

The Dauer Pathway

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The *C. elegans* dauer pathway represents the genetic, molecular and physiological mechanisms by which *C. elegans* tailors its life cycle to respond appropriately to environmental conditions. The L1 larva and the pre-dauer L2d predict whether their progeny will survive (at least to the dauer stage), and choose between rapid growth to the reproductive adult, or developmental arrest and dispersal. A mistaken decision either way has disastrous consequences for reproduction. Hence, the selective pressure to fine tune the system is strong, favoring the assessment of multiple indicators of future reproductive success, and comparing those indicators for consistency. Furthermore, the processes of dauer or non-dauer morphogenesis, which affect virtually every tissue, must be coordinated properly for survival. It has not been surprising that the genetic pathway has evolved into a gene network. Work in recent years has detailed the roles of modulatory genes and points of cross-talk with other pathways that coordinate cellular activities. The major components of the central protein growth factor signaling pathways are known, but the regulation of ligand synthesis is less understood, as are the roles of downstream effector genes for developmental arrest, morphogenesis and longevity. Systems biology approaches hold the key to sorting out the latter functions.

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RNAi screens for novel candidate insulin receptor-like proteins reveal a potent but unexpected role for EGF signaling in healthy aging

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The DAF-2 insulin receptor functions as an upstream receptor in a kinase cascade that includes AGE-1 (PI-3 kinase) and ultimately phosphorylates forkhead transcription factor DAF-16 to inhibit its transcriptional activity. Decreased *daf-2* signaling promotes DAF-16 transcriptional functions and causes lifespan extension that depends on *daf-16* activity. One interesting thing about the insulin signaling system of *C. elegans* is that there are 39 insulin-like ligands encoded by the genome, but only one clear insulin receptor homolog, DAF-2. Previously, bioinformatic analyses listed 54 putative insulin receptor-like proteins in nematodes. It should be noted, however, that receptor-related genes on this list are homologous in the extracellular ligand-binding domain and contain secretion signal sequences, but lack the transmembrane domain present on classic receptor kinases--so they actually look more like potential secreted proteins. We constructed or obtained 54 clones for these candidate insulin binding proteins and used these clones for RNAi inactivation to test effects on the locomotory healthspan of aging animals. We identified two candidates (*hpa-1* and *hpa-2*) for which RNAi confers **high** performance in **advanced** age (*hpa*) phenotype. We have also shown that RNAi directed against either candidate protein also extends lifespan. We conclude that *hpa-1* and *hpa-2* are two new genes that influence healthspan and aging.

Interestingly, when we looked more closely at HPA-1 and HPA-2 primary sequences, we found that both candidates more closely resemble EGF receptor than insulin receptor. We then showed that the healthspan-promoting effects are independent of the DAF-2 insulin-like receptor and the downstream insulin signalling transcription factor FOXO/DAF-16. These data prompted us to look at EGF pathway mutants for aging phenotypes. We found that a gain-of-function mutation in EGF receptor extends lifespan and locomotory healthspan, and modestly lowers age pigment levels. Loss-of-function in the EGF receptor has the opposite effect. EGF ligand LIN-3 is needed for the *hpa-1* and *hpa-2* RNAi phenotypes. We conclude that the EGF pathway impacts aging--with pathway activation being associated with longevity and healthy aging. We suggest a model in which HPA-1 and HPA-2 bind EGF ligand to restrict its availability in normal aging, when EGF is increased, animals age more gracefully.

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Parkinson's Disease associated factors LRK-1 and PINK-1 interact to regulate axon guidance and cell migration in *C.elegans*

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Parkinson's Disease is characterized by the preferential loss of projection neurons in the substantia nigra. Dominant mutations in α -synuclein and LRRK2 (leucine-rich-repeat kinase) and recessive mutations in Parkin, DJ-1, and PINK1 (PTEN-induced kinase) segregate with the familial variants of PD. Mutations in these genes have been linked to mitochondrial dysfunction and increased vulnerability to oxidative, as well as ER, stress. However, how these mutations promote the pathological processes, the first manifestations of which are cytoskeletal defects in the affected neurons, is not known at present. Studies in mammalian support the idea that PINK1 forms a protein complex with DJ-1 and acts in the same pathway as Parkin. Furthermore, a biochemical interaction between LRRK2 and Parkin was suggested recently. Taking these multiple functional interactions into account, we reasoned that the *C. elegans* homologues, LRK-1 and PINK-1, could contribute to overlapping biological functions. In order to identify common targets for the kinases we created a protein interaction network of PD-related proteins based on genetic and biochemical approaches. We identified an interaction network linking PINK-1 and LRK-1 to Rho GTPases and their targets and regulators, which are key players of cytoskeletal regulation. We show that antagonistic activities of PINK-1 and LRK-1 regulate the activities of two members of the *C. elegans* Rho family to affect neuronal migrations and neurite pathfinding. Our data suggest that the combinatorial activity of both kinases is involved in controlling actin cytoskeleton dynamics in order to regulate neuronal integrity.

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A Whole-genome RNAi Screen for Genes Promoting Hypoxic Sensitivity

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The ability to survive in and recover from hypoxic environments varies widely across species and cell types. This trait is not only fundamentally important but has tremendous medical implications for diseases such as stroke, heart attack, and cancer. Yet, a systematic identification of the genes controlling hypoxic sensitivity has not been reported. Using the Ahringer library, a whole genome RNAi screen identified 198 genes as necessary for normal organismal hypoxic sensitivity. The hypoxia resistance (Hyp-r) phenotype was confirmed for all clones with at least four-independent trials and was greater than four standard deviations from vector controls. Inserts of all Hyp-r clones were sequence verified. BLASTs of all overlapping 25 bp target sequences against the whole-genome identified potential off-target RNAi, which were then directly tested. The 198 Hyp-r genes fell into several functional classes. The largest class encoded signaling proteins, including several serpentine receptors and second messengers. Both general and specific transcription factors were implicated. As expected inhibition of translation and intermediary metabolism promoted hypoxic survival. Surprisingly, knockdown of ubiquitination pathway genes promoted survival. Several lifespan genes were among the 198. For most genes, the Hyp-r phenotype was suppressed in an *rrf-1(lf)* background indicating the importance of somatic expression. Finally, microarray experiments and subsequent qRT-PCR revealed that the transcript abundance of a subset of the Hyp-r genes was controlled by hypoxia. We are now determining the mechanisms whereby select genes regulate hypoxic survival and are testing for a similar role by mammalian orthologs.

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Natural Variation in Pathogen Resistance Due to Polymorphism in the *npr-1* Gene

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We observed that there is natural variation in the susceptibility to infection by the pathogenic bacteria *Pseudomonas aeruginosa* between the wild strains N2 and CB4856, with the CB4856 strain exhibiting increased susceptibility to pathogen killing. Using a recombinant inbred line collection, we found that a region containing *npr-1* is the only region determining pathogen susceptibility between these two strains. NPR-1 is a neuropeptide Y receptor-like protein that has been implicated in the related set of behaviors of social feeding, clumping, bordering, and aerotaxis in the presence of food, as well as adaptation to ethanol. Analysis of *npr-1* null mutants in the N2 background revealed a pathogen susceptibility phenotype that is stronger than that seen in the CB4856 strain. Quantitative RT-PCR of pathogen response genes suggests that NPR-1 functions independently of previously described immune-signaling pathways, indicating that NPR-1 may modulate nematode immunity through neuroimmune mechanisms. (KCR and ECA contributed equally to the study)

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An Adult Reproductive Diapause in *C. elegans*

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The study of the *dauer* diapause in *C. elegans* has led to the identification of numerous mechanisms that control aging and stress resistance across species. Here, we present the discovery of a starvation induced adult reproductive diapause. Entry into this diapause is absolutely dependent upon the nuclear receptor NHR-49, a close relative of the mammalian HNF4 α . Adult diapause formation does not require DAF-16/FoxO signaling, however, showing that the mechanism of adult diapause establishment is distinct from that of dauer formation. Arrested adults are able to survive starvation for at least 30 days and yet still recover to produce offspring and age normally when food is restored. There are two extraordinary features of this diapause: first, although starvation leads to the progressive apoptotic death of almost the entire germline, a small population of stem cells is preserved. Second, upon restoration to food, these surviving stem cells faithfully regenerate a functional germline. We propose a “dispensable germline” hypothesis for adult diapause maintenance and survival; whereby differentiated germ cells are terminated during starvation as a means of sustaining somatic tissues and germline stem cells. This work presents a novel physiological phenomenon by which stem cells are protected during prolonged starvation, and establishes *C. elegans* as a model for investigating the impact of nutritional diapause on adult stem cell survival and recovery.

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The Stress of Misfolded Proteins: A Systems Approach to Heat Shock and Proteostasis in Disease and Aging

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The long-term health of the cell is inextricably linked to proteostasis, a complex network of molecular interactions that maintains the health of proteins. An imbalance in proteostasis, whether caused by environmental or physiological stress, ageing, or the chronic expression of disease-associated proteins (mutant SOD1, huntingtin, polyQ, or A β) can be restored by genes that control lifespan (Daf-16) and the heat shock response (Hsf-1). How such folding networks and stress responses are integrated at the molecular and cellular level to the organism has not been examined. The response of *C. elegans* to heat shock requires the AFD thermosensory neurons and animals harboring mutations in AFD function are defective for induction of the heat shock response in other somatic cells. This AFD requirement is highly selective for activation of heat shock genes by cadmium is unaffected in mutant animals. This reveals that transmission of the heat shock signal is cell non-autonomous and requires active neuronal signaling which serves to integrate temperature-dependent behavioral, metabolic, and stress-specific responses. We draw similar conclusions from a forward genetic screen for enhancers of protein misfolding in body wall muscle cells that identified a mutation in *unc-30* that regulates GABAergic signaling. Whether caused by mutations in the GABAergic or cholinergic pathways or by small molecule agonists and antagonists, an imbalance in cholinergic signaling enhances misfolding of polyQ and other metastable proteins in post-synaptic body wall muscle cells.

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Functional Regulation of Nicotinamide Levels and NAD⁺ Biosynthesis by a Secreted *C. elegans* Nicotinamidase

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NAD⁺ is central to cellular metabolism and an obligate co-substrate for NAD⁺ consuming enzymes, including sirtuins and PARPs. Like Sir2, the founding member of the sirtuins, *C. elegans* SIR-2.1 extends lifespan. Sirtuins and PARPs require NAD⁺ for activity and are inhibited by a byproduct of their NAD⁺ hydrolysis, nicotinamide. Thus, regulation of NAD⁺ biosynthesis and its metabolite nicotinamide are relevant to understanding the regulation of sirtuins and other NAD⁺ consumers.

Nicotinamide is recycled to form NAD⁺ via a salvage pathway. While the biological role of this pathway is studied in yeast, its impacts on the biology of a multicellular organism are unknown. To understand the biological functions of the NAD⁺ salvage pathway and to probe the consequences of perturbations in NAD⁺ salvage to the cell and to the physiology and development of the organism, we study the salvage pathway in *C. elegans*.

NAD⁺ salvage begins with a nicotinamidase in yeast and invertebrates such as *C. elegans*. Mutations in the nicotinamidase PNC-1 have revealed novel roles for the salvage pathway. Temporal development of the gonad is delayed in *pnc-1* mutants. Additionally, 4 uterine cells necrose, and the hermaphrodites are egg-laying defective. The temporal defect and the cell necrosis are strikingly separable, arising as the result of product depletion and substrate buildup in the absence of enzyme activity, respectively. Our results highlight the dual biological functions of the pathway in promoting NAD⁺ biosynthesis and in modulating nicotinamide levels.

The enzymatic progression from nicotinamide to NAD⁺ is not conserved with vertebrates, which begin with another enzyme, Nampt. While the enzymatic activities of Nampt and nicotinamidase are distinct, they are believed to perform homologous functions because they similarly impact NAD⁺ biosynthesis and nicotinamide concentrations. Nampt can rescue *pnc-1* mutants, demonstrating homologous biological function of vertebrate and invertebrate NAD⁺ salvage pathways.

The *pnc-1* locus encodes two isoforms, PNC-1a, which has a signal peptide, and PNC-1b. PNC-1a is expressed in the head muscle and only 11 additional cells, including the 2 ASK neurons. PNC-1a (but not PNC-1b) rescues all phenotypes when expressed only in the ASK neurons, demonstrating the function of the secreted isoform. Thus, not only are Nampt and nicotinamidase similar in biological function, their functional conservation extends to activity as an extracellular enzyme.

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Heat Shock Treatment Reduces Beta Amyloid Toxicity *In Vivo* By Diminishing Oligomers

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Heat shock response, mediated by heat shock proteins, is a highly conserved physiological process in multicellular organisms for maintenance of cellular homeostasis. Expression of heat shock factors and subsequent heat shock protein plays a role in protection against proteotoxicity in invertebrate and vertebrate models. Proteotoxicity due to β -amyloid peptide ($A\beta$) oligomerization has been linked to the pathogenesis of Alzheimer's disease. In a transgenic *C. elegans* model of AD, intracellular expression of human $A\beta$ is associated with elevated oxidative stress, accumulation of $A\beta$ aggregation and progressive paralysis behavior. Small heat shock protein HSP16.2 has been reported to co-localize with $A\beta$ in the transgenic *C. elegans*, and its over expression suppressed the paralysis. Heat shock response in *C. elegans* is a neuronal controlled behavior. Transcription factor HSF-1, which regulates heat shock response by up regulation of heat shock proteins in *C. elegans*, has been coupled to normal aging and age-related diseases. Recently, it also has been associated with dietary restriction and insulin-like signaling in protection against proteotoxicity. However, the functional link between sHSP and $A\beta$ on protection against $A\beta$ toxicity remains unknown. Previously, we demonstrated that progressive paralysis induced by expression of human $A\beta_{1-42}$ in transgenic *C. elegans* was alleviated by $A\beta$ oligomer inhibitors *ginkgo biloba* extract and its constituents (Wu, et al., J. Neurosci 2006, 26:13102-13). In this study, we apply a protective heat shock treatment (2h, 35°C) to $A\beta$ *C. elegans* and reveal a significant decrease of $A\beta$ toxicity and $A\beta$ oligomers in the transgenic worms. This data is consistent with the view that co-expression of HSP16.2 in $A\beta$ *C. elegans* lessened $A\beta$ toxicity in these worms. In addition, it provides new insight into the role of non-invasive physical treatment and endogenous chaperone proteins in regulation of $A\beta$ aggregation and toxicity.

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The Role of Ubiquitin-Conjugating Enzymes in Polyglutamine Protein Aggregation: Analysis by Real-Time *in vivo* Fluorescence Imaging

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Our lab has employed time-lapse fluorescence microscopy in combination with RNAi (RNA interference) to examine the role of the ubiquitin-proteasome system in polyglutamine protein aggregation in *C. elegans*. Genetic expansions of polyglutamine repeat regions underlie several progressive human neurodegenerative diseases, including Huntington's disease. The protein products of these mutant genes are often prone to intracellular aggregation, a hallmark of many neurodegenerative diseases. Inability of the ubiquitin-proteasome system to degrade aggregation-prone proteins may contribute to neuronal cell loss. Using a transgenic line of *C. elegans* developed by Morimoto et al. that expresses an aggregation-prone stretch of glutamine repeats fused to GFP (green fluorescent protein), we have shown that knockdown of specific E2 ubiquitin-conjugating (UBCs) enzymes alters the size and frequency of polyglutamine-GFP aggregates, and that *ubc-1*, *ubc-13*, and *uev-1* are required for ubiquitin colocalization to aggregates (Howard et al., *BMC Cell Biology* 2007, **8**:32). Further work is being undertaken to determine if these UBCs are required for colocalization of non-polyglutamine proteins to polyglutamine aggregates. Using time-lapse fluorescence microscopy, the formation of insoluble polyglutamine aggregates from soluble fluorescent fusion protein can be observed in real-time in *C. elegans* larvae. Initial aggregate formation is rapid, completing in approximately 40 minutes. By analyzing videos of animals treated with RNAi, the roles of individual UBCs in the dynamics of polyglutamine aggregation can be investigated.

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Clk and caloric restriction regulated genes with aging phenotypes

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Caloric restriction extends life span in *C. elegans* acting through a genetic pathway shared with Clk class and certain mitochondrial genes. This pathway is genetically distinct from the insulin-like signaling pathway. We are interested the extent to which the target genes of these pathways are distinct or convergent. Using full genome microarrays, gene expression changes in Clk and caloric restriction worms were identified. Both *eat-2* and axenic media caloric restriction were examined. Several thousand genes show strong, reproducible expression changes in these experiments.

Microarray studies have identified gene expression changes during normal aging and of long-lived insulin-like signaling pathway mutants. We compared expression changes in these experiments with Clk/caloric restriction gene expression by gene and at the pathway level and identified both shared and distinct effector genes and cellular pathways in the different genetic classes of long-lived mutants. Also, a number of known aging genes have expression altered in the direction that extends lifespan while others have lifespan shortening expression changes. The net result of extended lifespan in Clk and caloric restricted worms is the result in part of complex cooperative and opposing changes in known aging genes.

Genes with significant expression changes in these long lived worms may affect the aging process. To test this the ability of genes individually to alter lifespan is being assayed. Candidate aging gene expression was knocked down using RNAi and then lifespan assays were performed. Over a hundred genes were screened and several were found to extended lifespan. These aging genes are being tested for interaction with the insulin-like signaling pathway and caloric restriction pathways.

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A Pharmacological Approach to Studying Dietary Restriction in *C. elegans* Using Metformin, a Proposed DR Mimetic

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Limiting overall number of calories consumed can extend lifespan in yeast, nematodes, flies, rodents and primates. In higher organisms, the dietary restriction (DR) condition is associated with lowered incidence of age-related disease and increased overall vigor, a clear healthspan extension. Metformin, a drug currently used to treat type-2 diabetes, is a proposed human DR mimetic. We find that metformin can confer significant health benefits in *C. elegans*, extending median lifespan and youthful locomotory ability in a dose-dependent manner. We also find that energy sensor AMPK kinase is required for metformin-associated lifespan increase. Metformin can still extend midlife viability in mutants deficient in insulin signaling, supporting that metformin does not act through the canonical insulin signaling pathway and downstream transcription factor FOXO/DAF-16. By contrast, metformin cannot further extend median lifespan of DR-constitutive mutant *eat-2*, defective in feeding. Instead, *eat-2* mutants are hypersensitive to metformin, which appears toxic at high doses in this DR mutant. Interestingly, metformin also induces multiple features that characterize DR animals, including specific age pigment profiles (lipofuscin and advanced glycation endproducts) profiles, low fat stores, and low fecundity. Taken together, our data suggest that metformin induces a DR state in *C. elegans*, a condition that could explain associated healthspan extension. Our results thus support that metformin acts as an AMPK-dependent DR mimetic across species boundaries, and suggest the *C. elegans* model can be exploited to reveal details of *in vivo* metformin mechanism of action as well as to screen for improved drug efficacy with potential anti-aging application.

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A Novel ¹³C Isotope Labeling Strategy Identifies Insulin/IGF Receptor Mutations that Selectively Modulate de novo Fatty Acid Synthesis and Longevity

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Although studies in *C. elegans* have identified numerous genes and pathways involved in fat storage, it is now important to determine how these factors affect in vivo lipid metabolism. We developed a ¹³C-isotope assay to quantify the contribution of dietary fat absorption and de novo fatty acid synthesis to triglyceride and phospholipid synthesis in *C. elegans*. We employed this method to define the impact of insulin/IGF signaling on fat storage and membrane synthesis. Worms harboring the long-lived *daf-2(e1370)* insulin receptor mutation contained significantly higher levels of synthesized fats in triglycerides and phospholipids. Elevated fat synthesis was completely dependent upon the DAF-16/FoxO transcription factor. Intriguingly, other long-lived alleles of *daf-2* did not increase de novo fatty acid synthesis, suggesting that site-specific mutations in the insulin receptor can selectively modulate longevity and fat metabolism. Additionally, we have begun to employ similar labeling strategies to monitor the turnover of fatty acid molecules found in triglyceride stores as well as phospholipid membranes. These stable isotope-labeling assays permit measurement of nutrient flux in *C. elegans* and present a strategy for combining quantitative metabolic studies with genetic analysis.

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Discovery of *C. elegans* signalling molecules that regulate development and behavior using Differential Analysis by 2D-NMR Spectroscopy

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Small molecule signals play an important role in *C. elegans* biology. Recent studies show that the ascarosides ascr#2 and ascr#3 differentially regulate development and mating behavior (Butcher et al., 2007 and Srinivasan et al, in press). Additionally, *daf-22(m130)* mutant worms are dauer-defective and do not have dauer pheromone activity, suggesting that they might be deficient in the synthesis of the dauer pheromone (Golden and Riddle, 1985). We hypothesized that they might not make ascr#2 and ascr#3 and also be defective with regard to the biosynthesis of other signalling molecules. In order to address this question, we used Differential Analysis by 2D-NMR Spectroscopy (DANS), a method developed in our lab (Schroeder et al, 2007). This method allows for the detection and identification of small molecules from crude mixtures without extensive activity-guided fractionation. As expected, we were able to detect ascr#2 and ascr#3 in significant amounts in wild-type derived extracts, but not in *daf-22* extracts. Our analysis also revealed four novel ascarosides (ascr#5-8), including an aromatic derivative (ascr#8). We prepared synthetic samples of all four compounds and are currently investigating their biological properties. Preliminary results show that ascr#8 strongly attracts males but, in contrast to ascr#2 and ascr#3, has little, if any, dauer-inducing activity. Our results show that DANS is a useful tool for correlating genetic information with small molecule signals from crude metabolic mixtures.

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The Target of Rapamycin (TOR) pathway antagonizes *pha-4/FoxA* to control development and aging

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FoxA factors are critical regulators of embryonic development and post-embryonic life. *C. elegans pha-4* encodes a FoxA transcription factor that is required to establish the foregut in embryos, and to control growth and longevity after birth (1). Loss of *pha-4* causes severe defects in organogenesis and mis-expression of PHA-4 induces ectopic foregut cells (1). These data show that regulation of *pha-4/Foxa* is critical for normal development, but little is known about the regulation of FoxA proteins in any animal. We conducted a genetic screen to identify mutations that can suppress a partial loss of *pha-4* function and identified the AAA+ ATPase homologue *ruvb-1* (2).

We have found that *ruvb-1* is a component of the TOR pathway. The most striking phenotype associated with *ruvb-1* homozygous mutants is an arrest during the third larval stage. Previous studies had found that mutations that disrupt insulin signaling (*daf-2/insR*), and the TOR kinase pathway (*let-363/TOR*, *daf-15/raptor*), lead to a dauer or L3 arrest (3). *ruvb-1* larvae lack phenotypes typically associated with *daf-2/insR* mutants and are similar to those of *let-363/TOR* animals. Both *ruvb-1* and *let-363/TOR* control nucleolar size and promote localization of box C/D snoRNPs to nucleoli, suggesting a role in rRNA maturation. Similar to *ruvb-1*, inactivation of *let-363/TOR* suppressed the lethality associated with reduced *pha-4*.

The TOR pathway controls protein homeostasis and also contributes to longevity (4). Reduced TOR extends lifespan independent of insulin signaling and does not require *daf-16/Foxo* (5,6,7). We find that *pha-4* is required to extend adult lifespan in response to reduced TOR signaling, and our data reveal that regulation of lifespan by TOR does not simply reflect changes in protein biosynthesis. The data suggest that *pha-4* and the TOR pathway antagonize one another to control post-embryonic development and adult longevity.

1) Mango, Wormbook (2007).

2) Updike and Mango, Genetics (2007).

3) Hu, Wormbook (2007).

4) Antebi, PLOS Genet. (2007).

5) Hansen et al., Aging Cell (2007).

6) Henderson et al., Curr.Biol. (2001).

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Dietary Nutrients Affect Lifespan, Fat Storage, and Feeding Behavior in *C. elegans*

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The optimum amounts of macronutrients in human diets is currently a subject of great interest. Diets emphasizing fat restriction, low carbohydrates or high protein content are promoted in popular books, but little is known about their long-term metabolic effects. We found that various *E. coli* strains commonly used to maintain *C. elegans* in most laboratory studies have distinct nutrient compositions. Nematodes maintained on these strains show significant differences in lifespan, fat storage, and feeding behavior.

While all *E. coli* strains have a relatively high protein content, carbohydrate content varies in strains depending on whether or not they secrete a polysaccharide capsule. When growing on the capsule-secreting strain HB101, we found that wild type *C. elegans* show increased quiescence, reduced fat stores, and a shorter lifespan compared to *C. elegans* growing on the capsule-less strain OP50. Gene expression studies indicate an increase in fat metabolism in *C. elegans* growing on OP50, which may induce mitochondrial metabolism and a hormetic response that enables an extended lifespan. We find that the differences lifespan among worms grown on the two bacterial strains depends on efficient feeding, since *eat-2* mutants show equally extended lifespans on both bacterial strains. We also find that AAK-2, a homolog of the large subunit of AMP-dependent kinase (AMPK), is necessary for the increased lifespan on OP50. Fat storage depends on feeding, since *eat-2* shows indistinguishable low fat stores on both bacterial strains. Furthermore, mutants in the peptide transporter encoded by *opt-2* store equally high amounts of fat when growing on both bacterial strains. Our studies indicate that various nutrient sensing pathways feed back to influence feeding behavior and metabolism, which in turn affect fat storage and lifespan.

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A spontaneous mutant of *E. coli* that increases *C. elegans* lifespan through changes in bacterial metabolism

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The relationship between diet and lifespan is a key but complex question in the biology of ageing. Dietary manipulation can be confounded by the diversity of microbes that inhabit the gastrointestinal tracts of all animals. These microbes can produce essential compounds that cannot be synthesized by the host. *C. elegans* is a unique model of ageing because we have complete control over its intestinal microbes. Previous studies have suggested that the metabolism of the *E. coli* diet is a determinant of *C. elegans* ageing^{1,2}. While testing various RNAi feeding clones for their effects on lifespan, we discovered a clone that increases *C. elegans* lifespan by 30-50%. However, this lifespan effect was not due to RNA interference but instead to spontaneous mutation of the HT115(DE3) feeding strain because the effect remained after losing the RNAi plasmid and the plasmid failed to increase lifespan when retransformed into the parent HT115(DE3).

We found that the longevity-inducing bacterial strain could not grow on minimal medium unless supplemented with glycine. This requirement allowed us to screen for rescued growth using a plasmid library of DNA from the parent HT115(DE3). Sequencing in the mutant bacteria identified the mutation as a transposon insertion in *aroD*, a gene that encodes an enzyme in the shikimic acid pathway. Either transformation with the *aroD* construct or addition of shikimic acid to the bacteria restored *C. elegans* lifespan to normal. The shikimic acid pathway is only found in microbes and plants and is used to make several aromatic compounds including the aromatic amino acids and folate. Animals lack this pathway and depend on their diet for many of its products.

We have begun to investigate known mechanisms of lifespan extension in the lifespan increase caused by the mutant bacteria. Firstly, it is independent of *daf-2* and *daf-16*. In addition, worms grown on the mutant bacteria showed no obvious developmental delay suggesting that the lifespan extension is not due to gross dietary restriction. Previous studies have suggested that *E. coli* deficient in respiration causes *C. elegans* to live longer². Supplemented with glycine, our mutant could grow with succinate as the sole carbon source suggesting it is competent for respiration. We are currently testing other potential mechanisms of lifespan extension. This study shows how *C. elegans* lifespan can be modulated by single gene mutations of its bacterial diet.

1. Lenaerts *et al.* J Gerontol. 2008 63:242, 2 Saiki *et al.* Aging Cell 2008 7:291

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The Deadly Sweet Tooth

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We and others have found that worms fed 2% glucose have a shortened lifespan. In this talk, I will discuss what we know about the underlying mechanism, and why we think that glycerol might play an important role in the response to glucose, and also in the coordination of the aging rates of different tissues in the animal.

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EAK-7 is a novel regulator of nuclear DAF-16 activity

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The FoxO transcription factor DAF-16 regulates metabolism, development, stress responses and longevity in *C. elegans*. The DAF-2 pathway negatively regulates DAF-16/FoxO via AKT-mediated phosphorylation, resulting in nuclear exclusion. However, a nonphosphorylatable, constitutively nuclear form of DAF-16/FoxO (DAF-16^{AM}) does not promote dauer formation or lifespan extension in a wild-type background but is sufficient to do so in a *daf-2(e1370)* background[1], indicating that a branch of DAF-2 signaling represses nuclear DAF-16/FoxO. In order to isolate DAF-2 effectors that modulate nuclear DAF-16/FoxO transcriptional activity rather than DAF-16/FoxO localization, an enhancer of *akt-1* (*eak*) screen was performed that identified the novel, conserved gene *eak-7*. We demonstrate that loss of *eak-7* function is sufficient to drive dauer formation in animals expressing DAF-16^{AM}. Although loss of *eak-7* does not promote DAF-16/FoxO nuclear localization, loss of *eak-7* leads to dramatic up-regulation of the direct DAF-16/FoxO target gene *sod-3* in an *akt-1* null background. *eak-7(lop)* mutants exhibit a variety of phenotypes that are characteristic of DAF-2 pathway mutants. *eak-7* mutants have a weakly penetrant dauer phenotype and strongly enhance the dauer phenotypes of the PI3K/AKT pathway mutants. They also exhibit greater resistance to heat, ultraviolet radiation and oxidative stress than their wild-type counterparts. *eak-7* mutants are long-lived relative to wild-type animals. Lifespan extension of *eak-7* mutants is dependent upon both *daf-16* and *smk-1*, a recently characterized regulator of DAF-16 activity. We also find that various stresses induce *eak-7* message levels in *C. elegans* as well as in human cultured cells. Because EAK-7 negatively regulates DAF-16/FoxO activity, the up-regulation of *eak-7* may be necessary to attenuate DAF-16/FoxO-mediated responses to stress. These data implicate EAK-7 as a potential effector of DAF-2 that modulates nuclear DAF-16/FoxO activity in parallel to the canonical PI3K/AKT pathway.

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Evolution and Significance of DAF-16 signalling in the *Caenorhabditis* genus

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In nematodes, lifespan and stress resistance have been shown to have both a gender specific and species specific variability and previous work has suggested that there is a common molecular mechanism or a “molecular link” which governs these processes. Using various *Caenorhabditis* species we are investigating whether the transcription factor DAF-16, a downstream component of the Insulin like/IGF-1 signalling pathway, acts as this “molecular link”. DAF-16 is orthologous to the FOXO family of transcription factors in mammals and is thought to play a key role in stress response, immune response and ageing by controlling expression of several hundred genes.

We have shown that hermaphrodite species within the *Caenorhabditis* genus seem to age faster and possess a weaker immune response in comparison to the male/female (gonochoristic) species. This work focuses on establishing that it is the difference in expression of the DAF-16 transcription factor and its activity which is responsible for the difference in immune response and hence the difference in ageing among the various *Caenorhabditid* species. We propose that this could also explain the evolutionary conundrum of post-reproductive ageing since enhanced immunity would also increase lifespan even after the animal has reproduced.

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The Parkin co-regulated gene encodes a protein, PCRG-1, localizes to sensory cilia and regulates lifespan

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Caenorhabditis elegans senses environmental cues through ciliated sensory neurons located primarily in the head and tail. Mutants with defective cilia have impaired sensory perception, and often, this correlates with an increased lifespan and dauer defects.

Here we demonstrate that the *C. elegans* orthologue of mammalian *Pacrg*, the gene which shares a promoter element with Parkin, a ubiquitin ligase implicated in Parkinson's disease, is required for specifying normal lifespan. Although the *C. elegans* PACRG protein (PCRG-1) localizes specifically to ciliated neurons, abrogating its function causes none of the known or canonical ciliary phenotypes, including increased longevity or defects in chemotaxis, osmo-avoidance, mating, or dauer formation. In addition, the mutant animals are morphologically sound, develop normally to the adult stage, and exhibit a typical brood size. Surprisingly, *pcrg-1* mutants have a reproducible and significant reduction in lifespan, a first for a gene encoding a *bone fide* ciliary protein. This defect does not stem from a general or non-specific loss of viability, since animals lacking both *pcrg-1* and the insulin-like receptor gene *daf-2* have a lifespan indistinguishable from that of the very long-lived *daf-2* single mutant. Epistasis analyses reveal genetic interactions between *pcrg-1* and several ciliary mutants, and suggest that PCRG-1 functions upstream of the insulin-like receptor DAF-2 in the insulin-like signaling pathway. Consistent with these data, *pcrg-1* mutants have decreased levels of DAF-16, the transcription factor implicated in DAF-2 dependant lifespan control. Our findings therefore reveal that a ciliary axonemal protein is required for controlling a specific insulin-dependant program that regulates longevity in *C. elegans*.

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Long Lived Mitochondrial (Mit) Mutants Utilize a Novel Metabolism

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The *C. elegans* Mit mutants have reduced electron transport chain (ETC) functionality yet, surprisingly, are long lived. It has remained a mystery how mutants deficient in energy production can exhibit life extension. Several hypotheses have been proposed such as reduced ROS production and the activation of a hormetic response. We have previously proposed that the Mit mutants exploit alternative energy production pathways that as a consequence, alter lifespan indirectly. In this investigation we have begun to formally map the energy metabolism of two Mit mutants, *clk-1* and *isp-1*. We have established novel methodology to characterize the excreted *C. elegans* metabolome as an indicator of internal metabolic events. Using HPLC-UV, GC-MS and PCA we have compared metabolic profiles of the Mit mutants directly to aerobically and anaerobically cultured wild type animals, as well as short lived mitochondrial mutants, *mev-1* and *ucr-2.3*. We have shown that the long lived Mit mutants exhibit a metabolism that is distinct from both short lived mitochondrial mutants as well as aerobic wild type animals. Furthermore, the Mit mutant metabolic signature shows only minor overlap with the profile of anaerobically cultured wild type animals. Our study suggests Mit mutants employ a unique metabolism that is possibly fundamental to their longevity response.

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Caenorhabditis elegans lifespan is profoundly modulated by its diet of E. coli: What are the roles of E. coli metabolism and coenzyme Q?

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Coenzyme Q_n is a fully substituted benzoquinone containing a polyisoprene tail of distinct numbers (n) of isoprene groups. Q is an essential component of respiratory electron transport and a potent lipid soluble antioxidant. Extended lifespans are observed in *C. elegans clk-1* mutants with defects in Q biosynthesis. Intriguingly, mice heterozygous for a *clk-1* gene disruption also show increased lifespan. *C. elegans* fed *E. coli* devoid of Q₈ have a significant life span extension when compared to *C. elegans* fed a standard “Q-replete” *E. coli* diet. These results initially suggested that the lack of Q is the important parameter affecting lifespan. However, recent results indicate that feeding respiratory incompetent *E. coli*, whether Q-replete or Q-less, produces a robust lifespan extension in *C. elegans*. *C. elegans skn-1* mutants fed the respiratory incompetent *E. coli* diets also show lifespan extension, suggesting that the response is independent of a dietary restriction effect. The respiratory incompetent *E. coli* diet may be imposed well after the worms reach adulthood, including post-reproductive adults, indicating that lifespan extension operates independently of worm development. Curiously, *C. elegans* fed the Q-less *E. coli* diet seem to exhibit more pronounced lifespan extension than when fed respiratory incompetent *E. coli* that are Q-replete. *C. elegans ttx-3* mutants, harboring defects in the gene encoding a LIM homeodomain transcription factor, fail to distinguish the Q-less from the Q-replete respiratory defective *E. coli*. The difference in life span extension mediated by the two respiratory deficient *E. coli* diets depends on normal food seeking behavior exhibited by N2 *C. elegans*. The data suggest that the life span extension of N2 worms fed Q-less *E. coli* diet is caused by a combination of respiratory deficiency of the *E. coli* and the food seeking behavior of *C. elegans*.

This work was supported by NIA Grant AG19777, and by the Ellison Medical Foundation.

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Characterizing Two *coq-3* Mutants in *Caenorhabditis elegans*

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Coenzyme Q (referred to simply as Q) is a lipophilic component of the electron transport chain in mitochondria. The biosynthesis of Q is known to include several steps, including two *O*-methyltransferase reactions catalyzed by COQ-3. The *coq-3* gene is well-conserved among various organisms, from *Homo sapiens* to *E. coli*, where the gene is termed *ubiG*. Expression of *C. elegans* COQ-3 restores respiratory growth and Q biosynthesis in an *E. coli ubiG* mutant, and in a *S. cerevisiae coq-3* mutant. In *C. elegans*, *coq-3* mutants demonstrate decreased fertility, activity, survival and behavior. One mutant, *coq-3(qm188)*, possesses a deletion of the third and fourth exons. Sequencing of the *coq-3(qm188)* mRNA transcript shows a spliced product containing exons 1, 2 and 5 in a complete reading frame. Another mutant, *coq-3(ok506)*, is missing exon 3 only; however, the spliced mRNA is frameshifted and is predicted to generate an early stop codon. Interestingly, *coq-3(qm188)* mutants are sterile on a Q-replete bacterial diet of OP50, whereas *coq-3(ok506)* mutants generate approximately 100 larvae that arrest at the L1 stage. Both mutant strains are sterile and when fed the Q-deficient bacteria strain GD-1. Neither the *coq-3(qm188)*, nor the *coq-3(ok506)* mutant is rescued, however, when grown on media containing NovaSol Q10, a lipid-soluble coenzyme Q10 delivery system. RT-PCR conducted on these strains shows mRNA product of the predicted sizes for both strains. Sequencing of these transcripts reveals no cryptic splice sites. The *coq-3* gene is situated in a three-gene operon. Transcripts of the flanking genes in the operon from the *coq-3(qm188)* mutant allele are present, as evaluated via RT-PCR. Currently we are optimizing small-scale quinone analysis in lipid extracts of 50 adult worms via HPLC-MS/MS.

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Autophagy Genes Affect in the Extension of Lifespan by Dietary Restriction in *C. elegans*

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Dietary restriction (DR) extends lifespan in multiple organisms, at least in part, by down-regulation of the TOR pathway (1,2). In *C. elegans*, both DR and TOR inhibition reduce protein translation (1) and extend lifespan via a DAF-16/FOXO independent mechanism (3-5). Even though inhibition of protein translation extends lifespan (1, 6-8), it remains unclear if this is the sole process by which DR promotes longevity or whether this is also an independent avenue of regulation. Besides protein translation, TOR also regulates autophagy. This cellular process of cytoplasmic degradation is generally induced following nutritional deprivation (9). To better understand the molecular mechanism underlying DR, we have addressed the role of autophagy in this process in *C. elegans* (10).

We find that DR and TOR inhibition trigger autophagy in this organism, and that inhibiting genes required for autophagy during adulthood prevents DR and TOR inhibition from extending lifespan. The longevity response to DR in *C. elegans* is not a passive consequence of reduced energy levels, because it is known to require transcription, in part through the PHA-4 transcription factor (11). Interestingly, we find that the autophagic response to DR also requires PHA-4 activity, indicating that autophagy in relation to lifespan, too, is a transcriptionally-regulated response to food limitation. Finally, in spite of the rejuvenating effect that autophagy is predicted to have on cells, we find that autophagy does not appear to be sufficient to extend lifespan. Long-lived *daf-2* insulin/IGF-1 receptor mutants require both the transcription factor DAF-16/FOXO (12) and the autophagy for longer life. Taken together with our finding that autophagy takes place in the absence of DAF-16, we conclude that autophagy is necessary but not sufficient for lifespan extension in the context of insulin/IGF-1 signaling. Perhaps, by recycling cellular components, autophagy provides raw material for new protein construction, and transcription factors such as DAF-16 program the cells to recycle these components into cell-protective longevity proteins.

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1. Hansen et al., 2007; 2. Kapahi et al., 2004; 3. Lakowski and Hekimi, 1998; 4. Hourthod et al., 2003; 5. Vellai et al., 2003; 6. Henderson et al., 2006; 7. Pan et al., 2007; 8. Syntichaki et al., 2007; 9. Levine and Klionsky, 2004; 10. Hansen et al., 2008; 11. Panowski et al., 2007; 12. Kenyon, 2005

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IRE-1 Promotes the Longevity of *C. elegans* Insulin/IGF-1-Receptor Mutants by Reducing the Levels of Insulin and ER Stress

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Long-lived *C. elegans* mutants that have reduced insulin/IGF-1 signaling (IIS) express many cell-protective genes and are resistant to oxidative stress. In this study we asked whether the genes that mediate the ER stress response might play a role in their longevity as well. Our findings suggest that the ER stress-protective protein IRE-1 contributes to the increased longevity of IIS mutants in two ways. First, IRE-1 acts independently of its known target, the transcription factor XBP-1, to reduce insulin levels in animals with reduced IIS. Second, IRE-1 acts with XBP-1 to increase ER stress resistance and longevity. Paradoxically, reducing IIS enhances ER stress resistance in an IRE-1/XBP-1 dependent fashion, but at the same time reduces the level of XBP-1 and the expression of *hsp-4*, a known XBP-1 target gene. Thus reducing IIS allows lower levels of XBP-1 to generate a more effective response to ER stress and to produce a robust extension of lifespan.

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C. *Elegans* Learning and Memory as Measures of Age-Related Neuronal Decline

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In humans, aging often is associated with a decline in cognitive function. The neurological changes associated with normal aging are subtle compared to those of age-related neurodegenerative diseases, such as Alzheimer's disease. Progress toward an understanding of the molecular mechanisms underlying the initiation and progression of age-related neuronal decline could be hastened by the development of experimental systems that quickly test early and true symptoms (rather than the correlative downstream effects) of neuronal decline and disease. In contrast to muscle degradation, the nervous system of *C. elegans* is structurally remarkably well-preserved, leaving open the question of how to define age-related changes in neuronal function. To solve this problem, we have established a novel system to study associative learning, short-term associative memory, and long-term associative memory in *C. elegans*: through chemotaxis assays, we have measured worms' ability to learn a positive association of a neutral chemoattractant with food. We have found that long-term, but not short-term, associative memory is dependent on *crh-1*, the *C. elegans* homolog of the transcription factor CREB. Furthermore, transcriptional analysis identified CREB targets that are changed during long-term memory training, which include genes involved in vesicle trafficking, ion channels, synaptogenesis, RNA binding, and uncharacterized genes, many of which, we have shown to be required for long-term memory. LTAM training induces CREB-reporter GFP expression in the SIA and AIY interneurons, a pattern that differs from CREB activation during starvation. Additionally, downstream targets of CREB are activated in other neurons, including glia, likely through CREB's activation of the JNK pathway. When applying our assays for cognitive function to aging worms, we find that worm learning and long-term associative memory decreases with age and is influenced by longevity pathways. Our assays will allow quick screening of mutations and conditions that improve age-related associative learning and memory decline.

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Ascaroside Signalling and Lifespan in *C. elegans*

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Key developmental pathways in *C. elegans* appear to be regulated by several sets of endogenous small molecule signals. Recent analyses in our laboratory characterized eight *C. elegans*-produced glycosides of the dideoxysugar ascarylose. Among these, the three ascarosides ascr#1, ascr#2, and ascr#3 had previously been shown to induce dauer formation in *C. elegans*; in addition, ascr#2 and ascr#3 synergize with ascr#4 as sex pheromones (Kawano et al., 2005; Butcher et al., 2007, Srinivasan et al., in press). Identification of these endogenous compounds presents a unique opportunity by providing small-molecule tools for dissecting developmental pathways. Here, we show that ascarosides ascr#2 and ascr#3 strongly increase thermo tolerance in *C. elegans*. Wild type worms grown on NGM medium containing nanomolar concentrations of these ascarosides survived up to six times longer at 35°C compared to untreated controls. We also found that animals exposed to ascr#2 and ascr#3 on minimal growth medium had up to three-fold increased lifespan. Notably, 50 day old adult worms grown on minimal medium containing ascr#2 and ascr#3 carried small numbers of fully developed or over-developed embryos. When re-exposed to favourable conditions, these animals gave rise to few, but viable and reproductive progeny. We currently investigate the impact of these endogenous signaling molecules on thermotolerance and life span extension in a comprehensive set of mutant strains.

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Heterochronic Genes and the Control of Dauer Formation

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Animals monitor a variety of environmental cues including seasonal variation in day length as well as more unpredictable and stress-inducing fluctuations in temperature and nutrient availability, and they respond to these cues through adjustments in metabolism, physiology, and behavior. Dauer larva formation in *C. elegans* is one of the best-studied responses to environmental stress, and a particularly interesting aspect of this response is how dauer formation is coordinated with the concomitant suspension of the continuous developmental timing pathway. More than 20 heterochronic genes have been identified that time events during continuous reproductive development; certain of these genes also modulate the reversible interruption of developmental time imposed by dauer diapause. *lin-42*, the worm *period* homolog, is one gene that controls temporal development and mediates dauer entry. *lin-42* loss-of-function mutants initiate multiple postembryonic events too early during reproductive development, including vulval cell divisions, gonad migration, and terminal differentiation of the hypodermis. These animals are also sensitized toward dauer formation and constitutively form dauer larvae under conditions where wild-type animals do not. Our work positions LIN-42 as a key component of the dauer switch and demonstrates a functional connection between it and the ligand-gated nuclear hormone DAF-12, suggesting that these two proteins are intimate partners in controlling the dauer decision. miRNAs, including the *let-7* family members *mir-48*, *mir-84* and *mir-241*, are also key players in the heterochronic gene pathway and here too, studies reveal ties to the dauer pathway. Although single gene deletions of the three *let-7* “sisters” reveal little phenotype, roles for these genes in the timing pathway are revealed by gain-of-function mutations, overexpression studies and analysis of multiply mutant animals. Overexpression of *mir-48* causes a strong precocious phenotype in the hypodermis and vulva, and the animals are dauer defective. Suppressors of these phenotypes have been identified, and on their own, these mutations can cause retarded heterochronic defects. These mutations are likely to define genes involved in miRNA expression, processing or function, possibly including target genes. An obvious challenge now is to decipher the mechanistic interactions among the heterochronic genes and to determine how they help integrate external signals to halt development for dauer formation.

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The Heterochronic Gene *mab-10* Acts with *lin-29* to Regulate Terminal Differentiation in Hypodermal Lineages

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The study of heterochronic mutants has revealed a complex genetic pathway that regulates the timing of many developmental events in *C. elegans*. Heterochronic mutants fall into two classes: precocious mutants, which prematurely express later developmental fates, and retarded mutants, which reiterate earlier developmental fates. Both classes can alter the timing of the larval-to-adult transition. This transition comprises four events: seam cell fusion, generation of an adult-specific cuticle, exit of seam cells from the cell cycle and exit from the molting cycle.

Like retarded *let-7* and *lin-29* mutants, *mab-10* males undergo a supernumerary molt approximately 18 hours after the larval-to-adult transition (C. Link, Worm Breeder's Gazette 10, 92, 1988). To understand further the regulation of the larval-to-adult transition, we have extended the analysis of *mab-10* and found that both *mab-10* males and hermaphrodites enter lethargus as adults and often execute a supernumerary molt. While the seam cells of *mab-10* mutants fuse appropriately at the end of the L4 stage and generate a relatively normal adult cuticle, the seam cell nuclei of *mab-10* mutants inappropriately undergo extra rounds of division. We conclude that *mab-10* is required both for the prevention of seam cell divisions and for the cessation of molting, two of the four events that occur during the larval-to-adult transition. By contrast, the C2H2 zinc finger protein LIN-29 is required for all four events.

We cloned *mab-10* and found that it encodes the only *C. elegans* member of the conserved NAB (NGFi-Alpha Binding) family of transcription factors. Recent studies of mice have implicated the NAB family of proteins in regulating the terminal differentiation of specific stem cell lineages (Le et al., Nature Neurosci. 8, 932, 2005). NAB proteins are believed to act as co-factors for C2H2 zinc fingers to regulate differentiation. Our genetic analyses and co-localization and *in vitro* binding experiments suggest that MAB-10 functions as a cofactor for the C2H2 zinc finger transcription factor LIN-29 to regulate specifically the exit of the seam cells from the cell cycle and the cessation of molting, but not seam cell fusion or adult cuticle synthesis. We propose that the regulation of developmental stage in *C. elegans* and the regulation of terminal differentiation in mammalian stem cell lineages share a common mechanism controlled by a conserved heterochronic pathway.

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The Roles of *lin-28* and *lin-46* in the Heterochronic Pathway

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The heterochronic pathway controls the appropriate timing of cell fate decisions and has been demonstrated to affect, among other tissues, lateral hypodermal-seam cell lineages by altering the timing of cell division and differentiation. In particular, three miRNA genes *mir-48*, *-84* and *-241* (*3-mirs*), belonging to *let-7* family negatively regulate *hbl-1* to control the timing of L2 cell fate decisions. However, additional factors influence this regulation. Specifically, *lin-28* and *lin-46* act oppositely to control cell fates via *hbl-1*. We found that *lin-28* positively regulates *hbl-1* through its 3'UTR. Our genetic analysis reveals that a negative factor other than *3-mirs* acts on *hbl-1* and that *lin-28* opposes that factor in some way. In trying to identify this factor, we examined *ain-1* (ALG-1 interacting protein), which resembles GW182, a human protein involved in the miRNA pathway. An *ain-1(0)* mutant highly enhances the retarded phenotype of *3-mirs(0)* animals. However, *lin-28(0)* precocious phenotype is epistatic to the retarded phenotype of *3-mirs(0); ain-1(0)* animals. The missing negative regulator may still be another miRNA gene. Candidates include *lin-4*, *let-7*, and their less well-characterized relatives (*mir-237*, *mir-793 – 795*, respectively) and the Pumilio-related gene *puf-9*. Importantly, we have found that LIN-28 protein binds selectively to miRNA precursors, as was found for its human homologue, suggesting *lin-28* may inhibit miRNAs directly. Another player is *lin-46* which encodes a putative scaffolding protein. LIN-46 protein accumulates in briefly at each stage, and can form cytoplasmic foci resembling P-bodies. *lin-46* is known to enhance *3-mirs(0)*, and *lin-28(0)* is *not* epistatic to that quadruple mutant phenotype. We observed that *lin-46(0)* greatly enhances the retarded phenotype of *ain-1(0)*, and tentatively conclude that the two genes act in parallel pathways. *hbl-1(RNAi)* is epistatic to *lin-46; ain-1* double mutants and moreover, a *hbl-1::gfp::hbl-1* reporter expression in *lin-46; ain-1* mutants is similar to its expression in *3-mirs(0)* animals. These observations suggest that *lin-46* acts upstream of *hbl-1*, but whether its mechanism involves miRNAs is not yet clear.

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Screens for Chemical Suppressors and Enhancers of Dauer Formation

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Genetic screens for mutants in dauer formation have identified many genes and multiple pathways involved in this developmental process. We have hypothesized that new components of these pathways that may have been missed in genetic screens can be identified by screening chemical libraries for small molecules that affect dauer formation. In addition since many of the genes initially identified for their role in dauer formation also exhibit lifespan phenotypes this approach may also identify new determinants of adult lifespan.

We have screened a number of small chemical libraries and have identified compounds that either suppress or enhance dauer formation in *daf-2(e1368)*. Of three compounds that suppress *daf-2* dauer formation one is a natural product that has no known activity in other systems, while a second is a substrate in the biosynthetic pathway for the DAF-12 ligand. The third compound is a synthetic antagonist of the mammalian cannabinoid receptor, of which there are no known orthologs in worms. The activity of this compound suggests the existence of a novel endocannabinoid signaling pathway that interacts with insulin signaling in *C. elegans* to modify dauer development. We are beginning to define this nematode endocannabinoid pathway using a number of different approaches.

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A screen of putative DAF-16/FOXO target genes for novel SynDaf genes reveals a connection between dauer formation and innate immunity

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The DAF-16/FOXO transcription factor is the major downstream output of the insulin/IGF1R signaling pathway in *C. elegans* for lifespan and dauer formation. To determine the effects of DAF-16 target genes on dauer formation, we tested previously identified candidate genes putatively regulated by DAF-16. We used RNAi in a sensitized background [*eri-1(mg366); sdf-9(m708)*], which enhances RNAi and constitutive dauer formation (Daf-c). Among 515 genes tested, 22 have a synthetic Daf-c (SynDaf) phenotype with *sdf-9*. Two of these genes are previously identified SynDaf genes. Five other genes are involved in processes known to affect dauer formation. Two of the 22 genes are known to participate in innate immunity, and six more are predicted to be involved. The latter result suggests that disrupting the immune response may contribute to dauer formation. When grown on bacteria that are pathogenic to *C. elegans*, *daf-8* and *sdf-9* show a stronger Daf-c phenotype. This indicates that dauer formation is a response to pathogen exposure, in addition to the well-known environmental cues of population density, food supply and temperature.

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HLH-13, a novel DAF-16 regulatory target, suppresses recovery from dauer and L1 arrest

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Insulin/IGF-signaling (IIS) affects longevity, stress resistance and metabolism in worms, flies, and mammals. The Forkhead transcription factor DAF-16 is the major downstream effector of DAF-2/insulin receptor signaling, and is responsible for the activation and repression of genes that mediate the diverse effects of IIS. To identify DAF-16-regulated target genes, I used quantitative reverse-transcription PCR (qRT-PCR) to survey a set of 115 *C. elegans* genes containing a DAF-16 binding site at their promoter. One of the DAF-16 regulatory target genes identified is *hlh-13*, the predicted ortholog of the mammalian transcription factor Ptf1a, a critical determinant of pancreatic development.

A GFP reporter construct of *hlh-13* is expressed in all dopaminergic neurons, several unidentified ventral cord neurons during the L2/L3 developmental stages, and has DAF-16-responsive expression in the hypodermis. Since *hlh-13* represents a putative new DAF-16 transcriptional target, we tested whether loss of *hlh-13* may result in phenotypes commonly associated with insulin signaling, such as alterations in lifespan, stress resistance, and dauer formation. The lifespan of an *hlh-13* deletion mutant *hlh-13(tm2279)* is identical to controls in both a wild-type and IIS mutant background. *hlh-13(tm2279)* also does not exhibit differences in resistance to heat stress or oxidative stress (paraquat) compared to controls. However, we found that the *hlh-13(tm2279)* mutant worms recover faster from dauer arrest in a *daf-2(e1370)* mutant background at a sensitive temperature of 23C. The *hlh-13* mutant worms also recover faster from an extended L1 arrest, and are able to recover after a longer arrest than control worms. In addition, they are more resistant to an extended period of starvation than control worms in an IIS mutant background. *hlh-13* may regulate recovery from arrest and resistance to starvation by altering metabolism or fat storage. qRT-PCR of the *hlh-13* mutant worms shows lower levels of *acs-2*, F25H5.3, and *fat-5*, genes involved in mitochondrial beta-oxidation, glycolysis, and fatty acid desaturation, respectively. Also, staining with the lipophilic dye Nile Red shows altered fat storage in an *hlh-13(tm2279);daf-2(e1370)* double mutant. These results suggest intriguing roles for HLH-13 in dauer recovery and metabolism, and further studies are needed to fully understand the mechanisms involved in vivo.

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A Predictive Model for Small Molecule Accumulation and Bioactivity in *C. elegans*

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We are using *C. elegans* as a model system to discover new small molecule tools for biological analyses. One obstacle to finding such tools is the large number of xenobiotic defense factors encoded by the worm genome (Lindblom et al., 2006). For example, in a screen of 3,265 small molecules that are bioactive in other systems, we found only 64 (2%) that induce robust phenotypes in the worm (Kwok et al., 2006). We hypothesized that this hit rate is relatively low because most small molecules fail to accumulate in worms. To test this, we developed an HPLC-based assay to measure small molecule accumulation in worm tissue. We surveyed 1,018 compounds from the Spectrum library (Microsource Inc.) for accumulation in whole worms after 6 hours of incubation in 40uM of the small molecules. To ensure confidence in our assignments we established a detection limit of 18uM. Of the 361/1018 compounds that satisfied this criterion, only 25 (6.9%) accumulate in worms. Notably, 2 out of 25 accumulating molecules induce robust phenotype in the worm, compared to 0 out of 336 non-accumulating molecules. We also assayed 25 nematicides obtained from our other small molecule screens, and found that 17 (68%) accumulate. These results support our hypothesis that worms are generally resistant to small molecule accumulation, and show that accumulating small molecules are enriched for bioactivity in *C. elegans*.

Next, the accumulation of an additional 77 compounds from our other screens was assayed, for a complete dataset of 463 small molecules. We used this dataset to build a predictive structure-based model of accumulation. We compiled 4,166 structural descriptors of the small molecules in our dataset, and built a model using a Bayesian Classifier machine learning method. Five-fold cross validation of the model estimates a prediction accuracy of $75.36 \pm 2.04\%$. We used the model to rank 9,742 compounds of a DIVERSet library (Chembridge Corp.), of which 48 induce robust phenotype in the worm. Encouragingly, 12 of the top-scoring 200 molecules induce phenotype, representing a 12.2-fold enrichment for bioactivity compared to the entire library ($p < 10^{-9}$). None of the bottom-scoring 200 molecules induce phenotype. These data demonstrate that our model is effective at predicting small molecule accumulation and bioactivity in *C. elegans*. We hope to use this model to increase our efficiency at identifying new small molecule probes for biological analysis, and to aid the development of potential drug leads using *C. elegans* as a model.

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The 3- β -Hydroxysteroid Dehydrogenase (3 β HSD) Family Member HSD-1/ EAK-2 Acts in the Dafachronic Acid Biosynthetic Pathway to Inhibit Nuclear DAF-16/FoxO Activity

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How transcription factors interact functionally to regulate dauer arrest is poorly understood. The EAK pathway promotes reproductive development by acting in parallel to AKT-1 to inhibit the activity of the FoxO transcription factor DAF-16. *eak-2* is allelic to the conserved 3 β HSD family member *hsd-1*, which was first identified as an enhancer of the *ncr-1* dauer arrest phenotype. In contrast to AKT-1, which promotes the cytoplasmic retention of DAF-16/FoxO, HSD-1 does not affect DAF-16/FoxO subcellular localization but inhibits nuclear DAF-16/FoxO activity. In *hsd-1; akt-1* double mutants, both DAF-16/FoxO and DAF-12 are required for maximal expression of the direct DAF-16/FoxO target gene *sod-3*. Preliminary results suggest that HSD-1 has 3 β HSD activity *in vitro*. We propose that HSD-1 acts in parallel to AKT-1 to regulate the expression of a subset of DAF-16/FoxO target genes via dafachronic acids and DAF-12. 3 β HSD family members may interact similarly with insulin signaling to regulate FoxO target gene expression in mammals.

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Investigation of endogenous RNAi processes affecting stress response in *C. elegans*

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Stress response and aging are influenced by a variety of processes such as translational regulation, insulin signaling, mitochondrial function, and others. Gene expression profiling performed on *zfp-1(ok554)* and *rde-4(ne301)* mutants related to RNAi suggested that short RNAs may play a role in regulating stress response and longevity in *C. elegans*. We are investigating this possibility further and focusing on the characterization of *zfp-1* gene function. *zfp-1* gene encodes a chromatin protein required for a strong RNAi response in *C. elegans*. It was also characterized as a DAF-16 target, and loss of *zfp-1* has been shown to shorten lifespan in *C. elegans*. We have found that *zfp-1* mutants are stress-sensitive and that upregulation of DAF-16 targets, such as *sod-3*, upon stress is impaired in this mutant background, despite the fact that it does not affect DAF-16::GFP nuclear localization upon stress. Furthermore, *zfp-1* suppresses stress resistance associated with the translational initiation factor mutation *ife-2*. These results suggest that *zfp-1* is working downstream of both insulin signaling and translational regulation. We are interested in finding direct targets of ZFP-1 responsible for the observed phenotype. Surprisingly, we have found that many canonical RNAi mutants such as *rde-1*, *rde-4* and others are resistant to oxidative stress. We also found that wild type (N2) worms fed on bacteria producing dsRNA, independent of the gene being knocked down, are similarly more stress resistant. We are testing a possibility that competition between exogenous and endogenous RNAi pathways is responsible for the observed effects.

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Functional Characterization Of Ubiquilin And Erasin Proteins In *Caenorhabditis elegans*: Demonstration Of Their Role In Regulating ER Stress And Aging

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An accumulation of misfolded proteins in cells and tissues is associated with a number of human diseases, particularly age-related diseases. It is not surprising that cells have therefore evolved a number of quality control systems to monitor and maintain fidelity of protein folding, a sophisticated set of which is present in the endoplasmic reticulum (ER), the organelle where over one third of all proteins are synthesized. An accumulation of misfolded proteins in the ER activates a series of signaling pathways collectively called the unfolded protein response (UPR) that function to eliminate the misfolded proteins and restore proper protein homeostasis. One set of genes that are upregulated during UPR are those involved in ER-associated degradation (ERAD), the process by which misfolded proteins in the ER are retro-translocated from the ER to the cytosol or nucleoplasm for degradation by the proteasomes. Inefficient clearance of misfolded proteins from the ER leads to ER stress, which appears to be responsible for causing many human diseases. We have identified ubiquilin and erasin as components of a novel complex involved in ERAD. Using *Caenorhabditis elegans* as a model system, we demonstrate that ubiquilin and erasin are UPR genes upregulated during tunicamycin-induced ER stress and that their upregulation requires the IRE-1 branch of the UPR. Loss of ubiquilin or erasin by RNA interference (RNAi) resulted in activation of the UPR and increased accumulation of polyubiquitinated proteins. Moreover, loss of ubiquilin ultimately led to a shortened lifespan in worms. We also demonstrate the involvement of ubiquilin in a worm model of Huntington's disease. We show that overexpression of mRFP-tagged ubiquilin prevented and rescued a motility defect caused by expression of the human huntingtin exon 1 fragment containing toxic polyglutamine tracts while loss of ubiquilin by RNAi exacerbated this defect. Our results strongly support a role for ubiquilin and erasin as key components in the ER stress response to maintain protein homeostasis. Methods to regulate expression of ubiquilin and erasin may provide a therapeutic strategy to a variety of diseases caused by ER stress.

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Survival from Hypoxia by Inactivation of Aminoacyl-tRNA-Synthetases

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The sensitivity of an organism and its cells to hypoxic injury varies widely across species and cells types. These determinants of hypoxic sensitivity control fundamental emergent organismal traits such as habitat range and hibernation; additionally, they offer potential avenues to therapies for major human diseases such as cancer, myocardial infarction, and stroke. To identify modulators of hypoxic sensitivity, we performed a clonal mutagenesis screen in *C. elegans* for mutations that confer resistance to hypoxic death. A profoundly hypoxia resistant mutant was isolated in the screen and mapped to a genomic interval containing *rrt-1*, which encodes an arginyl-tRNA synthetase. Transformation rescue, identification of a missense mutation, and phenocopy of the hypoxia resistance by RNAi confirmed the assignment of the mutation as a reduction-of-function allele of *rrt-1*. Acute reduction in *rrt-1* function either before or after the hypoxic insult increased survival. *rrt-1* acted within somatic cells to control organismal hypoxic sensitivity, and knockdown protected neurons and myocytes. Aminoacyl-tRNA-synthetases, such as RRT-1, are a highly evolutionarily conserved family of proteins, each functioning to aminoacylate its cognate tRNA. RNAi knockdown of twenty-two genes of this family conferred significant hypoxia resistance. Thus, modulation of the activity of aminoacyl-tRNA-synthetases is a potent mechanism for controlling hypoxic sensitivity in *C. elegans*.

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SKN-1, Systemic Detoxification, and Aging in *C. elegans*

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Various lines of evidence suggest that oxidative damage plays a major role in aging. Our lab has shown that the *C. elegans* transcription factor SKN-1 orchestrates a major conserved defense against oxidative damage (Phase 2 detoxification). In response to stress, SKN-1 accumulates in intestinal nuclei and activates Phase 2 genes. This response requires p38 signaling, and its constitutive activation is blocked by GSK-3. SKN-1 is also directly inhibited by the insulin/IGF-1-like signaling (IIS) pathway in the intestine. *skn-1* contributes to increases in stress resistance and longevity that are seen when IIS signaling is reduced, and promotes longevity when overexpressed in intestinal nuclei, indicating that SKN-1 and the Phase 2 system are important in IIS effects on lifespan.

More recently, we have used RNAi screening to identify other mechanisms that prevent constitutive Phase 2 gene activation. This work has identified new biochemical mechanisms and cellular pathways that affect SKN-1, and has also uncovered existence of SKN-1- and p38-independent pathways of Phase 2 gene regulation.

Using microarrays, we have identified genes that are regulated by SKN-1 under normal and stress conditions. SKN-1 is required for expression of numerous Phase 2 and other classes of genes, but also suppresses expression of a smaller cohort of genes that decrease stress resistance and lifespan. Many genes in each group seem to be direct SKN-1 targets. A subgroup of SKN-1 targets are induced in response to arsenite, but treatment with a lipid-soluble peroxide leads to activation of an overlapping but smaller set of SKN-1 targets, and major SKN-1-independent classes of detoxification genes. We conclude that under normal conditions SKN-1 not only defends directly against free radical damage, but also inhibits regulatory genes that reduce stress resistance. SKN-1 and Phase 2 genes integrate different signals in response to particular stresses, allowing plasticity in organismal responses to toxins.

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Nutritional Control of Gene Expression During L1 Growth and Arrest

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Upon hatching, L1 larvae must initiate feeding and growth in order to execute post-embryonic development. When larvae hatch in the absence of food they persist in a stress resistant, developmentally arrested state (L1 arrest). We seek to identify the pathways that regulate L1 arrest and to determine how they accomplish developmental arrest and stress resistance by characterizing their effects on gene expression. We characterized mRNA expression genome-wide in a pair of bifurcating time series starting in the late embryo and proceeding through the hatch in the presence and absence of food (*E. coli*). Known regulators of energy homeostasis are up-regulated during L1 arrest (PI3K, TOR, AMPK, and SIR-2), and expression dynamics suggest that L1 arrest serves as a developmental checkpoint and that arrested L1s are poised for rapid recovery. Steroid hormone metabolism genes and numerous nuclear hormone receptors are up-regulated early in L1 arrest and lipid and fatty acid metabolism genes are up-regulated late. Reporter gene analysis demonstrates nutritional control of transcription and indicates that the response to starvation is anatomically complex while suggesting that the sensory neurons and gut are most affected. This analysis shows that in maintaining homeostasis physiological mechanisms contribute as much to gene regulation as development.

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Manganese Uptake by *C. elegans* NRAMP2 Orthologues leads to Dopaminergic Neurodegeneration and Dopamine-dependant Toxicity

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Manganese (Mn) is essential for development and vital enzymatic activities. However, occupational exposure to Mn has been implicated in a Parkinsonian-like syndrome called manganism, primarily affecting dopaminergic neurons.

Here we demonstrate that the nematode *C. elegans* readily takes up Mn resulting in lethal osmoregulation defects at high concentrations. Among three divalent metal transporter 1 (DMT1, also called NRAMP2) orthologues, *smf-1* and *smf-3* mutants were found to take up less Mn than wildtype and showed hyper-resistance to the metal exposure, while *smf-2* mutants were hypersensitive, exhibiting higher Mn content. Accordingly, expression pattern analysis revealed that *smf-1* and *smf-3* were strongly expressed in the intestine and localized at its apical membrane likely responsible for most Mn uptake, while *smf-2* was found in epithelial pharyngeal cells and localized in intracellular compartments unlikely involved in Mn uptake.

Concomitantly, we proved that sub-lethal doses of Mn lead to selective and dose-dependent degeneration of *C. elegans* dopaminergic neurons. Moreover, using mutants affecting dopamine metabolism or release, we established that high extracellular dopamine levels sensitize to manganese exposure, whereas low levels are protective, suggesting that dopamine directly contributes to manganese toxicity.

Our work validates *C. elegans* as a suitable model for manganese toxicology studies. Moreover it unravels a potential toxic role for dopamine in dopaminergic system disorders such as manganism and Parkinson's disease.

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WDR-23, a novel WD40 repeat protein, is a ubiquitin ligase substrate adaptor that regulates the stress tolerance and longevity factor SKN-1

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Transcription of phase II detoxification genes in mammals is activated predominantly by the NF-E2-related factor Nrf2. The activity and abundance of Nrf2 is controlled by direct interaction with the kelch repeat protein Keap1, which functions as a substrate recognition subunit for the cullin ubiquitin ligase CUL3. In *Caenorhabditis elegans*, the Nrf2 homologue SKN-1 activates expression of phase II detoxification genes. Despite sequence and functional similarities between SKN-1 and Nrf2, *C. elegans* does not express a Keap1 homologue. We searched for novel regulators of SKN-1 by performing a genome-wide RNAi screen for genes that repress transcription of the phase II detoxification gene *gst-4*. RNAi of proteasome components, the CUL-4/DDB-1 ubiquitin ligase complex and the novel WD40 repeat protein WDR-23 activate *gst-4* expression in the absence of stress. WDR-23 is expressed in intestinal, hypodermal, and neuronal cell nuclei and interacts with DDB-1 and SKN-1. Loss of function of WDR-23 causes localization of SKN-1 to intestinal cell nuclei and increases longevity and stress resistance. These data are consistent with a model in which WDR-23 functions as a substrate-recognition subunit of the CUL-4/DDB-1 ubiquitin ligase complex. We hypothesize that binding of SKN-1 to WDR-23 targets it for ubiquitylation by the CUL-4/DDB-1 complex and subsequent destruction by the proteasome. Our studies have elucidated a novel mechanism for regulation of SKN-1 activity in *C. elegans* that is remarkably similar to Keap1 regulation of Nrf2 in mammals. Despite little to no sequence similarity, *C. elegans* WDR-23 and mammalian Keap1 regulate homologous stress-activated transcription factors by direct interaction and recruitment to cullin ubiquitin ligases.

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Lab: Strange

Uncoupling Mechanisms Required for Oxygen Deprivation Survival and Longevity in *C. elegans*

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Stress resistance and longevity occur from single gene mutations, suggesting that these two traits are regulated by similar mechanisms. Others have shown that mutations in the insulin-like signaling pathway (*daf-2(e1370)*) or alterations that affect germline (*glp-1(q158)*) lead to increased lifespan. We show that these same mutations lead to severe oxygen deprivation survival (anoxia), indicating that stress resistance and longevity are under control of DAF-16 or signals involving the reproductive tissue. Further analysis of mutations that enhance long-term anoxia survival indicates that there are distinct genetic and physiological factors affecting anoxia responses that do not affect longevity. For example, the DAF-16-regulated glycolytic genes, *gpd-2/3*, are required for the *daf-2(e1370)* animals' long-term anoxia and high temperature anoxia survival phenotype but not the longevity phenotype. Furthermore, we have identified mutants with reproductive phenotypes, such as the *fog-2(q71)* animals, which have an enhanced ability to survive long-term anoxia but are known to not be long lived. Additionally, not all *daf-2* mutants that are long lived will survive long-term anoxia. Thus, data suggests that modulation of distinct components of the insulin signaling pathway or specific changes in reproductive physiology differentially regulate anoxia survival and longevity. Therefore, we hypothesize that the mechanisms required for anoxia stress responses and longevity are distinct and can be uncoupled. To test this hypothesis we are conducting further analysis on the functional role of *gpd-2/3*. First, we are determining if *gpd-2/3* function is required for other long-term anoxia survival mutants. Second, we are using a transcriptional reporter (*Pgpd-2/3::GFP*) to analyze *gpd-2/3* expression in anoxia exposed animals, aging adults or *daf-16(mu86)* null mutants. Finally, we tested whether transgenic over expression of GPD-2/3 is sufficient to increase long-term anoxia survival or enhance lifespan. This research will further our understanding of the relationship between oxygen deprivation resistance and longevity.

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osm-8* encodes a mucin-like protein that negatively regulates osmotic stress responses via the transmembrane protein *ptr-23

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Osmoregulation is involved in many physiological and pathophysiological processes, including cell growth, apoptosis, immunity, and renal function. The molecular mechanisms by which animal cells and tissues sense and signal osmotic disturbances are poorly understood. Following exposure to hypertonic stress, *C. elegans* synthesizes the organic osmolyte glycerol. Glycerol accumulation is preceded by the rapid transcriptional activation of the glycerol biosynthetic enzyme *gpdh-1* in the intestine and hypodermis, which can be conveniently visualized in live animals with a *gpdh-1p::GFP* reporter. Using this GFP assay, we have screened for mutants that positively and negatively regulate *gpdh-1p::GFP* expression. One such mutant, *osm-8(n1518)*, is resistant to acute and chronic osmotic stress (*osr* phenotype), exhibits strong constitutive expression of the *gpdh-1p::GFP* reporter, and contains high glycerol levels under isotonic conditions. We cloned *osm-8* and found it encodes a secreted mucin-like protein. Mucins are highly glycosylated extracellular proteins that line the apical surface of most non-keratinized epithelial cells. Recent genetic studies in yeast have implicated mucins as ‘osmo-sensors’, but our findings are the first to suggest a role for mucins in animal osmoregulation. Based on *osm-8* GFP reporters and cell-type specific rescue experiments, *osm-8* is likely secreted from the apical membrane of the hypodermis to exert its effects on osmosensitive signaling pathways. To define the genes involved in these pathways, we performed an RNAi screen for genes that suppress *osm-8* mutant phenotypes (*osr* and high *gpdh-1* expression). We found that RNAi of the Patched-Related homolog *ptr-23* suppressed the *osr* and high *gpdh-1* expression phenotypes of *osm-8*, as well as two other *osr* mutants, *osm-7* and *osm-11*. *ptr-23(RNAi)* had no effect on other stress-induced reporters, suggesting that *ptr-23* is specifically involved in osmosensitive signaling. We propose that *osm-8* may form part of an ‘osmo-sensor’ complex that mechanically tethers the extracellular matrix to the hypodermal cell membrane, possible via interactions with *ptr-23*. Disrupting this linkage, either through osmotically-induced cell shrinkage or *osm-8* mutations, results in the constitutive activation of signaling through *ptr-23*. Our data show that mucins in both yeast and *C. elegans* can regulate osmosensitive signal transduction and suggest that mucins might also play similar roles in vertebrate osmosensing.

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Dual Functions of EGL-9: Defining the Molecular Mechanisms by which EGL-9 inhibits HIF-1 Transcriptional Activity

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In mammals and in *C. elegans*, the hypoxia inducible transcription factor (HIF-1) mediates most of the gene expression changes induced by hypoxia (Jiang H *et al.*, 2001 PNAS 98: 7916; Shen *et al.*, 2005 JBC 280: 20580). EGL-9 has a central role in HIF regulatory networks. EGL-9 hydroxylates the proline in the LXXLAP motif of HIF-1, using oxygen as a co-substrate. This promotes HIF-1 degradation through a ubiquitination/proteasome pathway that requires the VHL-1 E3 ligase. When oxygen levels are low, EGL-9 hydroxylase activity is inhibited, and HIF-1 is stabilized (Epstein *et al.* 2001 Cell 107: 43). In prior studies, we have shown that EGL-9 functions through at least one additional pathway to inhibit HIF-1. In animals carrying a deletion mutation in *vhl-1*, loss-of-function mutations in *egl-9* have no effect on HIF-1 protein levels but dramatically up-regulate expression of HIF-1 target genes (Shen *et al.*, 2006 Genetics 174: 1205). In the past year, we have conducted a series of molecular genetic experiments to distinguish between alternative hypotheses for EGL-9 function. Our data support a model in which EGL-9 has two distinct functions: (1) hydroxylation and destabilization of HIF-1 via the well-characterized VHL-1 pathway; and (2) repression of HIF-1 transcriptional activity by a novel pathway that does not require HIF-1 hydroxylation.

We gratefully acknowledge funding from the NIH (GM078424).

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IRE-1 and HSP-4 Play Key Roles in Energy Homeostasis via Novel Fasting-induced Lipases in *C. elegans*

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The endoplasmic reticulum (ER) is an organelle associated with lipid metabolism. However, the involvement of the ER in nutritional status-dependent lipid homeostasis is unclear. We demonstrate that IRE-1, an ER protein involved in the unfolded protein response, and HSP-4, an ER chaperone, regulate the expression of novel fasting-induced lipases, *fil-1* and *fil-2*, for fat granule hydrolysis under short term starvation in *C. elegans*. Failure to hydrolyze intestinal fat granules during fasting impaired motility. Glucose supplementation rescued the motility defect, demonstrating the importance of *ire-1/hsp-4*-dependent lipid homeostasis for energy supply from stored fat droplets during fasting. Our data suggest that IRE-1 and HSP-4 are key nutritional sensors that modulate FIL-1 and FIL-2 expression to maintain energy homeostasis in *C. elegans*.

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The Mediator Subunit MDT-15 Integrates the Genomic Responses to Fasting, Xenobiotic Toxins, Heavy Metals, and Oxidative Stress

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The multi-subunit complex Mediator is a conserved transcriptional coregulator for transcription factors (TFs) such as Nuclear Hormone Receptors (NHRs). Although Mediator is globally required for Pol_{II}-dependent transcription, some of its subunits confer specific biological functions. Others and we found that the *C. elegans* Mediator subunit MDT-15 cooperates with the TFs SBP-1 [1] and NHR-49 [2] to control certain aspects of lipid biology. Importantly, MDT-15 affects worm health and life span by controlling fatty acid desaturation. Here, we show that MDT-15 is prominently involved in systemic detoxification. Specifically, depletion of MDT-15 protein or mutation of the *mdt-15* gene abrogates the induction of (i) Phase 2 detoxification genes such as *cyp-35C1* in response to the hydrophobic toxin fluoranthene, (ii) of metal responsive genes such as the metallothionein *mtl-1* in response to cadmium, and (iii) of oxidative stress response genes such as glutathione-S-transferases (*gst*) in response to arsenite. Moreover, *mdt-15(-)* animals are hypersensitive to fluoranthene. Intriguingly, MDT-15's role in stress response is selectively related to dietary uptake, as MDT-15 functional defects do not abrogate the diet-independent response to heat-shock. Taken together with our finding that MDT-15 and NHR-49 coordinate a sector of the fasting response, we propose that MDT-15 integrates several regulatory pathways to monitor both the availability and quality of ingested materials. Thus, in addition to maintaining normal health and longevity by assuring appropriate fatty acid desaturation, we hypothesize that MDT-15 also contributes to normal life span by governing metabolism and elimination of toxic compounds. Finally, some MDT-15 targets such as *gst* genes are regulated by insulin/IGF-1 signaling in *C. elegans*, and *mdt-15* potentially as well [3]; hence, MDT-15 may affect lipid and detoxification metabolic pathways in the context of insulin/IGF-1 signaling.

1. Yang et al, 2006, Nature.
2. Taubert et al, 2006, Genes & Development.
3. Murphy et al, 2003, Nature.

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Lab: Yamamoto

***Caenorhabditis elegans* genes NUMR-1 and NUMR-2 confer increased resistance to cadmium toxicity and are essential for certain neuromuscular functions**

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Cadmium is a known human carcinogen and while several signaling cascades have been found to be involved in cadmium-induced gene transcription, the molecular mechanisms by which cadmium activates these pathways are not well established. Cadmium has been shown to elevate intracellular calcium levels, which could result in the activation of multiple signaling cascades. Here we report the functional analysis of two cadmium responsive genes in the nematode *Caenorhabditis elegans*, *numr-1* and *numr-2*, whose expression, at least in part, may be regulated by alterations in intracellular calcium levels. In the absence of metal, constitutive expression of *numr-1/-2* was developmentally regulated. Expression of *numr-1/-2* was maximal in intestinal nuclei during the L1 larval stage and minimal in adult nematodes. However, when adult nematodes were exposed to metal, *numr-1/-2* expression increased dramatically in pharyngeal and intestinal nuclei. Interestingly, in *C. elegans* the intestinal cells experience endogenous endoplasmic reticulum (ER) stress during early larval stages of development and cadmium is a toxicant that induces ER stress. It has been proposed that cadmium can increase intracellular calcium levels by inducing release of calcium from ER stores. We found that when adult nematodes carrying a NUMR-1::GFP translational fusion were exposed to thapsigargin or a calcium ionophore, A23187, *numr-1* expression dramatically increased throughout intestinal nuclei. This suggested to us that alterations in intracellular calcium levels might regulate *numr-1/-2* expression. Calcium signaling also plays an important role in egg-laying and feeding in *C. elegans*. In accordance with the idea that variations in calcium levels might act as a signal to regulate *numr-1/-2* expression, loss of *numr-1/-2* activity produced animals with egg-laying defects and decreased feeding. We also found that decreased *numr-1/-2* expression increased sensitivity to cadmium; while over expression of *numr-1* increased resistance to cadmium stress and influenced life span. Taken together, our data suggest that *numr-1* and *numr-2* are involved in normal development, certain neuromuscular functions, and resistance to cadmium toxicity. Thus, *numr-1/-2* may be important in integrating environmental inputs to mediate specific cellular responses.

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Biochemical Interactions And Genetic Analyses Of ABC Transporters Required For Heavy Metal Detoxification

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Understanding the cellular mechanisms of heavy metal (e.g. cadmium [Cd²⁺], mercury [Hg²⁺] and lead [Pb²⁺]) detoxification is critical for the cure and prevention of heavy metal-caused diseases, such as neurodegenerative conditions, dysfunction of vital organs, and cancer. Among the major contributors to heavy metal detoxification are ATP-binding cassette (ABC) transporters. The specific family members involved, and their functions in evolutionarily diverse organisms, however, are not understood. The recently discovered acute requirement of a half-molecule ABC transporter, CeHMT-1, in Cd²⁺ detoxification in the nematode worm, *Caenorhabditis elegans*, provides a powerful approach to study the mechanisms, structural requirements, and components of the CeHMT-1 metal detoxification pathway. Unique structural features of CeHMT-1 (it is the only half molecule ABC protein in *C. elegans* possessing the hydrophobic N-terminal extension (NTE)) define the HMT-1 subfamily in different species, including humans. Although it is suggested that half molecule ABC transporters must associate with other family members and/or other cellular proteins, how HMT-1 proteins detoxify heavy metals and how this subfamily of ABC transporters functions is unknown.

To understand the mechanism of the CeHMT-1 functional activity, we study its genetic and biochemical interactions, its tissue-specific expression and subcellular localization. Towards this goal we have established that CeHMT-1 forms higher oligomeric complex and localizes to the lysosomal-like compartment in intestinal cells. To identify putative interacting partners of CeHMT-1, we have screened 30 from 60 ABC transporters in *C. elegans* genome and identified six ABC transporters in addition to CeHMT-1 that are involved in metal detoxification. Their role as compared to CeHMT-1 in providing heavy metal tolerance will be discussed. In addition, we will present data demonstrating that our screens have the potential to identify new pathways, and to ascribe functions to known pathways, not previously determined to be involved in heavy metal tolerance and, thus, will contribute to our understanding of cellular resources for metal detoxification.

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Bacterial pathogens, inflammation and disease resistance mechanisms in *C. elegans*

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An increasing number of bacterial species with pathogenic effects on *C. elegans* have been identified and studied, either by testing known pathogens from other systems, or by the discovery of nematode-specific diseases. These investigations shed light on basic and potentially conserved aspects of innate immunity: bacterial infection, host detection, response to infection and mechanisms of antibacterial defense. We have focused mainly on the coryneform pathogen *Microbacterium nematophilum*, which attacks the worm by rectal infection and causes a conspicuous tail swelling, akin to an inflammatory response. Genetic analysis of the infection has identified multiple host genes involved in infection, many of which affect properties of the rectal epithelia and the hypodermis. Some of these also affect infection and pathogenesis by other bacteria. A specialized rectal version of the ERK MAP kinase cascade is required for the swelling effect and for amelioration of infection, which is potentially lethal. The rectal inflammation response can also be elicited by other Gram-positive bacteria, such as some strains of *Staphylococcus* and a new species of *Leucobacter*; the latter was isolated from a naturally infected Japanese population of *C. elegans* (collected by Marie-Anne Felix). These different infections exhibit both common and pathogen-specific features.

Microarray analysis has revealed the induction of a variety of genes after infection by *M. nematophilum*, notably those belonging to lysozyme and C-type lectin gene families. Expression analysis indicates that many are expressed in the alimentary tract (pharynx, gut or rectum), and some in complex gene-specific patterns within these tissues. RNAi knockdown and/or gene deletion knockouts demonstrate that several of these genes contribute significantly to antibacterial defence.

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Novel Innate Immunity Gene and Pathway Discovery Using Comparative Genomics

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In response to infection, humans mount an immediate innate immune response and a slower but more specific adaptive immune response. The innate response involves the action of phagocytic and cytotoxic cells that fight the infection. Therefore, the innate immune system plays a vital role in host defense. However mis-regulation of innate immunity can also contribute to a variety of immunological diseases, including asthma and sepsis. Thus, the identification of genes that regulate innate immunity is critical to understanding host defense and to identifying potential targets for treatment of infectious and immunological diseases. While many genes that regulate the response to Gram negative lipopolysaccharide (LPS) have been discovered, it is unclear how many other genes regulate this response. To identify novel regulators of the innate immune response to LPS (and other microbial toxins), we developed assays in two model systems to inhibit candidate genes by RNA-interference and monitor the subsequent immune response. Both models utilize a Gram negative bacterial stimulus. In one *in vivo* assay, the nematode *C. elegans* was stimulated with *E. coli* and production of antimicrobial proteins was monitored. In the second *in vitro* assay, mouse macrophages were stimulated with LPS and cytokine production was monitored. Genes that altered innate immune responsiveness in these systems were validated using mutant nematode models and are currently being tested in mutant murine models. These assays have led to the discovery of 11 genes that regulate the innate immune response in both systems and the identification of a novel protein interaction network with a conserved role in innate immunity regulation. These genes represent potential therapeutic targets for infectious and immunological diseases.

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Pre-exposure of *C. elegans* to enteropathogenic *E. coli* promotes increased survival through activation of longevity and innate immunity pathways

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Enteropathogenic *E. coli* (EPEC) is a human gastrointestinal pathogen that primarily causes the mortality of infants through diarrhea and dehydration in developing nations. We previously showed that EPEC kills *C. elegans* using a secreted toxin in an EPEC::*Caenorhabditis elegans* model to study EPEC virulence and host response. *C. elegans*' initial response to EPEC exposure is to escape the lawn and avoid subsequent contact. Here, we report that this brief exposure and avoidance behavior to EPEC activates protective pathways that “condition” *C. elegans* and promote increased survival upon subsequent exposure to lethal EPEC. Conditioning requires genes in the dopamine signaling (*cat-2* and *dop-3*), in the innate immunity signaling (*sek-1* and *pmk-1*) and the insulin/IGFR1 signaling pathways (*daf-2* and *daf-16*). Additionally, we show that *pmk-1* and *daf-16*, upon EPEC exposure, initiate the transcription of two downstream genes *spp-1* and *aqp-1* to mediate the conditioning response. Finally, although other stressors (heat shock, heavy metals, starvation), can condition *C. elegans* to survive exposure to EPEC, the stressors do so via *daf-16*, but not through sensory mechanisms (e.g. *dop-3*), indicating the specificity of conditioning. Together, our findings suggest that *C. elegans* uses dopaminergic neurons to detect EPEC, and then signals the coordinate regulation of longevity and immunity pathway genes to allow survival to subsequent exposure. The avoidance period may therefore be necessary for the protective responses to develop. Thus, being naturally exposed to various pathogens in the soil, “conditioning” may have developed to ensure the increased survival of *C. elegans* in pathogen rich environments.

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Innate Cellular Defenses Against Pore-Forming Toxins

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Our laboratory is studying the response of *C. elegans* to pore-forming toxins (PFTs). PFTs are the largest class of protein virulence factors and are made by many of the most important bacterial pathogens. *C. elegans* is virtually the only whole organism model for studying the effects of these toxins. Studies in *C. elegans* revealed the first cellular pathway that defends cells against PFTs, namely the p38 mitogen-activated protein kinase (MAPK) pathway. p38 MAPK has been implicated as being important in the innate defenses of *C. elegans* against many pathogenic and toxic attacks.

We performed genome-wide RNAi screening with the Ahringer library to reveal genes involved in defending against the PFT Cry5B—ie looking for animals that were more intoxicated than wild-type on a sub-lethal dose of PFT. We found over 200 genes involved in this process. One interest we have is how these genes relate (upstream or downstream) to the p38 MAPK pathway.

We have taken several approaches to analyze these data, including analysis of gene-gene interaction networks that emerge from the RNAi data. One surprising direction some of our data pointed us in was with regards to the unfolded-protein response (UPR). We discovered that the UPR, which protects the endoplasmic reticulum (ER) from stresses associated with accumulation of unfolded proteins, is activated in *C. elegans* upon exposure to PFTs. This activation extends to known downstream targets of the pathway and is not unique to the nematode, as we found that mammalian cells have the same response. Based on the response of UPR mutant animals to PFT, we found that activation of the UPR is functionally relevant for cellular defenses. In fact, we discovered that the UPR and the p38 MAPK pathways become sequentially linked together when animals are exposed to PFT. We were able to fill in an important and expected gap in our knowledge as to how the p38 MAPK pathway works.

Here we will discuss our data and current models of the UPR and p38 pathways, as well as on-going efforts to characterize cellular defenses to PFTs, including the discovery of a fatty acid biosynthetic pathway and a membrane trafficking pathway involved in these defenses.

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Vibrio cholerae* hemolysin is Responsible for Lethal Infection in *Ceanorhabditis elegans

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Vibrio cholerae (VC), the causative agent of cholera, is responsible for devastating epidemics across the world. The major virulence factor underlying the pathogenesis of cholera is cholera toxin (CT). However, CT negative VC non-O1 and non-O139 strains and CT deletion vaccine mutant strains are still able to cause severe diarrheal disease through mechanisms that are currently unclear. Additional virulence factors such as zonula occludens toxin (*zot*), accessory cholerae toxin (*ace*), and hemolysin (*hlyA*), are implicated in cholera pathogenesis. VC causes lethal infection in the nematode *C. elegans* via a CT-independent process (1, 2), providing an excellent model to determine the roles of the other virulence factors in pathogenesis. We fed CVD110, a VC vaccine strain that is deficient in several virulence factors (3), to the worms, and observed an attenuated killing. CVD110 is deficient in zonula occludens toxin (*zot*), accessory cholerae toxin (*ace*), and hemolysin (*hlyA*) as well as the CT genes *ctxA* and *ctxB*. These data suggest that the participation of one or more of these factors was/were responsible for the lethality in *C. elegans*. To test the hypothesis that *hlyA* was responsible for lethality in the *C. elegans* model, the effect of strain CVD109, which has an intact *hlyA* and otherwise genetically identical with CVD110, was evaluated. We found that the presence of intact *hlyA* locus was sufficient to promote same level of killing as the wild type VC. Furthermore, worms fed with a VC strain carrying a single locus deletion of *hlyA* gene, found to be attenuated in *C. elegans* lethality, a result which is similar to that observed for CVD110. Together, our data strongly suggest that *Vibrio cholerae hly A* is responsible for killing of *C. elegans*. *hemolysin A* is a pore forming exotoxin whose role in VC pathogenesis is not fully understood. Further studies in *C. elegans* host model will help us understand the contribution of hemolysin virulence in VC pathogenesis.

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***Pseudomonas aeruginosa* subversion of *Caenorhabditis elegans* immunity by neuronal INS-7-mediated activation of the DAF-2 insulin-like signaling pathway**

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Host immune suppression is a very potent pathogenesis mechanism employed by a variety of pathogens. We provide genetic evidence from host gene expression and protein localization studies within the whole animal that the human bacterial pathogen *Pseudomonas aeruginosa* is able to suppresses *C. elegans* immune defense. We show that the ability of *P. aeruginosa* to suppress the expression of a subset of host immune-effector genes requires the bacterial quorum sensing system and is mediated by the host insulin-like signaling pathway. Suppression of immune genes is associated with induced expression of a DAF-2 insulin receptor agonist, INS-7, and the ability of *P. aeruginosa* to translocate DAF-16 out of the nucleus of the intestine. The immunomodulatory effect of INS-7 is mediated by the neuronal function of *ins-7* and its secretion from dense core vesicles. We will discuss a model of how a bacterial pathogen is able to suppress host immunity by affecting the activity of the neuroendocrine system.

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Tissue-specific TIR-1/NSY-1/SEK-1-dependent MAPK Activation Confers Pathogen Resistance Through Neuroendocrine Signaling in *C. elegans*

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A conserved TIR-1/NSY-1/SEK-1/PMK-1 MAPK pathway is required for immunity in *C. elegans*. We investigated the tissue-specific functions of TIR-1/NSY-1/SEK-1-dependent MAPK signaling in response to pathogen infection. We determined that TIR-1/NSY-1/SEK-1-dependent MAPK signaling in the intestine provides a major contribution to pathogen resistance, but does not completely restore pathogen resistance. Surprisingly, we defined an additional tissue-specific role for TIR-1/NSY-1/SEK-1-dependent MAPK signaling in chemosensory neurons in resistance to pathogen killing. We are currently defining the neuroendocrine signaling circuitry that functions downstream of TIR-1/NSY-1/SEK-1-dependent MAPK signaling to confer pathogen resistance in *C. elegans*.

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Neuroimmune regulation of antimicrobial peptide expression via a non-canonical TGF- β signalling pathway in *C. elegans*

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Following sterile injury or infection by the fungal pathogen *Drechmeria coniospora*, *C. elegans* mounts a p38/MAPK-dependent response in the epidermis. This leads to the production of NLP antimicrobial peptides. We have found that a second group of putative antimicrobial peptides of the Caenacin (CNC) family are regulated independently of the p38 pathway. We focused on the regulation of *cnc-2* that is only induced by infection and not wounding. We found that a non-canonical TGF- β signalling pathway controls its expression. It involves the TGF- β ligand DBL-1, its heterodimeric receptor SMA-6/DAF-4 and the SMAD protein SMA-3, but not SMA-2 or SMA-4, which are otherwise required for the known functions of DBL-1. We also show that neuronal expression of *dbl-1* controls *cnc-2* expression in the epidermis in a dose-dependent paracrine fashion. Our results lead to a model where antifungal defenses are coordinately regulated by a cell-autonomous p38 cascade and a cytokine-like TGF- β signal from the nervous system to the epidermis, each regulating distinct sets of antimicrobial peptide genes.

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Genetic Dissection of Pathogenic Interactions of *Lysobacter enzymogenes* with *C. elegans*

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Lysobacter enzymogenes infects lower plants, fungi and nematodes but not higher plants or mammals. *L. enzymogenes* produces copious amounts of lytic enzymes including chitinases, β -1, 3-glucanases, and proteases while the recently sequenced genome of *L. enzymogenes* has revealed the presence of a type III secretion system (T3SS). We investigated the contributions of lytic enzymes and the T3SS to disease by feeding *C. elegans* with wild-type and mutant strains of *L. enzymogenes*.

Wild-Type *L. enzymogenes* induced cuticle shrinkage, nuclear fragmentation, autophagy and cell death in *C. elegans*. Worms inoculated with either the *clp*- mutant (defective in lytic enzymes) or the Δ T3SS strain displayed a reduced mortality compared to wild type *L. enzymogenes*. Unlike infection with the wild-type strain or the *clp*- mutant, worms infected with the Δ T3SS strain did not develop autophagic vacuoles (although they still displayed cuticular shrinkage). Worms inoculated with the *clp*-/ Δ T3SS double mutant displayed no abnormal effects, grew to maturity and gave rise to viable offspring. These results indicate that both the Clp-dependent pathway and the T3SS pathway contribute to virulence of *L. enzymogenes* on *C. elegans*. We are currently conducting a screen to identify the nature and targets of T3SS effectors.

To identify the mechanism of cell death induced by *L. enzymogenes*, we studied the response of *C. elegans* mutants that are defective in different host programmed cell death pathways. These studies, coupled with pharmacological approaches indicate that susceptibility to *L. enzymogenes* in *C. elegans* is independent of the type I PCD pathway and the necrotic cell death pathway, but is dependant on the autophagic cell death pathway that is itself the target for T3SS effectors. Our data indicate that the engulfment pathway may also contribute to the expression of cell death in response to *L. enzymogenes*. We are currently using additional *C. elegans* mutants to investigate the contributions of other cell death pathways to host susceptibility.

These results provide support for the development of *C. elegans* as a new multicellular model for molecular genetic dissection of the mechanisms of *L. enzymogenes* induced cell death. These studies will lay the foundation for identification of targets and inhibitors that can prevent *L. enzymogenes* induced cell death. This approach should also throw light on the genetic and molecular basis for broad host range specificity of *L. enzymogenes*.

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The G-protein Coupled Receptor FSHR-1 is Required for the *C. elegans* Innate Immune Response

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Innate immunity is an ancient defense system used by vertebrates and invertebrates to prevent or limit infection by pathogenic microorganisms. Innate immune responses are triggered by detection of pathogens using specific receptors. Multiple unrelated receptors in diverse species employ leucine-rich repeat (LRR) domains to bind molecular patterns associated with infection. *C. elegans* uses defense pathways conserved with vertebrates; however, it is unknown which receptors are used to detect pathogens. We screened all LRR-containing transmembrane receptors in worms and identified the G-protein coupled receptor (GPCR) FSHR-1 as an important component of the *C. elegans* immune response to bacterial pathogens. *fshr-1* null mutant worms succumb to infection by Gram-negative and Gram-positive pathogens more quickly than wild-type worms, but have a normal lifespan on non-pathogenic bacteria. FSHR-1 is necessary and sufficient in the intestine, the primary site of exposure to ingested pathogens, for its role in immune defense. FSHR-1 signals in parallel to the known immunity pathways defined by the p38 MAPK PMK-1 and the insulin/IGF receptor DAF-2. FSHR-1 regulates the transcriptional induction of a set of pathogen response genes. Some, but not all, of these putative antimicrobial effectors are also regulated by the p38 MAPK pathway, suggesting that these two signaling pathways converge.

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TPA-1 is Required for the Anti-fungal Response in the *C. elegans* Epidermis

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The spores of the nematophagous fungus *Drechmeria coniospora* adhere to the cuticle of *C. elegans*. They produce penetration tubes that transverse its cuticle and develop into trophic hyphae that colonize the epidermis. *C. elegans* responds to this infection by up-regulating many genes in the epidermis, including certain members of the *nlp* gene family that encode putative antimicrobial peptides (AMPs). This induction can be visualized using reporter genes, such as *pnlp-29::GFP*. In addition to infection, sterile injury and high salt also markedly increase expression of *pnlp-29::GFP*.

We performed a direct genetic screen for mutants that do not show up-regulation of the AMP gene *nlp-29* upon infection. Among the alleles isolated, two (*fr1* and *fr3*) failed to complement each other. Both block the response to infection and injury, but show a normal response to osmotic stress. We identified the mutations as new alleles of *tpa-1*. This well-studied gene encodes a protein kinase C (PKC), homologous to mammalian PKC delta. PKC can be activated by diacylglycerol (DAG), or by the DAG mimetic phorbol 12-myristate 13-acetate (PMA). As expected, PMA alone strongly induced the expression of *nlp-29* in wild-type worms, but not in *tpa-1* mutants. The PMA-induced expression of *nlp-29* was similarly blocked in *tir-1*, *nsy-1*, *sek-1* and *pmk-1* mutants. These genes all encode components of a p38 MAPK cascade that we have shown to regulate *nlp-29* expression after infection. These results and further epistasis analyses place the p38 MAPK pathway downstream of the PKC TPA-1. We also found that the induction of *nlp-29* after infection was blocked by mutations in the genes *egl-8* and *plc-3*, which encode phospholipases C that act up-stream of *tpa-1*. The response also requires the G-protein alpha subunit gene *gpa-12*. This suggests that activation of a G-protein coupled receptor may initiate the response to fungal infection.

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