array of the genes identified in our screen provides an unprecedented glimpse of cellular machineries that regulate body fat content and deposition. The concordance between the list of *C. elegans* and mammalian fat regulatory and obesity genes suggests ancient and common pathways of body fat regulation. Importantly, over 50% of the *C. elegans* fat regulatory genes identified in our screen have mammalian homologs that have not previously been implicated in regulation of fat storage. It is likely that homologs of many of these newly identified *C. elegans* fat regulatory genes also control mammalian body weight.



Increased Nile Red fat in daf-2 animals on the right

An interesting feature of the fat RNAi screen and the aging RNAi screen is that there is essentially no overlap between the genes detected. It seems that even though daf-2 co regulates fat storage and longevity and even though there is a connection between metabolism and aging in many systems, the genes that regulate fat storage do not cause increased longevity. Now that we are testing for genes that are necessary for long life in a daf-2 mutant, we may find more overlap.

13. **Regulation of fat storage by the C. elegans homolog of tubby.**

During the last decade, major progress on the genetics of metabolism has been realized through the study of the nematode *Caenorhabditis elegans*. The biochemical functions of many of these genes is now known and because they are related to processes implicated in vertebrates, have potential general significance to obesity. The clearest example of such a biochemical convergence is the finding that an insulin-like signaling pathway regulates longevity and metabolism in *C. elegans* (54). *daf-2, age-1,* and *pdk-1* mutants that are components of the *C. elegans* insulin signaling pathway live 2x to 3x longer than wild type and accumulate much larger stores of fat. This insulin-like signaling pathway is part of a global endocrine system that controls whether the animals grow reproductively or arrest at the dauer diapause stage.

We have also tested thousands of genes by RNAi of *C. elegans* and have discovered hundreds of promising candidates that may determine how fat is stored and used in a variety of animals. The findings represent the first survey of an entire genome for all genes that regulate fat storage. We identified about 300 worm genes that when inactivated decrease fat content and about 100 genes that when inactivated caused increased fat storage. This list of 400 genes constitutes the metabolism and communications pathway that controls the how much fat is stored in an animal body. The identified genes were very diverse, including both the expected genes involved in fat and cholesterol metabolism as well as other new candidates, including some that are expected to function in the central nervous system.

There are human counterparts to about 200 of these genes and in some cases the genes encode proteins that are attractive for the development of drugs. Thus, we suggest that some of the genes identified could point the way for designing drugs to treat obesity and its associated diseases such as diabetes.

We found that some of the identified genes were effective at regulating fat levels in all strains of *C. elegans* and others could only regulate fat in certain worm obesity syndromes caused by neural defects. This is significant because at this early stage of understanding the complex regulation of human fat, it is already clear that our brains play a major role: signals from fat cells, for example, the well known hormone leptin, are sensed by regions deep in our brains to in turn control feeding. Some obese people have defects in the ability to send or receive these signals of fat storage and

have a continuous voracious appetite. Even in the usual middle age spread, the difference between staying slim or gaining 20 pounds over 20 years is a difference of about one part in a 1000 of food intake/energy balance. That is, a non-obese person eats exactly what he or she metabolizes, whereas a run of the mill obese person eats .1% too much. This highlights the fine tuning of our consumption of food to our energy balance. Subtle defects in the control of feeding by our brains and the complex interplay between our brain and our fat are the root cause of most obesity.

The difference between this current worm screen and the past discoveries of fat regulatory genes in humans is the systematic nature of the worm screen. Essentially every worm gene has now been tested, rather than the less comprehensive traditional genetic approach that depends more on who walks into the clinic and then careful genetic mapping within the family of that patient, to explore the detailed workings of fat storage. Our study has given us a glimpse of every gene that regulates fat in a worm, and we hypothesize, many in humans.

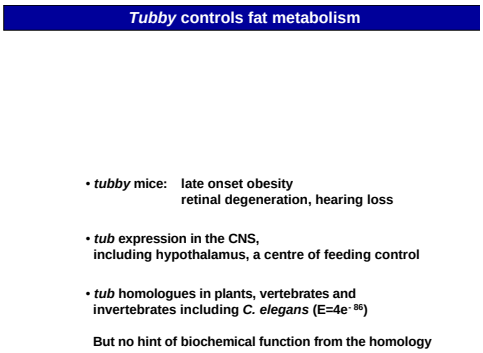
The reason that the worm can be used to discover these fat regulatory genes is that the common ancestor to worms and humans, about 600 million years ago, also stored fat and regulated its feeding and metabolism based on communication between its stored fat and the brain centers that control feeding. Both the worm and humans have inherited this complex system from our common ancestor. It is likely that failure of these circuits is one of the underlying causes of obesity. The challenge now is to unravel these regulatory pathways and prioritize the relevant genes in animal models, such as the worm, but also in the mouse for example

The mammalian *tub* gene encodes an evolutionarily conserved protein that is highly expressed in the central nervous system, notably in the hypothalamus, a center of feeding control. Loss of function mutations in *tub* lead to late-onset obesity, retinal degeneration and hearing loss in *tubby* mice. The phenotypes of *tubby* mice resembles that of patients with Alstrom and Bardet-Biedl syndromes although no mutation in the human *tub* homologue has been found that is associated with late onset obesity and neurodegeneration. Nevertheless, mutations in Tulp1, a *tubby* gene family member, is the cause of one form of human retinitis pigmentosa (RP14), thus underlying the importance of this gene family in human health and disease. Consistent with the action of tulp1 in ciliated sensory neurons, we showed that the worm ortholog of tubby is also expressed in ciliated sensory neurons. It is possible that tubby in the hypothalamus is also expressed in neurons of this type; one tantalizing possibility is that these hypothalamic neurons sense fat levels. Despite the conservation of the *tubby* gene family and the well documented phenotypes of *tubby* mice, the cellular role and molecular function of the TUB protein family remain to be ascertained. The TUB-like protein 1 (TULP1) has been implicated in intracellular vesicular transport of rhodopsin in the retina. It has been reported that TUB can be phosphorylated by the insulin receptor, and its subcellular localization can be modulated by G-protein coupled receptor signaling. However, it is unclear how these observations are related to the role of TUB in regulation of body weight.

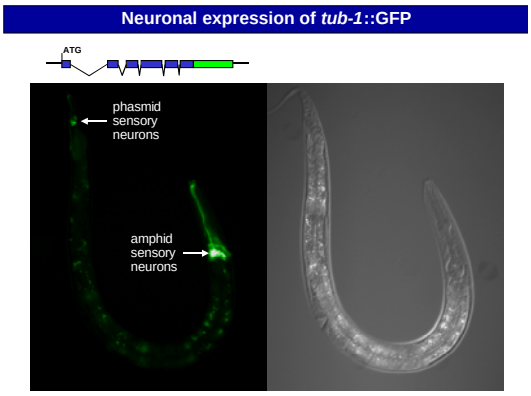
To better understand the molecular function of TUB will clearly require the identification of additional genes that act in the pathway with tub, both upstream and downstream of *tub*. These *tub* pathway genes may mediate *tub* function in the nervous system or act distally in peripheral tissues to affect energy balance and fat metabolism in response to neuronal *tub* activity. There is a single *tub* homologue in *C. elegans*, thus avoiding complications from functional redundancy when multiple gene family members are present. More importantly, *C. elegans* is amenable to large scale genetic and functional genomic screens which is not feasible in mice. It has been shown that genes that govern fat storage and metabolism are highly conserved from *C. elegans* to human. Therefore, identification of *tub* pathway genes in *C. elegans* may provide targets for intervention of obesity in human. In fact the genetic analysis we have done so far has revealed that tubby in C. elegans sensory neurons couples to peroxisomal beta oxidation in the fat storage tissues. This is the first evidence of a possible central role of perosisomes in fat storage and or signaling. This genetic analysis has also revealed that a C. elegans homolog of a human Bardet-Biedl syndrome gene also acts with tubby in control of fat storage, proving once again of the homology between the worm and mammalian fat storage circuits. The C. elegans Bardet-Biedl homolog bbs-1 is also highly synergistic with defects in peroxisomal beta oxidation, once again pointing to a connection between ciliated sensory neurons and signals to fat cells to control types of beta oxidation. We have a large collection of mutants that synergize with the peroxisomal pathway mutants, which should reveal other signaling components of this pathway, both in the neuroendocrine cells as well as in the fat storage depots.

The fat signaling genes identified by C. elegans genetics may reveal components of insulin, tubby, and other signaling pathways in mammals that are important for the understanding and eventual treatment of obesity and diabetes. It is clear that it is at least in part a genetic disease, but is likely to be multifactoral based on pedigree analysis.

The mammalian *tub* gene encodes an evolutionarily conserved protein that is highly expressed in the central nervous system, notably in the hypothalamus, a center of feeding control. *tub* is also transcribed in the spiral ganglion of the inner ear and in the photoreceptor cells of the retina. Loss of function mutations in *tub* lead to late-onset obesity, retinal degeneration and hearing loss in *tubby* mice. The obesity phenotype of *tubby* mice is evident at 8 to 12 weeks of age and *tubby* mice eventually weigh twice as much as wild-type control. This is in contrast to other established murine obesity models such as the *obese* and *diabetes* mice which harbor mutations in the leptin and leptin receptor genes respectively, where the obesity phenotype is evident at postnatal period. The phenotypes of *tubby* mice resembles that of patients with Alstrom and Bardet-Biedl syndromes although no mutation in the human *tub* homologue has been found that is associated with obesity and neurodegeneration. Nevertheless, mutations in *Tulp1*, a *tubby* gene family member, is the cause of one form of human retinitis pigmentosa (RP14), thus underscoring the importance of this gene family inhuman health and disease.



Immunoelectron microscopy analyses using anti TULP1 antibodies on wt mouse retinal sections show that TULP1 is distributed uniformly throughout the photoreceptor inner segments, perikarya, and synaptic terminals but is excluded from the outer segments and the nuclei. In addition, rhodopsin sorting to the outer membrane is not normal in the tulp1 homozygous knockout mouse and in the tulp1 tubby double homozygous mutant mouse. Thus tubby and its homologs is implicated in sorting membrane proteins from the inner segment to the outer segment of ciliated neurons. Interestingly, recent studies suggest that the Bardet-Biedl syndrome obesity is due to defects in ciliated neurons as well. Thus it is likely that tubby in *C. elegans* and humans causes obesity due to its action in ciliated neuron function in both species. What does not emerge from these studies is what extracellular signal these ciliated neurons respond to in the case of fat storage regulation---is it small molecule measures of fat levels, for example fatty acids or cholesterol derivatives? Also not clear at this point is what the output of these ciliated neurons is - is it a neuropeptide analogous to NPY?



Despite the conservation of the *tubby* gene family and the well documented phenotypes of *tubby* mice, the cellular role and molecular function of the TUB protein family remain to be ascertained. The TUB-like protein 1 (TULP1) has been implicated in intracellular vesicular transport of rhodopsin in the retina. It has been reported that TUB can be phosphorylated by the insulin receptor, and its subcellular localization can be modulated by G-protein coupled receptor signalling (95). However, using C. elegans tubby and serotonin-deficient mutants, we have not been able to repeat in C. elegans reports that tubby is nuclear localized in the low serotonin signaling. We think it is more likely that these mammalian studies, which used over-expressed genes, discovered artifactual coupling of tubby to serotonin pathways, and that the *C. elegans* genetics we report will reveal a more accurate view of tubby molecular function.

To better understand the molecular function of TUB will clearly require the identification of additional upstream and downstream genes that act in the pathway with *tub*. These *tub* pathway genes may mediate *tub* function in the nervous system or act distally in peripheral tissues to affect energy balance and fat metabolism in response to neuronal *tub* activity. There is a single *tub* homologue in *C. elegans*, thus avoiding complications from functional redundancy when multiple gene family members are present.

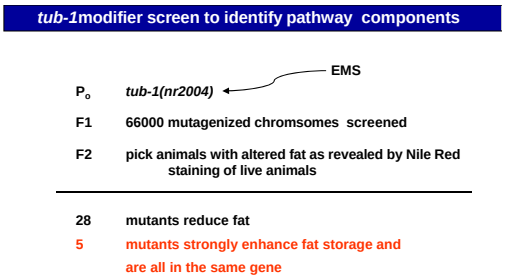
A single predicted open reading frame of the *tubby* gene family, originally designated F10B5.4 and renamed *tub-1*, was identified in the genomic sequence of *C. elegans*. As is the case for other members of this gene family, sequence conservation is evident only in the carboxy-terminal half of the protein.

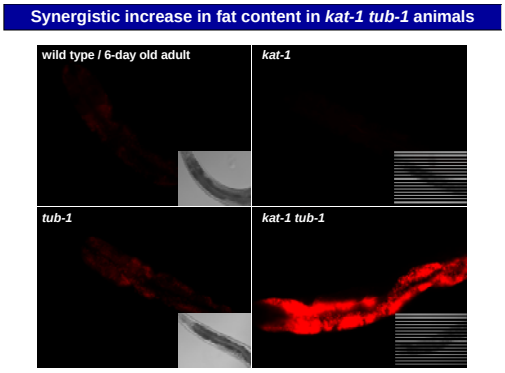
To determine the expression pattern of *tub-1*, we generated a *tub-1*::GFP fusion gene that bears 1.5 kb of the *tub-1* 5' regulatory region, the entire *tub-1* coding sequence, including introns, and inserted the coding sequence of green fluorescence protein (GFP) immediately 5' to the initiator codon of *tub-1*. Expression of the N-terminally tagged *TUB-1*::GFP fusion protein rescued the enhanced fat accumulation phenotype of *kat-1 tub-1* mutant animals (see later), suggesting that the fusion protein is faithfully expressed in tissues where endogenous *TUB-1* is found and is

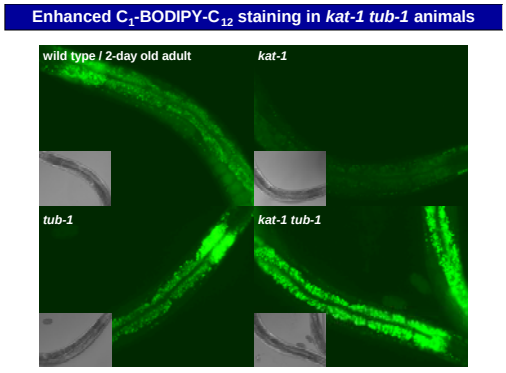
targeted to the correct subcellular compartments. *TUB-1*::GFP expression was observed in a subset of neurons, including high levels of expression in the ciliated amphid neurons in the head and in the ciliated phasmid neurons in the tail. Lower levels of expression were observed in neurons whose locations are consistent with those of other ciliated neurons in the animal. Some of the GFP expressing neurons in the head anterior to the amphid neurons were tentatively identified as the inner and outer labial neurons. In addition, GFP expression was observed in neurons identified as ADE (a pair of bilaterally symmetric neurons in the deirid sensillum), AQR (an asymmetric neuron in the head), PDE (a pair of bilaterally symmetric neurons in the postdeirid sensillum), and PQR (an asymmetric neuron in the tail). The *TUB-1*::GFP fusion protein is localized to the neuronal cell body, the dendrites and the ciliated endings but is excluded from the nucleus.

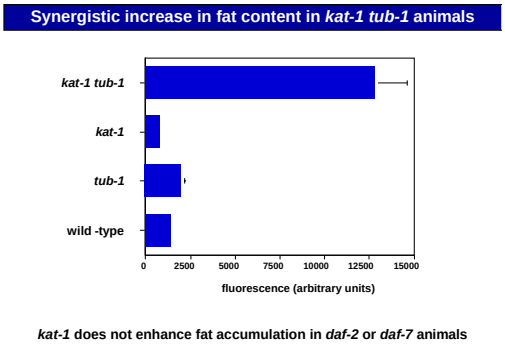
To determine the function of *tub-1*, we used PCR-based screening approaches to isolate two independent *tub-1* deletion mutations. The *tub-1(nr2004)* deletion, isolated from a strain carrying a *Tc1* transposon insertion allele, removes 3.2 kb spanning the entire *tub-1* open reading frame. The *tub-1(nr2044)* deletion, isolated from a library of animals mutagenized with diepoxyoctane, removes 2.2 kb spanning all but the first of the six exons in the gene. *tub-1(nr2004)* and *tub-1*(nr2044) are most likely molecular null alleles and they display similar mutant phenotypes. We focused our analysis on *tub-1(nr2004)*.

Tubby mutant mice suffer from degeneration of ciliated neurons in the retina and inner ear where *tubby* is highly expressed. Given the expression of *tub-1* in ciliated neurons in *C. elegans*, we wondered if *tub-1(nr2004)* mutant animals have any structural defect in their ciliated sensory neurons. We stained *tub-1(nr2004)* animals with the vital dye DiI which is taken up by 12 amphid and 4 phasmid sensory neurons that have ciliated endings exposed to the environment. We found that *tub-1(nr2004)* animals are not dye filling defective in their entire life cycle, including post-reproductive adults, which suggests that *tub-1* is not necessary for the differentiation or maintenance of structural integrity of ciliated sensory neurons in *C. elegans*. Next we examined whether *tub-1(nr2004)* mutant animals accumulate more lipid, thus mimicking the obesity phenotype of the *tubby* mutant mice. To monitor the lipid content of live animals, we used the vital dye nile red that fluorescence in neutral lipid particle in the intestine, the major lipid storage organ in *C. elegans*. Indeed, Nile Red fluorescence is elevated by 1.5-fold in *tub-1(nr2004)* animals when compared to wild-type control at both reproductive and post-reproductive stages. Such increase in lipid accumulation of *tub-1(nr2004)* animals was verified by staining with a BODIPY labeled fatty acid analogue (C1-BODIPY-C12). Taken together, our results suggest an evolutionarily conserved role of *tub-1* and *tubby* in the control of lipid homeostasis from ciliated neurons.









kat-1 does not enhance fat accumulation in daf-2 or daf-7 animals

Obesity in *tub-1*(-) animals defective in peroxisomal fatty acids β-oxidation

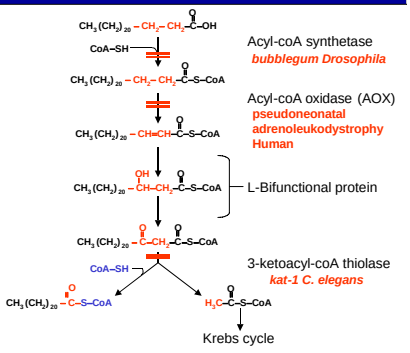
To explore the pathway by which tub-1 regulates fat storage, we performed a forward genetic screen for mutants that synergistically cause dramatic increases in fat in a tub-1 mutant but not in wild type animals. We identified five mutants that confer a dramatic, *tub-1(nr2004)* dependent increase in lipid accumulation as indicated by staining with the vital dye Nile Red. These five mutants, *mg368*, *mg399*, *mg400*, *mg401* and *mg402*, define a single gene, *kat-1*, and are phenotypically indistinguishable. For example, *kat-1(mg368) tub-1(nr2004)* double mutant animals display up to 6-fold increase in lipid

content when compared with *tub-1*(-) or *kat-1(mg368)* mutant animals, and a 9-fold increase in lipid content relative to wild-type control. The severity of the mutant phenotype is age dependent and is most prominent in post-reproductive animals. A similar phenotype was observed with a second allele of *tub-1*, in *mg368 tub-1(nr2044)* animals and other alleles of *kat-1*. The enhanced lipid accumulation phenotype of *kat-1 tub-1(nr2004)* animals were verified by staining with a BODIPY labeled fatty acid analogue. These *kat-1* alleles are recessive.

Despite excessive accumulation of lipid, reproductive development and brood size of *kat-1(mg368)*; *tub-1(nr2004)* animals is not compromised. The pharyngeal pumping rate and defecation cycle of well-fed animals also appeared to be normal indicating perhaps the increase in lipid accumulation is not due to increase in food consumption or retention.

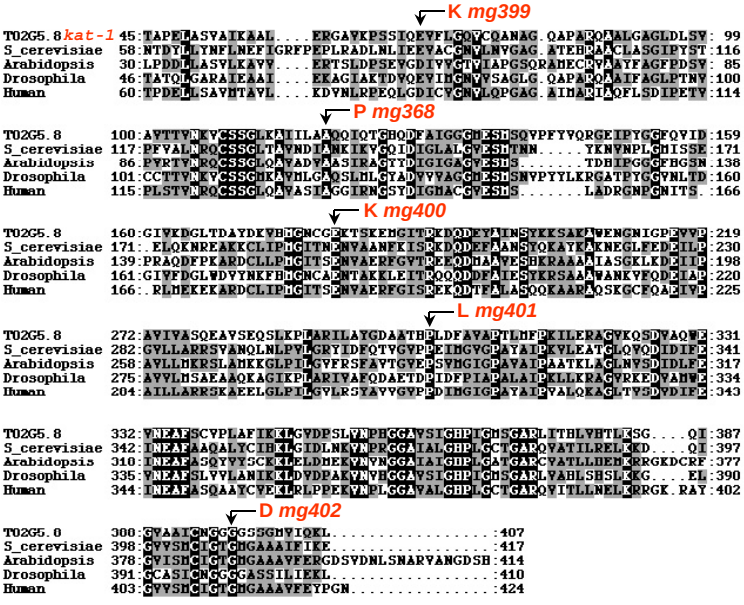
By SNP (single nucleotide polymorphism) mapping and cosmid rescue, we cloned the gene mutated in *mg368* and found that it encodes a peroxisomal 3-ketoacyl-coA thiolase (*kat-1*, T02G5.8), an enzyme involved in β-oxidation of very long chain fatty acids. Peroxisomal 3-ketoacyl-coA thiolase is a highly conserved enzyme that can be found from yeast to human. The *C. elegans* KAT-1 is 30% identical and 48% similar to human ACAA1 (NP_001598). Sequencing of *kat-1* coding region in all five mutant alleles revealed mis-sense mutations that lead to substitution of highly conserved amino acid residues. The *mg368* mutation converts alanine 119 to proline that probably leads to a severe loss of function, since it disrupts an α-helix that is critical for the architecture of the catalytic site and dimerization property of the enzyme, based on the crystal structure of its yeast homologue, Pot1p.

Peroxisomal β-oxidation



The KAT-1 enzyme acts at the last step in the β-oxidation of fatty acids. Inactivation of KAT-1 may potentially lead to accumulation of fatty-acyl-coA and other β-oxidation intermediates.

Sequence alignment of 3-ketoacyl-coA thiolase family members

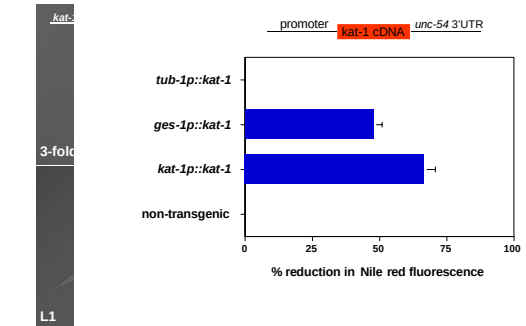


kat-1 expression in non-neuronal tissues

To determine the expression pattern of *kat-1*, a transcriptional GFP fusion gene, *kat-1*p::GFP was constructed. Expression of *kat-1p::GFP* was detected in the intestine from late embryogenesis, and in the body wall muscle, hypodermis and phaynx from larval stage 2 (L2). We also observed weak and inconsistent *kat-1p::GFP* expression in at least two pairs of unidentified head neurons. This suggests a distinct role of *kat-1* in the intestine or indicates that peroxisomal β-oxidation may be used early in development of this tissue.

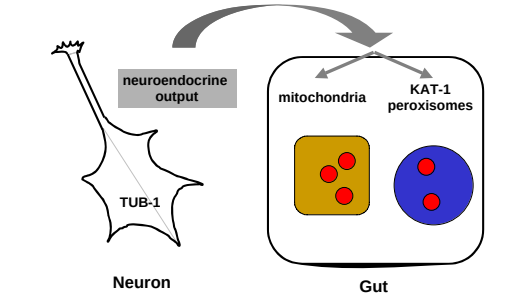
What is the molecular basis of synergism between *tub-1* and *kat-1* loss of function mutations? Our working hypothesis is that *tub-1* mutation causes a change in the neuropeptide output of ciliated neurons to then change the type of beta oxidation in the intestine where fat is stored. But it is also possible that both tub-1 and kat-1 act in the ciliated neurons to change the spectrum of fat derivatives in that neuron. For example, *kat-1* may affect *tub-1* activity or function by modulating lipid content of *tub-1* expressing neurons. If the kat-1 activity in the intestine is key, restoration of *kat-1* function in the intestine would be expected to rescue the excessive lipid accumulation phenotype of *kat-1 tub-1* mutant animals since the intestine is the major site of lipid storage in *C. elegans*. If kat-1 acts in the ciliated neurons, neuronal expression of *kat-1* may be key for suppressing the *kat-1 tub-1* mutant phenotype. To test these hypotheses, tissue-specific promoters were used to direct *kat-1* expression in the intestine (*ges-1* promoter), muscle (*unc-54* promoter), hypodermis (*dpy-7* promoter) and neurons (*tub-1* promoter) in *kat-1 tub-1* mutant animals.

Tissue specific rescue of *kat-1 tub-1* mutant animals



It is interesting that there is no significant overlap in the expression patterns of *kat-1* and *tub-1*. Expression of *kat-1* under the control of its own promoter reduced the nile red staining of *kat-1 tub-1* animals by 70%. Gut expression of *kat-1*, driven by the ges-1 promoter, leads to a 50% reduction. Furthermore, expression of *kat-1* under the control of the *tub-1* promoter did not reduce nile red staining of *kat-1 tub-1* animals. Taken together, this indicates that loss of *kat-1* activity in the gut is responsible for the fat accumulation phenotype in the *kat-1 tub-1* animals. In addition, the distinct sites of action of *kat-1* and *tub-1* suggests that global lipid homeostasis may be controlled by *tub-1* expression neurons which emit neuroendocrine signals to *kat-1* expressing peripheral tissues.

A model of lipid homeostasis in wild-type animals

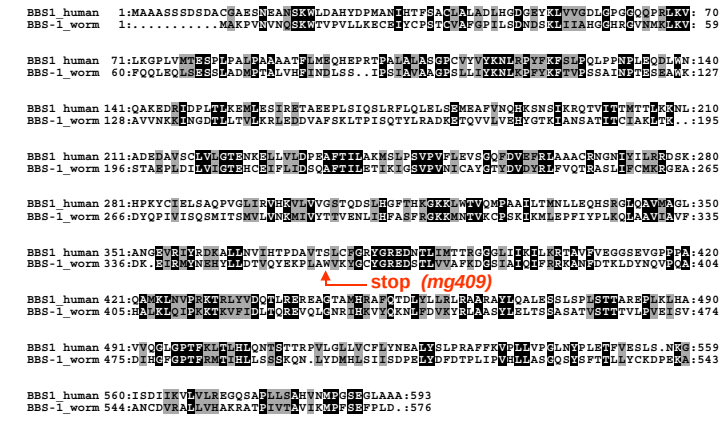


Cloning of *C. elegans* orthologue of Bardet-Biedl syndrome gene BBS1

The specificity of the genetic interaction between *tub-1* and *kat-1* is demonstrated by two observations. First, multiple independent alleles of *kat-1* were isolated from the *tub-1(nr2004)* genetic modifier screen. In particular, no other isolates from this screen yielded the same lipid accumulation phenotype as *kat-1 tub-1* mutant animals. Second, the *kat-1(mg368)* mutation fails to enhance lipid accumulation of *daf-2* and *daf-7* mutant animals, two previously characterized models of enhanced fat storage. Such specific genetic interaction between *tub-1* and *kat-1* led us to speculate that additional components of the *tubby* pathway may be identified in a *kat-1(mg368)* modifier screen. To this end, we screened 79200 haploid genomes after EMS mutagenesis of *kat-1(mg368)* animals, for mutants that show synergistic increase in lipid accumulation in a *kat-1(mg368)* dependent manner. We recovered 41 independent mutants, and report here the cloning of *mg409*. *mg409*; *kat-1(mg368)* mutant animals show up 5-fold increase in lipid accumulation over wild-type post-reproductive adult animals. The *mg409* and *kat-1(mg368)* mutations are highly synergistic since *mg409* animals show a mere 1.5-fold increase in lipid accumulation when compared to wild-type. In addition, *mg409* mutant animals are dye filling defective (Dyf) indicating a failure in ciliated neuron differentiation. Accordingly, *mg409* animals have a small body size (Sma) and display excessive dwelling behavior, two phenotypes that had been attributed to sensory deficits.

We cloned the gene mutated in *mg409* by SNP mapping and found that it is orthologous to human BBS1, a gene mutated at high incidence in patients suffering from Bardet-Biedl syndrome, a complex genetic disease where obesity is one of the prominent clinical features. The *mg409* mutation converts the codon for tryptophan 359 to a stop. Introduction of a transgene spanning the genomic region of *bbs-1* rescued the dye filling defective and excessive lipid accumulation phenotypes of *bbs-1(mg409)*; *kat-1(mg368)* animals. Similar to *tub-1*, *bbs-1* is also expressed in ciliated neurons in *C. elegans*. It is formally possible that *tub-1* and *bbs-1* may function in two parallel pathways that genetically interact with *kat-1*. However, *bbs-1(mg409)*; *tub-1(nr2004)* animals do not accumulate more neutral lipid when compared to *bbs-1(mg409)* and *tub-1(nr2004)* single mutant animals. This argues that *bbs-1* and *tub-1* function in the same genetic pathway.

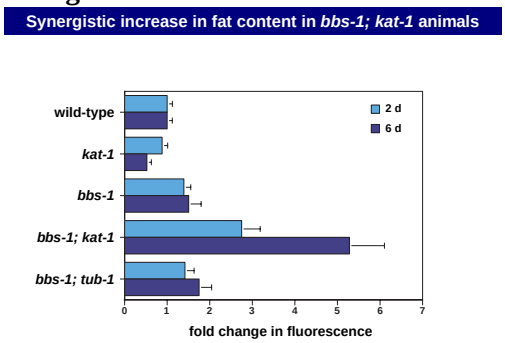
Alignment of BBS-1 and its human orthologue



The structural defect of ciliated neurons due to a loss of *bbs-1* function may cause excessive lipid accumulation in *kat-1*(-) animals. Alternatively, *bbs-1* may act in concert with *tub-1* to control lipid accumulation, independent of its role in ciliated neuron differentiation. Besides *bbs-1*, a number of genes are known to be required for the differentiation and maintenance of ciliated neurons. To determine whether structural defects of ciliated neurons *per se* can lead to excessive lipid accumulation in *kat-1(mg368)* animals, we examined *kat-1(mg368)*; *che-2(e1033)* and *kat-1(mg368)*; *osm-5(p813)* animals. While *che-2(e1033)* and *osm-5(p813)* single mutant animals do not accumulate excessive lipid, *kat-1(mg368)*; *che-2(e1033)* and *kat-1(mg368)*; *osm-5(p813)* animals show a similar phenotype as *bbs-*

1(mg409); *kat-1(mg368)* mutants. As 2-day old adult animals, they accumulate up to 3-fold more lipid than wild-type animals as indicated by nile red staining.

Taken together, control of lipid accumulation and energy balance depend on structural and functional integrity of ciliated sensory neurons in *C. elegans* as exemplified by *tub-1* and *bbs-1* mutant animals. The mammalian hypothalamus, a center of feeding control, is found to possess primary cilia. Therefore, it is conceivable that cilia malformation or dysfunction may contribute to human obesity. **The important point of the worm genetic analysis of tubby and our finding that ciliated neurons regulate fat metabolism in mammals and the worm are the following:**



a. sensory neurons that may be ancestrally related in worms and mammals may sense fat stores to in turn control feeding behavior and metabolism.

b. the worm genetic analysis of tubby may reveal what the receptors for such fat signals are and how they couple to tubby and how cGMP signaling then couples it to neuropeptide outputs to in turn regulate fat storage in adipocytes in mammals and in the intestine in the worm.