An Insulin-like Signaling Pathway Affects Both Longevity and Reproduction in *Caenorhabditis elegans*

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ABSTRACT

Mutations in *daf-2* and *age-1* cause a dramatic increase in longevity as well as developmental arrest at the dauer diapause stage in *Caenorhabditis elegans*. *daf-2* and *age-1* encode components of an insulin-like signaling pathway. Both *daf-2* and *age-1* act at a similar point in the genetic epistasis pathway for dauer arrest and longevity and regulate the activity of the *daf-16* gene. Mutations in *daf-16* cause a dauer-defective phenotype and are epistatic to the diapause arrest and life span extension phenotypes of *daf-2* and *age-1* mutants. Here we show that mutations in this pathway also affect fertility and embryonic development. Weak *daf-2* alleles, and maternally rescued *age-1* alleles that cause life span extension but do not arrest at the dauer stage, also reduce fertility and viability. We find that *age-1(hx546)* has reduced both maternal and zygotic *age-1* activity. *daf-16* mutations suppress all of the *daf-2* and *age-1* phenotypes, including dauer arrest, life span extension, reduced fertility, and viability defects. These data show that insulin signaling, mediated by DAF-2 through the AGE-1 phosphatidylinositol-3-OH kinase, regulates reproduction and embryonic development, as well as dauer diapause and life span, and that DAF-16 transduces these signals. The regulation of fertility, life span, and metabolism by an insulin-like signaling pathway is similar to the endocrine regulation of metabolism and fertility by mammalian insulin signaling.

GENETIC screens for mutants with increased life spans have identified genes that may regulate the aging process (Guarente 1996; Kenyon 1996). In many cases, the mutations are pleiotropic, affecting life span in conjunction with altered metabolism, stress resistance, or reproduction (Friedman and Johnson 1988a; Kennedy *et al.* 1995; Vanfleteren and De Vreese 1995). An inverse correlation between life span and metabolism rate has been noted in phylogenetic comparisons of life span (Finch 1990). Additionally, regimens that alter metabolism such as caloric restriction extend the maximum life span in rats and mice (Finch 1990; Masoro *et al.* 1991). In the nematode *Caenorhabditis elegans* two genes known to regulate aging, *daf-2* and *age-1*, also affect the rate of metabolism (Klass 1983; Friedman and Johnson 1988a,b; Kenyon *et al.* 1993; Dorman *et al.* 1995; Larsen *et al.* 1995; Morris *et al.* 1996; Kimura *et al.* 1997). As well, mutations in these genes result in developmental arrest and metabolic shift at the dauer larval stage (Gottlieb and Ruvkun 1994; Larsen *et al.* 1995; Malone *et al.* 1996; Kimura *et al.* 1997) (reviewed in Riddle 1988; Riddle and Albert 1997).

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The *C. elegans* dauer larva is a specialized third larval stage adapted for survival in nonoptimal environmental conditions. In response to high levels of a continuously secreted pheromone and low amounts of food, that is, unfavorable growth conditions, animals form a dauer larva (Golden and Riddle 1984). The dauer larva is metabolically shifted and stress resistant (Cassada and Russell 1975; reviewed in Riddle 1988; Thomas 1993; Riddle and Albert 1997; Kimura *et al.* 1997). Animals arrested at the dauer stage can live up to eight times as long as a nondauer. Further, the time an animal spends as a dauer does not affect the postdauer life span (Klass and Hirsch 1976). When conditions become more favorable, animals molt and reenter the life cycle at the fourth larval stage (L4). These recovered animals are nearly indistinguishable from animals that have not arrested at the dauer stage (Riddle and Albert 1997; reviewed in Riddle 1988).

Genes that affect dauer formation (daf) fall into two classes: genes that mutate to a dauer-constitutive phenotype, where animals enter dauer inappropriately, and dauer-defective mutants, where, even under unfavorable growth conditions, animals will not arrest as a dauer. Based on genetic epistasis analysis, these genes have been ordered into a pathway (Riddle *et al.* 1981; Vowels and Thomas 1992; Thomas *et al.* 1993; Gottlieb and Ruvkun 1994). *daf-2* and *age-1* are placed in a similar branch of the dauer pathway since they show unique and similar epistasis behavior (Riddle *et al.*

1981; Vowels and Thomas 1992; Kenyon *et al.* 1993; Gottlieb and Ruvkun 1994; Malone and Thomas 1994; Dorman *et al.* 1995; Larsen *et al.* 1995).

Unlike all other *daf* genes, weak or conditional alleles of *daf-2* and *age-1* exhibit an increase in the postdauer life span (Kenyon *et al.* 1993; Dorman *et al.* 1995; Larsen *et al.* 1995; Morris *et al.* 1996). Therefore, these *daf-2* and *age-1* mutants affect life span without the associated developmental arrest at the dauer stage, suggesting that dauer arrest can be decoupled from life span extension in these animals. Both *daf-2* and *age-1* have been shown by genetic epistasis analysis to regulate *daf-16* activity: mutations in *daf-16* suppress the dauer arrest and longevity phenotypes of both *daf-2* and *age-1* (Riddle *et al.* 1981; Vowels and Thomas 1992; Kenyon *et al.* 1993; Gottlieb and Ruvkun 1994; Dorman *et al.* 1995).

Molecular characterization of *daf-2* and *age-1* indicates that an insulin-like signaling pathway regulates diapause and longevity in *C. elegans: daf-2* encodes a member of the insulin receptor family and *age-1* encodes the catalytic subunit of phosphatidylinositol-3-kinase (PI-3-kinase), a molecule that has been shown to act downstream of the mammalian insulin receptor (reviewed in Kahn 1994; Morris *et al.* 1996; Kimura *et al.* 1997). Insulin plays a central role in regulating mammalian metabolism. Not only is *daf-2* related to the mammalian insulin receptor by sequence homology, but it also regulates metabolism: decreased DAF-2 signaling induces metabolic changes (Kimura *et al.* 1997).

In a genetic screen for long-lived mutants, *age-1 (hx546)* was recovered (Klass 1983; Friedman and Johnson 1988a,b). *age-1(hx546)* fails to maternally complement *age-1(mg44)*, which is associated with a molecular lesion in a PI 3-kinase gene (Morris *et al.* 1996). Moreover, genetic mapping places *age-1(hx546)* in the approximate region of the PI 3-kinase gene affected by the *age-1* alleles *mg44*, *mg109*, and *m333* (Gottlieb and Ruvkun 1994; Malone *et al.* 1996; Morris *et al.* 1996). However, sequencing of the PI 3-kinase coding region of *age-1 (hx546)* failed to reveal a mutation (Morris *et al.* 1996). Therefore, there is some doubt whether *age-1(hx546)* affects this PI-3-kinase gene or identifies a closely linked maternal enhancer mutation. In this study, we further mapped *age-1(hx546)* to refine its genetic map position to the same interval as the PI-3-kinase gene. We also show that *age-1(hx546)* affects both maternal and zygotic *age-1* activity and that putative null mutations in the PI 3-kinase gene enhance *age-1(hx546)*. We find that complete lack of zygotic and maternal *age-1* leads to dauer arrest, whereas lack of maternal or reduction of zygotic *age-1* activity leads to an increase in life span in the absence of dauer arrest. These data suggest that normal senescence depends on phosphatidylinositol signaling from both maternal and zygotic AGE-1.

We show that at the nonpermissive temperature, *daf-2* mutants show markedly reduced fertility as well as partially penetrant embryonic lethal phenotypes. Similarly, we show that maternally rescued *age-1* null mutants cause the same spectrum of phenotypes. The increased life span and dauer constitutive phenotypes (Riddle *et al.* 1981; Vowels and Thomas 1992; Kenyon *et al.* 1993; Gottlieb and Ruvkun 1994; Dorman *et al.* 1995) as well as the decreased fertility and embryonic lethal phenotypes of *daf-2* and *age-1* mutants are suppressed by *daf-16* mutations. Thus, the *daf-2* and *age-1* regulation of life span and reproduction uses the same pathway as the dauer arrest pathway. We argue that both the aging and reproductive effects of *daf-2* and *age-1* mutations derive from the metabolic changes induced by insulin-like signaling defects.

MATERIALS AND METHODS

Methods and strains: All strains used were maintained and handled as described in Brenner (1974) and Sulston and Hodgkin (1988). Animals were grown on standard NG agar plates supplemented with 4 μm streptomycin and 10 μm nystatin to minimize mold. In this study, the mutations used were LGI: *dpy-5(e61)*, *daf-16(m27*, *mg51*, *mg52*, *mg53*, and *mg54)*; LGII: *unc-4(e120)*, *sqt-1(sc13)*, *age-1(hx546*, *mg44*, *mg109*, and *m333)*, *lin-29(n333)*, *mnC1*; LGIII: *daf-7(e1372)*, *daf-2(e1370*, *e1391)*, *dpy-17(e164)*, *unc-32(e189);* LGIV: *unc-5(e53)*; LGV: *dpy-11(e229)*, *osm-3(p802)*; LGX: *daf-12(m20); lon-2(e678)*.

Mapping between *unc-4* and *sqt-1*: *age-1(hx546)* was three-factor mapped in the 1.4 map unit (Acedb) *unc-4 sqt-1* interval. *age-1(hx546)* males were obtained by heat shock and mated into *unc-4(e120) sqt-1(sc13)* hermaphrodites at 20°. Non-Sqt-non-Unc progeny were picked to separate plates. From these plates, recombinants (Sqt-non-Unc and Unc-non-Sqt animals) were picked to individual plates and allowed to self-fertilize to isolate a homozygous strain.

Mapping between *sqt-1* and *lin-29*: *age-1(hx546)* was three-factor mapped in the 1.2 map unit (Gottlieb and Ruvkun 1994) *sqt-1 lin-29* interval. *age-1(hx546)* males were obtained by heat shock and mated with *sqt-1(sc13) lin-29(n333)/mnC1* hermaphrodites at 20°. Non-Sqt-non-Lin progeny were picked to separate plates. From plates that segregated Sqt Lin progeny, recombinants (Sqt-non-Lin and Lin-non-Sqt animals) were picked to individual plates and allowed to self-fertilize to isolate a homozygous strain.

Complementation test: *age-1(hx546)* males were mated with *sqt-1(sc13) age-1(mg44)* hermaphrodites at 20°. F1 Non-Sqt Non-Daf cross progeny were picked to separate plates. Individual heterozygotes were singled to plates at 25°, 20°, or 15° and transferred daily (20° and 25°) or every 2–3 days (20° and 15°). Progeny of the *age-1(hx546)/sqt-1(sc13) age-1(mg44)* strain were counted and scored for dauer and non-dauer after either 3 days (25°), 4 days (20°) or 7 days (15°). For *sqt-1(sc13) age-1(mg44)/sqt-1(sc13)age-1(mg44)* progeny from *sqt-1(sc13) age-1(mg44)/sqt-1(sc13)age-1(mg44)* mothers, hermaphrodites were allowed to lay eggs for 6-14 hours and then removed from the plate, because these animals tend to be egg-laying defective such that their eggs hatched within them. Plates were scored for dauer and nondauer after either 3 days (25°), 4 days (20°) or 7 days (15°).

Testing recombinants for maternal *age-1* activity: *unc-4(e120) age-1(m333)/mnC1* males were mated with all of the recombinant hermaphrodites with the exception outlined below. Wild-type hermaphrodite cross-progeny were singled to 25°

and their progeny scored for whether *unc-4(e120) age-1 (m333)* homozygous progeny arrest as dauer larvae. For the Unc-non-Sqt recombinants, *sqt-1(sc13) age-1(mg44)/mnC1* males were obtained by heat shock and were mated with the recombinants. For the Lin-non-Sqt recombinants, *sqt-1(sc13) age-1 (mg44)/mnC1; him-8(e1489)* males were mated with the homozygous Lin recombinants. Prior to mating, Lin recombinants were opened at the vulva with an injection needle.

Isolation of new *daf-16* alleles: *daf-2(e1370); daf-12(m20)* animals were grown at 15° and then placed in a bleach solution to isolate eggs. Animals were then grown until the L4 and mutagenized with ethylmethanesulfonate, then placed at 15° and allowed to grow for one full generation. Gravid F1s were grouped together and then placed in a bleach solution to isolate F2 eggs. The eggs were placed in S medium overnight at 25° on a rocking platform. Each 15 ml tube contained a different set of animals and remained separate. The synchronous preparations of F2 larvae were placed on large plates seeded with bacteria at 25° and 2–3 days later examined for any animals that failed to arrest at the dauer stage. Each individual plate from independent mutagenized parents on which a nondauer suppressor mutant was isolated was named *mg51*, *mg52*, *mg53*, *mg54*. Only one suppressor mutant was studied from each egg pool; thus the suppressor mutants are independent.

Mapping and complementation tests of *mg51, mg52, mg53, mg54*: Because *daf-16* had been previously identified as a suppressor of *daf-2* (Riddle *et al.* 1981; Vowels and Thomas 1992; Gottlieb and Ruvkun 1994), *mg51*, *mg52*, *mg53*, and *mg54* were first tested for complementation of *daf-16(m27)* as follows: *daf-16(m27)*; *daf-2(e1370)* males were mated into each of the mutant strains at 25°. Two and three days later the mating plate was examined for the presence of dauers and nondauers.

The newly isolated mutants were also genetically mapped. At 15°, *daf-2(e1370)* males were mated with *mg51*, *daf-2(e1370), daf-12(m20)* hermaphrodites and male cross-progeny were picked. These *mg51/+*; *daf-2(e1370)*; *daf-12(m20)* males were mated with each of the following hermaphrodite strains; Chromosome *I dpy-5(e61); daf-2(e1370)*; Chromosome *II sqt-1 (sc13); daf-2(e1370)*; Chromosome *III daf-2(e1370) unc-32 (e189)*; Chromosome *IV daf-2(e1370); unc-5(e53)*; Chromosome *V daf-2(e1370); dpy-11(e229)*; Chromosome *X daf-2 (e1370); lon-2(e678)*. The cross progeny were either *daf-2 (e1370)*; *mg51/+*; *marker/+*; *daf-12(m20)/+* or *daf-2(e1370)*; *marker/mg51*; *daf-12(m20)/+*. From these plates, nonmarked individual progeny were singled at 15° and allowed to self-fertilize. From this brood, a total of 50 L4s or young adults homozygous marker progeny were singled to 25° from 2–3 individual plates and their broods examined for dauers and nondauers. If the marker was unlinked to the suppressor, then the progeny were either marked dauers, marked arrested larvae (*daf-2; daf-12*), or marked dauers and nondauers. If the marker was linked to the suppressor then all of the animals would be dauers, unless there was recombination between the marker and the suppressor.

Life span assays: Life span assays were done at 25°. Adult hermaphrodites were picked (5–6 per plate) from each strain and allowed to undergo one full generation at 15° or 20° to ensure the animals being tested had not starved or gone through dauer. From these plates, individual L4 or young adult animals were picked to plates at 25°. Occasionally, individual L4 or young adult animals were picked from a well-seeded nonstarved plate as well. Day 1 of the life span was the day that the animal was picked to 25°. Therefore, life spans are either post-L4 or post-young adult. Animals were transferred to new plates every 1–2 days while producing progeny. After egg production ceased, animals were transferred to new plates every 4–7 days. Animals were tapped with a pick every 2–4 days and were scored as dead when they did not move after repeated taps with the pick. Animals that did not have any progeny, were egg-laying defective such that their eggs hatched within them, or had crawled off the plate, were not included in the study. For each analysis, N2 and *age-1 (hx546)* were also tested for life span as controls, to account for any changes in the incubator environment.

Fertility measures: Adult hermaphrodites were picked (5–6 per plate) from each strain and allowed to undergo one full generation at 15° or 20° to ensure the parental strain had not starved or gone through dauer. From these plates, individual L4 or young adult animals were singled to individual plates at 25°. The parental animals were transferred daily to fresh plates and the number of eggs laid overnight was counted. Plates at 25° were scored for dauers, nondauers, dead eggs and arrested larvae two days later. Dauers that had crawled off the side of the plate were counted as well. Plates at 15° were scored for dauers, nondauers, dead eggs, and arrested larvae 5–6 days later.

Strain constructions: *daf-16(m27); daf-2(e1391): daf-16(m27)* males were mated into *daf-2(e1391)* hermaphrodites at 15°. Cross-progeny were singled to individual plates at 25° and allowed to self-fertilize. The F2 brood plates segregated dauers and nondauers. From one plate, sixteen dauers were singled to 15° and allowed to recover. When the F2 animals developed into fertile adults, they were shifted again to 25° and allowed to self-fertilize. The progeny were then scored for dauers and nondauers. Many nondauers were singled onto separate plates from a plate segregating both dauers and nondauers [*daf-16(m27)/+*; *daf-2(e1391)* parent]. A non-dauer that produced all nondauers at 25° and established the strain was tested to confirm the presence of *daf-2* in the strain by crossing to *daf-2(e1370)* males and looking for dauer cross-progeny in the F1 at 25°.

daf-16(m27); daf-2(e1370); osm-5(p802): daf-16(m27); daf-2(e1370) males were mated with *daf-2(e1370); osm-5(p805)* hermaphrodites at 15°. Cross-progeny were singled to individual plates at 25° and allowed to self-fertilize. The F2 brood plates segregated dauers and nondauers. However, since the *daf-2(e1370); osm-5(p805)* strain had been previously reported to result in 50–75% arrested L1s at 25° (Vowels and Thomas 1992), cross-progeny plates that lacked arrested L1s and had nondauers were picked for further analysis. Of the eight cross-progeny plates, two plates had nondauers and few arrested larvae. From these plates, 48 animals were singled to individual plates and allowed to self-fertilize. Each individual plate was then tested for the presence of *osm-5(p802)* by testing for the ability to take up 3-3′-dioctadecyloxacarbocyanine perchlorate (DiO) as described in Perkins *et al.* (1986).

RESULTS

Fine mapping of *age-1(hx546)*: Animals carrying the *age-1(hx546)* allele live 2–3 times as long as wild-type animals, and arrest at the dauer stage at temperatures above which animals are standardly cultivated (Klass 1983; Friedman and Johnson 1988a,b; Dorman *et al.* 1995; Malone *et al.* 1996; Morris *et al.* 1996). The strong *age-1* alleles, *mg44*, *mg109*, and *m333*, are associated with amino acid substitutions or nonsense mutations in the PI-3-kinase gene that maps between *sqt-1* and *lin-29* (Gottlieb and Ruvkun 1994; Larsen *et al.* 1995; Morris *et al.* 1996; Figure 1; Table 1). *age-1(hx546)* fails to maternally complement, but zygotically complements molecular null mutations in the PI-

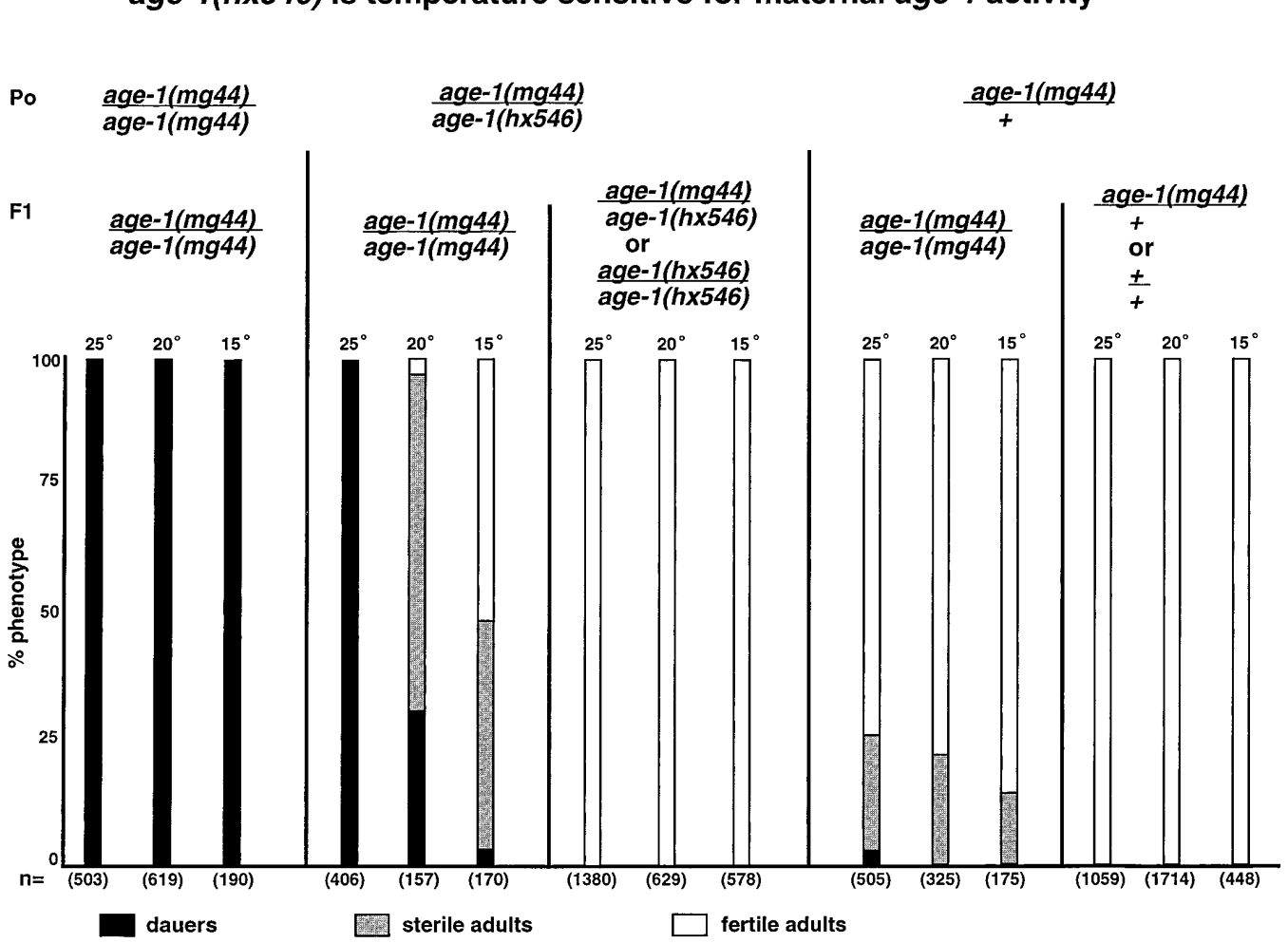

Figure 1.—*age-1(hx546)* fails to provide maternal *age-1* activity at all temperatures. In this experiment, *age-1(mg44)* was marked in cis with *sqt-1(sc13).* The + chromosome in this figure is the balancer *mnC1. age-1(hx546)* males were mated with *sqt-1(sc13) age-1(mg44)* hermaphrodites at 20°. F1 Non-Sqt Non-Daf cross-progeny were picked to separate plates. Individual heterozygotes were singled to plates at either 25°, 20° or 15°. Animals were then counted and scored for dauer and nondauer as outlined in materials and methods. In this figure, progeny of *age-1(mg44)/age-1(mg44)* parents are on the left, progeny of *age-1(mg44)/age-1(hx546)* parents are in the middle, and progeny of *age-1(mg44)/+* parents are on the right. In each data set, the left column represents data collected at 25°, the middle column is data at 20°, and the right column is data from animals grown at 15°. At 25°, 94% of the animals arrested as dauers, and 6% of the animals arrested as L1s or dead eggs; from *age-1(mg44)/age-1(hx546)* parents, all Sqt animals arrested as dauers except one fertile adult was a recombinant *sqt-1(sc13) age-1(mg44)/sqt-1(sc13) age-1(hx546);* from *age-1(mg44)/+* parents, all fertile *age-1(mg44)/age-1(mg44)* adults gave rise to all Sqt dauers in the F3 except for three recombinants *sqt-1(sc13) age-1(mg44)/sqt-1(sc13).* At 20°, from *age-1(mg44)/age-1(hx546)* parents, all fertile *age-1(mg44)/age-1(mg44)* adults gave rise to all Sqt dauers in the F3 except for one recombinant *sqt-1(sc13) age-1(age-1(mg44)/sqt-1(sc13) age-1(hx546);* from *age-1(mg44)/+* parents, all fertile *age-1(mg44)/age-1(mg44)* adults gave rise to all Sqt dauers in the F3 except for one recombinant *sqt-1(sc13) age-1 mg44)/sqt-1(sc13).* At 15°, from *age-1(mg44)/age-1(hx546)* parents; from *age-1(mg44)/+* parents all fertile *age-1(mg44)/age-1(mg44)* adults gave rise to all Sqt dauers in the F3 except for one recombinant *sqt-1(sc13) age-1(age-1(mg44)/sqt-1(sc13) age-1(hx546);* from *age-1(mg44)/+,* all fertile *age-1(mg44)/age-1(mg44)* adults gave rise to all Sqt dauers in the F3 except for one recombinant *sqt-1(sc13) age-1(mg44)/sqt-1(sc13).* The genotype of the recombinants was determined by picking individual animals from the original nondauer Sqt and then examining its brood. The complementation tests were performed as outlined in materials and methods.

3-kinase (Morris *et al.* 1996; Figure 1; data not shown). *age-1 (hx546)* has also been mapped to the *sqt-1 lin-29* interval where the PI-3-kinase gene is located (Malone *et al.* 1996; Morris *et al.* 1996). However, no PI-3-kinase coding sequence change has been detected in *age-1(hx546)* (Morris *et al.* 1996). Thus, the genetic evidence that *age-1(hx546)* affects the PI-3-kinase gene activity has not been confirmed by molecular analysis. To further substantiate that *age-1(hx546)* affects the same PI-3-kinase gene affected by *mg44, mg109,* and *m333,* we studied genetic interactions between these known PI-3-kinase mutations and *age-1(hx546).* We also mapped *age-1(hx546)* further to delimit its genetic map position to the PI-3-kinase genetic region.

TABLE 1

Three factor map of *age-1(hx546)*

Genotype of heterozygote	Recombinant phenotype	Recombinant genotype	Maternally complements *age-1(m333)*[a]	Life span	*age-1* position
age-1/unc-4 sqt-1	Unc non-Sqt	*unc(−) sqt(+) age(hx546)*	No	Long	Right of *unc-4*
	Sqt non-Unc	*unc(+) sqt(−) age(+)*	Yes	Short	Right of *sqt-1*
age-1/sqt-1 lin-29	Sqt non-Lin	*sqt(+) age(+) lin(+)*	Yes	Short	Left of *lin-29*
	Sqt non-Lin[b]	*sqt(+) age(hx546) lin(+)*	No	Long	Between *sqt*-1 and *lin-29*
	Lin non-Sqt[b]	*sqt(+) age(hx546) lin(−)*	No		
	Lin non-Sqt[b]	*sqt(+) age(+) lin(−)*	Yes		Between *sqt-1* and *lin-29*

age-1(hx546) was mapped in the *unc-4(e120) sqt-1(sc13)* interval as described in materials and methods. Recombinants were tested for complementation of *age-1(m333)* for the Sqt-non-Uncs and of *age-1(mg44)* for the Unc-non-Sqts as described in materials and methods. 3/3 Sqt-non-Age by complementation test were also short-lived, with mean life spans of 9.7 ± 0.7 (n = 25), 9.7 ± 0.4 (n = 31), and 8.1 ± 0.4 days (n = 29). 1/1 Unc-non-Sqt failed to complement *age-1(mg44)* and was long-lived with a mean life span of 18.5 ± 1.8 days (n = 21). Animals with mutations in either *sqt-1(sc13)* or *unc-4(e120)* lived similar to wild-type with a mean life span of 11.4 ± 0.4 (n = 34) and 10.8 ± 1.3 days (n = 13), respectively. All values are mean ± SE.

[a] Some recombinants were tested for complementation with *age-1(mg44)* as outlined in materials and methods.

[b] Some of this data was previously published in Morris *et al.* (1996).

age-1(hx546) lacks material *age-1* activity but does have zygotic *age-1* activity. Mating *age-1(hx546)* males into *age-1(mg44)* hermaphrodites, the cross-progeny *age-1(hx546)/age-1(mg44)* develop as nondauers. Therefore, *age-1(hx546)* provides sufficient zygotic *age-1* activity to allow the animals to develop as nondauers, because the unmated *age-1(mg44)/age-1(mg44)* hermaphrodites would produce a brood of all nondauers. However, in the next generation, after self-fertilization, the *age-1(mg44)* homozygous progeny of this *age-1(hx546)/age-1(mg44)* heterozygote will arrest as dauers. Therefore, *age-1(hx546)* fails to supply maternal *age-1* activity but does supply zygotic activity (Morris *et al.* 1996). Recombinants were analyzed in this complementation test.

age-1(mg44), a putative null allele of *age-1*, substitutes a stop codon at amino acid position 405 (Trp405Amber) that is predicted to truncate AGE-1 upstream of the kinase domain (Morris *et al.* 1996). Animals with only maternally contributed *age-1* activity, *age-1(mg44)*/*age-1(mg44)* progeny of *age-1(mg44)*/+ mothers live twice as long as wild type animals but do not arrest at the dauer stage (Morris *et al.* 1996; Figure 1). However, animals bearing no maternal or zygotic *age-1* activity [progeny of *age-1(mg44)*/*age-1(mg44)* long-lived mothers] arrest at the dauer stage [Gottlieb and Ruvkun 1994; Morris *et al.* 1996; Figure 1; in all the experiments reported below, *age-1(mg44)* is marked in *cis* with *sqt-1(sc13)*]. Therefore, the putative null phenotype of *age-1* is maternal-effect dauer-constitutive and zygotic long-lived (Gottlieb and Ruvkun 1994; Morris *et al.* 1996; Figure 1).

age-1(hx546) was originally reported to map to the left of *sqt-1*, close to *unc-4* (Friedman and Johnson 1988a,b). Our data conflicts with this mapping. We collected recombinants between *age-1(hx546)* in *trans* to an *unc-4 sqt-1* chromosome. All of the Sqt-non-Unc (14/14) recombinants were non-Age; animals bearing each of these recombinant chromosomes maternally complemented *age-1(mg44)* (Table 1). However, all of the Unc-non-Sqt (3/3) recombinants were Age: they failed to supply maternal *age-1* activity. This is consistent with *age-1(hx546)* mapping to the right of *sqt-1* or within 0.29 map units ($P < 0.05$) to the left of *sqt-1*.

Recombinants were also tested for life span (Table 1; all of the life span values are mean ± SE). 3/3 Sqt-non-Unc non-Age recombinants that maternally complemented *age-1(m333)* mutants had life spans similar to wild-type (8.9 ± 0.2 days, n = 170). The mean life spans of the recombinants were 9.7 ± 0.7 (n = 25), 9.7 ± 0.4 (n = 31), and 8.1 ± 0.4 days (n = 29). An Unc-non-Sqt Age recombinant that failed to complement *age-1(mg44)* maternally was long-lived relative to wild type with a mean life span of 18.5 ± 1.8 days (n = 21). Animals that carry the marker mutations *sqt-1(sc13)* or *unc-4(e120)* had mean life spans similar to wild type, of 11.4 ± 0.4 (n = 34) and 10.8 ± 1.3 days (n = 13), respectively.

In summary, we found a total of 17 recombinants in the *unc-4 sqt-1* interval. All of the Unc-non-Sqt recombinants fail to maternally complement *age-1(mg44)* and a recombinant tested for life span was long-lived. The Sqt-non-Unc recombinants maternally complemented *age-1(m333)* and the three recombinants tested for life span were short-lived (Table 1).

We also collected recombinants between a *sqt-1 lin-29* chromosome in *trans* to *age-1(hx546).* For the 17 Sqt-non-Lin recombinants, 3/17 were Sqt-Age and 14/17 were Sqt-non-Age (Morris *et al.* 1996; Table 1). Of the 6 Lin-non-Sqt recombinants, there were 4/6 Lin-Age recombinants and 2/6 Lin-non-Age recombinants (Morris *et al.* 1996; Table 1). The Lin-non-Age non-Sqt recombinants, as well as the other mapping in Table 1, help to eliminate the possibility that the *age-1(hx546)* mutation maps to the left of *sqt-1*, as previously reported (Friedman and Johnson 1988a,b). Of the total 23 recombinants collected in the *sqt-1 lin-29* interval, 5 were between *sqt-1* and *age-1* and 18 were between *age-1* and *lin-29* (Morris *et al.* 1996; Table 1). In addition,

the *age-1(hx546)* failure to maternally rescue *age-1(mg44)* and the long life span are linked in these 35 recombinants over 2.3 map units. Therefore, all of the genetic mapping data is consistent with the *age-1(hx546)* lesion lying in the PI-3-kinase gene product. It is possible that the *age-1(hx546)* mutation is in a region that has not been sequenced, such as in a promoter or enhancer element (Quiring *et al.* 1994; Ortiz-Lopez *et al.* 1997). Sequencing of the *age-1* flanking regions in *age-1(hx546)* may identify such an element.

***age-1(hx546)* affects maternal *age-1* gene activity at all temperatures:** *age-1(hx546)* fails to contribute maternal *age-1* activity at 25°: 100% of the *age-1(mg44)* homozygous progeny of *age-1(hx546)/age-1(mg44)* heterozygotes arrest at the dauer stage (Morris *et al.* 1996; Figure 1). This is the same as the control *age-1(mg44)* daughters of *age-1(mg44)* parents (Gottlieb and Ruvkun 1994; Morris *et al.* 1996; Figure 1). The dauer larvae produced in both cases have the dark intestine, cuticular remodeling, and arrest of the molting cycle characteristic of this stage (reviewed in Riddle 1988; Riddle and Albert 1997; Gottlieb and Ruvkun 1994). In contrast, only 1% of *age-1(mg44)* progeny of *+/age-1(mg44)* parents arrest development as dauer larvae at 25° (Figure 1).

age-1(hx546) supplies sufficient zygotic activity to allow reproductive development: *age-1(hx546)/age-1(mg44)* progeny of *age-1(hx546)/age-1(mg44)* mothers form fertile adults, like *+/age-1(mg44)* progeny (Morris *et al.* 1996; Figure 1). In addition, in the absence of *age-1* maternal contribution, zygotic *age-1* expression from *age-1(hx546)* is sufficient to allow reproductive development: mating *age-1(hx546)* males with *age-1(mg44)* hermaphrodites, the cross-progeny *age-1(hx546)/age-1(mg44)* develop as reproductive adults rather than dauers. Therefore, at 25° *age-1(hx546)* expresses sufficient zygotic but not maternal levels of *age-1* for nondauer growth.

age-1(hx546) supplies some *age-1* maternal gene activity at lower temperatures (Figure 1). At 20°, 100% of *age-1(mg44)* progeny from *age-1(mg44)* hermaphrodites arrest as dauers. However, fewer of the *age-1(mg44)* progeny of *age-1(hx546)/age-1(mg44)* arrest development as dauer larvae at 20°; most continue development as dauer-like animals that are dark, developing into sterile adults, and 4% of *age-1(mg44)* progeny of

TABLE 2

The affect of maternal and zygotic *age-1* genotype on dauer formation and longevity

Parent *age-1 (+ or 0)*	Zygote tested	Maternal/zygotic dose	Reproductive development *vs.* dauer arrest	Life span
age-1 (+) / *age-1 (+)*	*age-1 (+)* / *age-1 (+)*	$M^{+}Z^{+}$	Reproductive development	Normal
age-1 (0) / *age-1 (0)*	*age-1 (0)* / *age-1 (0)*	$M^{0}Z^{0}$	Dauer	
age-1 (0) / *age-1 (0)*	*age-1 (0)* / *age-1 (+)*	$M^{0}Z^{+/2}$	Reproductive development	Normal
age-1 (0) / *age-1 (+)*	*age-1 (0)* / *age-1 (0)*	$M^{+/2}Z^{0}$	Reproductive development	Long
age-1 (0) / *age-1 (+)*	*age-1 (0)* / *age-1 (+)*	$M^{+}Z^{+}$	Reproductive development	Normal
age-1 (+ or 0 or hx546)				
age-1 (+) / *age-1 (+)*	*age-1 (+)* / *age-1 (+)*	$M^{+}Z^{+}$	Reproductive development	Normal
age-1 (hx546) / *age-1 (hx546)*	*age-1 (hx546)* / *age-1 (hx546)*	$M^{hx546}Z^{hx546}$	Reproductive development	Long
age-1 (hx546) / *age-1 (hx546)*	*age-1 (hx546)* / *age-1 (+)*	$M^{hx546}Z^{+}$	Reproductive development	Normal
age-1 (0) / *age-1 (hx546)*	*age-1 (0)* / *age-1 (hx546)*	$M^{0/hx546}Z^{0/hx546}$	Reproductive development	Long
age-1 (0) / *age-1 (hx546)*	*age-1 (0)* / *age-1 (0)*	$M^{0/hx546}Z^{0/0}$	Dauer	
age-1 (0) / *age-1 (+)*	*age-1 (0)* / *age-1 (hx546)*	$M^{+}Z^{0/hx546}$	Reproductive development	Long
age-1 (0) / *age-1 (0)*	*age-1 (0)* / *age-1 (hx546)*	$M^{0}Z^{0/hx546}$	Reproductive development	Long

0 represents a putative null allele *age-1(mg44)* (Morris *et al.* 1996). Life span values are shown in Table 3.

age-1(hx546)/*age-1(mg44)* parents are fertile (Figure 1). Therefore, *age-1(hx546)* maternal activity is temperature sensitive.

At 15°, *age-1(hx546)* shows considerable maternal *age-1* activity, although less than wild-type levels. Fifty-five percent of *age-1(mg44)* progeny of *age-1(hx546)*/*age-1(mg44)* parents are fertile (Figure 1). Because the *age-1(mg44)*/*age-1(mg44)* progeny of *age-1(hx546)*/*age-1(mg44)* heterozygous parent do not all arrest as dauers at lower temperatures, the *age-1(hx546)* mutant has sufficient maternal *age-1* gene activity at lower temperatures to develop as a reproductive adult. There is still, however, a decrease in maternal *age-1(hx546)* activity: 45% of *age-1(mg44)* progeny of *age-1(hx546)*/*age-1(mg44)* parents arrest development without reproduction. However, most of the arrested animals develop to sterile adults rather than dauers. This suggests that decreases in maternal *age-1* activity cause developmental arrest as a sterile adult, whereas complete lack of both zygotic and maternal *age-1* activity results in arrest as a dauer larva.

The data suggest that *age-1(hx546)* is a temperature-sensitive allele. Alternatively, it is possible that the reduced level of maternal *age-1(hx546)* activity is sufficient at lower temperatures. This difference is not due to the intrinsic temperature sensitivity of the dauer pathway because, at all these temperatures, 100% of the progeny of parents carrying null *age-1* alleles arrest as dauers (Gottlieb and Ruvkun 1994; Morris *et al.* 1996; Figure 1). Similar results were observed at all three temperatures when another marker, *unc-4*, was used to mark two other *age-1* alleles, *m333*, and *mg109* (data not shown). Both *mg44* and *m333* are molecular null alleles as they have premature stop codons, while *mg109* is a missense mutation in a conserved region just outside the kinase domain (Morris *et al.* 1996).

Decreased zygotic *age-1(hx546)* activity confers long life: Animals lacking both zygotic and maternal *age-1* activity will arrest development as dauers [*age-1(mg44)*/*age-1(mg44)* progeny from *age-1(mg44)*/*age-1(mg44)* mothers]. Lack of only maternal *age-1* activity does not affect life span or development; *age-1(mg44)*/+ animals derived from *age-1(mg44)*/*age-1(mg44)* mothers develop as nondauers with normal senescence (Tables 2 and 3; Figure 2A). Thus, *age-1* is completely zygotically rescued (Gottlieb and Ruvkun 1994; Morris *et al.* 1996; Tables 2 and 3; Figure 2A). Animals lacking only zygotic *age-1* activity [*age-1(mg44)*/*age-1(mg44)* progeny of *age-1(mg44)*/+ mothers] develop into fertile adults but show a more than two-fold increase in life span (Gottlieb and Ruvkun 1994; Morris *et al.* 1996; Tables 2 and 3; Figure 2A). These data indicate that reducing either maternal or zygotic *age-1* activity by half has no effect on longevity or development as measured in our assays (Tables 2 and 3; Figure 2, A and B).

age-1(hx546) animals show an increase in life span but do not arrest at the dauer stage under normal growth conditions (Klass 1983; Friedman and Johnson 1988a,b; Dorman *et al.* 1995; Malone *et al.* 1996; Morris *et al.* 1996). Like other *age-1* alleles, *age-1(hx546)* is zygotically rescued for life span: *age-1(hx546)*/+ ani-

TABLE 3

***age-1* (*hx546*) has reduced maternal and zygotic *age-1* activity**

Paternal genotype	Maternal genotype	Zygotic genotype	Age phenotype		Life span relative to wild type
			Dauer formation	Life span (days)	
	+/+	+/+	nondauer ($n > 1000$)	8.9 ± 0.2 ($n = 170$)	1.0
	mg44/mnC1	*mg44/mg44*	nondauer ($n = 505$)[a]	20.7 ± 0.2 ($n = 26$)	2.2
	mg44/mnC1	*mg44/mnC1*	nondauer ($n > 1000$)	10.4 ± 0.8 ($n = 22$)	1.2
	mg44/mg44	*mg44/mg44*	dauer ($n = 505$)		
	hx546/hx546	*hx546/hx546*	nondauer ($n > 1000$)	15.6 ± 0.7 ($n = 108$)	1.8
	hx546/hx546[b]	*hx546/hx546*[b]	nondauer ($n > 100$)	14.8 ± 0.9 ($n = 40$)	1.7
+/+	*mg44/mg44*	*mg44/+*	nondauer ($n > 100$)	10.7 ± 0.5 ($n = 35$)	1.2
+/+	*hx546/hx546*	*hx546/+*	nondauer ($n > 50$)	12.0 ± 1.2 ($n = 20$)	1.4
hx546/hx546	*mg44/mg44*	*hx546/mg44*	nondauer ($n > 100$)	19.8 ± 1.1 ($n = 54$)	2.2
	mg44/hx546	*mg44/mg44*	dauer ($n = 406$)		
	mg44/hx546	*mg44/hx546*	nondauer ($n > 1000$)	19.3 ± 0.9 ($n = 32$)	2.2
	mg44/hx546	*hx546/hx546*	nondauer ($n > 300$)	19.1 ± 1.2 ($n = 21$)	2.2
hx546/hx546	*mg44/mnC1*	*hx546/mg44*	nondauer ($n > 100$)	15.8 ± 1.4 ($n = 30$)	1.8
hx546/hx546	*mg44/mnC1*	*hx546/mnC1*	nondauer ($n > 100$)	10.7 ± 0.6 ($n = 23$)	1.2
+/+	*mg44/mnC1*	*mg44/+*	nondauer ($n > 100$)	9.7 ± 0.6 ($n = 31$)	1.0
+/+	*mg44/mnC1*	+/*mnC1*	nondauer ($n > 100$)	10.0 ± 0.9 ($n = 21$)	1.1

Some of the data presented here has been previously published in Morris *et al.* (1996).

[a] 1% of the animals formed dauer larvae and 20% formed sterile adults.

[b] *age-1(hx546)* backcrossed strain that is marked with *sqt-1(sc13)*.

All values are mean ± SE.

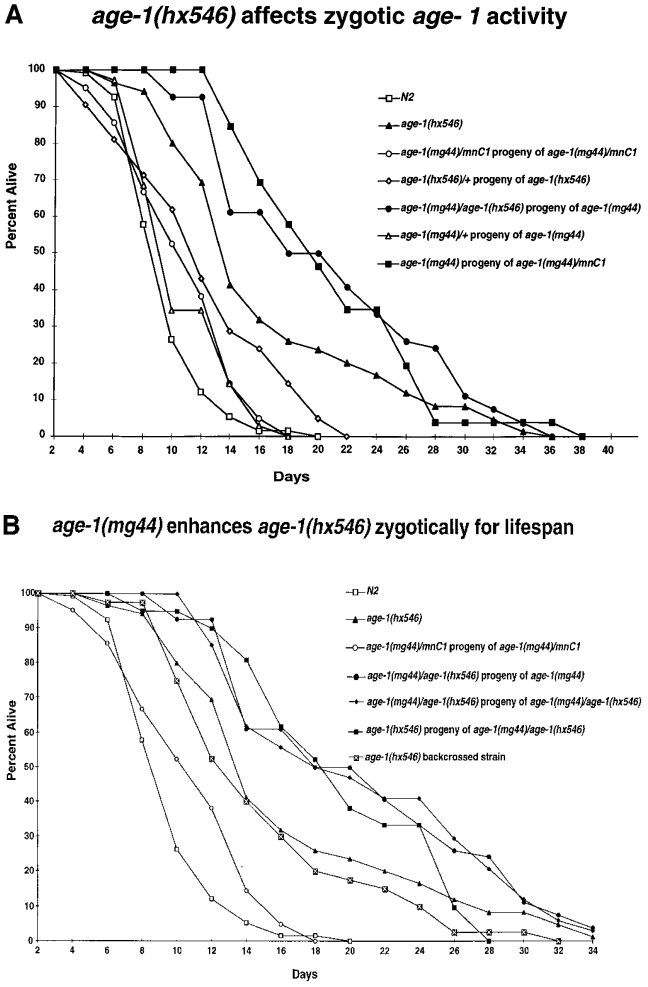

Figure 2.—(A) *age-1(hx546)* affects zygotic *age-1* activity for life span. The mean life span and the number of animals examined are listed in Table 3. *age-1(hx546)*/*age-1(mg44)* hermaphrodites do not arrest at the dauer stage but the animals have an increased life span. In contrast, *age-1(mg44)*/+ progeny of *age-1(mg44)*/*age-1(mg44)* have a life span that is similar to +/+. Therefore, *age-1(hx546)* is defective in zygotic *age-1* activity. Life span assays were performed as outlined in materials and methods. Animals were grown at 20° until the L4 stage, when they were transferred to 25°. (B) *age-1(mg44)* enhances *age-1(hx546)* both maternally and zygotically. *age-1(mg44)*/*age-1(hx546)* progeny from *age-1(mg44)*/*age-1(hx546)* mothers have similar life spans to *age-1(hx546)*/*age-1(mg44)* hermaphrodite progeny of *age-1(mg44)*/*age-1(mg44)* mothers mated with *age-1(hx546)* males. This suggests that the *age-1(mg44)*/*age-1(hx546)* heterozygote may completely lack *age-1* activity for life span. These data also suggest that *age-1(hx546)* has reduced but non-null zygotic gene activity for life span and *age-1(mg44)* enhances *age-1(hx546)* both maternally and zygotically. The small difference in *age-1(hx546)*/*age-1(hx546)* hermaphrodites from *age-1(mg44)*/*age-1(hx546)* when compared to the *age-1(hx546)* strain itself is not significant ($P > 0.05$). *hx546*/*hx546** represents a backcrossed strain. Life span assays were performed as outlined in materials and methods. Animals were grown at 20° until the L4 stage, when they were transferred to 25°. The mean life span for each strain and the total number of animals examined is shown in Table 3.

mals from *age-1(hx546)*/*age-1(hx546)* mothers have wild-type life spans (Tables 2 and 3; Figure 2A). *age-1(hx546)*, however, does affect zygotic *age-1* activity in the control of senescence. *age-1(hx546)* in *trans* to a putative null allele of *age-1* supplies sufficient zygotic activity in the absence of any maternal activity to allow reproductive development in *age-1(hx546)*/*age-1(mg44)* progeny of *age-1(mg44)*/*age-1(mg44)* mothers, but these animals show a more than two-fold increase in longevity (Tables 2 and 3; Figure 2, A and B). In fact, *age-1(hx546)* is zygotically enhanced for life span extension by *age-1(mg44)* (Figure 2, A and B and Tables 2 and 3): *age-1(hx546)* has a mean life span of 15.6 ± 0.7 days while *age-1(hx546)*/*age-1(mg44)* animals have a mean life span of 19.8 ± 1.1 days. However, this is not due to the fact that *age-1(mg44)* is dominant: *age-1(mg44)*/+ progeny of *age-1(mg44)*/*age-1(mg44)* mothers show reproductive development and normal life span (Tables 2–4; Figure 2A).

These results suggest that *age-1(hx546)* is a maternal enhancer of *age-1(mg44)*. In addition, *age-1(mg44)* is a zygotic enhancer of *age-1(hx546)*. These data along with the genetic mapping results presented in Table 1 and Figure 1 further endorse that *age-1(hx546)* is a mutation in the PI-3-kinase.

***daf-2* and *age-1* have pleiotropic effects on reproduction and viability:** *daf-2(e1370)* and *daf-2(e1391)* are temperature-sensitive dauer-constitutive alleles that substitute conserved amino acids in the kinase domain of the DAF-2 insulin-like receptor protein (Kimura *et al.* 1997). When shifted to the nonpermissive temperature after the larval stage 1 temperature-sensitive period (tsp) for dauer formation (Swanson and Riddle 1981), several *daf-2* mutant animals are sterile (data not shown), while the remaining animals have a severely reduced fertility (Table 4). Wild-type animals grown at 20° until the young adult stage, and then shifted to 25°, had a mean brood size of 278 ± 20.4 with no embryonic lethality. However, *daf-2(e1370)* animals, shifted from 15° to the nonpermissive temperature (25°) at the young adult stage, had a severely reduced mean brood size of 67 ± 8.2 (Table 4). Moreover, 8.9% of the eggs laid resulted in dead eggs or arrested L1/L2 larvae. Most of the L1/L2 larvae recovered to form fertile adult hermaphrodites when shifted to 15°.

At the permissive temperature, *daf-2(e1370)* and *daf-2(e1391)* animals produce nearly wild-type brood size (Table 5). As well, less than 1% of the progeny of these *daf-2* mutants arrest at embryonic and larval stages. Thus, only at the nonpermissive temperature do the *daf-2* mutant alleles significantly reduce fertility and viability of progeny.

We further examined whether or not the decreased fertility was due to a mutation unrelated to *daf-2*. We constructed a *daf-2(e1370) dpy-17(e164)* double mutant to eliminate any closely linked as well as unlinked mutations. This strain was then crossed to wild-type and a Daf-non-Dpy recombinant was collected in order to iso-

TABLE 4

Decreased brood size and viability in long-lived *daf-2* and *age-1* mutants

Genotype	Stage shifted to 25°	Total progeny after upshift[a]	% Progeny arrest/lethal	Life span at 25° (days)[a]
+/+	L4	256 ± 15.6 (14)	0	8.9 ± 0.2 (170)
	Young adult	280 ± 20.4 (8)	0	9.1 ± 1.1 (41)
daf-2(e1370)	L4	44 ± 7.3 (21)	15.4	21.6 ± 2.5 (18)
	Young adult	67 ± 8.2 (8)	8.9	18.3 ± 1.1 (34)
daf-2(e1391)	L4	32 ± 5.2 (13)	10.5	33.8 ± 2.2 (42)
	Young adult	50 ± 4.1 (8)	2.5	35.1 ± 2.6 (37)
daf-2(e1370)	L4	71 ± 7.0 (15)	9.3	22.7 ± 2.0 (22)
backcrossed	Young adult	88 ± 8.0 (8)	6.9	21.7 ± 2.0 (21)
age-1(hx546)	L4	189 ± 6.2 (10)	0	15.6 ± 0.7 (108)
age-1(mg44)	L4	76 ± 7.3 (11)	5.8	20.7 ± 0.2 (26)

For each worm, the total number of eggs laid were counted (including dauers, nondauers, arrested larvae, and dead eggs) and averaged to obtain the total progeny after upshift. The % arrest/dead eggs was measured, and the total number of animals that arrested or died was divided by the total number of progeny, as in Hengartner *et al.* (1992). Previously, it has been shown that *age-1(mg44)* and *age-1(hx546)* have a reduced brood size at 25° (Gottlieb and Ruvkun, unpublished observations; Friedman and Johnson 1988a,b). Many of the *age-1(mg44)* animals at 25° die from internal hatching of the progeny, as they are slightly egg-laying defective (Gottlieb and Ruvkun 1994; our unpublished observations). These animals were not included in this experiment. *age-1(mg44)/age-1(mg44)* animals from *age-1(mg44)/mnC1* mothers were singled to plates and the total number of progeny produced counted. These *age-1(mg44)/age-1(mg44)* animals from *age-1(mg44)/age-1(mg44)* mothers all arrested as dauers.

[a] Values (total progeny and life span) are mean ± SE (*n*).

late a twice backcrossed homozygous *daf-2* strain. This resulted in a slight increase in brood size and a slight decrease in the associated larval arrest/dead eggs (Tables 4 and 5). However, the resulting backcrossed *daf-2 (e1370)* strain still exhibited a reduction in the brood size with associated larval arrest/dead eggs when shifted either as young adult (88 ± 8 total progeny with 6.9% arrest/dead eggs) or as L4 animals (71 ± 7 total progeny with 9.3% arrest/dead eggs). Because the reduction in fertility, the arrested larvae and dead eggs are seen in two *daf-2* alleles as well as in a backcrossed strain; these data suggest that *daf-2* controls function in other than dauer formation and that the reduction in brood size is indeed a phenotype of the *daf-2* mutation itself.

Because *age-1* and *daf-2* show many similar defects, we examined the fertility of *age-1* at 25°. *age-1(mg44)* homozygous animals also have severely reduced fertility at 25° with a mean of 76 total progeny (Table 4). Moreover, 5.8% of the total brood result in either dead eggs or larval arrest similar to *daf-2* mutants. This reduction in fertility is not specific to this allele. *age-1(hx546)* also

TABLE 5

***daf-2* mutants have wild-type brood sizes and reduced lethality at 15°**

Genotype	Temperature for assay	Total progeny[a]	% Progeny arrest/lethal	Life span (days)[a]
+/+	15	360 ± 17.4 (8)	0.4	17.0 ± 1.0 (21)
	25	256 ± 15.6 (14)	0	8.9 ± 0.2 (170)
daf-2(e1370)	15	293 ± 16.7 (7)	0	39.5 ± 1.6 (37)
	25	44 ± 7.3 (21)	15.4	21.6 ± 2.5 (18)
daf-2(e1391)	15	243 ± 18.0[b] (8)	0.5	45.3 ± 3.2 (32)
	25	32 ± 5.2 (13)	10.5	33.8 ± 2.2 (18)
daf-2(e1370)	15	240 ± 19.4 (7)	0.2	29.7 ± 1.5 (36)
Backcrossed	25	71 ± 7.0 (15)	9.3	22.7 ± 2.0 (22)

For each worm, the total number of eggs laid were counted (including dauers, nondauers, arrested larvae, and dead eggs) and averaged to obtain the total progeny. The % arrest/dead eggs was measured and the total number of animals that arrested or died was divided by the total number of progeny, as in Hengartner *et al.* (1992).

[a] Values are mean ± SE (*n*).

[b] *daf-2(e1391)* forms 0.7% dauers at 15° (*n* = 14/1000).

shows a 26% reduction in fertility compared to wild-type N2 at 25° (Friedman and Johnson 1988a,b; Table 4). Therefore, insulin-like signaling through the AGE-1 PI-3-kinase gene also regulates reproduction, embryonic development, and dauer formation.

The amount of time that *daf-2* mutants are allowed to develop at the permissive temperature affects fertility. As shown in Table 4, *daf-2(e1370)* mutants shifted to 25° at L4 have a mean brood size of 44 with 15.4% of the total brood either arrested as L1/L2 larvae or dying as eggs. Whereas, if *daf-2(e1370)* animals are shifted as young adults, the brood size increases to a mean of 67 with 8.9% arrest/lethality. A similar trend is shown for both *daf-2(e1391)* and the *daf-2(e1370)* backcrossed strain (Table 4). This indicates that *daf-2* acts late in development to affect reproduction and progeny viability and, therefore, has activity well past the temperature-sensitive period for dauer formation.

In the life span assays, we noted the days that the animals laid progeny. In general, wild-type N2 animals produced progeny for the first four days of its 8-day life span. Therefore, approximately half of the wild-type animal's adult life span is postreproduction. However, in *daf-2* mutants, progeny are produced very late into the life span. This suggests that *daf-2* animals do not live very long postreproduction; instead they show extended reproductive life spans albeit with lower fertility. They produce progeny up to the day they die. It is interesting to note that *daf-2(e1391)* animals exhibit a fourfold increase in life span, which is higher than reported for other *daf-2* alleles (Kenyon *et al.* 1993; Dorman *et al.* 1995; Larsen *et al.* 1995; Table 4).

Longevity defects in *daf-2* and *age-1* are not due to reduced fertility: We examined whether the increase in longevity in *daf-2* mutants was associated with the reduced brood size of these mutants. Previously it had been reported that *daf-2(e1370)* had a wild-type brood size but a twofold increase in life span at 15° (Larsen *et al.* 1995). Therefore, we compared *daf-2(e1370)*, *daf-2(e1391)*, and the backcrossed *daf-2(e1370)* mutants for total progeny produced as well as life span at 15° (Table 5). All three strains had nearly wild-type brood sizes (Table 5). However, several of the strains had increased life spans (Table 5). This suggests that the life span defect in *daf-2* mutants is unrelated to the number of eggs produced, similar to the results with *daf-2(e1370)* of Larsen *et al.* (1995).

Because we observed differences in brood size when animals were shifted to the nonpermissive temperature at different stages of development, we examined whether life span was also affected in a similar manner. In Figure 3, we plot the life spans of wild-type N2, *daf-2(e1370)*, *daf-2(e1391)*, and *daf-2(e1370)* backcrossed when shifted as L4 and young adult. For all strains there was no significant difference in life span between the L4 and adult temperature-shifted animals, suggesting again that there is no correlation between the number of progeny produced and life span.

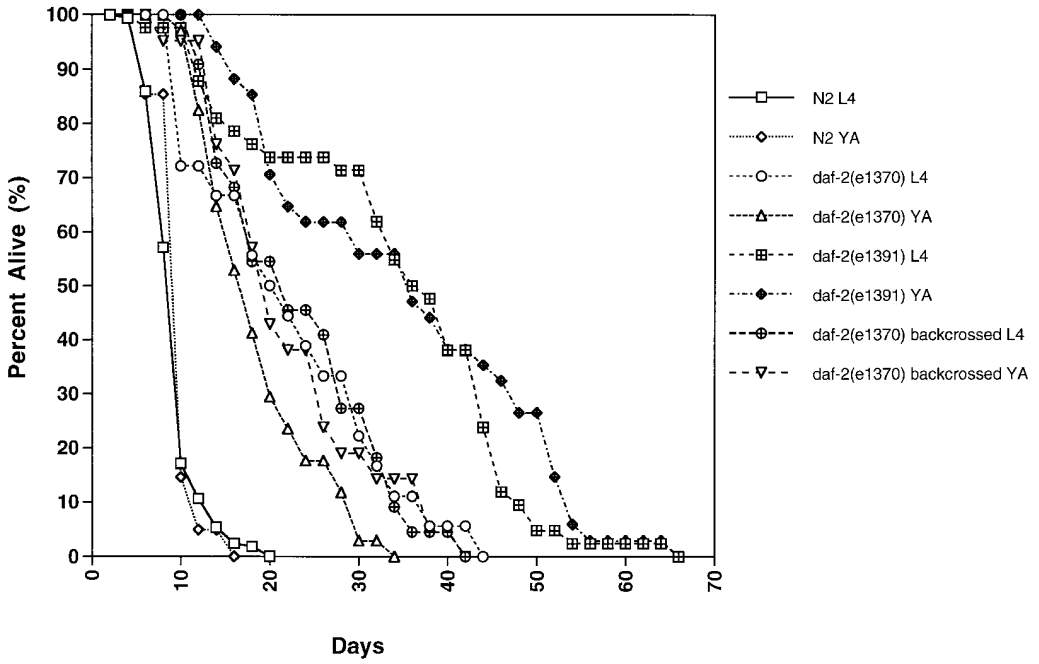

Figure 3.—Time of upshift does not affect longevity of temperature sensitive *daf-2* mutants. Animals were grown at 15° until either the L4 stage or young adult (YA), when they were transferred to 25°, except for N2 which was grown at 20° and then shifted to 25°. Mean life spans for each strain are shown in Table 4. For each strain, multiple life span assays were done. The total number of animals counted in each assay are the following: for N2 L4 shift, $n = 170$; for N2 young adult shift, $n = 36$; for *daf-2(e1370)* L4 shift, $n = 18$, with an additional 34 animals (65%) that died from internal hatching of the progeny and young adult shift, $n = 34$, with an additional 39 animals (53%) that died from internal hatching of the progeny; for *daf-2(e1391)* L4 shift, $n = 42$, with an additional 29 animals (41%) that died from internal hatching of the progeny and young adult shift, $n = 34$, with an additional 29 animals (46%) that died from internal hatching of the progeny; for *daf-2(e1370)* backcrossed strain, $n = 22$, with an additional 33 animals (60%) that died from internal hatching of the progeny and young adult shift $n = 21$, with an additional 21 animals (50%) that died from internal hatching of the progeny. Animals continued to produce progeny late into the life span. *daf-2(e1391)* continued to produce progeny up to Day 46 of the life span. Life span assays were performed as outlined in materials and methods.

***daf-2* reduced brood size and lethality/arrest is suppressed by *daf-16*:** Because *daf-16* suppresses all known *daf-2* and *age-1* phenotypes (Riddle *et al.* 1981; Vowels and Thomas 1992; Kenyon *et al.* 1993; Gottlieb and Ruvkun 1994; Dorman *et al.* 1995), we examined whether a *daf-16* mutation could restore wild-type fertility to *daf-2* and *age-1* mutants. The brood size of the *daf-16(m27); daf-2(e1370)* double mutant is significantly increased to a mean brood size of 189 (Table 6). *daf-16(m27)* mutants have a mean brood size of 202, which is slightly decreased from wild-type N2 of 256 (Table 6). *daf-16(m27)* only partially suppresses the dauer arrest phenotype of *daf-2(e1391)* (Table 6). The *daf-16(m27); daf-2(e1391)* double mutant has a mean brood size of 155, which is many more than *daf-2 (e1391)*, but is still decreased compared to wild type. Thus, similar to all other phenotypes associated with *daf-2* (Riddle *et al.* 1981; Vowels and Thomas 1992; Gottlieb and Ruvkun 1994; Dorman *et al.* 1995), the fertility defect of *daf-2* is at least partially suppressed by *daf-16* (Table 6).

The *daf-2* larval arrest and embryonic lethal phenotype is completely suppressed by a mutation in *daf-16*. Even though *daf-16(m27)* is a partial suppressor of the dauer arrest and brood size defects of *daf-2(e1391)*, it potently suppresses the arrest/lethality associated with *daf-2(e1391)* mutants at 25°, because none of the progeny laid arrested as L1/L2 or died as eggs in the double mutant (Table 6).

We further examined the ability of *daf-16* to suppress the aging defects in both *daf-2(e1370)* and *age-1(mg44)* at 25°. L4 animals were shifted from 15° to 25° and life span was determined. The mean life span of both *daf-16(m27); daf-2(e1370)* and *daf-16(m27); sqt-1 (sc13) age-1(mg44)* were similar to wild type (data not shown). Therefore, *daf-16* completely suppresses the life span defects in both *age-1* and *daf-2* mutants.

Both *daf-2* and *age-1* show a synthetic lethality with the mutations affecting the amphid processes (*che/osm* class of mutations; Vowels and Thomas 1992; Gottlieb and Ruvkun 1994). At the *daf-2(e1370)* nonpermissive temperature, the mean brood size of *daf-2(e1370); osm-3(p802)* mutants was 27, with 26% of the brood arresting as larvae or dying as eggs, when animals are shifted as young adults. Most of the arrested larvae could be recovered and form fertile adults if shifted to the permissive temperature, suggesting that the animals indeed are arrested and this is not a larval lethal interaction. The triple mutant, *daf-16(m27); daf-2 (e1370); osm-3(p802)* has a wild-type brood size with no associated arrest (data not shown). Therefore, *daf-16* can suppress all the phenotypes of *daf-2*.

***daf-16* is the major downstream target of *daf-2*:** In mammalian cells, insulin receptor couples to many downstream targets that mediate several different signaling cascades, including PI-3-kinase, Grb-2, pp60 c-src, Shc and PLC gamma (reviewed in Kahn 1994; Kahn and Weir 1994; Bonfini *et al.* 1996). We wanted to determine if there were other negatively regulated downstream targets of the *daf-2* insulin-like signaling pathway other than *daf-16*. Therefore, we screened for suppressors of the *daf-2; daf-12* arrest phenotype.

Double mutants of both *daf-2* and *daf-12* at the nonpermissive temperature and *age-1* and *daf-12* result in an arrest at the L1 and L2 stage (Vowels and Thomas 1992; Gottlieb and Ruvkun 1994). *daf-16* can completely suppress the growth arrest phenotype of both the *daf-2; daf-12* and *age-1; daf-12* double mutants (Gottlieb and Ruvkun 1994). The *daf-2(e1370); daf-12 (m20)* double mutant strain provides for an easy selection for *daf-2* or *daf-12* suppressors. We screened 7000 genomes for suppressors of the *daf-2; daf-12* arrest at 25° and found four independent alleles that allow reproductive development at 25°; *mg51*, *mg52*, *mg53*, and *mg54*. All

TABLE 6

daf-2* reduced brood size and lethality is suppressed by *daf-16

Genotype	Phenotype at 25°	Total number of progeny laid[a]	% Progeny arrest/lethal	Number of parents
+/+	Nondauer	256 ± 15.6	0	14
daf-2(e1370)	Dauer	44 ± 7.3	15.4	21
daf-2(e1391)	Dauer	32 ± 5.2	10.5	13
daf-16(m27)	Nondauer	202 ± 6.7	0	6
daf-16(m27); daf-2(e1370)	Nondauer	189 ± 15.8	0.1	8
daf-16(m27); daf-2(e1391)	Nondauer[b]	155 ± 15.6	0	10
daf-16(m27); age-1(mg44)[b]	Nondauer	183 ± 21.8	0.1	6

For each worm, the total number of eggs laid were counted (including dauers, nondauers, arrested larvae and dead eggs) and averaged to obtain the total progeny after upshift. The % arrest/dead eggs was measured and the total number of animals that arrested or died was divided by the total number of progeny, as in Hengartner *et al.* (1992).

[a] Values are mean ± SE.

[b] 34% of the *daf-16(m27); daf-2(e1391)* animals arrested as dauers and dauer-like animals after 3 days at 25°. Most of the dauers failed to maintain dauer by the following day.

four alleles map in the *daf-16* genetic interval near *dpy-5* on Chromosome *I*, and failed to complement *daf-16*, and were further characterized for dauer formation and suppression (Gottlieb and Ruvkun 1994; Ogg *et al.* 1997; S. Gottlieb and G. Ruvkun, unpublished observations). This suggests that the major target for *daf-2* is *daf-16*, because only alleles of *daf-16* were identified. Other mutations that may suppress *daf-2* are relatively rare or may not exist.

DISCUSSION

An insulin-like signaling pathway controls *C. elegans* longevity, metabolism, and fertility: Mutations in *daf-2* or *age-1* affect life span (Klass 1983; Friedman and Johnson, 1988a,b; Kenyon *et al.* 1993; Dorman *et al.* 1995; Larsen *et al.* 1995; Morris *et al.* 1996; this study). We have shown that mutations in either of these two genes also affect fertility and embryonic development, and that all of these phenotypes are regulated via outputs to *daf-16*. The molecular identity of both *daf-2* and *age-1* as part of an insulin-like signaling pathway (Morris *et al.* 1996; Kimura *et al.* 1997) suggests how these genes coordinately control longevity, metabolism, and fertility. The similar phenotypes and genetic epistasis results of *daf-2* and *age-1* position them at the same point in the dauer genetic pathway, consistent with their molecular identities in a similar signal transduction pathway (Riddle *et al.* 1981; Vowels and Thomas 1992; Gottlieb and Ruvkun 1994; Dorman *et al.* 1995; Larsen *et al.* 1995; Morris *et al.* 1996; Kimura *et al.* 1997). Therefore, we suggest that dauer diapause, longevity, reproduction, and embryonic development are regulated by an insulin-like signaling cascade through the DAF-2 insulin receptor family member and the AGE-1 PI-3-kinase.

Animals that arrest at the dauer diapause stage, such as *daf-2* and *age-1* mutants, show a decrease in the rate of metabolism, suspend development of both somatic and germline tissues, and age more slowly, allowing the animals to survive long periods under suboptimal conditions (reviewed in Riddle 1988; Thomas 1993; Riddle and Albert 1997). In this study, we have shown that *daf-2* and *age-1* animals have a decline in fertility when shifted to high temperatures even as young adults. It is possible that decreased insulin signaling in adult *daf-2* animals causes metabolic changes as seen by the change in fat in *daf-2* mutants shifted to a nonpermissive temperature at this stage (Kimura *et al.* 1997). Indeed, both *age-1* and *daf-2* mutants show a metabolic shift as adults as examined by changes in the levels of many metabolic enzymes (Vanfleteren and De Vreese 1995). Alternatively, the L4 or young adult temperature-shift experiment may uncover a defect, for example, in gonadogenesis, that may have occurred much earlier.

Mutations in *daf-2* affect embryogenesis, reproduction, longevity, and dauer arrest. We have shown that several *daf-2* alleles cause a small percentage of the animals to arrest as larvae or die as eggs. None of the *daf-2* alleles generated so far are null alleles (Kimura *et al.* 1997). Therefore, it is possible that the null phenotype of *daf-2* is more severe than nonconditional dauer arrest (*i.e.*, embryonic or larval arrest). Indeed, null mutants of the mouse or human insulin receptor result in runaway lipolysis and associated ketoacidosis and neonatal death (Krook *et al.* 1993; Wertheimer *et al.* 1993; Accili *et al.* 1996; Joshi *et al.* 1996). The developmental arrest caused by vertebrate insulin receptor mutations may be due to the metabolic defects caused by lack of insulin signaling, or the arrest may be caused more directly by the lack of insulin growth factor responses. We suggest that *daf-2* and *age-1* also regulate growth of the germ line and embryo, as well as metabolism.

All of the phenotypes caused by mutations in either *age-1* or *daf-2*, including the embryonic lethality and fertility defects, are suppressed by mutations in *daf-16* (Riddle *et al.* 1981; Vowels and Thomas 1992; Kenyon *et al.* 1993; Gottlieb and Ruvkun 1994; Larsen *et al.* 1995; Dorman *et al.* 1995; this study); *daf-16* is the major downstream target of insulin-like signaling in *C. elegans*. *daf-16* encodes a forkhead type transcription factor (Ogg *et al.* 1997). Therefore, insulin-like signaling pathways couple to the transcriptional activity of DAF-16 to in turn control fertility and metabolism. Because all of the *daf-2* and *age-1* mutant phenotypes are suppressed by *daf-16* mutations, the other possible signaling outputs from the *daf-2* insulin-like signaling cascade, for example, to glucose transport or metabolic enzyme activities, are not as germane to longevity and fertility as the *daf-16* transcriptional output.

Our results do not support a model that fertility decline is the cause of longevity increase. We find that life span in these mutants can be decoupled from their effects on brood size, similar to previous reports of *daf-2 (e1370)* by Larsen *et al.* (1995). Therefore, the increased longevity seen in *daf-2* and *age-1* mutants is not due to the suspension of fertility. This is in contrast to studies in Drosophila where high rates of egg production decreased life span in females (Partridge *et al.* 1987). Moreover, in female flies, life span was increased by exposure to higher temperature, and this correlated with a decrease in egg production at these higher temperatures (Smith 1958). Further studies revealed that life span of females can be increased by reducing the rate of egg-laying (Smith 1958).

In *C. elegans*, two studies that examine the relationship between mating and life span find that mating can reduce male life span (Van Voorhies 1992). Mating to hermaphrodites either reduces (Gems and Riddle 1996) or has no effect (Van Voorhies 1992) on life span. However, this reduction in life span is not due to an effect on either egg or sperm production, but rather a cost of copulation (Gems and Riddle 1996). In

Drosophila, female life span is also decreased by courting (Chapman 1992) and receipt of male accessory fluid (Chapman *et al.* 1995); male life span is shortened by sexual activity (Partridge and Farquhar 1981).

In wild-type animals, half of the life span is postreproductive, which is significantly different from *daf-2* mutants where progeny can be produced up until the day of death. Brood size is limited by the number of sperm in a wild-type hermaphrodite (Hodgkin and Barnes 1992). Adult hermaphrodites will lay unfertilized eggs at the end of the brood, and in fact brood size is significantly increased when hermaphrodites are mated to wild-type males (Hodgkin and Barnes 1992). Because *daf-2* mutants have such a small brood size, it is possible that they continue to have progeny up until they die because they do not run out of sperm. However, we have noted that even with the small brood size, *daf-2* animals also lay unfertilized eggs (H. A. Tissenbaum and G. Ruvkun, unpublished results). We hypothesize that the fertility defects in *daf-2* and *age-1* mutants are due to the metabolic shift towards energy storage so that fewer oocytes and sperm are generated.

***age-1(hx546)* affects maternal and zygotic *age-1* activity:** *age-1(hx546)* animals are long-lived compared to wild type (Klass 1983; Friedman and Johnson 1988a,b; Dorman *et al.* 1995; Malone *et al.* 1996; Morris *et al.* 1996; this study). *age-1(hx546)* has reduced zygotic *age-1* activity but *age-1(hx546)* is not dauer constitutive at most temperatures, because the zygotic *age-1* gene activity of this allele is sufficient to allow nonarrested development (Klass 1983; Friedman and Johnson 1988a,b; Malone *et al.* 1996; Morris *et al.* 1996; this study). However, the major defect in *age-1 (hx546)* is in maternal contribution of *age-1* activity: at high temperature, *age-1(hx546)* shows no more *age-1* activity than the *age-1(mg44)* molecular null allele (Morris *et al.* 1996; this study). Thus the long life span of *age-1(hx546)* is caused by a defect in maternally contributed *age-1* activity as well as a decline in zygotic *age-1* activity.

The *age-1* alleles (*mg109*, *mg44*, *m333*), including those that are predicted to truncate the AGE-1 protein, show a dauer-constitutive phenotype that can be rescued by wild-type maternal gene activity (Gottlieb and Ruvkun 1994; Morris *et al.* 1996). In addition, both *age-1(m333)* and *age-1(mg44)* allele, which are probable null alleles, show a dramatic longevity increase when maternal but not zygotic *age-1* gene activity is supplied (Larsen *et al.* 1995; Morris *et al.* 1996; this study). These data show that the AGE-1 PI 3-kinase homologue functions in the particular signaling pathway that controls dauer developmental arrest, senescence, and reproduction and may not be consistent with a more general AGE-1 requirement. In addition, this shows that maternal AGE-1-mediated phosphatidylinositol signaling is sufficient to rescue lack of zygotic AGE-1 signaling for arrest at the dauer stage but not for decreased senescence. Because in *age-1(hx546)*, reduced maternal AGE-1 phosphatidylinositol signaling also leads to increased longevity, these data suggest that normal senescence depends on phosphatidylinositol signaling from both maternal and zygotic AGE-1.

***age-1(hx546)* is modulated by temperature:** We have examined the maternal contribution of *age-1(hx546)* at several temperatures to establish that this allele is temperature sensitive for maternal activity. Dauer formation is modulated by temperature (Golden and Riddle 1984); however, *age-1(mg44)* produces 100% dauers at all temperatures (Gottlieb and Ruvkun 1994; this study). Therefore, the fact that *age-1(mg44)*/*age-1(hx546)* hermaphrodites produce 100% dauers only at 25° can be attributed to temperature sensitivity of the *age-1(hx546)* allele for maternal *age-1* activity.

Temperature input to dauer formation has been previously reported for *daf-2*. Analysis of a *daf-2* allelic series suggests that the level of *daf-2* signaling is modulated by temperature (Malone and Thomas 1994; Kimura *et al.* 1997). Most *daf-2* alleles are temperature sensitive, including alleles isolated in genetic screens that would allow the recovery of nontemperature sensitive mutations (Gottlieb and Ruvkun 1994; Malone and Thomas 1994). Substitutions of DAF-2 amino acid residues conserved across phylogeny cause more penetrant dauer arrest at all temperatures than substitutions of nonconserved residues (Kimura *et al.* 1997). Accordingly, *daf-2* mutants that are likely to have the least gene activity [*e.g.*, *daf-2(mg43)*] arrest development at the dauer stage independent of growth temperature (Gottlieb and Ruvkun 1994; Kimura *et al.* 1997).

Temperature input to the parallel DAF-7 (TGF-β) and *daf-11* signaling pathways have also been detected, though unlike *daf-2* and *age-1*, even null alleles of *daf-7* are temperature sensitive (Ren *et al.* 1996; Riddle and Albert 1997). Indeed, expression from a *daf-7* reporter gene construct was shown to be modulated by temperature as well (Ren *et al.* 1996; Schackwitz *et al.* 1996). Therefore, we propose that temperature sensory input modulates either the level of the DAF-2 ligand produced or the response to that ligand. Given the dependence of metabolism rate on cultivation temperature, and the temperature extremes encountered by animals, it is not surprising to find such an explicit connection between temperature sensation and diapause/metabolism control. Indeed, we have found that a dedicated temperature-sensing neural pathway is involved in regulating temperature input to the dauer pathways (Hobert *et al.* 1997).

Dauer formation itself is modulated by temperature and this occurs during L1 and part of L2 for *daf-2* mutants (Swanson and Riddle 1981; Golden and Riddle 1984). We have shown that temperature also affects *daf-2* mutants as adults. Therefore, *daf-2* mutants are either temperature sensitive for the brood size defect, or insulin-like signaling in *C. elegans* is regulated in part by temperature. This is in contrast to other *daf-c* mutants

like *daf-4* and *daf-7*, where dauer formation is modulated by temperature such that they form a higher percentage of dauers at higher temperatures and yet they show other phenotypes at all temperatures: *daf-7* is egg-laying defective and *daf-4* is small (reviewed in Riddle 1988; Thomas 1993; Riddle and Albert 1997).

***daf-2* and *age-1* affect senescence:** In animals carrying weak *daf-2* or *age-1* alleles, conditions that induce a longevity increase and a reduction in fertility and also cause a metabolic shift to fat storage (Kimura *et al.* 1997; this study). This suggests the possibility that the dauer program has been partially induced. Therefore, we interpret the longevity enhancement by *daf-2* and *age-1* mutants as a consequence of the dauer metabolic shift and associated shift from reproduction at higher temperatures. At 15°, the dauer program is sufficiently induced to extend life span but not to cause a fertility defect. An alternative and more complex model is that *daf-2* and *age-1* via *daf-16* affect multiple parallel endocrine outputs that affect metabolism, longevity, and fertility.

It is still unclear, however, why animals bearing mutations in *daf-2* and *age-1* live longer. One of the main theories of aging is the oxidative stress/free radical hypothesis whereby the progressive and irreversible accumulation of oxidative damage leads to declines in viability (reviewed in Harman and Talbert 1985; Finch 1990; reviewed in Schneider and Rowe 1990; Sohal and Weinruch 1996). This theory argues the following: (1) the level of oxidative damage increases with age; (2) lower levels of oxidative damage within and among a species are associated with longer life expectancy; and (3) regimens such as caloric restriction that prolong life also are associated with declines in oxidative damage (Sohal and Weinruch 1996). *age-1* and *daf-2* mutant animals are hyperresistant to oxidative stress (Anderson 1982; Larsen 1993) and both *age-1* and wild-type dauer larvae have elevated levels of superoxide dismutase, a key enzyme involved in protection from oxidative damage (Larsen 1993; Vanfleteren and De Vreese 1995). Moreover, dauer larvae do not feed, indicating that the increased life span may be induced by a metabolic shift analogous to the increase in life span seen with caloric restriction. The involvement of insulin-like control of metabolism in this pathway is consistent with the correlation of metabolism level and senescence (Kimura *et al.* 1997). Life span extension in *daf-2* and *age-1* mutants may be in part due to their resistance to oxidative damage as seen by the increase in levels of superoxide dismutase and other enzymes (Anderson 1982; Larsen 1993; Vanfleteren 1993; Vanfleteren and De Vreese 1995) and, in addition, due to a metabolic shift away from a state of high respiration. Consistent with the connection between metabolism and longevity, *clk-1* mutations cause an increase in life span, and *clk-1* encodes for a protein involved in regulation of cellular respiration, mitochondrial biogenesis, and gluconeogenesis (Lakowski and Hekimi 1996; Ewbank *et al.* 1997). Therefore, it is possible that life span is intrinsically linked to the metabolic control.

Identifying new genes that regulate longevity may help to uncover how life span is itself regulated. We suggest that longevity is intrinsically linked to metabolism in *C. elegans* similar to mammalian studies linking caloric restriction and longevity. Molecular identification of other genes in the pathway may help to uncover the link between metabolism and longevity.

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