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Correspondence and requests for materials should be addressed to J.S.W. (e-mail: jsw1@itsa.ucsf.edu).

Increased dosage of a *sir-2* gene extends lifespan in *Caenorhabditis elegans*

Heidi A. Tissenbaum & Leonard Guarente

Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA

In *Caenorhabditis elegans*, mutations that reduce the activity of an insulin-like receptor (*daf-2*)¹ or a phosphatidylinositol-3-OH kinase (*age-1*)² favour entry into the dauer state during larval development³ and extend lifespan in adults^{3–6}. Downregulation of this pathway activates a forkhead transcription factor (*daf-16*)^{7,8}, which may regulate targets that promote dauer formation in larvae and stress resistance and longevity in adults⁹. In yeast, the *SIR2* gene determines the lifespan of mother cells, and adding an extra copy of *SIR2* extends lifespan¹⁰. Sir2 mediates chromatin silencing through a histone deacetylase activity that depends on NAD (nicotinamide adenine dinucleotide) as a cofactor^{11–13}. We

have surveyed the lifespan of *C. elegans* strains containing duplications of chromosomal regions. Here we report that a duplication containing *sir-2.1*—the *C. elegans* gene most homologous to yeast *SIR2*—confers a lifespan that is extended by up to 50%. Genetic analysis indicates that the *sir-2.1* transgene functions upstream of *daf-16* in the insulin-like signalling pathway. Our findings suggest that Sir2 proteins may couple longevity to nutrient availability in many eukaryotic organisms.

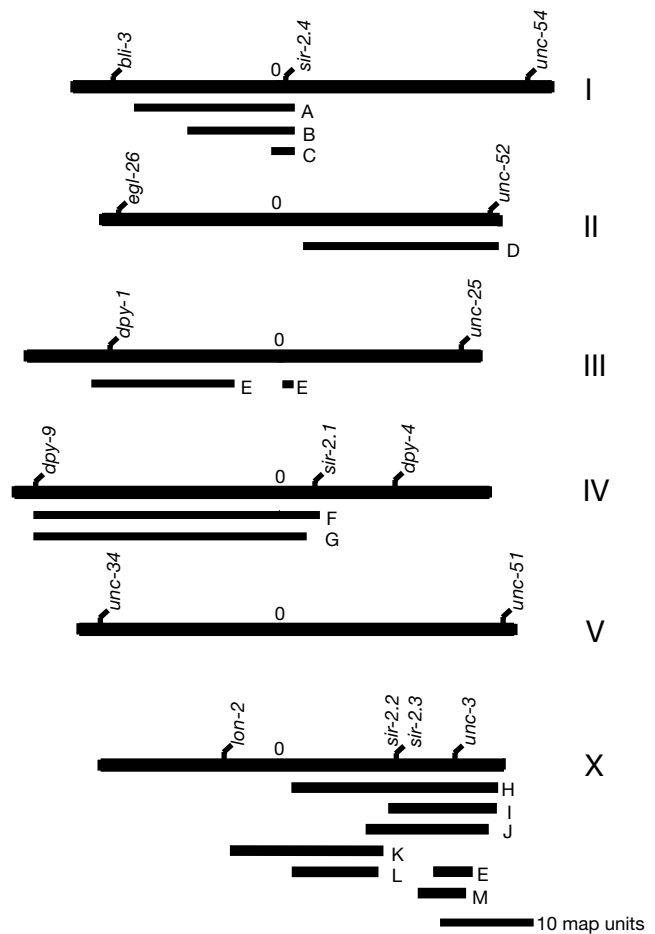


Figure 1 Genetic map of duplication strains. Genetic map showing some of the duplications tested for lifespan. Chromosome numbers are indicated to the right and drawn to scale according to WormBase (<http://www.wormbase.org/>) and Acedb (<http://elegans.swmed.edu/>). Chromosome sizes are as follows: I, 14,808,446 bp; II, 15,205,541 bp; III, 13,755,072 bp; IV, 17,452,072 bp; V, 21,150,598 bp; X, 17,727,995 bp. Thick line indicates chromosome; thin line represents duplication(s). In many cases, duplications were grouped together and assigned a letter A–M, as the endpoints were within one map unit. Mean lifespans of the duplication strains (in days) were tested in four trials. Trial 1: N2, 15.2 ± 0.5; KR1293-B, 11.7 ± 0.3; KR1725-C, 14.7 ± 0.3; KR1722-C, 15.8 ± 0.6; SP75-J, 14.0 ± 0.5; KR1704-C, 14.0 ± 0.4; TY1912-L, 15.8 ± 0.6; DR1786-F, 17.3 ± 1.0; SP116-I, 16.8 ± 0.5, SP117-H = 13.7 ± 0.6. Trial 2: N2, 15.1 ± 0.3; KR1732-C, 17.3 ± 0.5; RW6011-D, 17.6 ± 1.0; DR1786-F, 21.1 ± 1.0; TY1909-K, 14.0 ± 0.6; SP125-M, 18.8 ± 1.0. Trial 3: N2, 17.8 ± 0.6; MT070-G, 14.6 ± 0.6; DR907-G, 16.6 ± 0.7; Trial 4: N2, 18.1 ± 0.8; KR1108-A, 18.7 ± 0.8; KR1110-A, 19.7 ± 0.7; KR1112-A, 18.7 ± 0.9; KR1236-C, 15.3 ± 0.6; KR1284-B, 12.8 ± 0.4; KR1280-B, = 10.2 ± 0.3; KR1548-C, 14.5 ± 0.4; KR1282-B, 10.9 ± 0.6; KR1815, 12.5 ± 1.0; SP1911-E, 14.3 ± 0.4 (see also Supplementary Information Table 1). *sir-2.2* and *sir-2.3* are adjacent to each other on the X chromosome. E is SP1911-*mnDp1*; this free duplication contains regions on both chromosome III (where it has a large deletion) and the X chromosome²⁷. The duplication in strain KR1815, *hDp30*, has not been precisely mapped. An additional nine strains that cover chromosome I and the X chromosome have been tested and do not display a significant increase in lifespan (not shown).

The *C. elegans* genome has four genes with similarity to yeast *SIR2* (ref. 14). Of these, we term the most related *sir-2.1* (31% identity to yeast *Sir2* in the conserved core domain). Other *sir-2* genes (*sir-2.2*, *sir-2.3* and *sir-2.4*) are between 10 and 20% identical in their conserved domains. To test whether these *sir-2* genes might regulate lifespan, we obtained strains with duplications of many chromosomal regions, including duplications covering these loci. Duplications are stably maintained in *C. elegans*, and the dosage of genes that they carry is increased by 50% (ref. 15). In total, we examined strains containing 35 different duplications, covering about half of the *C. elegans* genome (Fig. 1). Most of these strains, including those with extra copies of *sir-2.2*, *sir-2.3* and *sir-2.4*, exhibited lifespans similar to or shorter than the parental strain (see Supplementary Information Table 1). Notably, strain DR1786 containing the free duplication *mDp4* showed a significant extension in both mean and maximum lifespan (Fig. 2a; and Supplementary Information Table 1). This chromosome IV duplication includes the *sir-2.1* locus. Another chromosome IV duplication (*mDp1*; present in strains DR907 and MT7070) includes about 90% of the *mDp4* duplication, but is missing the region carrying the *sir-2.1* locus, and does not extend lifespan (Fig. 2a). Therefore, we inferred that *sir-2.1* or one of the other genes carried uniquely by *mDp4* extended the lifespan.

To test whether *sir-2.1* was responsible for this extension, we injected a 2.2-kilobase (kb) genomic fragment of *sir-2.1* into the N2 parental strain along with the transformation marker gene *rol-6* and identified three independent transgenic lines (see Methods). All three lines containing *sir-2.1* (*geEx1*, *geEx2* and *geEx3*) showed significantly extended lifespans ($P < 0.05$; mean lifespan 20.9 ± 0.3 , 27.4 ± 1.0 and 22.4 ± 0.4 days, respectively) compared with animals transgenic for *rol-6* (*su1066*) alone (mean lifespan 18.2 ± 0.3 days) or the N2 parent (mean lifespan 17.6 ± 0.2 days; Fig. 2b).

We derived stably integrated lines from animals carrying the *SIR2* extrachromosomal array (see Methods). Integrants derived from both *geEx1* (*geIn1*, *geIn2*) and *geEx2* (*geIn3*) had a several-fold increase in *sir-2.1* copy number, as determined by Southern blotting, compared with the N2 control (data not shown). These lines exhibited the same long lifespans as lines containing extrachromosomal arrays (*geEx1*, 20.9 ± 0.3 days; *geIn1*, 21.6 ± 0.3 ; *geIn2*, 21.5 ± 0.5 ; *geEx2*, 27.4 ± 1.0 ; *geIn3*, 27.6 ± 0.4 ; Fig. 2c). We conclude that, as in yeast, increasing the dosage of a *SIR2* gene extends the lifespan of *C. elegans*.

We considered whether *sir-2.1* extends lifespan by acting through one of the known *C. elegans* pathways. The extension in lifespan by mutations in the insulin-like signalling pathway (*daf-2*, *age-1* or *pdk-1*) is abolished in strains mutant in the downstream gene *daf-16* (refs 3, 6, 16); however, a second class of mutations that extend lifespan in *C. elegans*, termed *clk*, affects metabolic rate, characterized by a slowing in rhythmic behaviours and an increase in the time

Table 1 The *sir-2.1* transgene synergizes with TGF- β signalling mutants for dauer formation

Genotype	% dauer formation		Lifespan of array
	15°C (n)	20°C (n)	
<i>daf-4(m63)</i>	0.7 (449)	10.0 (981)	—
<i>daf-4(m63)</i> + pRF4	1.0 (209)	11.6 (353)	18.2
<i>daf-4(m63);geEx2</i>	15.0 (301)	48.0 (229)	27.4
<i>daf-4(m63);geEx3</i>	12.4 (431)	29.2 (380)	22.4
<i>daf-1(m40)</i>	0.6 (179)	2.7 (670)	—
<i>daf-1(m40)</i> + pRF4	0 (692)	0 (350)	18.2
<i>daf-1(m40);geEx2</i>	0 (30)	29.5 (285)	27.4
<i>daf-1(m40);geEx3</i>	1.3 (79)	13.0 (339)	22.4

Adult hermaphrodites were allowed to lay eggs for 4–18 h at 15°C after which they were removed and the plate was placed at either 15°C or 20°C. Eggs and L1 larvae were counted. Either 3 (20°C) or 6 (15°C) d later, plates were scored for dauer, nondauer, roller and nonroller. Data are the results of at least two completely independent trials. n, number of animals tested. As *daf-1* mutations are a maternal effect, hermaphrodites were allowed to lay eggs at 20°C for this mutant.

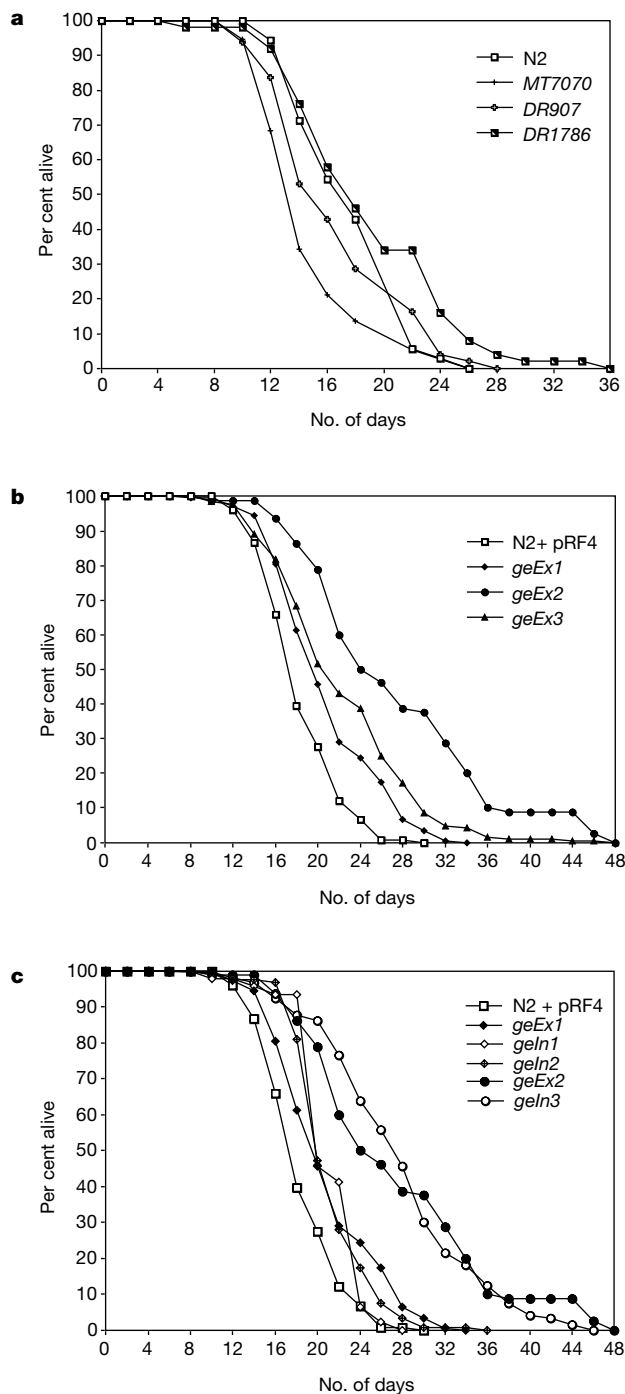


Figure 2 Increased dosage of *sir-2.1* extends lifespan. **a**, Lifespan curve shows that duplication of the *sir-2.1* region extends *C. elegans* lifespan. Data for each strain (mean \pm s.e. (number of trials); n, number of animals scored): N2, 17.6 ± 0.2 (13), $n = 451$; MT7070, 14.6 ± 0.6 (1), $n = 38$; DR907, 16.6 ± 0.7 (1), $n = 49$; DR1786, 19.4 ± 0.6 (2), $n = 50$; $P < 0.05$ compared with N2. **b**, Lifespan curve shows introduction of *C. elegans sir-2.1* transgene extends lifespan. Data for each strain (mean \pm s.e. (number of trials); n, number of animals scored): N2 + pRF4, 18.3 ± 0.3 (3), $n = 142$; *geEx1*, 20.9 ± 0.3 (6), $n = 214$; *geEx2*, 27.4 ± 1.0 (4), $n = 80$; *geEx3*, 22.4 ± 0.4 (6), $n = 209$. $P < 0.05$ for all three extrachromosomal array lines when compared with wild type. Also, there was no significant effect of *rol-6* on lifespan (mean lifespan 18.2 ± 0.3 with *rol-6*; 17.6 ± 0.2 wild-type alone). **c**, Integration of extrachromosomal array shows similar lifespan extension to the free array. Data for each strain (mean \pm s.e. (number of trials); n, number of animals scored). N2 + pRF4, 18.3 ± 0.3 (3), $n = 142$; *geEx1*, 20.9 ± 0.3 (6), $n = 214$; *geIn1*, 21.6 ± 0.3 (2), $n = 46$; *geIn2*, 21.5 ± 0.5 (3), $n = 121$; *geEx2*, 27.4 ± 1.0 (4), $n = 80$; *geIn3*, 27.5 ± 0.4 (7), $n = 276$.

of development from embryo to adulthood. Mutations in this class are not suppressed by *daf-16* (ref. 17).

We thus tested whether *sir-2.1* may be acting in this pathway by determining the effect of a mutation in *daf-16* on *sir-2.1*-mediated longevity. The extrachromosomal array or integrated *sir-2.1* was crossed into a background containing *daf-16(mgDf50)*—a large deletion that eliminates most of the *daf-16* coding region⁷. *daf-16(mgDf50)* animals displayed a shorter mean lifespan than did wild-type animals (14.6 ± 0.1 days in *daf-16(mgDf50)* versus 17.6 ± 0.2 in N2) similar to other alleles of *daf-16* (refs 7, 8). The lifespan of *geIn3* animals was 27.6 ± 0.4 days, and was reduced to 13.7 ± 0.2 in *daf-16(mgDf50);geIn3* animals, similar to the lifespan of

daf-16(mgDf50) mutants alone (Fig. 3a). Similarly, when *daf-16(mgDf50)* was crossed to any of the three extrachromosomal arrays, lifespan was reduced to the level of *daf-16* mutant alone (Fig. 3a, legend). Therefore, the lifespan extension of the *sir-2.1* transgene requires *DAF-16* activity, suggesting that *sir-2.1* acts in the insulin-like signalling pathway.

If *sir-2.1* acts in this pathway, the transgene should not further extend the lifespan of a *daf-2* mutant, in which the pathway has been inactivated. *sir-2.1* transgenic arrays were thus crossed into a *daf-2(e1370)* mutant background. The *daf-2* mutation increased lifespan markedly, similar to previous data^{3,5,6,18} (Fig. 3b). However, no further extension was observed in a strain bearing the transgene and *daf-2(e1370)*; that is, the lifespan of the *daf-2(e1370);geIn3* was similar to that of *daf-2(e1370)* animals (Fig. 3b). Thus, these experiments indicate that the *sir-2.1* transgene functions to extend lifespan in the insulin-like signalling pathway.

Many mutations in the insulin-like signalling pathway show other pleiotropies, including dauer formation, a reduced brood size and early larval lethality^{4,18–20}. Therefore, we determined whether *sir-2.1* extrachromosomal transgenic animals displayed any pleiotropic phenotypes. Brood size and time of development from hatching through the four larval stages to adulthood were normal in these animals when compared with wild-type animals carrying a *rol-6(su1066)* extrachromosomal array (see Supplementary Information Table 2). Furthermore, there was little dauer formation at 27 °C or at lower temperatures in any of the transgenic *SIR2* strains compared with wild-type animals with or without the *rol-6(su1066)* extrachromosomal array (data not shown). Thus, the *sir-2.1* transgene by itself does not affect fertility, early development or dauer formation.

Mutations in several genes in the insulin-like signalling pathway including *daf-2*, *unc-31* and *unc-64* also synergize with mutations in a parallel, transforming growth factor- β (TGF- β) signalling pathway, represented by *daf-4* and *daf-1* (refs 7, 20). *daf-1* and *daf-4* are type I and type II TGF- β receptors, and mutations in either of these genes cause a temperature-sensitive dauer-constitutive phenotype in larvae but do not affect lifespan in adults^{5,21}. Thus, to establish further that *sir-2.1* is linked to the insulin-like signalling pathway, we crossed the *sir-2.1* transgenic lines into *daf-4(m63)* or *daf-1(m40)* mutants.

We observed a striking increase in dauer formation at the permissive temperature of 20 °C in *sir-2.1* transgenic animals in combination with either *daf-4* or *daf-1* mutations (Table 1). Moreover, the tendency to form dauers correlated with the lifespan extension of the two transgenic lines tested. For example, at 20 °C 48% of the *daf-4;geEx2* animals formed dauers (*geEx2* increases lifespan 1.5 times), whereas 29% of the *daf-4;geEx3* animals formed dauers (*geEx3* increases lifespan 1.2 times). Even at 15 °C, both *daf-4;geEx2* and *daf-4;geEx3* animals showed a significant increase in dauer formation (Table 1). Thus, like mutations of other components of the insulin-like signalling pathway, the *sir-2.1* transgene synergizes with mutations in the TGF- β signalling pathway. As predicted, the *sir-2.1* transgene did not synergize with *daf-2(e1370)* for dauer formation (data not shown). These findings further bolster the claim that *sir-2.1* exerts its effect on the insulin-like signalling pathway. Evidently the effect of the *sir-2.1* transgene alone is too subtle to trigger dauer formation without the sensitizing *daf-1* or *daf-4* mutations.

In yeast, *Sir2* may act at the interface of nutrient availability and survival, by extending lifespan in response to calorie restriction²², and also by allowing haploid cells to mate and thus sporulate when starved. In *C. elegans*, *sir-2.1* may also couple environmental conditions to survival mechanisms; that is, the decision to entry into dauer in larvae, and the determination of lifespan in animals that proceed to adulthood. The mechanism by which *sir-2.1* functions may be the silencing of genes upstream of *daf-16* in cells that respond to the *DAF-2* ligand, the silencing of genes that result in

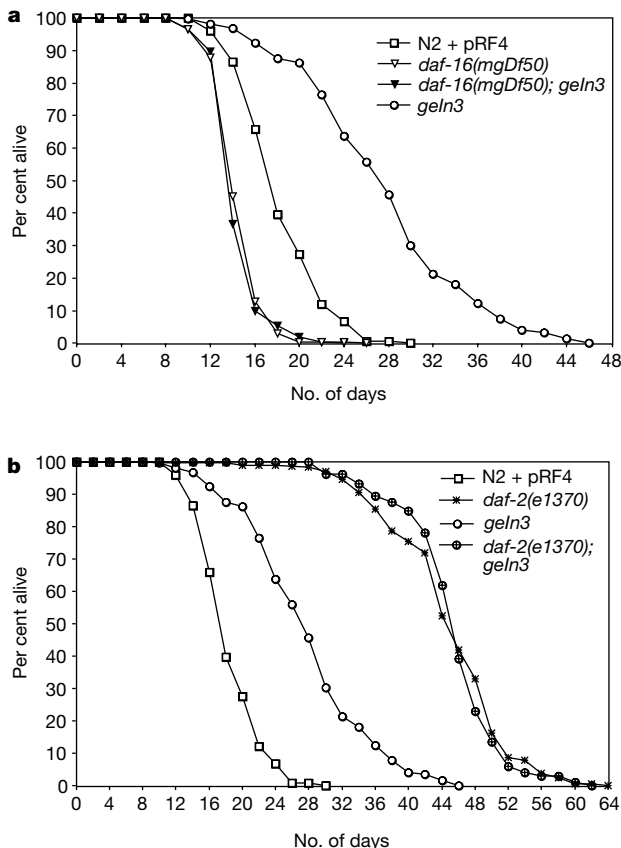


Figure 3 *sir-2.1* functions in the dauer signalling pathway. **a**, Lifespan curve shows that *daf-16* suppresses the lifespan extension of *sir-2.1* transgene. Data for each strain (mean \pm s.e. (number of trials); *n*, number of animals scored): N2 + pRF4, 18.3 ± 0.3 (3), *n* = 142; *daf-16(mgDf50)*, 14.6 ± 0.1 (5), *n* = 274; *daf-16(mgDf50);geIn3*, 13.7 ± 0.2 (4), *n* = 204; *geIn3*, 27.5 ± 0.4 (7), *n* = 276. Similar findings were seen with the extrachromosomal array lines: *daf-16(mgDf50);geEx1*, 14.2 ± 0.5 (1), *n* = 40; *daf-16(mgDf50);geEx2*, 14.0 ± 0.3 (1), *n* = 29; *daf-16(mgDf50);geEx3*, 14.4 ± 0.4 (1), *n* = 47. This experiment was repeated on different independent isolates from both *daf-16(mgDf50);geEx1* and *daf-16(mgDf50);geEx3* with similar results (not shown). **b**, Lifespan curve shows that *daf-2* does not synergize with *sir-2.1* transgene for lifespan extension. Data for each strain (mean \pm s.e. (number of trials); *n*, number of animals): N2 + pRF4, 18.3 ± 0.3 (3), *n* = 142; *daf-2(e1370)*, 44.7 ± 0.4 (6), *n* = 293; *daf-2(e1370);geIn3*, 45.0 ± 0.5 (2), *n* = 105; *geIn3*, 27.5 ± 0.4 (7), *n* = 276. Similar values were seen with another independent isolate of *daf-2(e1370);geIn3* (not shown). When crossed into a *daf-2* background, *geEx1* and *geEx3* showed similar findings with the means similar to *daf-2(e1370)* alone: *daf-2(e1370);geEx1* = 41.9 ± 1.1 (2), *n* = 63; *daf-2(e1370);geEx3*, 47.2 ± 1.4 (1), *n* = 36. This experiment was repeated on different independent isolates from both *daf-2(e1370);geEx1* and *daf-2(e1370);geEx3* with similar results (not shown). Side by side comparisons showed that the *rol-6(su1066)* extrachromosomal array did not affect the mean or maximum lifespans of the *daf-2(e1370)* mutant (not shown).

production of the ligand in signalling cells, or both. Overexpression of *sir-2.1* would promote longevity and predispose animals to dauer formation by hyper-repressing these genes.

It seems remarkable that replicative ageing in yeast mother cells and postmitotic ageing in the soma of adult worms are both regulated by Sir2 proteins. In yeast, one beneficial effect of Sir2 appears to occur in the ribosomal DNA, where Sir2-silencing represses recombination and may also coordinate rRNA synthesis and growth rate. In *C. elegans*, silencing by Sir2 may couple nutrient availability to the level of signalling through the insulin-like signalling pathway. It will be of interest to determine whether Sir2 proteins regulate the rate of ageing in still higher eukaryotes, which contain both dividing and postmitotic cells in their soma. If so, SIR2 genes may provide a link that coordinates ageing in these two kinds of tissues to nutrient availability in an underlying and pervasive regulatory mechanism. □

Methods

Duplication strains

All strains were maintained and handled as described^{23,24}. Duplication strains were maintained by picking individual worms and examining the brood for proper segregants.

Transgenic worms

A 2.2-kb polymerase chain reaction (PCR) fragment containing the complete *sir-2.1* coding sequence and 400 base pairs (bp) upstream and 300 bp downstream was injected at 50 ng μl^{-1} along with pRF4 at 100 ng μl^{-1} (ref. 25) to obtain stable extrachromosomal transgenic lines. Lines were maintained by picking roller animals.

Integration

For each of *geEx1*, *geEx2* and *geEx3*, 10 L4 hermaphrodites were placed on 10 plates subjected to gamma irradiation from a Gammacell 220⁶⁰Cosource at 4,000 rads. We then placed 5 animals onto each of 20 plates at 20°C and allowed the plates to starve such that no more bacteria remained. Each of the 20 plates was then chunked onto a fresh plate. After 2 d, a total of 20 worms was singly picked to a plate from every parent plate for a total of 400 worms per gamma irradiation. After 4 d, plates were scored for the presence of rollers and non-rollers. We kept any plates with all rollers, and followed them for an integrated line. From these plates a total of five individual hermaphrodites were singled to new individual plates and scored 4 d later for all rollers. Integrants segregated all rollers on all of the five plates.

Strain construction

For *daf-2*, *daf-2(e1370)*, males were mated to the transgenic SIR2 lines at 15°C. After 5–7 d, we transferred putative roller cross progeny to individual plates at 25°C, and scored the plates 3 d later for the presence of dauers. Roller dauers were returned to 15°C to recover and singled to individual plates. For integrated lines, 20 animals were transferred from the brood of the recovered dauers to individual plates and their progeny scored for the presence of 100% rollers.

For *daf-16*, *daf-16(mgDf50)* males were mated to the *sir-2.1* lines at 15°C. After 5–7 d, putative roller cross progeny were singled to individual plates at 15°C. We singled 12 animals to plates from each of the F₁ plates and allowed them to lay eggs. After 4–5 d, PCR was done of the F₂ parent to determine the *daf-16* genotype using primers SO77 and SO78 (ref. 7). Strains were maintained by transferring roller animals to new plates.

For *daf-1/daf-4*, wild-type males were mated to either *daf-1(m40)* or *daf-4(m63)* hermaphrodites at 15°C. After 5–7 d, non-Daf F1 male cross progeny were mated to the transgenic SIR2 lines at 15°C. After 5–7 d, 10 putative roller cross progeny hermaphrodites were singled to plates at 25°C. After 3 days, plates segregating roller dauer progeny were kept. Roller dauers were then picked off the plate and allowed to recover individually on a plate at 15°C to establish the strain. We maintained strains by transferring roller animals to new plates.

Lifespan and development assays

Lifespan assays were done at 20°C. Adult hermaphrodites were picked (4–10 per plate) from each strain and allowed to undergo one full generation at 15°C or 20°C. From these plates, we picked individual L4s or young adults to plates at 20°C containing 400 $\mu\text{g ml}^{-1}$ 5'-fluoro-2'-deoxyuridine (FUDR), which blocks DNA synthesis and causes animals to lay eggs that do not develop and eliminates the need to transfer animals throughout the lifespan assay²⁶. Animals were tapped every 2–4 d and were scored as dead when they did not move after repeated taps with a pick. A limited number of experiments were carried out on plates without FUDR by transferring adult animals every 1–2 d to new plates and these revealed the same, long lifespans of *sir-2.1* transgenic animals. All statistical tests were done using JMP 4.0 software.

Timing of development was scored for each strain at 20°C. Plates were scored every 12–18 h for developmental stage. No noticeable differences were observed comparing the roller strains with and without the *sir-2.1* transgene. For 27°C dauer formation, animals

were allowed to lay eggs at 27°C for 4 h. We scored plates 2 d later for the presence of dauers and non-dauers. Both wild-type and *unc-31(e928)* animals were used as controls for dauer induction.

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Correspondence and requests for materials should be addressed to L.G. (e-mail: leng@MIT.edu).