A Feedforward Loop Governs The Relationship Between 
Lipid Metabolism And Longevity 

Nicole K. Littlejohn, Nicolas Seban and Supriya Srinivasan*

Department of Neuroscience and The Dorris Neuroscience Center, 
The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA 

* Corresponding Author and Lead Contact: supriya@scripps.edu (S.S.)
SUMMARY

The relationship between lipid metabolism and longevity remains unclear. In particular although fat oxidation is essential for weight loss, whether it is remains beneficial when sustained for long periods, and the extent to which it may alter lifespan remains an important unanswered question. Here we develop an experimental handle in the *C. elegans* model system, that uncovers the mechanisms that connect long-term fat oxidation with longevity. We find that sustained β-oxidation, via activation of the conserved triglyceride lipase ATGL-1, triggers a feedforward transcriptional loop that involves the mito-nuclear transcription factor ATFS-1, and a previously unknown and highly conserved repressor of ATGL-1 called HLH-11/AP4. This feedforward loop orchestrates the dual control of fat oxidation and lifespan protection, shielding the organism from life-shortening mitochondrial stress in the face of continuous fat oxidation. Thus, we uncover one mechanism by which fat oxidation can be sustained for long periods without deleterious effects on longevity.
INTRODUCTION

The relationship between lipid metabolism, adiposity and lifespan remains unclear. Support for this assertion comes from several sources. On the one hand, accumulation of lipid deposits in ectopic tissues is associated with age-associated illnesses including diabetes, hepatic and pancreatic steatosis and other metabolic illnesses that shorten lifespan (Conte et al., 2019a; Schmeisser et al., 2019; Shulman, 2014). Converting the excess lipid deposits to energy via a cascade of metabolic reactions: lipolysis of triglyceride lipids, fatty acid breakdown via beta-oxidation and increased electron transport chain activity in the mitochondria, have been proposed to benefit health and longevity (Bonawitz et al., 2007; Conte et al., 2019b; Vatner et al., 2018). In this context, increased metabolic activity and mitochondrial respiration increase lifespan (Speakman et al., 2004). On the other hand, long-term caloric restriction and decreased metabolic rate reduce electron transport chain activity in the mitochondria and increase lifespan (Burkewitz et al., 2015; Durieux et al., 2011; Mattison et al., 2017). This paradoxical observation, first made in rats (McCay et al., 1935) and then extended to several species including C. elegans (Kimura et al., 1997; Lakowski and Hekimi, 1998) and primates (Mattison et al., 2017; Redman and Ravussin, 2011), argues for the opposite: that decreased metabolic and mitochondrial activity increases longevity.

Additional data supporting a non-linear relationship between lipid metabolism, fatty acid oxidation and longevity come from studies of the systemic insulin and TGF-beta signaling pathways, which are master regulators of physiology in C. elegans. Loss of the insulin/insulin-like growth factor (IGF-1) receptor daf-2, or of the transforming growth factor-β (TGFβ) signal daf-7, each result in substantially increased adiposity and body fat stores, but with a concomitant increase in lifespan (Greer et al., 2008; Kimura et al., 1997; Ogg et al., 1997). This non-linearity between adiposity and longevity is echoed in human observational studies that repeatedly suggest either no relationship (Kuk and Ardern, 2009; Reynolds et al., 2005), or an
inverse association between adiposity and mortality in late life (Fontaine et al., 2003; Stevens et al., 1998; Zheng and Dirlam, 2016). A resolution to this dichotomy might stem from considering the nature of experimental interventions. The majority of C. elegans studies showing a relationship between reduced mitochondrial function and increased longevity come from manipulations that decrease fat oxidation and metabolic rate (Dillin et al., 2002; Durieux et al., 2011; Lee et al., 2003). However, the converse has not been tested. In other words, the consequences of a sustained increase in fat oxidation on longevity in non-disease states has remained unexplored. Furthermore, fundamental mechanisms that connect lipid metabolism with longevity regulation at an organismal level still remain poorly understood. To this end, we considered it fruitful to conduct an investigation into the effects of sustained fat loss and increased mitochondrial respiration on longevity.

The neuromodulator serotonin (5-hydroxytryptamine; 5-HT) is a major regulator of metabolism, behavior and physiology in many species. In C. elegans, 5-HT is synthesized by the rate-limiting enzyme tryptophan hydroxylase (TPH-1) in only a few head neurons (Sze et al., 2000) but has wide-ranging effects across the organism including lipid metabolism (Srinivasan et al., 2008) behavioral responses to food (Cunningham et al., 2012), reproduction (Tanis et al., 2008), and pathogen avoidance (Zhang et al., 2005). 5-HT is a powerful stimulator of fat loss in the intestine (Noble et al., 2013), wherein the majority of lipids are stored and metabolized (Srinivasan, 2015). 5-HT-elicited fat loss occurs by activating the mitochondrial beta-oxidation pathway (Srinivasan et al., 2008), in which stored triglyceride lipids are oxidized to usable energy (Salway, 1999). Thus, approaches that increase neuronal 5-HT would have the potential to serve as a system to test the effects of sustained fat oxidation on lifespan. A major caveat is that genetic or pharmacological approaches that globally augment or decrease serotonin signaling lack specificity, leading to confounding and counterregulatory behavioral and metabolic effects that become difficult to disentangle. In this regard, an experimental approach
that allows the selective manipulation of metabolic rate via increased fat oxidation would represent a valuable methodological advance.

We had recently defined a role for a tachykinin peptide called FLP-7 that couples neuronal signaling with fat oxidation in the intestine. FLP-7 is the *C. elegans* ortholog of the mammalian family of tachykinin peptides and is released from secretory neurons in response to fluctuations in neuronal 5-HTergic signaling. We discovered FLP-7 in the context of deciphering the neural circuit for 5-HT-mediated fat mobilization. In recognizing that the *C. elegans* gut is not directly innervated but that 5-HT, a neural signal, exerts profound effects on lipid metabolism in the intestine, we had sought to define the neuroendocrine mechanisms that allow communication from the nervous system to the gut. A genetic screen followed by molecular genetic analyses identified FLP-7 as the causative neuroendocrine secreted factor (Palamiuc et al., 2017). FLP-7 is secreted from the ASI neurons in which it is necessary and sufficient, and activates the G-protein coupled receptor NPR-22 (ortholog of mammalian tachykinin receptor NK2R), in the intestinal cells (Palamiuc et al., 2017). FLP-7/NPR-22 signaling transcriptionally activates the enzyme adipocyte triglyceride lipase (ATGL; ATGL-1 in *C. elegans*), a highly conserved lipolytic enzyme that regulates the first step in the fat oxidation and energy production cascade: the hydrolysis of triglycerides to free fatty acids.

The tachykinin signaling pathway thus defines the core neuroendocrine axis by which the metabolic effects of neuronal 5-HT signaling are relayed to the intestine. Critically, we previously showed that FLP-7/NPR-22 signaling does not alter behaviors or physiological outputs associated with global 5-HT signaling (Palamiuc et al., 2017). Here we leverage this knowledge and generate a *C. elegans* transgenic line that expresses the FLP-7 tachykinin peptide from the ASI neurons to specifically and selectively recapitulate all of the effects of neuronal 5-HT on driving fat oxidation without concomitant behavioral effects. In principle, the
FLP-7 transgenic line represents a potent experimental tool for us to address, in a non-disease context, the question of whether neuronally-driven sustained fat oxidation has an effect on longevity.

RESULTS

An experimental handle to generate sustained fat oxidation in the intestine

The FLP-7/NPR-22 tachykinin neuron-to-intestine signaling pathway is triggered by increases in neuronal 5-HT (Figures 1A-C), as previously published (Palamiuc et al., 2017). We tested several measures to ascertain the extent to which the FLP-7 transgenic line (henceforth flp-7tg) recapitulates the effects of neuronal 5-HT signaling on metabolic parameters in the intestine.

First, we observed that flp-7tg worms have a significant reduction in intestinal fat stores (Figures 1D, E), recapitulating that seen with 5-HT treatment (Figures 1F, G). The fat reduction elicited by flp-7tg is dependent on the presence of the intestinal triglyceride lipase ATGL-1 (Figures 1D, E; note that the atgl-1 null mutant is inviable), as was noted for 5-HT itself (Figures 1F, G) (Noble et al., 2013; Palamiuc et al., 2017). In previous work, we uncovered major components of the mitochondrial beta-oxidation pathway that functionally connect ATGL-1 activity to the electron transport chain (ETC) that underlies mitochondrial respiration (Noble et al., 2013; Srinivasan et al., 2008). Accordingly, FLP-7-stimulated fat loss is accompanied by increased basal and maximal respiration that are both abrogated upon RNAi-mediated inactivation of atgl-1 (Figures 1H, I). Together, Figure 1 shows that increased mitochondrial respiration in flp-7tg animals results from conversion of stored triglycerides to energy via beta-oxidation and ETC activity. Thus, the flp-7tg line fully recapitulates the metabolic effects of genetic and pharmacological manipulation of neuronal 5-HT signaling without altering other 5-HT-mediated behaviors (Horvitz et al., 1982; Loer and Kenyon, 1993; Palamiuc et al., 2017; Song and Avery, 2012; Sze et al., 2000; Waggoner et al., 1998) and can serve as a valuable experimental handle to examine the long-term effects of sustained fat oxidation and increased ETC activity.
Intestinal fat oxidation via ATGL-1 induction evokes a mitochondrial stress response

Several lines of evidence have suggested that mild reductions in mitochondrial respiration can lead to a sustained increase in lifespan via decreased ROS production, hormesis, or other mechanisms. We examined \textit{flp-7\textsuperscript{tg}} animals to test whether the observed increase in mitochondrial respiration (Figures 1H, I) might evoke the opposite effect on longevity. However, we found that \textit{flp-7\textsuperscript{tg}} animals had nearly the same lifespan as wild-type animals, with a non-significant difference (\textit{p}=0.08 by Log-rank Test) in survival probability statistics (Figures 2A, B).

Next, we considered whether the increased mitochondrial respiration (Figures 1H, I) might evoke a systemic stress response. To test this possibility, we examined the effects of 5-HT administration or FLP-7 secretion on a variety of well-established stress reporters in \textit{C. elegans} including those involved in the cytoplasmic heat shock response (\textit{hsp-70} and \textit{hsp-16.2}), oxidative stress (\textit{sod-3}), nutrient stress (DAF-16 nuclear localization), ER stress (\textit{hsp-4}) and mitochondrial stress (\textit{hsp-60}). In the \textit{flp-7\textsuperscript{tg}} animals (Figures 2C-E) and in those treated with 5-HT (Figure S1), we observed a two-fold induction of an \textit{hsp-60::GFP} transgene, the canonical reporter for the mitochondrial stress response. Other stress responses were absent. The induced mitochondrial \textit{hsp-60}-mediated stress response was seen predominantly in the intestine (Figure 2C), and was wholly dependent on the presence of \textit{atgl-1} because RNAi-mediated inactivation of \textit{atgl-1} ameliorated \textit{hsp-60} induction as judged by the reporter assay (Figure 2D) as well as by directly measuring \textit{hsp-60} transcripts by qPCR (Figure 2E). The increased mitochondrial respiration in \textit{flp-7\textsuperscript{tg}} also depended on \textit{atgl-1}-dependent utilization of fat reserves (Figures 1H, I), suggesting the surprising result that fat oxidation evokes a mitochondrial stress response.
The mitochondrial stress response factor ATFS-1 sustains fat oxidation via ATGL-1

All known mitochondrial stress pathways that induce *hsp-60* require the mito-nuclear transcription factor called ATFS-1 (Nargund et al., 2012). Under normal conditions, ATFS-1 is transported into mitochondria and degraded. However, under conditions that induce mitochondrial stress, ATFS-1 is stabilized and translocated to the nucleus where it initiates a range of transcriptional responses that allow adaptation to the stressor (Lin and Haynes, 2016; Nargund et al., 2015). To determine whether the FLP-7-induced mitochondrial stress response required *atfs-1*, we measured *hsp-60* induction in its absence. As shown in Figures 2F and G, *hsp-60* was no longer induced by FLP-7 in the *atfs-1(tm4525)* null mutants. We also noted that the combined loss of *atgl-1* and *atfs-1* completely suppressed the *hsp-60* induction, which recapitulates the loss of each gene alone with no additive or synergistic effects (Figures 2F, G). This finding was replicated with 5-HT-stimulated *hsp-60* induction that was completely suppressed with either *atgl-1* or *atfs-1* inactivation (Figure S1). This result suggested the possibility that *atgl-1*-dependent fat oxidation and *atfs-1*-dependent mitochondrial stress may function in a linear pathway.

Despite our knowledge about the role of ATFS-1 in mitochondrial biology, it had not previously been associated with fat oxidation *per se*. To our surprise, we found that both 5-HT and FLP-7-induced fat oxidation were partially suppressed in *atfs-1* null mutants (Figures 3A, B and Figure S2A). The absence of *atfs-1* also suppressed the transcriptional induction of *atgl-1* by FLP-7 and 5-HT as judged by measuring the *atgl-1* reporter *in vivo* (Figures 3C, D), and by direct measurement of *atgl-1* transcripts by qPCR (Figures 3E and S2B). As in the case of the *hsp-60*-mediated stress response (Figures 2D, E), loss of both *atfs-1* and *atgl-1* also did not lead to a further suppression of FLP-7-induced fat loss (Figures 3A, B), suggesting again that they function in a linear pathway. However, these data also indicated that the effects of secreted FLP-7 on the intestine are intertwined: on the one hand the mitochondrial stress response...
requires \textit{atgl-1} and fat oxidation (red arrow; Figure 3F), and on the other hand fat oxidation requires the mito-nuclear stress response transcription factor \textit{atfs-1} (green arrow; Figure 3F). Published ChIPseq and microarray studies of the ATFS-1 transcription factor did not suggest a direct induction of \textit{atgl-1} by ATFS-1 (Nargund et al., 2015; Nargund et al., 2012). We also did not find the putative cis-binding site of ATFS-1 within a 5 kb region upstream of the \textit{atgl-1} transcriptional start site. Yet, ATFS-1 is required for both the induction of \textit{atgl-1} expression (Figures 3C-E), and the ensuing fat loss (Figures 3A, B). Thus, ATFS-1 likely regulates \textit{atgl-1} transcription by an indirect mechanism.

\textbf{The conserved transcription factor HLH-11 governs fat oxidation via direct control of ATGL-1}

To identify direct transcriptional regulators of \textit{atgl-1 in vivo}, we began by conducting an RNAi-based screen of the ~900 transcription factors in \textit{C. elegans} (Fuxman Bass et al., 2016). We used the \textit{Patgl-1::GFP} reporter we had previously developed (Noble et al., 2013) and screened for genes that regulate \textit{atgl-1} under basal conditions, as well as for those that were essential for 5-HT or FLP-7-stimulated \textit{atgl-1} induction. Our top hit was a gene called \textit{hlh-11}, the sole conserved ortholog of the mammalian transcription factor AP4 (Lee et al., 2009). We obtained and outcrossed a null mutant for \textit{hlh-11(ok2944)}, which when crossed into the \textit{Patgl-1::GFP} reporter line showed significant \textit{in vivo} induction of \textit{atgl-1} in the intestine (Figures 4A, B). These results were reinforced by measuring transcript levels of \textit{atgl-1} mRNA by qPCR, which were significantly greater in \textit{hlh-11} null mutants (Figure 4C). We also noted that the extent of \textit{atgl-1} induction by FLP-7 was matched by the absence of \textit{hlh-11} mutants without a further increase in \textit{flp-7};\textit{hlh-11} animals (Figures 4A-C). These data suggested that in wild-type animals, HLH-11 functions as a repressive transcription factor that suppresses \textit{atgl-1} under basal conditions. In turn, this predicts that a metabolic phenotype may result from \textit{hlh-11} removal.
hlh-11 null mutants showed a dramatic ~70-80% decrease in body fat stores relative to wild-type and phenocopied the FLP-7 transgenic line (Figures 4D, E). flp-7\textsuperscript{tg};hlh-11 animals also had significant reductions in body fat stores and resembled either single mutant alone. Removal of atgl-1 by RNAi completely suppressed fat oxidation under all conditions (Figures 4D, E). Further, the fat loss phenotype of hlh-11 null mutants was accompanied by a significant increase in basal and maximal respiration that was also abrogated in the absence of atgl-1 (Figures 4F, G). Together, these data show that loss of the transcription factor hlh-11 constitutively increases atgl-1 gene expression, fat oxidation and energy expenditure.

To test whether HLH-11 was also instructive in regulating atgl-1 expression and subsequent fat metabolism, we generated an HLH-11 overexpression line (henceforth HLH-11\textsuperscript{ox}) fused to GFP using the endogenous 3 kb promoter (Lee et al., 2009). We observed robust nuclear HLH-11 expression in the intestine (Figure 5A), suggesting a plausible model for the interaction between HLH-11 and atgl-1. Remarkably, HLH-11\textsuperscript{ox} animals showed an ~40% increase in body fat stores compared to wild-type animals and showed a near-complete suppression of the fat loss elicited in flp-7\textsuperscript{tg} animals (Figures 5B, C). The increase in fat stores was accompanied by a corresponding decrease in maximal respiration, which was not further decreased upon removal of atgl-1 (Figure 5D). The fat accumulation and metabolic output in HLH-11\textsuperscript{ox} and flp-7\textsuperscript{tg};HLH-11\textsuperscript{ox} animals were accompanied by a significant decrease in atgl-1 mRNA, as judged by qPCR (Figure 5E). Thus, HLH-11 plays an instructive role as a negative regulator of atgl-1 expression and influences fat oxidation and mitochondrial respiration in the intestine.

The cis-binding site of HLH-11 has been precisely mapped to an 8-mer (Lee et al., 2009), but has not yet been functionally tested. We identified two HLH-11 cis-binding sites at 389 and 1,149 bp upstream of the atgl-1 transcriptional start site (Figure 5F). To test for direct
binding of the atgl-1 promoter by HLH-11, we conducted chromatin immunoprecipitation experiments followed by qPCR (ChIP-qPCR) of the transgenic line in which HLH-11 was fused to GFP. Relative to wild-type animals, we observed a substantial and significant increase in atgl-1 promoter regions bound to HLH-11 (Figure 5G), thus the transcription factor HLH-11 binds directly to the atgl-1 promoter. Finally, we generated an atgl-1 reporter line that lacked both hlh-11 cis sites (Δcishlh-11) and observed a ~ 2-fold increase in atgl-1 expression (Figures 5H, I). Taken together, the conserved transcription factor HLH-11 is a direct repressor of atgl-1 expression, fat oxidation, and mitochondrial respiration.

The mito-nuclear transcription factor ATFS-1 promotes fat oxidation via HLH-11 regulation

Next, we wished to examine how hlh-11 itself is regulated in the context of the FLP-7 pathway. Because atgl-1 is induced by FLP-7 (Figures 3C-E) but repressed by HLH-11 (Figures 4A-C), we hypothesized that FLP-7 signaling itself might repress hlh-11. We found that relative to wild-type, the flp-7tg line had reduced HLH-11GFP in the nuclei of the intestinal cells (Figures 6A, B), suggesting that hlh-11 is downregulated by secreted FLP-7. This observation was corroborated by measuring hlh-11 transcripts using qPCR (Figure 6C). These experiments establish that neuronally-secreted FLP-7 negatively regulates intestinal HLH-11 that directly binds to and represses ATGL-1 transcription.

However, we had originally identified HLH-11 in light of our data suggesting that the mito-nuclear transcription factor ATFS-1 was indirectly required for FLP-7-mediated atgl-1 induction and fat oxidation in the intestine (Figure 3). In seeking a greater understanding of how ATFS-1 may be connected to HLH-11, we serendipitously found that a previous report had listed hlh-11 as one of the direct targets of ATFS-1 (Nargund et al., 2015). To test the possibility of an interaction between ATFS-1 and hlh-11 in the context of FLP-7-mediated fat oxidation and
mitochondrial stress, we measured hlh-11 transcripts in the absence of atfs-1. We found that atfs-1 removal blunted FLP-7-mediated hlh-11 repression (Figure 6C), suggesting that FLP-7-mediated hlh-11 repression requires atfs-1. In flp-7g animals, inactivation of atgl-1, a condition that would simultaneously block fat oxidation and atfs-1 activation, also blunted this hlh-11 repression, suggesting a connection between fat oxidation, atfs-1 induction, and hlh-11 repression. Loss of both atfs-1 and atgl-1 diminished the FLP-7-dependent suppression of hlh-11 to a similar extent as loss of either gene alone (Figure 6C). Thus, the repression of hlh-11 by neuronal FLP-7 signaling occurs via ATFS-1, which is stabilized during fat-oxidation-induced mitochondrial stress (Figure 3). These results suggest the interesting possibility of a feedforward loop (Figure 6D): repression of hlh-11 by FLP-7 permits atgl-1-dependent fat oxidation, which induces atfs-1 and in turn represses hlh-11 to sustain and augment fat oxidation. Predictions from this model are two-fold. On the one hand, the ATFS-1/HLH-11 interaction should modulate the hsp-60-dependent mitochondrial stress response, and on the other, it should also regulate the atgl-1 transcriptional response (Figure 6D).

A feedforward loop orchestrates the relationship between fat oxidation, mitochondrial stress and longevity.

To functionally test the feedforward loop model (Figure 6D), we studied the relationships between HLH-11 and ATFS-1 in the context of FLP-7 signaling. First, we predicted that HLH-11, a transcription factor that suppresses atgl-1 and fat oxidation, would also regulate the mitochondrial stress response, in an atgl-1-dependent manner. Accordingly, we found that overexpression of HLH-11, which represses atgl-1 mRNA (Figure 5D), showed a significant decrease in hsp-60 mRNA (Figure 6E). Thus, HLH-11 decreases not only fat oxidation and mitochondrial respiration (Figures 5B, C, E), but also the ensuing stress response. In contrast, hlh-11 null mutants, which constitutively stimulate fat oxidation and mitochondrial respiration by modulating atgl-1 transcription (Figure 4), significantly increased hsp-60 mRNA (Figure 6E).
Effects of HLH-11 overexpression and absence were identical in the presence and absence of FLP-7 secretion (Figure 6E). Also, the induction of hsp-60 upon loss of hlh-11 was fully suppressed by removal of either atgl-1, atfs-1, or both, suggesting that HLH-11 mediated mitochondrial stress is a direct consequence of fat oxidation (Figure 6E). Thus, rather than being independent of one another, HLH-11-mediated control of fat oxidation and energy expenditure evoke a stress response as a direct consequence of these mitochondrial functions.

Second, we tested the relationship between ATFS-1 and its transcriptional target hlh-11, with respect to atgl-1 expression (refer to model in Figure 6D). As expected, hlh-11 mutants increased atgl-1 expression with and without increased FLP-7 secretion as judged by atgl-1 reporter expression (Figure 6F) as well as qPCR (Figure 6G). Also as predicted, atfs-1 loss alone does not lead to appreciable changes in atgl-1 (Figures 6F, G); this is because atfs-1 is non-functional in wild-type animals (Nargund et al., 2012). In contrast, hlh-11;atfs-1 double mutants resembled hlh-11 mutants alone, thus during increased FLP-7 secretion, atfs-1-dependent suppression of atgl-1 induction requires hlh-11 repression (Figures 6F, G). This result again suggested that rather than being a simple consequence of fat oxidation, the mitochondrial stress response is an integral component of sustained fat loss via atgl-1 transcriptional regulation.

We were curious whether disrupting the HLH-11/ATGL-1/ATFS-1 feedforward loop (Figure 6D) would shed light on the relationship between sustained fat oxidation and longevity. To this end, we measured the consequences of these transcriptional changes on physiological parameters. flp-7tg animals have reduced fat stores because of increased atgl-1-dependent fat oxidation (Figure 1). In the context of flp-7tg animals, hlh-11 mutants also show augmented fat loss, whereas atfs-1 removal blocked fat oxidation (Figures 7A, B). In accordance with the increased atgl-1 transcript levels (Figures 6F, G), we found that hlh-11;atfs-1 double mutants
also resembled *hlh-11* single mutants alone in their fat phenotypes, suggesting that the suppression of fat oxidation in *atfs-1* mutants is dependent on the presence of *hlh-11* (Figure S3). Thus, ATFS-1-dependent fat oxidation requires *hlh-11* repression of *atgl-1* (Figures 7A, B); HLH-11 acts genetically downstream of ATFS-1. We had already noted that increased fat oxidation via augmented FLP-7 secretion in *flp-7* animals lead to no appreciable change in lifespan (Figures 2A, B). One explanation for this result is that FLP-7 signaling induces ATFS-1 activation because of mitochondrial fat oxidation. In addition to its role in repressing *hlh-11*, ATFS-1 has hundreds of additional targets which in combination have been postulated to serve mitochondrial recovery functions (Lin and Haynes, 2016; Nargund et al., 2012). Thus, in the context of *flp-7* animals, concomitant with the fat oxidation, our data suggest that an ATFS-1-dependent mechanism is simultaneously evoked, thus protecting lifespan. In testing this idea, we found that in *flp-7* animals removal of both *hlh-11* and *atfs-1* led to a significant decrease in both median and maximal lifespan (p<0.001, Figures 7C, F) that was not seen in *hlh-11;atfs-1* mutants (Figure 7D), *hlh-11* (Figure 7E) or *atfs-1* mutants (Tian et al., 2016). We reasoned that in *hlh-11;atfs-1* mutants alone (that is, without increased FLP-7 secretion) we observed modest fat loss (Figure S3) that is not sufficient to shift lifespan in either direction (Figure 7D). Even though *hlh-11* mutants show a substantial increase in fat oxidation (Figures 4 D, E), the presence of *atfs-1* (Figure 6E) prevents lifespan shortening.

Why did loss of *hlh-11;atfs-1* in the *flp-7* animals alone lead to a decrease in longevity? Our model (Figure 7G) is consistent with the following interpretation: FLP-7 secretion serves as the neuronal cue to trigger fat oxidation by repressing *hlh-11* expression that in turn de-represses *atgl-1* (red pathway, Figure 7G). The resulting increase in fat oxidation and mitochondrial respiration generates an ATFS-1-mediated mitochondrial response, as judged by induction of the mitochondrial stress sensor *hsp-60*. We propose that ATFS-1 induction serves as a second cue from the mitochondria to further repress *hlh-11*, which in turn augments fat.
oxidation via ATGL-1 in a feedforward loop (green pathway, Figure 7G). The atgl-1/atfs-1/hlh-11 feedforward loop serves the dual functions of sustaining fat oxidation and protecting lifespan. Although each influences the other, the hlh-11/atgl-1 arm of the pathway primarily drives fat loss, whereas the atfs-1/hlh-11 arm of the pathway primarily protects lifespan.

**DISCUSSION**

In this study, we wished to address the question of whether, in a non-disease context, sustained fat oxidation and increased mitochondrial respiration have an effect on lifespan. We initiated these studies because the relationship between fat oxidation and longevity, although of major significance, has remained opaque. We report here that sustained fat oxidation evokes a mitochondrial stress response that functions to simultaneously augment fat oxidation and confer longevity protection (modeled in Figure 7G). We have found that the dual control of fat oxidation and lifespan protection emerges from a feedforward transcriptional loop and shields the organism from life-shortening mitochondrial stress in the face of continuous fat oxidation. Thus, sustained fat oxidation does not shorten lifespan because a mitochondrial response protects both. Under normal or wild-type conditions when fat oxidation levels are not high, this loop remains latent.

In this study, we have uncovered a regulatory pathway that is initiated in the nervous system and functions in the intestine, the major seat of metabolic and longevity regulation. The conserved transcription factor HLH-11 functions as the nexus of this signaling pathway and receives two signals: one non-cell-autonomous via neuronal FLP-7 that transmits sensory signals to initiate a metabolic response, and the other cell-autonomous via the mito-nuclear transcription factor, ATFS-1, that coordinates the mitochondrial stress response. HLH-11 is a direct transcriptional repressor of the triglyceride lipase atgl-1: loss of hlh-11 increases atgl-1-dependent fat oxidation, and overexpression of hlh-11 has the opposite effect. Thus, controlling
*hlh-11* levels can serve as an excellent surrogate for titrating intestinal fat stores in future efforts. Interestingly, the mammalian ortholog of HLH-11, called AP4, is widely distributed along the epithelial cells the mammalian intestine, as is ATGL (Jung and Hermeking, 2009; Obrowsky et al., 2013).

Signaling loops as a network feature of biological systems are not uncommon; three predominant types have been described in the literature: negative feedback loops, positive feedback loops, and feedforward loops (Hornung and Barkai, 2008; Reeves, 2019). Negative feedback loops are commonly found in metabolic pathways such as glycolysis, in which a signal input (glucose) initiates a biochemical cascade that must be eventually be turned off as the pathway reaches capacity (Salway, 1999). Thus, negative feedback loops are sensitive to the external input and serve to diminish output over time. In contrast, positive feedback loops serve to amplify external signals over time, and as the network amplifies, become insensitive to the external stimulus (Abdel-Sater, 2011; Doncic and Skotheim, 2013). Such networks states have typically been described as unstable, and noted in deleterious physiological settings, such as an uncontrolled drop in blood pressure leading to death (Doncic and Skotheim, 2013; Goldstein and Kopin, 2017). Feedforward regulation is a network motif that is distinct from the above states. Network modeling approaches show that feedforward loops remain sensitive to the external input, are stable yet reversible, and are used to balance tradeoffs (Abdel-Sater, 2011; Jesty and Beltrami, 2005; Mitrophanov and Groisman, 2008). Here, we identify the broad molecular features of one such feedforward network state: the *hlh-11/atgl-1/atfs-1* signaling loop, which remains sensitive to input from neuronal FLP-7, is stable and reversible, coordinates multiple physiological outputs, and balances the tradeoff between sustained fat oxidation and longevity. Such a feedforward motif may be a general conserved feature in balancing multiple physiological parameters.
ACKNOWLEDGMENTS

Strains were provided by Knockout Consortium at Tokyo Women’s Medical University as well as the CGC, which is funded by the NIH Office of Research Infrastructure Programs (P40 OD010440). This work was supported by research grants to S.S. from the NIH/NIDDK (R01 DK095804) and NIH/NIA (AG056648). N.K.L. was supported by a fellowship from the American Heart Association (17POST33660740) and a Dorris Scholar Award from the Dorris Neuroscience Center, The Scripps Research Institute.

AUTHOR CONTRIBUTIONS

Conceptualization, S.S, N.K.L., and N.S.; Methodology, S.S., N.K.L. and N.S.; Investigation, N.K.L. and N.S.; Resources, S.S., N.K.L. and N.S.; Formal analysis, N.K.L. and N.S.; Visualization, N.K.L.; Writing, S.S. and N.K.L.; Project administration, S.S., Supervision, S.S.; Funding Acquisition, S.S.

DECLARATION OF INTEREST

The authors declare no competing interests.

METHODS

Materials Availability

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Supriya Srinivasan (supriya@scripps.edu).

Worm maintenance and strains

Nematodes were cultured as previously described (Brenner, 1974). N2 Bristol was obtained from the Caenorhabditis Genetic Center (CGC) and used as the wild-type reference strain. All
mutant and transgenic strains used in the study are listed in the Supplemental Table 1. The hlh-11(ok2944) strain was provided by the C. elegans Gene Knockout Project at OMRF, which is part of the International C. elegans Gene Knockout Consortium. The atfs-1(tm4525) strains were generously provided by Cole Haynes and originally obtained from the National BioResource Project (Tokyo, Japan). Animals were synchronized for experiments by hypochlorite treatment and then hatched L1 larvae were seeded on plates with the appropriate bacteria. All experiments were performed on day 1 adults.

Cloning and transgenic strain construction

The hlh-11 promoter and gene were cloned using standard PCR techniques and Gateway Technology™ (Life Technologies) from N2 lysates. The final plasmid (Phlh-11::hlh-11GFP) encoded an HLH-11GFP fusion. 25 ng/µL of Phlh-11::hlh-11GFP plasmid was injected with 10 ng/µL of Pmyo-2::mCherry as a co-injection marker and 65 ng/µL of an empty vector to maintain a total injection mix concentration of 100 ng/µL. A well-transmitting transgenic line with consistent expression was integrated using the UV psoralen 2400 (Stratagene) and backcrossed 4 times before experimentation. To generate a plasmid of the atgl-1 promoter lacking the two hlh-11 cis-binding sites (Patgl-1Δcishlh-11::GFP), the Q5® site-directed mutagenesis kit (New England Biolabs) was used on the Patgl-1::GFP plasmid previously generated (Noble et al., 2013). The primers used are listed in the Supplemental Table 2. 25 ng/µL of the Patgl-1Δcishlh-11::GFP plasmid, 50 ng/µL of rol-6 co-injection marker, and 25 ng/µL of empty vector were injected into wild-type worms. A transgenic line was selected based on consistency of expression and transmission.

5-HT treatment

5-HT hydrochloride powder (Alfa Aesar) was dissolved in 0.1 M HCl and was added to plates for a final concentration of 5 mM as previously described (Palamiuc et al., 2017).
RNAi

RNAi experiments were conducted as previously described (Kamath and Ahringer, 2003; Palamiuc et al., 2017). Plates were seeded with HT115 bacteria containing vector or the relevant RNAi clone four days prior to seeding larvae.

Oil Red O staining

Oil Red O staining was performed as described and validated (Hussey et al., 2018; Hussey et al., 2017; Noble et al., 2013; Palamiuc et al., 2017; Witham et al., 2016). Briefly, animals were washed off plates with PBS and incubated on ice for 10 min. Animals were fixed as described (Noble et al., 2013), after which they were stained in filtered oil Red O working solution (60% oil Red O in isopropanol: 40% water) overnight. For all genotypes and conditions, approximately 2,000 animals were fixed and stained, and about 100 animals were visually examined. Then, 15-20 animals were chosen blindly and imaged. All experiments were repeated at least 3 times. Wild-type animals were always included as controls within each experiment.

Image acquisition and quantitation

Black and white images of oil Red O stained animals were captured using a 10X objective on a Zeiss Axio Imager microscope. Images were quantified using ImageJ software (NIH) as previously described (Noble et al., 2013). All reported results were consistent across biological replicates. Fluorescent images of reporters for FLP-7 secretion were captured using a 20X objective on a Zeiss Axio Imager microscope. The first pair of coelomocytes was imaged. mCherry fluorescence intensity in one of the two imaged coelomocytes was quantified and normalized to the area of the coelomocyte GFP as previously described and validated (Palamiuc et al., 2017). For fluorescence imaging of gene expression reporter lines (animals with integrated Patgl-1::GFP, Phsp-60::GFP, or Phlh-11::hlh-11GFP transgenes), an equal
number of animals were chosen blindly and lined up side by side. Images were take using a 10X or 20X objective on a Nikon Eclipse 90i microscope. Fluorescence intensity for all chosen animals was quantified for each condition and normalized to area of the animals excluding the head as indicated in the figure legend. Images were quantified using ImageJ software (NIH).

**Oxygen consumption**

Oxygen consumption rates (OCR) was measured using the Seahorse XFe96 Analyzer (Agilent) as previously described (Hussey et al., 2018). Briefly, adult animals were washed with M9 buffer and approximately 10 animals per well were placed into a 96-well plate. 5 measurements were taken for baseline, then at 37 min FCCP (50 µM) was injected to measure maximal OCR. Lastly, sodium azide (40 mM) was injected at 62 min to measure residual OCR. Afterwards, the number of worms per well was counted, and OCR values were normalized to number of worms per well. Basal OCR was calculated by averaging all measurements prior to FCCP (50 µM) addition, and maximal OCR was calculated by averaging the first two measurements after FCCP injection. In all genotypes tested, we did not observe any changes in worm size, growth or developmental stage.

**Chromatin immunoprecipitation-qPCR**

Chromatin immunoprecipitation was done as previously described (Mukhopadhyay et al., 2008). 50,000 synchronized D1 adult animals were washed with PBS three times and fixed with 1.1% formaldehyde for 15 min. Animals were partially lysed using a Dounce homogenizer. Fixative was quenched with 2.5 M glycine for 20 min. Animals were then washed and incubated in HEPES lyses buffer (50mM HEPES-KOH pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% (wt/vol) sodium deoxycholate, 1% (vol/vol) Triton X-100, 0.1% (wt/vol) SDS, 1 mM PMSF, and diluted protease inhibitor cocktail). Animal suspensions were sonicated using the Sonic Dismembrator Model 100 (Fischer Scientific) for 10 sec and then place on ice for 2 min, which was repeated
eight times. An aliquot of lysate was kept for input DNA analysis. 3 mg of protein was subjected to a pre-clearing step by incubating samples with prewashed salmon sperm DNA/protein-A agarose beads (Millipore Sigma) for 1 hour. The supernatant was then incubated with GFP-Trap® coupled to magnetic agarose beads (Bulldog Bio) overnight at 4°C. As a negative control, wild-type animals, which lack GFP expression, were used. Bead-GFP-Trap-DNA complex was washed three times. Then precipitated DNA was eluted from the beads, and the cross-link was reverse overnight at 65°C. Precipitated chromatin and the input samples were treated with proteinase K, and the DNA was purified using the PCR Purification kit (Qiagen). Purified DNA was then subjected to quantitative PCR using three sets of primers targeting the promoter region of *atgl-1*, and *act-1* primers were used as a negative control. 3 technical replicates were used per group, and the entire ChIP-qPCR procedure was performed three times for a total of 3 biological replicates. All primer sequences are provided in the Supplemental Table 2.

**qPCR**

Total RNA was isolated from D1 adult animals using TRizol™ (Invitrogen) and purified as previously described (Palamiuc et al., 2017). cDNA was made using iScript™ Reverse Transcription Supermix for RT-qPCR kit (BioRad) following the manufacturer’s instructions. SsoAdvanced™ Universal SYBR Green Supermix (BioRad) was used for performing qPCR according to the manufacturer’s instructions. Data was normalized to *act-1* mRNA. Primers sequences are listed in the Supplemental Table 2. Fold change was calculated following the Livak method (Livak and Schmittgen, 2001). Each group had at least 3 biological replicates in which worms were harvested and RNA was isolated from each biological replicate separately. For each qPCR plate, at least 2 technical replicates were included per sample.
**Lifespan**

All lifespan experiments were performed at 20°C (Keith et al., 2014). Approximately one hundred L4 larvae per group were transferred to NGM plates seeded with OP50, which was recorded as day 0. Animals were transferred to new plates every other day until egg laying stopped. Surviving and dead animals were counted every other day until all animals were dead. Animals were considered dead when they did not respond to a gentle stimulation with a platinum wire. Bagging, exploding, and contaminated animals were excluded from analysis.

**STATISTICAL ANALYSES**

Each assay was powered for sample size and statistical test based on the following: (i) pilot studies to assess the strength of the phenotype; (ii) minimum number of animals needed to detect significant differences (p<0.05). For each type of assay, we used the same number of animals for each genotype or condition so as not to over-power or under-power comparisons. The sample size, statistical method and significance for each experiment is listed in the corresponding figure legend. All actual p-values are given in Supplemental Table 3. Wild-type animals were included as controls for every experiment. Error bars represent standard error of the mean (sem). Student’s t-test, one-way ANOVA, Log-Rank Test, and two-way ANOVA were used as indicated in the figure legends. Bonferroni’s correction for multiple comparisons was used for all ANOVAs.
REFERENCES

Abdel-Sater, K.A. (2011). Physiological Positive Feedback Mechanisms. American Journal of Biomedical Science & Research 3, 145-155.

Bonawitz, N.D., Chatenay-Lapointe, M., Pan, Y., and Shadel, G.S. (2007). Reduced TOR signaling extends chronological life span via increased respiration and upregulation of mitochondrial gene expression. Cell metabolism 5, 265-277.

Brenner, S. (1974). The genetics of Caenorhabditis elegans. Genetics 77, 71-94.

Burkewitz, K., Morantte, I., Weir, H.J., Yeo, R., Zhang, Y., Huynh, F.K., Ilkayeva, O.R., Hirschey, M.D., Grant, A.R., and Mair, W.B. (2015). Neuronal CRTC-1 governs systemic mitochondrial metabolism and lifespan via a catecholamine signal. Cell 160, 842-855.

Conte, M., Martucci, M., Sandri, M., Franceschi, C., and Salvioli, S. (2019a). The Dual Role of the Pervasive "Fattish" Tissue Remodeling With Age. Frontiers in endocrinology 10, 114.

Conte, M., Ostan, R., Fabbri, C., Santoro, A., Guidarelli, G., Vitale, G., Mari, D., Sevini, F., Capri, M., Sandri, M., et al. (2019b). Human Aging and Longevity Are Characterized by High Levels of Mitokines. The journals of gerontology Series A, Biological sciences and medical sciences 74, 600-607.

Cunningham, K.A., Hua, Z., Srinivasan, S., Liu, J., Lee, B.H., Edwards, R.H., and Ashrafi, K. (2012). AMP-activated kinase links serotonergic signaling to glutamate release for regulation of feeding behavior in C. elegans. Cell metabolism 16, 113-121.

Dillin, A., Hsu, A.L., Arantes-Oliveira, N., Lehrer-Graiwer, J., Hsin, H., Fraser, A.G., Kamath, R.S., Ahringer, J., and Kenyon, C. (2002). Rates of behavior and aging specified by mitochondrial function during development. Science (New York, NY) 298, 2398-2401.
Doncic, A., and Skotheim, J.M. (2013). Feedforward regulation ensures stability and rapid reversibility of a cellular state. Molecular cell 50, 856-868.

Durieux, J., Wolff, S., and Dillin, A. (2011). The cell-non-autonomous nature of electron transport chain-mediated longevity. Cell 144, 79-91.

Fontaine, K.R., Redden, D.T., Wang, C., Westfall, A.O., and Allison, D.B. (2003). Years of life lost due to obesity. Jama 289, 187-193.

Fuxman Bass, J.I., Pons, C., Kozlowski, L., Reece-Hoyes, J.S., Shrestha, S., Holdorf, A.D., Mori, A., Myers, C.L., and Walhout, A.J. (2016). A gene-centered C. elegans protein-DNA interaction network provides a framework for functional predictions. Molecular systems biology 12, 884.

Goldstein, D.S., and Kopin, I.J. (2017). Homeostatic systems, biocybernetics, and autonomic neuroscience. Autonomic neuroscience : basic & clinical 208, 15-28.

Greer, E.R., Perez, C.L., Van Gilst, M.R., Lee, B.H., and Ashrafi, K. (2008). Neural and molecular dissection of a C. elegans sensory circuit that regulates fat and feeding. Cell metabolism 8, 118-131.

Hornung, G., and Barkai, N. (2008). Noise propagation and signaling sensitivity in biological networks: a role for positive feedback. PLoS computational biology 4, e8.

Horvitz, H.R., Chalfie, M., Trent, C., Sulston, J.E., and Evans, P.D. (1982). Serotonin and octopamine in the nematode Caenorhabditis elegans. Science (New York, NY) 216, 1012-1014.

Hussey, R., Littlejohn, N.K., Witham, E., Vanstrum, E., Mesgarzadeh, J., Ratanpal, H., and Srinivasan, S. (2018). Oxygen-sensing neurons reciprocally regulate peripheral lipid metabolism via neuropeptide signaling in Caenorhabditis elegans. PLoS genetics 14, e1007305.
Hussey, R., Stieglitz, J., Mesgarzadeh, J., Locke, T.T., Zhang, Y.K., Schroeder, F.C., and Srinivasan, S. (2017). Pheromone-sensing neurons regulate peripheral lipid metabolism in *Caenorhabditis elegans*. PLoS genetics 13, e1006806.

Jesty, J., and Beltrami, E. (2005). Positive feedbacks of coagulation: their role in threshold regulation. Arteriosclerosis, thrombosis, and vascular biology 25, 2463-2469.

Jung, P., and Hermeking, H. (2009). The c-MYC-AP4-p21 cascade. Cell cycle (Georgetown, Tex) 8, 982-989.

Kamath, R.S., and Ahringer, J. (2003). Genome-wide RNAi screening in *Caenorhabditis elegans*. Methods (San Diego, Calif) 30, 313-321.

Keith, S.A., Amrit, F.R., Ratnappan, R., and Ghazi, A. (2014). The *C. elegans* healthspan and stress-resistance assay toolkit. Methods (San Diego, Calif) 68, 476-486.

Kimura, K.D., Tissenbaum, H.A., Liu, Y., and Ruvkun, G. (1997). daf-2, an insulin receptor-like gene that regulates longevity and diapause in *Caenorhabditis elegans*. Science (New York, NY) 277, 942-946.

Kuk, J.L., and Ardern, C.I. (2009). Influence of age on the association between various measures of obesity and all-cause mortality. Journal of the American Geriatrics Society 57, 2077-2084.

Lakowski, B., and Hekimi, S. (1998). The genetics of caloric restriction in *Caenorhabditis elegans*. Proceedings of the National Academy of Sciences of the United States of America 95, 13091-13096.
Lee, S.S., Lee, R.Y., Fraser, A.G., Kamath, R.S., Ahringer, J., and Ruvkun, G. (2003). A systematic RNAi screen identifies a critical role for mitochondria in C. elegans longevity. Nature genetics 33, 40-48.

Lee, S.U., Song, H.O., Lee, W., Singaravelu, G., Yu, J.R., and Park, W.Y. (2009). Identification and characterization of a putative basic helix-loop-helix (bHLH) transcription factor interacting with calcineurin in C. elegans. Molecules and cells 28, 455-461.

Lin, Y.F., and Haynes, C.M. (2016). Metabolism and the UPR(mt). Molecular cell 61, 677-682.

Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods (San Diego, Calif) 25, 402-408.

Loer, C.M., and Kenyon, C.J. (1993). Serotonin-deficient mutants and male mating behavior in the nematode Caenorhabditis elegans. Journal of Neuroscience 13, 5407-5417.

Mattison, J.A., Colman, R.J., Beasley, T.M., Allison, D.B., Kemnitz, J.W., Roth, G.S., Ingram, D.K., Weindruch, R., de Cabo, R., and Anderson, R.M. (2017). Caloric restriction improves health and survival of rhesus monkeys. Nature communications 8, 14063.

McCay, C.M., Crowell, M.F., and Maynard, L.A. (1935). The effect of retarded growth upon the length of the life span and upon the ultimate body size. Nutrition (Burbank, Los Angeles County, Calif), 63–79.

Mitrophanov, A.Y., and Groisman, E.A. (2008). Positive feedback in cellular control systems. BioEssays : news and reviews in molecular, cellular and developmental biology 30, 542-555.

Mukhopadhyay, S., Lu, Y., Shaham, S., and Sengupta, P. (2008). Sensory signaling-dependent remodeling of olfactory cilia architecture in C. elegans. In Developmental Cell, pp. 762-774.
Nargund, A.M., Fiorese, C.J., Pellegrino, M.W., Deng, P., and Haynes, C.M. (2015). Mitochondrial and nuclear accumulation of the transcription factor ATFS-1 promotes OXPHOS recovery during the UPR(mt). Molecular cell 58, 123-133.

Nargund, A.M., Pellegrino, M.W., Fiorese, C.J., Baker, B.M., and Haynes, C.M. (2012). Mitochondrial import efficiency of ATFS-1 regulates mitochondrial UPR activation. Science (New York, NY) 337, 587-590.

Noble, T., Stieglitz, J., and Srinivasan, S. (2013). An integrated serotonin and octopamine neuronal circuit directs the release of an endocrine signal to control C. elegans body fat. Cell metabolism 18, 672-684.

Obrowsky, S., Chandak, P.G., Patankar, J.V., Povoden, S., Schlager, S., Kershaw, E.E., Bogner-Strauss, J.G., Hoefler, G., Levak-Frank, S., and Kratky, D. (2013). Adipose triglyceride lipase is a TG hydrolase of the small intestine and regulates intestinal PPARalpha signaling. The Journal of Lipid Research 54, 425-435.

Ogg, S., Paradis, S., Gottlieb, S., Patterson, G.I., Lee, L., Tissenbaum, H.A., and Ruvkun, G. (1997). The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in C. elegans. Nature 389, 994-999.

Palamiuc, L., Noble, T., Witham, E., Ratanpal, H., Vaughan, M., and Srinivasan, S. (2017). A tachykinin-like neuroendocrine signalling axis couples central serotonin action and nutrient sensing with peripheral lipid metabolism. Nature communications 8, 14237.

Redman, L.M., and Ravussin, E. (2011). Caloric restriction in humans: impact on physiological, psychological, and behavioral outcomes. Antioxidants & redox signaling 14, 275-287.
Reeves, G.T. (2019). The engineering principles of combining a transcriptional incoherent feedforward loop with negative feedback. Journal of biological engineering 13, 62.

Reynolds, S.L., Saito, Y., and Crimmins, E.M. (2005). The impact of obesity on active life expectancy in older American men and women. The Gerontologist 45, 438-444.

Salway, J.G. (1999). Metabolism at a glance, 2nd edition edn (Blackwell Science).

Schmeisser, S., Li, S., Bouchard, B., Ruiz, M., Des Rosiers, C., and Roy, R. (2019). Muscle-Specific Lipid Hydrolysis Prolongs Lifespan through Global Lipidomic Remodeling. Cell reports 29, 4540-4552.e4548.

Shulman, G.I. (2014). Ectopic fat in insulin resistance, dyslipidemia, and cardiometabolic disease. The New England journal of medicine 371, 1131-1141.

Song, B.M., and Avery, L. (2012). Serotonin activates overall feeding by activating two separate neural pathways in Caenorhabditis elegans. The Journal of neuroscience : the official journal of the Society for Neuroscience 32, 1920-1931.

Speakman, J.R., Talbot, D.A., Selman, C., Snart, S., McLaren, J.S., Redman, P., Krol, E., Jackson, D.M., Johnson, M.S., and Brand, M.D. (2004). Uncoupled and surviving: individual mice with high metabolism have greater mitochondrial uncoupling and live longer. Aging cell 3, 87-95.

Srinivasan, S. (2015). Neuroendocrine Control of Body Fat in Caenorhabditis elegans. Annual Reviews in Physiology 77.

Srinivasan, S., Sadegh, L., Elle, I.C., Christensen, A.G., Faergeman, N.J., and Ashrafi, K. (2008). Serotonin regulates C. elegans fat and feeding through independent molecular mechanisms. Cell metabolism 7, 533-544.
Stevens, J., Cai, J., Pamuk, E.R., Williamson, D.F., Thun, M.J., and Wood, J.L. (1998). The effect of age on the association between body-mass index and mortality. The New England journal of medicine 338, 1-7.

Sze, J.Y., Victor, M., Loer, C., Shi, Y., and Ruvkun, G. (2000). Food and metabolic signalling defects in a Caenorhabditis elegans serotonin-synthesis mutant. Nature 403, 560-564.

Tanis, J.E., Moresco, J.J., Lindquist, R.A., and Koelle, M.R. (2008). Regulation of serotonin biosynthesis by the G proteins Galphao and Galphaq controls serotonin signaling in Caenorhabditis elegans. Genetics 178, 157-169.

Tian, Y., Garcia, G., Bian, Q., Steffen, K.K., Joe, L., Wolff, S., Meyer, B.J., and Dillin, A. (2016). Mitochondrial Stress Induces Chromatin Reorganization to Promote Longevity and UPR(mt). Cell 165, 1197-1208.

Vatner, D.E., Zhang, J., Oydanich, M., Guers, J., Katsyuba, E., Yan, L., Sinclair, D., Auwerx, J., and Vatner, S.F. (2018). Enhanced longevity and metabolism by brown adipose tissue with disruption of the regulator of G protein signaling 14. Aging cell 17, e12751.

Waggoner, L.E., Zhou, G.T., Schafer, R.W., and Schafer, W.R. (1998). Control of alternative behavioral states by serotonin in Caenorhabditis elegans. Neuron 21, 203-214.

Witham, E., Comunian, C., Ratanpal, H., Skora, S., Zimmer, M., and Srinivasan, S. (2016). C. elegans Body Cavity Neurons Are Homeostatic Sensors that Integrate Fluctuations in Oxygen Availability and Internal Nutrient Reserves. Cell reports 14, 1641-1654.

Zhang, Y., Lu, H., and Bargmann, C.I. (2005). Pathogenic bacteria induce aversive olfactory learning in Caenorhabditis elegans. Nature 438, 179-184.
Zheng, H., and Dirlam, J. (2016). The Body Mass Index-Mortality Link across the Life Course: Two Selection Biases and Their Effects. PLoS ONE 11, e0148178.
FIGURE LEGENDS

Figure 1. Neuronal FLP-7/tachykinin secretion as an experimental tool to model sustained fat oxidation in vivo. A. Model depicting the C. elegans tachykinin neuroendocrine axis. Neuronal FLP-7/tachykinin is secreted from the ASI neurons in response to 5-HT signaling. Secreted FLP-7 acts on its cognate receptor NPR-22 (ortholog of the mammalian neurokinin 2 receptor NK2R) in the intestine. FLP-7/NPR-22 signaling transcriptionally activates the conserved lipase ATGL-1, which is the rate-limiting enzyme that converts stored triglycerides to free fatty acids destined for beta-oxidation. B. An assay to detect and quantify secreted FLP-7mCherry that was previously validated (Palamiuc et al., 2017). Representative images of vehicle or 5-HT treated wild-type animals bearing a FLP-7mCherry fusion protein (red) expressed from ASI neurons and coelomocytes countermarked with GFP (CLM::GFP). FLP-7mCherry is secreted and non-specifically endocytosed by the coelomocytes. Left panels, GFP expression in coelomocytes; center panels, secreted FLP-7mCherry in coelomocytes; Right panels, merge. C. The intensity of FLP-7mCherry within the coelomocytes was quantified and normalized to the area of CLM::GFP. Data are presented as percent of vehicle ± sem (n=21). *, p<0.05 by Student’s t-test. D. Representative images of wild-type and flp-7 animals on vector or atgl-1 RNAi fixed and stained with Oil Red O. E. Quantification of lipid droplets in the depicted conditions, presented as a percentage of wild-type controls (n=16-20). ns, not significant. *, p<0.05 by two-way ANOVA. F. Representative images of wild-type animals on vector or atgl-1 RNAi, treated with vehicle or 5-HT, fixed and stained with Oil Red O to measure fat content. G. Quantification of lipid droplets in the depicted conditions, presented as percent of wild-types treated with vehicle ± sem. (n=18-20). ns, not significant. *, p<0.05 by two-way ANOVA. H, I. Oxygen consumption rate (OCR) of wild-type or flp-7 animals fed vector or atgl-1 RNAi. Basal OCR (H) was quantified prior to addition of FCCP (50 µM) and maximal OCR (I) was determined following FCCP stimulation. Data are presented as pmol/min/worm ± sem. (n=10-20
wells, each containing approximately 10 worms) ns, not significant. *, p<0.05 by two-way ANOVA.

**Figure 2. Sustained fat oxidation in the intestine stimulates a mitochondrial stress response.**

**A.** Lifespans of wild-type (n=101) and *flp-7*\(^{tg}\) (n=101) were assessed by counting the number of alive and dead animals every other day until all animals had died. Data are plotted as the percentage of animals that survived on any given day, relative to the number of animals alive on Day 1 of adulthood. p-value = 0.08 by the Log-Rank Test. **B.** Percent deaths of wild-type and *flp-7*\(^{tg}\) animals over the span of days 14-20 (green), days 21-27 (blue), and days 28-31 (black). Data are presented as percent death for each range of days. **C.** Representative images of wild-type and *flp-7*\(^{tg}\) bearing an integrated *Phsp-60::GFP* transgene. Scale bar, 100 µm. **D.** GFP intensity was quantified and normalized to the area of each animal, fed vector or atgl-1 RNAi, expressed relative to wild-type vector ± sem. (n=30). ns, not significant. *, p<0.05 by two-way ANOVA. **E.** *hsp-60* mRNA was measured via qPCR in the groups indicated. *act-1* mRNA was used as a control. Data are presented as fold change relative to wild-type vector ± sem. (n=4-6). ns, not significant. *, p<0.05 by two-way ANOVA. **F.** The fluorescence intensity of *hsp-60* expression was quantified in the conditions indicated in the figure panel. Data are presented as a percent of wild-type vector ± sem. (n=30). ns, not significant. *, p<0.05 by two-way ANOVA. **G.** qPCR of *hsp-60* mRNA. Data are presented as fold change relative to wild-type vector ± sem. (n=4-6). ns, not significant. *, p<0.05 by two-way ANOVA. See also Figure S1.

**Figure 3. The ATFS-1 mediated mitochondrial stress response is required to sustain fat oxidation.**

**A.** Representative images of wild-type and indicated genotypes on vector or atgl-1 RNAi fixed and stained with Oil Red O. **B.** Quantification of lipid droplets in the depicted conditions, presented as a percentage of wild-type vector ± sem. (n=20). ns, not significant. *, p<0.05 by two-way ANOVA. **C.** Representative images of wild-type and *flp-7*\(^{tg}\) bearing an
integrated Patgl-1::GFP transgene. Scale bar, 100 µm. D. The fluorescence intensity of atgl-1 expression is quantified and presented as percent of wild-type vector ± sem. (n=29-30). ns, not significant. *, p<0.05 by two-way ANOVA. E. qPCR of atgl-1 mRNA. act-1 mRNA was used as a control. Data are presented for the indicated genotypes as fold change relative to wild-type ± sem. (n=4). ns, not significant. *, p<0.05 by one-way ANOVA. F. As indicated by the data, model depicting reciprocal regulatory relationship between the fat-burning enzyme ATGL-1 that triggers the hsp-60 mitochondrial stress response (red arrow), and the stress sensor ATFS-1 that is required for fat oxidation (green arrow). See also Figure S2.

Figure 4. A genetic screen identifies the conserved transcription factor HLH-11 as a negative regulator of ATGL-1. A. Representative images of wild-type, flp-7⁰, and flp-7⁰;hlh-11 animals bearing an integrated Patgl-1::GFP transgene. Scale bar, 100 µm. B. The fluorescence intensity of atgl-1 expression was quantified and normalized to the area of each animal for each indicated genotype. Data are presented as percent of wild-type vector ± sem. (n=26-30). ns, not significant. *, p<0.05 by one-way ANOVA. C. qPCR of atgl-1 mRNA in the indicated genotypes. act-1 mRNA was used as a control. Data are presented as fold change relative to wild-type ± sem. (n=4). ns, not significant. *, p<0.05 by one-way ANOVA. D. Representative images of animals of the indicated genotypes on vector or atgl-1 RNAi, fixed and stained with Oil Red O to measure fat content. E. Quantification of lipid droplets in the depicted conditions, presented as percent of wild-types treated with vehicle ± sem. (n=18-20). ns, not significant. * p<0.05 vs wild-type and $ p<0.05 vs vector by two-way ANOVA. F, G. OCR of wild-type or hlh-11 animals on vector or atgl-1 RNAi were measured over time. Basal OCR (F) was quantified prior to addition of FCCP (50 µM) and maximal OCR (G) was determined following FCCP stimulation. Data are presented as pmol/min/worm ± sem. (n=15 wells, each well containing approximately 10 worms). ns, not significant. *, p<0.05 by two-way ANOVA.
Figure 5. HLH-11 is a direct repressor of atgl-1 transcription. A. Representative image of a transgenic animal expressing the HLH-11GFP fusion protein. GFP was clearly visible in the nucleus of intestinal cells (arrowheads). Scale bar, 100 µm. B. Representative images of animals of the indicated genotypes, fixed and stained with Oil Red O to measure fat content. C. Quantification of lipid droplets in the depicted conditions, presented as percent of wild-types ± sem. (n=18-20). ns, not significant. D. Maximal OCR of wild-type, HLH-11\textsuperscript{ox}, and flip-7\textsuperscript{do};HLH-11\textsuperscript{ox} animals on vector or atgl-1 RNAi. Data are presented as pmol/min/worm ± sem. (n=15 wells each containing approximately 10 worms). ns, not significant. *, p<0.05 vs wild-type and $, p<0.05 vs vector RNAi by one-way ANOVA. E. qPCR of atgl-1 mRNA in wild-type, HLH-11\textsuperscript{ox}, and flip-7\textsuperscript{do};HLH-11\textsuperscript{ox}. act-1 mRNA was used as a control. Data presented as fold change relative to wild-type ± sem. (n=4-6). ns, not significant. *, p<0.05 by one-way ANOVA. F. Schematic of the promoter region of atgl-1. There are two hlh-11 cis-sites (grey): one is 389 bp upstream of the atgl-1 transcription start site and the second binding site is another 752 bp upstream. For ChIP-qPCR, 3 primer sets were designed. Set #1 (light grey) flank the distal HLH-11 binding site, set #2 (dark grey) targets the region between the binding sites, and set #3 (black) flanks the proximal binding site relative to the transcriptional start site. G. Wild-type animals and animals bearing the Phlh-11::hlh-11GFP transgene were subjected to ChIP-qPCR. act-1 mRNA was used as a control. Data are presented as fold change relative to wild-type. ChIP-qPCR was performed using 3 technical replicates, and the experiment was repeated three times. nd, not detected. *, p<0.05 by Student’s t-test. H. Representative images of transgenic animals bearing the Patgl-1::GFP (left panel) and those bearing the Patgl-1\textsuperscript{Δcishlh-11}::GFP transgene (right panel), which lacks both HLH-11 binding domains. Scale bar, 100 µm. I. The fluorescence intensity of atgl-1 expression was quantified, normalized to number of worms, and presented as percent of wild-type ± sem. (n=23-26). *, p<0.05 by Student’s t-test.
Figure 6. HLH-11-ATFS-1 interaction integrates HSP-60 mitochondrial stress response and ATGL-1 transcriptional status. A. Representative images of wild-type and flip-7tg animals bearing an integrated Phlh-11::hlh-11GFP transgene. DIC (left) and GFP (right) in the first two pairs of intestinal cells shown in representative animals. Scale bar, 25 µm. B. The fluorescence intensity of hlh-11 expression was quantified and normalized to the area of the first two pairs of intestinal cells. Data are presented as percent of wild-type vector ± sem. (n=26-30). *, p<0.05 by Student’s t-test. C. qPCR of hlh-11 mRNA in wildtype and flip-7tg animals fed vector or atgl-1 RNAi. act-1 mRNA was used as a control. Data are presented as fold change relative to wild-type vector ± sem. (n=4-6). ns, not significant. *, p<0.05 by two-way ANOVA. D. Model for interaction between HLH-11, ATGL-1 and ATFS-1. In wild-type (left panel), HLH-11 is constitutively on, which represses ATGL-1 (red arrow) and keeps fat oxidation low. In this state, mitochondrial stress is not activated, hsp-60 levels are low and ATFS-1 is not induced, keeping HLH-11 levels high. The FLP-7 neuronal signal (right panel) represses HLH-11, which de-represses ATGL-1, triggering fat oxidation. In turn, this generates an ATFS-1-dependent mitochondrial stress response as observed by the hsp-60 induction (blue arrow). ATFS-1 represses HLH-11 (green arrow), providing a feedforward cue to match fat oxidation with mitochondrial capacity. E. qPCR of hsp-60 mRNA in groups indicated in the figure panel. Data are presented as fold change relative to wild-type vector ± sem. (n=3-6). ns, not significant. *, p<0.05 vs wild-type on vector RNAi and $, p<0.05 vs vector RNAi by two-way ANOVA. F. The fluorescence intensity of atgl-1 expression was quantified and presented as percent of wild-type vector ± sem; groups as indicated in the figure panel. (n=26-30). * p<0.05 vs wild-type on vector and $ p<0.05 vs vector by two-way ANOVA. G. qPCR of atgl-1 mRNA in the genotypes indicated on the right. Data presented as fold change relative to wild-type ± sem. (n=3-6). *, p<0.05 vs wild-type by one-way ANOVA.

Figure 7. HLH-11 and ATFS-1 balance the tradeoff between sustained fat oxidation and longevity. A. Representative images of animals of the indicated genotypes on vector or atgl-1
RNAi, fixed and stained with Oil Red O to measure fat content. **B.** Quantification of lipid droplets in the depicted conditions, presented as percent of wild-type treated with vehicle ± sem. (n=18-20). ns, not significant. *, p<0.05 vs wild-type and $, p<0.05 vs vector by two-way ANOVA. **C-E.** Lifespan for each indicated genotype was assessed by counting the number of alive and dead animals every other day until all animals had died. Data are plotted as the percentage of animals that survived on any given day relative to the number of animals alive on Day 1 of adulthood. Number of animals used for each genotype are indicated in the figure panels. **p<0.001 by Log-Rank Test. Median and maximal lifespans are given in Supplemental Table 1. **F.** Table of median and maximum lifespans for each genotype. Data are presented in days. **G.** Model depicting a feedforward loop that coordinates fat oxidation with longevity. 5-HT-mediated FLP-7 secretion from neurons stimulates lipid metabolism in the intestine via repression of the conserved transcription factor HLH-11, which de-represses ATGL-1. Increased ATGL-1-dependent fat oxidation and mitochondrial respiration activates a mitochondrial stress response, as seen by induction of hsp-60. Simultaneously, the mito-nuclear transcription factor, ATFS-1, represses HLH-11 providing a feedforward cue to sustain and augment HLH-11-dependent fat oxidation. On the one hand, overexpression of HLH-11 or absence of ATGL-1 blocks fat oxidation. On the other hand, during high fat oxidation and mitochondrial respiration, loss of the ATFS-1-HLH-11 feedforward signal attenuates lifespan. Thus, the feedforward signaling loop orchestrates the balance between sustained fat oxidation, mitochondrial stress, and lifespan. See also Figure S3.
**Figure 1**

A

5-HT

\[ \text{FLP-7}^{\text{ASI}} \]

Intestine

\[ \text{NPR-22} \]

\[ \text{ATGL-1} \]

B

CLM::GFP

FLP-7mCherry

merge

C

Ratio FLP-7mCherry/CLM::GFP (% of vehicle)

D-E

Fat Content (% of wildtype on vector)

F-G

Fat Content (% of wildtype on vector)

H-I

Basal OCR (pmol/min/worm)

Max OCR (pmol/min/worm)

---

The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under a CC-BY 4.0 International license.
Figure 3

A

wild-type

flp-7tg

atfs-1 (tm4525) (tm4525)

vector RNAi

atgl-1 RNAi

B

Fat Content (% of wild-type vector)

$ p<0.05$ vs vector

ns

C

wild-type

flp-7tg

Patgl-1::GFP

D

Patgl-1::GFP Fluorescence (% of wildtype on vector)

E

qPCR of atgl-1 mRNA

F

5-HT

FLP-7ASI

Intestine

Stored lipids

Fat oxidation

Mito respiration

ATGL-1

ATFS-1

hsp-60

Mito stress response
Figure 4

A

wild-type  
$h lh-11$ (ok2944)  
$flp-7^t g$;  
$h lh-11$ (ok2944)

Patgl-1::GFP

B

Patgl-1::GFP Fluorescence  
(% of wildtype on vector)

\[
\begin{array}{c}
\text{vector RNAi} \\
\text{atgl-1 RNAi}
\end{array}
\]

C

qPCR of atgl-1 mRNA

\[
\begin{array}{c}
\text{vector RNAi} \\
\text{atgl-1 RNAi}
\end{array}
\]

D

wild-type  
$flp-7^t g$ (ok2944)  
$h lh-11$ (ok2944)

Fat Content  
(% of wildtype)

\[
\begin{array}{c}
\text{vector RNAi} \\
\text{atgl-1 RNAi}
\end{array}
\]

E

$\text{\$ p<0.05 vs vector}$

$\text{\$}$

$\text{\$}$

$\text{\$}$

F

Basal OCR  
(pmol/min/worm)

\[
\begin{array}{c}
\text{vector RNAi} \\
\text{atgl-1 RNAi}
\end{array}
\]

G

Maximal OCR  
(pmol/min/worm)

\[
\begin{array}{c}
\text{vector RNAi} \\
\text{atgl-1 RNAi}
\end{array}
\]
Figure 5

A

Phlh-11::hlh-11GFP

B

wild-type

HLH-11ox

wild-type

flp-7
g

C

Fat Content (% of wildtype)

D

Max OCR (pmol/min/worm)

E

qPCR of atgl-1 mRNA

F

TGTAGTC

hlh-11 cis-site

752 bp

R1 R2 R3

389 bp

TGTAGTC

hlh-11 cis-site

F1 F2 F3

G

ChIP-qPCR of patgl-1 (anti-GFP)

* p<0.05 vs wild-type

H

Patgl-1::GFP

Patgl-1^cishlh-11::GFP

I

Patgl-1::GFP Fluorescence (% of wildtype)
### Figure 6

**A**
- Wild-type
- flip-7

**B**
- Phlh-11::HLH-11GFP Fluorescence (% of wild-type)

|          | wild-type | flip-7          |
|----------|-----------|-----------------|
| Vector   | 1.0       | 0.5             |
| flip-7   | 0.5       | 0.25            |

**C**
- qPCR of hlh-11 mRNA

|          | wild-type | flip-7          |
|----------|-----------|-----------------|
| Vector   | 1.0       | 0.5             |
| flip-7   | 0.5       | 0.25            |

**D**
- HLH-11
type
- flip-7

**E**
- qPCR of hsp-60 mRNA

|          | wild-type | flip-7          |
|----------|-----------|-----------------|
| Vector   | 1.0       | 0.6             |
| flip-7   | 0.6       | 0.4             |

**F**
- Palgl-1::GFP Fluorescence (% of wild-type on vector)

|          | vector RNAi | atfs-1 RNAi |
|----------|-------------|-------------|
| wild-type| 100         | 50          |
| flip-7   | 50          | 25          |

**G**
- qPCR of atgl-1 mRNA

|          | vector RNAi | atfs-1 RNAi |
|----------|-------------|-------------|
| wild-type| 1.0         | 0.5         |
| flip-7   | 0.5         | 0.25        |

* p<0.05 vs wild-type on vector
$ p<0.05 vs vector

**Legend**
- ATGL-1
type
- ATFS-1
type

**Note**
- This preprint was not certified by peer review and is made available under a CC-BY 4.0 International license.
Figure 7

A

B

$ p<0.05 \text{ vs vector}$

C

D

E

F

Genotypes

| Genotypes                     | Median Lifespan (days) | Maximum Lifespan (days) |
|--------------------------------|------------------------|-------------------------|
| wild-type                     | 19                     | 27-29                   |
| hhl-11(ok2944)                | 19                     | 29                      |
| hhl-11(ok2944); atfs-1(tm4525)| 19                     | 26                      |
| hhl-11(ok2944); atfs-1(tm4525)| 16                     | 23                      |

G

Intestine

5-HT

FLP-7ASI

HLH-11

ATGL-1

ATFS-1

 Stored lipids

Fat oxidation

Mito respiration

Longevity

hsp-60

Mito stress response
**Figure S2; Related to Figure 3.** 5-HT drives fat metabolism via ATFS-1.  

A. Fat content of wild-type and *atfs-1(tm4525)* animals treated with vehicle or 5-HT. Data are presented as percent of wild-type treated with vehicle ± sem. (n=10-16). *, p<0.05 by two-way ANOVA.

B. qPCR of *atgl-1* mRNA in wild-type or *atfs-1* null animals treated with vehicle or 5-HT. Data are presented as fold change relative to wild-type animals treated with vehicle ± sem. (n=5); ns, not significant. *, p<0.05 as indicated and $, p<0.05$ vs vector RNAi by two-way ANOVA.
Figure S1; Related to Figure 2. Induction of mitochondrial stress by 5-HT. qPCR of hsp-60 mRNA in wild-type worms treated with vehicle of 5-HT and fed vector, atgl-1, or atfs-1 RNAi. Data are presented as fold change relative to vehicle treatment on vector RNAi ± sem. (n=6) ns, not significant. *, p<0.05 vs vehicle and $, p<0.05 vs vector RNAi by two-way ANOVA.
Figure S3; Related to Figure 7. Reduced fat content with loss of both hlh-11 and atfs-1.
A. Fat content was measure in wild-type, hlh-11, atfs-1, and hlh-11;atfs-1 null animals fed vector or atgl-1 RNAi. Representative images are shown. B. Fat was quantified and data are presented as percent of wild-type animals fed vector RNAi ± sem. (n=6-12); ns, not significant. *, p<0.05 as indicated and $, p<0.05 versus vector RNAi by two-way ANOVA.
Supplemental Table 1. List of *C. elegans* strains used in this study.

| Strain    | Genotype                                      | Backcrossed |
|-----------|-----------------------------------------------|-------------|
| SSR1164   | N2;Pdaf-7::flp-7mCherry;Punc-122::GFP            | 3X          |
|           | N2;Phsp-60::GFP                                |             |
| SSR1488   | N2;Pdaf-7::flp-7mCherry;Punc-122::GFP;Phsp-60::GFP |             |
|           | atfs-1(tm4525)V                                 |             |
| SSR1481   | atfs-1(tm4525);Pdaf-7::flp-7mCherry             | 1X          |
|           | atfs-1(tm4525);Phsp-60::GFP                     |             |
| SSR1571   | atfs-1(tm4525);Pdaf-7::flp-7mCherry;Punc-122::GFP;Phsp-60::GFP | 1X          |
| SSR1564   | N2;Patgl-1::GFP                                | 7X          |
| SSR1503   | N2;Pdaf-7::flp-7mCherry;Punc-122::GFP;Patgl-1::GFP | 7X          |
| SSR726    | hlh-11(ok2944)III                              | 7X          |
| SSR1560   | hlh-11(ok2944);Pdaf-7::flp-7mCherry;Punc-122::GFP |             |
| SSR1563   | hlh-11(ok2944);Patgl-1::GFP                    | 7X          |
| SSR1536   | hlh-11(ok2944);Pdaf-7::flp-7mCherry;Punc-122::GFP;Patgl-1::GFP | 7X          |
| SSR1530   | N2;Phlh-11::hlh-11GFP                           | 4X          |
| SSR1550   | N2;Pdaf-7::flp-7mCherry;Punc-122::GFP;Phlh-11::hlh-11GFP | 5X          |
| SSR1567   | N2;Patgl-1Δcishlh-11::GFP                       |             |
| SSR1399   | hlh-11(ok2944);atfs-1(tm4525)                   |             |
| SSR1538   | hlh-11(ok2944);atfs-1(tm4525);Pdaf-7::flp-7mCherry;Punc-122::GFP |             |
| Primer | Forward | Reverse |
|--------|---------|---------|
| Primers for cloning the promoter of *hlh-11* | ggggacaactttgtatagaaaaggcttggtgtggttcgattgcag | ggggacgctttttgtacaaactgtcttcagtttctactatgatctacgcttg |
| Primers for cloning the *hlh-11* gene | ggggacaagtttgtacaaaaaaggcttggttcgattgcag | ggggacgctttttgtacaaactgtcttcagtttctactatgatctacgcttg |
| Primers to remove the upstream *hlh-11 cis-site* in the *Patgl-1::GFP* plasmid | gcactaactatatatatgttcgttcttcag | cctctgtctcgaacgc |
| Primers to remove the downstream *hlh-11 cis-site* in the *Patgl-1::GFP* plasmid | aatgattacataaagtcag | atcttgctatgaatgtacc |
| qPCR primers for *act-1* | gtatggagtccgccgga | ctctcatggtgttgaggggaaa |
| qPCR primers for *atgl-1* | ctacactgcaatgggaatct | gtgggtcatccatatccaaata |
| qPCR primers for *hlh-11* | gccgagaagaagataaatcgcag | gttgacatttcgatttcgatttc |
| qPCR primers for *hsp-60* | cgacgctgtacgaggaatgaa | gcctttcgtacccagacttagag |
| ChIP-qPCR primers set 1 for *atgl-1* | cggtggtacacagtacttc | tggctagctgttagtgacag |
| ChIP-qPCR primers set 2 for *atgl-1* | cgccctacacagtccagc | ccgcaggtgcgtgatgaat |
| ChIP-qPCR primers set 3 for *atgl-1* | gtatgggcctccgagacag | ggtaccatactgtcaggaacgc |
Supplemental Table 3. List of all p-values and 95% Confidence Interval (CI) of the difference between means for all Figures.

| Figure | Group Comparison | p-value   | 95% CI of difference between means |
|--------|------------------|-----------|------------------------------------|
| 1C     | vehicle vs 5-HT  | 0.013     | 11.4 to 91.5                       |
| 1E     | wild-type vector RNAi vs flp-7g vector RNAi | p<0.001 | -80.1 to -60.0                     |
| 1E     | wild-type atgl-1 RNAi vs flp-7g atgl-1 RNAi | 0.0675   | -41.5 to 1.0                       |
| 1G     | vehicle vector RNAi vs 5-HT vector RNAi | p<0.001 | -75.5 to -55.2                     |
| 1G     | vehicle atgl-1 RNAi vs 5-HT atgl-1 RNAi | 0.9322   | -7.9 to 12.5                       |
| 1H     | wild-type vector RNAi vs flp-7g vector RNAi | 0.002    | 1.9 to 6.2                         |
| 1H     | wild-type atgl-1 RNAi vs flp-7g atgl-1 RNAi | 0.504    | -5.7 to 2.9                        |
| 1I     | wild-type vector RNAi vs flp-7g vector RNAi | 0.009    | 1.92 to 11.8                       |
| 1I     | wild-type atgl-1 RNAi vs flp-7g atgl-1 RNAi | 0.302    | -5.8 to 1.9                        |
| 1I     | flp-7g vector RNAi vs flp-7g atgl-1 RNAi | p<0.001 | 4.4 to 13.2                        |
| 2A     | wild-type vs flp-7g | 0.0825   | 0.4 to 1.8*                       |
| 2D     | wild-type vector RNAi vs flp-7g vector RNAi | p<0.001 | 86.0 to 155.7                     |
| 2D     | wild-type atgl-1 RNAi vs flp-7g atgl-1 RNAi | 0.0044   | 11.2 to 80.9                       |
| 2D     | flp-7g vector RNAi vs flp-7g atgl-1 RNAi | p<0.001 | -117.1 to -47.5                    |
| 2E     | wild-type vector RNAi vs flp-7g vector RNAi | 0.0258   | 1.0 to 1.5                         |
| 2E     | wild-type atgl-1 RNAi vs flp-7g atgl-1 RNAi | 0.6506   | -0.4 to 0.6                       |
| 2E     | flp-7g vector RNAi vs flp-7g atgl-1 RNAi | 0.0029   | 0.5 to 1.5                         |
| 2F     | wild-type vector RNAi vs wild-type atgl-1 RNAi | 0.0491   | -0.8 to -0.01                     |
| 2F     | wild-type vector RNAi vs flp-7g vector RNAi | p<0.001 | 96.2 to 145.6                     |
| 2F     | flp-7g vector RNAi vs flp-7g atfs-1 RNAi | p<0.001 | -164.1 to -114.7                   |
| 2F     | flp-7g vector RNAi vs flp-7g;atfs-1 atgl-1 RNAi | p<0.001 | -175.8 to -126.5                   |
| 2F     | wild-type atfs-1 RNAi vs flp-7g;atfs-1 atgl-1 RNAi | 1.0000   | -25.6 to 23.8                     |
| 2F     | atfs-1 atgl-1 RNAi vs flp-7g;atfs-1 atgl-1 RNAi | 0.9507   | -32.2 to 17.1                     |
| 2G     | wild-type vector RNAi vs flp-7g;atfs-1 RNAi | 0.0258   | 0.1 to 1.5                         |
| 2G     | flp-7g vector RNAi vs flp-7g;atfs-1 RNAi | 0.0030   | -1.6 to -0.4                       |
| 2G     | flp-7g vector RNAi vs flp-7g;atfs-1 atgl-1 RNAi | 0.0014   | -1.7 to -0.5                       |
| 2G     | wild-type atfs-1 RNAi vs flp-7g;atfs-1 atgl-1 RNAi | 0.9889   | -1.0 to 0.8                       |
| 2G     | atfs-1 atgl-1 RNAi vs flp-7g;atfs-1 atgl-1 RNAi | 0.9753   | -1.0 to 0.8                       |
| S1     | vehicle vector RNAi vs 5-HT vector RNAi | 0.0455   | 0.1 to 0.6                         |
| S1     | vehicle vector RNAi vs vehicle atgl-1 RNAi | 0.0019   | -0.9 to -0.3                       |
| S1     | 5-HT vector RNAi vs 5-HT atgl-1 RNAi | 0.0002   | -1.2 to -0.6                       |
| S1     | vehicle vector RNAi vs vehicle atfs-1 RNAi | 0.0038   | -0.8 to -0.2                       |
| S1     | 5-HT vector RNAi vs 5-HT atfs-1 RNAi | p<0.001 | -1.1 to -0.6                       |
| S1     | vehicle atgl-1 RNAi vs 5-HT atgl-1 RNAi | 0.7123   | -0.4 to 0.3                       |
| S1     | vehicle atfs-1 RNAi vs 5-HT atfs-1 RNAi | 0.3579   | -0.3 to 0.1                       |
| 3B     | wild-type vector RNAi vs flp-7g vector RNAi | p<0.001 | -83.2 to -57.0                     |
| 3B     | atfs-1 vector RNAi vs flp-7g;atfs-1 vector RNAi | 0.9967   | -16.1 to 10.1                     |
| 3B     | wild-type vector RNAi vs wild-type atgl-1 RNAi | p<0.001 | 17.2 to 43.4                     |
| 3B     | flp-7g vector RNAi vs flp-7g atgl-1 RNAi | p<0.001 | 7.0 to 93.2                       |
| 3B     | atfs-1 atgl-1 RNAi vs flp-7g;atfs-1 atgl-1 RNAi | 0.3357   | -7.2 to 45.8                       |
| 3D     | wild-type vector RNAi vs flp-7g vector RNAi | p<0.001 | 19.6 to 56.0                       |
| 3D     | flp-7g vector RNAi vs flp-7g atfs-1 RNAi | p<0.001 | -65.1 to -28.8                     |
| 3D     | wild-type atfs-1 RNAi vs flp-7g atfs-1 RNAi | 0.6733   | -23.2 to 9.2                       |
|   | Comparison                                      | p-value | Effect Size |
|---|------------------------------------------------|---------|-------------|
| 3E | wild-type vs \(flp-7^g\)                      | 0.0002  | 0.6 to 1.8  |
| 3E | \(flp-7^g\) vs \(flp-7^g; atfs-1\)           | p<0.001 | -1.9 to -0.8|
| 3E | \(atfs-1\) vs \(flp-7^g; atfs-1\)           | 0.9489  | -0.7 to 0.5 |
| S2A| wild-type vehicle vs wild-type 5-HT           | p<0.001 | -84.9 to -55.3|
| S2A| wild-type 5-HT vs \(atfs-1\) 5-HT             | p<0.001 | 11.8 to 41.4|
| S2A| \(atfs-1\) vs wild-type 5-HT                 | p<0.001 | -54.3 to -24.7|
| S2B| wild-type vehicle vs wild-type 5-HT           | 0.0341  | 0.1 to 1.3   |
| S2B| wild-type 5-HT vs \(atfs-1\) 5-HT             | 0.0406  | 0.1 to 1.4   |
| S2B| \(atfs-1\) vehicle vs \(atfs-1\) 5-HT        | 0.8302  | -0.5 to 0.6  |
| 4B | wild-type vs \(flp-7^g\)                      | p<0.001 | 20.2 to 55.5 |
| 4B | wild-type vs \(hlh-11\)                      | p<0.001 | 26.3 to 57.9 |
| 4B | \(hlh-11\) vs \(hlh-11; flp-7^g\)           | 0.3587  | -25.8 to 5.8 |
| 4C | wild-type vs \(flp-7^g\)                      | 0.0083  | 0.3 to 2.1   |
| 4C | wild-type vs \(hlh-11\)                      | 0.0077  | 0.3 to 2.1   |
| 4C | \(hlh-11\) vs \(hlh-11; flp-7^g\)           | 0.9159  | -1.1 to 0.7  |
| 4E | wild-type vector RNAi vs \(flp-7^g\) vector RNAi | p<0.001 | -82.4 to -57.8|
| 4E | wild-type vector RNAi vs \(hlh-11\) vector RNAi | p<0.001 | -80.5 to -55.2|
| 4E | \(hlh-11\) vector RNAi vs \(flp-7^g\) vector RNAi | 0.5673  | -20.4 to 4.9 |
| 4E | wild-type vector RNAi vs wild-type \(atgl-1\) RNAi | p<0.001 | 18.0 to 42.6 |
| 4E | \(flp-7^g\) vector RNAi vs \(flp-7^g\) \(atgl-1\) RNAi | p<0.001 | 67.8 to 92.4 |
| 4E | \(hlh-11\) vector RNAi vs \(hlh-11\) \(atgl-1\) RNAi | p<0.001 | 65.6 to 90.9 |
| 4E | \(flp-7^g; hlh-11\) vector RNAi vs \(flp-7^g; hlh-11\) \(atgl-1\) RNAi | p<0.001 | 75.1 to 99.7 |
| 4E | \(hlh-11\) \(atgl-1\) RNAi vs \(hlh-11\) \(flp-7^g\) \(atgl-1\) RNAi | 1.0000  | -10.9 to 13.7|
| 4F | wild-type vector RNAi vs \(hlh-11\) vector RNAi | 0.037   | 0.2 to 6.4   |
| 4F | wild-type \(atgl-1\) RNAi vs \(hlh-11\) \(atgl-1\) RNAi | 0.509   | -1.4 to 2.6  |
| 4F | \(hlh-11\) vector RNAi vs \(hlh-11\) \(atgl-1\) RNAi | 0.002   | 1.9 to 7.6   |
| 4G | wild-type vector RNAi vs \(hlh-11\) vector RNAi | 0.009   | 1.9 to 11.8  |
| 4G | wild-type \(atgl-1\) RNAi vs \(hlh-11\) \(atgl-1\) RNAi | 0.302   | -5.9 to 1.9  |
| 4G | \(hlh-11\) \(atgl-1\) RNAi vs \(hlh-11\) \(atgl-1\) RNAi | p<0.001 | 4.4 to 13.2  |
| 5C | wild-type vs \(flp-7^g\)                      | p<0.001 | -82.1 to -58.0|
| 5C | wild-type vs \(HLH-11^{ox}\)                | p<0.001 | 37.2 to 61.6 |
| 5C | \(flp-7^g\) vs \(flp-7^g; HLH-11^{ox}\)     | 0.0078  | 95.0 to 119.1|
| 5C | \(HLH-11^{ox}\) vs \(flp-7^g; HLH-11^{ox}\) | 0.1520  | -27.9 to 2.9 |
| 5D | wild-type vector RNAi vs \(HLH-11^{ox}\) vector RNAi | 0.0426 | -8.6 to -0.2 |
| 5D | \(HLH-11^{ox}\) vector RNAi vs \(flp-7^g; HLH-11^{ox}\) vector RNAi | 0.3660 | -2.8 to 7.2 |
| 5D | \(HLH-11^{ox}\) vector RNAi vs \(HLH-11^{ox}\) \(atgl-1\) RNAi | 0.8072 | -4.1 to 5.2 |
| 5E | wild-type vs \(HLH-11^{ox}\)                | 0.0472  | -0.8 to -0.01|
| 5E | wild-type vs \(flp-7^g; HLH-11^{ox}\)      | 0.0379  | -0.8 to -0.03|
| 5G | wild-type vs \(HLH-11^{ox}\) set #1        | 0.0052  | 6.7 to 18.5  |
| 5G | wild-type vs \(HLH-11^{ox}\) set #2        | 0.0165  | 3.2 to 15.3  |
| 5G | wild-type vs \(HLH-11^{ox}\) set #3        | p<0.001 | 13.4 to 27.7 |
| 5L | wild-type vs \(\Delta cishlh-1\)            | p<0.001 | 103.8 to 135.0|
| 6B | wild-type vs \(flp-7^g\)                      | 0.0042  | -96.8 to -19.2|
| 6C | wild-type vector RNAi vs \(flp-7^g\) vector RNAi | 0.0170 | -0.7 to -0.1 |
| 6C | \(flp-7^g\) vector RNAi vs \(flp-7^g; atfs-1\) vector RNAi | 0.0280 | 0.1 and 0.2 |
| 6C | \(atfs-1\) vector RNAi vs \(flp-7^g; atfs-1\) vector RNAi | 0.1532 | 0.1 and 0.3 |
| 6C | \(flp-7^g\) vector RNAi vs \(flp-7^g\) \(atgl-1\) RNAi | 0.0413 | 0.01 to 0.3 |
| 6C | wild-type \(atgl-1\) RNAi vs \(flp-7^g\) \(atgl-1\) RNAi | 0.2278 | -0.5 to 0.2 |
| 6E | wild-type vector RNAi vs \(flp-7^g\) vector RNAi | 0.0258 | 0.1 to 1.5 |
| 6E | wild-type vector RNAi vs \(HLH-11^{ox}\) vector RNAi | 0.0462 | -1.0 to -0.01|
| 6E | wild-type vector RNAi vs \(flp-7^g; HLH-11^{ox}\) vector RNAi | 0.0443 | -1.0 to -0.01|
| Group Description                                                                 | p Value | Median Survival CI |
|----------------------------------------------------------------------------------|---------|--------------------|
| 6E wild-type vector RNAi vs hlh-11 vector RNAi                                    | 0.0007  | 0.5 to 1.7         |
| 6E hlh-11 vector RNAi vs hlh-11;atfs-1 vector RNAi                              | 0.0009  | -2.0 to -0.6       |
| 6E flp-7⁰;hlh-11 vector RNAi vs flp-7⁰;hlh-11;atfs-1 vector RNAi                 | 0.0136  | 0.2 to 2.0         |
| 6E wild-type vector RNAi vs wild-type atgl-1 RNAi                               | 0.0491  | -0.8 to -0.01      |
| 6E flp-7⁰ vector RNAi vs flp-7⁰ atgl-1 RNAi                                     | 0.0029  | 0.5 to 1.5         |
| 6E hlh-11 vector RNAi vs hlh-11 atgl-1 RNAi                                    | 0.0074  | -1.7 to -0.5       |
| 6E flp-7⁰;hlh-11 vector RNAi vs flp-7⁰;hlh-11 atgl-1 RNAi                       | 0.0001  | -1.9 to -0.7       |
| 6F wild-type vector RNAi vs flp-7⁰ vector RNAi                                   | p<0.001 | 22.0 to 53.6       |
| 6F flp-7⁰ vector RNAi vs flp-7⁰ atfs-1 RNAi                                     | p<0.001 | -62.6 to -31.2     |
| 6F wild-type vector RNAi vs hlh-11 vector RNAi                                  | p<0.001 | 20.8 to 49.4       |
| 6F wild-type vector RNAi vs flp-7⁰;hlh-11 vector RNAi                           | p<0.001 | 17.9 to 46.4       |
| 6F flp-7⁰ atfs-1 RNAi vs flp-7⁰;hlh-11 atfs-1 RNAi                             | p<0.001 | 23.9 to 51.9       |
| 6F wild-type vector RNAi vs hlh-11 atfs-1 RNAi                                  | p<0.001 | 9.4 to 44.1        |
| 6F wild-type vector RNAi vs flp-7⁰;hlh-11 atfs-1 RNAi                           | p<0.001 | 14.6 to 42.9       |
| 6F hlh-11 atfs-1 RNAi vs flp-7⁰;hlh-11 atfs-1 RNAi                             | p<0.001 | 13.9 to 64.8       |
| 6G wild-type vs flp-7⁰                                                          | 0.0122  | 0.3 to 2.1         |
| 6G wild-type vs hlh-11                                                           | 0.0077  | 0.3 to 2.1         |
| 6G wild-type vs flp-7⁰;hlh-11                                                    | 0.0397  | 0.03 to 2.0        |
| 6G flp-7⁰ vs flp-7⁰;atfs-1                                                     | 0.0041  | -2.3 to -0.4       |
| 6G flp-7⁰;atfs-1 RNAi vs flp-7⁰;hlh-11;atfs-1                                   | 0.0095  | 0.2 to 2.1         |
| 6G wild-type vs hlh-11;atfs-1                                                   | 0.0431  | 0.02 to 1.4        |
| 6G wild-type vs flp-7⁰;hlh-11;atfs-1                                            | 0.0285  | 0.1 to 2.0         |
| 6G hlh-11;atfs-1 RNAi vs flp-7⁰;hlh-11;atfs-1                                   | 0.5000  | -0.4 to 1.0        |
| 7B flp-7⁰;atfs-1 vector RNAi vs flp-7⁰;hlh-11;atfs-1 vector RNAi                 | p<0.001 | -85.3 to -57.9     |
| 7B flp-7⁰ vector RNAi vs flp-7⁰ atgl-1 RNAi                                     | p<0.001 | 66.4 to 93.9       |
| 7B flp-7⁰;hlh-11 vector RNAi vs flp-7⁰;hlh-11 atgl-1 RNAi                       | p<0.001 | 73.7 to 101.1      |
| 7B flp-7⁰;atfs-1 vector RNAi vs flp-7⁰;atgl-1 RNAi                             | p<0.001 | 29.3 to 56.7       |
| 7B flp-7⁰;hlh-11;atfs-1 vector RNAi vs flp-7⁰;hlh-11;atfs-1 vector RNAi          | p<0.001 | 77.8 to 105.3      |
| 7C wild-type vs flp-7⁰;hlh-11;atfs-1                                            | p<0.001 | 0.5 to 1.9*        |
| 7D wild-type vs hlh-11;atfs-1                                                   | 0.1166  | 0.4 to 1.6*        |
| 7E wild-type vs hlh-11                                                          | 0.4056  | 0.2 to 1.7*        |
| S3B wild-type vector RNAi vs hlh-11 vector RNAi                                 | p<0.001 | -83.4 to -52.3     |
| S3B hlh-11 vector RNAi vs hlh-11;atfs-1 vector RNAi                            | p<0.001 | 26.8 to 57.9       |
| S3B atfs-1 vector RNAi vs hlh-11;atfs-1 vector RNAi                            | 0.0010  | -37.1 to -5.9      |
| S3B wild-type vector RNAi vs wild-type atgl-1 RNAi                             | p<0.001 | 14.7 to 45.8       |
| S3B hlh-11 vector RNAi vs hlh-11 atgl-1 RNAi                                   | p<0.001 | 62.7 to 93.8       |
| S3B atfs-1 vector RNAi vs atfs-1 atgl-1 RNAi                                   | p<0.001 | 3.3 to 34.4        |
| S3B hlh-11;atfs-1 vector RNAi vs hlh-11;atfs-1 atgl-1 RNAi                     | p<0.001 | 19.9 to 51.0       |

* indicates the 95% CI for the ratio of median survival