A Developmental Timing MicroRNA and Its Target Regulate Life Span in C. elegans

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The microRNA lin-4 and its target, the putative transcription factor lin-14, control the timing of larval development in Caenorhabditis elegans. Here, we report that lin-4 and lin-14 also regulate life span in the adult. Reducing the activity of lin-4 shortened life span and accelerated tissue aging, whereas overexpressing lin-4 or reducing the activity of lin-14 extended life span. Life-span extension conferred by a reduction in lin-14 was dependent on the DAF-16 and HSF-1 transcription factors, suggesting that the lin-4–lin-14 pair affects life span through the insulin/insulin-like growth factor–1 pathway. This work reveals a role for microRNAs and developmental timing genes in life-span regulation.

Life span is highly variable among species, and it has become clear that a genetic program of senescence in the soma is responsible for this variation (1). Recent studies have suggested that gene expression changes in the aged adult are developmentally timed at the transcriptional level. For example, in the aged adult are developmentally timed suggest that gene expression changes in damage has begun (well before the accumulation of molecular with age can be detected in young adulthood, allowing for this variation (2). Thus, conserved genes may act temporally to initiate a program of aging that starts early in adult life (3, 4). We hypothesized that if such an aging program exists, it may be controlled by mechanisms similar to those used in developmental timing (3). The heterochronic genes of C. elegans constitute one such genetic pathway that regulates developmental timing (5–7).

Heterochronous genes, such as lin-4 and lin-14 (5, 8, 9), are temporal identity genes that affect the fate choices that cells make at specific times during development, and mutations in heterochronic genes result in temporal alterations to stage-specific patterns of cellular development (6, 7). Expression of the lin-4 microRNA (miRNA) is up-regulated near the end of the first larval stage, and lin-4 binds with imperfect complementarity to the 3’UTR of its target, lin-14, to prevent its translation (8–11) and allow stage two larval cell fates to occur. The molecular function of lin-14 is unknown, but it encodes a nuclear protein (12) that associates with DNA (13) and has sequence similarities to transcription factors (fig. S1). LIN-14 is down-regulated in the hypodermis at the first to second larval-stage transition (12), but its expression persists weakly in other tissues throughout larval development (13) and into adulthood (fig. S2). Similarly, lin-4 is up-regulated in the adult (14, 15) (fig. S2). Although the roles of lin-4 and lin-14 during larval development have been extensively studied, the function of these genes in the adult has not been investigated. Therefore, we tested whether genes that direct the timing of early developmental events may also function in the adult to regulate the timing of later processes, such as life span and aging.

We assayed heterochronous mutants for life-span length and found that mutations in lin-4 and lin-14 resulted in aging defects. Animals with a loss-of-function (lf) mutation in lin-4 displayed a life span that was significantly shorter than that of the wild type (Fig. 1A), suggesting that lin-4 is required to prevent premature death. Conversely, overexpressing lin-4 from an extrachromosomal array led to a lengthened life span (Fig. 1C). This result demonstrates that the lin-4(1f) mutant did not die prematurely solely as the result of an unrelated, general pathology, but rather that lin-4 functions to extend life span. Consistent with our lin-4 data, we found that a 1f mutation in a target of lin-4, lin-14, produced the opposite life-span phenotype. Animals carrying a temperature-sensitive 1f mutation in lin-14 had a 31% longer life span than the wild type (Fig. 1B).

Fig. 1. lin-4 and lin-14 mutants have opposite life-span phenotypes. (A) Red, survival of wild-type (N2) animals on control bacteria containing empty vector (mock RNAi); blue, lin-4(e912)lf; mock RNAi; pink, lin-14(RNAi); light blue, lin-4(e912)lf; lin-14(RNAi) at 20°C. N2: n = 69, m = 14.6, lin-4(e912)lf: n = 56, m = 6.9, P < 0.0001*. N2;lin-14(RNAi): n = 68, m = 18.7, P < 0.0001*. lin-4(e912)lf;lin-14(RNAi): n = 72, m = 16.6, P < 0.0001*. (B) A lin-4(1f) mutation extends life span when grown and assayed at the restrictive temperature of 25°C. N2: n = 57, m = 9.5. lin-14(n179)lf: n = 58, m = 12.5, P < 0.0001*. lin-4(e912)lf;lin-14(n179)lf: n = 51, m = 9.0, P = 0.0906*. (C) lin-4 overexpression extends life span. Three lines overexpressing (oe) lin-4 are shown in purple, blue, and green; wild-type animals are in red. N2: n = 64, m = 15.8. lin-4/oe line 3.3: n = 54, m = 18.3, P < 0.0001*. lin-4/oe line 3.14: n = 62, m = 17.7, P = 0.0023*. lin-4/oe line 4.9: n = 37, m = 17.8, P = 0.0113*. (D) A lin-14 gain-of-function mutant, n355, has a short-lived phenotype similar to that of the lin-4(e912)lf mutant. Red, wild-type animals; green, lin-4(e912)lf; blue, lin-14(n355)lf. N2: n = 59, m = 15.9. lin-4(e912)lf: n = 85, m = 7.7, P < 0.0001*. lin-14(n355)lf: n = 94, m = 5.9, P < 0.0001*. All experiments were repeated at least once with similar effects. n, number of animals observed in each experiment. m, mean adult life span (days). *P values refer to experimental strain and N2 control animals in a single experiment, and #P values refer to a strain on control and experimental RNAi treatment in a single experiment.
phenotype produced by the lin-14(lf) lesion was reproduced by RNA interference (RNAi) of lin-14 (Fig. 1A). Thus, lin-14 normally acts to promote a short life span. A lin-14 gain-of-function (gf) mutant (16), which lacks the lin-4 complementary sites in the lin-14 3’ untranslated region (UTR) and overexpresses LIN-14 at later stages (12), closely phenocopied the short-lived phenotype of the lin-4(gf) mutant (Fig. 1D). Additionally, lin-14(RNAi) suppressed the short life span of the lin-4(e912)gf mutant (Fig. 1A). Taken together, the data suggest that the major role of lin-4 in regulating life span is to repress its target, lin-14.

To determine whether the short life span of lin-4(lf) mutants is due to accelerated aging or to an unrelated, pleiotropic cause, we monitored the accumulation of intestinal autofluorescence in adult animals. Intestinal autofluorescence, which is caused by lysosomal deposits of lipofuscin, accumulates over time in the aging animal and is an established marker for aging (17). In agreement with its short life span, the lin-4(lf) mutant accumulated intestinal autofluorescence more rapidly than the wild type (Fig. 2, A and B). These results resemble those found for the short-lived strain with a daf-16(lf) mutation (fig. S3, A and B). daf-16 encodes a FOXO transcription factor that regulates life span through insulin-like signaling (1, 18–20). The premature lipofuscin accumulation caused by lin-4(lf) was suppressed when combined with the lin-14(n179)lf lesion (Fig. 2, A and B), consistent with the ability of lin-14(lf) to suppress the short life span of the lin-4(gf) mutant. In contrast to lin-4(lf), the lin-14(n179)lf mutant displayed a slower rate of intestinal autofluorescence accumulation as compared with the wild type (Fig. 2, C and D), in agreement with...
its extended life span. The decreased rate of gut autofluorescence accumulation is similar to that observed in the long-lived daf-2(lf) mutant (Fig. S3, C and D) (17). daf-2 encodes an insulin/insulin-like growth factor-1 (IGF-1) receptor that lies upstream of daf-16 in insulin-like signaling (18, 20, 21).

The stress response of the lin-4(lf) and lin-14(lf) strains was also examined. C. elegans mutants that display life-span phenotypes also display altered responses to stress treatments, including heat shock (22, 23). For instance, the long-lived daf-2(lf) mutant is highly tolerant to heat shock, and this heightened stress resistance is believed to be essential for life-span extension (22). In accordance with its life-span phenotype, the lin-4(e912)lf mutant displayed a greater sensitivity to heat shock as compared with the wild type (fig. S4B), whereas the lin-14(n179)lf mutant displayed a greater resistance to heat shock as compared with the wild type (fig. S4C).

To rule out the possibility that life-span modulation directed by lin-14 and lin-4 is merely due to their role in larval development, we examined the effect of reducing the function of lin-14 only in the postmitotic adult. RNAi-mediated inhibition of lin-14 expression after the final larval molt extends the life span of wild-type animals, similar to the extension observed when animals were exposed to lin-14(RNAi) just after hatching (Fig. 3A). Additionally, growing the lin-14(n179)lf mutant at the permissive temperature until young adulthood and then shifting to the restrictive temperature also produced an extended life span (Fig. 3B). These results demonstrate that lin-14 functions in the adult to restrict life span. Furthermore, the short life span of the lin-4(e912)lf mutant was also rescued to a significant extent when exposed to lin-14(RNAi) only during adulthood. This result supports the idea that the lin-4(e912)lf accelerated-aging phenotype is not due to developmental abnormalities or an unrelated pleiotropic cause. Thus, the lin-4 miRNA appears to suppress senescence in C. elegans through repression of lin-14 in the adult.

We tested whether lin-4 and lin-14 extend life span by acting through one of the known C. elegans life-span regulatory pathways, such as the insulin/IGF-1 signaling pathway. Several insulin/IGF-1 signaling pathway members regulate life span through mechanisms dependent on the downstream DAF-16/FOXO and HSF-1 transcription factors (1, 18–21, 24, 25). As with lin-4, inhibiting daf-16 or hsf-1 activity shortens life span, whereas elevating their activity lengthens life span (25, 26). The daf-16(mu86) null mutant strain, when treated with lin-14(RNAi), did not display an extended life span (Fig. 4B), nor did lin-14(n179)lf; daf-16(RNAi) animals (Fig. 4A). These data demonstrate that daf-16 is required for the lin-14(lf)-mediated longevity phenotype. lin-4(lf) animals grown on daf-16(RNAi) had shortened life-span lengths that are identical to that of the lin-4(lf) strain grown on mock RNAi (Fig. 4C), indicating that lin-4 and lin-14 genetically interact with daf-16. However, the lin-4(lf) mutant had a shorter life span than the daf-16(lf) mutant, indicating that lin-14 does not exert its effect on life span by negative regulation of DAF-16 alone. Consistent with this idea, the lin-4(lf); hsf-1(RNAi) animals had a short life span, indicating that the lin-4(lf)-mediated longevity phenotype is dependent on hsf-1 (fig. S4A) as well as on daf-16.

To further explore the possibility that lin-4 and lin-14 might function through the insulin/IGF-1 pathway, we analyzed their interactions with the daf-2-insulin/IGF-1 receptor. Consistent with previous studies, daf-2(RNAi) animals had a significant extension in life span compared with wild-type animals (Fig. 4C) (25). This life-span extension was significantly reduced by the lin-4(e912)lf lesion (Fig. 4C), such that lin-4(e912)lf; daf-2(RNAi) animals displayed life spans similar to those of the wild type. This phenotype is different from that of the hsf-1(lf) mutation, which wholly abolishes the life-span extension conferred by daf-2(lf) and results in a shortened life span (25). An epistatic relationship between lin-4 and daf-2 cannot be determined because the daf-2 allele is non-null. However, our data suggest that a wild-type copy of daf-2 is necessary for the short life span phenotype conferred by daf-16(RNAi). The life span of the daf-2(e1370)lf mutant was modestly extended by lin-14(RNAi) (Fig. 4B), and lin-14(n179)lf; daf-2(RNAi) animals also displayed an extended life span as compared with daf-2(RNAi) animals (Fig. 4A). Null alleles were not used for either analysis, and thus concrete epistatic relationships cannot be determined. However, our data support a model whereby lin-4 and lin-14 modulate life span through the canonical daf-2 insulin/IGF-1 pathway. Alternatively, lin-4 and lin-14 may converge onto the DAF-16/FOXO transcription factor in a pathway parallel to the daf-2 insulin/IGF-1 pathway to control aging.
In key *C. elegans* adult tissues, the lin-4 miRNA may act to suppress the translation of lin-14, preventing lin-14 from affecting the transcription of a yet unidentified factor that regulates or interacts with the daf-2 insulin/IGF-1 pathway. By demonstrating that lin-4 and lin-14, two key temporal regulators of development, also influence the rate of aging, we provide support for the theory that life span is affected by an innate, programmed timing mechanism. However, our data are also consistent with an alternative theory of aging, antagonistic pleiotropy, which posits that genes with primary roles in development can later secondarily influence life span (27). miRNAs are important regulators of development, apoptosis, and metabolism (28–31), and our work demonstrates that a miRNA can regulate aging, possibly through the insulin-like signaling pathway. It is possible that the mammalian lin-4 miRNA homologs, the miR-125 family, may regulate processes responsible for life-span determination in vertebrates.

**References and Notes**

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**Supporting Online Material**

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Materials and Methods

Figs. S1 to S4

Table S1

References and Notes

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**fgf20 Is Essential for Initiating Zebrafish Fin Regeneration**

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Epimorphic regeneration requires the presence or creation of pluripotent cells capable of reproducing lost organs. Zebrafish fin regeneration is mediated by the creation of blastema cells. Here, we characterize the *devoid of blastema* (*dob*) mutant that fails fin regeneration during initial steps, forms abnormal regeneration epithelium, and does not form blastema. This mutation has no impact on embryonic survival. *Dob* results from an *fgf20a* null mutation, Y148S. *Fgf20a* is expressed during initiation of fin regeneration at the epithelial-mesenchymal boundary and later overlaps with the blastema marker *msxb*. Thus, *fgf20a* has a regeneration-specific requirement, initiating fin regeneration, and controlling blastema formation.

Vertebrate regeneration is of scientific and medical interest. Although acute tissue regeneration in humans is limited, other vertebrates possess extraordinary regenerative capabilities. Zebrafish are amenable to genetic analyses and regenerate an impressive array of structures, including spinal cord, optic nerve, heart, and fins (1–3). Zebrafish fin regeneration is marked by five stages: regeneration epithelialization, mesenchymal disorganization, blastema formation, regenerative outgrowth, and termination. Although genetic analyses have enhanced our understanding of fin regeneration (1, 4–6), the specific signaling factor(s) that initiate regeneration and blastema formation are unknown.

To discover genes that initiate regeneration, we treated zebrafish with *dob* C. The first stage of regeneration, formation of regeneration epithelium, appeared abnormal. At 6 and 12 hours post-amputation (hpa), *dob* regenerates demonstrated a thickened regeneration epithelium (Fig. 2A). Epithelial proliferation levels in *dob* at 6 and 12 hpa were similar to wild type (Fig. S1A). Therefore, thickened regeneration epithelium likely results from aberrant epithelial migration (9, 10).

To determine whether *dob* resulted from a primary defect in wound healing, we performed a longitudinal incision along the caudal fin and allowed healing at 33°C. The wild-type response to this injury is nonregenerative, as the wound is covered by epithelium and leaves a silt down the fin. We found no difference in the timing, histochemistry, or bromodeoxyuridine (BrdU) immunohistochemistry of wound-healing between wild type and *dob* (Fig. S3B). The possibility remains that the *dob* mutant may have a subtle defect in wound-healing not identified by our observations.

We expected that *dob* would also disrupt embryogenesis (1, 4–6). However, at 33°C, *dob* viability was comparable with wild type (Fig. S1A). Half (23/46) of *dob* adults developed asymmetric caudal fin lobes when heat-shocked as embryos, yet all wild type (41/41) developed symmetric fin lobes (Fig. S1B). The total size of wild-type and *dob* caudal fins was comparable (Fig. S1B). Therefore, there appears to be an incompletely penetrant patterning defect in *dob* (8). Survival of *dob* adults at 33°C was also comparable to wild type (Fig. S1C). These data suggest a regeneration-specific requirement for *dob*.

To determine the cellular nature of *dob* regenerative failure, we examined histology of regenerates at 33°C. The first stage of regeneration, formation of regeneration epithelium, appeared abnormal. At 6 and 12 hpa, *dob* regenerates demonstrated a thickened regeneration epithelium (Fig. 2A). Epithelial proliferation levels in *dob* at 6 and 12 hpa were similar to wild type (Fig. S3A). Therefore, thickened regeneration epithelium likely results from aberrant epithelial migration (9, 10).

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