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The *C. elegans* MicroRNA *mir-71* Acts in Neurons to Promote Germline-Mediated Longevity through Regulation of DAF-16/FOXO

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SUMMARY

The life span of *Caenorhabditis elegans* is controlled by signaling between the germline and the soma. Germ cell removal extends life span by triggering the activation of the DAF-16/FOXO transcription factor in the intestine. Here we analyze microRNA function in *C. elegans* aging and show that the microRNA *mir-71* functions to mediate the effects of germ cell loss on life span. *mir-71* is required for the life span extension caused by germline removal, and overexpression of *mir-71* further extends the life span of animals lacking germ cells. *mir-71* functions in the nervous system to facilitate the localization and transcriptional activity of DAF-16 in the intestine. Our findings reveal a microRNA-dependent mechanism of life span regulation by the germline and indicate that signaling among the gonad, the nervous system, and the intestine coordinates the life span of the entire organism.

INTRODUCTION

Genetic studies of *C. elegans* have identified numerous genes that function in highly conserved pathways to control aging (Kenyon, 2010). For example, in an insulin-like signaling pathway the DAF-2 insulin/IGF-1 receptor homolog activates a conserved phosphatidylinositol 3-kinase pathway to shorten life span by inhibiting the activity of DAF-16, a FOXO family transcription factor (Tatar et al., 2003). DAF-16 promotes longevity by regulating the expression of a number of targets, including antioxidant, antimicrobial, and metabolic enzymes (Lee et al., 2003a; Murphy et al., 2003; Oh et al., 2006).

The reproductive systems of worms and possibly also those of flies and mammals regulate life span (Kenyon, 2010). For example, when the germline of *C. elegans* is removed either by laser microsurgery or by mutations that block germ cell proliferation, animals live up to 60% longer than control animals (Arantes-Oliveira et al., 2002; Hsin and Kenyon, 1999). This life span extension requires the activities of DAF-16 and of the steroid hormone receptor DAF-12 (Hsin and Kenyon, 1999). In animals lacking germ cells, DAF-16 accumulates specifically in the intestinal nuclei and activates the transcription of stress-related and metabolic genes (Lin et al., 2001; Wang et al., 2008; Yamawaki et al., 2008). Upon germline removal, the somatic gonad promotes longevity by triggering a pathway involved in the biosynthesis of the endogenous ligands for DAF-12 (Gerisch et al., 2001, 2007; Yamawaki et al., 2010).

MicroRNAs, a class of small noncoding RNAs, have emerged as critical posttranscriptional regulators of gene expression in diverse biological processes (Ambros, 2004; Stefani and Slack, 2008). The first microRNAs discovered, the products of the *C. elegans* genes *lin-4* and *let-7*, control the timing of developmental events (Ambros and Horvitz, 1984; Chalfie et al., 1981; Reinhart et al., 2000). *C. elegans* microRNAs also control cell-fate specification, embryonic development, physiology, behavior, neural synaptic activity, and longevity (Alvarez-Saavedra and Horvitz, 2010; Boehm and Slack, 2005; Chang et al., 2004; Simon et al., 2008; Yoo and Greenwald, 2005). In this study we performed a comprehensive search for microRNA genes that regulate *C. elegans* life span by determining the life spans of mutants for most known microRNA genes. We show that the microRNA *mir-71* acts in the nervous system to mediate the effects of the germline on longevity.

RESULTS

mir-71 Functions to Promote Longevity and Stress Resistance and Delay Aging

We have previously described the isolation and initial characterization of a large collection of strains that carry deletions in most of the 115 known *C. elegans* microRNA genes (Miska et al., 2007). To identify microRNAs that function in the aging process, we tested these microRNA mutants for defects in aging and longevity. Specifically, we determined the life spans of 81 mutant strains that carried deletions in 90 microRNA genes. We found that most microRNAs are dispensable for normal life span (see Table S1 available online). Strikingly, two independently isolated deletion mutants of *mir-71* each displayed a severe decrease in life span of about 40% (Figure 1A). Since longevity and resistance to stresses are frequently coupled, we subjected *mir-71* mutant adults to various stresses. We found that *mir-71* mutants showed increased sensitivity to both heat shock and oxidative stress (Figures S1A–S1C). A transgene that contained a wild-type copy of the *mir-71* genomic locus rescued both the short life span

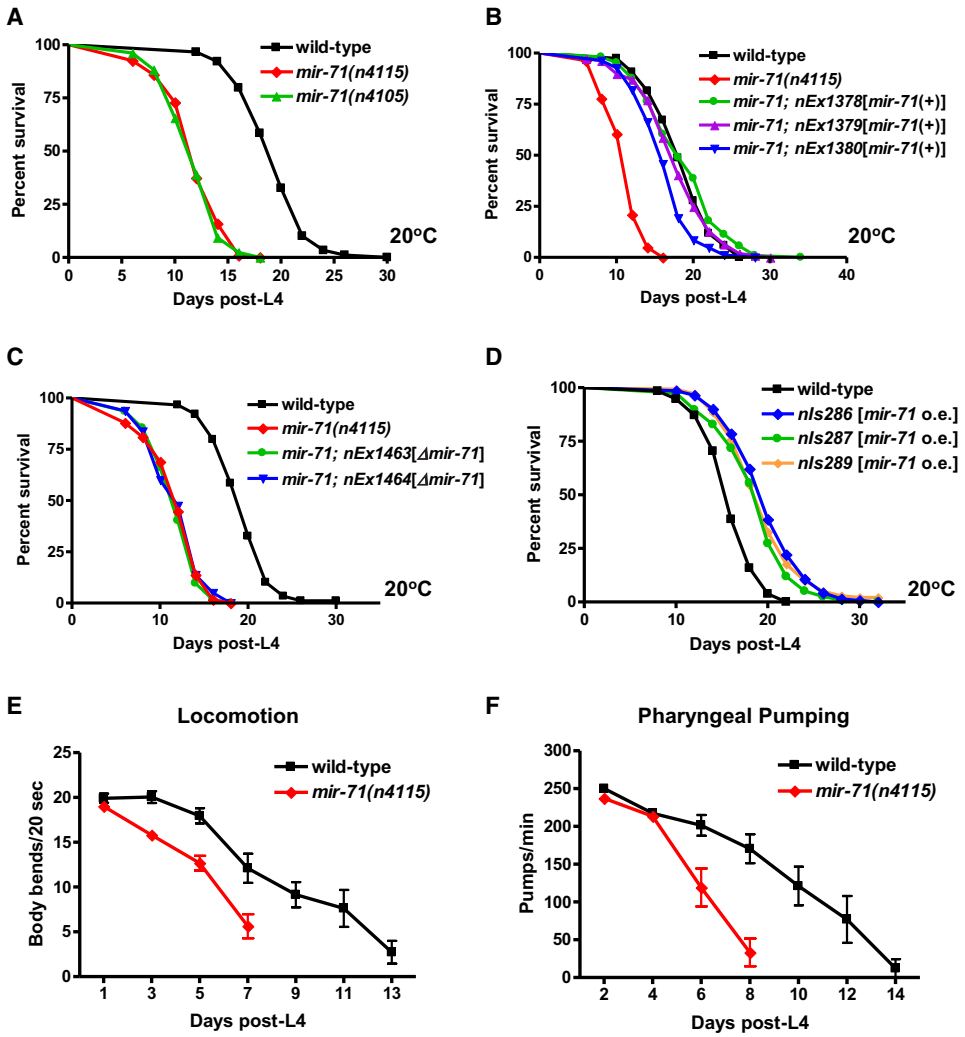

Figure 1. The MicroRNA *mir-71* Functions to Promote Longevity and Delay Aging

(A) *mir-71* mutants have a short life span ($p < 0.0001$; 40% reduction in mean life span).

(B) A genomic fragment containing the *mir-71* locus rescued the life span defect of *mir-71(n4115)* mutants ($p < 0.0001$; 45%–70% mean life span extension compared to *mir-71(n4115)*).

(C) A genomic fragment lacking the mature *mir-71* sequence failed to rescue the life span defect of *mir-71(n4115)* mutants ($p > 0.1$ compared to *mir-71(n4115)*).

(D) Extra copies of *mir-71* extended life span ($p < 0.0001$; 15%–25% mean life span extension).

(E) Young day 1 *mir-71* adults showed normal levels of locomotion ($p > 0.1$; mean 19.0 body bends/20 s) compared to wild-type day 1 adults (mean velocity, 19.9 body bends/20 s). At day 7, *mir-71* adults showed a 50% decrease in locomotion ($p < 0.01$; mean 5.6 body bends/20 s) compared to wild-type adults of the same age (mean 12.1 body bends/20 s) (wild-type, n = 11; *mir-71(n4115)*, n = 12). Error bar, standard error of the mean (SEM).

(F) Young *mir-71(n4115)* adults showed normal levels of pharyngeal pumping compared to the wild-type. The pumping rate of day 2 *mir-71* adults was only slightly reduced ($p < 0.01$; mean pumping rate 237.1 pumps/min) compared to the wild-type (mean pumping rate 250.0 pumps/min), and day 4 *mir-71* adults had a pumping rate ($p > 0.1$; mean pumping rate 213.3 pumps/min) indistinguishable from that of the wild-type (mean pumping rate 217.3 pumps/min). At day 8, *mir-71* adults showed a large decrease in pumping rate ($p < 0.0001$; mean pumping rate 33.2 pumps/min) compared to wild-type adults of the same age (mean pumping rate 170.5 pumps/min) (wild-type, n = 11; *mir-71(n4115)*, n = 12). Error bar, standard error of the mean (SEM). All experiments were repeated at least once with similar effects. Mean life span values and statistical analyses of life span assays are shown in Table S2. p values refer to the experimental strain and corresponding wild-type control animals unless otherwise noted.

(Figure 1B) and the heat-stress sensitivity of *mir-71* mutants (Figure S1D). Removing the 22 bp sequence of the mature *mir-71* microRNA from this transgene abolished its rescuing activity (Figure 1C). Taken together, these results indicate that *mir-71* is required for normal life span and normal responses to heat and oxidative stress. In agreement with our findings, a recent study of the temporal patterns of microRNA expression during aging reported that *mir-71* is upregulated in aging adults and promotes longevity and stress resistance (de Lencastre et al., 2010).

To determine whether overexpression of *mir-71* is sufficient to extend *C. elegans* life span, we integrated extrachromosomal arrays carrying the *mir-71* locus into the genome and determined the life spans of multiple resulting transgenic lines. We

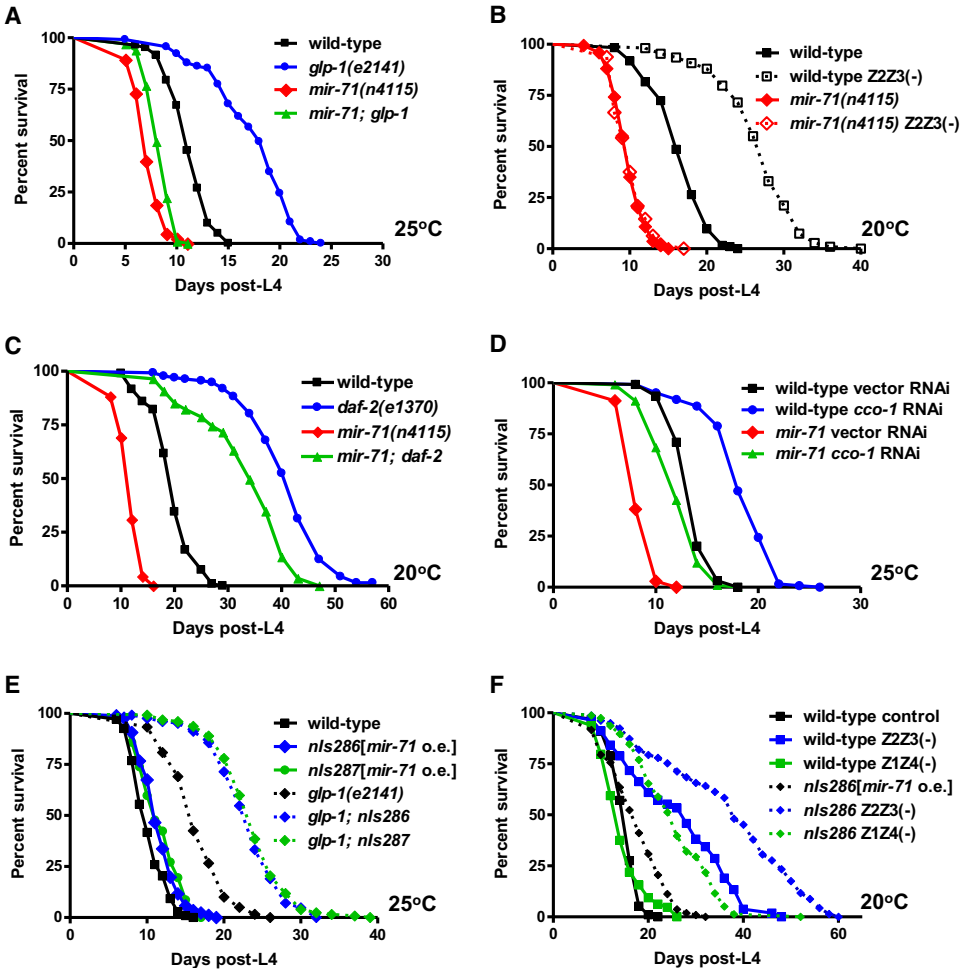

Figure 2. *mir-71* Mediates the Effects of Germ Cell Loss on Longevity

(A) Loss of *mir-71* function suppresses the long life span of germline-deficient *glp-1(e2141)* mutants. Germline removal by *glp-1(e2141)* resulted in a 55% extension of mean life span in otherwise wild-type animals ($p < 0.0001$) compared to a 15% extension in *mir-71(n4115)* mutants ($p < 0.0001$).

(B) Loss of *mir-71* function fully suppresses the increased longevity of germline-ablated animals. Ablation of germline precursor cells Z2 and Z3 resulted in a 60% extension of wild-type mean life span ($p < 0.0001$), while it had no effect ($p > 0.5$) on *mir-71(n4115)* mutant life span.

(C) Loss of *daf-2* function extended both wild-type life span ($p < 0.0001$; 100% mean life span extension) and the life span of *mir-71(n4115)* mutants ($p < 0.0001$; 180% mean life span extension).

(D) *cco-1* RNAi extended both wild-type life span ($p < 0.0001$; 35% mean life span extension) and the life span of *mir-71(n4115)* mutants ($p < 0.0001$; 45% mean life span extension).

(E) Extra copies of *mir-71* modestly extended the life span of intact animals at 25°C ($p < 0.0003$, 14%–15% mean life span extension), whereas it caused a robust extension on the life span of germline-deficient *glp-1(e2141)* animals at 25°C ($p < 0.0001$, 40%–45% mean life span extension).

(F) Extra copies of *mir-71* caused a robust life span extension on the life span of both germline-ablated (Z2 and Z3) ($p < 0.0001$, 40% mean life span extension compared to wild-type Z2Z3[−] animals) and somatic gonad-ablated (Z1 and Z4) animals ($p < 0.0001$, 75% mean life span extension compared to wild-type Z1Z4 [−] animals). All experiments were repeated at least once with similar effects. Mean life span values and statistical analyses of life span assays are shown in Table S2.

found that extra copies of *mir-71* modestly extended life span by 20% (Figure 1D), suggesting that *mir-71* functions to promote longevity and that the short life span of *mir-71* mutants is not the result of nonspecific pathology. Consistent with this hypothesis, aging *mir-71* mutant adults showed a premature reduction in the rates of both locomotion and pharyngeal pumping (Figures 1E and 1F), two behaviors that normally decline with age (Huang et al., 2004). These results indicate that loss of *mir-71* causes an accelerated aging phenotype and suggest that *mir-71* acts to delay aging.

mir-71 Mediates the Effects of Germ Cell Loss on Life Span

To test if *mir-71* functions in one of the pathways known to control *C. elegans* aging, we assayed genetic interactions between *mir-71* and various longevity genes. When temperature-sensitive *glp-1* mutants grow at the restrictive temperature, they fail to develop mature germ cells and as a result live 60% longer than wild-type animals (Figure 2A) (Arantes-Oliveira et al., 2002). Interestingly, we found that mutations in *mir-71* strongly suppressed the long life span of germline-deficient

glp-1 mutants (Figure 2A). To confirm that *mir-71* is required for the life span extension caused by germ cell loss, we used laser microsurgery to ablate the germline precursor cells Z2 and Z3 in wild-type and *mir-71* mutant animals. Whereas germline ablation resulted in a robust life span extension of wild-type animals, it failed to extend the life span of *mir-71* mutants (Figure 2B). These results indicate that *mir-71* is required for the increased longevity of germline-less animals and suggest that *mir-71* mediates the effects of germ cell loss on life span.

To examine if *mir-71* is specifically required for germ cell loss to extend life span, we tested whether deletion of *mir-71* suppresses the long life span of animals with compromised insulin/IGF signaling or defective mitochondrial function. Partial loss-of-function mutations of the *daf-2* Insulin/IGF receptor homolog cause animals to live twice as long as the wild-type (Figure 2C) (Kenyon et al., 1993). We observed that loss of *daf-2* function similarly extended the life span of *mir-71* mutants more than 2-fold (Figure 2C). RNAi that reduced the levels of *cco-1* or *T02H6.11* or a loss-of-function mutation of *isp-1* (all three of these genes encode enzymes that function in mitochondrial respiration [Dillin et al., 2002; Feng et al., 2001; Lee et al., 2003b]) extended the life span of *mir-71* mutants to a similar degree as in a wild-type background (Figure 2D and Figures S2A and S2B). In addition, *mir-71* mutants could extend life span in response to dietary restriction (Figure S2C). Taken together, these results indicate that *mir-71* is specifically required for the life span extension caused by germline removal.

Extra Copies of *mir-71* Further Extend the Life Span of Germline-less Animals

To examine the effect of *mir-71* overexpression on the life span of germline-deficient animals, we introduced the integrated *mir-71* transgenes into *glp-1* mutants. While extra copies of *mir-71* resulted in a modest life span extension in germline-intact animals, overexpression of *mir-71* extended the life span of germline-deficient *glp-1* animals by more than 40% (Figure 2E). In agreement with this observation, ablation of the germline precursor cells Z2 and Z3 further extended the life span of *mir-71* overxpressors by about 40% compared to germline-ablated wild-type controls (Figure 2F). Interestingly, extra copies of *mir-71* also robustly extended the life span of somatic gonad-ablated animals, indicating that the somatic gonad is not required for *mir-71*-mediated life span extension in the absence of the germline (Figure 2F). This effect was specific to germline-deficient animals, as extra copies of *mir-71* caused little or no effect on the long life span of either *daf-2* mutants or animals with defective mitochondrial respiration, respectively (Figure S3). These results indicate that *mir-71* overexpression is sufficient to extend further the life span of germline-less animals and suggest that the presence of the germline limits the life span-promoting activity of *mir-71*.

mir-71 Is Broadly Expressed during Development and Adulthood

To identify the tissue(s) in which *mir-71* functions to regulate longevity, we monitored the spatial pattern of *mir-71* expression using a gfp transcriptional reporter. We found that a P*mir-71::gfp* reporter was broadly expressed; stronger signal was detected in the intestine, body wall muscles, and neurons during larval development and adulthood, and weaker expression was observed in the hypoderm of adult animals (Figure 3A) (Martinez et al., 2008). Germline removal did not affect the expression pattern of this *Pmir-71::gfp* reporter during development and adulthood (Figure S4).

mir-71 Functions in Neurons to Promote Germline-Mediated Longevity

To test if *mir-71* functions in the intestine, muscles, neurons, or hypoderm to mediate the effects of germ cell loss on life span, we generated genetic mosaics that lacked *mir-71* gene function in a subset of cell lineages. Genetic mosaics were produced by the spontaneous loss of an extrachromosomal array that carries the only wild-type gene copy of *mir-71* as well as gfp reporters that serve as cell lineage markers (Yochem and Herman, 2003). We identified two classes of genetic mosaics: *mir-71* AB(−) mosaics that lacked *mir-71* function in the AB lineage, which generates almost the entire nervous system and most of the hypoderm, but retained *mir-71* function in the P1 lineage, which gives rise to the intestine, muscles, gonad, and part of the hypoderm; and *mir-71* E(−) mosaics that lacked *mir-71* function in the E lineage (a subset of the P1 lineage), which generates the entire intestine, but retained *mir-71* function in the AB lineage (Figure 3B and the Experimental Procedures). As expected, germline removal in animals that carried the *mir-71*-expressing array in presumably all tissues caused extension of life span by 60% (Figure 3C). We observed that the life span of *mir-71* AB(−) mosaics was extended by only 23% by germline removal, indicating that the activity of *mir-71* in the AB lineage was largely required for the effect of germ cell loss on life span (Figure 3D). On the other hand, we found that the life span of *mir-71* E(−) mosaics was extended by 60% by germline removal, indicating that *mir-71* function in the AB lineage is sufficient to fully restore the effect of germ cell loss on life span, whereas intestinal *mir-71* activity is dispensable (Figure 3E). Therefore, our mosaic analysis shows that *mir-71* activity in the AB lineage, which generates almost all neurons and most of the hypoderm, is both necessary and sufficient for germline-mediated longevity.

Since *Pmir-71::gfp* expression was detected in neuronal and hypodermal cells, we hypothesized that *mir-71* likely acts in either the nervous system or the hypoderm to mediate the extension of life span that occurs in the absence of the germline. To distinguish between those alternatives, we performed tissue-specific rescue experiments. Expression of *mir-71* under the control of the ubiquitous *rpl-28* promoter strongly rescued the short life span of *mir-71; glp-1* mutants (Figures 4A and 4B). Similarly, driving expression of *mir-71* in neurons alone, using the pan-neuronal *unc-119* and *rab-3* promoters, resulted in strong rescue of the life span defect of *mir-71* mutants; their life span was extended by germline removal by more than 40% or 30%, respectively (Figure 4C and Figure S5). By contrast, hypodermal-specific expression of *mir-71* failed to restore the effect of germ cell loss on life span (Figure 4D). Taken together, these results indicate that *mir-71* functions in the nervous system to promote germline-mediated longevity.

mir-71-Mediated Life Span Extension in Germline-less Animals Depends on *daf-16*

The long life span of animals that lack germ cells depends on the activities of DAF-16 and a pathway that signals through the

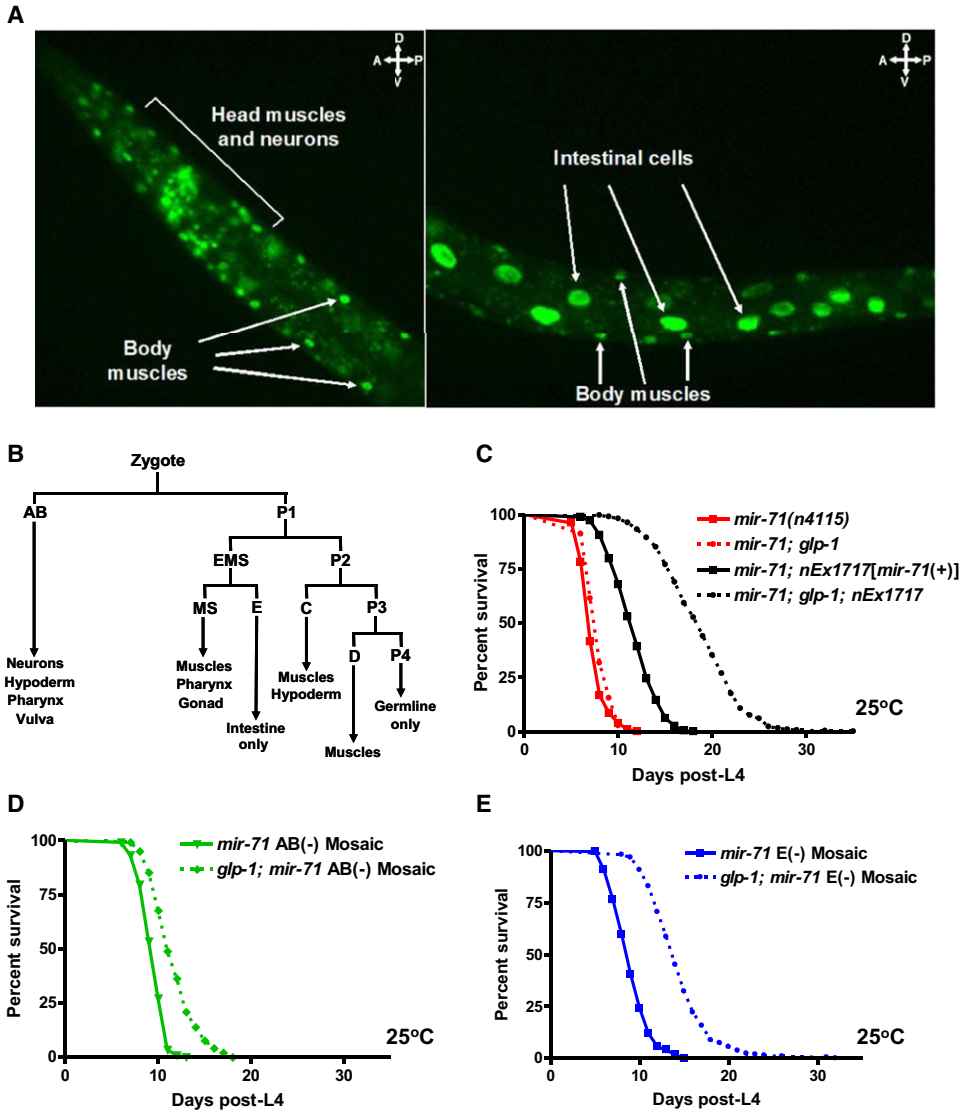

Figure 3. *mir-71* Functions in the AB Lineage to Regulate the Life Span of Germline-Deficient Animals

(A) A P*mir-71::gfp* reporter was strongly expressed in the intestine, body wall muscles, and neurons during adulthood.

(B) Shown is a cell-lineage diagram indicating tissues produced by the early blastomeres of *C. elegans*.

(C–E) *mir-71* functions primarily in AB-derived tissue(s) to mediate the effects of germline on life span. Germline removal had a minor effect on the life span of *mir-71* mutants ($p < 0.0001$; 7% mean life span extension), whereas it robustly extended the life span of animals that carried the *mir-71*-expressing array presumably in all tissues ($p < 0.0001$; 60% mean life span extension). Whereas *mir-71* E(−) mosaics fully responded to germline removal ($p < 0.0001$; 60% mean life span extension), the life span of *mir-71* AB(−) mosaics was only modestly extended by germ cell loss ($p < 0.0001$; 23% mean life span extension). Mean life span values and statistical analyses of life span assays are shown in Table S2.

DAF-12 steroid hormone receptor (Gerisch et al., 2007; Hsin and Kenyon, 1999). It is thought that signals from the germline regulate the subcellular localization and transcriptional activity of DAF-16, while signals from the somatic gonad control the biosynthesis of DAF-12 ligands (Lin et al., 2001; Yamawaki et al., 2008, 2010). To test whether *mir-71* genetically interacts with either *daf-16* or *daf-12* to regulate life span upon germ cell removal, we examined the effect of *mir-71* overexpression in loss-of-function mutants of *daf-16* or *daf-12*. Importantly, we observed that *mir-71*-mediated life span extension was fully suppressed by a null allele of *daf-16* but was unaffected by complete loss of *daf-12* function (Figures 5A–5C and Figure S6). That *daf-12* is dispensable for *mir-71* to promote the life span of germline-less animals is not surprising, given that the somatic gonad is also not required (Figure 2F). These results indicate that *mir-71* acts upstream of or in parallel to *daf-16* to promote germline-mediated longevity.

Intestinal *daf-16* Function Is Sufficient for *mir-71* to Promote Germline-Mediated Longevity

To identify the tissue(s) in which *daf-16* activity is required for *mir-71*-mediated life span extension, we assayed whether

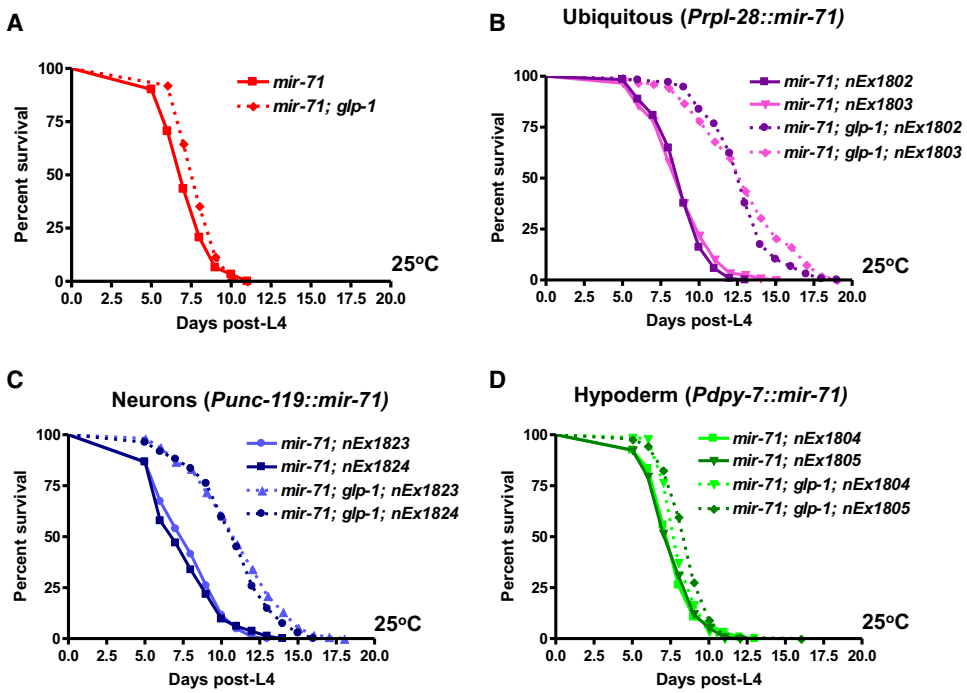

Figure 4. Expression of *mir-71* in Neurons Alone Was Sufficient to Promote Germline-Mediated Longevity

(A–D) Driving *mir-71* expression either ubiquitously (using the *rpl-28* promoter) or in the nervous system of *mir-71* mutants (using the pan-neuronal *unc-119* promoter) resulted in strong rescue ($p < 0.0001$; 40%–45% mean life span extension). On the other hand, hypodermal-specific expression of *mir-71* (using the *dpy-7* promoter) failed to rescue the life span defect of *mir-71; glp-1* mutants ($p < 0.0001$; 5%–10% mean life span extension). All experiments were repeated at least once with similar effects. Mean life span values and statistical analyses of life span assays are shown in Table S2.

tissue-specific expression of DAF-16 is sufficient for *mir-71* overexpression to extend the life span of germline-deficient animals. We found that driving *daf-16* expression only in neurons had little or no effect on the life span of *daf-16; glp-1* animals overexpressing *mir-71* (Figure 5D). By contrast, intestinal expression of DAF-16::GFP fully rescued *mir-71*-mediated life span extension, indicating that the activity of DAF-16 in the intestine is sufficient for *mir-71* to promote longevity in animals lacking germ cells (Figure 5E). In short, our results suggest that upon germline removal, *mir-71* functions in the nervous system to promote life span extension by facilitating the activation of *daf-16* in the intestine.

mir-71 Promotes the Localization and Transcriptional Activity of DAF-16 in the Intestine of Germline-less Animals

When the germline is removed, DAF-16 accumulates in the nuclei of intestinal cells and promotes the expression of several target genes (Lin et al., 2001; Yamawaki et al., 2008). Our findings raise the intriguing possibility that *mir-71* regulates the expression, subcellular localization, or transcriptional activity of DAF-16 in the intestine of germline-deficient animals. To distinguish among these alternatives, we first examined the expression and subcellular localization of a functional DAF-16::GFP fusion protein. Interestingly, we observed that loss of *mir-71* function partially blocked the accumulation of DAF-16::GFP in the intestine of germline-deficient adults without affecting the overall levels of DAF-16 in the presence or absence of germ cells (Figures 6A–6C). This effect is specific, since the nuclear translocation of DAF-16 in response to heat shock and in *daf-2* mutants was not dependent on *mir-71* (Figure S7). These results suggest that *mir-71* specifically facilitates the translocation of DAF-16 to the intestinal nuclei of animals lacking germ cells.

To examine whether the absence of *mir-71* affects the ability of DAF-16 to activate its targets, we examined the expression of *Psod-3::gfp*, a well-characterized and widely used sensor of DAF-16 activity (Libina et al., 2003; Yamawaki et al., 2008). As previously shown, we found that *Psod-3::gfp* expression was upregulated primarily in the intestine of germline-deficient *glp-1* adults (Figure 6D) (Yamawaki et al., 2008). Importantly, we found that loss of *mir-71* function partially blocked the induction of *Psod-3::gfp*, suggesting that *mir-71* is required for the transcriptional activation of DAF-16-dependent gene targets in the intestine (Figure 6D). To directly test this, we measured by qRT-PCR the transcript levels of a number of genes known to be upregulated in the intestine of germline-deficient animals in a *daf-16*-dependent manner. The induction of superoxide dismutase *sod-3*, the triglyceride lipase *K04A8.5*, and the glyceraldehyde 3-phospate dehydrogenase *gpd-2* is totally dependent on DAF-16, while the upregulation of the putative steroid dehydrogenase *dod-8* and the nicotinamide nucleotide transhydrogenase *nnt-1* is partially *daf-16* dependent (Wang et al., 2008; Yamawaki et al., 2008). We found that loss of *mir-71* function in animals lacking germ cells resulted in 50% reduction in the levels of *sod-3* and *K04A8.5*;

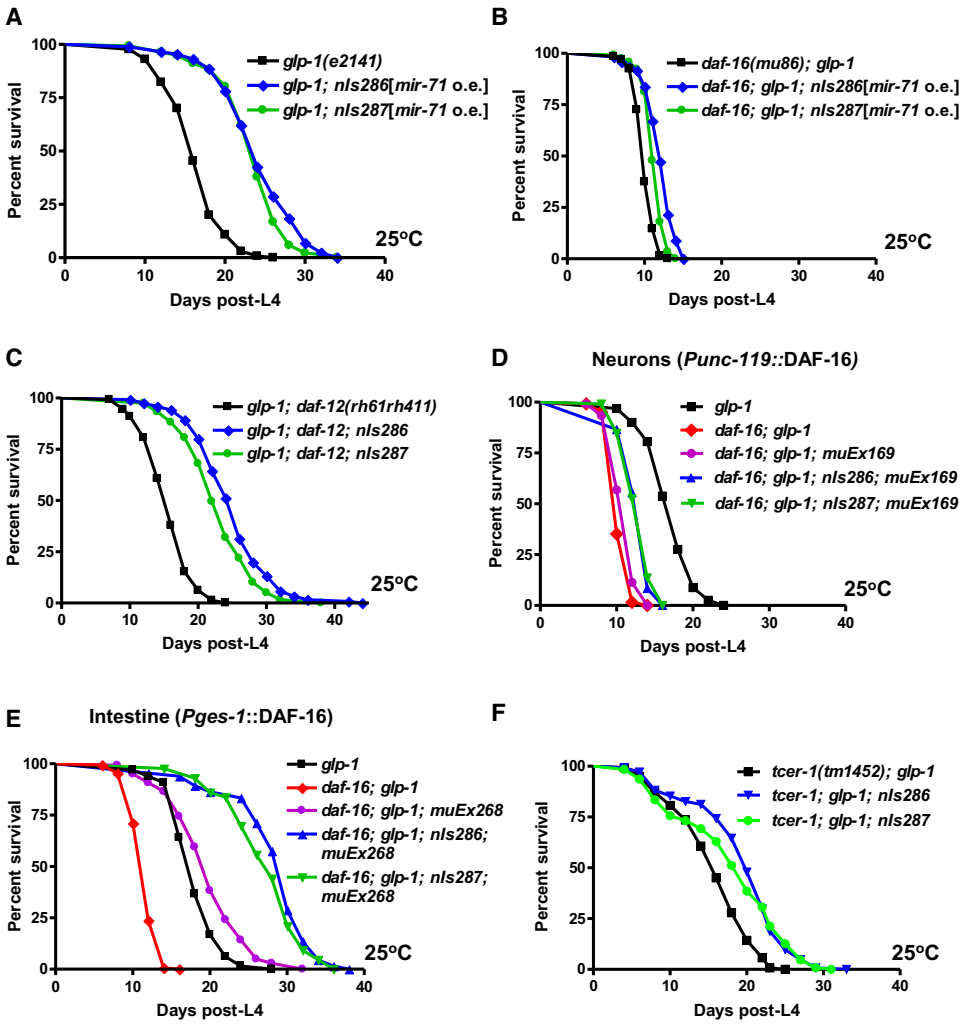

Figure 5. *mir-71* Functions Upstream of or Parallel to DAF-16 to Promote the Longevity of Germline-Deficient Animals

(A) Extra copies of *mir-71* caused a robust extension of the life span of germline-deficient *glp-1(e2141)* animals ($p < 0.0001$, 40%–45% mean life span extension).

(B) Loss of *daf-16* function suppressed *mir-71*-mediated life span extension in germline-deficient *glp-1(e2141)* animals. Extra copies of *mir-71* only slightly extended the life span of germline-deficient *daf-16*; *glp-1* animals ($p < 0.0001$, 13%–20% mean life span extension).

(C) Loss of *daf-12* function did not suppress *mir-71*-mediated life span extension in germline-deficient *glp-1(e2141)* animals. Extra copies of *mir-71* caused a robust extension on the life span of germline-deficient *glp-1*; *daf-12* animals ($p < 0.0001$, 45%–60% mean life span extension).

(D) Expression of DAF-16 in neurons was not sufficient for *mir-71* overexpression to substantially extend the life span of germline-deficient *glp-1(e2141)* animals. Extra copies of *mir-71* caused only a slight extension on the life span of germline-deficient *glp-1* animals that express *daf-16* only in neurons ($p < 0.0001$, 15% mean life span extension).

(E) Expression of DAF-16 in the intestine was sufficient for *mir-71* overexpression to substantially extend the life span of germline-deficient *glp-1(e2141)* animals. Extra copies of *mir-71* caused a robust extension on the life span of germline-deficient *glp-1* animals that express *daf-16* only in the intestine ($p < 0.0001$, 40% mean life span extension).

(F) Deletion of *tcer-1* partially suppressed *mir-71*-mediated life span extension in germline-deficient *glp-1(e2141)* animals. Extra copies of *mir-71* only modestly extended the life span of germline-deficient *tcer-1(tm1452)*; *glp-1* animals ($p < 0.0001$, 15%–25% mean life span extension). All experiments were repeated at least once with similar effects. Mean life span values and statistical analyses of life span assays are shown in Table S2.

the levels of *gpd-2*, *dod-8*, and *nnt-1* were not significantly affected (Figure 6F). By contrast, the levels of *sod-3* and *K04A8.5* were not affected by the absence of *mir-71* in germline-intact animals (Figure 6E). Taken together, our results suggest that *mir-71* promotes germline-mediated longevity by regulating the localization and transcriptional activity of DAF-16.

DISCUSSION

In this study we report a systematic analysis of microRNA genes in the regulation of aging of an entire organism. We have established that the microRNA gene *mir-71* is a critical factor in mediating the effect of germ cell loss on life span: *mir-71* is necessary for the life span extension caused by germline removal and

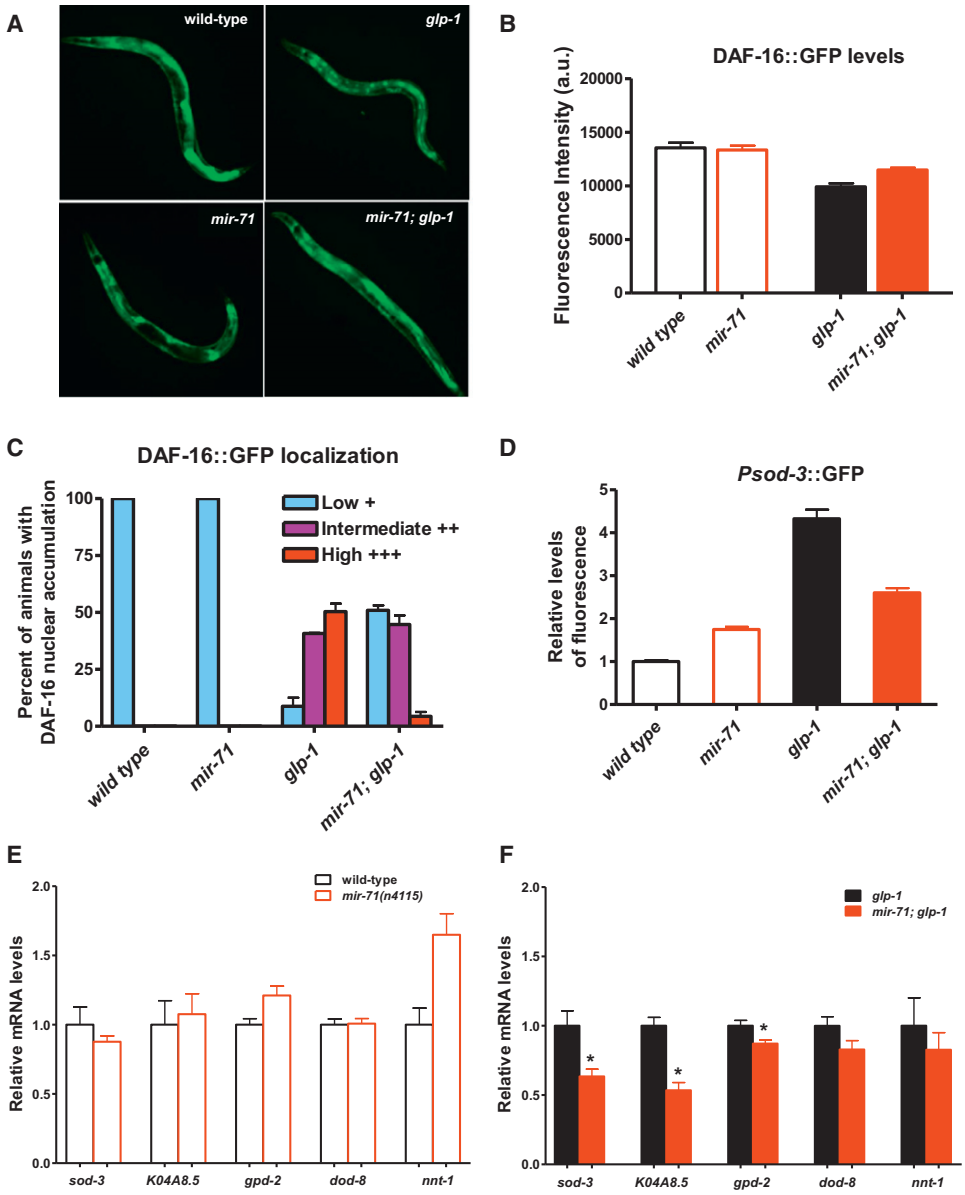

Figure 6. *mir-71* Facilitates the Localization and Transcriptional Activity of DAF-16 in the Intestine of Animals Lacking Germ Cells

(A–C) Loss of *mir-71* function partially affected the nuclear accumulation of DAF-16::GFP in the intestine of germline-deficient *glp-1(e2141)* animals. (A) Representative images for each genotype. (B) Loss of *mir-71* function does not cause major changes in overall levels of DAF-16::GFP expression in day 2 adults (n = 20). Error bar, standard error of the mean (SEM). (C) Intestinal DAF-16::GFP nuclear accumulation was assessed by measuring the number of nuclei from the images of day 2 adults (low accumulation, <10 nuclei; intermediate accumulation, 10–20 nuclei; high accumulation, >20 nuclei) (n = 78 for each genotype). Error bar, standard error of two biological replicates. All strains contain the *daf-16(mu86)* allele and the functional *muIs109*[*Pdaf-16::DAF-16::GFP*] reporter.

(D) Loss of *mir-71* function blocked the induction of *Psod-3::gfp* in day 3 germline-deficient *glp-1(e2141)* animals ($p < 0.0001$; 40% reduction in mean GFP fluorescence intensity) (wild-type, n = 32; *mir-71(n4115)*, n = 35; *glp-1(e2141)*, n = 40; *mir-71(n4115); glp-1(e2141)*, n = 40). Error bar, standard error of the mean (SEM).

(E) Loss of *mir-71* function did not affect the expression of DAF-16 targets in intact animals. mRNA levels of DAF-16 target genes *sod-3*, *K04A8.5*, *gpd-2*, *dod-8*, and *nnt-1* were measured by qRT-PCR in wild-type and *mir-71(n4115)* day 2 germline-intact adults. Loss of *mir-71* function did not affect the levels of *sod-3*, *K04A8.5*, *gpd-2*, and *dod-8*, while it resulted in a 50% increase in the levels of *nnt-1* ($p < 0.05$). mRNA levels are relative to wild-type levels. Error bars, standard error of four biological replicates.

(F) Loss of *mir-71* function reduced the expression of a subset of DAF-16 targets in germline-less animals. mRNA levels of DAF-16 target genes *sod-3*, *K04A8.5*, *gpd-2*, *dod-8*, and *nnt-1* were measured by qRT-PCR in germline-deficient *glp-1* and *mir-71; glp-1* day 2 adults. Loss of *mir-71* function in germline-defective *glp-1* animals resulted in a 50% decrease in the levels of *sod-3* ($p < 0.05$) and *K04A8.5* ($p < 0.01$) and a small reduction in the levels of *gpd-2* ($p < 0.05$). mRNA levels are shown relative to *glp-1(e2141)* levels. Error bar, standard error of four biological replicates.

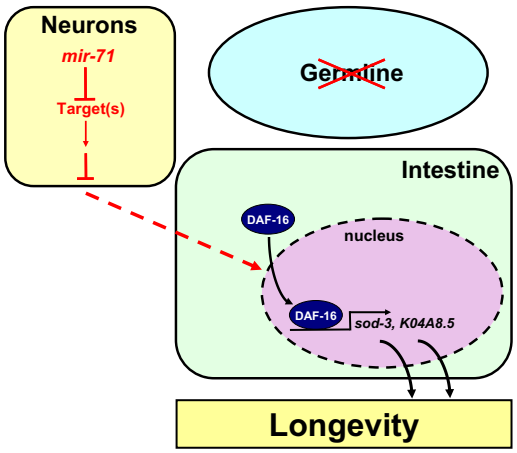

Figure 7. A Model for the Regulation of Germline-Mediated Longevity by the *mir-71* MicroRNA

We propose that *mir-71* functions in neurons to posttranscriptionally inhibit the expression of a factor(s) involved in the production or modification of a signal that controls the localization and activity of DAF-16 in the intestine.

promotes the longevity of animals lacking germ cells by regulating the localization and transcriptional activity of DAF-16. We propose a model in which *mir-71* functions in the nervous system to mediate the production or modification of a life span-extending signal that promotes the intestinal expression of key DAF-16-dependent target genes (Figure 7). According to this model, *mir-71* could inhibit posttranscriptionally the expression of a neuronal factor(s) that antagonizes cell-nonautonomously the activity of DAF-16 in the intestine (Figure 7).

Several approaches using bioinformatics have been developed to help identify direct targets of microRNAs. Although these algorithms have identified a large number of predicted microRNA targets, experimental validation of most of these putative targets is lacking (Hammell et al., 2008; Lall et al., 2006; Lewis et al., 2005). Recently, de Lencastre et al. (2010) implicated *cdc-25.1* as a potential target of *mir-71* function. We have tested if *cdc-25.1* is a biologically significant target of *mir-71* in controlling germline-mediated longevity in genetic epistasis experiments; our preliminary results do not support this hypothesis (our unpublished observations). Given our finding that *mir-71* activity in neurons is important for germline-mediated longevity, it will be interesting to test experimentally whether predicted *mir-71* targets known to be highly enriched in the nervous system function to mediate the effects of germline on life span.

Our results indicate that the germline strongly suppresses the ability of *mir-71* to promote longevity, since extra copies of *mir-71* have only a modest effect on the life span of intact animals. When the germline is removed, *mir-71*-mediated life span extension requires the intestinal activity of *daf-16*, whereas life span extension does not depend on the presence of the somatic gonad or the *daf-12* pathway. Since *mir-71* expression is not regulated by the germline, we postulate that *mir-71*-mediated life span extension requires factors that are triggered by germline removal and act on DAF-16 function. Previous studies have shown that germline removal triggers the upregulation of TCER-1, a transcription elongation factor that acts to promote the transcriptional activity of DAF-16 in the intestine (Ghazi et al., 2009). We found that *mir-71*-mediated life span extension is partially dependent on *tcer-1* gene function (Figure 5F). Thus, *mir-71* might act with TCER-1 and possibly other factors to promote intestinal DAF-16 activity in germline-deficient animals.

Current evidence suggests that the rate of aging at least in *C. elegans* and *Drosophila* is coordinated through communication and signaling among different tissues. For example, tissue-specific manipulations of insulin/IGF-1 signaling in the intestine or the nervous system have been shown to regulate the life span of the whole organism, while genetic ablation of a set of neurons can affect the ability of worms to extend life span in response to dietary restriction (Bishop and Guarente, 2007; Broughton et al., 2005; Hwangbo et al., 2004; Libina et al., 2003; Wolkow et al., 2000). In addition, a recent study showed that perturbation of mitochondrial function can modulate *C. elegans* aging in a cell-nonautonomous fashion (Durieux et al., 2011). Furthermore, the germline of *C. elegans* is thought to send signals that inhibit the life span of the entire animal (Kenyon, 2010), while the somatic gonad is thought to be involved in the production of a steroid hormone that promotes the life span of animals lacking germ cells (Yamawaki et al., 2010). Tissue-specific rescue experiments suggested that the somatic gonad might act through the hypoderm, the endocrine XXX cells, or sensory neurons to promote longevity (Yamawaki et al., 2010). Our finding that *mir-71* activity in neurons is sufficient to promote longevity underscores the importance of the nervous system and neuroendocrine signaling for the control of germline-mediated longevity. Based on our results, we suggest that *mir-71* functions cell-nonautonomously in neurons to promote DAF-16 activity in the intestine. In short, our results implicate the nervous system in life span control upon germ cell removal and support a model in which signaling among the germline, the somatic gonad, the intestine, and the nervous system coordinates the rate of aging of the whole organism.

EXPERIMENTAL PROCEDURES

Strains

Strains were cultured as described (Brenner, 1974) and maintained at 20°C unless specified otherwise. Strains that contained the *glp-1(e2141ts)* allele were maintained at 15°C. *mir-71(n4115)* was outcrossed eight times, *mir-71(n4105)* six times, and *nIs286*, *nIs287*, and *nIs289* four times to the wild-type. A list of the strains used in this study is provided in the Supplemental Information.

Rescue Experiments and Transgenic Animals

For rescue experiments and mosaic analysis, we amplified a 3 kb fragment surrounding the *mir-71* locus (2 kb upstream of and 1 kb downstream of the *mir-71* locus) from wild-type genomic DNA using PCR and cloned this fragment into the PCRII-TOPO (Invitrogen) vector. We used site-directed ligase-independent mutagenesis to generate a control plasmid in which the mature microRNA sequence was deleted (Chiu et al., 2004). To generate the P*mir-71::gfp* reporter, we amplified by PCR the 2 kb upstream region of the *mir-71* locus present in the rescuing construct and cloned this fragment into the pPD96.62 (Adgene) vector.

For tissue-specific rescue experiments, we substituted the gfp coding sequence of pPD95.75 with the *mir-71* precursor sequence (pPD95.75-*mir-71*pr). Subsequently, we cloned either the *rpl-28* promoter fragment (ubiquitous expression) from pPD129.57 or the *unc-119* promoter fragment (pan-neuronal expression) from *Punc-119::gfp* (Nakano et al., 2010) or the *dpy-7* promoter

fragment (hypodermal expression) from *Pdpy-7::2Xnls::yfp* (Myers and Greenwald, 2005) into pPD95.75-*mir-71*pr.

Germline transformation experiments were performed as described (Mello et al., 1991). For rescue experiments, injection mixes contained plasmids at 5 ng/μl (for *mir-71* and for a *mir-71* control plasmid with the *mir-71* mature sequence deleted), 20 ng/μl of pTG96 (*Psur-5::gfp*) as a cotransformation marker, and 80 ng/μl of 1 kb DNA ladder (Invitrogen) as carrier DNA. For mosaic analyses, *mir-71(n4115)* hermaphrodites were injected with a mix that contained 5 ng/μl of *mir-71* rescuing construct, 50 ng/μl of P*osm-6::gfp* construct (Collet et al., 1998), 50 ng/μl of *Pges-1::gfp* construct (Bishop and Guarente, 2007), and 80 ng/μl of 1 kb DNA ladder. For tissue-specific rescue experiments, *mir-71(n4115)* hermaphrodites were injected with a mix that contained 20 ng/μl of *Prpl-28::mir-71* construct or 100 ng/ul *Punc-119::mir-71* construct or 100 ng/ul *Prab-3::mir-71* construct or 20 ng/ul *Pdpy-7::mir-71* construct along with 100 ng/ul pRF4 and 80 ng/μl of 1 kb DNA ladder. We generated the *Pmir-71::gfp* transgenic strain by injecting *lin-15AB(n765)* hermaphrodites with 30 ng/μl of *Pmir-71::gfp* construct, 33 ng/μl p*lin-15*(EK), and 80 ng/μl of 1 kb DNA ladder.

nIs286, *nIs287*, and *nIs289*, integrants of the *Ex*[*mir-71*(+) + pTG96] transgene, and *nIs298*, an integrant of the *Ex*[P*mir-71::gfp*] transgene, were isolated after a standard γ-ray integration screen and were backcrossed twice to the wild-type before analysis (Mello et al., 1991).

Life Span Analyses

Unless stated otherwise, life span assays were performed by standard methods at 20°C using NGM plates seeded with OP50 bacteria containing 25 μM FUdR. In life span experiments assaying strains that carried the *glp-1(e2141ts)* mutation, animals (*glp-1* and control germline-intact controls) were raised at 25°C during embryogenesis and were either kept at 25°C on plates without FUdR throughout the analysis or shifted to 20°C after the L4 stage and for the rest of the life span analysis. Statistical analysis was performed with GraphPad Prism 4 software, which uses the log-rank (Mantel-Cox) method to calculate p values. RNAi life span assays were performed according to the standard feeding protocol (Kamath et al., 2003).

Behavioral Assays

Single animals were maintained on individual NGM plates throughout adulthood and were transferred to fresh plates seeded with OP50 bacteria before locomotion or pumping rates were counted. Locomotion rates were determined by counting body bends per 20 s of animals moving on a fresh bacterial lawn using a dissecting microscope. Pumping rates were assayed by counting the number of movements per min of the rear bulb of the pharynx of animals within the bacterial lawn using a dissecting microscope.

Laser Ablation Experiments

Laser ablations of germline precursor cells (Z2 and Z3) and somatic gonad precursor cells (Z1 and Z4) of newly hatched L1 larvae were performed as described previously (Avery and Horvitz, 1987). At adulthood, the absence of a germline was determined using a dissecting microscope. Intact controls were anesthetized and recovered from the same sodium azide agarose pads as experimental animals.

Mosaic Analysis

We used *mir-71(n4115); nEx1717* and *mir-71(n4115); glp-1(e2141); nEx*1717 animals to generate *mir-71* genetic mosaics. *nEx1717* is an extrachromosomal array containing a genomic copy of *mir-71* as well as lineage-specific markers (see Rescue Experiments and Transgenic Animals). Approximately 200,000 progeny of each of *mir-71(n4115); nEx1717* and *mir-71(n4115); glp-1(e2141); nEx*1717 animals were raised at 25°C until the L4 stage and then were screened using a fluorescence dissecting microscope (Olympus) for mosaic animals in which either the AB-specific marker *Posm-6::gfp* (expressed in ciliated neurons) or the E-specific marker *Pges-1::gfp* (expressed in the intestine generated by the P1 lineage) was absent. AB(−) mosaics (*Posm-6::gfp* negative, *Pges-1::gfp* positive) lost the *mir-71* locus in the AB lineage and presumably retained *mir-71* locus in the P1 lineage. E(−) mosaics (*Posm-6::gfp* positive, *Pges-1::gfp* negative) lost the *mir-71* locus in the E lineage, presumably retained *mir-71* locus in the AB lineage, and might or might not have carried the *mir-71* locus in the MS and P2 lineages. Thus E(−) mosaics probably included a mix of E(−), EMS(−), and P1(−) mosaic animals. Mosaics were selected as L4 larvae and were transferred to 20°C for life span analysis. *mir-71(n4115); nEx1717* and *mir-71(n4115); glp-1(e2141); nEx*1717 controls (array present in all cells) and *mir-71(n4115)* and *mir-71(n4115); glp-1(e2141)* controls (array lost in all cells) underwent the same procedure. They were selected using the fluorescence dissecting microscope in parallel with the mosaic animals and were exposed to UV radiation for approximately the same time.

GFP Fluorescence Microscopy and Quantification

Animals were anaesthetized on agarose pads containing 20–50 mM NaN3. Images were taken with a CCD digital camera using a 5× objective on a Zeiss Axioskop microscope. For each trial, exposure time was calibrated to minimize the number of saturated pixels and was kept constant though the experiment. The ImageJ software was used to quantify mean fluorescence intensity per worm as measured by intensity of each pixel in the selected area. No expression of the transgene was visible in embryos prior to egg laying.

Quantitative RT-PCR Analysis

Germline-deficient *glp-1(e2141ts)* and wild-type N2 animals were raised at 25°C until the L4 stage and then shifted to 20°C. On day 2 of adulthood, animals were collected for RNA extraction. RNA extraction, purification, and reverse transcription and qPCR were carried as described (Andersen et al., 2008). Data were generated from four biological replicates. mRNA levels of *snb-1* and *rpl-26* were used for normalization (Curran et al., 2009). Primer sequences are available upon request.

Statistical Analysis

Error bars represent the standard error of the mean (SEM). p values were calculated using the unpaired Student's t test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures, two tables, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.cmet.2012.02.014.

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