# Uncoupling Obesity from Insulin Resistance

## A Genetic Screen for Fat Content Suppressors using a *C. elegans* model of Insulin Resistance

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**Abstract:**

Insulin resistance and obesity are the two major risk factors for metabolic syndrome. It has long been thought that shutting down the insulin signaling pathway should promote fat burning similar to starvation scenarios. It is observed however that patients with insulin resistance do not always seem to lose weight. Instead, excess fat accumulation persists despite their insulin resistant state. This creates a vicious cycle, where persistent obesity continues to promote insulin resistance often resulting in type two diabetes.

Genetic mouse models lacking the insulin receptor in adipose tissue do not follow this disease model. Instead, they more predictably fail to accumulate fat stores. The model organism, *C. elegans*, presents many advantages in determining how fat dysregulation and adult-onset insulin resistance are linked. Strains carrying a temperature sensitive mutation of the daf-2 insulin receptor become obese when shifted to the non-permissive temperature as adults. A reverse-genetic screen was conducted to find gene inactivations, which suppress the obese phenotype in this model. We identified a number of novel metabolic regulators. We further identified one, hlh-16, which shares homology with an eye-development gene in humans. We propose this transcription factor may also regulate lipid metabolism, and present evidence that it effects transcriptional levels of fatty-acid elongase, elo-2.

**Introduction:**

Multicellular organisms obtain nutrients from their diets and circulate them throughout the body. The production and secretion of insulin, or insulin-like peptides in lower metazoans, serve to signal nutritional availability at the cellular and organismal levels. The resulting signaling cascade is remarkably conserved from nematodes, to flies, to mice and humans. It serves to promote nutritional uptake and upregulate synthesis and growth pathways. Excess nutrients are stored as fat, and utilized as energy sources in times of nutritional deprivation. Excessive fat storage results in a compensatory shutdown of the insulin signaling pathway, the result of which leads to metabolic dysregulation. Interestingly, insulin resistance continues to promote fat accumulation.

It is estimated that 22.9% of the United States population has metabolic syndrome[1]. Metabolic syndrome is defined as a group of risk factors associated with the development of pathologies including type-two diabetes (T2D), heart disease, non-alcoholic fatty liver disease, and certain cancers. The most prevalent symptoms of metabolic syndrome are obesity and insulin resistance. These symptoms are not independent of one another, as obesity itself puts an individual at severe risk of developing insulin resistance. Excessive nutrient uptake increases insulin signaling. In an attempt to dampen chronic signaling, cells engage compensatory mechanisms to shut down the insulin signaling pathway[2–6]. Over time, this could result in a diabetic disease state, whereby and organism stops producing insulin altogether. In turn, insulin resistance alters metabolic homeostasis and promotes ectopic fat storage and altered lipid metabolism. Superficially, it is a paradox that insulin resistant individuals would sustain excess fat mass. Insulin signaling activates a transcriptional program that promotes lipid storage; a lack of insulin promotes the breakdown of macromolecular stores to generate energy units and

building blocks. Insulin resistance should therefore confer a permanent state of starvation that promotes fat break down. Instead, in the disease state of insulin-resistance, we see sustained fat storage. It has been speculated that chronic lipolysis generates FFAs that, when abundant, do themselves trigger lipid storage to prevent lipid toxicity[2]. The precise molecular mechanism resulting in sustaining lipid content in an organism with impaired insulin signaling has yet to be fully defined.

Insulin Signaling and Insulin Resistance in *C. elegans*

*C. elegans* hatch from eggs fertilized and laid by hermaphroditic adults. Hatchlings progress through four larval stages (L1-L4), before reaching adulthood. *C. elegans* can undergo an alternate lifecycle in times of overcrowding, high stress, or nutrient deprivation known as dauer. The dauer state is a lethargic state of low energy consumption, cessation of eating, increased accumulation of lipid stores, and development of a thicker cuticle. Developing animals integrate cues from pheromones and nutrients and selectively enter dauer from the L2 stage, emerging as L4s once conditions become more favorable. A series of genetic screens were conducted in a number of laboratories to identify genetic mutations that confer a constitutive dauer phenotype, (daf-c,) regardless of environmental conditions. From these screens the daf-2 gene was identified and cloning revealed it shared remarkable homology with human insulin receptor. DAF-2 is the only insulin receptor in *C. elegans*, and regulates the function of both IR and IGF in the animal[7,8]. The FOXO homolog, DAF-16, is a member of the dauer defective (daf-d) gene class, and its nuclear entry and function is required for dauer entry. Double daf-2;daf-16 mutants fail to enter dauer; in fact daf-16 mutation is sufficient to rescue all daf-2 conferred phenotypes [8,9].

All isolates of the daf-2 mutation, except one, are temperature sensitive. As such, the opportunity for dauer can be bypassed by raising animals at the permissive temperature, and shifting them to the non-permissive temperature after the developmental stage for dauer entry has passed. Studies conducted under such conditions model adult-onset insulin resistance, and the associated phenotype of obesity noted as increased TAG stores (compared to wild type controls) can be detected using histochemistry, Coherent Anti-Stokes Raman Scattering (CARS) microscopy, and biochemical techniques [10].

Metabolic pathways of *C. elegans* and humans share remarkable conservation. The canonical insulin signaling pathway, the mTOR pathway, and both cell-autonomous as well as endocrine and neuronal regulators of lipid metabolism ranging from lipogenesis, to lipolysis, and oxidation have been studied in depth in worms. Homologs of the major regulators of these pathways in humans have been identified in *C. elegans*.

Anatomical differences between mammals and nematodes provide both advantages and disadvantages in translating studies from one system to the other. *C. elegans* do not have many of the organ systems higher organisms have developed. Their intestinal cells, of which there are a set number, constitute the major lipid storage organ in the animal[10,11]. In that sense, the intestinal cells (and to some degree the hypodermis) are functionally analogous to adipose tissue in mammals; the exception being that "adipogenesis", or the generation of additional fat-storage cells is not possible. Instead, "obesity" is limited to the expansion of both the quantity and size of lipid droplets within the cells. The intestine also functions as the liver would in other systems, converting fat to glycogen during periods of fasting, as well as providing proteins to package and solubilize fats so they can be exported and circulated throughout the organism. The organismal complexity of metabolism however is maintained in *C. elegans*. Energy demands from the

germline, muscle, and other cell types are supplied by the intestinal glycogen and fat reserves in fasting conditions. Nutrient availability and demand are signaled through integrated hormonal and neurological cues throughout the organism[10,12,13].

In mice, genetic insulin resistance in the adipose tissue, as seen in FIRKO mice, actually protects against obesity and improves glucose and lipid homeostasis[14–17]. Insulin receptor knockout in the muscle however decreases muscle mass and increases fat mass[18,19]. Unlike in mouse models, whole-animals genetic impairment of insulin signaling through insulin receptor knock-down or mutation in *C. elegans* is possible. Daf-2 mutation promotes fat gain, phenotypically similar to the disease state of obesity-induced insulin resistance[11].

The research here aims to identify novel genetic regulators of persistent obesity despite insulin resistance, and the mechanisms by which they affect lipid metabolism and homeostasis. Most importantly, our goal is to identify metabolic modulators that prevent obesity and weight gain following the development of insulin resistance, but which do not affect normal metabolism in the insulin-sensitive state

**Materials and Methods:**

Strains: N2 was used as the wild type strain. The following mutant strains were used: rrf-3(pk1426), daf-2(e1368); rrf-3(pk1426), hlh-16(ot711), hlh-16(ot711); daf-2(e1368), daf-16 (mu86); daf-2(e1368), daf-2 (e1370); sod-3::GFP+rol-6, N2; sod-3::GFP+rol-6, daf-16(mu96); daf-2 (e1370); daf-16p::GFP::DAF-16+rol-6, unc-119(tm4063); hlh-30::TY1::EGFP::3xFLAG+unc-119(+)

RNAi assay: Prior to all RNAi experiments, animal populations were synchronized by bleaching gravid adults and allowing the surviving eggs to hatch in S Buffer and subsequently arrest as L1s for 20-26 hours at 20 degrees. Synchronized, arrested populations were then seeded onto NGM media containing IPTG and Carbenicillin (50ug/mL). Assays following the initial screen were conducted on plates which also contained 5ug/mL chloramphenicol. Plates were seeded with the HT115 strain of *E. coli* harboring the L440 vector containing the sequence to knock down the gene of interest. RNAi clones were obtained from both the Vidal and Ahringer libraries, and sequence verified. The original screen was conducted in 96 well format, as previously described. Follow-up experiments were done on 6cm plates seeded with the RNAi clone of interest. RNAi cultures were inoculated in LB containing 50ug/mL carbenicillin, grown 16 hours at 37 degrees, concentrated to 10X, and 250uL was seeded onto plates and allowed to dry and induce overnight. Animals were seeded from minimal media next day. Unless otherwise noted, animals were grown at the permissive temperature of 15 degrees for ~65 hours, and were shifted to the non-permissive (25 degree) temperature as L4s. Animal were then harvested for assessment as young adults approximately 24 hours after temperature shift.

Fat content staining: Young adult animals were washed from plates using S Buffer and allowed to gravity settle. The buffer was exchanged for 60% isopropanol, which resulted in

whole animal fixation. The isopropanol was then exchanged for a working solution of Oil Red O. Stock Oil Red O was made to 0.5% in 100% isopropanol and allowed to equilibrate for at least 1 night. The solution was then filtered and diluted to 60% in water, and again equilibrated for at least 1 night. Oil Red O solution was freshly filtered prior to use. Animals were resuspended in the Oil Red O solution and incubated overnight at 25 degrees in a wet chamber to prevent evaporation and subsequent dye precipitation. The next day, the Oil Red O was exchanged for S Buffer + 0.01% trition. Animals were imaged within 2 days, and most times same day.

Imaging: Images were taken on a Nikon Eclipse Ti microscope. Flourescence images were captured using a Hamamatsu Orca-Flash 4.0 camera, while color images were acquired on a Nikon DS-Ri2.

Image Analysis: Images were analyzed using the Worm Toolbox for Cell Profiler, as previously described. Cell Profiler Analyst was used to classify animal populations as either fat or wild-type. Each day's data was analyzed separately and compared to internal experimental controls (animals fed vector only or daf-16 RNAi constructs) to control for potential differences in experiment or imaging conditions.

qRTPCR: Animals were harvested from plates in S Buffer and allowed to gravity settle before buffer was removed and animals were flash frozen in liquid nitrogen. RNA extraction was conducted using TRIzol reagent. cDNA was transcribed in reactions normalized to total RNA content, as determined by nanodrop quantification. MMLV and OligodTs were purchased from LifeTechnologies; AM2044 and AM5730G, repectively. qRTPCR reactions were then made with SYBRgreen, and the appropriate primer set. Data are normalized to transcript levels in rrf-3 animals as well as either Y45F10D.4 or ama-1 internal control expression levels.

**Results:**

A high throughput, reverse genetic screen was conducted in *C. elegans*. The goal was to identify genes that suppressed obesity in the insulin resistant, daf-2 mutant, background. Synchronized populations of animals were seeded on RNAi expressing bacteria in 96 well format as L1s. Animals were grown to L4 stage at the permissive temperature, then shifted to 25 degrees to activate the daf-2 mutation. Young adult animals were harvested, fixed, and stained for fat content using Oil Red O. This was done in the RNAi sensitive background, rrf-3. Images were then analyzed and quantitated using the Worm Toolbox for Cell Profiler[20,21]. Cell Profiler Analyst was then used to classify representative animals as either "fat" or "wild-type" in respect to fat staining quantity and distribution. As previously reported, the values for Original Blue Intensity, as well as Mean Intensity of Fat objects proved to be the most robust differential variables identified in all data sets[21]. The identified rules for differentiation were used to score all animal populations within a data set, and a threshold of 90% "wild-type" E-score was placed. All hits were then scored by eye. The final list of hits (Table 1) is comprised of all RNAi treatments which suppressed the fat phenotype of daf-2; rrf-3 animals compared to same-day vector-fed control animals. The effect of these gene inactivations on non-insulin resistant (rrf-3 control) animals was then observed, and hits were ranked with priority being placed on treatments that did not affect rrf-3 fat levels. Gene inactivations which did affect rrf-3 fat levels are most likely general regulators of lipid metabolism, rather than representing genes specifically responsible for altered lipid metabolism in a daf-2 background.

We decided to focus on one hit, hlh-16, for further analysis. Hlh-16 is a basic helix-loop-helix transcription factor, which has not been previously implicated in metabolism. It shares homology with the BHLHE23 (also known as BETA4 or BHLHB4) transcription factor in

humans. BHLHE23 also has not been previously implicated in a metabolic context, but rather has been shown to be important for retina development and eye health[22–25]. As nematodes have no organ structure homologous to eyes, the presence of this gene in this organism must be related to some other biological function.

We first confirmed the daf-2 fat suppression phenotype, following the same experimental design as before, but on a smaller scale in 6sm plates. Indeed, hlh-16 RNAi was sufficient to suppress daf-2 mutation mediated fat content increase. In addition, the daf-2; hlh-16 double mutant showed wild-type fat levels. Neither hlh-16 RNAi nor the genetic mutant alone showed altered fat content from that of wild-type animals, suggesting hlh-16 lowers fat accumulation specifically in a daf-2 background (Figure 1).

**Comment [K1]:** I think this addresses ur earlier comment for fig 1

We next asked whether hlh-16 was functioning downstream of the insulin receptor but upstream of daf-16 nuclear translocation. DAF-16 suppression has proven sufficient to suppress all known daf-2 phenotypes, including fat accumulation (as shown by our data in Table 1)[8,9]. To ask if hlh-16 knockdown prevented daf-16 expression or nuclear translocation, we fed RNAi to animals carrying an integrated transgene, daf-16p::DAF16::GFP (a translational fusion of the daf-16 gene to GFP under control of its native promoter). Hlh-16 RNAi did not prevent expression of the transgene nor did it prevent its nuclear translocation in the daf-2 background (Figure 2).This suggests hlh-16 does not act to suppress daf-2 fat content increase by prohibiting DAF-16 translation or localization.

**Comment [CD2]:** Add a sentence or two at the end giving some interpretation….what does this indicate…same for the rest of the paragraphs discussing figures.

We next asked whether hlh-16 knock-down prevented daf-16 function by assessing the expression of one of its target genes, SOD-3[26,27]. Hlh-16 RNAi did not prevent SOD-3 expression (Figure 3), and therefore does not inhibit daf-16 function.

Finally we asked if the effect of knocking-down of hlh-16 on suppression of fat increase was dependent on daf-16. We fed daf-2;daf-16 double mutants RNAi against hlh-16. We found that the double mutants following hlh-16 knockdown did not exhibit the daf-2 fat phenotype, and concluded that the genes are not acting antagonistically to one another. Instead, hlh-16 knockdown seemed to further decrease fat content within the daf-2; daf-16 double mutants, suggesting daf-16 and hlh-16 either function within the same pathway, possibly even correlating the same genes, or else act independently to regulate fat accumulation in a daf-2 background (Figure 4).

**Comment [K3]:** Again, does this suffice?

Hlh-30 is a transcription factor in worms which has been studied in the context of its sequence and functional homology to the human TFEB[28,29]. TFEB regulates autophagy, lysosomal biogenesis, and lysosmal lipases[30,31]. In fact, hlh-30 is homologous to all transcription factors within the basic helix-loop-helix zip family of proteins in humans, demonstrating significant gene family expansion from worms to mammals (Table 2). Another member of this family, MITF, is functionally known to regulate eye development[32,33]. We hypothesized that hlh-16 may interact with hlh-30 in the nematode, thus affecting lipid metabolism, while in higher mammals, the homologs may have diversified to play more important roles to interact together in the context of eye development. We so far have tested whether hlh-16 affects expression of an hlh-30 translational fusion. Hlh-16 RNAi feeding does not prevent expression of the transgene in the wild-type background, nor does it prevent the transcription factor's nuclear localization when daf-2 RNAi is also fed to the transgenic animals (Figure 5). We concluded that hlh-16 does not act through translation or nuclear exclusion of the MITF homolog, hlh-30.

All *C. elegans* with penetrant hypomorphic mutations in the insulin receptor, daf-2, show increased lipid accumulation compared to wild type animals. In 2008, Perez and Van Gilst,

through a C13 labeling technique, determined that many of the daf-2 mutant alleles have remarkable increased levels of de novo lipid synthesis compared to wild type animals[34]. Daf-2 mutants show upregulation of fat-5, fat-6, and fat-7 as well as elo-2 [35]. Transcriptional levels of these fatty acid desaturases and elongases, as well as the acetyl CoA carboxylase, acs-2, are mediated by the SREBP homolog, SBP-1 [36]. To determine if hlh-16 inactivation suppressed fat accumulation by changing the expression or localization of SBP-1, we fed RNAi to animals carrying an integrated transgene, sbp-1p::SBP-1::GFP (a translational fusion of the daf-16 gene to GFP under control of its native promoter). Hlh-16 RNAi did not affect expression or nuclear localization of the transgene when also fed RNAi against daf-2 (Figure 6). Hlh-16 does not prohibit lipogenesis through sbp-1 regulation.

We began to test by qPCR what genes related to metabolism may be under the direct or indirect transcriptional regulation of hlh-16. We tested candidate genes in pathways including lipogenesis, lipolysis, and beta oxidation.

So far, we have determined that the fatty acid elongase, elo-2 may be regulated by hlh-16. Elo-2 has been shown to be upregulated in a daf-16 dependent manner in the daf-2 mutant, and be under transcriptional control of sbp-1, although expression and localization of these gene products are unaffected by hlh-16 RNAi. Furthermore, suppression of elo-2 by RNAi has been shown to be sufficient to prevent fat content increase in the daf-2 background[36,37]. RNAi of hlh-16 returns elo-2 expression levels to that of wild-type animals (Figure 7).

**Discussion:**

Altered Lipid Metabolism in the Insulin Resistant State:

Risk factors for the development of insulin resistance include aging, anti-HIV therapies, and obesity. Meanwhile, insulin resistance itself does not always lead to a depletion of fat stores as one would expect if insulin signaling was simply tuned down, like in the case of starvation. Instead, we observe in many models of obesity induced insulin resistance, increased fat content of an individual is persistent; the result being a continuing cycle of cause and effect. While, as described above, we know some of the effects obesity has on insulin resistance and vice versa, current therapeutic approaches only address the symptoms but not the roots of the problem. This is mostly due to the lack of understanding of the molecular link between obesity and insulin resistance. The safest treatment is a modification of diet and exercise regimens. However, these interventions do not always prove effective on their own and require lifestyle modifications sometimes outside of a patient's physical, economical, or social capabilities.

While methods of insulin resistance diagnosis are improving, current therapeutic approaches to treat insulin resistance take a sledgehammer to the metabolic imbalances within an individual, and only further upset organismal regulation of energy homeostasis. PPAR agonists, such as thiazolidinedione (TZD), are thought to act by promoting lipogenesis. While effective in increasing insulin sensitivity, this also promotes fat mass gain, an initial risk factor for the development of insulin resistance. AMPK activators, like 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) and metformin, trigger a plethora of cellular starvation responses, among them increased lipolysis, risk of lactic acidosis resistance [1] and appetite. [1]. Thus, while current therapeutics treat insulin resistance in the short term, their method of action only worsens the underlying metabolic dysregulation, perpetuating a vicious cycle.

This research aims to identify novel regulators of insulin resistance persistence of obesity and the mechanisms by which they affect lipid metabolism and homeostasis. Most importantly, our goal is to identify the genes that promote obesity and weight gain even in the absence of insulin signaling, as potential targets for the treatment of metabolic syndrome.

Taking advantage of the simplicity with which animals can be grown powerful genetic tools available in *C. elegans*, including RNAi feeding, we were able to conduct a high-throughput, quantitative reverse genetic screen[38–40]. Our screen identified 64 genes which reduce the fat content specifically in a daf-2 background. These represent potential novel targets for therapies to combat lipid dysregulation in insulin resistant state.

Our data indicate the presence of three homologs of the guanalyl cyclase, atrial natriuretic factor (ANF), among our hits. ANF has recently been proposed as a theraputic target for diabeties[41–45]. There is precedence for redundant function of the guanalyl cyclases in *C. elegans*[46]. Studies of the role of ANF in *C. elegans* present an opportunity to determine its metabolic function without cardiac implications/specifically looking at lipid regulation, as nematodes have no cardiovascular system. Specifically, one could address how increases of cGMP and PKG affecting lipid metabolism. Mammalian studies have shown that activated PKG can increase lipolysis through its action on perilipin[4,47]. Nematodes however do not have perilipin, so ANF function must have an alternative effect of lipid metabolism[48,49]. ANF also upregulates transcription of PGC1 and UCP1[42]. This promotes energy /fat burning, which is decreased in obese/insulin resistant humans. The worm situation seems interestingly paridoxial to this. That is, RNAi against ANF-R homologs (and presumably loss of gyanayl cyclase function) suppresses obesity in daf-2 animals.

We also investigated hlh-16 and its role in lipid metabolism. We have determined that hlh-16 is acting downstream of daf-16 and identified one metabolically relevant gene, elo-2, to be downregulated in the daf-2 background following hlh-16 knock down. We currently do not know if this target is under direct or indirect regulation by hlh-16, and directed qCHIP data would need to be obtained to determine this. There are surely more targets of the hlh-16 transcription factor, and we plan on conducting RNAseq experiments in the future to find all direct and indirect targets. mRNAseq in *C. elegans* can be done on entire populations of animals, and so we will send the most promising genetic backgrounds which suppress insulin-resistant induced obesity by altering lipid metabolism in daf-2 animals for a full transcriptional analysis. To determine which genes are affected by our RNAi treatments, both wild type and daf-2 mutant animals will be fed vector control or the suppressors, then sequenced. To determine which of these genes may affect our fat phenotype, we will compare the transcripts affected by our RNAi treatment in the daf-2 mutant background to the levels seen in the wild-type background under the same treatment and identify genes differentially expressed in daf-2 that return to wild-type levels after suppressor RNAi treatment. Genes found to be downregulated by hlh-16 will be knocked down at the level of the whole organism, as well as specifically in the intestine, to determine if they act cell autonomously or systemically. Overexpression lines can easily be constructed in *C. elegans* as well of genes found to be upregulated in our suppressed genetic background.

I have identified transcription factors, phosphatases, and kinases which, when inactivated, suppress insulin resistance induced obesity in daf-2 *C. elegans*. Future characterization of these genes in the context of metabolism to uncover the molecular mechanisms they control *in vivo* will inform us on the mechanisms that enable the persistance of obesity and lipid dysregulation

in the absence of insulin signaling, and has the potential to unveil therapeutic targets to break the vicious link between insulin resistance and obesity.

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**Figures:**

| Library Screened | Worm Gene | Seq name | Mammalian Homolog(s) | daf-2 fat phenotype suppression |
|---|---|---|---|---|
| Transcription Factors | | | | |
| | ztf-6 | W06H12.1 | HIC2 | complete |
| | aha-1 | C25A1.11 | AHA-1 | complete |
| | ceh-8 | ZK265.4 | ARX, RAX | complete |
| | ceh-38 | F22D3.1 | HNF6, ONECUT2/3 | complete |
| | F01D4.5 | F01D4.5 | TCF20 | complete |
| | crh-2 | C27D6.4 | CREB | complete |
| | daf-16 | R13H8.1 | FOXO | complete |
| | ceh-14 | F46C8.5 | LHX3 | complete |
| | hlh-16 | DY3.3 | BHLHE23, OLIG3 | complete |
| | pax-3 | F27E5.2 | PAX3 | complete |
| | gfl-1 | M04B2.3 | YEATS4 | complete |
| | daf-16 | R13H8.1 | FOXO | complete |
| | ceh-2 | C27A12.5 | EMX1/2 | complete |
| | dmd-7 | W06H12.1 | DMRT2 | complete |
| | lin-39 | C07H6.7 | hox (a5, a6, b6, c4, b4, a4, d4, b5, …) | partial |
| | gei-17 | W10D5.3a | pias 1, 3, 4 | partial |
| | mgl-2 | F45H11.4 | metabotropic glutamate receptor | partial |
| | pad-1 | Y18D10A.13 | protein dopey | partial |
| | | R102.6 | none | partial |
| | mab-9 | T27A1.6 | Brachyury | partial |
| | ceh-17 | D1007.1 | ARX, Phox2 | partial |
| | rnt-1 | B0414.2 | RUNX | partial |
| | egl-18 | F55A8.1 | GATA 4, 3, 2 | partial |
| | | F35B3.7 | | partial |
| | ntl-4 | C49H3.5a | CCR4-NOT transcription complex subunit 4 | partial |
| | zip-1 | Y75B8A.35 | none | partial |
| | tab-1 | F31E8.3 | brain specific homeobox protein | partial |
| | lin-48 | F34D10.5 | transcription factor Ovo-like 2 isoform 1 | partial |
| | | R05D11.6 | none | partial |

| | | | | |
|---|---|---|---|---|
| | pop-1 | W10C8.2 | transcription factor 7-like 2 | partial |
| | tbx-11 | F40H6.4 | Tbox brain protein 1 | partial |
| | | T22D1.6 | none | partial |
| | cep-1 | F52B5.5 | none | partial |
| Phosphotases | | | | |
| | pgp-5 | C05A9.1 | ATP-binding cassette sub-family B member 5 | complete |
| | pho-13 | F07H5.9 | HGNC:ACP2 | complete |
| | eak-6 | F10G8.4 | receptor-type tyrosine-protein phosphatase kappa isoform f | complete |
| | unknown | F55H12.5 | none | complete |
| | rfc-2 | F58F6.4 | unknown | complete |
| | ptp-4 | K04D7.4 | receptor-type tyrosine-protein phosphatase gamma isoform X2 | partial |
| Kinases | | | | |
| | hpk-1 | F20B6.8 | homeodomain-interacting protein kinase 1 isoform 2 | complete |
| | old-2 | ZK938.5 | fibroblast growth factor receptor 3 isoform 2 | complete |
| | rskn-2 | C54G4.1 | ribosomal protein S6 kinase alpha-5 isoform a | complete |
| | unc-22 | ZK617.1 | titin isoform X5 | complete |
| | F31B12.3 | frm-9 | none | complete |
| | ver-3 | F59F3.1 | vascular endothelial growth factor receptor 1 isoform 1 precursor | complete |
| | pnk-1 | C10G11.5 | HGNC:PANK2 | complete |
| | unc-89 | C09D1.1 | titin | complete |
| | **gsk-3** | Y18D10A.5 | GSK3 | partial |
| | cdk-2 | K03E5.3 | cyclin-dependent kinase 3 | partial |
| | dkf-1 | W09C5.5 | serine/threonine-protein kinase D3 | partial |
| | gcy-19 | C17F4.6 | atrial natriuretic peptide receptor 2 precursor | partial |
| | gcy-23 | T26C12.4 | atrial natriuretic peptide receptor 1 precursor | partial |
| | hil-8 | T05E8.2 | none | partial |
| | mom-4 | F52F12.3 | mitogen-activated protein kinase kinase kinase 7 | partial |
| | pmk-1 | B0218.3 | mitogen-activated protein kinase 14 isoform 2 | partial |
| | unknown | C35E7.10 | tyrosine-protein kinase Fer isoform X1 | partial |
| | unknown | K08H2.5 | tau-tubulin kinase 1 | partial |
| | unknown | F23C8.7 | tyrosine-protein kinase Fes/Fps isoform 2 | partial |
| | unknown | C34B2.3 | tau-tubulin kinase 2 | partial |
| | unknown | C03C10.2 | tau-tubulin kinase 1 | partial |

| | | | | |
|---|---|---|---|---|
| | unknown | C56C10.6 | tau-tubulin kinase 1 isoform X1 | partial |
| | unknown | Y32H12A.7 | uncharacterized aarF domain-containing protein kinase 2 | partial |
| | unknown | R05H5.4 | tyrosine-protein kinase Fes/Fps isoform 2 | partial |
| | gcy-15 | ZC239.7 | atrial natriuretic peptide receptor 2 isoform X2 | partial |

Table 1: 64 daf-2 obesity suppressors were identified in the high throughput Oil Red O screen. These genes, when in activated by RNAi feeding, reduced fat content in daf-2 animals without affecting fat elvels in wild-type controls. An E-score threshold of 90% as determined by CellProfiler Analyst was used.

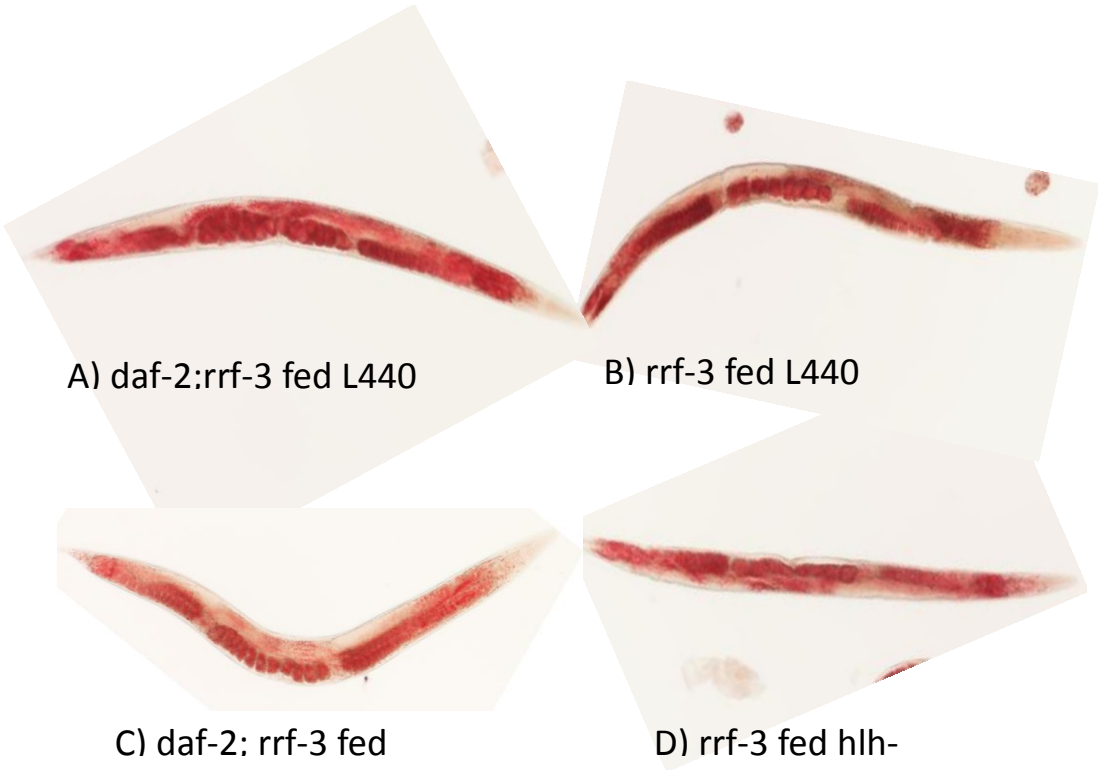



Figure 1.1: Conformation of hlh-16 knockdown effect on daf-2 Oil Red O staining. Fat content of daf-2 animals fed hlh-16 RNAi is reduced compared to vector fed daf-2 animals, without changing wild-type control fat levels.

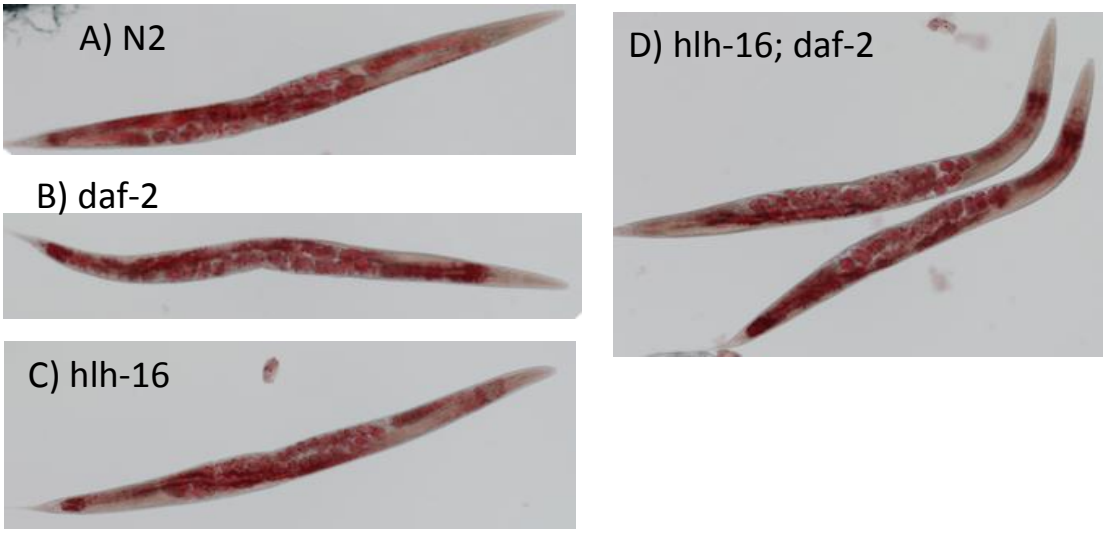



Figure 1.2: hlh-16; daf-2 double mutant also shows reduced fat content versus daf-2 mutant alone, without affecting wildtype fat levels in the single mutant.

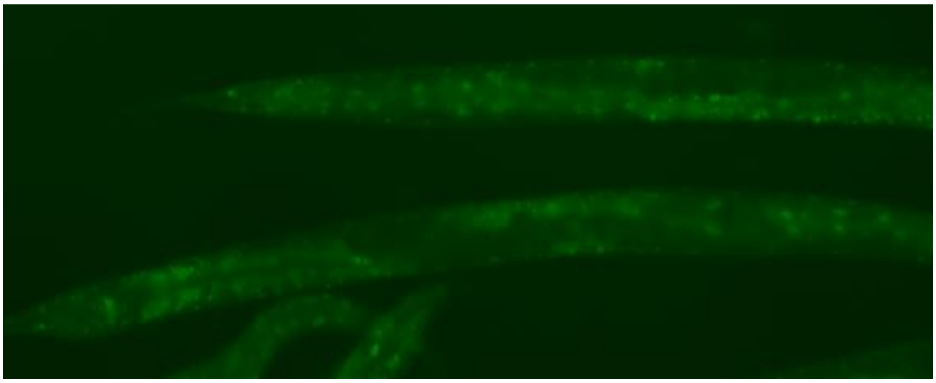
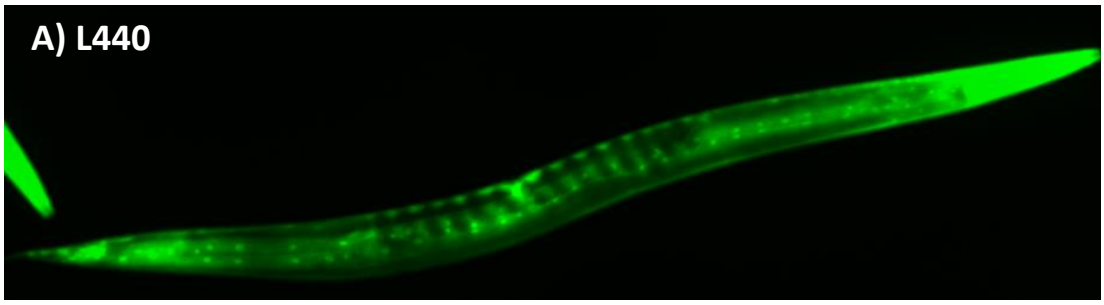
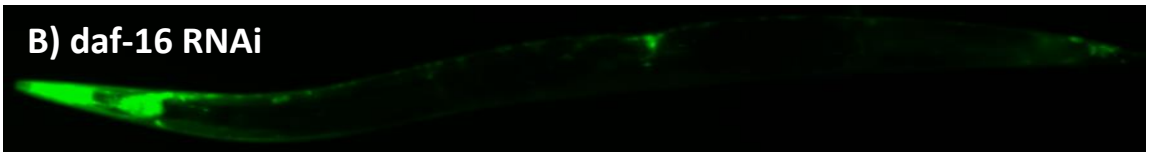


Figure 2: DAF-16::GFP still localizes to the nucleus in the daf-2 mutant background after feeding hlh-16 RNAi. Hlh-16 RNAi does not change daf-2 trangene expression levels or pattern compared to daf-2 mutant alone.



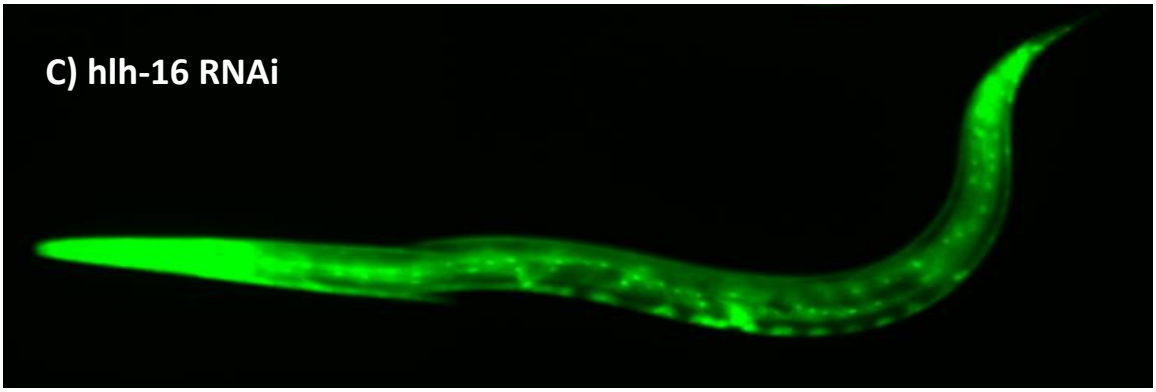
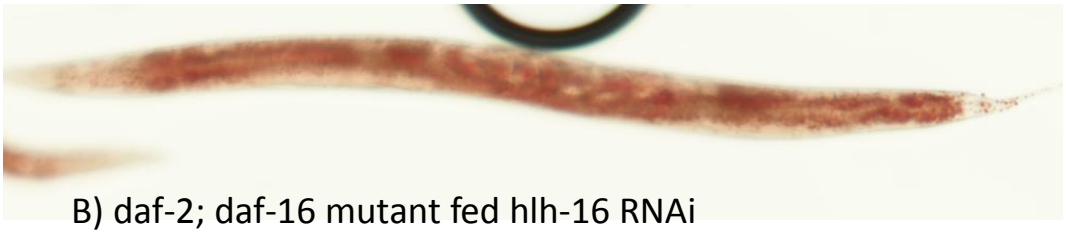




Figure 3: SOD-3::GFP, a transcriptional target of daf-16, is still expressed after feeding hlh-16 RNAi to daf-2 mutant animals. Hlh-16 does not prevent expression of daf-16 targets.

A) daf-2; daf-16 mutant fed L440 (vector control)



B) daf-2; daf-16 mutant fed hlh-16 RNAi

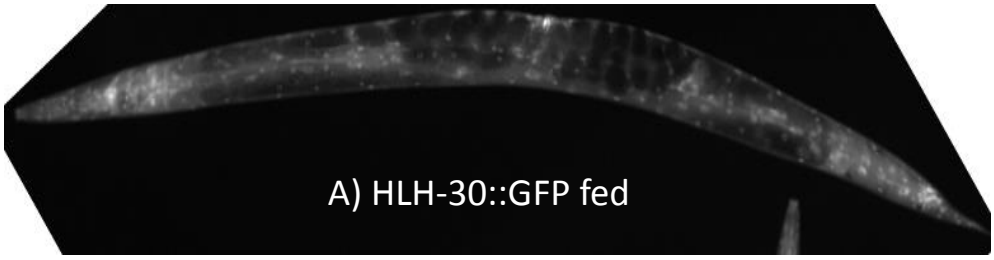
Figure 4: hlh-16 RNAi is not additive to daf-16 mutation on daf-2 obesity suppression. Hlh-16 is liking working in the same genetic pathway as daf-16.

| Query | Result | BLASTp |
|---|---|---|
| *H. sapiens* | *C. elegans* | E value |
| MITF | hlh-30 | e-27 |
| TFE3 | hlh-30 | e-25 |
| TFEB | hlh-30 | e-23 |
| USF2 | hlh-30 | e-09 |
| USF1 | hlh-30 | e-07 |

| *C. elegans* | *H. sapiens* | BLASTp |
|---|---|---|
| hlh-30 | MITF | e-29 |
| hlh-30 | TFE3 | e-28 |
| hlh-30 | TFEB | e-26 |
| hlh-30 | USF2 | e-10 |
| hlh-30 | USF1 | e-07 |

Table 2: Homology of human bHLHzip family of transcription factors to hlh-30 . While hlh-30 is traditionally described in the literature as functionally homologous to TFEB, it may be ancestral to the mammalian eye-maintenance gene, MITF.

*When BLASTp of H. sapiens gene is entered, hlh-30 is not only the top hit, but the only hit with an E value less than 0.



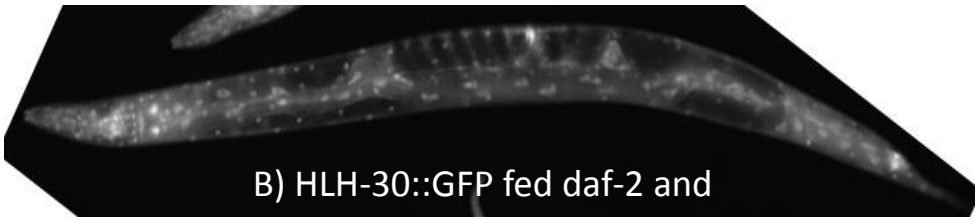
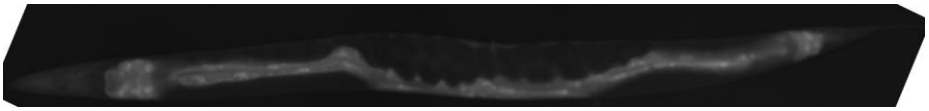




Figure 5: hlh-16 RNAi does not affect hlh-30 expression or nuclear localization following daf-2 knockdown.

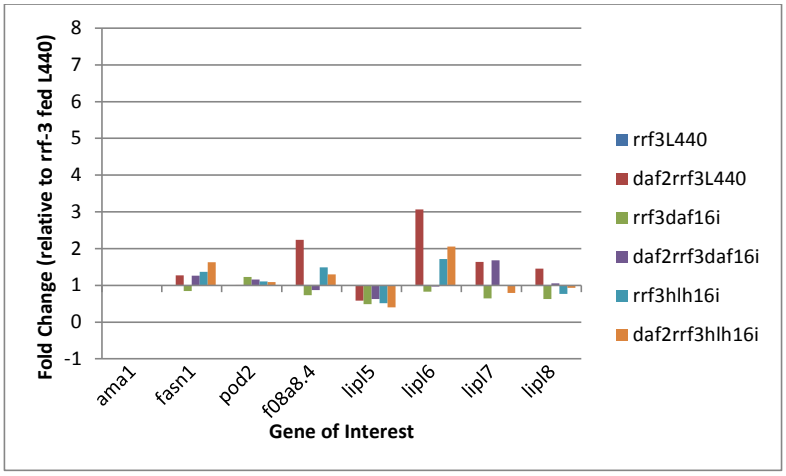


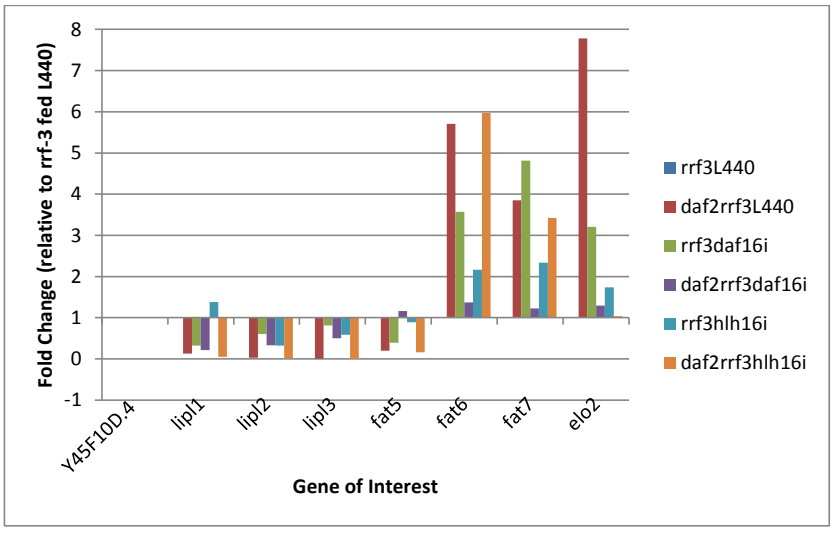
A) SBP-1::GFP fed L440 (vector control)

B) SBP-1::GFP fed daf-2 RNAi

C) SBP-1::GFP fed daf-2 and hlh-16

Figure 6: hlh-16 RNAi does not affect the expression pattern on SBP-1::GFP following daf-2 knockdown. Hlh-16 inactivation does not suppress daf-2 fat content increases by altering sbp-1 expression.


Fold Change (relative to rrf-3 fed L440)
8
7
6
5
4
3
2
1
0
-1
ama1
fasn1
pod2
f08a8.4
lipl5
lipl6
lipl7
lipl8
Gene of Interest
rrf3L440
daf2rrf3L440
rrf3daf16i
daf2rrf3daf16i
rrf3hlh16i
daf2rrf3hlh16i


Fold Change (relative to rrf-3 fed L440)
8
7
6
5
4
3
2
1
0
-1
Y45F10D.4
lipl1
lipl2
lipl3
fat5
fat6
fat7
elo2
Gene of Interest
rrf3L440
daf2rrf3L440
rrf3daf16i
daf2rrf3daf16i
rrf3hlh16i
daf2rrf3hlh16i

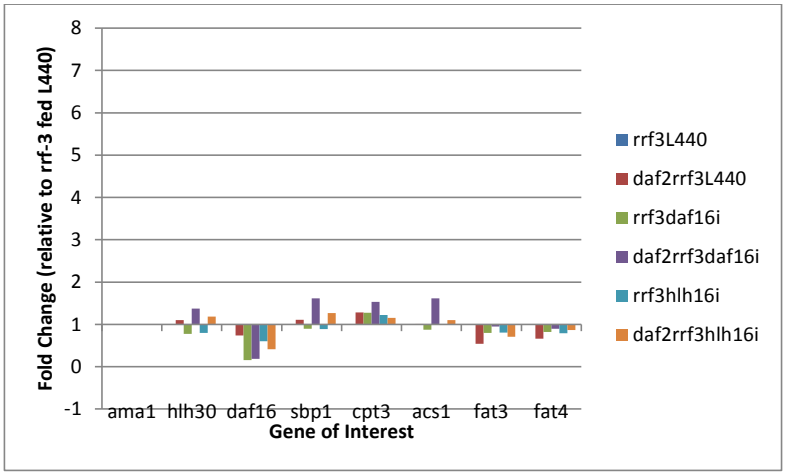



Figure 7: qPCR results. A panel of genes involved in lipid metabolism were screened. Elo-2 was shown to be upregulated in a daf-16 and hlh-16 dependent manner in daf-2 animals. Hlh-16 RNAi treatment reduced elo-2 levels to that of wildtype.

**Appendix:**

Papers Published during Master's Degree:

- Carolina Wählby, Annie Lee-Conery, Mark-Anthony Bray, Lee Kamentsky, Jonah Larkins-Ford, Kate Sokolnicki, Matthew Veneskey, **Kerry Michaels**, Anne E. Carpenter, and Eyleen J O'Rourke***.** (2014) "High and low throughput quantitative analysis of lipid metabolism in *C. elegans*", Methods.
- Likhite, N., Jackson, C., Liang, M., Lei, P., Wood, J., Birkaya, B., **Michaels, K.L.**, Andreadis, S., Stewart D. Clark, S., Yu, M., Ferkey, D.M. (2015) The Protein Arginine Methyltransferase PRMT-5 Regulates Dopamine Receptor Signaling in *C. elegans*. Science Signaling.