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

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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 (Aran tes-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; Reinhardt 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
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...
To identify the tissue(s) in which 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 overexpressors 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 Pmir-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
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 germ-line-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...
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-phosphate dehydrogenase gpd-2 is totally dependent on DAF-16, while the upregulation of the putative steroid dehydrogenase dod-8 and the nicotinamide nucleotide transhydrogenase nth-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; and the glyceraldehyde 3-phosphate dehydrogenase gpd-2 is totally dependent on DAF-16, while the upregulation of the putative steroid dehydrogenase dod-8 and the nicotinamide nucleotide transhydrogenase nth-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; and the nicotinamide nucleotide transhydrogenase nth-1 is partially daf-16 dependent.

**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.
the levels of gpd-2, dod-8, and nnt-1 were not significantly affected (Figure 6F). By contrast, the levels of sod-3 and K04AB.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 germine 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.
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 tcdr-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). Tissuespecific 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 nls286, nls287, and nls289 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 Pmir-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-71pr). 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.

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

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 PGK96 (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 Posm-6::gfp construct (Collet et al., 1998), 50 ng/μl of Pgces-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/μl Punc-119::mir-71 construct or 100 ng/μl Prab-3::mir-71 construct or 20 ng/μl Pdpy-7::mir-71 construct along with 100 ng/μl prf4-3 and 80 ng/μl of 1 kb DNA ladder. We generated the Pmir-71::gfp transgenic strain by injecting lin-15ABn765) hermaphrodites with 30 ng/μl of Pmir-71::gfp construct, 33 ng/μl piprin-15(EK), and 80 ng/μl of 1 kb DNA ladder. nls286, nls287, and nls289, integrants of the Ex[mir-71+] + PГӨ96] transgene, and nls289, an integral of the Ex[Pmir-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 gfp-1(e24114) mutation, animals (gfp-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 assessed 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 precursors (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(e24114); nEx1717 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(e24114); nEx1717 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(e24114); nEx1717 controls (array present in all cells) and mir-71(n4115) and mir-71(n4115); glp-1(e24114) 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 anesthetized on agarose pads containing 20–50 mM Na3N. 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 throughout 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(e24114) 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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REFERENCES


