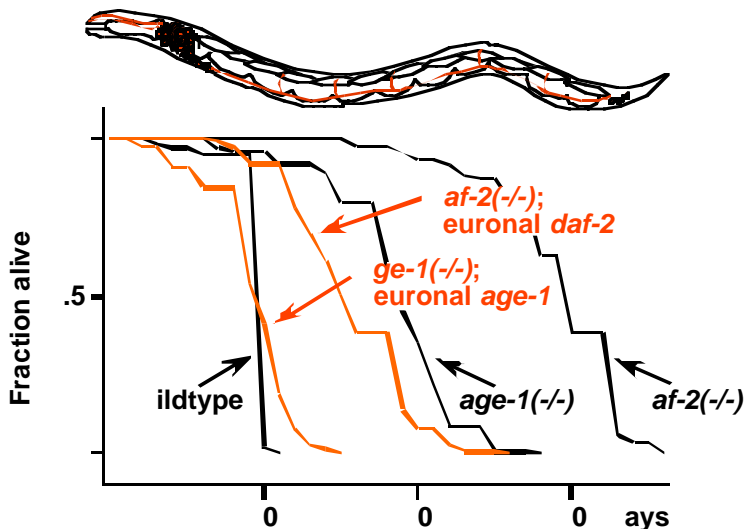


2. Neural regulation of lifespan and metabolism

C. *elegans* lifespan is regulated by insulin-like signaling in the nervous system Cathy Wolkow, Koutarou Kimura, Mingsum Lee

An insulin-like signaling pathway controls *C. elegans* aging, metabolism and development. Mutations in the *daf-2* insulin receptor-like gene or the downstream *age-1* phosphoinositide 3-kinase gene extend adult lifespan two to three-fold. To identify tissues where this pathway regulates aging and metabolism, *daf-2* pathway signaling was restored to only neurons, muscle or intestine. Insulin-like signaling in neurons alone is sufficient to specify wild type lifespan, but muscle or intestinal signaling is not. However, restoring *daf-2* pathway signaling to muscle rescues metabolic defects, thus decoupling regulation of lifespan and metabolism. These findings point to the nervous system as a central regulator of animal longevity.

Lifespan: Neuronal *daf-2* signaling is sufficient



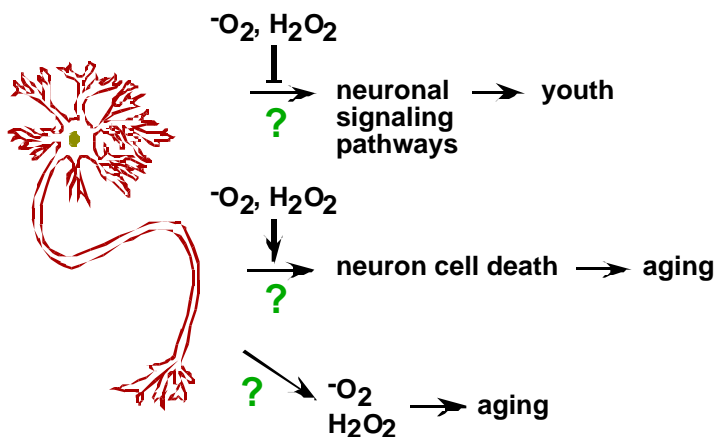
live longer than reproductive adults. One reasonable hypothesis is that free radicals generated as byproducts of metabolism damage cellular components. The lower level of free radicals in *daf-2* insulin-like signaling mutants is essential for lifespan extension: the lifespan extension in a *daf-2* mutant requires the activity of a cytosolic catalase *ctl-1*.

The cells where *daf-2* pathway signaling is required for signaling normal lifespan are not known. For example, insulin-like signaling may regulate metabolism and free radical production directly in aging skin or muscle. Alternatively, these pathways may act in key signaling centers that then coordinately control the senescence of the entire organism. In addition, it is not clear whether insulin/IGF-I regulation of lifespan is simply co-regulated with metabolism, or whether the metabolic shifts are mechanistically connected to the lifespan regulation. 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 *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. Rescue of the *age-1* and *daf-2* mutant phenotypes (long lifespan, metabolic changes and dauer arrest) was tested in these transgenic animals that can transduce insulin-like signals only in specific cell types.

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-114* promoter. Neuronally-restricted *age-1* expression fully restored wild-type adult lifespan to an *age-1* (*mg44*) null mutant (Figure). 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, although not as completely as the comparable *age-1* rescued animals, but to the same extent as ubiquitous *daf-2* expression from the *dpy-30* promoter. The long *daf-2(e1370)* lifespan is also rescued when *daf-2* is expressed from the *unc-119* promoter, another neuron-specific promoter. 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 from

Models for neuronal control of lifespan



the *ges-1* promoter, the major site of fat storage, does not rescue lifespan as efficiently as neural expression of these genes.

The conclusion that it is the expression of *age-1* or *daf-2* within the nervous system that rescues aging depends on the *unc-14* or *unc-119* promoters driving expression only in neurons. Expression level differences between the neural, muscle, and intestinal promoters also do not appear to account for the much more potent lifespan rescue by transgenes expressed from the neuronal-specific promoters. We observe high levels of GFP expression from the muscle-specific *unc-54* promoter, relative

to the other promoters used. Consistent with this observation, *unc-54* is more represented in the 100,000 sequence *C. elegans* EST database, which contains 90 *unc-54* ESTs compared to 14 *unc-14* ESTs and 2 *ges-1* ESTs. Thus it is the activation of the *daf-2* pathway in the nervous system in particular, rather than high expression in any tissue, that rescues the longevity extension of *daf-2* pathway mutations.

The more potent regulation of longevity by the *daf-2* pathway from the nervous system could either represent distinct outputs from some or all neurons, or simply that neuronal expression activates the pathway in more cells (302 neurons in the adult) than when the pathway is expressed in muscles or intestines (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 intestine. Further analysis of animals with *daf-2* pathway signaling restored to restricted neuronal subtypes 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. There are precedents for insulin signaling in the mammalian nervous system. While the target tissue responses to insulin are better known, there are feeding and metabolic responses to insulin in the mammalian brain. In addition, insulin receptor signaling defects in the neurosecretory beta cells of the mouse pancreas cause profound metabolic defects, also suggestive of a role for insulin signaling in neuronal tissues.

How does *daf-2* signaling from neurons control lifespan? *C. elegans* dauer larvae express high levels of the free radical-scavenging enzymes, catalase and SOD. The expression of catalase and Mn-SOD are transcriptionally regulated by DAF-16, the major target of *daf-2* pathway signaling. Furthermore, mutations in *ctl-1* cytosolic catalase reduce the lifespan of *daf-2* mutants, showing that *ctl-1*, and possibly other free radical-scavenging enzymes, are required for long lifespan. Neurons may be particularly sensitive to free radical damage during aging. In fact, overexpression of Cu/Zn superoxide dismutase (SOD) in only motorneurons 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.

Neuronal DAF-2 activity may maintain relatively low levels of free radical scavenging enzymes, such as SOD-3 and CTL-1, by antagonizing the DAF-16 transcription factor. 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.

Food and metabolic signaling defects in a *C. elegans* serotonin synthesis mutant Ji Ying Sze (now on the faculty at UC Irvine), in collaboration with Martin Victor and Yang Shi at Harvard Medical School.

Serotonin is synthesized by an enzymatic pathway from tryptophan: Tryptophan hydroxylase (TPH) catalyzes the rate-limiting first step, and the dual functional 5HTP/L-dopa decarboxylase then matures 5-hydroxytryptophan to serotonin. There is one probable orthologue of mammalian tryptophan hydroxylase in the *C. elegans* genome, ZK1290.2, which we call *tph-1*. Two other *C. elegans* aromatic amino acid hydroxylase (AAAH) gene family members are B0432.5, a probable tyrosine hydroxylase that catalyzes dopamine synthesis and is expressed in dopaminergic neurons, and K08F8.4, a probable phenylalanine hydroxylase that is not expressed in neurons.

A green fluorescent protein gene fusion to *tph-1* (*tph-1::GFP*) is expressed in *C. elegans* serotonergic neurons: NSM, ADF, the hermaphrodite-specific HSN, male-specific CP, and rarely, AIM and RIH. We generated a *tph-1* deletion mutation which a likely null mutation by sib-screening mutagenized animals with PCR primers from the *tph-1* gene. *tph-1(mg280)* homozygous mutant animals are viable, but show a variety of behavioural and metabolic defects. Consistent with the molecular prediction that the *tph-1(mg280)* mutation eliminates the gene activity, mutant animals accumulate no detectable serotonin as determined by anti-serotonin immunofluorescence.

tph-1(mg280) animals display several behavioural defects that are associated with starvation: on a bacterial lawn, the mutant pumps slower and retains more eggs than wild-type. The depression of pharyngeal pumping and egg-laying is similar to that caused by starvation of wild-type animals.

Food level not only modulates *C. elegans* motor outputs, it also affects a metabolism-regulating neurosecretory axis. We find 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. The dauer arrest phenotype is independent of temperature, suggesting that temperature modulation of dauer arrest may in part be serotonergic. While only a minority of the *tph-1(mg280)* animals arrest at the dauer stage, most L2 and L3 stage animals accumulate larger stores of fat than wild-type animals.

There are serotonin inputs to both the TGF-beta and insulin-like dauer control pathways. There is a serotonergic input to the DAF-7 pathway at the level of production of the DAF-7 TGF-beta neuroendocrine signal: 1) Using an integrated *daf-7::GFP* fusion gene, fewer *tph-1(mg280)* animals express *daf-7::GFP*, and express at a lower level than wild type. Consistent with the up-regulation of DAF-7 expression by serotonin, *tph-1(mg280)* strongly enhances dauer arrest of *daf-7(e1372)* at low temperature,. Conversely, high *tph-1* gene dosage potently interferes with dauer arrest induced by *daf-7(e1372)* at the non-permissive temperature. There is serotonergic input to the parallel insulin-like signaling pathway as well as the DAF-7 TGF-beta pathway. Mutations in either *daf-16*, which bypasses the need for *daf-2* insulin like signaling, or *daf-3*, which bypasses the need for DAF-7 TGF-beta signaling, suppress the dauer arrest and fat accumulation phenotypes of *tph-1(mg280)*. Further evidence for serotonergic input to the insulin-like pathway comes from an analysis of *tph-1(mg280)* reproductive longevity. Like *daf-2* insulin-receptor mutants *tph-1(mg280)* hermaphrodites have an extended reproductive lifespan that is dependent on *daf-16* gene activity. The expression or release of any of the *C. elegans* insulins may be responsive to serotonin. In support of serotonin input to insulin signaling, in mammals serotonin enhances insulin synthesis, release, as well as target tissue sensitivity.

The dauer arrest and metabolic shift of *tph-1(mg280)* is probably not the result of the slower pharyngeal pumping and caloric restriction: thirteen different *eat* mutants which have slower pumping rates do not arrest at the dauer stage or synergize with *daf* mutants for dauer

arrest. In addition, the longevity phenotypes of *eat* mutants is not suppressed by *daf-16*, showing that these mutants do not act in the insulin-like pathway.

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 may be decoupled from such food and temperature inputs. Serotonin signaling has been implicated in the control of mammalian feeding and metabolism. A mouse knockout of the 5HT2C subtype receptor gene causes hyperphagia and weight gain, that eventually leads to type 2 diabetes. Conversely, addition of receptor-specific serotonin agonists, or serotonin reuptake inhibitors such as fluoxetine reduces appetite and promotes weight loss. Serotonin signaling in mammals has also been implicated in body temperature regulation by the hypothalamus. Since body temperature regulation is tied to metabolic rate, this suggests a coupling of serotonin to neuroendocrine outputs such as insulin, as we find in *C. elegans*. Thus mammalian 5HT levels may be regulated by nutrition and other food sensory cues to in turn regulate feeding behaviours, as well as neuroendocrine signals analogous or homologous to *C. elegans* DAF-7 TGF- beta and insulin-like hormones.

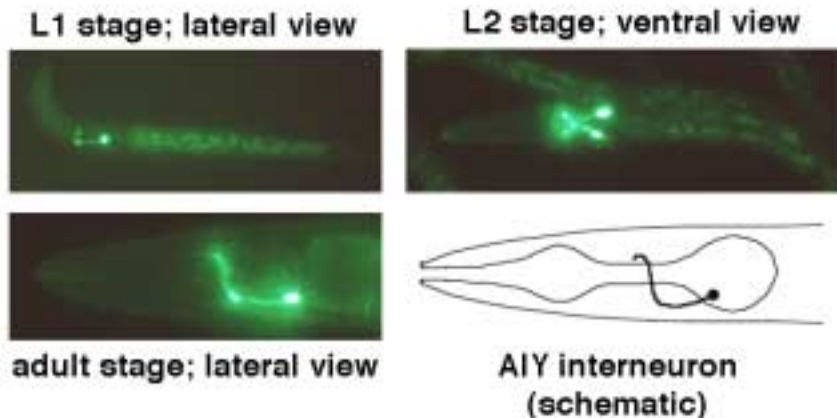
The unifying feature of many of the *C. elegans* serotonergic responses is the coupling of food sensation to various motor and endocrine outputs. Food is a major reward of many animal behaviours, both innate and learned. The ancient coupling of serotonin levels to food suggests a deep mechanistic connection between the role of satiety as a reward in mammalian associative learning and the finding that serotonin has a role in modification of synaptic signaling in the invertebrate *Aplysia*.

Thermosensory input to endocrine control of metabolism

Oliver Hobert (now on the faculty at Columbia University)

Temperature is a potent regulator of dauer arrest. All mutations in the DAF-7 TGF-beta pathway, including *daf-7* null mutations are temperature sensitive. We showed that there is

***ttx-3* is expressed in the AIY interneuron**

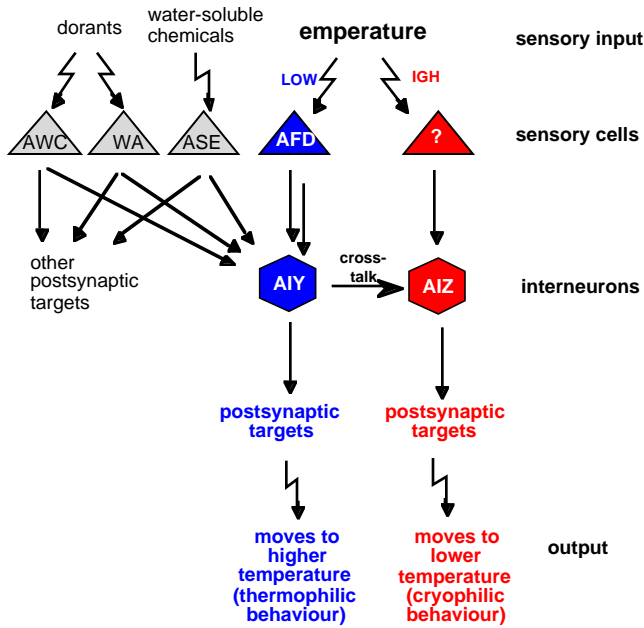


explicit temperature sensory input to this endocrine pathway. A neural pathway couples thermosensory inputs to motor as well as metabolic control. This pathway includes the AFD thermosensory neurons which connect to the interneurons AIY and AIZ, which connect to command motor neurons to

trigger thermophilic and cryophilic movement, respectively. *ttx-3* is required for the function of the AIY interneuron in the *C. elegans* neural pathway that mediates thermoregulation. *ttx-3* encodes a LIM homeodomain protein that is expressed specifically in the AIY interneuron. In *ttx-3* mutant animals, the AIY interneuron is generated, but exhibits patterns of abnormal axonal outgrowth. The TTX-3 LIM homeodomain protein is likely to regulate the expression of target genes required late in AIY differentiation for the specific function of this interneuron in the thermoregulatory pathway.

The *ttx-3* thermosensory pathway couples thermal information to the dauer regulatory pathway. Unlike *daf-7* single mutant animals, *daf-7;ttx-3* double mutant animals can bypass the dauer stage to reproduce. At 15° the opposite effect is manifested: *daf-7; ttx-3* mutants form significantly more dauers at 15° than *daf-7* mutants alone. These results argue that the temperature sensitivity of dauer arrest is due to an AIY-mediated enhancement of dauer arrest

Thermotactic response pathways in *C.elegans*



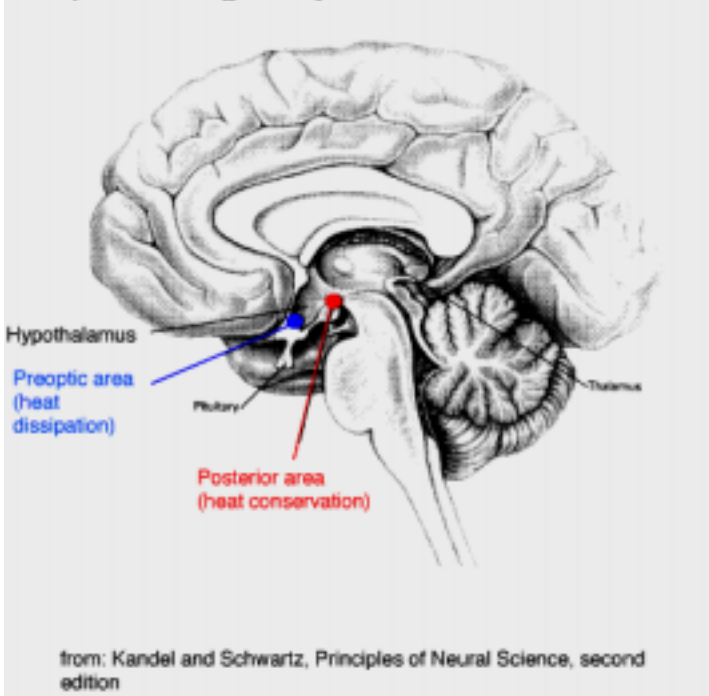
at high temperature and suppression of dauer arrest at low temperature. The *ttx-3* mutation decouples AIY from this thermoregulation of

dauer arrest. However, *ttx-3* does not suppress *daf-2* mutants with defects in the response to insulin-like signaling, suggesting that the suppression of dauer formation at low temperature is due to an increase in insulin signaling and the enhancement of dauer formation at high temperature is due to a decrease in insulin signaling. Consistent with the model that temperature modulation of dauer arrest occurs via the insulin like pathway, strong *daf-2* and *age-1* alleles arrest at the dauer stage at all temperatures.

The antagonistic high and low temperature processing pathways of the *C. elegans* thermotactic response pathway is similar to the organization of the vertebrate hypothalamus which contains distinct warm and cold temperature processing units. In fact, the probable vertebrate *TTX-3* homologue *Lhx2* is required for the development of the mouse diencephalon, the precursor of the hypothalamus. These data suggest that *ttx-3* and *Lhx2* may share an evolutionarily related role in the specification of a thermosensory information processing pathway.

Because thermal input to the dauer arrest depends on *daf-2* insulin like signaling, signals from the thermoregulatory AIY and AIZ interneurons may couple to metabolic control via insulin-like secretory neurons. Such a coupling of thermosensory input to metabolic control by the *daf-2* insulin-like signaling pathway may be homologous to the hypothalamic modulation of autonomic input to the pancreatic beta cells.

Temperature regulatory centers in the human brain



TGF-beta neuroendocrine signal from the ASI dauer regulatory neuron Scott Kennedy, Weiqing Li, and Garth Patterson (now on the faculty at Rutgers University)

In addition to the *daf-2/age-1/daf-16* insulin-like signaling pathway, *C. elegans* dauer arrest is also regulated by the *daf-7/daf-1/daf-4/daf-8/daf-14/daf-3* TGF-beta signaling pathway. The signals in these two pathways are not redundant: animals missing either of these two signals shift their metabolism and arrest at the dauer stage. The DAF-2/AGE-1/DAF-16 and the DAF-7 TGF-beta ke pathways are not sequential. Null mutations in *daf-16* do not suppress mutations in the TGF-beta athway but completely suppress the dauer arrest and metabolic shifts

induced by *daf-2* and *age-1* mutations, and null mutations in *daf-3* do not suppress *daf-2* or *age-1* mutations in the insulin-like pathway, but do suppress all upstream TGF-beta signaling mutations.

daf-7 encodes a TGF-beta neuroendocrine signal that is produced by the ASI neuron. A GFP fusion to *daf-7* is expressed by the sensory neuron ASI during reproductive development, whereas *daf-7* expression in this neuron is inhibited by dauer-inducing pheromone. Using genetic screens, we have identified a number of signal transduction components from the pheromone receptor to the transcription of *daf-7*. We are currently analysing at a molecular level a number of the genes so identified.

daf-1 encodes a ser/thr receptor kinase that is most similar to mammalian type I activin receptor proteins, and *daf-4* encodes a ser/thr receptor kinase that is most similar to mammalian type II activin receptor kinase proteins. *daf-8* and *daf-14* encode proteins that are related to Smad proteins (unpublished data from the Thomas and Riddle labs), which couple TGF-beta signals from receptor kinases to the control of transcription in the nucleus. These genes constitute a canonical TGF-beta signaling cascade: based on biochemical analysis of mammalian TGF-beta signaling, binding of DAF-7 is expected to induce dimerization of receptors to produce an active signalling kinase that phosphorylates cytoplasmic DAF-8 and DAF-14 Smad proteins, and causes them to relocate to the nucleus, where they act as transcription factors.

daf-3 is a unique TGF-beta signalling pathway gene in that it is antagonized, rather than activated by the *daf-7* TGF-beta-related pathway. A *daf-3* null allele completely suppresses the dauer constitutive phenotype of mutations in *daf-1*, *daf-4*, *daf-7*, *daf-8*, and *daf-14*. Thus mutations in *daf-3* bypass the need for any of the DAF-7 signal transduction pathway genes, suggesting that the major function of this signaling pathway is to antagonize DAF-3 gene activity. In the absence of DAF-7 signaling, DAF-3 activity is unregulated to induce dauer arrest. We showed that *daf-3* encodes a Smad protein most closely related to vertebrate DPC4, which is a cofactor for Smad1, Smad2 and Smad.

The expression pattern of functional DAF-3/Green Fluorescent Protein (GFP) and DAF-4/GFP fusion genes suggests that these TGF-beta signal transduction proteins could function in target tissues that are responsive to the neuroendocrine DAF-7 signal. These fusion genes are expressed in most tissues of the animal, including the intestine and hypodermis which shift to fat storage metabolism in dauer larvae. The observation that DAF-4 and DAF-3 are expressed in many of the same cells is consistent with a model that DAF-4/DAF-1 signalling to the downstream DAF-8 and DAF-14 Smads directly regulates DAF-3 gene activity. The dauer promoting DAF-3 homooligomers may be disrupted when DAF-3 heterooligomerizes with a phosphorylated DAF-8 and/or DAF-14.

The genes regulated by the Fork head/Smad transcriptional regulatory complex may correspond to the *C. elegans* homologues of mammalian genes, such as those that encode PEPCK, GAPDH, and the Glut4 glucose transporter, that are known to be transcriptionally regulated by insulin. *C. elegans* homologues of these genes are found in the genome sequence and studies are underway to explore their regulation in various *daf* mutants.

Genetic pleiotrophies also suggest that the insulin-like and TGF-beta like pathways may regulate distinct aspects of dauer arrest. For example, only mutations in the insulin-like pathway genes cause dramatic increases in longevity. The increase in longevity induced by decreased insulin-like signaling activity are suppressed by mutations in *daf-16* but not by mutations in *daf-3*. This suggests that the transcriptional program for longevity does not depend on TGF-beta transcriptional cofactors in the same manner that the metabolic switch to fat storage depends on both pathways. It is possible that DAF-16 alone regulates longevity. On the other hand, *daf-7* null mutants, as well as *daf-1* and *daf-4* receptor mutants and *daf-8* and *daf-14* Smad mutants cause dauer arrest which is associated with longevity increase, so the TGF beta pathway may also have outputs to longevity control.

Pheromone Regulation of *daf-7* expression

Scott Kennedy

A major regulator of the decision to enter the dauer stage is the level in the environment of the secreted dauer promoting pheromone. The dauer pheromone controls the expression level of the TGF-b like ligand DAF-7 in the ASI amphid neuron (ref the Thomas lab and Riddle lab paper here). We are studying the molecular mechanism by which this extracellular

pheromone signal regulates *daf-7* transcription. *daf-11* is an important regulator of *daf-7* expression; in *daf-11* mutants, a *daf-7*GFP fusion gene is expressed at much lower levels. *daf-11* encodes a transmembrane guanylyl cyclase, an enzyme that converts GTP to the intracellular second messenger cGMP. We have also found that loss of function mutations in the α subunit of a cyclic nucleotide gated ion channel *tax-4* also cause a loss of DAF-7 expression. The dauer and low *daf-7p::GFP* phenotypes of *daf-11* but not *tax-4* mutants are reversed by exogenous addition of cGMP analogues. Interestingly, in the cilia mutants, even though *daf-11* induced dauer arrest is suppressed, the low level of expression of *daf-7*GFP is not suppressed. This suggests that the ciliated endings are coupled to more outputs than *daf-7*, and that the cilia mutants do not suppress *daf-11* by producing more cGMP or otherwise activating the signaling pathway to *daf-7*. This suggests that pheromone signaling in *C. elegans*, like mammalian photoreceptor signaling, involves the second messenger cGMP and a cGMP regulated ion channel.

To discern the signal transduction elements upstream and downstream of cGMP to *daf-7*, we have undertaken two genetic screens. The first screen seeks to identify mutations that both suppress the *daf-11* Daf-c phenotype and lead to a reversion in *daf-7p::GFP* expression. From an initial screen of 250,000 haploid genomes we have identified two mutations: **daf-7 regulator** *drg-1(mg296)* and *drg-2(mg297)* that satisfy these criteria. *drg-1(mg296)* is a recessive mutant that blocks dauer pheromone downregulation of *daf-7p::GFP*. We are now mapping *drg-1* and *drg-2* for molecular analysis. Our second genetic screen seeks to identify mutations that both suppress the *tax-4* Daf-c phenotype and also lead to *daf-7p::GFP* expression. From an initial screen of 10,000 haploid genomes we have identified two mutations: *drg-3(mg318)* and *drg-4(mg319)* that satisfy these criteria. Progress on the characterization, mapping, and identification of these mutations will be reported.