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Highlight:
- REGE-1, related to the RNase MCPIP1/Zc3h12a/Regnase-1, controls fat in *C. elegans*
- REGE-1 degrades mRNA encoding the transcription factor ETS-4
- ETS-4 induces expression of fat metabolic and defense genes
- REGE-1 and ETS-4 form a dynamic, auto-regulatory module controlling body fat
Ribonuclease-Mediated Control of Body Fat

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SUMMARY

Obesity is a global health issue, arousing interest in molecular mechanisms controlling fat. Transcriptional regulation of fat has received much attention, and key transcription factors involved in lipid metabolism, such as SBP-1/SREBP, LPD-2/C/EBP, and MDT-15, are conserved from nematodes to mammals. However, there is a growing awareness that lipid metabolism can also be controlled by post-transcriptional mechanisms. Here, we show that the Caenorhabditis elegans RNase, REGE-1, related to MCP1p1/Zc3h12a/Regnase-1, a key regulator of mammalian innate immunity, promotes accumulation of body fat. Using exon-intron split analysis, we find that REGE-1 promotes fat by degrading the mRNA encoding ETS-4, a fat-loss-promoting transcription factor. Because ETS-4, in turn, induces reg-1 transcription, REGE-1 and ETS-4 appear to form an auto-regulatory module. We propose that this type of fat regulation may be of key importance when, if faced with an environmental change, an animal must rapidly but precisely remodel its metabolism.

INTRODUCTION

Remodeling energy metabolism is critical for development, tissue homeostasis, and the etiology of numerous diseases including obesity and cancer (Folmes et al., 2012; Longo and Mattson, 2014; Ward and Thompson, 2012). Such remodeling can be induced by adverse environmental stimuli leading to a state of suspended animation or torpor. For example, hibernating animals use stored lipids, rather than carbohydrates, to fuel survival (Dark, 2005). In the wild, the Caenorhabditis elegans species populates temperate climates (Barrière and Félix, 2005), indicating that these animals are capable of surviving spells of cold. Indeed, when adapted to a decreasing temperature, C. elegans survives the exposure to near-freezing temperatures (Murray et al., 2007; Ohta et al., 2014).

In this study, we observed that C. elegans also utilize fat while in cold. Using cold sensitivity as the readout, we identified a putative RNase, REGE-1, related to the mammalian MCP1p1/Zc3h12a/Regnase-1, as a factor critical for C. elegans body fat accumulation. The examples of post-transcriptional regulation of fat remain few and are limited to specific microRNAs, whose functionally relevant mRNA targets remain, in most cases, unclear (Amer and Kulyté, 2015; Rottiers and Näär, 2012). In animals, microRNAs repress their mRNA targets through translational repression and/or exonucleolytic degradation (Wilczynska and Bushell, 2015). By contrast, MCP1p1 is a PIN-domain endonuclease (Iwasaki et al., 2011; Matsushita et al., 2009; Xu et al., 2012), suggesting a possible requirement for the endonucleolytic mRNA degradation in C. elegans body fat regulation. Indeed, we demonstrate that REGE-1 controls body fat by targeting the 3’ UTR of an mRNA encoding a fat-loss-promoting transcription factor, ETS-4. Interestingly, while REGE-1 inhibits ETS-4, ETS-4 promotes the expression of REGE-1. Such an auto-regulatory module is particularly well suited for a dynamic control of gene expression. It may be of key importance when, faced in the wild with an environmental change, an animal must rapidly remodel its fat metabolism to maximize its chances for survival.

RESULTS

Cold-Sensitivity Screen Reveals REGE-1 as a Factor Promoting Body Fat

Extending the initial observations by others that C. elegans, when adapted to a decreasing temperature, can survive exposure to near-freezing temperature (Murray et al., 2007; Ohta et al., 2014), we found that the animals can do so for many days, without a major impact on fecundity or lifespan (Figures S1A–S1C). To what degree the C. elegans response to cold is analogous to that of hibernating animals is not clear. Nevertheless, consistent with fueling cellular processes by fat in hibernators, we observed a gradual decline in the fat levels in “hibernating” C. elegans (Figure S1D). This observation prompted us to use cold sensitivity as a readout to uncover new regulators of fat stores. We performed a genome-wide RNAi screen, searching for genes essential at 4°C but non-essential at 20°C (Figure 1A). This approach uncovered factors implicated in diverse cellular processes (Figures 1B and S1E). Importantly,
the depletion of several factors caused a “pale” appearance of animals already at 20 °C, indicating reduced stores of body fat. Indeed, some of these factors have established roles in fat metabolism (Figure 1B). For example, the mediator complex component MDT-15 regulates expression of many fatty acid metabolism genes (Taubert et al., 2006), and FAT-6/-7 are fatty acid desaturases previously implicated in cold survival (Murray et al., 2007; Watts and Browse, 2000). In addition, knockdown of the hitherto uncharacterized gene C30F12.1 resulted in pale animals. C30F12.1 protein is a member of the CCCH-type zinc-finger family (Liang et al., 2008) and is related to the mammalian MCPIP1/Zc3h12a/Regnase-1, a PIN-domain endonuclease (Xu et al., 2012), which directly degrades specific mRNAs such as those encoding pro-inflammatory cytokines (Iwasaki et al., 2011; Matsushita et al., 2009). Because the RNase and CCCH zinc-finger domains are highly conserved between C30F12.1 and Zc3h12a/MCPIP1/Regnase-1 (Figures S2 and 3A), we named C30F12.1 as REGE-1 (REGnasE-1).

REGE-1 Is a Putative RNase Primarily Expressed in the Intestine

To study REGE-1 in more detail, we generated a deletion allele, rege-1(rrr13), predicted to cause a severe truncation of the protein (Figure 3A). Consistent with the RNAi-mediated depletion, rege-1(rrr13) animals were viable but displayed reduced fat (Figure 2D), suggesting that, to promote fat accumulation, REGE-1 functions in the soma.

REGE-1 Promotes Body Fat through the Transcription Factor ETS-4

Assuming that REGE-1 functions similarly to Regnase-1, depleting REGE-1 would be expected to primarily affect the levels of mature but not nascent transcripts. It has recently been shown that comparing exonic and intronic expression across conditions (exon-intron split analysis [EISA]) can be used to quantify the levels of nascent and mature transcripts in standard RNA-sequencing (RNA-seq) experiments (Gaidatzis et al., 2015). We thus performed RNA-seq on animals subjected to control or rege-1 RNAi and compared changes in the levels of nascent RNAs (Δintron) with the changes in mRNAs (Δexon). Although most of the observed changes in phenotypes were rescued by a single-copy-integrated, GFP-tagged REGE-1, expressed under the control of endogenous promoter and 3’ UTR (Figures 3B and S3). This REGE-1::GFP was mostly expressed in the intestinal cells adjacent to the pharynx (Figure 3C), suggesting that REGE-1 functions in the intestine, the primary fat-storing organ of C. elegans (Srinivasan, 2014; Watts, 2009). Mammalian Regnase-1 is a cytoplasmic RNase that, by an internal cleavage, induces the degradation of specific mRNA targets (Iwasaki et al., 2011; Matsushita et al., 2009; Xu et al., 2012). Investigating the putative RNase domain of REGE-1, we noticed that the residues critical for the RNase activity of Regnase-1 are conserved in REGE-1 (Figures S2 and 3D), suggesting that REGE-1 may also function as an RNase.

**REGENE-1 SCREEN**

(A) Schematic description of the genome-wide RNAi screen for cold sensitivity. Only those RNAi clones that induced death following the incubation at 4 °C, but not at 20 °C, were investigated further. (B) Factors identified in the cold-sensitivity screen. Factors whose depletion results in “pale” animals, suggesting reduced fat levels, are shown in bold. This group includes a putative RNase, C30F12.1. See also Figure S1.

However, the loss of fat also occurred in REGE-1-depleted, germine-less (glp-1) animals (Figure 2D), suggesting that, to promote fat accumulation, REGE-1 functions in the soma.
mRNA levels could be explained by differential transcription, we observed a small group of transcripts that were affected mainly at the level of mRNA, the most extreme example being ets-4 mRNA (Figures 4A, 4B, and S4A; Tables S1 and S2). Consistent with REGE-1-mediated regulation, we found, by western blot, that the GFP- and FLAG-tagged ETS-4 protein was more abundant upon rege-1 RNAi (Figure 4C). Importantly, we did not detect any obvious expression of this protein in wild-type animals, but observed its strong expression in the intestine nuclei upon rege-1 knockdown (Figure 4C), suggesting that REGE-1 and ETS-4 function in the same tissue. ETS-4 belongs to the ETS-domain family of transcription factors (Sharrocks, 2001). Prior to this work, ETS-4 was studied in the context of aging and ETS-4-regulated genes were reported (Thyagaraj et al., 2010). Interestingly, we found that transcripts reportedly activated by ETS-4 were enriched among the transcripts upregulated in REGE-1-depleted animals (data not shown). This suggested the possibility that the increased expression of ETS-4 may be largely responsible for the changes in gene expression observed in rege-1 animals. To test this, we compared changes in transcript levels between rege-1(rr13) and rege-1(rr13);ets-4(RNAi) animals with the changes in transcript levels between animals subjected to rege-1 or mock RNAi. Impressively, we found that the majority of changes in gene expression observed in the absence of REGE-1 were reversed by the additional depletion of ETS-4 (Figure 4D and Table S3), suggesting that ETS-4 is the major effector of REGE-1. If so, the depletion of ETS-4 would be expected to rescue the phenotypes observed in rege-1(rr13) animals. Indeed, we found that the RNAi-mediated depletion of ets-4 mRNA (but not of other putative targets marked in red in Figure 4A) restored both fat and developmental timing in rege-1(rr13) animals to the wild-type values (Figures 4E and S4B). By examining gene ontology terms associated with transcripts induced by ETS-4 in rege-1(rr13) animals (the transcripts marked in red in Figure 4D), we found two major classes of genes: genes implicated in lipid metabolism and in responses to pathogens (Figure S5A and Table S4). While the former category includes key genes functioning in the fatty acid degradation pathway (Figure S5B), the latter category contains many genes induced upon infection with bacterial or fungal pathogens (Figure S5C) (Engelmann et al., 2011).

REGE-1 Regulates ETS-4 Post-transcriptionally through the 3’ UTR

Regnase-1 is an RNA-binding protein (RBP) that targets 3’ UTRs of specific mammalian mRNAs. To test whether REGE-1 regulates ETS-4 expression by targeting the 3’ UTR of ets-4 mRNA, we produced strains carrying a single-copy-integrated ets-4::gfp, expressed from the endogenous ets-4 promoter and fused to either endogenous (ets-4) or unregulated (unc-54) 3’ UTR. We found that the depletion of REGE-1 caused increased expression of ETS-4::GFP only when this expression was controlled by the ets-4 3’ UTR (Figure 5A). Thus, ets-4 3’ UTR is required for the REGE-1-mediated regulation. To examine this further, we produced a strain expressing a reporter GFP (fused to H2B to accumulate signal in the nucleus, facilitating quantification) from a ubiquitous promoter (dpv-30) under the control of the ets-4 3’ UTR. In wild-type animals, we observed the expression of this reporter in various tissues and across development (reflecting promoter activity, data not shown) but not in the gut nuclei (Figure 5B). In contrast, in rege-1(rr13) mutants, this reporter GFP was additionally expressed in the gut nuclei (Figure 5B). Thus, the ets-4 3’ UTR is not only required but also sufficient for REGE-1-mediated regulation of ETS-4 expression. To examine which part of the ets-4 3’ UTR is targeted by REGE-1, we produced strains expressing the reporter GFP under the control of truncated variants of the ets-4 3’ UTR. We found that the fragment spanning the first one-third of the 3’ UTR (fragment F1, 357 nt) was sufficient for the repression of the reporter GFP in the gut nuclei and that this repression was alleviated upon rege-1 RNAi (Figure 5C). Examining this fragment more closely, we noticed a stretch of homology between the corresponding sequences from other nematode species (Figure S6). To test the functional significance of this fragment (fragment F1S, 115 nt), we “transplanted” it into the otherwise unregulated unc-54 3’ UTR. We found that this transplantation rendered the unc-54 3’ UTR sensitive to the regulation by REGE-1 (Figure 5D), suggesting that the RNA features required for REGE-1-mediated regulation are contained within the F1S fragment.
REGE-1 Induces an Endonucleolytic Cleavage within the ets-4 3' UTR

To test whether the putative RNase activity of REGE-1 is important for the regulation of ets-4 mRNA, we mutated, by genome editing, conserved amino acids (indicated in Figure 3D) in the RNase domain of the endogenous REGE-1 (generating the rrr13 allele of rege-1). As expected, mutating these residues (D231N, D313A, D314A, and D332A) resulted in increased levels of ets-4 mRNA (Figure 6A). If REGE-1 is indeed an RNase, its association with ets-4 mRNA is expected to be short lived. To examine this putative association, we used GFP-tagged and RNase domain-mutated REGE-1 (corresponding to the rrr13 allele). By immunoprecipitating this REGE-1 variant, followed by qRT-PCR-based detection of associated transcripts, we observed that, indeed, REGE-1 associated with ets-4 mRNA (Figure 6B). Based on the homology with Regnase-1 and the above experiments, REGE-1 is expected to induce an endonucleolytic cleavage within the F1S region of ets-4 3' UTR. Initially, we tested this by incubating immunoprecipitates of GFP-tagged, wild-type, and RNase domain-mutated REGE-1 with in vitro-produced RNA, corresponding to the fragment F1S. We observed that the incubation with the wild-type, but not the RNase domain-mutated, REGE-1 resulted in the degradation of the F1S RNA, but had no effect on unrelated RNA (100 nt of unc-54 3' UTR) (Figure 6C).

Finally, although detecting mRNA cleavage intermediates can be difficult, we used a modified 5' RACE (rapid amplification of 5' complementary cDNA ends) approach to amplify the 3' terminal cleavage intermediates of ets-4 mRNA. Rewardingly, this approach revealed that REGE-1 cleaves ets-4 mRNA within the F1S fragment of its 3' UTR (Figure 6D).

REGE-1 and ETS-4 May Form a Dynamic, Auto-regulatory Module

One advantage of post-transcriptional gene regulation is its speed and reversibility. Thus, the REGE-1/ETS-4 axis is potentially well suited to dynamically control ETS-4 levels. To test the dynamics of ETS-4 expression, we examined ets-4 mRNA levels during starvation, which was followed by refeeding. During starvation, ets-4 mRNA levels remained low for several days, increasing somewhat on the sixth day (Figure 7A). In contrast, upon refeeding, ets-4 levels were strongly increased. This increase was, however, transient, as the levels of ets-4 were back to the pre-starvation values only 1 day after refeeding (Figure 7A). With the changing levels of ets-4 mRNA, we expected to see the reciprocal changes in the levels of rege-1 mRNA. However, to our surprise we observed similar, rather than opposite, changes of rege-1 and ets-4 mRNAs (Figure 7A), suggesting their co-regulation. To test this further, we asked whether ETS-4...
affects the levels of *rege-1* mRNA. Indeed, depleting ETS-4 resulted in reduced expression of *rege-1* (Figure 7B). We observed this effect also using a GFP reporter, driven from the *rege-1* promoter and under control of unregulated (*unc-54*) 3’ UTR (Figure 7C), suggesting that ETS-4 induces (directly or indirectly) the transcription of *rege-1*. Thus, our findings suggest that REGE-1 and ETS-4 form an auto-regulatory module, which, upon an environmental change, is capable of rapidly adjusting its transcriptional output by altering ETS-4 levels.

**DISCUSSION**

We describe here a previously uncharacterized RNase, REGE-1, involved in the regulation of body fat. The mammalian Regnase-1 binds mRNAs by recognizing structured RNA, reportedly with the help of a distinct RBP, Roquin (Jeltsch et al., 2014). However, Regnase-1 and Roquin have been recently suggested to function in distinct subcellular compartments and by different molecular mechanisms (Mino et al., 2015). Moreover, in addition to the RNase and zinc-finger domains, Regnase-1-like proteins contain two additional domains, of which one has been recently reported to contribute, at least in vitro, to the RNase activity of Regnase-1 (Yokogawa et al., 2016). Thus, the problems of how exactly Regnase-1-like proteins achieve specificity for selected mRNA targets and whether additional mechanisms contribute to mRNA regulation are only partly understood. Nevertheless, our results suggest that degradation, induced by an endonucleolytic cleavage, is
the most plausible mechanism by which REGE-1 controls ets-4 mRNA. While REGE-1 degrades ets-4 mRNA, ETS-4 stimulates (directly or indirectly) the transcription of rege-1. Such an autoregulatory RNase/transcription factor module is well suited to a rapid and reversible regulation of gene expression. We hypothesize that, coupled to the positive transcriptional feedback, REGE-1-mediated degradation is more efficient at buffering changes in ETS-4 levels. Thus, upon an environmental cue, including but perhaps not limited to nutrient abundance, the reciprocal regulation of REGE-1 and ETS-4 might ensure a rapid and reversible regulation of gene expression. We hypothesize that increased expression of ETS-4 does not alter the module by primarily altering (levels or activity of) REGE-1 and/or ETS-4 will need to be established.

Finally, how precisely increased levels of ETS-4 stimulate fat loss remains an exciting problem for the future, with potential implications for obesity research. Our preliminary observations suggest that increased expression of ETS-4 does not alter feeding behavior, as monitored by pharyngeal pumping of rege-1 mutants (our unpublished data). Therefore, the loss of fat is unlikely to be caused by reduced food consumption. Interestingly, upon refeeding (following long-term fasting), when the intestine is challenged with increased nutrient uptake and utilization, we observed a rapid upregulation of the REGE-1/ETS-4 module, possibly indicating a role for the module in accelerating food utilization. The observed induction of lipid genes by ETS-4, including genes in the fatty acid degradation pathway, is consistent with this hypothesis. Other scenarios are, however, possible. Whether the mammalian Regnase-1...
(C) Immunoprecipitates, on beads, prepared as above, were incubated with labeled RNAs corresponding to either the F1S fragment of ets-4 unregulated (unc-54 supplemented with 2 mM isopropyl library was replicated in the 96-well format. Overnight cultures of this stock, for the genome-wide RNAi screen we used the Ahringer feeding library. The genome-wide RNAi Screen larval stage.

(a) The levels of ets-4 and rege-1 mRNAs were measured, by qRT-PCR, in animals carrying either wild-type or mutated (RNase mut) alleles of rege-1. The RNase mut allele, rege-1 (rrr21), was produced by changing four conserved residues in the RNase domain of REGE-1 (D231N, D313A, D314A, and D332A; see Figure 3D). Mutating these residues increased the levels of ets-4 mRNA (left); this increase was not due to a lower expression of rege-1 (right). The mRNA levels were normalized to the levels of act-1 (actin) mRNA. Error bars here and in subsequent panels represent SEM.

(B) Extracts of wild-type animals, which either did not express GFP-tagged REGE-1, or expressed wild-type or the RNase mut GFP-tagged REGE-1, were subjected to immunoprecipitation (IP) using anti-GFP antibodies. Left: Western blot of the immunoprecipitates, showing that the RNase mut REGE-1::GFP was not expressed at a higher level than the wild-type. REGE-1::GFP was detected with REGE-1 antibody. Right: Quantification of the indicated mRNAs, by qRT-PCR, in the immunoprecipitates. As indicated by the enrichment of ets-4 mRNA in the RNase mut REGE-1::GFP IP, REGE-1 associates with the ets-4 mRNA.

In contrast, in innate immune cells, Regnase-1 is well established to inhibit pro-inflammatory cytokines (Iwasaki et al., 2011; Matsushita et al., 2009) which, in turn, can stimulate adipocytes to lose fat (Gregor and Hotamisligil, 2011; Guilherme et al., 2008). Curiously, through ETS-4, REGE-1 regulates expression of many nematode defense genes, suggesting a conserved function of Regnase-1-like proteins in innate immunity. Although ETS-4 may control lipid and defense genes separately, perhaps depending on a particular environmental cue, it is possible that ETS-4-dependent fat loss is linked to an inflammatory response. If so, dissecting the underlying mechanism could provide important insights into the well-known, though insufficiently understood, connection between inflammation and lipid metabolism.

EXPERIMENTAL PROCEDURES

General Animal Handling and RNAi

Unless stated otherwise, animals were grown at 20°C as described previously (Brenner, 1974). RNAi of individual genes was performed by feeding animals with bacteria expressing double-stranded RNA, beginning from the L1 or L4 larval stage.

Genome-wide RNAi Screen

For the genome-wide RNAi screen we used the Ahringer feeding library. The library was replicated in the 96-well format. Overnight cultures of this stock, supplemented with 2 mM isopropyl β-D-1-thiogalactopyranoside, were used to seed 24-well plates. Ten to fifteen staged L4 N2 animals were transferred per well. Knockdown was conducted for about 40 hr and, after inspection for death or sterile animals, the animals were adapted at 10°C for 2 hr and then incubated at 4°C for 3 days. Afterward, the animals were incubated at 20°C for several hours to recover. Dead animals were scored and clones with more than four dead animals were retested at least three times. The RNAi clones were sequenced.

Oil Red O Staining, Image Processing, and Quantification

To visualize overall fat, we performed oil red O staining essentially as described previously (O’Rourke et al., 2009). All image-processing steps were carried out using the Fiji/ImageJ software suite (Schindelin et al., 2012). RGB images were stitched with the Grid/Collection stitching plug-in (Preibisch et al., 2009) and corrected for white balance by equalizing background mean values in the red, green, and blue channels. After conversion from RGB to HSB color space and background subtraction, red pixels were selected by thresholding the “H” (Hue) channel (only pixels with a Hue value between 0 and 7 are kept). A binary mask was created with the Saturation channel and applied to the thresholded image. After conversion to 32-bit, zero pixel values were replaced by NaN. The integrated density of all remaining pixels was used as an index of the amount of red staining in the animals (Fiji/ ImageJ macro available upon request).

Triacylglyceride Assay

Total triacylglyceride content was assayed essentially as described previously (Martorell et al., 2012), using the Triglyceride Quantification Colorimetric/Fluorometric Kit from Biovision (catalog no. K622-100). Animals were synchronized by hypochlorite treatment and grown on OP50 plates at 20°C to the L4 stage. RNAi knockdown was performed for 48 hr. After collecting 50 animals, and two washes in PBS, the animals were pelleted and resuspended in 300 µL of triacylglyceride buffer. Sonication was conducted with a Branson Digital Sonifier five times at 10% power for 30 s. Worm debris were excluded by centrifugation and supernatant heated twice to 95°C for 5 min. Amounts of the supernatant (50 and 25 µL) were used to assess triacylglyceride content according to the

Figure 6. REGE-1 Induces an Endonucleolytic Cleavage within the ets-4 3’ UTR

(a) The levels of ets-4 and rege-1 mRNAs were measured, by qRT-PCR, in animals carrying either wild-type or mutated (RNase mut) alleles of rege-1. The RNase mut allele, rege-1 (rrr21), was produced by changing four conserved residues in the RNase domain of REGE-1 (D231N, D313A, D314A, and D332A; see Figure 3D). Mutating these residues increased the levels of ets-4 mRNA (left); this increase was not due to a lower expression of rege-1 (right). The mRNA levels were normalized to the levels of act-1 (actin) mRNA. Error bars here and in subsequent panels represent SEM.

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unknown mechanism(s), to alter the level of ETS-4 and/or REGE-1. Because REGE-1 inhibits ETS-4 (by degrading ets-4 mRNA) and, conversely, ETS-4 promotes REGE-1 (by inducing, directly or indirectly, rege-1 transcription), a change in either protein eventually alters the abundance of ETS-4. This leads to altered expression of ETS-4 target genes, including fat catabolic and innate immunity genes, consequently leading to either gain (when ETS-4 is low) or loss (when ETS-4 is high) of fat.

Coherent Anti-Stokes Raman Spectroscopy
Animals were grown at 20°C from L1 to L4 stage and then transferred to RNAi plates. After 48 hr, animals were mounted on a slide with a drop of 2% agarose with 20 mM levamisole. The animals were then examined by CARS microscopy on a Leica TCS SP8 system with a CARS laser picomerald (optical parametric oscillator, >600 mW at 780–940 nm, pulse width 5–6 ps, 80 MHz; pump, >700 mW at 1,064 nm, pulse length 7 ps, 80 MHz) and with LAS AF software. The lasers were adapted to the symmetrical C-H stretch range by tuning the pump beam to 816.4 nm while keeping the Stokes beam constant at 1,064.6 nm. The output of both lasers was set to 1.3 W and the scan speed to 400 Hz. An image with the dimensions 145.31 x 145.31 μm (968 x 968 pixels) and stacks of approximately 30–50 sections with a step size of approximately 0.6 μm were collected. Only signals from epi-CARS (E-CARS) and epi-SHG (E-SHG) detectors were collected. Animals were imaged just below the pharynx with one stack per animal and approximately ten animals per condition. Each experiment was repeated three times. The number and size of lipid droplets in each stack were extracted with TRIzol as described previously (Arnold et al., 2014). Subsequently, RNA was depleted of rRNA using the Ribo-Zero Magnetic Kit (MRZ11124C) from epicenter and column purified with the RNA Cleanup & Concentrator from Zymo Research. Quality of RNA was monitored by Bioanalyzer RNA Pico chip. The library was prepared using the ScriptSeq v2 RNA-Seq Library Preparation Kit (Epigenetics).

Gene expression levels (exonic) from RNA-seq data were quantified as described previously (Hendriks et al., 2014). After normalization for library size, log2 expression levels were calculated after adding a pseudocount of 8 (y = log2[x + 8]). The rege-1 and ets-4 RNAI experiments were normalized separately. Intronic expression levels were quantified as previously described (Gaidatzis et al., 2015). EISA was performed for a subset of genes (n = 3,093) that showed an average expression level (considering all samples) of at least 4.5 on the exonic as well as the intronic level. Genes (n = 283) both upregulated upon rege-1 RNAI and downregulated upon ets-4 RNAI were subjected to gene ontology over-representation analysis using the PANTHER classification system (Mi et al., 2005) with default settings.

The genomic data have been deposited at the GEO with accession number GEO: GSE75163. See also Tables S1, S2, and S3.
qRT-PCR

RNA for qRT-PCR was extracted as described above. Three hundred nanograms of RNA was used for reverse transcription utilizing the QuantiTect Reverse Transcription Kit (Qiagen). The resulting cDNA was diluted 1:10 for further analysis. Five microliters of this dilution was used with equal volume of Express SYBR GreenER qPCR SuperMix w/ROX (Invitrogen) containing 0.2 μL of 10 mM gene-specific primers. The StepOne RT-PCR system combined with StepOne Software (Applied Biotechnologies) was used for analysis. The presented values are based on three biological replicates.

Western Blot Analysis

Western blot analysis was conducted as described previously (Arnold et al., 2014). Primary antibodies diluted in 4% milk/PBS-Tween 20 were mouse α-ACT-1 (1:2,000; MAB1501, Millipore) and mouse α-GFP (1:1,000; Roche). Secondary antibodies were horseradish peroxidase-coupled x-mouse (1:14,000; GM Healthcare), and the REGE-1 polyclonal rabbit antibody was raised against the first 119 amino acids (SDIX), used at 1:1,000.

Generation of rege-1(rrr13) and ets-4(rrr16) Alleles, GFP-Tagged REGE-1 and ETS-4, and GFP Reporter Lines

The rege-1(rrr13) and ets-4(rrr16) alleles were generated by CRISPR/Cas9 (Arrバレ et al., 2014; Katic et al., 2015). The mutations were verified by sequencing and outcrossed six times before analyzing. The ETS-4::GFP, REGE-1::GFP, and 3’ UTR reporter lines were constructed by MosSCI (Frokjaer-Jensen et al., 2008). In constructing the ets-4::gfp, we used the ets-4 promoter sequence (2,634 bp), ets-4 genomic sequence fused to the gfp, and the ets-4 3’ UTR (938 bp). In constructing the rege-1::gfp, the promoter sequence (2,989 bp) was fused to rege-1 CDS (1,908 bp) gfp, and rege-1 3’ UTR (262 bp). To check whether the F15 sequence is sufficient for REGE-1-mediated regulation, we inserted it into an otherwise unregulated 3’ UTR, unc54, between bp 164 and 165. The single guide RNA sequences and primers used for construct generation are provided in Supplemental Experimental Procedures.

Quantification of the ETS-4 3’ UTR GFP Reporter

Images for quantification of GFP intensity of reporter strains were acquired with an AxioImager.Z1 microscope (Zeiss) equipped with a 63x objective and an MFm camera (Zeiss). Signal intensity of a circular area of 52-pixels objective was measured in ImageJ and normalized to the background. In addition, 30–35 animals per strain were visually inspected for GFP expression.

Generation of Point Mutations in the REGE-1 RNase Domain

Four point mutations (D231N, D313A, D314A, and D332A) were introduced at the endogenous locus of C30F12.1, by CRISPR/Cas9 genome editing performed by Knudra Transgenics. The editing was performed in two steps: in the first D231N and then, simultaneously, D313A, D314A, and D332A. The obtained mutations, resulting in the rege-1(m271) allele, were verified by sequencing and the homozygous quadruple mutant was outcrossed three times to the wild-type.

Starvation and Refeeding

The assay was modified after Seidel and Kimble (2011). Animals synchronized by hypochloride treatment were hatched overnight in M9 and grown to young L4 stage. They were collected, washed two times with M9 buffer, and transferred to plates devoid of bacteria. For refeeding, the animals were transferred to bacteria-seeded plates. At indicated times, RNA was extracted and processed as described earlier.

REGE-1 Immunoprecipitation/ETS-4 qRT-PCR

Proteins were extracted from staged young adults as described for western blot analysis. Forty microliters of Chromotek GFP Trap A was washed twice in EB++ (EB + RNasin 5 μL/ml from Promega) and 3 mg of protein was added to a final volume of 1 mL. After incubation overnight on 4°C, the beads were washed four times with 600 μL of EB++ and RNA was extracted directly from the beads by adding 500 μL of TRIzol. After RNA extraction and subsequent qPCR analysis, fold enrichment was calculated as follows: Enrichment RNA immunoprecipitation (RIP) over input: ΔCt [normalized RIP] = Ct [RIP] – Ct [input] – log2(input dilution factor)); normalized to control/non-specific (NS) IP ΔΔCt [RIP/NS] = ΔCt [normalized RIP] – ΔCt [normalized NS]; fold enrichment: 2ΔΔCt [RIP/NS].

On-the-Beads RNAse Assay

Radioactively labeled RNAs were transcribed from PCR products. Templates for transcription were generated by PCR with an extended phase T3 RNA polymerase promoter (ATTAAACCTCTACTAAAGGGAGAA) appended to the 5’ end of the 5’ primer, and gel purified. Transcription was performed in 3–μL reactions containing 0.5 μL of template, 1.5 μL of sp32 UTP (3 μM), and 0.6 μL of 5x transcription buffer (Promega), 0.2 μL of RNasin (Promega), and 2.5 mM rATP, 2.5 mM rGTP, 2.5 mM rCTP, and 0.025 mM rUTP (adenosine, guanidine, cytidine, and uridine triphosphates; Roche) at 37°C for 3 hr. The reaction was stopped by adding 30 μL of Tris-EDTA and the RNA was purified on Sephadex G-25 columns (Roche) using the manufacturer’s instructions. REGE-1::GFP was immunoprecipitated as described above. The beads were washed additionally once with RNase assay buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM MgCl2, 2 mM DTT). RNA (103 cpm) was suspended in RNase assay buffer and incubated with immunoprecipitation beads for 15 min at room temperature. The tubes were centrifuged and the supernatant loaded on a pre-run 6% denaturing polyacrylamide gel. After electrophoresis at 200 V for 90 min, the gel was dried and autoradiographed.

Modified 5’ RACE

To identify the 3’-terminal cleavage product of ets-4 mRNA, we employed a modified 5’ RLM RACE protocol (Schmidt et al., 2015). Total RNA (3–5 μg) was ligated with 0.8 μg of 5’ RNA linker (GUUCAGAGUUCCAGUGCCGAUCCGAGUAUGAC) in a 10-μL reaction with 5 μL of T4 RNA ligase in 1x RNA ligase buffer (NEB) and 1.5 mM ATP. The ligated RNA sample was reverse transcribed with a gene-specific primer for ets-4 and SuperscriptIII reverse transcriptase (Thermo Fischer Scientific) using the manufacturer’s instructions. PCR was performed to obtain the cleaved product using a forward primer in 5’ RNA linker and ets-4 reverse primer upstream of the RT primer. PCR products were sequenced to determine the cleavage site.

ACCESSION NUMBERS

The WormBase ID for the gene C30F12.1 is: WBGene00016260.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2016.09.018.

AUTHOR CONTRIBUTIONS

R.C. wrote the manuscript and designed the experiments. C.H. designed and performed the majority of the experiments. Y.G. constructed ets-4 transgenic lines, demonstrated the regulation of rege-1 by ETS-4, and helped in analyzing the genomic data. R.V. performed the initial cold-sensitivity screen and analysis. P.K. assisted with the 5’ RACE and on-beads RNAse assay. A.N. assisted with constructing some transgenic strains. D.G. analyzed the RNA-seq data. E.B.H. and N.J.F. performed the CARs experiment. H.G. did homology analysis and modeling of the C. elegans RNase domain. All authors contributed to interpretation of the data and provided comments on the manuscript.

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