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The zinc-finger protein SEA-2 regulates larval developmental timing and adult lifespan in *C. elegans*

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SUMMARY

Like other biological processes, aging is regulated by genetic pathways. However, it remains largely unknown whether aging is determined by an innate programmed timing mechanism and, if so, how this timer is linked to the mechanisms that control developmental timing. Here, we demonstrate that *sea-2*, which encodes a zinc-finger protein, controls developmental timing in *C. elegans* larvae by regulating expression of the heterochronic gene *lin-28* at the post-transcriptional level. *lin-28* is also essential for the autosomal signal element (ASE) function of *sea-2* in X:A signal assessment. We also show that *sea-2* modulates aging in adulthood. Loss of function of *sea-2* slows the aging process and extends the adult lifespan in a DAF-16/FOXO-dependent manner. Mutation of *sea-2* promotes nuclear translocation of DAF-16 and subsequent activation of *daf-16* targets. We further demonstrate that insulin/IGF-1 signaling functions in the larval heterochronic circuit. Loss of function of the insulin/IGF-1 receptor gene *daf-2*, which extends lifespan, also greatly enhances the retarded heterochronic defects in *sea-2* mutants. Regulation of developmental timing by *daf-2* requires *daf-16* activity. Our study provides evidence for intricate interplay between the heterochronic circuit that controls developmental timing in larvae and the timing mechanism that modulates aging in adults.

KEY WORDS: *sea-2*, *lin-28*, Heterochronic genes, *daf-2*, *daf-16*, Aging, *C. elegans*

INTRODUCTION

Developmental timing mechanisms are integrated with various signaling pathways to achieve the synchrony and succession of stage-specific programs during animal development. In *C. elegans*, a network of heterochronic genes has been identified that control developmental timing of diverse post-embryonic cell lineages, best characterized by the development of a row of lateral hypodermal seam cells, which undergo stage-specific developmental programs during each of the four larval stages and terminally differentiate at the late L4 larval stage (Ambros, 2000; Rougvie, 2005; Moss, 2007). Mutations in heterochronic genes cause skipping or reiteration of stage-specific programs, resulting in premature or delayed terminal differentiation of seam cells. Adult animals, the somatic cells of which are post-mitotic, undergo a progressive decline in pharyngeal pumping and body movement, and also experience cell and tissue deterioration. Several mechanisms, including caloric restriction, the insulin/insulin-like growth factor 1 (IGF-1) endocrine system and the steroid hormone system, regulate aging in *C. elegans* (Guarente and Kenyon, 2000; Kenyon, 2005; Antebi, 2007). For example, reduced activity of *daf-2*, which encodes an insulin/IGF-1 receptor, extends the lifespan (Kenyon, 2005). The steroid hormone pathway, acting via the nuclear receptor DAF-12, also impacts the succession of developmental events in larvae (Fielenbach and Antebi, 2008). Two components of the heterochronic circuit, miRNA *lin-4* and its target *lin-14* (which encodes a nuclear transcription factor), also have a modest effect on adult lifespan (Boehm and Slack, 2005). *lin-4/lin-14* may influence lifespan by regulating metabolic outputs, and the insulin-like gene *ins-33* is a direct target of LIN-14 (Hristova et al., 2005). Nevertheless, it remains largely unknown whether genes controlling larval developmental timing also function in the adult and whether signals that modulate aging, such as insulin/IGF-1, also act within the heterochronic circuit.

MATERIALS AND METHODS

Strains

The following strains were used in this study: LGI, *lin-28(n719), daf-16(mu86)*; LGII, *lin-42(n1089), sea-1(gk799), sea-2(bp283), sea-2(tm4355), lin-4(e912), lin-29(n333)*; LGIII, *daf-2(e1370)*; LGIV, *lin-66(ku423), jcIs1(ajm-1::gfp), zIs356(daf-16::gfp)*; LGV, *lin-46(bp312), lin-46(bp284), wIs51(scm::gfp)*; and LGX, *daf-12(rh257), daf-12(rh61rh411), lin-14(n179), alg-1(gk214), ain-1(bp299)*. The genetic location for *muIs84(sod-3::gfp), bpIs124(dcap-1::rfp)* and *bpIs145(lin-28::gfp::lin-28 3′UTR(ΔLCE))* was not determined.

lin-46(bp312) contains a glycine to stop codon mutation at amino acid 249. *lin-46(bp284)* was isolated in a screen for mutants that enhanced the retarded heterochronic phenotype of *sea-2(bp283)*. *lin-46(bp284)* contains a leucine to phenylalanine mutation at amino acid 44 of LIN-46.

Isolation, characterization and cloning of *bp283*

Animals carrying the *scm::gfp* reporter were mutagenized and F2 progeny were examined for the number of SCM::GFP-positive cells. From ~4000 genomes screened, 10 mutations were isolated that caused altered number of seam cells. The *sea-2* mutants are cold sensitive. At 15°C, the retarded heterochronic defects are more severe. *ccDf2/sea-2(bp283)* mutants had an average of 22.5 seam cells (n=8) at 15°C, similar to 22.7 in *sea-2(bp283)* mutants. All experiments were performed at 20°C unless otherwise noted.

bp283 was mapped by three factor mapping. From the *sqt-2 lin-31* +/+ + *bp283* cross, 0 out of 32 Lin no Sqt recombinants carried *sea-2(bp283)*. From the *lin-31 clr-1 dpy-10* +/+ + + *bp283* cross, 0 out of 14 Lin non Clr recombinants carried *bp283*. From the *dpy-10 vab-19* +/+ + *bp283* cross, 17 out of 39 Dpy non Vab recombinants carried *bp283*. Single nucleotide polymorphism (SNP) mapping further located *bp283* between *pkp2115* (–6.31) and *pkp2051* (–4.92). Fosmids covering this region were used for transformation rescue experiments.

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Lineage analysis
Animals were placed on a 2% agarose pad in 5 μl of M9 and the seam cell division pattern was analyzed by microscopy. After observation, each animal was rescued and placed on a plate to recover. This procedure was repeated every 2 to 4 hours.

RNA interference
The PCR templates used for synthesizing RNA were: *sea-2* (K10G6, nt 9550-10438); *fox-1* (T07D1, nt 29314-30084); and *sex-1* (F44A6, nt 11213-12115). For RNA feeding of *hbl-1*, synchronized L1 worms were placed on RNAi plates and worms from the next generation were examined.

Reporter construction
Reporters for *sea-2* were constructed by a PCR fusion based approach. The fused PCR products were derived from two overlapping PCR fragments. One contained the promoter region and the entire ORF of *sea-2* (K10G6, nt3055-16856), the other contained *gfp* and the *unc-54* 3′UTR or the *sea-2* 3′UTR. The PCR products were co-injected with pRF4 [*rol-6(su1006)*] into wild type animals and at least two stable transgenic lines were analyzed. The *sea-2::gfp::unc-54* 3′UTR reporter displayed the same expression pattern as the reporter containing the s*ea-2* 3′UTR. We inserted a nuclear localization signal sequence (PKKKRK) at the N terminus of SEA-2 to determine whether SEA-2 acts in the nucleus or in the cytoplasm to specify the temporal fates of seam cells. However, the *sea-2::NLS::sea-2::gfp* transgene was expressed at a much lower level than *sea-2::gfp*, indicating the addition of the NLS destabilized SEA-2.

lin-28::gfp::lin-28 3′UTR(ΔLCE) contains the promoter and the entire ORF of *lin-28* (F02E9, nt 3769-7599), *gfp* and the *lin-28* 3′UTR (F02E9, nt 3235-3765) with a deletion of the LCE (F02E9, nt 3424-3438). Animals carrying the *lin-28::gfp::lin-28* 3′UTR(ΔLCE) extrachromosomal array were γ-ray irradiated and the resulting stable integrated line *(bpIs145)* was outcrossed two times.

Lifespan and heat stress assay
The lifespan assay was performed at 20°C. Animals that had just passed the final larval molt were transferred to new plates every 1-2 days until the end of reproduction and 2-4 days thereafter. Animals were scored as dead when they failed to response to gentle prodding. Worms with exploded vulva, or that had bagged (died from internal hatching) or crawled off the plate were excluded. Three independent assays were tested for each experiment. GraphPad Prism 5 was used for survival curves and statistical analysis.

The heat stress assay was performed at 32°C using 1-day-old adults. Animals were cultured and scored as described for lifespan assays. At least 100 animals were tested for each strain.

RNA isolation and real-time RT-PCR
Synchronized L1 and L3 animals were collected and total RNA was extracted from about 500 animals using Trizol reagent (Sigma) according to the manufacturer's protocol. Total RNA (2 μg) was reverse transcribed using an Invitrogen Superscript III kit. Quantitative PCR reactions were carried out using a SYBR RT-PCR kit (TaKaRa) and a Mastercycler ep realplex machine (Eppendorf). *eft-2* was used as an internal control (Bagga et al., 2005). The level of *lin-28* mRNA was normalized to the level of wild type L1 worms, which was set to 1. Error bars indicate the standard deviation (s.d.) of three independent experiments.

Primers used were: *lin-28* FW, TCGGAGTCTTGATGAAGGAG; *lin-28* RW, GAGACAGCCTTCTTACGACC; *eft-2* FW, ATGGTCAA-CTTCACGGTCGATG; *eft-2* RW: GATGGTAATACAACGCTCCTGC.

Fluorescence photography and quantification
Gut autofluorescence was photographed using a Zeiss Axioplan 2 imaging system and quantified by AxioVision Rel. 4.6. The same exposure time was used for different strains and also for animals of different ages.

Western blot
Lysates from synchronized animals were prepared as previously described (Seggerson et al., 2002), and endogenous LIN-28 was detected with diluted anti-LIN-28 serum (1:2000) and HRP-conjugated goat anti-rabbit secondary antibody. Anti-actin monoclonal antibody (A3853, Sigma) was used as a gel loading control.

Northern blot
To determine the level of *lin-4* RNA, total RNA isolation and northern analyses were performed as described (Grishok et al., 2001). Total RNA was isolated from synchronized L2 and L3 worms.

Tissue-specific expression
The following sequences were used to drive the expression of genomic coding region of *sea-2* and *unc-54 3′UTR* in various tissues: *Pceh-16* (C13G5, nt 4341-6883), *Pvha-6* (VW02B12L, nt 1023-2624), *Pmyo-3* (WRM061aH08, nt 24240-26523) and *Prgef-1*(F25B3, nt 10721-14265). At least three independent transgenic lines for each construct were examined.

RESULTS

Mutations in *sea-2* cause retarded heterochronic defects
Ten seam cells, aligned on each side of the animal, undergo asymmetric cell division at each of four larval stages (L1 to L4) with only one daughter retaining the seam cell identity. Certain seam cells also undergo one round of symmetric division with both daughter cells maintaining the seam cell fate at the L2 larval stage, increasing seam cell number from 10 at hatching stage to 16 from L2 stage onwards. All seam cells terminally differentiate at the late L4 stage, including cessation of cell division, fusion with neighboring seam cells and synthesis of adult-specific cuticular structures, called alae (Fig. 1A-C,G). In a genetic screen to identify mutants with defective seam cell development, we isolated a mutation, *bp283*, that increased the number of seam cells in young adults from 16 in wild-type animals to 20 (Table 1, Fig. 1D). Adult-specific alae were not completely formed in *bp283* mutant young adults, indicating a delay in terminal differentiation of seam cells (Fig. 1E,F). Analysis of seam cell lineages revealed that the L2 stage-specific proliferative division pattern was reiterated at the L3 larval stage in *bp283* mutants and certain seam cells failed to fuse and continued to divide at the L4/adult switch, resulting in an increase in the seam cell number and discontinuous alae (Fig. 1H). The seam cell developmental defects in *bp283* animals, as in other classic heterochronic gene mutants, were completely suppressed when animals developed through the alternate dauer larval stage (Table 1). Thus, *bp283* causes retarded heterochronic defects.

Using transformation rescue we found that a transgene containing a single gene, previously named *sea-2* (see below), rescued the heterochronic defects in *bp283* mutants (Fig. 1I). We sequenced cDNAs and found that *sea-2* encodes a 1727 amino acid protein. *bp283* contains an alanine to aspartate mutation at codon 1267 (see Fig. S1 in the supplementary material). *sea-2(tm4355)*, which deletes amino acids 294 to 590 of SEA-2, showed retarded heterochronic defects in sensitized genetic backgrounds (Table 1). Animals bearing *sea-2(bp283)* in trans to *ccDf2*, a deficiency that removes the *sea-2* locus, had an average of 20.2 seam cells (n=10), compared with 20.4 in *sea-2(bp283)* mutants. *sea-2(RNAi)* also caused retarded heterochronic defects, but did not further elevate the defects in *sea-2(bp283)* mutants (Table 1). These results indicate that *sea-2(bp283)* is probably a strong loss-of-function allele.

Bioinformatic analysis revealed that SEA-2 contains four CCHC zinc fingers and one CCHH zinc finger (see Fig. S1 in the supplementary material). The zinc fingers Z1 and Z5 of SEA-2 strongly bound to single stranded (ss) and double stranded (ds) RNA in an EMSA assay (see Fig. S2 in the supplementary material). Transgenes expressing truncated SEA-2 with a deletion

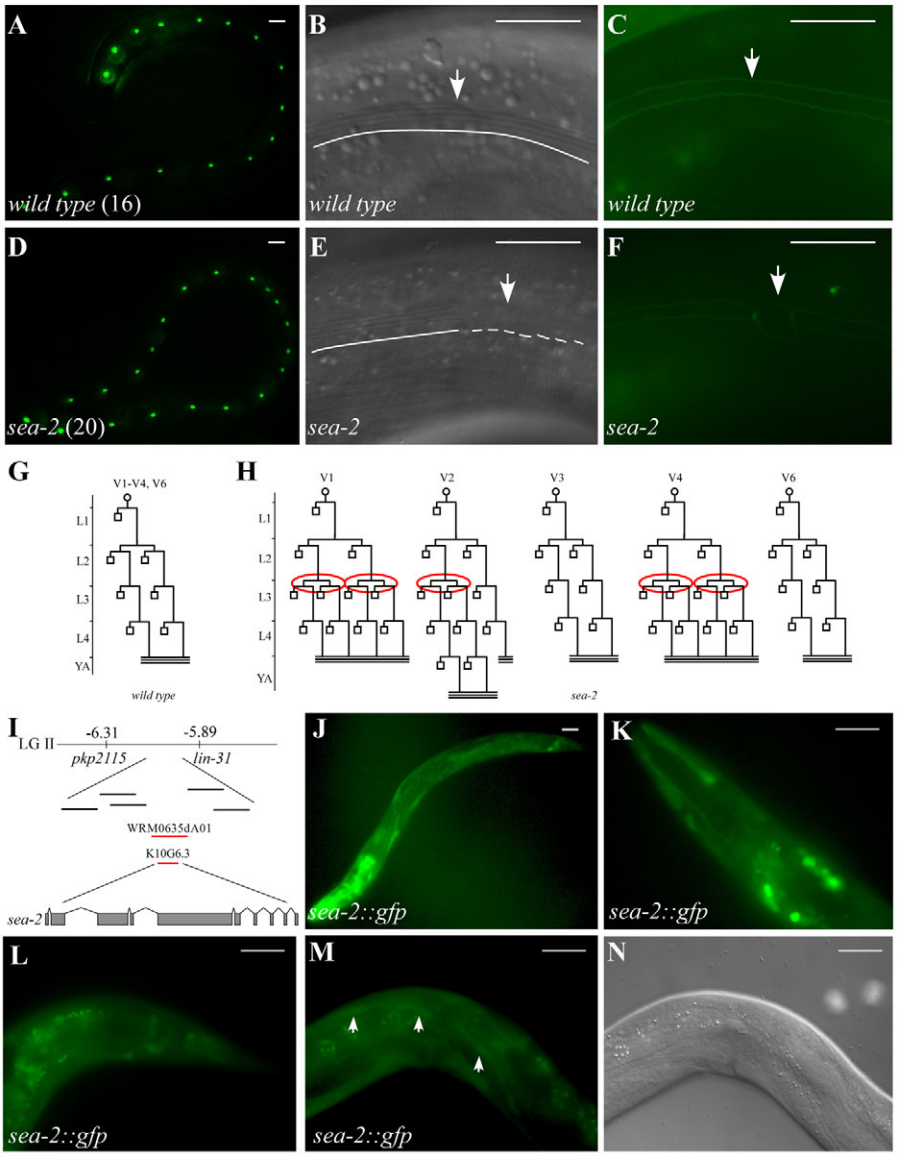

Fig. 1. Mutations in *sea-2* cause retarded heterochronic defects. (**A-C**) In a wild-type young adult animal, 16 seam cells are present on each side (A). Longitudinal cuticular ridges, known as alae, are synthesized by seam cells and run continuously from head to tail (arrow, B). Seam cells fuse together (arrow, C), marked by the adherens junction marker *ajm-1::gfp*. The number of seam cells is indicated in parentheses. Scale bar: 20 μm. (**D-F**) In *sea-2* mutant young adult animals, the number of seam cells is increased (D) and certain seam cells fail to terminally differentiate, resulting in gaps in the alae (arrow, E) and defective fusion with neighboring seam cells (arrow, F). Scale bars: 20 μm. (**G**) The seam cell lineage from the L1 to young adult stage in a wild-type animal. Squares represent the fusing daughter cells and the three horizontal lines at the bottom of the lineage stand for adult alae formation. (**H**) Seam cell lineage of a *sea-2* mutant grown at 15°C. Certain seam cells repeat the proliferative division pattern at the L3 stage (highlighted in red). Twelve *sea-2* animals were analyzed and the number of seam cells that repeated the L2 division pattern at the L3 stage varied among individual *sea-2* animals, which was consistent with the range of seam cell numbers present in *sea-2* mutants. (**I**) Cloning of *sea-2*. *sea-2* was mapped on chromosome II (LGII), close to *lin-31*. Fosmid WRM0635dA01 (in all seven transgenic lines examined) and the DNA fragment covering K10G6.3 (in all four transgenic lines examined) rescued the retarded heterochronic defects in *sea-2* mutants. The genomic structure of *sea-2* is shown at the bottom. (**J**) Expression of *sea-2::gfp* in an L3 larva. Scale bar: 20 μm. (**K-N**) Expression of *sea-2* in the head region (K), tail region (L) and seam cells (arrows in M). (N) Nomarski image of the animal shown in M. Scale bars: 20 μm.

of the first zinc finger (amino acid 319-339) or the fifth zinc finger (amino acid 1429-1449) failed to rescue the retarded heterochronic defects in *sea-2* mutants (see Table S1 and Fig. S3 in the supplementary material), suggesting that the RNA binding domains are important for SEA-2 function.

sea-2 is widely expressed

We constructed a reporter with *gfp* inserted at the C terminus of the *sea-2*-coding region to determine the expression pattern of *sea-2*. This translational reporter rescued the heterochronic defect in *sea-2* mutants (data not shown). *sea-2* was strongly expressed in various tissues, including seam cells, intestine cells, pharyngeal muscles and nerve ring neurons (Fig. 1J-N). SEA-2::GFP expression persisted into adulthood. SEA-2::GFP was diffusely localized in both cytoplasm and nucleus (Fig. 1J-M).

sea-2 functions cell-autonomously to specify temporal fates of seam cells

To determine whether *sea-2* acts cell-autonomously in controlling the stage specific fates of seam cells, we expressed *sea-2* using a seam cell-specific *ceh-16* promoter (Huang et al., 2009). *sea-2* mutants carrying a *ceh-16::sea-2* transgene had an average of 16.3 seam cells (see Table S1 in the supplementary material). Expression of *sea-2* in the intestine, body wall muscle cells or neurons failed to rescue the increased number of seam cells in *sea-2* mutants (see Table S1 and Fig. S3 in the supplementary material).

Mutations in *sea-2* enhance other retarded heterochronic mutations that cause reiteration of the L2 stage-specific fate

To further characterize the role of *sea-2* in specifying the L2/L3 progression, we examined the genetic interaction between *sea-2* and other heterochronic mutants. Other mutants that cause reiteration of the L2 stage fate at the L3 stage and an incomplete defect in the larval/adult switch phenotype include the recessive gain of function (*rh257*) or null allele (*rh61 rh411*) of *daf-12* and loss of function of *lin-46* (the gephyrin homolog), *lin-66* (a novel protein), *alg-1* (the Argonaute homolog), *ain-1* (the GW182 homolog) and *let-7* family miRNAs (Moss, 2007). *sea-2(bp283)* in combination with a mutation in each of these retarded heterochronic genes caused a dramatic increase in seam cell

Table 1. Role of *sea-2* and *daf-2* signaling in the heterochronic pathway

Genotype	Formation of alae (%) at the L3 stage (*n*)	Number of seam cells in young adults (*n*)	Formation of alae (%) at the young adult stage			
			No	Partial	Full	*n*
Wild type	0 (20)	16.0 (42)	0	0	100	46
sea-2(bp283)	0 (26)	20.4 (38)	0	6	94	69
sea-2(bp283) from dauer	ND	16.0 (26)	0	0	100	58
sea-2(bp283) 15°C	0 (14)	22.7 (24)	0	88	22	26
*sea-2(RNAi)**	ND	18.8 (26)	0	0	100	23
*sea-2(bp283); sea-2(RNAi)**	ND	20.6 (30)	0	7	93	27
lin-46(bp284)	ND	16.4 (40)	0	0	100	30
*sea-2(RNAi); lin-46(bp284)**	ND	26.7 (28)	0	100	0	18
sea-2(bp283); lin-46(bp284)	ND	27.1 (24)	0	64	36	22
sea-2(tm4355)	ND	16.4 (22)	0	0	100	20
sea-2(tm4355); lin-46(bp284)	ND	19.5 (28)	0	14	86	28
daf-12(rh257)	ND	33.7 (30)	0	100	0	33
sea-2(bp283); daf-12(rh257)	ND	43.4 (30)	0	100	0	19
daf-12(rh61rh411)	ND	17.8 (42)	0	0	100	35
sea-2(bp283); daf-12(rh61rh411)	ND	48.1 (32)	86	14	0	36
alg-1(gk214)	ND	17.8 (24)	0	43	57	21
sea-2(bp283); alg-1(gk214)	ND	34.9 (28)	100	0	0	18
ain-1(bp299)	ND	21.3 (32)	0	72	18	34
sea-2(bp283); ain-1(bp299)	ND	60 (29)	100	0	0	25
lin-66(ku423)	ND	37.1 (15)	100	0	0	15
sea-2(bp283); lin-66(ku423)	ND	90.2 (21)	100	0	0	24
lin-46(bp312)	ND	20.2 (42)	0	17	83	21
sea-2(bp283); lin-46(bp312)	ND	28.8 (24)	48	52	0	31
mir-48 mir-241(nDf51); mir-84(n4037)	ND	27.8 (25)	54	46	0	26
sea-2(RNAi); mir-48 mir-241(nDf51); mir-84(n4037)	ND	43.1 (26)	100	0	0	23
lin-28(n719)	100 (23)	10.7 (32)	0	0	100	20
lin-28(n719); sea-2(bp283)	100 (21)	11.4 (22)	0	0	100	21
hbl-1(RNAi)	53 (38)	ND	ND	ND	ND	ND
sea-2(bp283); hbl-1(RNAi)	18 (33)	ND	ND	ND	ND	ND
bpIs145†	ND	29.1 (32)	21	79	0	24
sea-2(bp283); bpIs145	ND	28.8 (28)	17	83	0	22
daf-2(e1370)	ND	16.3 (44)	0	0	100	22
sea-2(bp283); daf-2(e1370)	ND	27.8 (32)	0	42	58	31
daf-2(e1370); daf-12(rh61rh411)	ND	23.1 (36)	0	17	83	18
daf-2(e1370); alg-1(gk214)	ND	26.5 (28)	9	78	13	23
daf-16(mu86)	ND	16.1 (32)	0	0	100	29
daf-16(mu86); sea-2(bp283)	ND	20.0 (40)	0	4	96	26
daf-16(mu86); sea-2(bp283); daf-2(e1370)	ND	20.1 (34)	0	3	97	29

**sea-2(RNAi)* was delivered by injection.
†*bpIs145: lin-28::gfp::lin-28* 3′UTR(ΔLCE).
no, no alae; partial, alae gaps; full, complete alae; ND, not determined.
n, number of animal sides examined.
hbl-1 plays a secondary, opposite role during adulthood. *hbl-1(RNAi)* causes one or more breaks in adult alae and also abnormal nuclear division of seam cells at the young adult stage (Lin et al., 2003). Thus, the number of seam cells and the formation of alae in *hbl-1(RNAi)* animals were not examined.

numbers and a much more complete terminal differentiation defect (Table 1, Fig. 2A-D). For example, *sea-2; lin-66* double mutants had an average of 90 seam cells at the young adult stage, compared with 20 in *sea-2* and 37 in *lin-66* single mutants. In *sea-2; lin-66* mutants the L2 stage program was reiterated at both the L3 and L4 stages (Fig. 2E). The mutant alleles used are strong loss of function or null. The genetic interactions suggest that *sea-2* probably functions in parallel to these genes in specifying the L2/L3 switch.

We further examined the relationship between *sea-2* and other retarded heterochronic mutants. The *lin-4(e912)* null mutation leads to the reiteration of the L1 stage fate at subsequent larval stages. *lin-29* functions downstream in the heterochronic pathway in specifying the larval/adult switch (Ambros and Horvitz, 1984). We found that as in *lin-4* and *lin-29* single mutants, no alae were generated at the young adult stage in *sea-2 lin-4* and *sea-2 lin-29* double mutants (see Table S2 in the supplementary material). This is consistent with the hypothesis that *sea-2* functions upstream of *lin-29*.

The heterochronic defect in *sea-2* mutants is suppressed by loss of function of *lin-28*

To place *sea-2* in the heterochronic pathway, we examined the phenotype of *sea-2* mutants combined with precocious heterochronic mutants. *lin-28*, which encodes a protein with a cold shock domain and a CCHC zinc finger, specifies the L2 program (Moss et al., 1997). In *lin-28* mutants, L2-stage events are skipped and the larval/adult switch takes place at the L3 stage (Fig. 3A,B) (Ambros and Horvitz, 1984). The retarded heterochronic defects in *sea-2* mutants were completely suppressed by *lin-28*. *lin-28; sea-2* double mutants showed the same seam cell development phenotype as *lin-28* single mutants (Table 1; Fig. 3C,D). *hbl-1* (the Hunchback homolog) regulates the L2 fate and also the L4/adult switch. Loss of function of *hbl-1* causes a precocious heterochronic phenotype and also suppresses the retarded heterochronic defect in *let-7* family miRNAs mutants (Abrahante et al., 2003; Lin et al., 2003; Abbott et al., 2005). We found that *sea-2(bp283)* partially suppressed the precocious heterochronic defect in *hbl-1(RNAi)*

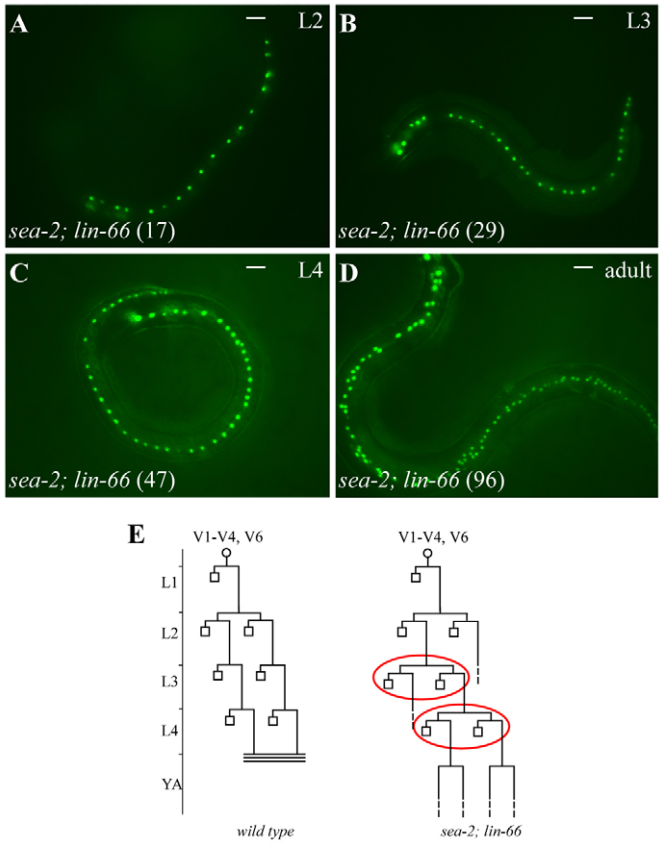

Fig. 2. Loss of function of *sea-2* causes strong synergistic heterochronic defects in *lin-66* mutants. (**A-D**) The number of seam cells in an L2 (A), L3 (B), L4 (C) and young adult (D) *sea-2; lin-66* double mutant. The number of seam cells is indicated in parentheses. Scale bars: 20 μm. (**E**) Schematic summary of the differentiation pattern of certain seam cells in *sea-2; lin-66* mutants. The L2 division pattern is repeated at the L3 and L4 stages in *sea-2; lin-66* mutants (highlighted in red). At the young adult stage, seam cells continue to divide and fail to form alae.

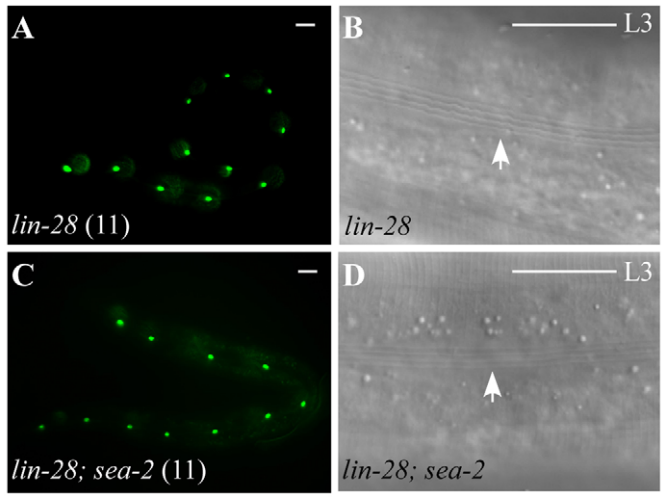

Fig. 3. Loss of function of *lin-28* completely suppresses *sea-2* heterochronic defects. (**A**,**B**) Loss of function of *lin-28* causes precocious heterochronic defects. In *lin-28* mutants, the L2 division pattern is skipped and thus fewer seam cells are present at the young adult stage (A) and alae are precociously formed at the L3 molt (arrow, B). Scale bars: 20 μm. (**C**,**D**) The retarded heterochronic phenotype in *sea-2* mutants is completely suppressed by loss of function of *lin-28*. The seam cell number is reduced (C) and alae are precociously formed at the L3 stage (arrow, D) in *lin-28; sea-2* mutants. Scale bar: 20 μm.

animals (Table 1). The *sea-2* mutation did not affect temporal expression of *hbl-1* (see Fig. S4 in the supplementary material). *sea-2* mutants also partially suppressed other precocious mutants, including *lin-14* and *lin-42* (see Table S2 in the supplementary material). These genetic analyses suggest that *sea-2* functions through *lin-28* in specifying the L2/L3 progression.

lin-28 is ectopically expressed in *sea-2* mutants

To understand how *sea-2* regulates *lin-28* activity, we examined expression of the translational fusion reporter *lin-28::gfp::lin-28* 3′UTR, which contains the *lin-28* coding and regulatory region. This transgene rescues the mutant phenotype of *lin-28(n719)* animals (Moss et al., 1997). *lin-28::gfp* is expressed in diverse cell types, including cells in the head, tail, muscles and seam cells. In wild-type animals, *lin-28::gfp* is expressed in L1 larvae, is detectable but diminished in L2 larvae and is almost undetectable from the L3 stage onwards (Fig. 4A,D; data not shown) (Moss et al., 1997). However, we found that in *sea-2* mutants high levels of LIN-28::GFP persisted in the head and tail at the L3 and L4 larval stages (Fig. 4B-D). The reporter also showed expression in seam cells in 87.5% of *sea-2* mutant L3 larvae (n=16) (Fig. 4E,F), whereas its expression was not detected in seam cells in wild type L3 larvae (n=15).

We further performed an immunoblot assay to examine the levels of endogenous LIN-28 protein using an anti-LIN-28 antibody. In wild-type animals, LIN-28 protein was present at the L1 larval stage, but was greatly reduced at the L3 larval stage (Fig. 4G) (Seggerson et al., 2002; Morita and Han, 2006). In *sea-2* mutants, levels of LIN-28 remained high in L3 larvae (Fig. 4G). Upregulation of LIN-28 in *sea-2* mutants was more prominent than that in *daf-12* mutants (Fig. 4G). We conclude that mutations in *sea-2* cause ectopic expression of *lin-28*.

sea-2 regulates *lin-28* expression at the post-transcriptional level

We next determined at what level *sea-2* regulates *lin-28* expression. No upregulation of *lin-28* mRNA was observed in L1 and L3 larvae in *sea-2* mutants (Fig. 4H); rather, levels of *lin-28* transcripts were even lower than wild type (Fig. 4H). This could be because wild-type SEA-2 affects the transcription or stability of *lin-28* or because high levels of LIN-28 in *sea-2* mutants negatively regulate the *lin-28* mRNA levels. To explore whether *sea-2* regulates *lin-28* expression at the post-transcriptional level via its 3′UTR, we examined expression of the *col-10::lacZ::lin-28* 3′UTR reporter (pKM50), in which the expression of *lacZ* is driven by the hypodermal cell-specific promoter *col-10* (Fig. 4I) (Morita and Han, 2006). In wild-type animals, expression of this reporter was strong in L1 larvae but absent in adults (Fig. 4I,J and data not shown). High expression persisted, however, in *sea-2* mutant adults (Fig. 4I,J). The *col-10::lacZ::unc-54* 3′UTR reporter, in which the *unc-54* 3′UTR was used instead of *lin-28* 3′UTR, was highly expressed in both wild type and *sea-2* mutants at the young adult stage (pKM53, Fig. 4J), suggesting that *sea-2* represses *lin-28* expression through its 3′UTR. To identify the SEA-2 response element, we examined the expression of a series of reporters with deletions of discrete regulatory elements in the *lin-28* 3′UTR. *sea-2(bp283)* dramatically increased expression of a reporter with a mutation in the putative

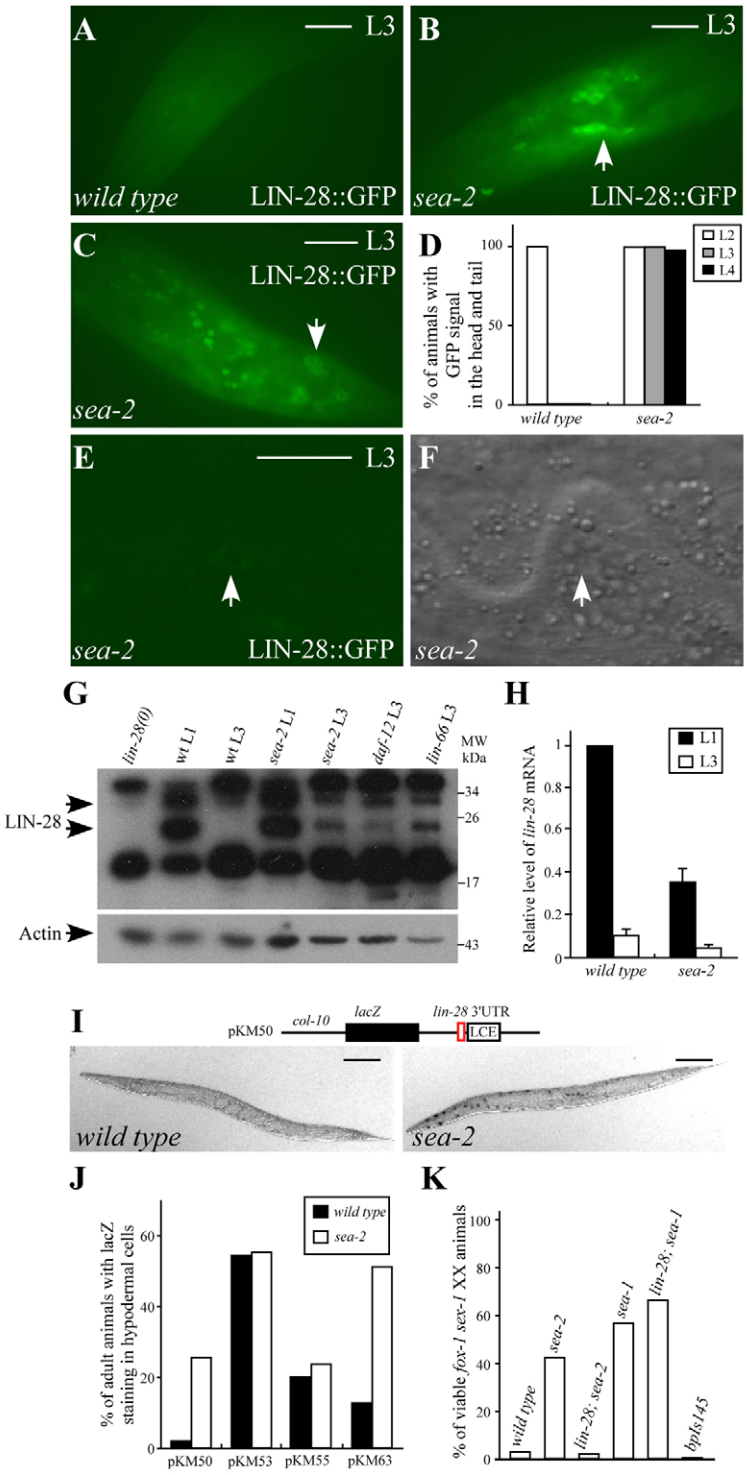

Fig. 4. *sea-2* regulates expression of *lin-28* at the post-transcriptional level through its 3′UTR. (**A**) In wild-type animals, the *lin-28::gfp* reporter is not detectable at the L3 stage in the head region (A) or the tail region (not shown). Scale bars: 20 μm. (**B**,**C**) High expression level of *lin-28::gfp* persists at the L3 stage in the head region (B) and the tail region (C) in *sea-2* mutants (arrow). Irregular fluorescence particles in C are gut autofluorescence. Scale bar: 20 μm. (**D**) Percentage of wild type and *sea-2* mutant animals expressing the *lin-28::gfp* reporter at different larval stages. Number of animals examined: wild type: L2 (*n*=22), L3 (*n*=34) and L4 (*n*=24); *sea-2*, L2 (*n*=29), L3 (*n*=34) and L4 (*n*=26). (**E**,**F**) Expression of the *lin-28::gfp* reporter in seam cells (arrow) in *sea-2* mutant L3 larvae. (F) DIC image of the seam cell shown in E. (E,F) Confocal images. Scale bar: 10 μm. (**G**) Western blot assay of endogenous LIN-28 protein using an anti-LIN-28 antibody. Arrows indicate LIN-28 protein bands due to alternative splicing as previously reported (Seggerson et al., 2002; Morita and Han, 2006). *lin-28(n719)* was used as a negative control. (**H**) Levels of *lin-28* mRNA, detected by quantitative RT-PCR, in wild type and *sea-2* mutants at the L1 and L3 stage. Consistent with published data, *lin-28* transcripts decrease from the L1 to the L3 stage in wild-type animals (Morita and Han, 2006). Error bars indicate the s.d. (**I**) Schematic structure of *col-10::lacZ::lin-28* reporter (pKM50). The DAF-12 response element (red box) and the *lin-4* complementary site LCE (box) are indicated. pKM50 is hardly expressed at the young adult stage in wild-type animals, but is strongly expressed in *sea-2* mutants. Scale bar: 100 μm. (**J**) Percentage of animals showing the expression of reporters in wild type and *sea-2* mutants. pKM53: *col-10::lacZ::unc-54* 3′UTR. pKM55 lacks the *lin-4* binding site in the *lin-28* 3′UTR. pKM63 [*col-10::lacZ::lin-28* 3′UTR (caaa to accc)] harbors a 4 bp substitution (caaa to accc) in the *lin-28* 3′UTR. Number of animals examined: wild type, pKM50 (*n*=309), pKM53 (*n*=398), pKM55 (*n*=220) and pKM63 (*n*=133); *sea-2*, pKM50 (*n*=265), pKM53 (*n*=432), pKM55 (*n*=278) and pKM63 (*n*=267). (**K**) *lin-28* is required for the role of *sea-2*, but not *sea-1*, in suppressing the XX lethality of *fox-1 sex-1*. Number of embryos examined: wild type (*n*=238), *sea-2* (*n*=232), *lin-28; sea-2* (*n*=219), *sea-1* (*n*=220), *lin-28; sea-1* (*n*=217) and *bpIs145* (*n*=158).

DAF-12 response element (pKM63) (Fig. 4J) (Morita and Han, 2006). However, *sea-2(bp283)* did not increase the expression of a *col-10::lacZ::lin-28* transgene lacking the *lin-4* binding site (pKM55) (Fig. 4J). A reporter lacking the *lin-4* complementary element (LCE), *lin-28::gfp::lin-28* 3′UTR(ΔLCE), is strongly expressed at late larval stages and also causes retarded heterochronic defects (see Fig. S5 in the supplementary material) (Moss et al., 1997). *sea-2(bp283)* did not further elevate reporter expression or the retarded heterochronic defects in animals carrying *lin-28::gfp::lin-28* 3′UTR(ΔLCE) (Table 1 and data not shown). Compared with wild-type animals, levels of *lin-4* remained unchanged at the L2 and L3 larval stage in *sea-2* mutants (see Fig. S5 in the supplementary material). These results indicate that *sea-2* probably acts through the LCE to regulate *lin-28* expression at the post-transcriptional level.

lin-28 is essential for the ASE function of *sea-2* in X:A signal assessment

sea-2 was previously identified as an autosomal signal element (ASE), loss of function of which suppresses the XX lethality phenotype caused by simultaneous depletion of the X signal

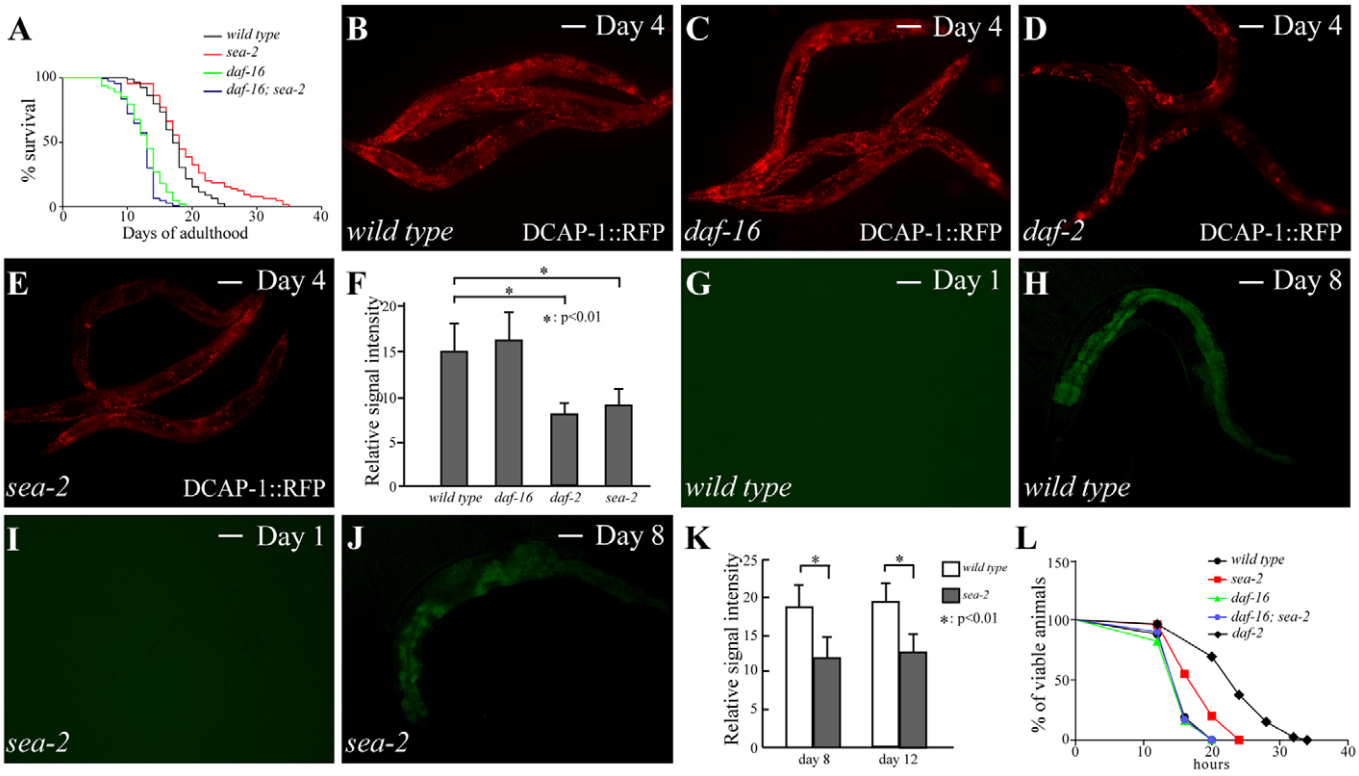

Fig. 5. *sea-2* mutants have extended lifespan. (**A**) *sea-2* mutants have extended lifespan. The long-lived phenotype of *sea-2* is suppressed by loss of function of *daf-16*. *P*=0.0027 (<0.01) when comparing N2 and *sea-2* mutants; *P*<0.001 when comparing *daf-16; sea-2* and *sea-2* mutants. (**B-E**) Expression of DCAP-1::RFP in 4-day-old adults. Animals were photographed on the same day under identical conditions. Scale bar: 100 μm. (**F**) Summary of relative signal intensity of DCAP-1::RFP. *P*=0.285 when comparing *daf-16* and wild type. *P*=0.006 when comparing *sea-2* and wild type. *P*=0.002 when comparing *daf-2* and wild type. (**G-J**) The gut autofluorescence intensity in *sea-2* mutants is weaker than in wild-type animals of the same age. Photographs were taken at 100× magnification. Scale bars: 100 μm. (**K**) Summary of signal intensity of lipofuscin autofluorescence in wild type and *sea-2* mutants. *P*=0.006 at day 8 when comparing *sea-2* and wild type, while *P*=0.003 at day 12. (**L**) *sea-2* mutants are more resistant to heat stress. The elevated stress resistance phenotype of *sea-2* mutants is suppressed by *daf-16*. *P*<0.001 when comparing wild type and *sea-2* mutants.

elements (XSEs) *sex-1* and *fox-1* (Meyer, 2005) (P. Nix and B. Meyer, personal communication). ASEs (also known as denominator elements) function with XSEs (also known as numerator elements) to communicate the ratio of X chromosomes to sets of autosomes (X:A signal) to determine sexual fate and to equalize expression of X-linked genes between hermaphrodites (XX) and males (XO) (Meyer, 2005). Thus, we determined whether *sea-2* also functions through *lin-28* to suppress the lethality of *fox-1 sex-1* mutants in XX animals. Simultaneously depleting the activity of *lin-28* in *sea-2; fox-1 sex-1* hermaphrodites caused XX animals to arrest during embryogenesis (Fig. 4K). *lin-28* has no effect on the suppression of the lethality of *fox-1 sex-1* by loss of function of another ASE, *sea-1* (Powell et al., 2005) (Fig. 4K). Mutations in other heterochronic genes, including *lin-14*, *daf-12* and *hbl-1* on the X chromosome, had no effect on the ASE function of *sea-2* (see Table S3 in the supplementary material). Loss of function of *fox-1 sex-1* still caused embryonic lethality in animals carrying the *lin-28::gfp::lin-28* 3′UTR(ΔLCE) transgene (Fig. 4K), indicating that *lin-28* is necessary but not sufficient to mediate the role of *sea-2* in the sex determination and dosage compensation pathways.

sea-2 mutants have an extended lifespan

We next examined whether *sea-2* functions in adult animals. Compared with wild-type animals, *sea-2(bp283)* mutants had a significant increase in lifespan (Fig. 5A; see Table S4 in the supplementary material). To determine whether *sea-2* mutants had a slower aging process, we examined two age-related markers, the accumulation of lipofuscin fluorescence in intestine cells (Garigan et al., 2002) and the accumulation of DCAP-1 (mRNA decapping enzyme)-labeled cytoplasmic processing bodies (P bodies). P bodies contain a variety of ribonucleoproteins and serve as sites for mRNA turnover and storage (Parker and Sheth, 2007). We found that DCAP-1 bodies gradually increased in hypodermal and muscle cells in aged wild-type animals (see Fig. S6 in the supplementary material). The accumulation of DCAP-1 bodies was slightly accelerated in short-lived *daf-16* mutants, and greatly decreased in long-lived *daf-2* mutants (Fig. 5B-D). *sea-2* mutants accumulated DCAP-1 bodies and lipofuscin fluorescence more slowly than wild-type animals (Fig. 5E-K). Many long-lived *C. elegans* mutants are resistant to heat stress (Kenyon, 2005). After heat shock treatment, *sea-2* mutants also survived longer than wild-type animals (Fig. 5L). The decreased rate of age-dependent reporter accumulation and elevated heat stress resistance confirm that loss of function of *sea-2* slows the rate of aging and extends the adult lifespan.

The extended lifespan in *sea-2* mutants depends on *daf-16*

DAF-16, a FOXO family transcription factor, is a master regulator of adult lifespan that integrates multiple inputs, including insulin/IGF-1 signaling, increased dosage of *sir-2.1* (the *C. elegans*

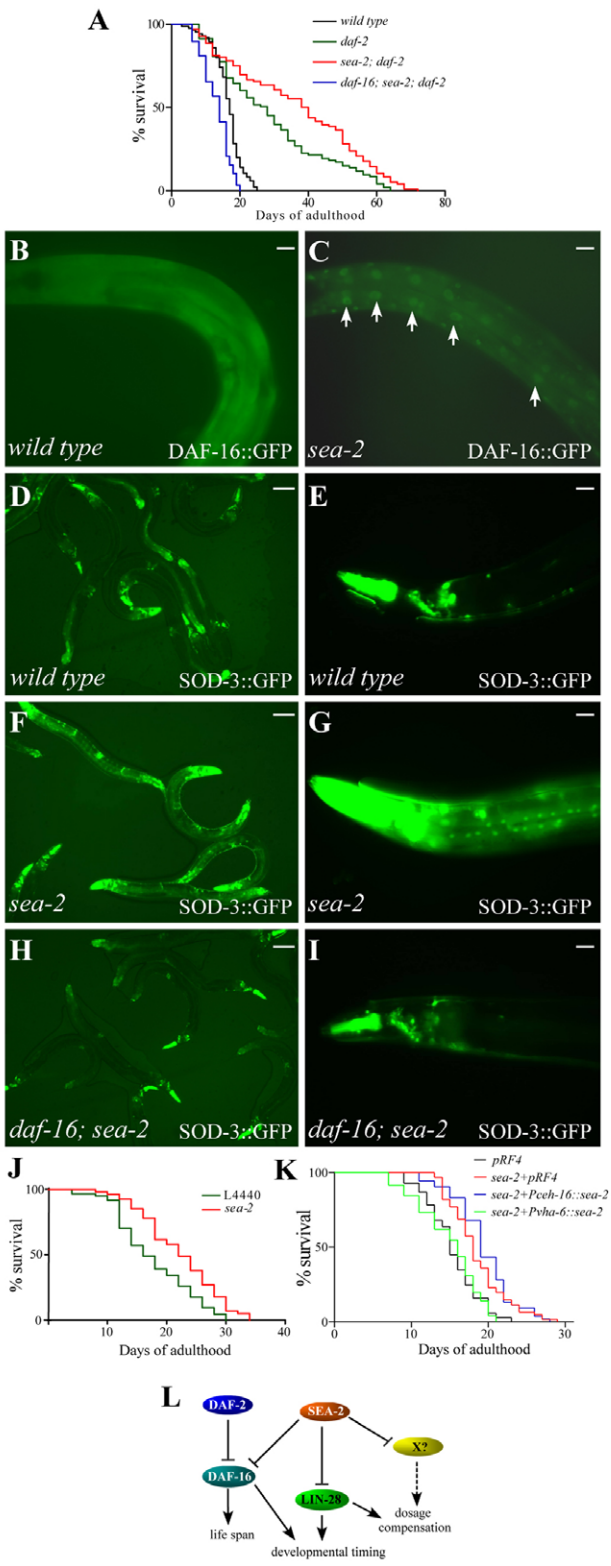

Fig. 6. *sea-2* regulates adult lifespan in a DAF-16 dependent manner. (**A**) Loss of function of *sea-2* extends the lifespan of *daf-2(e1370)* mutants. *P*=0.002 when comparing *sea-2; daf-2* and *daf-2*. (**B**) Wild-type animals show cytoplasmic localization of DAF-16::GFP. 0% of wild-type animals (*n*=73) show nuclear localization of DAF-16::GFP. (**C**) Loss of function of *sea-2* promotes nuclear localization of DAF-16::GFP (arrows). 82.7% of *sea-2* mutant animals (*n*=75) show nuclear localization of DAF-16::GFP. Scale bar: 20 μm. (**D**,**E**) Expression of *sod-3::gfp* in wild-type adult animals. Weak GFP signal is detected in the gut, pharynx and head neurons. (**F**,**G**) Loss of function of *sea-2* elevates *sod-3::gfp* expression in hypodermal and gut cells in *sea-2* adults. (**H**,**I**) Loss of function of *daf-16* suppresses the enhanced expression of *sod-3::gfp* in *sea-2* mutants. (D,F,H) Same magnification. Scale bars: 100 μm. (E,G,I) Same magnification. Scale bars: 20 μm. (**J**) Reduction of *sea-2* activity by RNAi feeding at the young adult stage extends the lifespan. L4440: feeding with the empty vector. *P*<0.001. (**K**) *sea-2* acts in the intestine to regulate the lifespan. Expression of *sea-2* in the intestine, but not in seam cells, rescues the extended lifespan phenotype in *sea-2* mutants. (**L**) Model for the role of *sea-2* in the pathways that control developmental timing, aging and dose compensation.

SIR2 NAD$^+$-dependent protein deacetylase homolog) and reduced activity of *lin-14* (Boehm and Slack, 2005; Lin et al., 1997; Ogg et al., 1997; Tissenbaum and Guarente, 2001). We thus investigated whether *sea-2* modulates lifespan through *daf-16*. *daf-16; sea-2* double mutants had the same lifespan as *daf-16* single mutants (Fig. 5A; see Table S4 in the supplementary material). The heat stress resistance of *sea-2* mutants was also abolished by the *daf-16* mutation (Fig. 5L). Thus, loss of activity of *daf-16* suppressed the longevity of *sea-2* mutants. Compared with *sea-2* and *daf-2* single mutants, the lifespan was further extended in *sea-2; daf-2* double mutants (Fig. 6A). The long-lived phenotype of *sea-2; daf-2* double mutants was also completely suppressed by loss of function of *daf-16* (Fig. 6A; see Table S4 in the supplementary material). Thus, *sea-2* probably acts in parallel to *daf-2* signaling and converges on *daf-16* in regulating lifespan.

In wild-type young adult animals, DAF-16 is diffusely localized in the cytoplasm (Fig. 6B). Reduced activity of insulin/IGF-1 signaling promotes nuclear translocation of DAF-16, which subsequently activates expression of genes involved in modulating stress resistance and aging (Lin et al., 1997; Ogg et al., 1997; Lee et al., 2001; Lee et al., 2003). In *sea-2* mutants, prominent nuclear localization of DAF-16 was observed in cells of the intestine (Fig. 6C), a tissue that is important for mediating the effect of DAF-16 on aging (Libina et al., 2003). Weak nuclear localization of DAF-16 was also evident in hypodermal and muscle cells (Fig. 6C). We further examined the expression of *sod-3*, a well-characterized target of DAF-16, in *sea-2* mutants (Libina et al., 2003). In wild-type young adult animals, *sod-3::gfp* was weakly expressed in intestinal, hypodermal and pharyngeal cells (Fig. 6D,E). Expression of *sod-3::gfp* was dramatically elevated in *sea-2* mutants (Fig. 6F,G), but this upregulation was completely abolished by reduced activity of *daf-16* (Fig. 6H,I). Therefore, loss of function of *sea-2* results in nuclear translocation of DAF-16 and activation of DAF-16 targets.

To investigate whether lifespan extension in *sea-2* mutants results from the retarded heterochronic defects at larval stages or is determined at the adult stage, we measured the lifespan of animals with reduced *sea-2* activity at different ages. RNAi inactivation of *sea-2* at early larval stages caused retarded heterochronic defects and also increased the lifespan (Table 1; data not shown). RNAi inactivation of *sea-2* in young adults had no effect on the temporal fate of seam cells, but still extended the lifespan (Fig. 6J; see Table S4 in the supplementary material). Therefore, *sea-2* regulates lifespan independent of its role in specifying developmental timing in larvae.

sea-2 acts in the intestine to regulate lifespan

We next expressed *sea-2* in a tissue-specific fashion to determine whether *sea-2* activity in any single tissue was sufficient to affect the lifespan. *sea-2* was specifically expressed in seam cells, neurons, body wall muscles or the intestine by fusing it with the *ceh-16*, *rgef-1*, *myo-3* or *vha-6* promoters, respectively (see Fig. S3 in the supplementary material). The lifespan of *sea-2* mutants carrying the transgene was measured. We found that expression of *sea-2* in the intestine, but not in other tissues, rescued the extended lifespan phenotype in *sea-2* mutants (Fig. 6K; see Table S5 in the supplementary material). Therefore, *sea-2* appears to act in the intestine to regulate adult lifespan.

Loss of function of *daf-2* causes heterochronic defects in larvae

Finally, we investigated whether insulin/IGF-1 signaling plays a role in the heterochronic circuit in larvae. *daf-2* and *daf-16* single mutants had the wild-type number of 16 seam cells and displayed no evident heterochronic defects (Table 1). However, *daf-2(e1370)* greatly enhanced the retarded heterochronic defects in *sea-2, daf-12* or *alg-1* mutants (Table 1). For example, the average number of seam cells was increased from 20 in *sea-2* mutants to 28 in *sea-2; daf-2* mutants and 42% of *sea-2; daf-2* double mutants showed defective terminal differentiation of seam cells, compared with 6% of *sea-2* single mutants. Loss of function of *daf-16* did not suppress the heterochronic defects in *sea-2* mutants (Table 1). However, *daf-16* completely suppressed the *daf-2*-dependent enhancement of the heterochronic defects in *sea-2* mutants (Table 1). Thus, *daf-16* also functions downstream of *daf-2* in controlling developmental timing in larvae. Expression of the *lin-28::gfp* reporter in *sea-2* mutants was not further elevated by simultaneous depletion of *daf-2* (see Fig. S7 in the supplementary material). The *hbl-1::gfp* reporter was also unchanged in *sea-2; daf-2* mutants (see Fig. S7 in the supplementary material). Thus, it remains to be determined whether *daf-2* signaling converges on *lin-28* or *hbl-1* in the heterochronic circuit.

DISCUSSION

Here, we demonstrated that the RNA-binding protein SEA-2 regulates the expression of *lin-28* in the heterochronic circuit, probably via the miRNA *lin-4* complementary element in the 3′UTR. SEA-2 may modulate the function of the miRNA-induced silencing complex (miRISC) at the *lin-28* 3′UTR. SEA-2 is not generally involved in modulating miRNA activity, because loss of function of *sea-2* has no effect on expression of two other miRNA targets, *hbl-1* (regulated by *let-7* family miRNAs) and *cog-1* (regulated by *lsy-6* miRNA) (see Fig. S4 in the supplementary material and data not shown). The role of *sea-2* in suppressing the XX lethality of *fox-1 sex-1* animals also requires the activity of *lin-28*. Mammalian Lin28 functions as a post-transcriptional repressor of the biogenesis of *let-7* family miRNAs (Heo et al., 2008), raising the possibility that LIN-28 mediates the biogenesis of miRNAs (e.g. *let-7*) that in turn play a role in sex determination and dose compensation. We also found that *sea-2* acts in parallel to *daf-2* insulin signaling to activate *daf-16*. Whether *sea-2* regulates adult lifespan through *lin-28* remains uncertain. *lin-28* mutants are short-lived (data not shown), which could be due to pleiotropic defects caused by loss of function of *lin-28*, including defective egg-laying. Animals carrying the *lin-28::gfp::lin-28* 3′UTR(ΔLCE) transgene that showed elevated levels of *lin-28* are also short-lived (data not shown). Overexpression of *sir-2.1* extends the adult lifespan in a manner dependent on DAF-16. However, *sea-2; sir-2.1(O/E)* animals are short-lived, which may be because double mutants display multiple defects such as bursting vulva (data not shown). Thus, the way in which *sea-2* converges on *daf-16* in regulating lifespan has yet to be determined. Consistent with a study showing that dauer arrest is decoupled from lifespan regulation, even though both processes are controlled by IGF-1 signaling (Dillin et al., 2002), *sea-2* mutation alone has no effect on dauer formation nor does it potentiate dauer formation in *daf-2* mutants (data not shown). Loss of function of *daf-16* had no effect on the role of *sea-2* either in the heterochronic pathway or in suppressing *fox-1 sex-1* lethality (see Table S3 in the supplementary material). Therefore, *sea-2* acts through distinct effectors to regulate developmental timing, dose compensation and lifespan (Fig. 6L). The multiple functions of *sea-2* during embryonic and larval development, such as specifying the temporal fate of seam cells and functioning as an ASE in dose compensation, indicate that wild-type *sea-2* has beneficial effects early in animal development. Thus, *sea-2* is favored by selection during evolution, even though *sea-2* mutants are long-lived. This provides support for the antagonistic pleiotropy theory of aging (Hughes and Reynolds, 2005).

Our study further supports the presence of an intrinsic timing mechanism that temporally initiates a program of aging in the adult, consistent with a coordinate shift in gene expression pattern early in adulthood in *C. elegans* and *Drosophila* (McCarroll et al., 2004). Here, we have revealed a novel function of *daf-2* insulin/IGF-1 signaling in controlling developmental timing at larval stages. Reduced activity of *daf-2* dramatically enhances retarded heterochronic defects by causing reiteration of the L2 stage fate at late larval stages. On the other hand, several components of the heterochronic circuit also influence the rate of aging. Reducing *lin-14* activity or overexpressing *lin-4* modestly extends lifespan in a *daf-16*-dependent fashion (Boehm and Slack, 2005). However, there is no correlation between the degree or type of heterochronic defect and the rate of aging. *lin-14* specifies the L1/L2 transition, while *sea-2* and *daf-2* control the L2/L3 switch. Loss of function of *lin-14* causes precocious, whereas *sea-2* and *daf-12* mutations cause retarded, heterochronic defects. *lin-14* and *sea-2* mutants have an extended lifespan, whereas null *daf-12* mutants have slightly shortened lifespans (Fisher and Lithgow, 2006). *sea-2*, *lin-14* and *daf-2* influence aging during adulthood in a way that is temporally separable from their role in determining developmental timing (Boehm and Slack, 2005; Dillin et al., 2002). Thus, the timing program that modulates the aging process in adults shares a subset of genes with the heterochronic circuit that functions at larval stages.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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