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Tissue-Specific Activities of *C. elegans* DAF-16 in the Regulation of Lifespan

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Summary

In *C. elegans*, the transcription factor DAF-16 promotes longevity in response to reduced insulin/IGF-1 signaling or germline ablation. In this study, we have asked how different tissues interact to specify the lifespan of the animal. We find that several tissues act as signaling centers. In particular, DAF-16 activity in the intestine, which is also the animal's adipose tissue, completely restores the longevity of *daf-16(−)* germline-deficient animals, and increases the lifespans of *daf-16(−)* insulin/IGF-1-pathway mutants substantially. Our findings indicate that DAF-16 may control two types of downstream signals: DAF-16 activity in signaling cells upregulates DAF-16 in specific responding tissues, possibly via regulation of insulin-like peptides, and also evokes DAF-16-independent responses. We suggest that this network of tissue interactions and feedback regulation allows the tissues to equilibrate and fine-tune their expression of downstream genes, which, in turn, coordinates their rates of aging within the animal.

Introduction

How signaling between tissues coordinates the physiology of an animal is a fundamental problem in endocrinology. The aging of *C. elegans* is controlled by an endocrine system that also regulates the lifespans of flies and mammals (Tatar et al., 2003). Reduction-of-function mutations affecting the insulin/IGF-1-like receptor DAF-2, or components of a downstream PI 3-kinase/PDK/AKT pathway, double the animal's lifespan (Kenyon et al., 1993; Kimura et al., 1997; Larsen et al., 1995; Morris et al., 1996; Paradis and Ruvkun, 1998). This lifespan extension requires DAF-16, a member of the FOXO-family of transcription factors (Kenyon et al., 1993; Larsen et al., 1995; Lin et al., 1997; Ogg et al., 1997). In the wild-type, the AKT-1 and AKT-2 proteins phosphorylate DAF-16, inhibiting its nuclear localization (Henderson and Johnson, 2001; Lee et al., 2001; Lin et al., 2001). In DAF-2 pathway mutants, DAF-16 accumulates in the nuclei of many cell types, where it leads to changes in the expression of a wide variety of metabolic, stress response, antimicrobial, and novel genes, and thereby extends lifespan (Lee et al., 2003; McElwee et al., 2003; Murphy et al., 2003).

Several findings suggest that interactions between tissues play an important role in establishing the animal's rate of aging. First, the *C. elegans* genome contains more than 35 insulin-like genes expressed in a variety of neurons and other tissues, and some of these have been implicated in lifespan regulation (Kawano et al., 2000; Li et al., 2003; Murphy et al., 2003; Pierce et al., 2001). In addition, in response to insulin-like ligands, cells that express the DAF-2 receptor are thought to produce (or stop producing) downstream signals or hormones, because *daf-2* acts cell non-autonomously to influence lifespan (Apfeld and Kenyon, 1998; Wolkow et al., 2000). For example, removing *daf-2* from either of the two blastomeres of the two-cell embryo lengthens the lifespan of the entire animal (Apfeld and Kenyon, 1998).

Recent studies suggest that the DAF-2 pathway may regulate multiple downstream signals. One potential signal is SCL-1, a member of the CRISP family of secreted proteins. *scl-1* expression is upregulated in *daf-2* mutants, and contributes to their longevity (Ookuma et al., 2003). The DAF-2 pathway also regulates several insulin-like genes. For example, expression of *ins-7*, which encodes a putative DAF-2 agonist, is upregulated by DAF-2 pathway activity (Murphy et al., 2003). In addition, a number of longevity genes encoding signaling proteins, as well as proteins that could be involved in synthesis of lipophilic hormones, are regulated in a *daf-16*-dependent manner (Lee et al., 2003; McElwee et al., 2003; Murakami and Johnson, 2001; Murphy et al., 2003).

DAF-2 (and by extension, DAF-16), has been thought to function primarily in the nervous system to influence lifespan. Mosaics lacking *daf-2* in AB, a lineage producing mainly ectodermal cell types (neurons and epidermis) are quite long lived, as was one mosaic lacking *daf-2* in a small group of neurons (Apfeld and Kenyon, 1998). In addition, expression of *daf-2* using neural promoters has been reported to shorten the lifespan of *daf-2* mutants to control levels (Wolkow et al., 2000). It seemed possible that DAF-16 might function in a different tissue to promote longevity in another situation: The lifespan of *C. elegans* is increased by removing the germline, and this lifespan extension, like that of DAF-2 pathway mutants, requires DAF-16 activity (Hsin and Kenyon, 1999). Curiously, whereas DAF-16 accumulates in the nuclei of many cell types in *daf-2* mutants, in germline-deficient animals DAF-16 accumulates primarily in intestinal nuclei (Lin et al., 2001). This finding has suggested that DAF-16 might function primarily in the intestine to promote longevity in these animals.

In this study, we have investigated the tissue-specificity of DAF-16 function. Surprisingly, we find that DAF-16 activity in neurons is sufficient to produce only a modest, 5%–20%, extension of lifespan in *daf-16(−); daf-2(−)* animals. However, we find that DAF-16 activity in the intestine is sufficient to extend the lifespans of these animals by 50%–60%, and can completely rescue the longevity of *daf-16(−)* germline-defective mutants. We also find that DAF-16 activity in signaling cells elicits two types of responses, one that requires DAF-16 activity in responding cells, and thus may involve feedback

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regulation of insulin production, and one that does not. Moreover, different tissues differ in their ability to send and respond to these signals. This intricate network of feedback regulation and cross-communication may coordinate the expression of downstream longevity genes in different tissues, and thereby specify the rate of aging of the animal as a whole.

Results

daf-16 Function in the DAF-2 Pathway: Tissue-Specific Expression

To investigate whether *daf-16* activity in any single tissue was sufficient to extend the lifespan of *daf-2* mutants, we expressed a DAF-16::GFP fusion in a tissue-specific fashion. As a control, we expressed the fusion under the control of the *daf-16* promoter in *daf-16(−); daf-2(−)* animals, and found that it almost completely rescued their longevity to *daf-16(+); daf-2(e1370)* levels (Figure 1A; Table 1). We then expressed *daf-16::gfp* specifically in neurons, muscle or intestine by fusing it to the *unc-119*, *myo-1*, or *ges-1* promoters, respectively. The fusions were expressed in the predicted tissue-specific fashion during development and most of adulthood at levels comparable to or higher than those observed in the *Pdaf-16::GFP::daf-16* control animals (Supplemental Figures S1–S3, available online at http://www.cell.com/cgi/content/full/115/4/489/DC1).

Because DAF-16 had been predicted to function primarily in neurons, we were surprised to find that in each of three independent lines, neuronal *daf-16* expression only increased lifespan 5%–20%, up to that of wild-type (Figure 1B; Table 1). We wondered whether our neuronal fusion might be toxic to the worm, thereby masking a longevity function. To test this, we expressed neuronal *daf-16* in *daf-16(+); daf-2(−)* animals. These animals lived as long as the *daf-2(−)* controls (Supplemental Figure S4A [available at above URL]; Table 1), arguing against this possibility. We also created a series of transgenic lines in which neuronal *daf-16::gfp* was expressed from very low to very high levels, and found that none of these lines lived longer than wild-type (Supplemental Figures S4B–S4D; Table 1). Finally, we confirmed that our neuronal *daf-16* fusions were functional, because they were able to induce a different *daf-16*-dependent response, dauer formation (see below). Thus we concluded that neuronal *daf-16* activity is sufficient to extend lifespan, but only modestly.

We found that expressing *daf-16* specifically in muscles produced no lifespan extension (Figure 1C; Table 1). In contrast, expressing *daf-16* in the intestine increased lifespan substantially, by 50%–60% (Figures 1D and 1E; Table 1). Injecting a higher concentration of the intestinal transgene did not further extend the lifspans of these animals (Figure 1E; Table 1). Therefore, we concluded that DAF-16 function in the intestine is sufficient to increase lifespan substantially, but that for full lifespan extension, its function in one or more other tissues is also required.

In addition to longevity, DAF-2 and DAF-16 also regulate dauer formation (Riddle and Albert, 1997). Dauer is an alternative, growth-arrested, juvenile state induced by food limitation and crowding (Golden and Riddle, 1982; Golden and Riddle, 1984). Strong *daf-2* mutants become dauers even in the presence of food (Riddle and Albert, 1997), and this requires *daf-16* (Gottlieb and Ruvkun, 1994; Larsen et al., 1995; Riddle et al., 1981; Vowels and Thomas, 1992). The *daf-2* mutation we used, *e1370,* causes dauer formation at 25°C. We found that expression of DAF-16::GFP in neurons was sufficient to promote dauer formation in *daf-16(mu86); daf-2(e1370)* mutants (Table 2). In contrast, muscle *daf-16::gfp* expression did not rescue dauer formation, and intestinal *daf-16::gfp* had only a small effect (Table 2). Thus, we concluded that neither muscle nor intestinal DAF-16 is necessary or sufficient for dauer formation, and that neuronal DAF-16 can be sufficient.

We also attempted to express *daf-16* in the epidermis. However, using either of two epidermal promoters (see Experimental Procedures), we were unable to generate transgenic lines expressing DAF-16::GFP at control or even very low levels. Instead, the GFP-expressing animals invariably died as embryos or young larvae. This suggests that expression of *daf-16* in the epidermis may be lethal unless accompanied by expression elsewhere.

daf-16 Function in the DAF-2 Pathway: Genetic Mosaic Analysis

A complementary way to investigate tissue specificity is to remove gene function from a subset of lineages, which generates genetic mosaics (Herman, 1984). *C. elegans* has an invariant cell lineage (Sulston and Horvitz, 1977; Sulston et al., 1983). The fertilized egg divides to produce the AB blastomere, which generates much of the ectoderm, including most of the epidermis and all but a few neurons; and the P_1 blastomere, which produces the intestine, muscle, reproductive system, and dorsal epidermis (see Figure 2A). In *C. elegans,* genetic mosaics can be produced by the spontaneous loss of an extrachromosomal array carrying the animal's only wild-type gene copy as well as genes that function as cell lineage markers (see Experimental Procedures). The markers we used allowed us to identify mosaics lacking *daf-16* in the entire AB lineage (AB-mosaics), the entire P_1 lineage (P_1-mosaics), or in either the EMS or E lineages (we refer to these as E(±MS)-mosaics; see Experimental Procedures and Figure 2) of otherwise *daf-16(+); daf-2(−)* animals. In two independent experiments, we found that both AB-mosaics and P_1-mosaics had extended lifespans (Figure 2B; Table 3). This implies that *daf-16* functions cell non-autonomously in both AB- and P_1-derived tissues to signal the *daf-16(−)* cells in the animal to age more slowly. The lifespan extension observed when DAF-16 was present only in the AB lineage (P_1-mosaics) was small, similar to that produced by DAF-16 expression in neurons alone (Figures 1B and 2B; Tables 1 and 3). This suggests that neurons may be the main AB-derived tissue in which DAF-16 activity can be sufficient to produce an extension in lifespan. Conversely, AB-mosaics, which contained *daf-16* in the entire P_1 lineage, had longer lifespans (Figure 2B; Table 3). Unexpectedly, the data suggested that nonintestinal as well as intestinal P_1-derived cells contributed to this longevity: E(±MS)-mosaics (in which DAF-16 was present in AB- and also some P_1-derived cells) lived longer than P_1-mosaics (in which DAF-16 was present only in AB) (Figure 2B; Table 3), and both of these mosaics lacked DAF-16 in the intestine. These experiments also

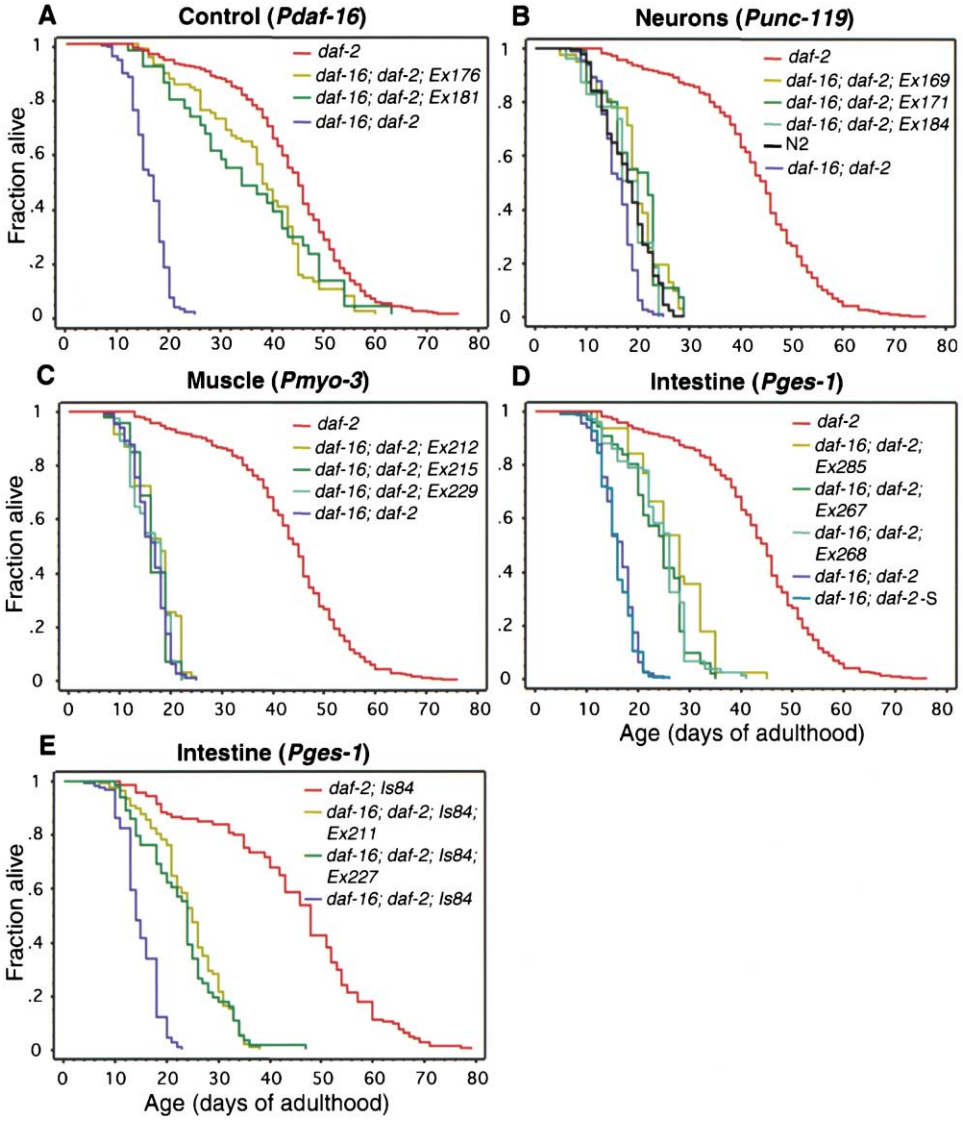

Figure 1. Effects of Tissue-Specific DAF-16 Activity on the Lifespans of *daf-16(mu86); daf-2(e1370)* Mutants

(A) DAF-16 driven by its own promoter can rescue the lifespan of the *daf-16(mu86); daf-2(e1370)* mutants to nearly *daf-2(e1370)* levels. (B) Neuronal DAF-16 has a small effect and (C) muscle DAF-16 has no effect on *daf-16(mu86); daf-2(e1370)* lifespan. (D and E) Intestinal DAF-16 can increase *daf-16(mu86); daf-2(e1370)* lifespan by 50%–60%.

The curves in this and subsequent figures represent the sum of animals examined in one or more experiments (see Table 1). Logrank (Mantel-Cox) statistics confirmed the hypothesis that animals in combined experiments behaved similarly. Transgenic animals carried the *rol-6* (pRF4) coinjection marker in (A), (B), (C), and (E), and *Podr-1::rfp* in (D). *Ex* and *Is* represent transgenes carried as extrachromosomal or integrated arrays. Transgenes are indicated by a Kenyon-lab allele number in each panel (the allele prefix *mu* is omitted). Transgenes (*Ex*) were injected into *daf-16(mu86); daf-2(e1370)* animals at 50 ng/μl, except *muEx181* in (A), which was injected at 30 ng/μl, and *muEx227* in (E), which was injected at 100 ng/μl. Each *muEx* represents an independent line (in this and subsequent figures). The *rol-6* coinjection marker alone had no effect on *daf-2(e1370)* ($p = 0.23$) or *daf-16(mu86); daf-2(e1370)* lifespan ($p = 0.25$), and the *Podr-1::rfp* coinjection marker had no effect on *daf-16(mu86); daf-2(e1370)* lifespan ($p = 0.15$). In (D), *daf-16; daf-2*-S represents non-RFP-expressing siblings selected under the fluorescent dissecting scope in parallel with transgenic animals. In (E), transgenic lines were crossed into *daf-16(mu86); daf-2(e1370)* animals carrying the integrated *sod-3::GFP* transgene (*muIs84*).

demonstrated that the combined effects of DAF-16 activity in the nonintestinal tissues are sufficient to increase lifespan substantially, since mosaics lacking DAF-16 specifically in the intestine (±MS) had lifespans that were only 17%–30% shorter than those of control *daf-16(+); daf-2(−)* animals.

We also tested for dauer formation at 25.5°C (see Experimental Procedures), and found that both AB- and P_1-mosaics were able to become dauers (Figure 2C). This suggests that *daf-16* does not act exclusively in neurons to regulate dauer formation, but in other tissues as well.

RNAi Analysis of DAF-16 Neuronal Activity

Both our tissue-specific expression and mosaic experiments indicated that *daf-16* expression in neurons is not sufficient to extend lifespan by more than about 20%. Because DAF-16 had been predicted to function

Table 1. Adult Lifespans of Animals with Tissue-Specific *daf-16* Expression

Genotype		Conc. injected transgene (ng/μl)	Mean lifespan ±SEM (days)	No. died/ total no. animals[#] (no. trials)	% control	*p* value against control	*p* value against specified group
Background	Transgene/Line						
daf-16(mu86); daf-2(e1370)	none		16.1 ± 0.2	420/741 (13)			
	Pdaf-16::gfp::daf-16/muEx176	50	36.9 ± 1.2	93/256 (3)	129%	<0.0001	<0.0001[†]
	muEx181	30	35.1 ± 2.4	32/257 (3)	118%	<0.0001	0.02[†]
	Punc-119::gfp::daf-16/ muEx169	50	19.7 ± 1.0	32/46 (1)	16.6%	0.009	0.45[ø]
	muEx171	50	19.4 ± 0.6	84/96 (1)	21.3%	<0.0001	0.001[ø]
	muEx184	50	17.8 ± 1.1	22/32 (1)	5.3%	0.75	0.042[ø]
	muEx213	1	15.6 ± 0.5	56/80 (1)	−4.8%	0.83	0.0002[ø]
	muEx214	1	17.2 ± 0.7	57/80 (1)	11.2%	0.009	0.64[ø]
	muEx239	1	16.4 ± 0.5	51/61 (1)	3.9%	0.14	0.0009[ø]
	muEx144	30	15.6 ± 0.5	75/92 (1)	−0.6%	0.39	0.002[ø]
	muEx145	30	15.9 ± 0.7	52/79 (1)	1.3%	0.39	0.007[ø]
	muEx284	100	18.1 ± 1.0	45/70 (1)	12.2%[ß]	0.001[ß]	0.37[ø]
	Pmyo-3::gfp::daf-16/muEx212	50	17.1 ± 0.5	67/90 (1)	4.3%	0.47	
	muEx215	50	16.3 ± 0.5	43/70 (1)	1.2%	0.65	
	muEx229	50	16.0 ± 0.4	82/101 (1)	1.3%	0.56	
daf-16(mu86); daf-2(e1370)[‡]	none		15.7 ± 0.3	112/209 (3)			
	Pges-1::gfp::daf-16/muEx285[¶]	50	26.8 ± 1.1	40/85 (1)	77.5%	<0.0001	<0.0001[†]
	muEx267[¶]	50	23.7 ± 0.8	53/126 (1)	47.2%	<0.0001	<0.0001[†]
	muEx268[¶]	50	24.1 ± 0.7	82/147 (1)	50.6%	<0.0001	<0.0001[†]
daf-16(mu86); daf-2(e1370); muIs84	none		14.9 ± 0.3	109/223 (3)			
	Pges-1::gfp::daf-16/muEx211	50	24.4 ± 0.7	95/166 (2)	64.1%	<0.0001	<0.0001[†]
	muEx227	100	23.0 ± 1.0	58/81 (1)	54.5%	<0.0001	<0.0001[†]
daf-2(e1370)	none		43.5 ± 0.5	659/858 (14)			
	Punc-119::gfp::daf-16/ muEx208	50	40.9 ± 1.3	54/71 (1)	0.5%	0.84	
	muEx209	50	42.4 ± 1.0	108/141 (1)	4.2%	0.64	
	muEx218	50	40.1 ± 2.4	30/71 (1)	−1.5%	0.86	
daf-16(mu86); mes-1(bn7) sterile	none		12.2 ± 0.2	178/364 (4)			
	Pdaf-16::gfp::daf-16/muEx248[¶]	50	23.1 ± 0.9	62/117 (1)	62.7%	<0.0001	0.53[¥]
	Punc-119::gfp::daf-16/ muEx245[¶]	50	15.1 ± 0.5	69/117 (1)	13.5%	0.0044	0.59[£]
	muEx246[¶]	50	15.1 ± 0.6	36/99 (1)	13.5%	0.0028	0.67[£]
	Pmyo-3::gfp::daf-16/muEx232[¶]	50	13.4 ± 0.4	71/98 (1)	3.9%	0.45	
	muEx236[¶]	50	12.2 ± 0.6	46/92 (1)	−5.4%	0.41	
	Pges-1::gfp::daf-16/muEx254[¶]	50	23.3 ± 0.9	97/197 (2)	77.9%	<0.0001	0.44[¥]

Transgenes were coinjected with the *rol-6* (pRF4) marker at 100 ng/μl, except in lines with a superscripted symbol[¶], in which transgenes were coinjected with the *Podr-1::rfp* marker at 100 ng/μl.

[#]Some animals were censored (see Experimental Procedures). The number of independent trials is in parentheses. The % difference between mean lifespans of transgenic animals and those of their respective controls is indicated in the sixth column. Transgenic animals in each group are compared to their respective non-transgenic controls (genotype indicated in the first column under "Background"). p values represent the probability that the estimated survival function of the experimental group of animals is equal to that of the control group. p values are determined using the logrank (Mantel-Cox) statistics. p values less than 0.05 are considered statistically significant, demonstrating that the two survival functions are different. In the eighth column, p values are against a group specified by a superscripted symbol: [†], *daf-2(e1370);* [ø], N2; [¥], *mes-1(bn7)* sterile; [£], *daf-16(mu86); mes-1(bn7)* fertile controls, which live 23.5% longer than *daf-16(mu86); mes-1(bn7)* sterile animals ($p < 0.0001$).

In (1)-trial experiments the "% control" and p values are relative to the controls assayed in parallel with the experiments (except in [ß], when they are relative to a cumulative *daf-16(mu86); daf-2(e1370)* control). In (2)- or more trial experiments "% control" and p values are relative to controls combined from 2 or more experiments. (We show cumulative statistics in this table and relevant figures because experimental animals compared to their respective controls assayed at the same time, and to cumulative controls, behaved similarly.) [‡]Non-transgenic siblings of transgenic animals that were selected under fluorescent dissecting scope.

primarily in neurons, we addressed this issue in a third way as well. Previously we found that treating wild-type animals with *daf-2* RNAi doubled lifespan, and that treating *daf-2(−)* animals with *daf-16* RNAi suppressed their 2-fold lifespan extension to wild-type levels (Dillin et al., 2002). This was unexpected, because neurons are known to be refractory to RNAi (Fraser et al., 2000; Kamath et al., 2000). To test directly whether these RNAi treatments affected neuronal gene expression, we established an in vivo assay for DAF-16 activity. The superoxide dismutase gene *sod-3* is thought to be a direct target of DAF-16 (Furuyama et al., 2000; Honda and Honda, 1999). Therefore we constructed strains containing a *Psod-3::gfp* transcriptional fusion (see Supplemental Experimental Procedures) and subjected them to RNAi. In the absence of RNAi, this fusion was expressed in only a few cells in wild-type (Figure 3A); in *daf-2(−)* mutants it was expressed in many more cells,

Table 2. Dauer/Larval Arrest Phenotype in Animals with Tissue-Specific *daf-16* Expression

Genotype		%L1 arrest	%L2-L2D arrest	%dauers	%dead embryos	%L3-adult	No. animals scored (no. trials)	%L1 arrest	%L2-L2D arrest	%dauers	%dead embryos	%L3-adult	No. animals scored (no. trials)
				25.5°C						27°C			
Background	Transgene/Line												
daf-16(mu86); daf-2(e1370)	none	0.4 ± 1.0	0.2 ± 0.4	0.1 ± 0.6	0	99.2 ± 1.1	1751(17)	0.8 ± 1.5	1.0 ± 1.4	0.8 ± 1.1	0	97.4 ± 2.3	1679(18)
	Pdaf-16::gfp::daf-16§/												
	muEx176	25.5	39.4‡	35.1	0	0	94(1)	52	43‡	4	1	0	74(1)
	muEx181	58	29‡	13	0	0	55(1)	68	30‡	2	0	0	97(1)
	Punc-119::gfp::daf-16§/												
	muEx169	11.1	7.4‡	63	1.2	17.3	81(1)	25.5	20‡	51	0	3.5	55(1)
	muEx171	20	28.6‡	25.7	5.7	20	70(1)	36.8	24.6‡	28.1	10.5	0	57(1)
	muEx184	nt	nt	nt	nt	nt	nt	26	25.5‡	36	0	12.5	80(1)
	muEx213	0	0	14.6	0	85.4	89(1)	0	3.5	19.3	0	77.2	57(1)
	muEx214	0	0	0	0	100	150(1)	5.5	9.7	1.4	1.4	81.9	72(1)
	muEx239	0	0.9‡	3.5	0	95.6	114(1)	5.8	9.7	7.7	0	75	52(1)
	muEx144	0.8	6‡	17.1	0	76.1	117(1)	32.8	16.9‡	33.6	1.7	15.1	119(1)
	muEx145	8	8‡	16	6	62	50(1)	7.7	38.5‡	36.5	3.8	13.5	52(1)
	muEx284	15.4	9‡	7	46	22.6	156(1)	15.3	18.4‡	19.4	40.8	6.1	98(1)
	Pmyo-3::gfp::daf-16/												
	muEx212	2	5	0	0	93	59(1)	1.9	53.7	0	7.4	37	54(1)
	muEx215	2	4	0	0	94	56(1)	18	27	0	0	55	44(1)
	muEx229	0	0	0	0	100	26(1)	0	8.8	0	0	91.2	34(1)
	muEx230	2.9	1.4	0	0	95.7	70(1)	12.9	24.7	0	2.4	60	85(1)
	Pges-1::gfp::daf-16/												
	muEx211	0	0	0	0	100	43(1)	14	29	0	0	57	125(1)
	muEx210	0	7	0	0	93	91(1)	22.9	39.6	18.8	0	18.8	48(1)
	muEx227	0	9.4	1.6	0	81	128(1)	1.5	23	2.5	0	73	79(1)
daf-2(e1370)¶	none	3.7 ± 3.7	5.2 ± 5.6‡	90.2 ± 7.2	0.9 ± 1.3	0	1728(17)	70.4 ± 8.9	3.2 ± 2.7‡	24.7 ± 8.5	1.7 ± 2.8	0	1255(12)
	Punc-119::gfp::daf-16§/												
	muEx209	14.5	30‡	42.2	13.3	0	90(1)	51.9	0	23.4	24.7	0	77(1)
	muEx218	29.4	13.7‡	36.3	20.6	0	102(1)	50.9	3.5‡	22.8	22.8	0	57(1)

Dauer/larval arrest phenotype was scored at 25.5°C and 27°C (see Experimental Procedures). Dauers produced under these conditions were complete, with the exception of a partially remodeled pharynx due to the absence of the *daf-16b* transcript (Lin et al., 2001), except in the *daf-2(e1370) background* (¶) with or without the *Punc-119::gfp::daf-16* transgene, in which all dauers formed were complete and had a fully remodeled pharynx.

All transgenes were injected with the *rol-6* (pRF4) coinjection marker (for concentrations of injected transgenes see Table 1; *muEx230* and *muEx210* were injected at 50 ng/μl). *rol-6* alone slightly enhanced the L2-L2D arrest phenotype of *daf-2(e1370)* at 25.5°C (which could be attributed to *rol-6* having a developmental delay phenotype) and embryonic lethality at 25.5°C and 27°C. *rol-6* did not enhance the L1 larval arrest phenotype.

Animals that resembled L2D, a pre-dauer L2 stage characterized by increased accumulation of fat granules in the epidermis, are denoted by the superscripted symbol ‡. % embryonic lethality was estimated as described in Experimental Procedures.

nt, not tested.

§In these animals early larval (L1, L2-L2D) and embryonic arrest phenotypes, which are associated with strong *daf-2(−)* alleles, were ehanced.

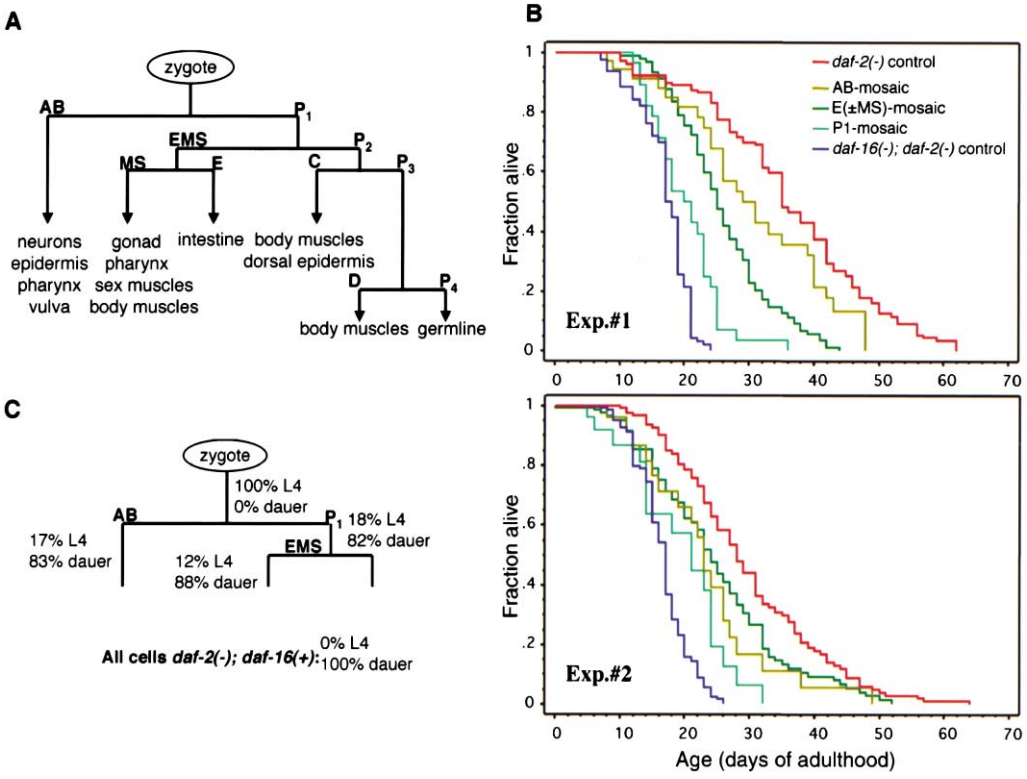

Figure 2. Lifespan and Dauer Formation of *daf-16* Genetic Mosaics

(A) Tissues produced by the early blastomeres of *C. elegans*. (B) Survival curves for *daf-16* genetic mosaics. Experiment #1, mosaic animals were selected at 20°C as L4 larvae; experiment #2, mosaic animals were selected as dauers or non-dauers at 25.5°C, dauers were allowed to exit dauer at 15°C. In both experiments, lifespans were determined at 20°C. *daf-2(−)* control: *daf-16(mu86); daf-2(e1370) ncl-1(e1865); muEx258*, where *muEx258* is an extrachromosomal array carrying a genomic DAF-16 clone, as well as markers used to identify mosaics (see Experimental Procedures). *daf-16(−); daf-2(−)* control: animals that have lost *muEx258* in all tissues. (C) Relative frequencies of dauer and L4 larvae among *daf-16* genetic mosaics in experiment #2 (see Experimental Procedures). We examined 85 AB-mosaics, 38 P_1-mosaics and 173 E(±MS)-mosaics. In experiment #1, approximately 15% of AB-mosaics formed dauers, whereas P_1-mosaics invariably developed into L4 larvae. These experiment #1 AB dauers took 2–6 days to recover and were asynchronous in reaching L4, suggesting that DAF-16 activity in AB might be necessary for dauer exit.

including many neurons (Figure 3B). When we subjected wild-type worms to *daf-2* RNAi, *Psod-3::gfp* was upregulated in nonneuronal tissues, but we did not detect upregulation in neurons (Figure 3A). Yet these animals lived twice as long as normal (Figure 3, legend). When we subjected *daf-2(−)* mutants to *daf-16* RNAi, *Psod-3::GFP* was downregulated in most tissues, but we did not detect downregulation in neurons (Figure 3B). Nevertheless, the lifespans of these animals were reduced to wild-type levels (Figure 3, legend). (We note that they lived longer than *daf-16; daf-2* double mutants, whose lifespans are about 20% shorter than normal [Lin et al., 2001], consistent with a modest longevity role for neuronal *daf-16*.) Together these experiments confirmed that the neurons of these animals were refractory to RNAi, and indicated that inhibiting either *daf-16* or *daf-2* activity can produce large changes in lifespan with no apparent change in neuronal *daf-16* activity.

DAF-16 Activity in Signaling Cells Can Increase DAF-16 Activity in Responding Cells

The expression of several insulin-like peptides is regulated by DAF-2 and DAF-16 (Murphy et al., 2003), suggesting that insulin-like peptides might function as downstream hormones to feedback regulate DAF-2 activity. If DAF-16 controls an insulin-like peptide or another hormone that signals through wild-type DAF-2 and DAF-16, we would not have been able to detect it in our tissue-specific expression or mosaic experiments because the potential responding cells were *daf-16(−); daf-2(−)*. Therefore, we designed an experiment that could detect such a signal by using the *sod-3::gfp* fusion to monitor DAF-16 activity. As a control, we introduced our intestinal, neuronal, or muscle-specific *daf-16* constructs into *daf-16(−); daf-2(−)* double mutants carrying this reporter. We found that *sod-3::gfp* was upregulated only in intestine, neurons, or muscle, respectively (Figure 4A). Thus, DAF-16 regulates *sod-3* expression cell autonomously. Next we asked whether increased DAF-16 activity in one tissue could lead to the upregulation of *sod-3* in other cells if the other cells were *daf-16(+); daf-2(+)*. We found that overexpressing *daf-16* in the intestines of *daf-16(+); daf-2(+)* animals increased *sod-3::gfp* expression not only in the intestine, but also in other tissues, including the epidermis (Figures 4B and 4F), head muscles (Figures 4C and 4G), and body muscles (Figure 4F), though not neurons (Figure 4C). Neuronal *daf-16* increased *sod-3::gfp* expression not only

Table 3. Adult Lifespan of *daf-16* Genetic Mosaics

daf-16(−) lineage	Mean lifespan ±SEM (days)	No. died/ total no. animals[#]	% control[‡]	*p* value against control	*p* value against specified group
Exp. 1					
all[*daf-16(−); daf-2(−)* control]	17.0 ± 0.4	96/146			
AB	30.7 ± 2.1	27/55	80.9%	<0.0001	0.023[$]
P1	20.2 ± 1.0	28/47	19%	0.0001	<0.0001[¶]
E(±MS)	25.9 ± 0.8	89/135	52.6%	<0.0001	<0.0001[$]
none[*daf-2(−)* control]	36.2 ± 1.2	116/169	113.3%	<0.0001	
Exp.2					
all[*daf-16(−); daf-2(−)* control]	16.6 ± 0.3	135/352			
AB	23.4 ± 2.2	19/65	41%	<0.0001	0.015[$]
P1	19.4 ± 1.7	17/42	15.1%	0.003	0.02[¶]
E(±MS)	24.8 ± 1.2	78/185	49.4%	<0.0001	0.005[$]
none[*daf-2(−)* control]	29.7±1.1	107/237	78.9%	<0.0001	

The statistical analyses performed here are as described in the Table 1 legend and in Experimental Procedures. For genotypes of strains see Figure 2B and Experimental Procedures.

[#] Some animals were censored (see Experimental Procedures).

[‡] % increase mean lifespan compared to *daf-16(−); daf-2(−)* control.

The first set of p values represents the probability that the estimated survival function is equal to that of *daf-16(−); daf-2(−)* controls. The second set of p values is against a group specified by a superscripted symbol: [$], *daf-2(−)* controls; [‡], E(±MS) mosaics.

in neurons (Figure 4C), but also to a small but significant extent in the epidermis (Figure 4F), body muscle (Figure 4F), and head muscles (Figures 4C and 4G). In contrast, muscle *daf-16* did not increase *sod-3::gfp* expression in the epidermis (Figure 4F) or neurons (data not shown).

Because *sod-3* is thought to be a direct target of DAF-16, we predicted that this increase in *sod-3* expression in responding cells would require DAF-16 activity in those cells. To test this, we examined *daf-16(−); daf-2(+); sod-3::gfp* animals that overexpressed *daf-16* in the intestine. As expected, we saw no *sod-3::gfp* induction in nonintestinal tissues (Figures 4D–4G). Together these findings indicated that DAF-16 acts in the intestine, and to a lesser extent in neurons, to control the production

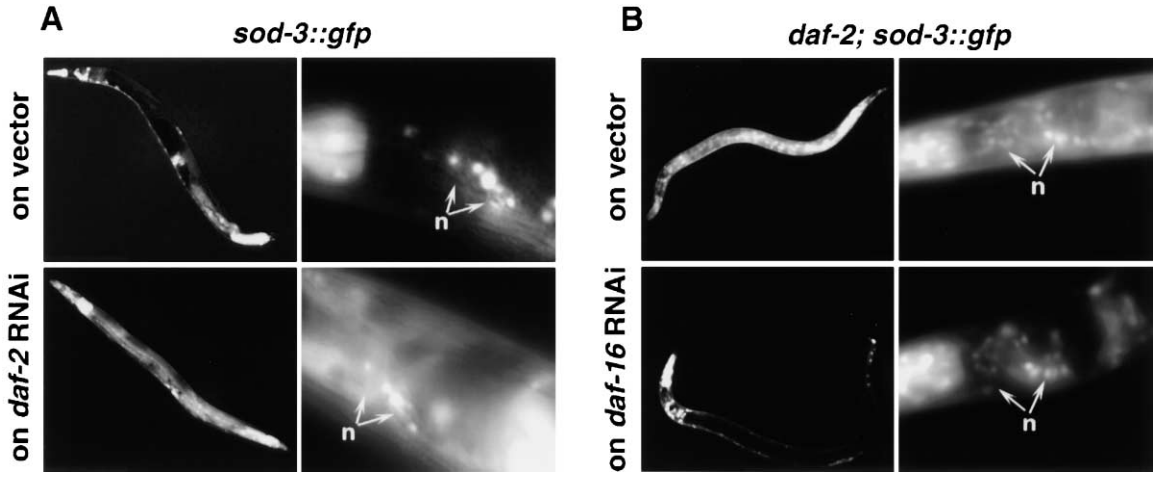

Figure 3. *daf-16* and *daf-2* RNAi Affect Lifespan without Affecting Neuronal DAF-16 Activity

Wild-type (A) or *daf-2(e1370)* (B) animals carrying an integrated *sod-3::gfp* transgene (*muIs84*) were grown on bacteria expressing *daf-2* or *daf-16* dsRNA, respectively. In both cases, they were compared to animals grown on control bacteria transformed with the vector alone. RNAi treatment had no detectable effect on *sod-3::GFP* expression in neurons (n; arrows point at groups of neurons in the head), although it dramatically affected the animals' lifespans (animals carrying *muIs84* on vector, mean lifespan m = 20.1 days; *muIs84* with *daf-2* RNAi m = 43.2 days; *daf-2; muIs84* on vector m = 38.2 days; *daf-2; muIs84* with *daf-16* RNAi m = 22.1 days). In (A), images of 2-day-old adults were taken at 100× magnification and 187 ms exposure time (left) or 1000× magnification and 27 ms exposure time (right). Note increase in overall fluorescence, arising from increased GFP expression in surrounding epidermal and other tissues, but no change in neuronal (n) GFP in *daf-2(RNAi)* animals. In (B), images of L4 larvae were taken at 200× magnification and 22 ms exposure time (left) or 1000× magnification and 7 ms exposure time (right). Note decrease in overall fluorescence, but no change in neuronal (n) GFP in *daf-16(RNAi)* animals. We observed similar results in animals up to 15 days old.

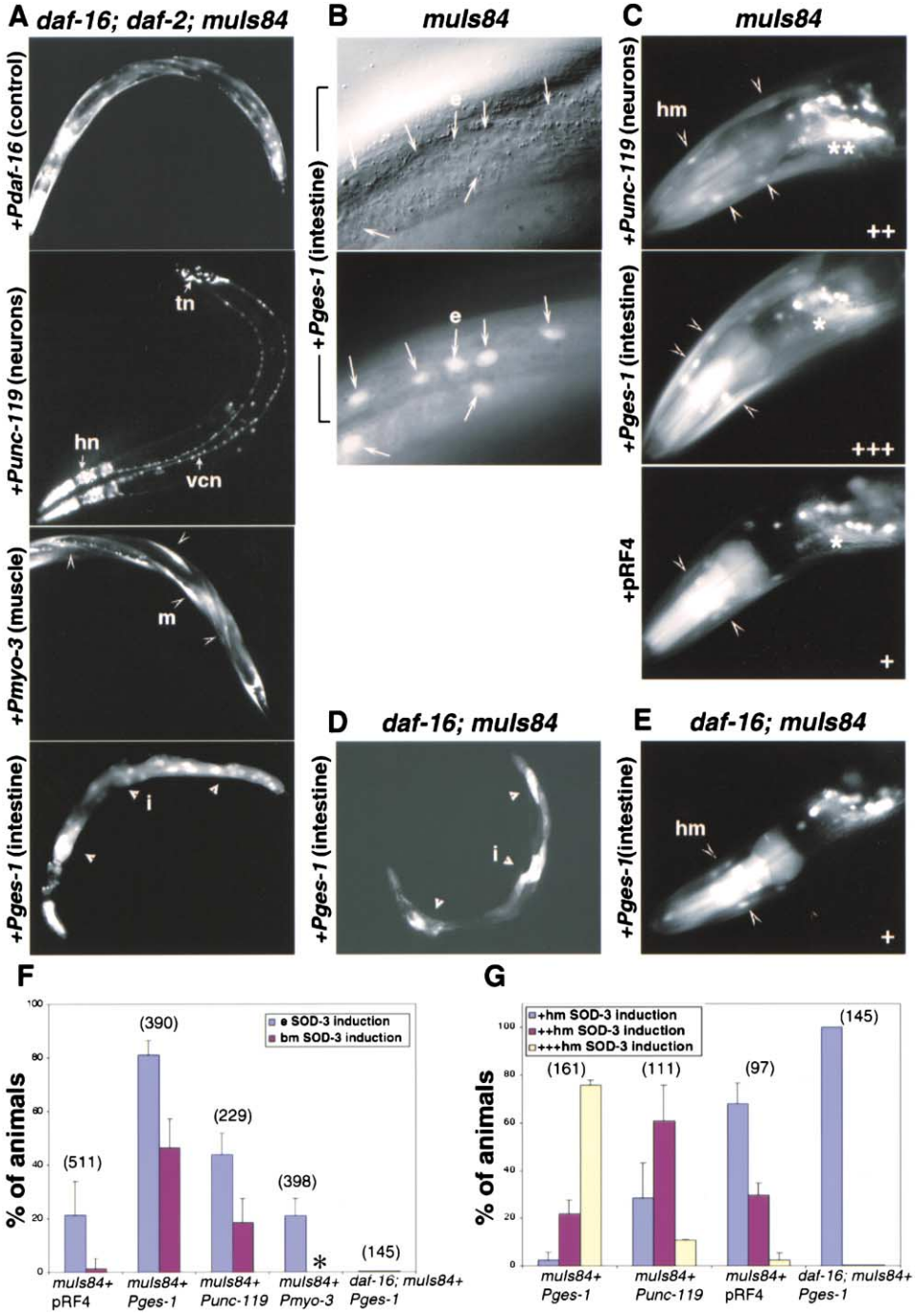

Figure 4. DAF-16 Regulates *sod-3* Expression both Autonomously and Non-autonomously

(A–E) The genotype of the strain is shown on top of each panel, the injected transgene on the side of the panel. (For specific strain information see Experimental Procedures.)

(A and D) SOD-3::GFP and DAF-16::GFP were distinguished as described in Experimental Procedures. (A) In *daf-16(mu86); daf-2(e1370); muls84* animals, DAF-16 regulates *sod-3* expression cell autonomously (*muls84*, *sod-3::gfp* transgene). From top to bottom: When driven by its own promoter, DAF-16 upregulates *sod-3::gfp* in most tissues (mean lifespan was 31.3 days). Neuronal DAF-16 upregulates *sod-3::gfp* only in neurons (hn, head neurons; tn, tail neurons; vcn, ventral cord neurons) (mean lifespan was 17.5 days). Muscle DAF-16 upregulates *sod-3::gfp* only in muscle (arrowheads point to muscles in the body, m) (mean lifespan was 15.5 days). Intestinal DAF-16 upregulates *sod-3::gfp* only in the intestine (arrowheads point to intestine, i) (mean lifespan was 24.4 days). Images of L4 larvae were taken at 200× magnification, 18 ms exposure time.

(B) When overexpressed in the intestine of wild-type worms carrying *muls84*, DAF-16 can induce *sod-3::gfp* in the epidermis (e, arrows point at epidermal nuclei). Images of 3-day-old adults were taken at 1000× magnification, Nomarski micrograph (top), fluorescent microscopy (bottom, 80 ms exposure time).

(C) Overexpression of DAF-16 in neurons (top) or the intestine (middle) of *muls84* animals can upregulate *sod-3::gfp* in the head muscles (hm;

of an intercellular signal that regulates DAF-16 activity in responding cells.

Intestinal *daf-16* Rescues the Longevity of *daf-16(−)* Germline-Deficient Animals

At 20°C, about 50% of *mes-1(bn7)* mutants lack the germline. These sterile animals live up to 50% longer than their fertile siblings and their longevity is *daf-16* dependent (Apfeld, 1999; Arantes-Oliveira et al., 2002). We found that expressing *daf-16* in the neurons or muscles of *daf-16(−); mes-1(bn7)* double mutants had little or no effect on lifespan (Figures 5A and 5B; Table 1). However, intestinal *daf-16::gfp* expression at levels comparable to the control completely restored their longevity (Figure 5C; Table 1), as did *daf-16::gfp* expression from the *daf-16* promoter (Figure 5D; Table 1). Thus *daf-16* activity in the intestine is sufficient to account for the longevity of germline-deficient animals.

Uncoupling Longevity and Stress Resistance

DAF-2-pathway mutants and germline-deficient animals are stress resistant (Arantes-Oliveira et al., 2002; Gems et al., 1998; Larsen, 1993; Lithgow et al., 1994, 1995; Vanfleteren, 1993), and DAF-16 upregulates many stress-response genes (McElwee et al., 2003; Murphy et al., 2003; Yu and Larsen, 2001) that contribute to *daf-2* longevity (Hsu et al., 2003; Murphy et al., 2003). It was curious that lifespan could be extended without the increased expression of the stress-response gene *sod-3* in every tissue (see Figure 4A). Stress resistance is thought to result from increased damage protection in all cells of the animal. Therefore, we reasoned that if *sod-3* were representative of all stress response genes (many of which, like *sod-3* [Furuyama et al., 2000], have *daf-16* binding sites in their promoters [Murphy et al., 2003]), then long-lived mosaics might not be stress resistant. However, we found that intestinal *daf-16* could increase the thermotolerance of *daf-16(−); daf-2(−)* double mutants by 59% (Supplemental Figure S5A). Thus, either *daf-16* can regulate stress resistance cell non-autonomously (in which case at least some stress-response genes do not require direct DAF-16 binding for their expression), or the intestine is particularly susceptible to stress.

We tested the heat resistance of a number of strains that had various degrees of lifespan extension, and found that there was not a consistent correlation between thermotolerance and lifespan. Altogether, mosaics that had lost *daf-16* within the P_1 lineage (that is, P_1 + E[±MS]-mosaics) lived 37% longer than normal. However, these animals were not stress resistant ($p = 0.58$ compared to *daf-16(−); daf-2(−),* Supplemental Figures S5B and S5D). Similarly, in *daf-16(−); mes-1(−)* sterile animals, intestinal *daf-16* could completely rescue lifespan (Figure 4C), whereas thermotolerance was only partially rescued (increased by 10%, $p = 0.009$ compared to *daf-16(−); mes-1(−)* sterile controls, Supplemental Figures S5C and S5D). Likewise, *daf-16(−); mes-1(−)* animals were very short-lived but exhibited much higher thermotolerance than the wild-type animals (Supplemental Figure S5D). Together these findings suggest that thermotolerance is not an absolute requirement for longevity, and that mechanisms that do not confer thermotolerance play an important role in lifespan regulation. This is consistent with the finding that many genes that have not been implicated in stress resistance contribute to the longevity of *daf-2* mutants (Lee et al., 2003; McElwee et al., 2003; Murphy et al., 2003).

Discussion

Although their genotypes are identical, different individuals within a population of *C. elegans* age at different rates (Garigan et al., 2002; Herndon et al., 2002). However, with the exception of the nervous system, which does not show signs of age (Herndon et al. 2002), within a single animal, the rates of decline of different tissues appear to be similar to one another (Garigan et al., 2002). Thus, epigenetic mechanisms must play an important role in coordinating the aging of the tissues. Our findings suggest that an elaborate signaling network may allow the animal to achieve this coordination.

Tissue-Specificity of DAF-16

In *daf-2* mutants, DAF-16 is expressed in many tissues. By expressing *daf-16* in specific tissues and carrying out genetic mosaic analysis, we found that *daf-16* activity in any of three tissues: neurons, intestine, or an unidentified nonintestinal P_1-derived tissue, was able to extend lifespan. If DAF-16 acted in a purely cell-autonomous fashion to influence only the lifespan of the cells in which it was expressed, then its activity in any one of several tissues or lineages (either AB or P_1) should not have enabled the animal to live longer than the longest-lived *daf-16(−); daf-2(−)* control animals. Therefore, we conclude that DAF-16 influences lifespan cell non-autonomously by regulating a signaling pathway that acts on many tissues of the animal.

GFP-intensities are denoted by ++ and +++ respectively) (compare to controls carrying the coinjection marker *rol-6* (pRF4) alone [bottom, +]). Note that neuronal *sod-3::gfp* is not upregulated in animals in which DAF-16 is overexpressed in the intestine (*) or the muscles (data not shown), and is only upregulated when DAF-16 is overexpressed in the neurons (**). Images of 3-day-old adults were taken at 630× magnification, 80 ms exposure time.

(D and E) The cell non-autonomous upregulation of *sod-3::gfp* requires DAF-16 in responding cells. In a *daf-16(mu86); muIs84* background, intestinal DAF-16 causes *sod-3::gfp* upregulation only in the intestine (D). It is not upregulated in the head muscles (E) (compare to [C]). Image of a 3-day-old adult was taken at 100× magnification (D), and 630× magnification (E), 80 ms exposure time.

(F) Percentage of animals overexpressing *daf-16* in specific tissues (indicated on the bottom of the graph) in which *sod-3::gfp* was upregulated non-autonomously in the epidermis (e) or the body muscle (bm). We did not include body muscle induction for *muIs84*+*Pmyo-3*, since in these animals DAF-16 is present in the muscles (*). Animals were scored under 1000× magnification in three or more experiments.

(G) Percentage of animals overexpressing *daf-16* in specific tissues (see bottom of the graph) with a certain intensity (+ to +++) of *sod-3::gfp* induction in the head muscles (hm) (examples of +, ++ and +++ can be seen in [C]). Animals were scored under 630× magnification in two or more experiments. In (F) and (G), the total number of animals scored for each genotype is indicated above each set of bars.

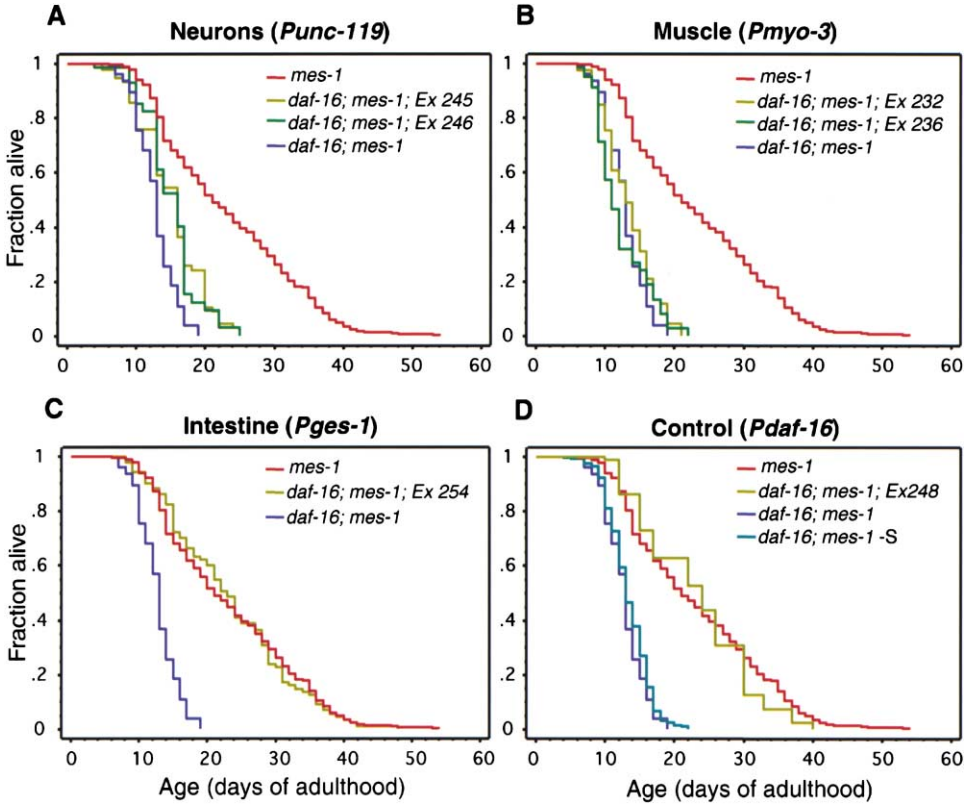

Figure 5. Intestinal *daf-16* Expression Rescues the Longevity of *daf-16(−)* Germline-Defective Animals

(A–D) Effects of tissue-specific *daf-16* expression on the lifespan of *daf-16(mu86); mes-1(bn7)* sterile animals. Only sterile animals were analyzed in these experiments. The coinjection marker, *Podr-1::rfp*, alone had no effect on the lifespan of *daf-16(mu86); mes-1(bn7)* animals ($p = 0.11$). Neuronal DAF-16 has a small effect (A) and muscle DAF-16 has no effect (B) on the lifespan of *daf-16(mu86); mes-1(bn7)* sterile animals. (C) Intestinal DAF-16 can rescue the lifespan of *daf-16(mu86); mes-1(bn7)* sterile animals to *mes-1(bn7)* sterile levels. (D) *daf-16* driven by its own promoter can rescue the lifespan of the *daf-16(mu86); mes-1(bn7)* mutants to *mes-1(bn7)* levels. *daf-16; mes-1*-S represents non-RFP-expressing sterile siblings selected under the fluorescent dissecting scope in parallel with transgenic animals. They behave similarly to *daf-16(mu86); mes-1(bn7)* animals not treated with fluorescence ($p = 0.1$).

Genetic mosaics expressing *daf-16* only in the AB lineage, which produces all but a few neurons, lived only about 20% longer than normal. Likewise, neuronal *daf-16* increased lifespan only by 5%–20%. Though unexpected, this is consistent with our previous observation (confirmed here as well, Supplemetnal Figure S2 available online at http://www.cell.com/cgi/content/full/115/4/489/DC1) that when driven by the *daf-16* promoter, *daf-16::gfp* is expressed at relatively low levels in the nervous system (Lin et al., 2001). Interestingly, *daf-16* expression in the nervous system increases markedly during dauer formation (Lin et al., 2001), and we found that neuronal *daf-16* expression was sufficient to induce dauer formation (Table 2). Thus, it is possible that DAF-16's activities in lifespan regulation and dauer formation are separated spatially as well as temporally (Dillin et al., 2002). Finally, we note that *daf-2* may function in a complex manner in the nervous system to regulate aging. In our previous *daf-2* mosaic analysis, we identified one very long-lived mosaic (ABalpppap) that lacked *daf-2* only in a small set of neurons (Apfeld and Kenyon, 1998). However, ABa-mosaics, which lacked *daf-2* in these and many other neurons as well, were not long-lived.

DAF-16 May Regulate Two Types of Downstream Signals

DAF-2 is the only insulin/IGF-1 receptor in *C. elegans,* and the longevity of *daf-2* mutants is dependent on DAF-16 (Gems et al., 1998; Kenyon et al., 1993; Larsen et al., 1995; Lin et al., 2001). Thus, the fact that tissue-specific DAF-16 activity can influence *daf-16(−); daf-2(−)* responding cells suggests that DAF-16 regulates a non-insulin-like downstream signal or hormone. In addition, we found that in a wild-type background, overexpression of DAF-16 in one tissue could upregulate DAF-16 activity in other cells. DAF-16 could potentially exert this effect by regulating an insulin-like signal. The expression of several insulin-like genes, including the putative DAF-2 agonist *ins-7*, is regulated by DAF-2 and DAF-16 (Murphy et al., 2003). Possibly DAF-16 activity in signaling cells stimulates DAF-16 activity in responding cells by downregulating *ins-7* or another DAF-2 agonist, or by upregulating a DAF-2 antagonist. Interestingly, we found that some tissues (for example, the intestine) were better able than others (neurons and muscles) to activate DAF-16 in responding cells. Conversely, some tissues (for example, the epidermis) were much more responsive than others (neurons) to this type of signaling. It will be

interesting to learn whether this pattern correlates with the pattern of any insulin gene expression, or with factors that influence insulin-sensitivity in responding cells.

Possible Solutions for Apparent Paradoxes

Our previous *daf-2* genetic mosaic analysis suggested that *daf-2,* and thus presumably *daf-16*, acts primarily in the AB lineage (Apfeld and Kenyon, 1998), whereas the mosaic analysis in this study suggests that *daf-16* acts primarily in the P_1 lineage to influence lifespan. How could this be? In the *daf-16* site-of-action experiments described here, the responding cells were *daf-16(−),* whereas in the *daf-2(−)* mosaic analysis, the responding cells were wild-type. Therefore, in the *daf-2* (but not the *daf-16*) mosaic analysis, lifespan could have been influenced by downstream signals that act through DAF-16. Perhaps one or more AB-derived tissues regulate such a signal. Our experiments suggest that, while neurons contribute to this type of signaling, the contribution from the intestine, a P_1-derived tissue, is even greater. Thus nonneuronal AB-derived cells may also produce such a signal. A number of insulin-like peptides are produced by nonneural tissues within the AB lineage, consistent with the model that this pathway involves insulin signaling.

How can we reconcile our findings with those of Wolkow et al. (2003), who proposed, based on the shortened lifespans of animals in which *daf-2* was expressed under the control of neural promoters in a *daf-2(e1370)* background, that DAF-2 functions primarily in the nervous system to regulate longevity? Our tissue-specific expression, mosaic analysis, and RNAi experiments each imply that inhibition of DAF-16 activity in neurons alone would not be sufficient to prevent *daf-2* mutants from living far longer than normal. We considered the possibility that the *daf-2(+)* neurons in the animals of Wolkow et al. produced a signal that could act on *daf-2(−)* intestinal and other cells to downregulate *daf-16* activity. However, using *daf-2* RNAi, we too created animals that appeared to be *daf-2(+)* in neurons but *daf-2(−)* in other cells, but, as assayed using the *sod-3::gfp* reporter, *daf-16* activity in the intestine and other nonneuronal cells was high in these animals, not low. Further experiments may resolve this apparent paradox.

Control of Downstream Gene Expression

We found that expression of *daf-16* in any of several tissues or lineages was sufficient to extend lifespan, indicating that *daf-16* regulates a downstream signal (or signals) that acts non-autonomously to influence tissue aging. Yet many genes that seem likely to affect longevity directly, including a wide variety of stress-response, antimicrobial, and metabolic genes, have binding sites for DAF-16 in their promoters. Moreover, our findings show that at least one of these genes, *sod-3,* requires the cell-autonomous activity of DAF-16 for its increased expression in DAF-2 mutants. It will be interesting to learn whether this is the case for other genes as well. If so, then the cell autonomous function of DAF-16 would be expected to contribute to the longevity of *daf-2* mutants. Perhaps this explains why none of our *daf-16* mosaics lived as long as control *daf-16(+)* animals.

Intestinal *daf-16* Plays an Important Role in Lifespan Regulation by the Germline

In long-lived germline-deficient animals, DAF-16 nuclear accumulation takes place primarily in the intestine (Lin et al., 2001; this study). Consistent with this, intestinal *daf-16* expression could completely restore the longevity of *daf-16(−)* germline-deficient animals. This suggests that DAF-16 may function primarily in the intestine to increase the lifespan of germline-deficient animals. Interestingly, animals that lack a germline live approximately as long as *daf-16(−); daf-2(−)* animals expressing *daf-16* only in the intestine. Thus, the germline pathway may function through an intestinal branch of the insulin/IGF-1 system.

It was interesting to find that the intestine plays such an important role in the regulation of longevity. It is possible that some of the stress-response and antimicrobial genes regulated by DAF-16 are particularly important in the intestine, which is relatively exposed to environmental toxins and pathogens. Previously, we showed that wild-type worms fed live but nonproliferating bacteria live about 30% to 40% longer than normal (Garigan et al., 2002). Genetic mosaics lacking DAF-16 specifically in the intestine (±MS) lived approximately 30% shorter than control *daf-16(+); daf-2(−)* animals. Perhaps the loss of cell-autonomous DAF-16 activity in the intestine contributes to this lifespan shortening. The intestine, which is the entire endoderm of the animal, is also the primary site of fat storage in *C. elegans,* and thus behaves as the animal's adipose tissue (Ashrafi et al., 2003; Kimura et al., 1997). FIRKO mice, which lack the insulin receptor in adipose tissue, are also long-lived (Blüher et al., 2003). This suggests that this tissue may have acquired a role in the regulation of longevity early in evolution. If so, then the mechanisms by which it exerts its effects on lifespan may also be conserved. Finally, it is possible that the intestine functions as the pancreas of *C. elegans* to produce insulin in response to food. The pancreas, too, is an endodermal organ, and its ability to produce insulin is regulated by the insulin receptor (Kulkarni et al., 1999). Likewise, we found that intestinally-expressed DAF-16 could modulate DAF-16 activity in responding cells, as would be expected if the DAF-2 pathway acts in intestinal cells to regulate the release of insulin-like peptides.

Conclusion

Together our findings suggest that an intricate signaling network regulates the lifespan of *C. elegans*. In response to their own levels of DAF-2 pathway activity, certain tissues engage in intercellular signaling that influences DAF-16 activity in responding cells. Because logically this is a feed-forward signaling system, it could have the effect of equalizing the levels of DAF-2 pathway activity among different cell types. In addition, DAF-16 activity causes some tissues to produce intercellular signals that act on target tissues in a DAF-16-independent fashion. By regulating DAF-16 activity in responding cells, the first signaling pathway may also influence the second pathway. In addition, both types of signals have the potential to act in an autocrine fashion on the signaling cells themselves.

The insulin/IGF-1 system affects many physiological processes, including dauer formation, reproduction, stress resistance, and metabolism as well as aging, and all of these processes may require close coordination between different tissues. This network of signaling may allow the animal to achieve this coordination. In addition, tissue-specific signaling may allow different tissues to make unique, or at least finely-tuned, contributions to the system as a whole; for example, by influencing expression of downstream genes. Finally, the use of interrelated signaling pathways that incorporate positive feedback regulation may allow the system to adjust rapidly and effectively to internal or environmental perturbations.

Experimental Procedures

For information on strain construction and thermotolerance assays see Supplemental Experimental Procedures available online at http://www.cell.com/cgi/content/full/115/4/489/DC1.

Molecular Biology

To generate *daf-16* tissue-specific promoter fusions, HindIII and SnaB1 sites were introduced immediately 5′ to the first ATG by PCR amplification of the *daf-16* cDNA construct (yk13f11, identified by Y. Kohara) using an inverse PCR strategy (HindIII is more proximal to the ATG). A HindIII GFP fragment from the L2911 plasmid (gift from A. Fire) was inserted into the HindIII site to generate an in-frame fusion of GFP immediately upstream of the N terminus of DAF-16a (pNL205). The SnaB1 site was used to insert the following tissue-specific promoter fragments: *Pdaf-16*, which contains 6 kb of the *daf-16* 5′ regulatory sequence amplified by PCR from cosmid R13H8 (pNL209); pan-neuronal *Punc-119*, which contains 2.2 kb upstream sequence of the *unc-119* gene (Maduro and Pilgrim, 1995), subcloned into pNL205 using blunt-ended XbaI/BamHI flanking sites, (pNL206); muscle *Pmyo-3*, which has 2.5 kb upstream regulatory sequence of this gene PCR-amplified from pPD93.97 (gift from A. Fire) (pNL212); intestinal *Pges-1*, which has 3.3 kb upstream sequence (Aamodt et al., 1991) PCR-amplified from pJM16 (pNL213); epidermal *Plin-26*, which has 4.6 kb upstream sequence (den Boer et al., 1998; Labouesse et al., 1994) PCR-amplified from pML006 (pNL208); epidermal (also expressed in neurons) *Punc-115,* which has 1.5 kb upstream sequence PCR-amplified as previously described (Lundquist et al., 1998) (pNL216). PCR-amplified *daf-16* coding sequence and all PCR-amplified promoter fragments, as well as the 5′ and 3′ junctions with the GFP, were subsequently sequenced. To generate transcriptional *Pges-1::gfp* fusion (pNL215), the HindIII/AgeI *Pmyo-3* fragment in pPD93.97 was replaced by the SnaBI *Pges-1* fragment from pNL213. To generate transcriptional *Psod-3::gfp* fusion (pJR2), 1.1 kb fragment, which includes the upstream sequence and the first exon of the *sod-3*, was amplified from the *C.elegans* genomic DNA and cloned into SphI/XbaI sites of pPD95.69 (gift from A. Fire).

Transgenic Animals

Standard techniques were used to perform germline transformation (Mello and Fire, 1995). Tissue-specific promoter constructs were injected into the *daf-16(mu86); daf-2(e1370)*, *daf-2(e1370)*, or *daf-16(mu86); mes-1(bn7)* animals to generate independent transgenic lines (indicated by a *muEx* number; see Table 1 for concentrations of injected transgenes). *Plin-26::gfp::daf-16* and *Punc-115::gfp::daf-16* were injected at concentrations ranging from 1 ng/μl to 100 ng/μl into *daf-16(mu86); daf-2(e1370)* with *rol-6* (pRF4) at 100 ng/μl and *Podr-1::rfp* at 100 ng/μl respectively as coinjection markers. Tissue-specific GFP expression was confirmed using Nomarski and fluorescent microscopy (see Supplemental Figures S1–S3). For *Plin-26::gfp::daf-16*, GFP expression was detectable in dead embryos only, as we failed to obtain any viable transgenic lines. For *Punc-115::gfp::daf-16*, epidermal GFP expression was observed in arrested L1 larvae and arrested embryos but only neuronal GFP expression was observed in transgenic animals that grew to adulthood.

Mosaic Analysis

We used a *daf-16(mu86); daf-2(e1370) ncl-1(e1865); muEx258* (CF1803) strain to generate *daf-16* genetic mosaics in two independent experiments (*daf-16(mu86)* is a null allele [Lin et al., 1997]); *muEx258* is an extrachromosomal array containing a genomic copy of *daf-16*, as well as lineage-specific markers; for strain construction information see Supplemental Experimental Procedures). In experiment #1, *daf-16(mu86); daf-2(e1370) ncl-1(e1865); muEx258* animals were grown continuously at 20°C and approximately 200,000 progeny of these animals were screened under the fluorescent dissecting scope for mosaic animals in which the AB-specific fluorescent marker, *Posm-6::gfp* (which is expressed in all ciliated neurons), or the E-specific fluorescent marker, *Pges-1::gfp* (which is expressed in the intestine generated by the P_1 lineage) was absent. In some animals, the loss of the array in the AB lineage was confirmed by looking at the cell-autonomous Ncl phenotype (enlarged nucleolus) using Nomarski optics (Hedgecock and Herman, 1995). Since the germline is generated by P_4, we distinguished P_1- from E(±MS)-mosaics by determining whether some of their progeny carried the array. E(±MS)-mosaics probably contained a mixture of E- and EMS-mosaics: we could not distinguish between the two because we did not have an MS-specific fluorescent marker on the array. Mosaics were selected as L4 larvae or, for some AB-mosaics, as dauers (∼15% of all AB-mosaics). Lifespan analysis was then initiated for L4 mosaics. Dauers were allowed to resume development at 15°C prior to the lifespan analysis.

In experiment #2, *daf-16(mu86); daf-2(e1370) ncl-1(e1865); muEx258* animals were moved from 15°C to 25.5°C as gravid P_0's, and approximately 400,000 progeny were screened as described above. Mosaic animals were selected as dauers or L4 larvae, which were moved to 20°C for lifespan analysis. Dauers were allowed to resume development at 15°C and, upon reaching the L4 stage, were moved to 20°C for lifespan analysis. *daf-2(−)* controls (array present in all cells), which were selected as dauers at 25.5°C, underwent the same treatment. In both experiments, the *daf-2(−)* and the *daf-16(−); daf-2(−)* (array lost in all cells) controls were selected under the fluorescent dissecting scope in parallel with the mosaic animals and were exposed to UV radiation for approximately the same time.

Lifespan Analysis

Lifespan assays were performed at 20°C and were initiated at the L4 larvae stage. Animals were transferred away from their progeny to new plates every other day until the end of the reproductive period (except when animals were sterile, in which case they were kept on the same plates). Strains carrying tissue-specific promoter fusions in *daf-16(mu86); daf-2(e1370)* background, as well as their controls, were grown at 15°C prior to lifespan analyses. Strains carrying tissue-specific promoter fusions in *daf-16(mu86); mes-1(bn7)* background and their respective controls were continuously grown at 20°C. To initiate lifespan assays in those animals, we picked synchronized L4 larvae and separated sterile animals from their fertile siblings one day later (based on the absence or presence of germ cells detectable under the dissecting scope; sterility was also confirmed by the lack of progeny in those animals). In all transgenic lines carrying the *Podr-1::rfp* coinjection marker, the transgenic animals and their nontransgenic siblings were separated under the fluorescent dissecting scope and were exposed to UV radiation for approximately the same time. In *daf-16(mu86); daf-2(e1370); muIs84* carrying *muEx211* or *muEx227*, animals in which *Pges-1::gfp::daf-16* was expressed more uniformly were selected for lifespan analysis based on the more uniform induction of *sod-3::gfp* in the intestine (detectable under fluorescent dissecting scope). When coinjected with *Podr-1::rfp*, *Pges-1::gfp::daf-16* was expressed at higher levels and was visible under the fluorescent dissecting scope, which allowed us to select animals with more uniform intestinal GFP expression directly. We used Statview 4.5 (SAS) software to carry out statistical analysis and to determine mean lifespans. Animals that crawled off the plate, "exploded" (i.e., had a gonad extruding through their vulva), or "bagged" (i.e., died from internal hatching) were censored at the time of the event and were incorporated into the data set as described (Lawless, 1982). Expressing *daf-16* in the *daf-16(mu86); daf-2(e1370)* background from *Pdaf-16, Punc-119*, and *Pges-1* promoters resulted in a higher proportion of "bagged"

animals (34%–66%, up to 18% and up to 24%, respectively) compared to *daf-2(e1370)*, *daf-16(mu86); daf-2(e1370)* and N2 controls (7%,4%, and 6%, respectively).

sod-3::gfp Induction Assays

In *daf-16(mu86); daf-2(e1370); muIs84* animals carrying the tissue-specific *daf-16* fusions (Figure 4A and Supplemental Figure S3), we distinguished between the GFP fluorescence obtained from the *sod-3::gfp* and *daf-16::gfp* transgenes based on the following criteria: tissue-specific *daf-16* induced much higher *sod-3::gfp* fluorescence (visible under a fluorescent dissecting microscope) than was obtained from the *daf-16::gfp* transgene (visible only under higher magnifications). In addition, in a *daf-2(−)* background, *sod-3::gfp* is found both in the cytoplasm and in the nuclei, whereas *daf-16::gfp* is predominantly nuclear. We used *sod-3::gfp* expression as a reporter for *daf-16* transcriptional activity.

In Figures 4B–4G, well-fed animals were grown at 15°C and transferred to 20°C as L4 larvae (20–30 per plate). As 3-day-old adults, these animals were mounted on 2% agarose slides (6–8 per slide), and the induction of the *sod-3::gfp* in different tissues was assayed using fluorescent and Nomarski microscopy.

Dauer Assays

To determine the relative frequencies of dauer/larval arrest and L4 larvae in *daf-16(mu86); daf-2(e1370)* animals carrying tissue-specific *daf-16*, we placed eggs (50–80 per plate) at 25.5°C or 27°C and scored dauer formation after ~48 and ~72 hr. We counted transgenic (Rol) animals that had reached the L4 stage after 48 hr. To distinguish between different stages of larval and dauer arrest among the transgenic animals, after 72 hr we mounted 10–15 animals per pad (2% agarose containing 1.5-3 mM NaN_3 as an anesthetic) and examined them under Nomarski microscopy. The presence of GFP was confirmed using fluorescent microscopy. Embryonic lethality was estimated based on the number of unhatched eggs on the plates (there was no embryonic lethality in *daf-16(mu86); daf-2(e1370)* animals). For *daf-2(e1370)* controls, relative frequencies of larval and dauer arrest were first scored at 48 hr and then confirmed at 72 hr.

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