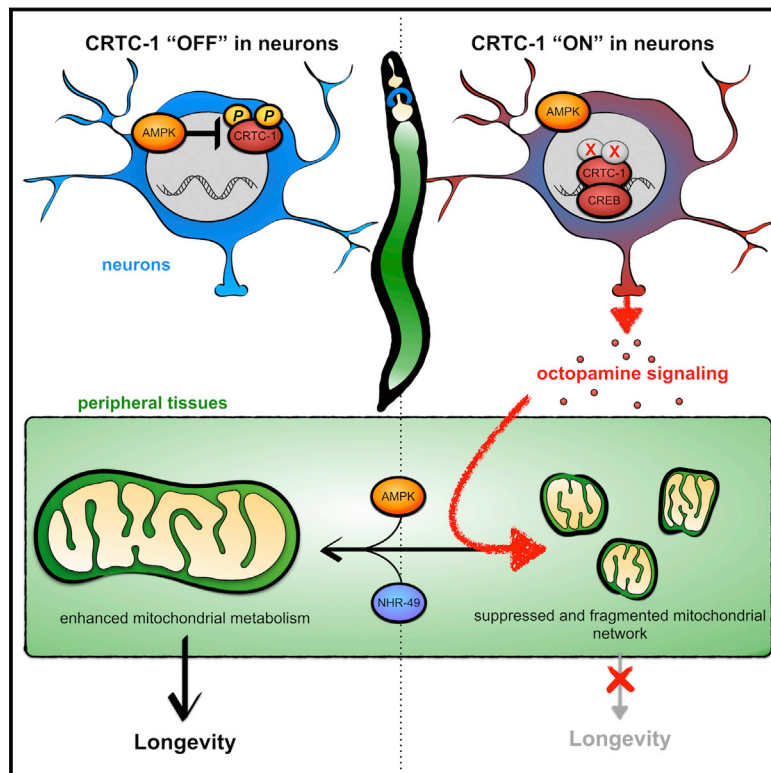


Neuronal CRTC-1 Governs Systemic Mitochondrial Metabolism and Lifespan via a Catecholamine Signal

Graphical Abstract



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In Brief

The energy sensor AMPK acts locally as well as cell nonautonomously to regulate longevity through neuronal CRTC-1-dependent remodeling of systemic mitochondrial metabolism.

Highlights

- CRTC-1 uncouples AMPK-mediated longevity from pleiotropic side effects
- AMPK promotes longevity via CRTC-1-dependent remodeling of mitochondrial metabolism
- Neuronal CRTC-1/CREB and NHR-49 antagonistically regulate longevity and metabolism
- Neuronal CRTC-1 regulates mitochondrial dynamics and lifespan via octopamine

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Neuronal CRTC-1 Governs Systemic Mitochondrial Metabolism and Lifespan via a Catecholamine Signal

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SUMMARY

Low energy states delay aging in multiple species, yet mechanisms coordinating energetics and longevity across tissues remain poorly defined. The conserved energy sensor AMP-activated protein kinase (AMPK) and its corresponding phosphatase calcineurin modulate longevity via the CREB regulated transcriptional coactivator (CRTC)-1 in *C. elegans*. We show that CRTC-1 specifically uncouples AMPK/calcineurin-mediated effects on lifespan from pleiotropic side effects by reprogramming mitochondrial and metabolic function. This pro-longevity metabolic state is regulated cell nonautonomously by CRTC-1 in the nervous system. Neuronal CRTC-1/CREB regulates peripheral metabolism antagonistically with the functional PPAR α ortholog, NHR-49, drives mitochondrial fragmentation in distal tissues, and suppresses the effects of AMPK on systemic mitochondrial metabolism and longevity via a cell-non-autonomous catecholamine signal. These results demonstrate that while both local and distal mechanisms combine to modulate aging, distal regulation overrides local contribution. Targeting central perception of energetic state is therefore a potential strategy to promote healthy aging.

INTRODUCTION

An organism's energy status is tightly coupled to its rate of aging, as low energy conditions increase longevity and disease resistance across the evolutionary spectrum (Burkewitz et al., 2014). Mechanisms that communicate energetic state between tissues to coordinate organismal health and longevity remain poorly understood, however, and must be defined in order to translate these effects to human therapeutics. AMP-activated protein kinase (AMPK) is a conserved energy sensor activated by increases in the AMP/ADP:ATP ratio, which signals low en-

ergy charge (Hardie et al., 2012). AMPK upregulates catabolic processes and shuts down energy-consuming processes to restore cellular energy homeostasis (Hardie et al., 2012). AMPK is also pro-longevity; activating AMPK in *C. elegans* and *Drosophila* increases healthy lifespan and mimics a low energy state in well-fed animals (Apfeld et al., 2004; Stenesen et al., 2013). *C. elegans* lacking AMPK activity fail to respond to low energy conditions, such as dietary restriction, that extend wild-type lifespan (Burkewitz et al., 2014).

Both AMPK and its effects on aging are conserved across eukaryotes (Hardie et al., 2012). Metformin, an indirect AMPK agonist, promotes healthy aging in *C. elegans* (Onken and Driscoll, 2010) and mice (Martin-Montalvo et al., 2013). Deregulation of AMPK results in age-onset human pathologies including cancer and neurodegenerative diseases (Hardie et al., 2012). AMPK signaling therefore plays a critical role linking energetics to pathology, making it an attractive target to treat or prevent multiple age-related diseases.

AMPK has both cell-autonomous effects on energetics through direct phosphorylation of metabolic effectors (Hardie et al., 2012) and cell-nonautonomous effects via integration of hormonal and neuroendocrine signals (Dagon et al., 2012; Lerner et al., 2009; Minokoshi et al., 2004). The extent to which AMPK promotes longevity locally via regulation of metabolism or distally via a secondary signal remains unclear. Additionally, AMPK's pro-longevity effects may not be universal in all tissues, as AMPK activation in certain cell types appears to increase risk for some diseases, and pleiotropic effects of AMPK activation unrelated to aging have detrimental physiological consequences (Burkewitz et al., 2014). Identifying downstream targets and processes regulated by AMPK that specifically mediate its role in longevity would therefore enhance our capacity to harness the link between energetics and aging for treatment of age-related pathologies.

Previously, we identified the cyclic AMP-responsive element binding protein (CREB)-regulated transcriptional coactivator (CRTC)-1 as a critical longevity target of AMPK and calcineurin in *C. elegans* (Mair et al., 2011). AMPK and calcineurin antagonistically regulate CRTC-1 phosphorylation status, thereby modulating its activity and effect on aging. CRTCs are transcriptional coactivators first discovered in mammals for their ability to

bind CREB and regulate its transcriptional activity (Altarejos and Montminy, 2011). Mammals possess 3 CRTC family members that act in distinct tissues, including neurons (CRTC1/2), liver (CRTC2), and adipose tissue (CRTC3), and aberrant regulation of CRTCs is implicated in multiple chronic diseases, including obesity, metabolic disease, and neurodegeneration (Altarejos and Montminy, 2011). *C. elegans* possess a single, highly conserved CRTC family member, CRTC-1. AMPK phosphorylates CRTC-1 directly, promoting 14-3-3 binding, cytosolic sequestration and inactivation. Blocking phosphorylation of CRTC-1 at conserved AMPK target sites, serines 76 and 179, renders it refractory to AMPK regulation and constitutively nuclear. CRTC-1^{S76A,S179A} blocks lifespan extension by AMPK activation or inhibition of the corresponding phosphatase calcineurin (Mair et al., 2011).

In this study we demonstrate that CRTC-1 specifically mediates the longevity output of AMPK. We perform transcriptomic analysis to elucidate genes downstream of AMPK/CRTC-1 signaling, which couple specifically to lifespan regulation. Through this approach we identify coordination of mitochondrial metabolism by CRTC-1 and the nuclear hormone receptor NHR-49, a functional PPAR α ortholog (Van Gilst et al., 2005). Notably, we demonstrate that these opposing transcriptional effectors act in the nervous system to regulate both longevity and systemic changes in metabolic transcription. NHR-49 is required for AMPK/calcineurin-mediated longevity, and limiting NHR-49 function to neurons is sufficient to mediate both longevity and regulation of metabolic genes in peripheral tissues. In addition, we demonstrate that neuronal CRTC-1 modulates AMPK/calcineurin-mediated longevity cell nonautonomously via regulation of the neurotransmitter/hormone octopamine. Neuron-specific activation of CRTC-1, like *nhr-49* loss, suppresses AMPK/calcineurin-mediated longevity and upregulates expression of key enzymes involved in octopamine synthesis. Correspondingly, neuronal CRTC-1^{S76A,S179A} has no effect on longevity in mutants deficient in octopamine synthesis. Together these data challenge the current paradigm that AMPK, CRTC-1 and NHR-49 act cell autonomously to regulate metabolism and longevity, and instead highlight their distinct role in communicating perception of energy status in neurons to systemic regulation of metabolism and lifespan.

RESULTS

CRTC-1 Is Specific to AMPK Longevity

We generated a *C. elegans* transgenic strain expressing a truncated AMPK α 2 catalytic subunit (AAK-2), which results in increased T172 phosphorylation and constitutively active (CA) AMPK (Mair et al., 2011). CA-AAK-2 increases *C. elegans* lifespan (Figure 1A), yet induces detrimental pleiotropic side effects including small body size and reduced reproductive capacity (Figures 1B and 1C). As shown previously, CRTC-1^{S76A,S179A} abolishes lifespan extension from AAK-2 activation (Figure 1D) without altering AMPK activity (Figure S1). In contrast to its role in longevity, CRTC-1^{S76A,S179A} does not suppress CA-AAK-2-mediated effects on growth (Figure 1E) or reproductive period (Figure 1F, Figure S1). The physiological effects of CRTC-1^{S76A,S179A} in CA-AAK-2 animals are therefore

specific to longevity and do not extend to non-aging-related traits.

Increased longevity is often coupled to increased stress resistance. To determine if this was the case for AMPK, we examined the effect of CA-AAK-2 on resistance to heat stress at 33°C. *C. elegans* lacking *aak-2* are sensitive to heat stress compared to wild-type (Apfeld et al., 2004). Conversely, activation of AMPK promotes heat resistance, as *C. elegans* expressing CA-AAK-2 show a 125% increase in median survival at 33°C compared to wild-type animals (Figure 1G; 27 and 12 hr, respectively). However, unlike its effect on AMPK longevity (Figure 1D), CRTC-1^{S76A,S179A} does not suppress heat resistance conferred by CA-AAK-2 (Figure 1H). Mechanisms that protect CA-AAK-2 animals from heat stress are thus separable from those which promote lifespan extension, and CRTC-1 represents a molecular switch that uncouples the longevity effects of AMPK from pleiotropy unrelated to aging (Figure 1I).

AMPK and CRTC-1 Coordinate Mitochondrial Metabolism to Regulate Longevity

We reasoned that CRTC-1 could be leveraged to identify the mechanisms by which AMPK increases lifespan. As CRTC-1^{S76A,S179A} suppresses only the longevity effects of AMPK activation, genes differentially expressed in CA-AAK-2 animals in a CRTC-1-dependent manner would be enriched for functions specific to AMPK-mediated longevity (Figure 1I). We defined CRTC-1-dependent genes as those differentially regulated in CA-AAK-2 (Figure 2A, yellow), or in CRTC-1^{S76A,S179A}; CA-AAK-2 double-transgenics (Figure 2A, red), but not in both (Figure 2A, orange).

We performed RNA-Seq analyses and identified 1,680 genes differentially expressed in worms with activated AAK-2, activated CRTC-1^{S76A,S179A}, or double-transgenics, relative to wild-type animals (Figures 2B, S2A, Table S2). AMPK induces small body size, reduced reproduction, and stress resistance independent of CRTC-1 (Figure 1), thus we predicted that AMPK-dependent/CRTC-1-independent genes (Figure 2B, orange region) would be associated with these phenotypes. Supporting this hypothesis, the gene ontology (GO) terms most enriched among those genes include processes involving germline differentiation, growth/development, and reproduction (Figure 2C, Table S3). Importantly, this tight association between phenotypes and functional enrichments within the transcriptomic changes validated our hypothesis that CRTC-1 could be used to filter out pleiotropic effects of AMPK activation unrelated to aging.

To identify processes specifically coupled to longevity, we focused on transcriptional changes induced by AMPK that are dependent on CRTC-1 activation status (Figure 2A). Of the 869 genes differentially expressed by AAK-2 activation in a CRTC-1-dependent manner, over 75% are differentially expressed when both AMPK and CRTC-1 are active (Figure 2B, red region). These genes are highly enriched for processes associated with metabolism, and more specifically, processes localized to mitochondria (Figure 2D, Table S3). We examined the directionality of CRTC-1-dependent gene expression changes, and found that suppression of AMPK longevity is associated with a broad down-regulation of mitochondrial metabolic processes (Figure 2D).

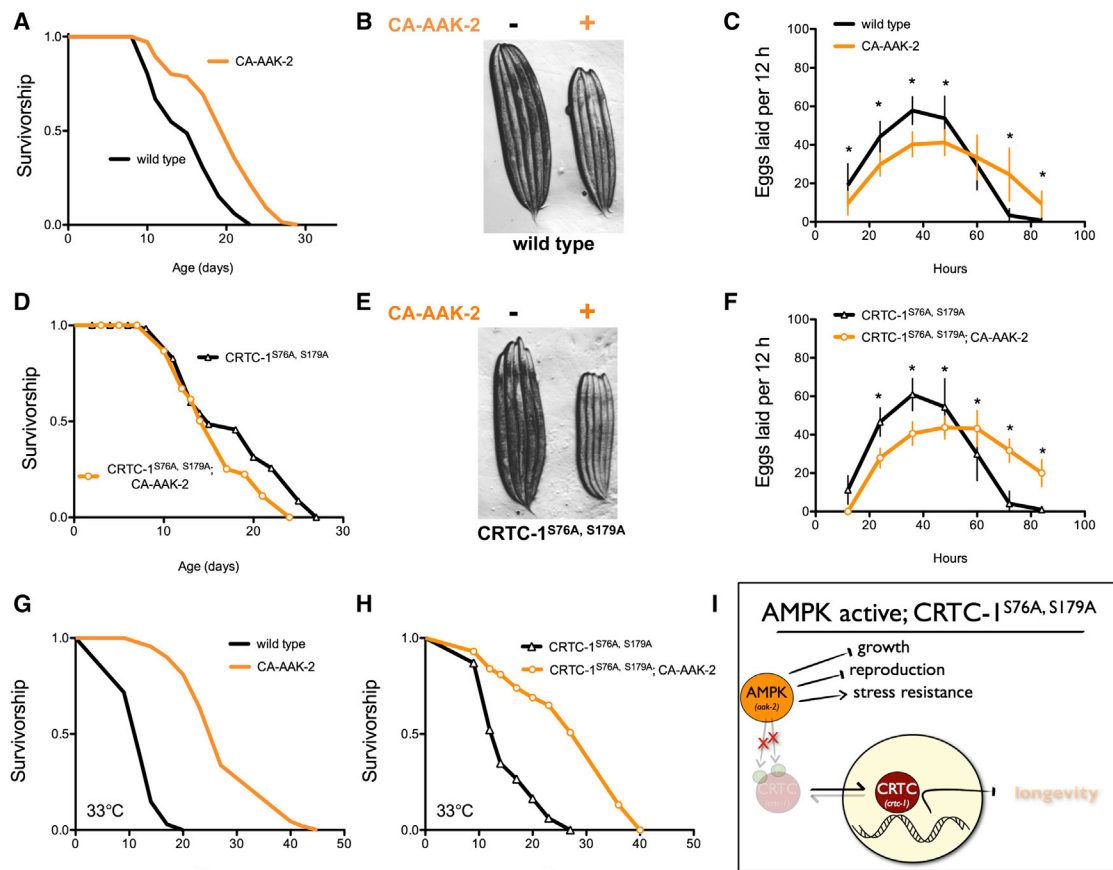


Figure 1. CRTC-1 Uncouples AMPK-Mediated Longevity from Other Pleiotropic Phenotypes

(A) AMPK activation (CA-AAK-2) extends lifespan in a wild-type *C. elegans* background. See Table S1 for lifespan statistics.

(B) CA-AAK-2 suppresses growth. Image of 5 wild-type (left) and 5 CA-AAK-2 (right) adult worms.

(C) Eggs laid per worm over successive 12 hr periods. Data are mean \pm SD for $n = 25$ –30 animals; * denotes $p < 10^{-4}$ via t test.

(D) CA-AAK-2 fails to promote longevity in a CRTC-1^{S76A,S179A} mutant background.

(E) CRTC-1^{S76A,S179A} does not suppress AMPK-mediated effects on growth.

(F) Eggs laid per worm over successive 12 hr periods (data are mean \pm SD for $n = 19$ –30 animals; * denotes $p < 10^{-4}$ via t test).

(G) and (H) Survival curves of AMPK and CRTC-1^{S76A,S179A} transgenic animals exposed to heat stress at 33°C. $n = 60$ –100 worms. $p < 10^{-5}$ via Log-rank (Mantel-Cox) analysis.

(I) CRTC-1 is a longevity-specific AMPK target that uncouples growth, reproduction, and stress resistance from lifespan extension.

To determine whether the transcriptional changes in metabolic genes ultimately alter metabolic function we performed metabolomic analyses. We measured organic acids, amino acids, and acylcarnitines, which represent metabolites of the major energy producing pathways, in CA-AAK-2 animals with and without CRTC-1^{S76A,S179A}. While there are few significant differences between CA-AAK-2 and CRTC-1^{S76A,S179A}, CA-AAK-2 in amino acids or acylcarnitines (Figures S2C and S2D, Table S4), the data show widespread differential regulation of TCA cycle intermediates (Figure 2E). Congruent with the changes observed at the transcriptional level, the TCA cycle intermediates exhibit a pattern consistent with altered mitochondrial metabolism being causal to AMPK/CRTC-1 regulation of longevity. Namely, TCA intermediates and associated organic acid levels are maintained or increased by AMPK activation, CRTC-1^{S76A,S179A} opposes these effects for several organic acids, including malate, citrate, and lactate (Figure 2E). These metabolomic data support a role

for AAK-2 and CRTC-1 in coordinating central metabolic processes and highlight a new role for CRTCs in mediating transcriptional links between AMPK status and mitochondrial metabolism. Although AMPK is a known sensor and regulator of mitochondrial function and biogenesis (Hardie et al., 2012), these data now specifically couple these processes to the role of AMPK in longevity assurance.

Transcriptional Regulation of Metabolism Is Required for AMPK and Calcineurin Longevity

To determine if the metabolic effects of CRTC-1 are causal to AMPK longevity, we searched for known alternative interventions that broadly regulate cellular metabolic processes to examine their effects on lifespan. Like AMPK, the nuclear hormone receptor and functional PPAR α ortholog, NHR-49, activates during low energy status such as fasting, and transcriptionally promotes genes required for mitochondrial function

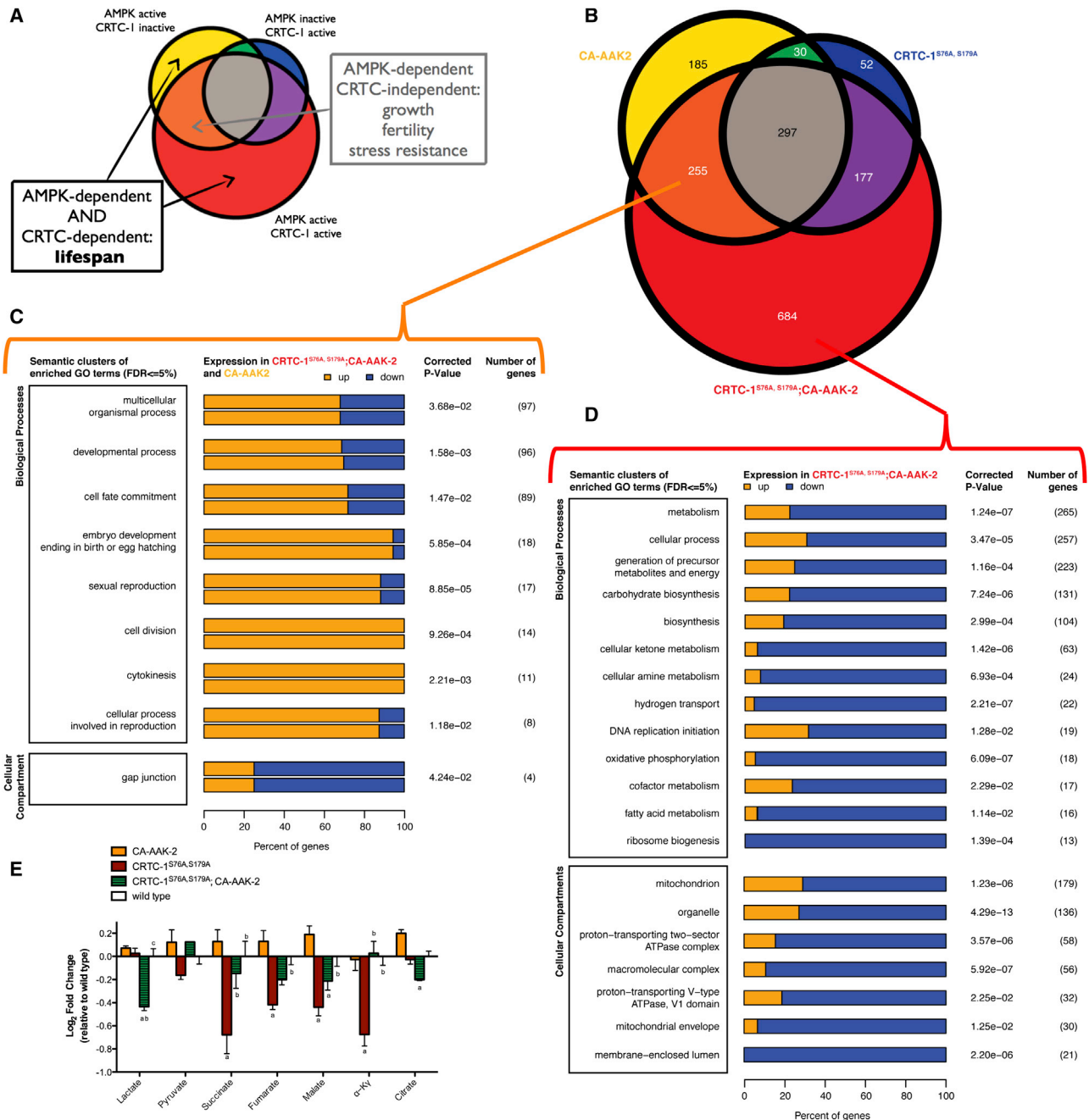


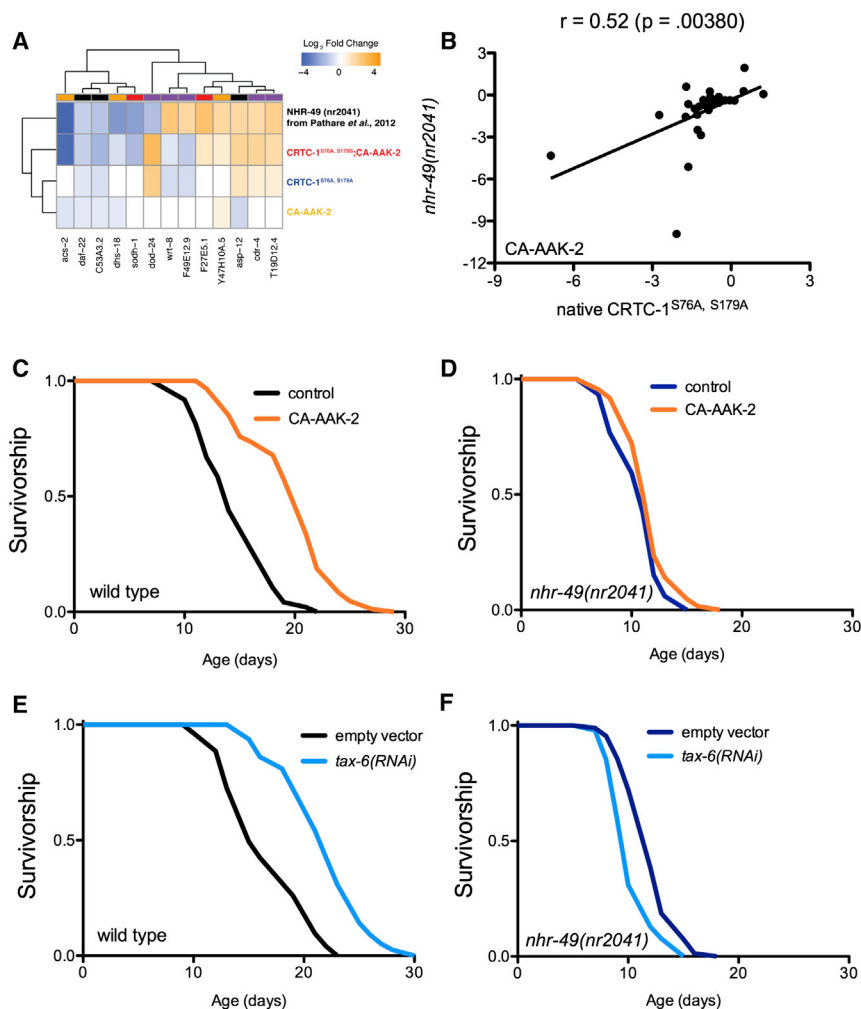
Figure 2. Leveraging CRTC-1 to Identify Longevity-Specific Processes Downstream of AMPK Reveals a Critical Role for Mitochondrial Metabolism

(A) Schematic Venn diagram illustrating how CRTC-1-specific gene expression filters AMPK-induced changes associated with longevity from those involved in other pleiotropic phenotypes.

(B) Venn diagram representing the number of differentially expressed (DE) genes identified through RNA-Seq analysis from each indicated genetic background relative to wild-type controls. See [Table S2](#) for complete list of DE genes.

(C and D) Clusters of enriched GO biological processes and cell compartments among the DE genes involved in CRTC-1-independent phenotypes (C, orange) or unique to the CRTC-1^{S76A, S179A}; CA-AAK-2 worms (D, red). Bars represent the percentage of genes within that category that are up- (orange) or downregulated (blue). The number of genes annotated within a cluster is tabulated, along with the smallest multiple-testing corrected p value for the observed enrichment attributed to a term within the cluster. See also [Figure S2](#) and [Table S3](#).

(E) Metabolomic analyses of transgenic strains to measure levels of organic acids (E), acylcarnitines, and amino acids ([Figure S2](#)). Two-way ANOVAs were performed with a Sidak multiple comparisons test after metabolite levels were normalized to total protein. Data are mean \pm SEM of $n = 2-5$ replicates per metabolite in each group. ^a $p \leq 0.05$ versus CA-AAK2, ^b $p \leq 0.05$ versus CRTC-1^{S76A, S179A}, ^c $p \leq 0.05$ versus CRTC-1^{S76A, S179A}; CA-AAK-2. See also [Table S4](#).



(Pathare et al., 2012). We compared genes differentially expressed in CRTC-1^{S76A,S179A}; CA-AAK-2 transgenic animals with genes previously reported to be differentially expressed in nematodes lacking functional *nhr-49* (Pathare et al., 2012). Of the 47 NHR-49-dependent genes identified by Pathare et al., we observe 30% overlap ($\chi^2 = 13.45$, $p = 10^{-5}$), suggesting that NHR-49 and CRTC-1 coordinately regulate shared metabolic targets (Figure 3A; Table S5). We selected a candidate list of 29 metabolic genes, including genes regulated by CRTC-1 from our RNA-Seq dataset and by NHR-49 from previously published data (Table S6), and validated the high degree of correlation between CRTC-1 activation and NHR-49 loss of function in a CA-AAK-2 background relative to wild-type (Figure 3B; $p < 0.01$). When AMPK is activated, loss of *nhr-49* mirrors activation of CRTC-1 to promote a transcriptional reprogramming of metabolic genes.

If transcriptional regulation of metabolism by CRTC-1 is causal to AMPK lifespan extension, we hypothesized that AMPK should also fail to promote longevity in *nhr-49* mutants, since they recapitulate similar transcriptional changes in metabolic processes. In support of this hypothesis, an *nhr-49(nr2041)* deletion allele

Figure 3. NHR-49 Is Required for AMPK- and Calcineurin-Mediated Longevity

(A) Heat map of genes differentially expressed in CRTC-1^{S76A,S179A}; CA-AAK-2 and *nhr-49(nr2041)* worms, demonstrating significant overlap in gene expression patterns. See also Table S5.

(B) Mean mRNA expression levels (average log₂ of fold change relative to wild-type worms) of 29 metabolic genes. In a CA-AAK-2 background, native CRTC-1^{S76A,S179A} (*crtc-1* promoter) correlates with whole-organism loss of NHR-49 (Pearson correlation coefficient, r), validating the comparison made in (A). Note $r = 0.66$; $p \leq 0.0001$ after 10% winsorization of strong outliers. See Table S6. (C) and (D) CA-AAK-2 extends lifespan in a wild-type background (C), but not in the absence of NHR-49 function (D). See Table S1 for lifespan statistics.

(E) and (F) *tax-6* RNAi extends lifespan in a wild-type background (E), but not in the absence of NHR-49 function (F). The genetic background in B–F is noted next to the origin.

suppresses lifespan extension via CA-AAK-2 (Figures 3C and 3D). Our previous work established that AMPK and calcineurin mediate longevity through a shared signaling pathway that converges on CRTC-1. Calcineurin, a protein phosphatase, directly opposes AMPK by dephosphorylating and activating CRTC-1. RNAi-mediated knockdown of *tax-6*, the catalytic subunit of calcineurin, mimics AMPK activation by increasing *C. elegans* lifespan in a CRTC-1-dependent manner (Mair et al., 2011) and additionally activates expression of the NHR-49-dependent target, *acs-2* (Figure S3).

Strikingly, *tax-6* RNAi also requires intact NHR-49 function to promote longevity (Figures 3E and 3F). These results suggest that AMPK/calcineurin signaling promotes a shift in metabolic programs by orchestrating the activity of opposing transcriptional effectors, CRTC-1 and NHR-49, and that this metabolic switch is required for longevity.

CRTC-1 Acts through CREB in Neurons to Mediate Longevity

Both *aak-2* and *nhr-49* are expressed ubiquitously (Mair et al., 2011; Van Gilst et al., 2005) and are believed to function as cell-autonomous regulators of metabolism. In contrast, *crtc-1* expression is limited to the intestine and neurons in *C. elegans* (Mair et al., 2011). We reasoned that CRTC-1 may directly regulate transcription of genes involved in metabolism in the intestine, one of the major organs of cellular metabolic activity and fat storage in *C. elegans*, and that this effect may be sufficient to stably modulate longevity. To test this hypothesis, we expressed CRTC-1^{S76A,S179A} from the *ges-1* promoter, limiting its expression to intestinal cells. Surprisingly, intestinal expression of CRTC-1^{S76A,S179A} has no effect on *tax-6* RNAi-mediated

longevity (Figures 4A and 4B). Ruling out the intestine as the site of action, we examined the role of neuronal CRTC-1 in AMPK/calci- neurin-mediated longevity. Expressing CRTC-1^{S76A,S179A} from the pan-neuronal *rab-3* promoter (Figure 4C, inset) fully sup- presses lifespan extension by both *tax-6* RNAi (Figure 4C) and AAK-2 activation (Figures 4D and 4E). Upon finding that selective CRTC-1 activation in neurons is sufficient for its effects on life- span, we asked whether activating AMPK in select tissues is also sufficient to promote longevity. We expressed CA-AAK-2 from pan-neuronal, muscle, and intestinal-specific promoters. AMPK activation is not sufficient for longevity in any of the individ- ual tissues tested (Figure S4A), suggesting this longevity mech- anism in *C. elegans* requires local AMPK-mediated programming of mitochondrial function in multiple tissues. Taken together these results indicate that CRTC-1 activity in neurons cell nonau- tonomously modulates AMPK/calci- neurin-mediated longevity. Moreover, signals downstream of neuronal CRTC-1 dominantly override the effects AMPK exerts locally in peripheral tissues.

CRTCs lack DNA-binding activity and depend on partner tran- scription factors for recruitment to DNA in order to stimulate gene transcription (Altarejos and Montminy, 2011). Though first identi- fied as CREB modulators, CRTCs also bind and regulate other bZIP transcription factor family members. To determine whether the effects of neuronal CRTC-1 on aging occur via CREB, we tested whether CREB was necessary for longevity suppression by CRTC-1^{S76A,S179A}. In animals lacking CRH-1, the *C. elegans* CREB ortholog, CRTC-1^{S76A,S179A} expressed under its endoge- nous promoter no longer suppressed CA-AAK-2-mediated longevity (Figures 4F and 4G). Additionally, neuronal expression of CRTC-1^{S76A,S179A} in *crh-1* null animals had no effect on life- span extension by *tax-6* RNAi (Figures 4H and 4I). Lifespan extension via AMPK/calci- neurin therefore requires inhibition of the CRTC-1/CRH-1 transcriptional complex in neurons.

Neuronal CRTC-1 Cell Nonautonomously Regulates Metabolic Genes

Since CRTC-1 regulates longevity through a systemic metabolic program (Figure 2) and neuron-limited CRTC-1 activation is suf- ficient to suppress longevity, we sought to determine if neuronal CRTC-1 is sufficient to produce the lifespan-related effects on metabolic transcription. Analyzing the same panel of 29 meta- bolic genes used previously (Figure 3B), we found that limiting CRTC-1^{S76A,S179A} expression to neurons recapitulates the ef- fects on peripheral metabolic genes seen in animals expressing CRTC-1^{S76A,S179A} from its native promoter (Figure 4J; $p < 0.0001$). Therefore, similarly to lifespan, CRTC-1 regulates meta- bolic cell nonautonomously from neurons. Finally, we deter- mined that neuron-specific activation of CRTC-1 mimics genetic deletion of *nhr-49* regarding peripheral expression of genes involved in cellular metabolism (Figure 4K; $p < 0.01$). Despite the presence of CA-AAK-2 in every background, AAK-2 acti- vated animals alone exhibit no correlation in gene expression with any of the double transgenics, indicating these transcrip- tional effects are all attributable to NHR-49 and CRTC-1 (Figures S4B–S4D; $p > 0.05$). Taken together, the striking overlap between gene expression profiles (Figure 4L; Table S6) reveals an antagonistic relationship between metabolic programs regu- lated by NHR-49 and neuronal CRTC-1.

NHR-49 Regulates Metabolism and Lifespan Cell Nonautonomously

NHR-49 has previously been characterized as a functional ortho- log of mammalian PPAR α , as *nhr-49* mutants fail to activate mitochondrial fatty acid oxidation (FAO) genes during starvation (Van Gilst et al., 2005). However, whether this effect is cell auton- omous is unknown, as the NHR-49 DNA-binding motif has not been determined and direct targets of NHR-49 remain elusive. To determine whether NHR-49 can mediate lifespan and meta- bolic cell nonautonomously, we selectively restored NHR-49 function to neurons in an *nhr-49* null background by driving expression with the *rab-3* promoter. As previously described, *C. elegans* subjected to 24 hr of fasting show strong upregulation of a key gene involved in beta-oxidation, acyl-CoA synthetase (ACS)-2, which is dependent on *nhr-49* (Figure 5A). Supporting a role for NHR-49 beyond cell-autonomous regulation of meta- bolic gene expression, neuronal rescue of NHR-49 is sufficient to restore both basal *acs-2* expression (Figure 5A) and the induc- tion of *acs-2* by fasting (Figure 5B). Since mRNA measurements were obtained using whole-animal preparations, we generated animals expressing GFP under control of the *acs-2* promoter to examine tissue-specific induction. Fasting for 24 hr induces expression of GFP in multiple tissues, including pharynx, mus- cle, and intestine (Figure 5C). This induction is abrogated by loss of *nhr-49* (Figure 5D). Remarkably, neuronal expression of *nhr-49* is sufficient to restore induction of *acs-2* in both neurons and distal tissues, including muscle and intestine, but not pharynx (Figure 5E).

Induction of *acs-2* expression in multiple tissues lacking NHR-49 suggests alternative transcription factors might respond to signals downstream of neuronal CRTC-1. To identify transcriptional regulators in this pathway, we revisited our RNA- Seq dataset and performed Hypergeometric Optimization of Motif EnRichment (HOMER) analysis of the ‘CRTC-1-dependent genes’ (Figure 2B, red section). We identified a motif with consensus TGATAACG or CGTTATCA enriched in the putative promoter regions of genes downstream of AMPK/CRTC-1 ($p = 1e-25$, found in 38% of targets versus 21% of background) (Figure S5A). The motif strongly resembles the GATA-like DAE (DAF-16 Associated Element), recently identified as the binding site for PQM-1 (Tepper et al., 2013). modENCODE ChIP-seq data also suggests that both DAF-16 and PQM-1 bind the *acs-2* promoter directly. To determine whether DAF-16 can regulate expression of *acs-2*, we examined the effect of fasting on our *acs-2P::GFP* reporter under control conditions, and in animals subjected to RNAi for *daf-16*, along with two other transcription factors known to mediate DR longevity, *skn-1* and *pha-4*. While inhibition of *skn-1* and *pha-4* does not alter induction of *acs-2* by fasting, *daf-16* RNAi significantly suppresses *acs-2* induction (Figures S5B and S5C). These data therefore suggest that DAF-16 and/or PQM-1 might be acting downstream of the neuronal CRTC-1 signal to modulate metabolic gene expression.

Given the ability of NHR-49 to cell nonautonomously regulate metabolic genes, we next asked whether, like CRTC-1, NHR-49 mediates longevity through its effects in neurons. Restoring NHR-49 function in neurons exclusively was sufficient to restore lifespan extension via both *tax-6* RNAi (Figures 5F–5H) and AMPK activation (Figures 5I–5K) in *nhr-49* deletion mutants.

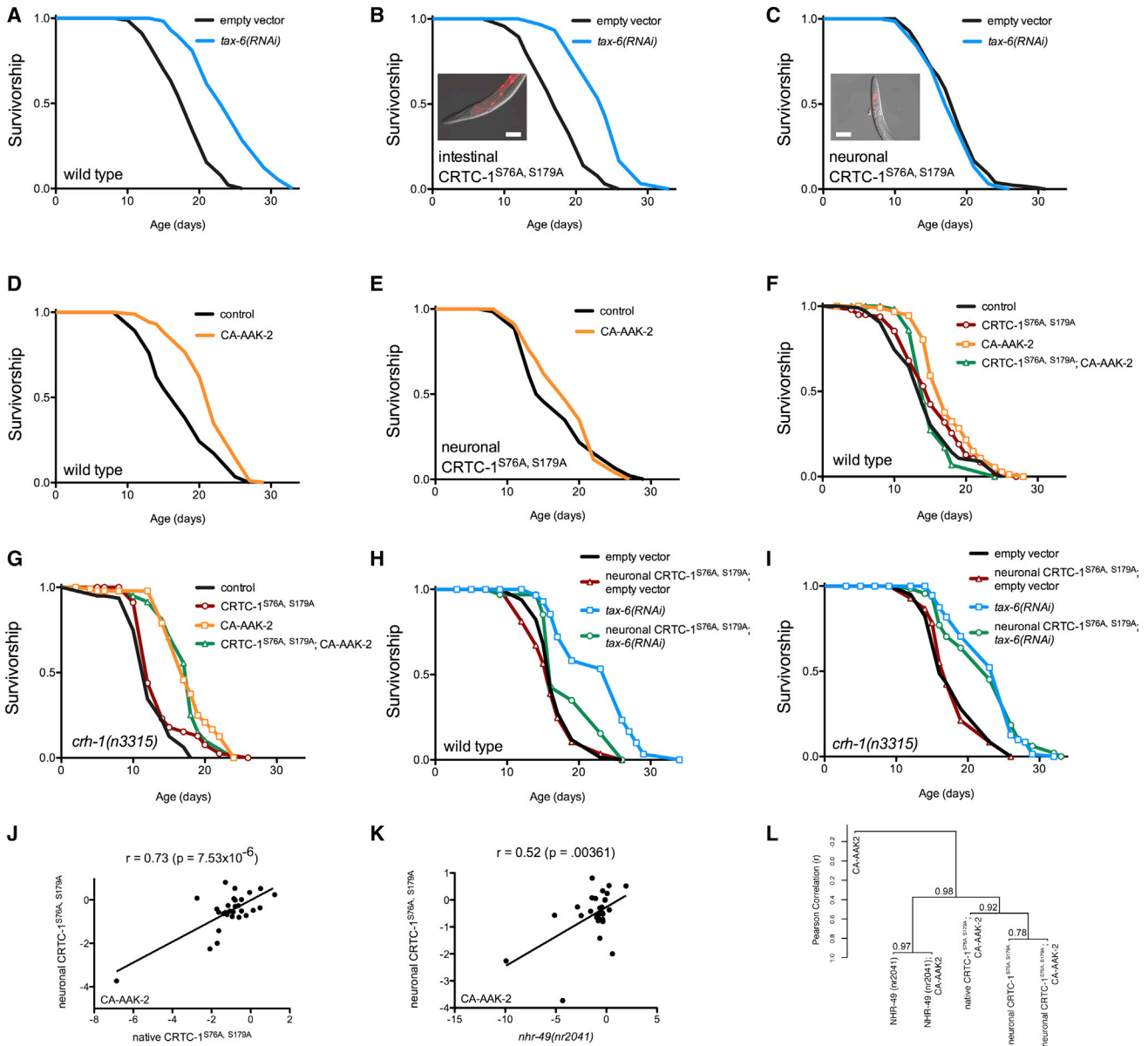


Figure 4. Neuronal CRT-1/CRH-1 Activation Suppresses Longevity Downstream of AMPK and Calcineurin Signaling and Cell Non-autonomously Regulates Metabolic Transcription

(A) *tax-6* RNAi increases longevity. See Table S1 for lifespan statistics. The genetic background in A – I is noted next to the origin.
 (B) Intestine-specific CRT-1^{S76A, S179A} (*ges-1* promoter) fails to suppress *tax-6* RNAi longevity. Inset: image of intestine-specific tdTOMATO-tagged CRT-1^{S76A, S179A}; scale bar, 100 μm.
 (C) Neuronal-specific (*rab-3* promoter) CRT-1^{S76A, S179A} fully suppresses *tax-6* RNAi-mediated longevity. Inset: image of neuron-specific tdTOMATO-tagged CRT-1^{S76A, S179A}; scale bar, 100 μm.
 (D and E) CA-AAK-2 extends lifespan in a wild-type background (D), but neuronal CRT-1^{S76A, S179A} suppresses AMPK-mediated longevity (E).
 (F and G) CA-AAK-2 extends lifespan and this effect is blocked by expressing CRT-1^{S76A, S179A} from its native promoter (F). In *crh-1* null mutants, CRT-1^{S76A, S179A} activation fails to suppress AMPK-mediated longevity (G).
 (H and I) Neuron-specific activation of CRT-1^{S76A, S179A} suppresses longevity mediated by *tax-6* RNAi (H), but neuronal CRT-1^{S76A, S179A} requires intact *crh-1* function to mediate its effects on aging (I).
 (J and K) Expression levels of 29 metabolic genes in CA-AAK-2 animals reveal that the effects of neuron-specific CRT-1^{S76A, S179A} correlate strongly with expression of CRT-1^{S76A, S179A} from its native promoter (J); and neuron-specific CRT-1^{S76A, S179A} activation correlates with the transcriptional effects of loss of NHR-49 function (K). *r* and *p* values were derived by Pearson correlation. See also Table S6.
 (L) Dendrogram summarizing similarities in metabolic gene expression between AMPK, CRT-1 and NHR-49 mutants. Strains were clustered by their pair-wise Pearson correlations using Ward’s minimum variance method. Vertical heights of branches indicate the degree of correlation (*r*; y axis). Multiscale bootstrap resampling *p* values on each branch were calculated via pvclust R package [http://www.sigmath.es.osaka-u.ac.jp/shimo-lab/prog/pvclust/].

However, unlike the effects of CRTC-1 on AMPK/calcineurin-mediated longevity, which is specific to neurons, the role of NHR-49 is more complex, as intestinal rescue can also partially reverse suppression of *tax-6* RNAi lifespan in *nhr-49* deletion mutants and is sufficient to restore *acs-2* induction exclusively in intestinal cells (Figures S6A and S6B). Additionally, while *nhr-49* overexpression in intestine does not affect *C. elegans* longevity, overexpression of *nhr-49* in neurons is sufficient to extend lifespan, further highlighting tissue-specific functions (Figures S6C and S6D).

Neuronal CRTC-1 Cell Nonautonomously Regulates Mitochondrial Dynamics

Recent studies of mitochondrial dynamics suggest that remodeling of the mitochondrial network itself may impact metabolic function (Liesa and Shirihai, 2013), and loss of NHR-49 has been shown to disrupt mitochondrial morphology and function (Pathare et al., 2012). Given that neuronal CRTC-1 and NHR-49 antagonistically regulate AMPK/calcineurin-mediated longevity and metabolic processes, we explored whether changes in mitochondrial architecture were involved in the metabolic reprogramming and longevity by AMPK/CRTC-1. To observe the mitochondrial network directly in distinct tissues, we employed nematodes expressing mRFP targeted to the outer mitochondrial membrane via fusion to TOM20. Typically, the mitochondria of muscle cells of young (day 1) adult worms are fused and tubular, running parallel among the myofilaments (Figure 6A). Activation of CRTC-1 exclusively in neurons of young adult worms, however, results in significant fragmentation of the mitochondrial network in muscle cells, demonstrating a cell-nonautonomous role for CRTC-1 in regulating mitochondrial dynamics (Figure 6B). This effect is consistent with the opposing transcriptional effects of NHR-49 and neuronal CRTC-1, as loss of *nhr-49* also causes mitochondrial fragmentation and altered morphology (Figures 6C–6E). To quantify the degree of fragmentation in these animals, we determined the ratio of mitochondrial area to perimeter in muscle cells of neuronal CRTC-1^{S76A,S179A} mutants and found a 56% reduction relative to control animals (Figure 6F; $p < 0.0001$). The area of muscle cells occupied by mitochondria was also decreased 30% upon neuronal CRTC-1 activation (Figure 6G; $p < 0.0001$), supporting CRTC-1-mediated suppression of mitochondrial function observed at the transcriptional and metabolomic levels (Figure 2).

CRTC-1 Mediates Lifespan via a Catecholamine Signal

Having determined neuronal CRTC-1 mediates longevity and mitochondrial function cell nonautonomously, we reasoned it might regulate a signal that relays energy status from neurons to coordinate aging and metabolism in peripheral tissues. Monoamine signals, e.g., dopamine and serotonin, act in nutrient-sensing pathways to regulate behavioral and peripheral metabolic changes conserved from nematodes to humans (Ashrafi, 2007). We therefore examined whether monoamine signaling might provide a potential mechanism by which neuronal CRTC-1 could regulate longevity and mitochondrial dynamics. Monoamines are secreted via exocytosis in dense core vesicles (DCVs), which in *C. elegans* requires the Ca^{2+} -dependent activator protein for secretion (CAPS), UNC-31 (Grishanin et al.,

2004). Null mutation of *unc-31* suppresses *acs-2* expression, as determined by our *acs-2P::GFP* reporter strain and qRT-PCR (Figures S7A–S7C), suggesting secreted signals within DCVs may mediate distal metabolic gene regulation.

In our RNA-Seq analysis we identified multiple enzymes involved in the synthesis of biogenic amines that were differentially mediated by AMPK and CRTC-1 (Figure 7A). We further examined the genes involved in monoamine synthesis by qRT-PCR and identified a catecholamine biosynthetic enzyme, tyramine beta-hydroxylase (TBH)-1, among the genes regulated by CRTC-1 that couples to AMPK longevity (Table S2). Downregulation of *tbh-1* expression by AMPK activation is attenuated by CRTC-1^{S76A,S179A} (Figure 7B). Acting in a pathway with tyrosine decarboxylase (TDC)-1, TBH-1 catalyzes the synthesis of octopamine, which functions as the invertebrate noradrenaline equivalent (Figure 7C). Analysis of the *tdc-1* and *tbh-1* promoters revealed putative cAMP response elements (not shown), suggesting that CRTC-1/CRH-1 may directly regulate transcription of these genes. As previously reported, expressing GFP downstream of the *tbh-1* promoter results in expression exclusively in two octopaminergic RIC neurons (Alkema et al., 2005), notably CRTC-1 also localizes to RIC neurons (Figure 7D) and thus may be capable of directly regulating octopamine signaling. We therefore hypothesized that octopamine may play a role in intercellular AMPK/CRTC-1 longevity signaling.

If octopamine mediates neuronal CRTC-1 signaling to other tissues, we reasoned it might be sufficient to generate the mitochondrial and metabolic phenotypes observed upon neuronal CRTC-1 activation. To test this hypothesis, we cultured nematodes expressing the mitoRFP reporter in the presence of exogenous octopamine. Strikingly, octopamine treatment elicits a similar degree of mitochondrial fragmentation in *C. elegans* muscle to that observed when CRTC-1 is activated neuronally, further suggesting that octopamine signaling may mediate CRTC-1 regulation of metabolism to peripheral tissues (Figures 7E–7G). In direct support of octopamine as a relay signal, *tax-6* RNAi increases expression of *acs-2*, but this effect is blunted in animals lacking TDC-1 or TBH-1 (Figure S7D).

To define the functional requirement of octopamine signaling in the modulation of aging by the AMPK/CRTC-1/NHR-49 pathway, we tested whether the suppression of longevity by neuronal CRTC-1^{S76A,S179A} or *nhr-49* deletion required either TDC-1 or TBH-1. As shown previously, AMPK activation robustly increases lifespan of wild-type animals and this effect is suppressed in worms expressing neuronal CRTC-1^{S76A,S179A} (Figure 7H) or *nhr-49(nr2041)* (Table S1). While suppression of lifespan by *nhr-49* deletion is independent of octopamine signaling (Figures S7E and S7F), the ability of neuronal CRTC-1^{S76A,S179A} to suppress longevity is completely abolished in animals harboring null mutations in either *tdc-1* (Figure 7I) or *tbh-1* (Figure 7J), both of which lack octopamine (Alkema et al., 2005). Confirming these findings, neuronal CRTC-1^{S76A,S179A} suppresses longevity mediated by *tax-6* RNAi (Figure 7K), and this suppression requires TDC-1 (Figure 7L) and TBH-1 (Figure 7M) function. The ability of CRTC-1 to regulate aging in *C. elegans* therefore requires functional octopamine signaling. Together these results suggest that neuronal CRTC-1 modulates AMPK/calcineurin-mediated longevity and metabolism via an

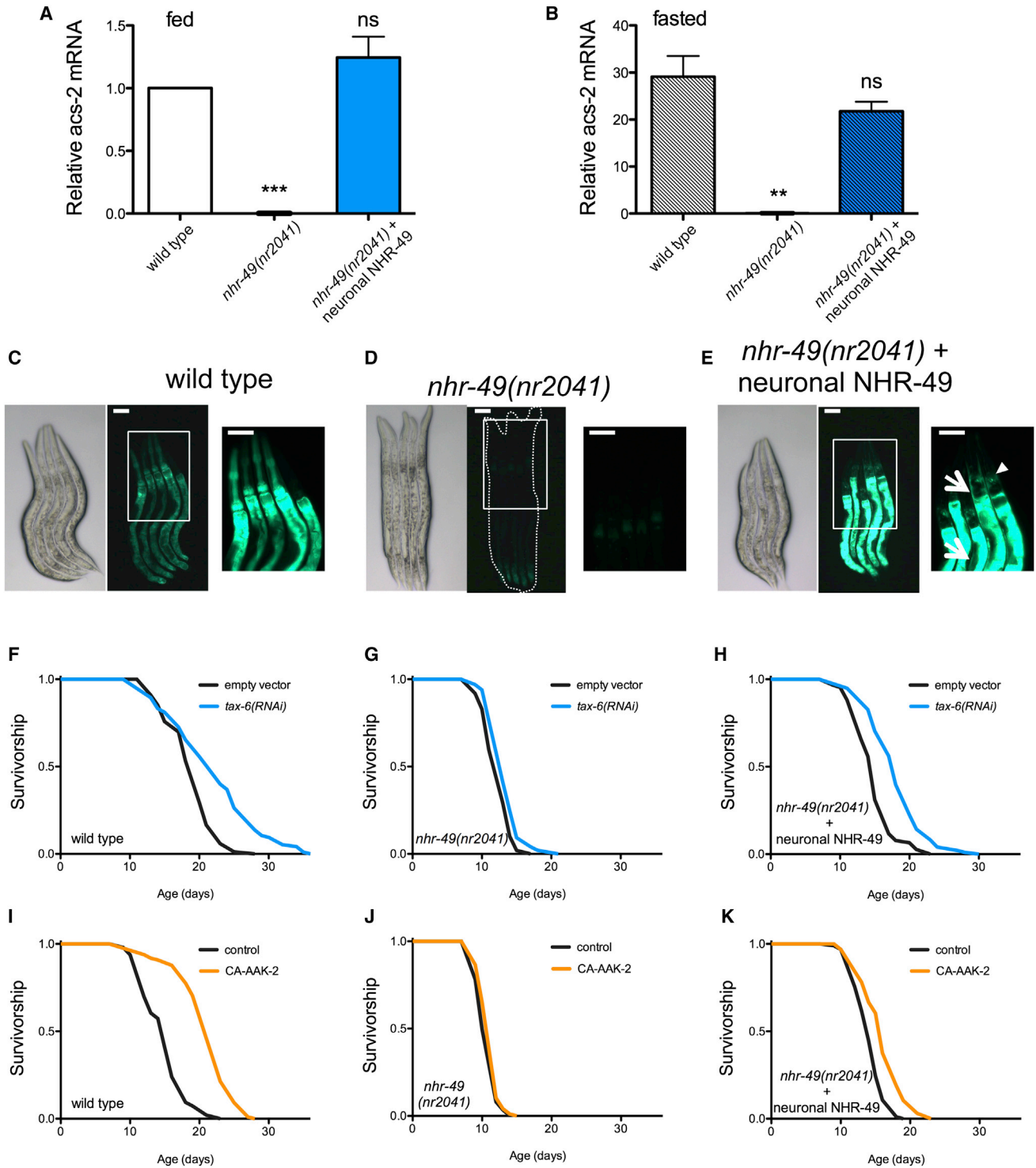


Figure 5. NHR-49 Regulates Mitochondrial Metabolism and Longevity Cell Nonautonomously from Neurons

(A) and (B) Analysis of *acs-2* transcript levels by RT-PCR in L4/young adult worms fed (A) and fasted 16 hr (B). Data are mean \pm SEM of 3-4 independent experiments. By 1-sample (A) or 2-sample (B) t test relative to fed wild-type animals, *** denotes $p < 0.001$; ** = $p < 0.01$; ns = $p > 0.05$. (C-E) Brightfield (left) and fluorescence (middle and right) imaging of L4-stage 16 hr fasted worms expressing GFP driven by the *acs-2* promoter. (C) Fasting activates the *acs-2* promoter ubiquitously in *C. elegans* (middle), and higher magnification reveals strongest expression in the intestine and pharynx (right).

(legend continued on next page)

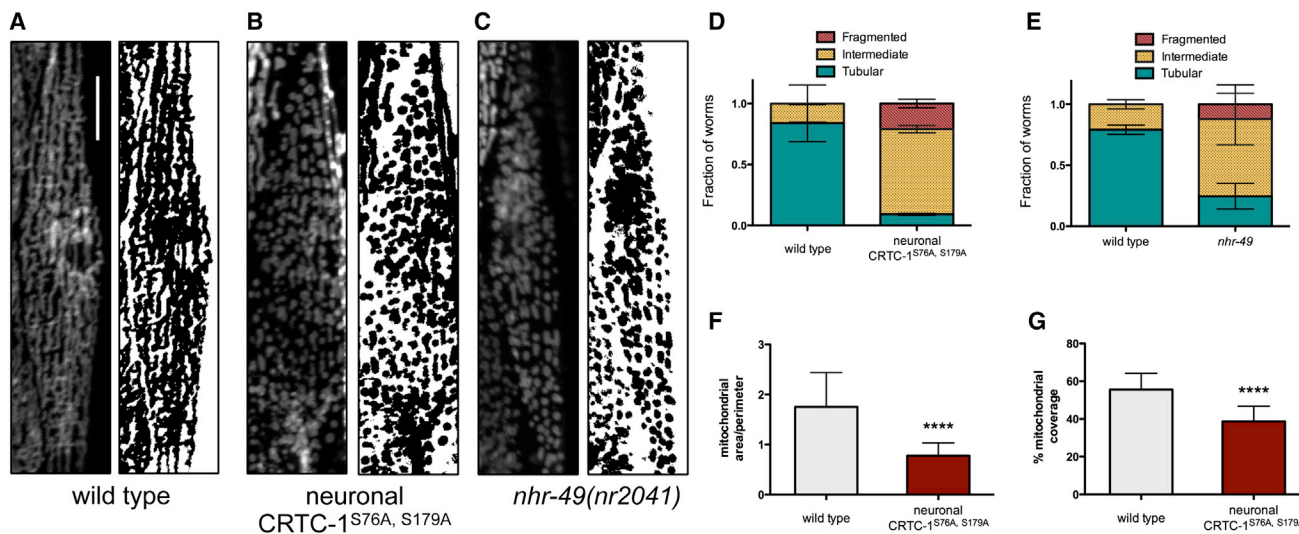


Figure 6. Neuronal CRTC-1 and NHR-49 Regulation of Mitochondrial Dynamics Mirrors Their Respective Roles in Longevity

(A–C) Fluorescence imaging (left) and binary representations (right) of mitochondrial networks in body wall muscle cells of day 1 adult worms. Neuronal CRTC-1^{S76A, S179A} (B) and *nhr-49* loss-of-function (C) induce fragmentation of the mitochondrial network in muscle cells relative to wild-type (A). Scale bars represent 20 μ m.

(D and E) Quantification of neuronal CRTC-1^{S76A, S179A} (D) and *nhr-49* loss-of-function (E) dependent mitochondrial fragmentation in a population of worms demonstrates loss of tubular morphology (mean \pm SD of $n = 3$ groups of 10–17 worms; $p = 0.001$ by *t* test).

(F) Quantification of the ratio between mitochondrial area and perimeter (Mean \pm SD from muscle cells of 32 worms; $p < 0.0001$ by *t* test).

(G) Neuronal CRTC-1^{S76A, S179A} activation decreases the area of muscle cells occupied by mitochondria (Mean \pm SD from muscle cells of 32 worms; $p < 0.0001$ by *t* test).

octopamine signal. Moreover, this cell-nonautonomous signal is dominant over the cell-autonomous effects of AMPK in peripheral tissues (Figure S7G).

DISCUSSION

These data challenge current thinking regarding strategies to translate the link between energetics and longevity for therapeutics; perception and cell-nonautonomous communication of energy status in neurons can override direct activation of pro-longevity factors in distal tissues and might therefore be targeted for healthy aging. Although AMPK and PPARs are both targeted peripherally to promote metabolic homeostasis in humans (Hardie et al., 2012; Wahli and Michalik, 2012), both can affect metabolism cell nonautonomously from the CNS (Bantubungi et al., 2012; Kocalis et al., 2012; Minokoshi et al., 2004). To date, the relative contributions of these local and distal effects to healthy aging have not been explored. Here, we demonstrate a cell-nonautonomous role these metabolic regulators play in coordinating energetics and longevity via effects on neuronal catecholamine signaling. AMPK locally and cell autonomously promotes remodeling of mitochondrial metabolic networks to increase longevity; however, AMPK must also inactivate CRTC-1-dependent transcription in neurons to systemically and cell nonautonomously

omously generate a permissive transcriptional landscape for its local metabolic programming. Critically, these cell-nonautonomous signals dominantly impact longevity, irrespective of AMPK activation and energetic state in receiving cells. Intriguingly, both neuronal activation of CRTC-1 and octopamine supplementation promote mitochondrial network fragmentation, suggesting dynamics of the mitochondrial network can be shaped from a distance and are critical for the ability of AMPK to promote longevity. However, this study also raises key questions going forward, including the sufficiency of the neuronal signal for longevity assurance, and how these mechanisms might translate to mammalian systems and therapeutics designed to promote metabolic homeostasis. Specifically, perhaps treatments targeting peripheral metabolic effectors to promote healthy aging will have reduced efficacy if cell-nonautonomous CNS signals remain discordant.

Although AMPK, PPARs, and CRTCs are key peripheral metabolic regulators, all have emerging roles in neuroendocrine control of organismal metabolism that may become dysfunctional with age or obesity. Early studies of CRTCs focused on regulation of glucose metabolism in the mammalian liver, but there are three CRTC family members in mammals, two of which are expressed in the nervous system. Though less studied, recently elucidated roles of neuronal CRTCs include regulating

(D) *nhr-49* mutants fail to activate *acs-2*. (E) Neuron-limited rescue of NHR-49 restores *acs-2* levels in neurons (arrowhead) and peripheral tissues (arrows). Boxes outline areas magnified in the right panels. Scale bars represent 50 μ m.

(F–I) Survival analysis demonstrating that *tax-6* RNAi (F) and CA-AAK-2 (I) extend lifespan in wild-type worms, but not worms lacking *nhr-49* (G, J). Restoring NHR-49 function selectively to neurons via the *rab-3* promoter rescues *tax-6* RNAi- (H) and CA-AAK-2-mediated longevity (K). Common genetic backgrounds are indicated next to the origin. See also Figure S5 and Table S1 for lifespan statistics.

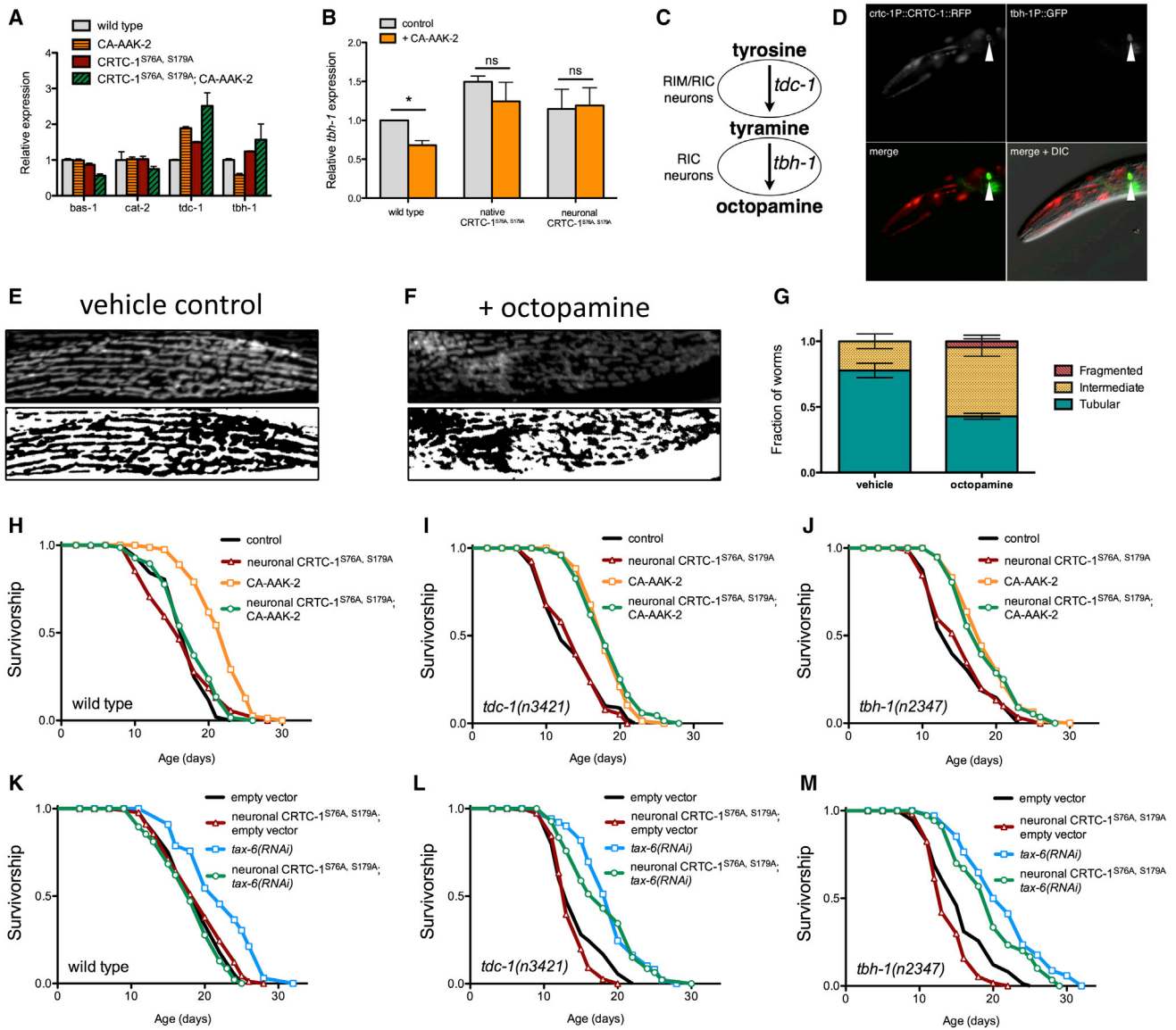


Figure 7. Neuronal CRTC-1 Regulates Longevity and Mitochondrial Function Cell Nonautonomously through Catecholamine Signaling
 (A) Normalized read counts for enzymes involved in synthesis of biogenic amines from the RNA-Seq analysis (see also Table S2).
 (B) qRT-PCR validating AMPK/CRTC-1 regulation of *tbh-1* transcript levels (Mean \pm SEM of mRNA levels extracted from 2-4 samples of 50-100 animals; * denotes $p < 0.05$ by t test).
 (C) The biosynthetic pathway of octopamine.
 (D) Fluorescence imaging of a worm co-expressing CRTC-1::tdTOMATO from the native *crtc-1* promoter (top left) and GFP driven by the *tbh-1* promoter reveals CRTC-1 expression in octopaminergic RIC neurons.
 (E and F) Fluorescence imaging (top) and binary representations (bottom) of the mitochondrial network in muscle cells of animals vehicle-treated (water) or grown on media with 5 mM octopamine (F).
 (G) Classifying worms by their mitochondrial morphology reveals a 45% decline in the fraction of worms with tubular mitochondria treated with octopamine versus control ($p < 0.001$ by t test; mean \pm SD of $n = 3$ samples of 11-19 worms).
 (H-M) Survival curves demonstrating that neuron-specific activation of CRTC-1^{S76A,S179A} suppresses both AMPK- (H) and calcineurin-mediated (K) lifespan extension. However, neuronal CRTC-1^{S76A,S179A} has no effect on AMPK or calcineurin-mediated longevity in animals lacking functional *tdc-1* (I, L) or *tbh-1* (J, M). Genetic backgrounds are noted next to the origin. See Table S1 for lifespan statistics.

expression of peptide signals and metabolic homeostasis in the periphery. Deletion of CRTC1, which is expressed primarily in the brain in mammals, results in hyperphagia and obesity in mice (Altarejos et al., 2008; Breuillaud et al., 2009). More recently,

knockout of TRPV1 pain receptors was shown to both promote metabolic fitness and extend lifespan through effects in mouse sensory neurons (Riera et al., 2014). Interestingly, TRPV1 mutant mice exhibit nuclear exclusion of CRTC1 and reduced

expression of a neuropeptide important for regulating glucose homeostasis. Although the requirement of neuronal CRT1 inhibition for the longevity effects associated with TRPV1 loss of function in mice remains untested, our results point to a potentially conserved, neuronally mediated mechanism by which CRTCs regulate systemic metabolic homeostasis and impact the aging process in worms and mice.

AMPK cell autonomously regulates numerous physiological processes known to play roles in aging, including autophagy, protein synthesis, mitochondrial biogenesis, and both lipid and glucose metabolism (Burkewitz et al., 2014). Our data indicate mitochondrial metabolism is causally associated with AMPK longevity. Moreover, they suggest that AMPK regulation of both longevity and metabolism can be divided into two components: acute remodeling of metabolic pathways through direct regulation of enzymatic activity, and long-term remodeling of cell function via transcriptional reprogramming. Surprisingly, the transcriptional effects of AMPK are induced via cell-nonautonomous signals that override local enzymatic effects; CRT1 transcription in neurons suppresses lifespan despite AMPK being constitutively active in all tissues. Cunningham et al. (2014) recently identified a cell-nonautonomous role for neuronal AMPK in modulating peripheral lipid storage in nematodes, which supports cell-nonautonomous effects of AMPK/CRT1 on metabolism. The role of AMPK in the central regulation of peripheral metabolism is conserved in mammals; AMPK integrates hormonal signals in the hypothalamus to control energy homeostasis, satiety, and metabolism (Minokoshi et al., 2004). Further, in response to changes in glucose levels, AMPK regulates CRT2 activity in the murine hypothalamus to modulate insulin signaling via IRS2 (Lerner et al., 2009). Communication between central and peripheral AMPK activity and its effect on metabolic homeostasis and aging in other organisms will be an exciting area for future research. While expressing truncated CA-AAK-2 in individual tissues of *C. elegans* in our study failed to promote longevity, work in *Drosophila* has shown that overexpressing wild-type AMPK in muscle or fat body (Stenesen et al., 2013) or activated AMPK mutants in brain or gut (Ulgherait et al., 2014) is sufficient to extend lifespan. Differential effects seen in *C. elegans* and *Drosophila* may be due to methods employed to generate tissue-specific strains. Moving forward, more work is needed to better elucidate how tissue-specific roles of AMPK coordinate to control longevity across different model systems.

Like AMPK and CRTCs, PPAR family transcription factors are best known for their primarily cell-autonomous roles in regulating metabolism, including lipid uptake, storage, and oxidation. Here, we demonstrate that the proposed worm PPAR α , NHR-49, acts antagonistically to CRT1/CREB, regulating the shift in metabolic and mitochondrial programming, and that neuronal *nhr-49* is sufficient for AMPK-mediated longevity (Figure 5). Notably, novel PPAR functions in the mammalian brain have also begun to emerge. The thiazolidinedione (TZD) class of anti-diabetic drugs is associated with weight gain, and two complementary studies identified brain PPAR γ as the critical mediator of TZD-induced effects on food intake, thermogenic energy expenditure, and peripheral glucose metabolism (Lu et al., 2011; Ryan et al., 2011). In addition, PPAR α null mice show increases in glucose turnover,

body weight, and adipogenesis that are not rescued by restoring hepatic PPAR α function. Pharmacologically activating PPAR α in the brain of these mice, however, decreases glucose usage in peripheral tissues (Knauf et al., 2006). How PPARs and NHR-49 function in neurons to systemically regulate metabolic homeostasis with age remains unknown and is an important scope for future work.

An exciting key finding of this study is the novel role of octopamine, the invertebrate equivalent to the catecholamine (nor)adrenaline, as a signal communicating energetic state between neuronal AMPK/CRT1 and the periphery to modulate longevity (Figure 7). Interestingly, there is precedent for both AMPK and CRTCs in the regulation of analogous bioamine pathways in mammals. In mice, AMPK α 2 suppresses sympathetic catecholamine release (Violet et al., 2003), while CRT1 enhances monoamine signaling in the prefrontal cortex (Violet et al., 2003). However, it remains unclear whether octopamine is acting as a neurotransmitter or a neuroendocrine signal to mediate longevity in *C. elegans*. Octopamine and 5-HT were recently shown to act through a positive regulatory loop in neurons to promote release of an unidentified endocrine factor capable of activating the nuclear hormone NHR-76 to regulate lipid oxidation in the *C. elegans* intestine (Noble et al., 2013). That at least 2 nuclear hormone receptors (NHR-49 and NHR-76) act downstream of octopamine suggests that perhaps octopamine regulates the release of a lipophilic hormone. Alternatively, given our finding that DAF-16/FOXO may be involved in metabolic transcription downstream of neuronal CRT1, octopamine may regulate the secretion of specific insulin-like peptides. Beyond serving as a signaling molecule between neurons, octopamine could also act as an endocrine molecule itself. *C. elegans* possesses three putative octopamine receptors, *ser-3*, *ser-6*, and *oct-1*, whose expression outside the nervous system has not been extensively examined. Interestingly, a small-molecule screen for drugs capable of extending *C. elegans* lifespan identified a molecule that was shown to be an antagonist of the SER-3 receptor (Petrascheck et al., 2007). Future studies characterizing the respective roles of each octopamine receptor will be enlightening in understanding how octopamine elicits metabolic and longevity-related responses in the periphery.

Although our studies point toward remodeling of mitochondrial metabolism as being required for AMPK longevity, they do not preclude a role for other cellular processes. AMPK and CRTCs are known regulators of autophagy (Egan et al., 2011; Seok et al., 2014), and autophagy is required for lifespan extension by AMPK activation in *Drosophila* (Ulgherait et al., 2014) and calcineurin inhibition in *C. elegans* (Dwivedi et al., 2009). Highlighting the role of inter-tissue communication in AMPK longevity, tissue-specific activation of AMPK in the fly promotes systemic tissue homeostasis via the autophagic effector, Atg1, which subsequently and cell nonautonomously promotes activation of autophagy in other tissues (Ulgherait et al., 2014). DAF-16/FOXO is a known regulator of autophagy and is directly regulated by both AMPK and calcineurin and required for their effects on longevity (Greer et al., 2007; Tao et al., 2013). We saw enrichment of the DAE element in genes downregulated when both AMPK and CRT1 were active, suggesting CRT1 might

remotely regulate DAF-16/FOXO activity and/or activate its transacting antagonist PQM-1 (Tepper et al., 2013). Understanding how neuronal CRTC-1 interacts with DAF-16 and/or PQM-1 and if they function intra- or intercellularly to modulate AMPK/calcineurin-mediated longevity will be informative.

In summary this study highlights “mito-centric” metabolism as the critical target of AMPK/CRTC-mediated effects on aging, and establishes that neurons are the causal and crucial site for CRTC-1-dependent regulation of longevity. Though both sensory perception of nutrient availability in neurons (Petrascheck et al., 2007) and organismal energy status (Burkewitz et al., 2014) are known to modulate aging, our data suggest an emerging paradigm: the optimal pro-longevity intervention requires coordination of energy perception in the neurons with accurately executed metabolic programs in peripheral tissues. Indeed, we show here that the pro-longevity, AMPK-mediated metabolic program in peripheral tissues is overridden when the regulatory link between AMPK and CRTC-1 is broken exclusively in neurons, completely suppressing all gains in longevity for the organism. If neuronal energy-sensing mechanisms are dominant, as our data indicate, selectively targeting central sensors and regulators of energy homeostasis may be sufficient to generate a peripheral metabolic program that promotes healthier aging.

EXPERIMENTAL PROCEDURES

Additional details are provided in the [Extended Experimental Procedures](#).

Lifespans

Lifespan experiments were performed on standard nematode growth media (NGM) at 20°C. Worms were synchronized by timed egg lays using gravid adults. When the progeny reached adulthood (~72 hr), 100 worms were transferred to fresh plates at 10–25 worms per plate and this was considered time = 0. Worms were transferred to fresh bacterial lawns every other day until the first deaths (10–14 days). Survival was scored every 1–2 days and a worm was deemed dead when unresponsive to 3 taps on the head and tail. Worms were censored due to contamination on the plate, leaving the NGM, eggs hatching inside the adult or loss of vulval integrity during reproduction. Only in the lifespans noted (TABLE S1), 5-Fluoro-2'-deoxyuridine (FUDR) was added to media to prevent excessive censoring. FUDR (100 µl; 1 mg ml⁻¹) was added 24 hr before picking worms to the plate on the first day of adulthood, and worms were transferred off FUDR-containing plates once reproduction had ceased (7 day), after which the assays continued normally.

RNA Sequencing

The experiment was performed with three biological replicates. Eggs were synchronized to L1 larvae overnight in M9 and 1,000 larvae were grown to L4 on NGM seeded with OP50-1 *E. coli*. Animals were collected and washed with M9 media to remove bacteria. Worms were then snap frozen in liquid nitrogen. RNA was extracted by five freeze/thaw cycles in Qiazol then purified by RNeasy mini kit (QIAGEN). RNA quality was checked using an Agilent Technologies 2100 Bioanalyzer. All samples had an RNA integrity number of 10. cDNA libraries were prepared from 4 µg of total RNA using the TruSeq RNA Sample Preparation v2 kit (Illumina). See [Extended Experimental Procedures](#) for more details of the data analysis.

Metabolomics

Synchronized L1 larvae were grown to L4 on NGM/OP50-1 before being washed off plates with M9, resuspended in 0.6% formic acid, snap-frozen, and thawed immediately before lysis by sonication. Aliquots were taken for total protein quantification, then an equal volume of acetonitrile was added to reach a final concentration of 0.3% formic acid and 50% acetonitrile. Samples

were then subject to metabolomic analysis as detailed in [Extended Experimental Procedures](#).

Mitochondrial Analysis

Mitochondria were analyzed in muscle cells from ≥ 10 d1 adult worms per genotype. Qualitative assessment of mitochondrial morphology was made by scoring worms based on three categories: tubular (interconnected mitochondrial network), intermediate (combination of interconnected network and isolated smaller mitochondria) or fragmented (mostly fragmented mitochondria). Quantitative assessments of percent mitochondrial coverage of the cell and mitochondrial area/perimeter ratio were made by measuring >30 muscle cells per genotype using a macro for ImageJ, as previously described (Dagda et al., 2009).

ACCESSION NUMBERS

The GEO accession number for the RNA-seq dataset in this paper is GSE58931.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, seven figures, and seven tables and can be found with this article online at <http://dx.doi.org/10.1016/j.cell.2015.02.004>.

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EXTENDED EXPERIMENTAL PROCEDURES

C. elegans Strains and Culture

MT9455 (*tbh-1*(n3247)X), MT9971 (*nls107*[*tbh-1*::GFP+*lin-15*(+)]III), MT10549 (*tdc-1*(n3421)II), STE68 (*nhr-49*(nr2041)I), CB928 (*unc-31*(e928)IV), BC168 (*unc-13*(s69)I) and N2 wild-type *C. elegans* strains were obtained from the *Caenorhabditis* Genetic Center, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). The *crh-1* mutant (*crh-1*(n3315)) was a gift from Mark Alkema. The strain expressing the mRFP mitochondrial marker was supplied by Sean Curran/Paul Sternberg. See Table S6 for the complete list of strains generated and employed in this study. Worms were grown and maintained on standard nematode growth media seeded with *E. coli* (OP50-1). *E. coli* bacteria was cultured overnight in LB at 37°C, after which 100 µl of liquid culture was seeded on plates to grow for 2 days at room temperature. RNAi experiments alternatively employed *E. coli* (HT115) expressing dsRNA against the gene noted or an empty vector control. Experiments with HT115 were performed identically except LB and NGM contained 100 µg ml⁻¹ Carbenicillin, and dsRNA expression was induced by addition of 100 µl IPTG (100 mM) 2 hr before worms were introduced to the plates.

RNA Isolation and qRT-PCR

Total RNA was isolated from 50 – 100 L4 stage animals. RNA was extracted using Qiazol reagent (QIAGEN) then column purified by RNeasy micro kit (QIAGEN). cDNA was generated using Quantitect Reverse Transcriptase kit (QIAGEN). Taqman real-time qPCR experiments were performed on a StepOne Plus instrument (Applied Biosystems) following the manufacturer's instructions. Data were analyzed with the comparative 2 $\Delta\Delta$ Ct method using the geometric mean of *cdc-42* and *Y45F10D.4* as endogenous controls. For each gene in each mutant strain, average fold-change relative to the wild-type was calculated and statistical significance evaluated with a t test. The following Taqman assays from Life Technologies were used: *acs-2* (Ce02486192_g1), *bas-1* (Ce02447109_g1), *cat-2* (Ce02426736_g1), *tbh-1* (Ce02403384_g1), *tdc-1* (Ce02435855_g1), and *tph-1* (Ce02435064_g1).

RNAi Constructs

The *tax-6* and *skn-1* RNAi constructs came from the Vidal RNAi library; *pha-4* and *daf-16* RNAi constructs came from the Ahringer RNAi library.

Microscopy

Worms were anaesthetized for 20 min in 0.2 mg ml⁻¹ tetramisole/M9, mounted on 2% agarose pads on glass slides under coverslips, and subsequently imaged on Zeiss Discovery V8 and Imager M2 microscopes with Axiocam cameras.

RNA-Seq Analysis

Experiment was performed with three biological replicates. Eggs were synchronized to L1 larvae overnight in M9 media and 1,000 larvae were then grown to L4 on a 100 mm NG plate seeded with OP50-1 *E. coli*. Animals were collected and washed with M9 media to remove bacteria. Worms were then snap frozen in liquid nitrogen. RNA was extracted by five freeze/thaw cycles in Qiazol then purified by RNeasy mini kit (QIAGEN). RNA quality was checked using an Agilent Technologies 2100 Bio-analyzer. All samples had an RNA integrity number of 10. cDNA libraries were prepared from 4 µg of total RNA using the TruSeq RNA Sample Preparation v2 kit (Illumina). 50-cycle paired-end sequencing was performed on an Illumina HiSeq 2000 by the Harvard Biopolymer Core. Read quality was evaluated with FASTQC (<http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/>). Adaptor sequences and poor quality bases (< 20) were trimmed and filtered with CUTADAPT (<http://code.google.com/p/cutadapt/>), resulting in a median of 44 million reads per replicate. These were aligned to the *C. elegans* genome (ce6, WS238) using TopHat version 2.0.8 (Kim et al., 2013), with a median 35 million reads mapped in proper pairs. The number of reads mapping to each gene was counted with htseq-count (<http://www-huber.embl.de/users/anders/HTSeq/doc/count.html>). Genes with less than 1 Count Per Million Reads (CPM) were discarded from further analysis. Counts were normalized for sequencing depth and RNA composition across all samples with edgeR (Robinson et al., 2010). Genes were tested for differential expression between each mutant strain and wild-type using edgeR's glm method. Briefly, negative binomial models were fitted and dispersion estimates obtained. These were then used to calculate average levels of change between conditions and determine differential expression, using the generalized linear model likelihood ratio test. For each comparison, genes with less than 5 CPM were filtered and those with at least 50% change and False Discovery Rate (FDR) of 1% or less were considered differentially expressed.

Functional Profiling

Lists of genes differentially expressed exclusively in CA-AAK-2; CRTG-1^{S76A, S179A} strain, in CA-AAK-2, or in both strains were compiled and submitted to DAVID v6.7 server (Huang et al., 2009) for functional annotation and enrichment analysis. For each list, enriched Gene Ontology (GO) (Ashburner et al., 2000) Biological Processes and Cellular Compartments with an FDR of 5% or less were considered significant. These terms were then clustered semantically using the ReviGO server (Supek et al., 2011).

Metabolomics

Metabolomic analysis of nematode lysate was performed as described for amino acids and acylcarnitines (An et al., 2004; Wu et al., 2004) and organic acids (Jensen et al., 2006). Raw data were normalized to protein levels, converted to log₂ fold change relative to wild-type, and subjected to two-way ANOVA with Sidak multiple comparisons test using GraphPad Prism software (La Jolla, CA).

Motif Discovery

Ab initio motif discovery was performed using HOMER (Hypergeometric Optimization of Motif EnRichment) (Heinz et al., 2010). Target sequences were defined from 700 bases upstream to 50 bases downstream of the transcription start site for genes differentially expressed exclusively in CA-AAK-2; CRTC-1^{S76A, S179A} strain. Background sequences were defined in the same way for all genes that do not respond to either mutation. Statistically enriched motifs 5 through 10 nucleotides long were identified and optimized.

Phospho-AMPK Western Blot

Sixty L4 animals were collected and washed with M9 media to remove bacteria. Worms were then snap frozen in liquid nitrogen. RIPA buffer supplemented with protease and phosphatase inhibitors was added then worms were lysed by sonication. Lysate was centrifuged at 14,000g for 5 min at 4 °C. 18 μg of total protein was resolved by SDS-PAGE and transferred to PVDF membrane. Western blot analysis was performed with Phospho-AMPKα Thr172 (Cell Signaling).

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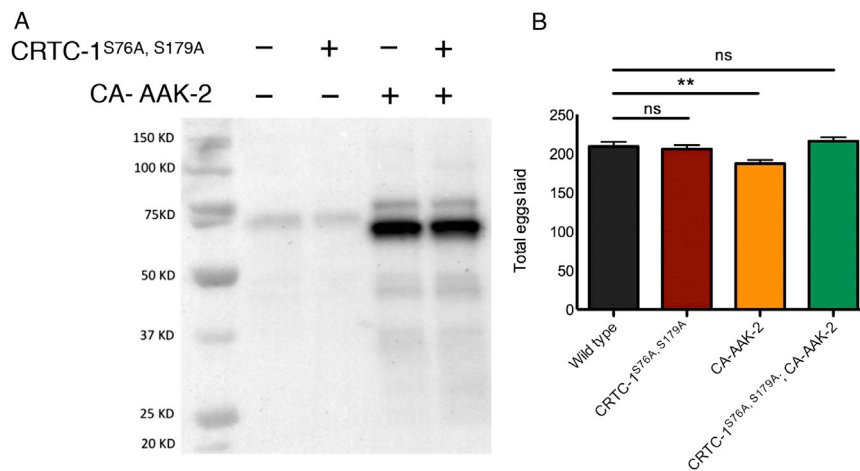


Figure S1. Effect of CRTC-1^{S76A,S179A} on AMPK Activation and Reproduction, Related to Figure 1

(A) Western blot with anti-AMPK pT172 indicating AMPK activation status is not altered by CRTC-1^{S76A,S179A}.

(B) Quantification of the total number of eggs laid by AMPK and CRTC-1^{S76A,S179A} mutant animals. Mean ± SEM for samples of 19-30 worms; ** represents $p < 0.01$ by t test.

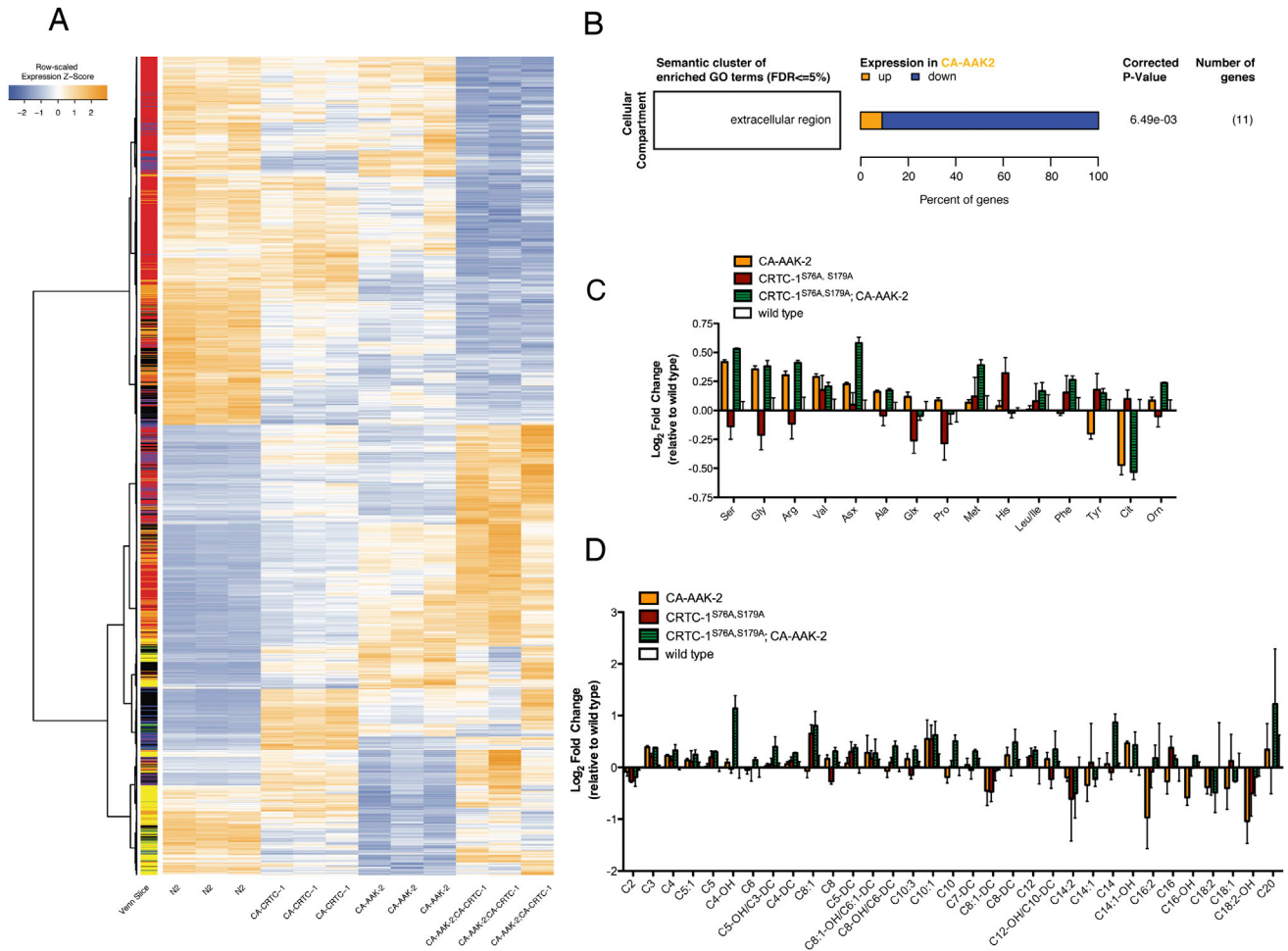


Figure S2. RNA-Seq Analysis Identifies Differentially Expressed Genes between Wild-Type Animals and AMPK α and CRTC-1^{S76A,S179A} Mutants, Related to Figure 2

(A) Heat map (clustered display of expression) of all differentially expressed genes, illustrating patterns of regulation across the different mutant strains. See also Table S2.

(B) Clusters of enriched GO categories among genes differentially expressed only in CA-AAK-2 mutants (yellow Venn region). Bars represent the percentage of genes within that category that are up- (orange) or downregulated (blue). The number of genes annotated within a cluster is tabulated, along with the smallest multiple-testing corrected p value for the observed enrichment attributed to a term within the cluster. See also Table S3.

(C–D) Targeted metabolomic analysis of amino acids (C) and acylcarnitines (D) in AMPK and CRTC-1^{S76A,S179A} mutant animals relative to wild-type. Data are mean \pm SEM of n=2-5 replicates per metabolite in each group. See also Table S4.

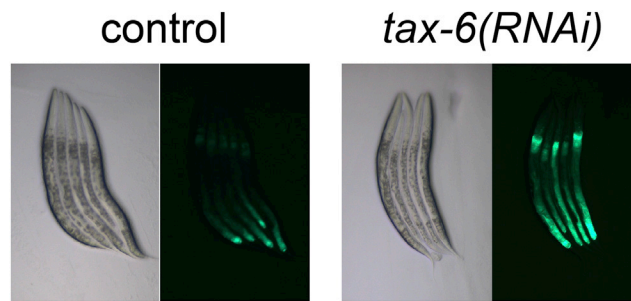


Figure S3. Calcineurin Signaling Regulates NHR-49-Dependent Transcription, Related to Figure 3

Fluorescence imaging of nematodes expressing *acs-2P::GFP* reveals that inhibiting calcineurin/*tax-6* activates *acs-2* transcription.

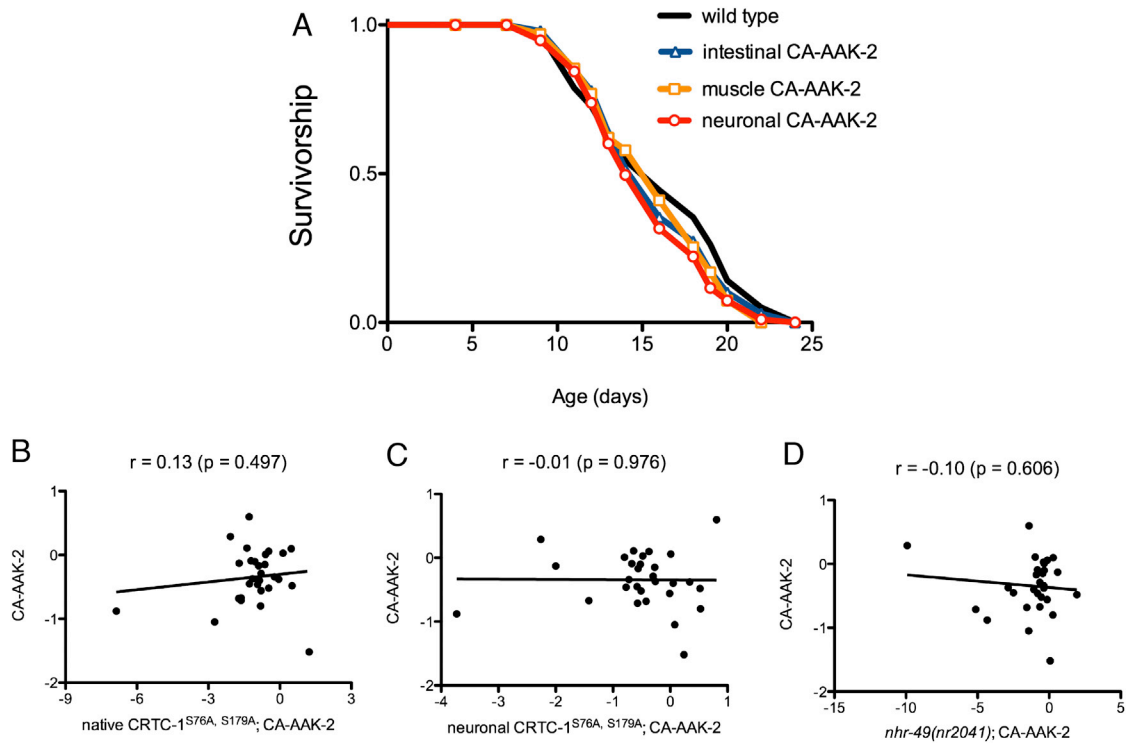


Figure S4. Tissue-Specific CRTC-1, Not AMPK, Is the Dominant Regulator of Longevity and Metabolic Transcription, Related to Figure 4
 (A) Lifespan curves demonstrating that tissue-specific expression of CA-AAK-2 in intestine, body wall muscle, and neurons is not sufficient to promote longevity. (B–D) Gene expression by activation of AMPK alone (y axis, CA-AAK-2) does not correlate with CA-AAK-2 in CRTC-1^{S76A,S179A} (B, C) or *nhr-49* (D) mutant backgrounds.

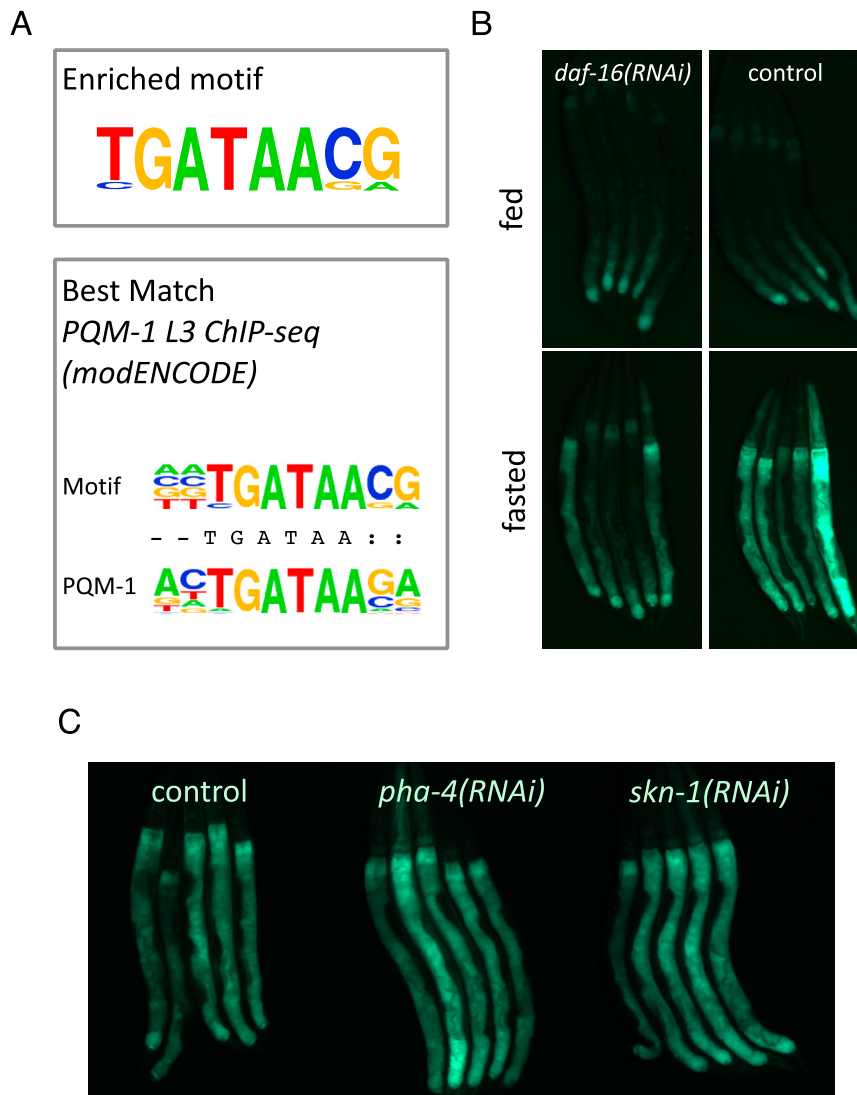


Figure S5. In Silico Analysis of Differentially Expressed Genes Identified by RNA-Seq Suggests DAF-16 May Regulate Transcription Downstream of AMPK/CRTC-1, Related to Figure 5

(A) *Ab initio* motif discovery with HOMER identifies one significantly enriched motif in the promoters of genes regulated downstream of AMPK/CRTC-1 (top). This motif closely resembles the DAF-16 associated element (DAE), a binding site for PQM-1 (bottom).

(B) Fluorescence imaging of worms expressing *acs-2P::GFP* before and after 5 hr of fasting reveals that DAF-16 regulates transcription of genes previously thought to be completely dependent on NHR-49.

(C) Other transcription factors known to play a role in longevity downstream of nutrient-sensing pathways do not alter the activation of the *acs-2P::GFP* reporter after 5 hr of fasting.

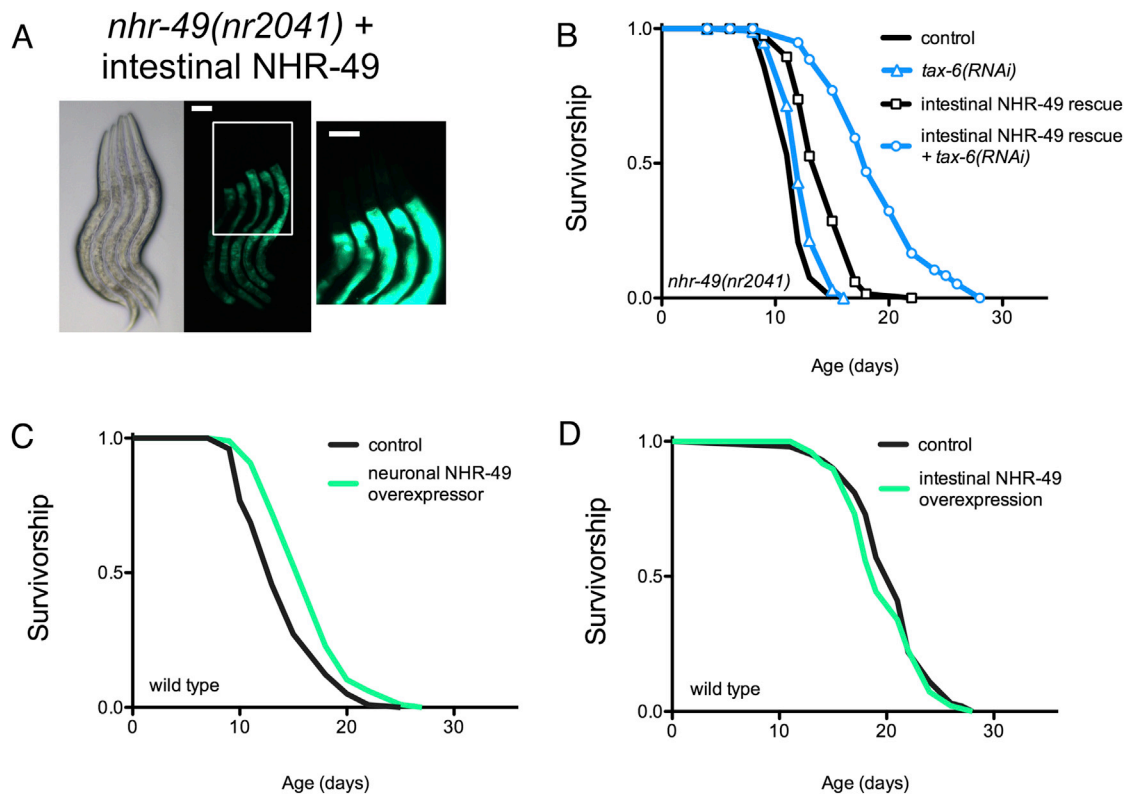


Figure S6. Intestinal NHR-49 Cell Autonomously Regulates *acs-2* Transcription and Is Partially Sufficient for Longevity Downstream of Calcineurin, Related to Figure 5

(A) Fluorescence imaging of *acs-2P::GFP* reporter reveals that unlike neuronal NHR-49, intestinal NHR-49 is only capable of rescuing *acs-2* transcription cell autonomously in the intestine. Scale bars are 50 μm . (Center: box represents the area imaged at greater magnification in the right panel).

(B) Lifespan curves demonstrate that rescuing NHR-49 selectively in the intestine restores the ability of *tax-6* RNAi to extend lifespan.

(C and D) Lifespan curves demonstrating that overexpression of NHR-49 from the pan-neuronal *rab-3* promoter increases longevity in otherwise wild-type worms (C), while overexpression from the *ges-1* intestinal promoter fails to extend lifespan (D).

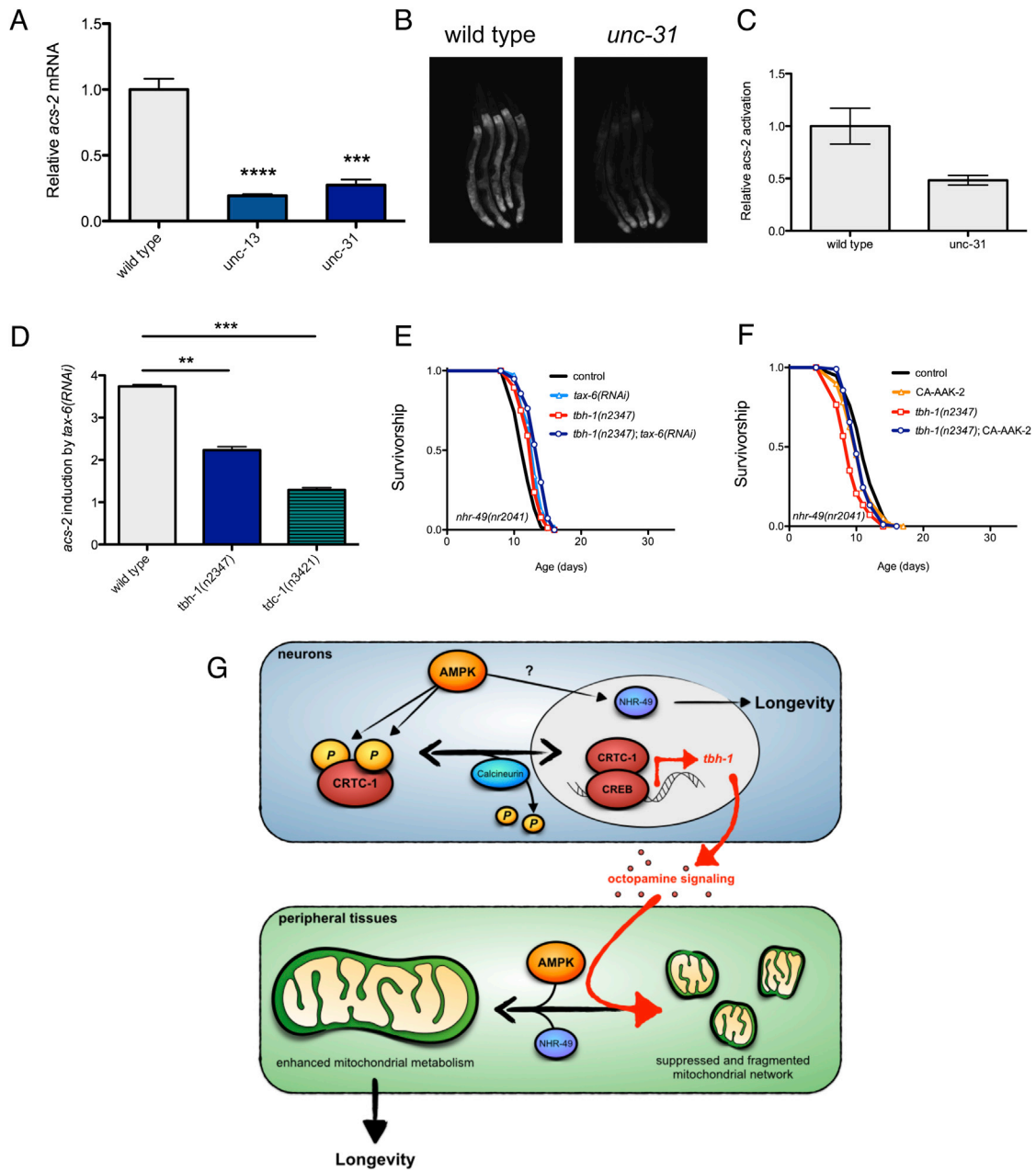


Figure S7. NHR-49 Regulation of Metabolism and Lifespan Lies Downstream of Neuronal Octopamine Signaling, Related to Figure 7

(A) Representative qRT-PCR analysis of *acs-2* mRNA levels reveals that mutants defective in release of synaptic vesicles exhibit decreased *acs-2* expression. Data are mean \pm SD from samples of 50+ worms; ***p = 0.0002, ****p < 0.0001 (t test).

(B and C) Representative fluorescence images of *acs-2::GFP* reporter demonstrating that animals deficient in synaptic vesicle release have impaired metabolic responses to 6 hr fasting in peripheral tissues (B). Fluorescence levels are quantified in (C). Mean \pm SEM from 3 samples of 5 worms; p = 0.0073 by t test.

(D) Representative qRT-PCR analysis reveals that octopamine signaling plays a role in the induction of *acs-2* transcription by calcineurin/*tax-6* RNAi. Values are normalized to wild-type control. Data are mean \pm SD from samples of 50+ worms per condition; **p = 0.0018, ***p.0004 (t test).

(E and F) Lifespan curves showing deficient octopamine signaling does not suppress the short lifespan of *nhr-49(nr2041)* mutants or reverse its effects on AMPK/calcineurin longevity.

(G) Model depicting AMPK/CRTC-1 regulation of mitochondrial metabolism and longevity.