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LET-60 RAS modulates effects of insulin/IGF-1 signaling on development and aging in *Caenorhabditis elegans*

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Summary

The DAF-2 insulin/insulin-like growth factor 1 (IGF-1) receptor signals via a phosphatidylinositol 3-kinase (PI3K) pathway to control dauer larva formation and adult longevity in *Caenorhabditis elegans*. Yet epistasis analysis suggests signal bifurcation downstream of DAF-2. We have used epistasis analysis to test whether the Ras pathway (which plays a role in signaling from mammalian insulin receptors) acts downstream of DAF-2. We find that an activated Ras mutation, *let-60(n1046gf)*, weakly suppresses constitutive dauer diapause in *daf-2* and *age-1* (PI3K) mutants. Moreover, increased Ras pathway signaling partially suppresses the *daf-2* mutant feeding defect, while reduced Ras pathway signaling enhances it. By contrast, activated Ras extends the longevity induced by mutation of daf-2, while reduced Ras pathway signaling partially suppresses it. Thus, Ras pathway signaling appears to act with insulin/IGF-1 signaling during larval development, but against it during aging.

Key words: aging; *C. elegans*; dauer larva; insulin/IGF-1 signaling; let-60/Ras.

Introduction

Aging in *Caenorhabditis elegans* is controlled by an insulin/IGF-1 signaling (IIS) pathway (Kenyon, 2005). The insulin/IGF-1 receptor is encoded by the gene *daf-2* (Kimura *et al*., 1997), mutation of which can more than double adult lifespan (Kenyon *et al*., 1993). In *C. elegans*, IIS also regulates formation of dauers, which are long-lived, stress-resistant, diapausal third-stage larvae. This dispersal stage normally forms in response to increased population density and reduced nutrition (Riddle & Albert, 1997). Severe mutational reduction of *daf-2* function results in constitutive formation of dauer larvae (the Daf-c phenotype).

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DAF-2 acts via a signal transduction pathway which includes the AGE-1 PI 3-kinase (Morris *et al*., 1996), and the AKT-1, AKT-2 and SGK-1 protein kinase B proteins (Paradis & Ruvkun, 1998; Hertweck *et al*., 2004). These protein kinases inactivate the FOXO transcription factor DAF-16 by phosphorylation. Mutation of *daf-16* suppresses all mutant phenotypes resulting from mutation of *daf-2*. PTEN phosphatases attenuate IIS by dephosphorylating phosphatidylinositol 3,4,5-trisphosphate (PIP3). *C. elegans* DAF-18 PTEN phosphatase is a negative regulator of signaling via DAF-2 and AGE-1 (Ogg & Ruvkun, 1998; Gil *et al*., 1999; Mihaylova *et al*., 1999; Rouault *et al*., 1999).

Epistasis analysis of insulin/IGF-1 signaling has been complicated by the fact that different *daf-2* mutant alleles interact in different ways with mutations in other genes. For example, mutation of *daf-12*, which encodes a nuclear hormone receptor (Antebi *et al*., 2000), suppresses *daf-2(m41)* Daf-c. Yet when added to *daf-2(e1370)* it has an enhancing effect, resulting in early larval arrest (Vowels & Thomas, 1992; Larsen *et al*., 1995) – even though in terms of Daf-c, *e1370* is a weaker allele than *m41* (Gems *et al*., 1998). Moreover, *daf-12(m20)* partially suppresses the *daf-2(m41)* longevity (Age) phenotype, yet enhances it in *daf-2(e1370)* (Larsen *et al*., 1995).

Such allele-specific interactions reflect the existence of distinct classes of *daf-2* allele. Class 1 mutants are Daf-c and Age; class 2 mutants exhibit these traits plus several others, including early larval arrest, reduced feeding (Eat) and production of progeny late in life (Gems *et al*., 1998). Class 1 mutations occur exclusively in the portion of the *daf-2* gene encoding the extracellular domain of the receptor, while class 2 mutations occur in both intracellular and extracellular domains (Kimura *et al*., 1997) (D. Patel and D. Gems, in preparation). Although weaker alleles are largely class 1, in terms of Daf-c and Age some class 1 alleles are more severe than most class 2 alleles. Thus, *daf-2* alleles do not fall into a single allelic series. This suggests that the *daf-2* gene contains several functional domains, which are differentially impaired in different mutants. This could reflect multiple signaling outputs from the DAF-2 receptor (Gems *et al*., 1998).

The occurrence of signal bifurcation from DAF-2 is supported by other *C. elegans* epistasis studies. For example, in severe *age-1* mutants, Daf-c is largely suppressed by gain-of-function mutations in *akt-1* and *pdk-1*, and a weak reduction-of-function mutation in *daf-18* (Fig. 1). If PI 3-kinase were the sole signaling output of DAF-2, then the same mutations should suppress Daf-c in the weak *daf-2(e1370)* mutant. In fact, they do not (Larsen *et al*., 1995; Paradis & Ruvkun, 1998; Gil *et al*., 1999; Paradis *et al*., 1999).

This is consistent with mammalian insulin and IGF-1 signaling, which have multiple signaling outputs from the receptors into

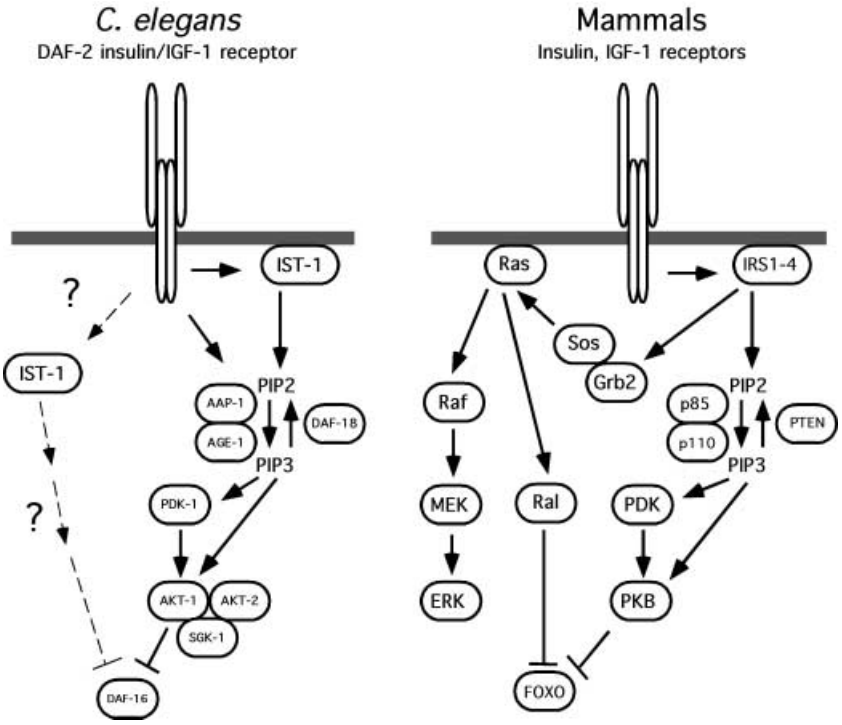

Fig. 1 Insulin/IGF-1 signaling in *C. elegans* and mammals. In *C. elegans* epistasis analysis suggests the presence of other pathways signaling downstream of DAF-2. Comparison with the mammalian insulin and IGF-1 signaling pathways suggests the Ras pathway as a candidate for such a pathway. There are other possible interactions between the insulin/IGF-1 receptor and Ras, for example via the docking protein Shc. A working hypothesis in this study is that DAF-2 signals via the Ras pathway as well as the PIP3 pathway. Note that IST-1 is the *C. elegans* orthologue of IRS. Abbreviations: AAP, phosphoinositide kinase adapter subunit. AGE, ageing alteration. AKT, akt (murine thymoma viral oncogene, named after AKT8 virus) kinase family. DAF, dauer formation abnormal. ERK, extracellular signal-regulated protein kinase. FOXO, forkhead box, subgroup O. Grb, growth factor receptor-bound protein. IRS, insulin receptor substrate. IST, insulin receptor substrate homolog. MEK, MAP kinase kinase or Erk kinase. PDK, 3-phosphoinositide-dependent kinase. PIP2, phosphatidylinositol 4,5-bisphosphate. PIP3, phosphatidylinositol 3,4,5-trisphosphate. PTEN, phosphatase and tensin homolog. PKB, protein kinase B. Ral, Ras-related GTPase homolog. Ras, rat sarcoma oncogene. Sos, son of sevenless.

the cell. These include signaling via the insulin receptor substrate (IRS) proteins to PI 3-kinase, or via the Shc and/or Grb2 docking proteins to the Ras ($p21^{ras}$) small GTPase (White & Kahn, 1994; Adams *et al.*, 2000; Finlayson *et al.*, 2003). $p21^{ras}$ acts as a switch in biological signaling, where Ras-GTP is the 'on' state and Ras-GDP the 'off' state. The PI 3-kinase pathway is the major signaling output of the insulin and IGF-1 receptors, while signaling through Ras plays a lesser role (Medema *et al.*, 1993; Yonezawa *et al.*, 1994; Krook *et al.*, 1997; Leahy *et al.*, 2004).

Notably, the C-terminal region of the *C. elegans* DAF-2 protein contains predicted binding motifs not only for the regulatory subunit of PI 3-kinase, but also for SEM-5 (Grb2) (Kimura *et al.*, 1997), which could be utilized to activate Ras. In the Ras pathway, the Grb2 adaptor protein binds via its SH2 domain to phosphotyrosyl residues in activated insulin receptor substrates (Fig. 1). The guanine nucleotide exchange factor (GEF) Sos (son-of-sevenless) binds to the SH3 domains of Grb2, thus bringing it (Sos) to the plasma membrane. Sos activates Ras by catalysing the exchange of GDP for GTP. Activated Ras binds to and activates the Raf serine/threonine kinase, which phosphoactivates the kinase MEK, which in turn phosphoactivates MAP kinase (ERK). The latter then phosphorylates multiple transcription factors. Depending on context, this can result in metabolic, differentiative or mitogenic responses (Schlessinger, 2000).

The Ras signaling pathway acts downstream of a number of receptor tyrosine kinases, including the EGF and PDGF receptors (White & Kahn, 1994; Adams *et al.*, 2000; Schlessinger, 2000). Insulin signaling via Ras can also lead to inhibition of the forkhead transcription factor FOXO4 (AFX) (Kops *et al.*, 1999). However, in this context, Ras acts via Ral, a related small GTPase, by means of activation of RalGEF, and not MAP kinase (Wolthuis & Bos, 1999; Essers *et al.*, 2004). Several sites of cross-talk between the Ras pathway and IIS have also been reported. For example, Ras can directly interact with and stimulate PI 3-kinase (Kodaki *et al.*, 1994; Rodriguez-Viciana *et al.*, 1997; Sheng *et al.*, 2001), and PKB can phosphorylate and thereby inhibit Raf (Rommel *et al.*, 1999; Zimmermann & Moelling, 1999; Guan *et al.*, 2000).

In *C. elegans*, Ras is encoded by the gene *let-60* (Han *et al.*, 1990; Han & Sternberg, 1990), and plays a role in many cellular processes. These include development of the excretory system and vulva, sex myoblast migration, progression through pachytene in meiosis I, and the function of chemosensory neurons (Sternberg & Han, 1998; Hirotsu *et al.*, 2000; Borland *et al.*, 2001).

To determine whether LET-60 Ras acts downstream of DAF-2 we have examined the effects on *daf-2* mutant phenotypes of mutations affecting PIP3 signaling and the Ras signaling pathway. Our results are consistent with a role for Ras signaling downstream of DAF-2.

Results

We have investigated *daf-2* function using epistasis analysis (Gems *et al.*, 2002). To understand *daf-2* allele-specific effects, we tested four *daf-2* mutant alleles: *m577* (weak class 1), *e1369* (severe class 1), *e1370* (weak class 2) and *e979* (severe class 2). All four show a temperature-sensitive dauer constitutive (Daf-c) phenotype. At higher temperatures *daf-2(e979)* also shows embryonic lethality and early larval (L1) arrest (Vowels & Thomas, 1992; Gems *et al.*, 1998). We have tested the effects on *daf-2*

Table 1 Effects of *daf-18(e1375)*, *pdk-1(gf)* and *akt-1(gf)* mutations on *daf-2* larval arrest

	25 °C							22.5 °C						
	72 h			96 h				80 h			104 h			
Genotype*	% dauer	% L4, adults	% other†	% dauer	% L4, adults	% other†	*n*	% dauer	% L4, adults	% other†	% dauer	% L4, adults	% other†	*n*
+	0	99	1				284	0	97	3				266
daf-18(e1375)	0	97	3				204	0	97	3				222
pdk-1(mg142)	0	98	2				286	0	99	1				262
akt-1(mg144)	0	97	3				258	0	96	4				219
daf-2(m577) (1)	96	0	4	96	0	4	216	12	87	1	0	99	1	226
daf-2(e1369) (1)	96	0	4	96	0	4	166	97	0	3	97	0	3	147
daf-2(e1370) (2)	90	0	10	90	0	10	190	85	0	15	85	0	15	137
daf-2(e979) (2)	0	0	100	0	0	100	150	96	0	4	96	0	4	199
daf-2(m577); daf-18	0	93	7				232	0	95	5				256
daf-2(e1369); daf-18	0	96	4				244	0	98	2				241
daf-2(e1370); daf-18	82	15	3	2	95	3	148	0	96	4				223
daf-2(e979); daf-18	0	0	100	0	0	100	135	19	77	4	19	77	4	156
daf-2(m577); pdk-1	75	21	4	27	69	4	178	0	97	3	0	97	3	280
daf-2(e1369); pdk-1	95	3	2	78	20	2	147	4	95	1	0	99	1	237
daf-2(e1370); pdk-1	92	6	2	5	93	2	231	0	97	3	0	97	3	197
daf-2(e979); pdk-1	97	0	3	97	0	3	174	100	0	0	100	0	0	179
daf-2(m577); akt-1	59	40	1	21	78	1	152	0	97	3	0	97	3	275
daf-2(e1369); akt-1	95	2	3	57	40	3	159	64	35	1	14	85	1	201
daf-2(e1370); akt-1	81	19	0	21	79	0	196	3	95	2	0	98	2	121
daf-2(e979); akt-1	11	0	89	11	0	89	101	98	0	2	84	14	2	143

**daf-2* allele class in parentheses.
†Dead eggs, arrested L1s or, rarely, L2s. *n*, sample size.

phenotypes of reduction-of-function (*rf*) alleles of *daf-18, sem-5, sos-1* and *let-60* and gain-of-function (*gf*) alleles of *pdk-1, akt-1, sos-1* and *let-60. sem-5* and *sos-1* encode a Grb2/Drk adaptor protein and a Ras-activating GEF, respectively (Clark *et al*., 1992; Chang *et al*., 2000).

Daf-c in class 1 *daf-2* alleles is fully suppressed by *daf-18(rf)* but not *pdk-1(gf)* or *akt-1(gf)*

The weak reduction-of-function mutation *daf-18(e1375)* fully suppresses Daf-c in *age-1(0)*, but not the weak class 2 allele *daf-2(e1370)* (Gottlieb & Ruvkun, 1994; Larsen *et al*., 1995; Gil *et al*., 1999). We found that both class 1 *daf-2* alleles tested were fully suppressed by *daf-18(e1375)* (Table 1). By contrast, there was little suppression of class 2 *daf-2* alleles: *daf-18(rf)* only weakly suppressed *daf-2(e1370)* at 72 h (25 °C), although by 96 h almost all dauers had recovered, consistent with previous findings. Moreover, *daf-18(rf)* did not suppress the embryonic and L1 arrest trait of *daf-2(e979)*.

In terms of Daf-c, *daf-2(e1369)* is a more severe allele than *e1370* or *e979*: at 15 °C these mutants form 55, 0 and 20% of dauers, respectively (Gems *et al*., 1998). Yet *daf-18(rf)* fully suppresses only *e1369* Daf-c (this study). Because DAF-18 removes PIP3, the unusual severity of the Daf-c phenotype in *daf-2(e1369)* may reflect reduced PI 3-kinase signaling. However, we also found that all four *daf-2* alleles were fully suppressed by the null mutation *daf-18(nr2037)* (25 °C; data not shown). Possibly, modulation of *daf-2* mutant phenotypes by signaling other than PIP3 is only detectable against a background of reduced PIP3 levels.

Gain-of-function alleles of *pdk-1* and *akt-1* are efficient suppressors of Daf-c in *age-1(0)* but not *daf-2(e1370)* (Paradis & Ruvkun, 1998; Paradis *et al*., 1999). In contrast to *daf-18(rf)*, we find that *pdk-1(gf)* and *akt-1(gf)* are poor suppressors of Daf-c in class 1 *daf-2* mutants (Table 1). Yet both *pdk-1(gf)* and *akt-1(gf)* caused some degree of suppression of the embryonic and L1 arrest trait of *daf-2(e979)*, which *daf-18(rf)* did not.

daf-18(rf), *pdk-1(gf)* and *akt-1(gf)* suppress Eat and late progeny production in *daf-2* mutants

pdk-1(gf) and *akt-1(gf)* are better suppressors than *daf-18(rf)* of *daf-2* embryonic and L1 arrest – which are class 2-specific phenotypes. Is this true of all class 2-specific mutant defects? Our evidence suggests not. We examined effects of *daf-18(rf)*, *pdk-1(gf)* and *akt-1(gf)* on reduced feeding (Eat) and production of late progeny in *daf-2(e1370)* adults (Larsen *et al*., 1995; Gems *et al*., 1998). To examine the Eat trait, animals were shifted from 20 °C to 25 °C at the fourth larval stage (L4), and pharyngeal pumping rates measured over a 5-day period. N2, *pdk-1(gf)*, *akt-1(gf)*, *daf-18(rf)* and *daf-2(m577)* strains showed a similar small decline in pumping rate over this period (data not shown). By contrast, pumping rate in *daf-2(e1370)* fell dramatically, as expected. *daf-18(rf)*, *pdk-1(gf)* and *akt-1(gf)* all partially suppressed the *daf-2* Eat trait (Fig. 2A).

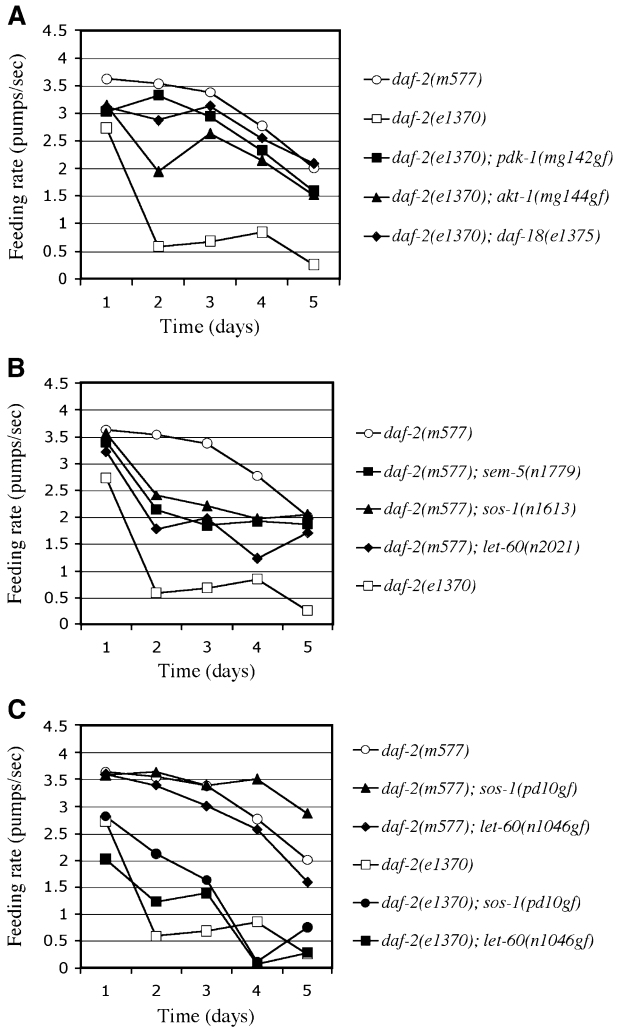

Fig. 2 Effects of insulin/IGF-1 and Ras signaling on pharyngeal pumping during the first 5 days of adulthood. (A) Suppression of the *daf-2(e1370)* Eat phenotype by *pdk-1(gf)*, *akt-1(gf)* and *daf-18(e1375)*. On days 2–5, the rate of pumping in all *daf-2(e1370)* double mutants was significantly higher than in the *daf-2(e1370)* single mutant [two-tailed Mann–Whitney test, $P < 0.0001$ except for *daf-2(e1370); akt-1(mg144gf)* where $P < 0.002$; $n = 21–37$ at all genotype/time points]. (B) Reduction-of-function mutations affecting Ras signaling significantly reduce pharyngeal pumping in *daf-2(m577)* (class 1) mutants on days 2–4 (two-tailed Mann–Whitney test, $P < 0.0001$ days 2 and 3; $P < 0.015$ day 4; $n = 16–37$ at all genotype/time points). (C) Weak suppression of the *daf-2(e1370)* Eat phenotype by gain-of-function mutations affecting the Ras pathway. Pumping rates were significantly higher relative to *daf-2(e1370)* on days 2 and 3 for *daf-2(e1370); let-60(n1046gf)* and *daf-2(e1370); sos-1(pd10gf)* [two-tailed Mann–Whitney test, $P < 0.05$; $n = 16–37$ at all genotype/time points except for *daf-2(e1370); sos-1(pd10gf)* on day 5 where $n = 7$].

Similarly, *daf-18(rf)*, *pdk-1(gf)* and *akt-1(gf)* all suppressed late progeny production by *daf-2(e1370)* (data not shown).

let-60(gf) (activated Ras) weakly suppresses Daf-c in *daf-2* and *age-1* mutants

We asked whether Ras functions downstream of the DAF-2 insulin/IGF-1 receptor as it does in mammals. Given that mutations which reduce *let-60*/Ras signaling are not dauer constitutive, we expected that this pathway would play at most a modulatory role in the control of dauer formation by IIS. We found that the activated Ras mutation *let-60(n1046gf)* weakly suppressed *daf-2* Daf-c in class 1 but not class 2 mutants (Table 2). *let-60(gf)* caused the majority of dauer larvae formed by *daf-2(m577)* (25 °C) and *daf-2(e1369)* (22.5 °C) to recover by 120 h.

This is consistent with action of LET-60/Ras in a second pathway downstream of DAF-2. This could signal in parallel to the AGE-1 catalytic subunit of PI 3-kinase (Morris *et al.*, 1996) (Fig. 1), in which case *let-60(gf)* should suppress the mutant effects of loss of function of *age-1*. Alternatively, the effects of *let-60(gf)* could be mediated through activation of AGE-1 via the Ras-interacting domain, as can occur in mammals (Kodaki *et al.*, 1994; Rodriguez-Viciana *et al.*, 1997; Sheng *et al.*, 2001). *C. elegans* AGE-1 does contain a predicted Ras-interacting domain (Morris *et al.*, 1996). We found that *let-60(gf)* can weakly suppress *age-1(mg44)*, a putative null allele (Morris *et al.*, 1996). At 22.5 °C, 75% of *age-1(0); let-60(gf)* larvae exited dauer by 96 h (Table 2). Thus, the weak influence of Ras signaling on IIS-associated dauer formation does not require AGE-1 PI 3-kinase.

Table 2 Effects of *let-60(gf)* on *daf-2* and *age-1* larval arrest

Genotype*	% L2d dauer	% L4, adults	% other	*n*
Trial 1 (25 °C)				
+	0	100	0	120
let-60(n1046gf)	0	100	0	180
daf-2(m577) (1)	98	0	2	189
daf-2(e1369) (1)	100	0	0	100
daf-2(e1370) (2)	100	0	0	173
daf-2(e979) (2)	0	0	100†	77
daf-2(m577); let-60(gf)	31	**69**	0	118
daf-2(e1369); let-60(gf)	98	0	2	92
daf-2(e1370); let-60(gf)	98	0	2	38
daf-2(e979); let-60(gf)	**90**	0	10	77
Trial 2 (22.5 °C)‡				
+	0	100	0	320
let-60(gf)	0	100	0	237
daf-2(e1369) (1)	100	0	0	157
daf-2(e979) (2)	100	0	0	189
daf-2(e1369); let-60(gf)	47	**53**	0	128
daf-2(e979); let-60(gf)	95	0	5§	83
age-1(mg44)	100	0	0	107
age-1(mg44); let-60(gf)	25	**75**	0	137

Instances of suppression highlighted in bold. Time of scoring: 25 °C, 120 h; 22.5 °C, 96–120 h.
**daf-2* allele class in parentheses.
†18% dead eggs, 82% arrested L1s.
‡Data compiled from two similar trials.
§2% dead eggs, 1% L1–3, very swollen, abnormal morphology, 2% L2d or dauer-like L3 within sheath.

sem-5(n1779) weakly enhances *daf-2(e1370)* Daf-c

If increased Ras signaling suppresses *daf-2* Daf-c, does reduced Ras signaling enhance it? We tested this using

Table 3 Weak mutual enhancement of *daf-2(rf)* and *let-60(rf)* or *sem-5(rf)*

Genotype	% L4, adults	% dauer	% L1 lethality	*n*
+	100	0	0	280
let-60(n2021)	15	0	85	72
sem-5(n1779)	100	0	0	179
daf-2(m577)	88	12	0	220
daf-2; let-60	0	0	100	82
daf-2; sem-5	55	35	10	304

Trials conducted at 22.5 °C, Daf-c scored at 80 h.

reduction-of-function alleles of *let-60*, and two other Ras pathway genes, *sem-5* (Grb2/Drk) and *sos-1* (GEF). In these genes, loss of function results in early larval (L1) lethality (Han *et al*., 1990; Clark *et al*., 1992; Chang *et al*., 2000). Therefore, we used weak reduction-of-function alleles, testing for effects on Daf-c in *daf-2(m577)* (class 1) and *daf-2(e1370)* (class 2). *sem-5* weakly enhanced the Daf-c phenotype of *daf-2(m577)*, doubling the proportion of dauer larvae formed (Table 3). This is consistent with signaling from DAF-2 to SEM-5. Unexpectedly, in *daf-2; let-60* animals, Daf-c was masked by increased levels of L1 arrest; a low level of L1 arrest was also seen in *daf-2; sem-5* populations (Table 3). In *daf-2; sos-1* animals, no enhancement of Daf-c or L1 arrest was seen (data not shown).

Ras signaling influences the *daf-2* feeding defect

Does Ras pathway signaling play a role in other *daf-2* mutant traits, such as reduced feeding (Eat) and production of late progeny? To address this, we tested whether reduced Ras signaling would induce these class 2-specific traits in a class 1 *daf-2* mutant (*m577*), and whether increased Ras signaling would suppress them in a class 2 mutant (*e1370*). To reduce Ras signaling, we used *sem-5(n1779)*, *sos-1(n1613)* and *let-60(n2021)*; to increase it, we used *sos-1(pd10gf)* and *let-60(n1046gf)*.

Ras pathway single mutants pumped at a similar rate to N2 and *daf-2(m577)* animals (data not shown). However, in *daf-2(m577); sem-5(rf)*, *daf-2(m577); sos-1(rf)* and *daf-2(m577); let-60(rf)* animals, the pumping rate was reduced (Fig. 2B). Moreover, there was a small but significant increase in pumping rate in *daf-2(e1370); sos-1(gf)* and *daf-2(e1370); let-60(gf)* animals relative to the *daf-2(e1370)* single mutant (Fig. 2C).

By contrast, altered Ras signaling had little effect on late progeny production. *let-60(gf)* did not suppress late progeny production by *daf-2(e1370)*, nor did *sem-5(rf)* and *let-60(rf)* cause *daf-2(m577)* animals to produce late offspring (data not shown). However, 4/55 *daf-2(m577); sos-1(rf)* animals did produce late offspring, while *daf-2(m577)* and *sos-1(rf)* single mutants did not (*n* = 71 and 16, respectively).

Ras signaling increases lifespan when insulin/IGF-1 signaling is reduced

Next we examined the effect of Ras signaling on the *daf-2* increased lifespan (Age) phenotype. *let-60(gf)* by itself caused a considerable reduction in lifespan (Fig. 3, Table 4). The effect of *let-60(gf)* on *daf-2* mutants was complex: in three of the four *daf-2* mutants tested, it increased maximum lifespan but not mean lifespan. The form of the survival curves suggests a bimodal distribution of population mortality. In each *daf-2; let-60(gf)* cohort there is a sharp increase in mortality early in life (particularly in week 2). This is followed by a drop in mortality, leaving a longer-lived population subset which outlive the *daf-2* cohorts in 3/4 cases. The *let-60(gf)* mutation is highly pleiotropic and its various phenotypic effects show variable penetrance. We suggest that this leads to early death in a subset of the population, probably from causes unrelated to normal *C. elegans* aging. In those that escape premature death, *let-60(gf)* appears to enhance the *daf-2* Age phenotype.

To confirm this, we censored the deaths that occurred during the first 2 weeks of adulthood, and then compared mortality in *daf-2* and *daf-2; let-60(gf)* populations. The effects of *let-60(gf)* on *daf-2* mean lifespan in the long-lived subset were: *m577*, +2.3%, $P = 0.78$ (log rank test); *e1370*, +67.6%, $P = 0.0002$; *e1369*, +30.2%, $P = 0.0004$; and *e979*, +43.8%, $P < 0.0001$. This implies that LET-60 Ras contributes to the *daf-2* mutant Age phenotype.

If this conclusion is correct, reduction of Ras pathway signaling should suppress the *daf-2* Age phenotype. We therefore tested effects of *sem-5(n1779)*, *sos-1(n1613)* and *let-60(n2021)* on lifespan, alone or in combination with *daf-2(m577)* (Fig. 2, Table 4). None of the Ras pathway mutations alone extended lifespan; *let-60(rf)* and *sem-5(rf)* each slightly reduced lifespan in one of the two trials ($P < 0.05$). However, all three Ras pathway mutations partially suppressed the *daf-2* Age phenotype ($P < 0.0001$ in all cases) (Fig. 3, Table 4). This implies that wild-type activity of these genes contributes to the *daf-2* Age phenotype. Taken together, these findings point strongly to the unexpected conclusion that Ras pathway signaling acts with IIS during larval development, but in opposition to it during aging.

L1 arrest in *daf-2* and *let-60* mutants entails different mechanisms

Severe mutations in either *daf-2* or *let-60* can result in L1 arrest, yet this is unlikely to involve the same mechanism. *let-60(0)* mutants die at the L1 stage with a fluid-filled morphology (Han *et al*., 1990). We find that this is distinct from the *daf-2(e979)* L1 arrest. *e979* L1 larvae appeared morphologically normal under Nomarski microscopy and most had arrested at the four-cell gonad stage, similar to L1s hatching in the absence of food (5/5 N2 animals hatched in the absence of food and 15/16 L1-arrested *e979* animals with food present arrested at the four-cell gonad stage). In *e979* L1s, pharyngeal pumping was weak and sporadic (5.5 ± 4 pumps min^{-1}, compared to 173.6 ± 13

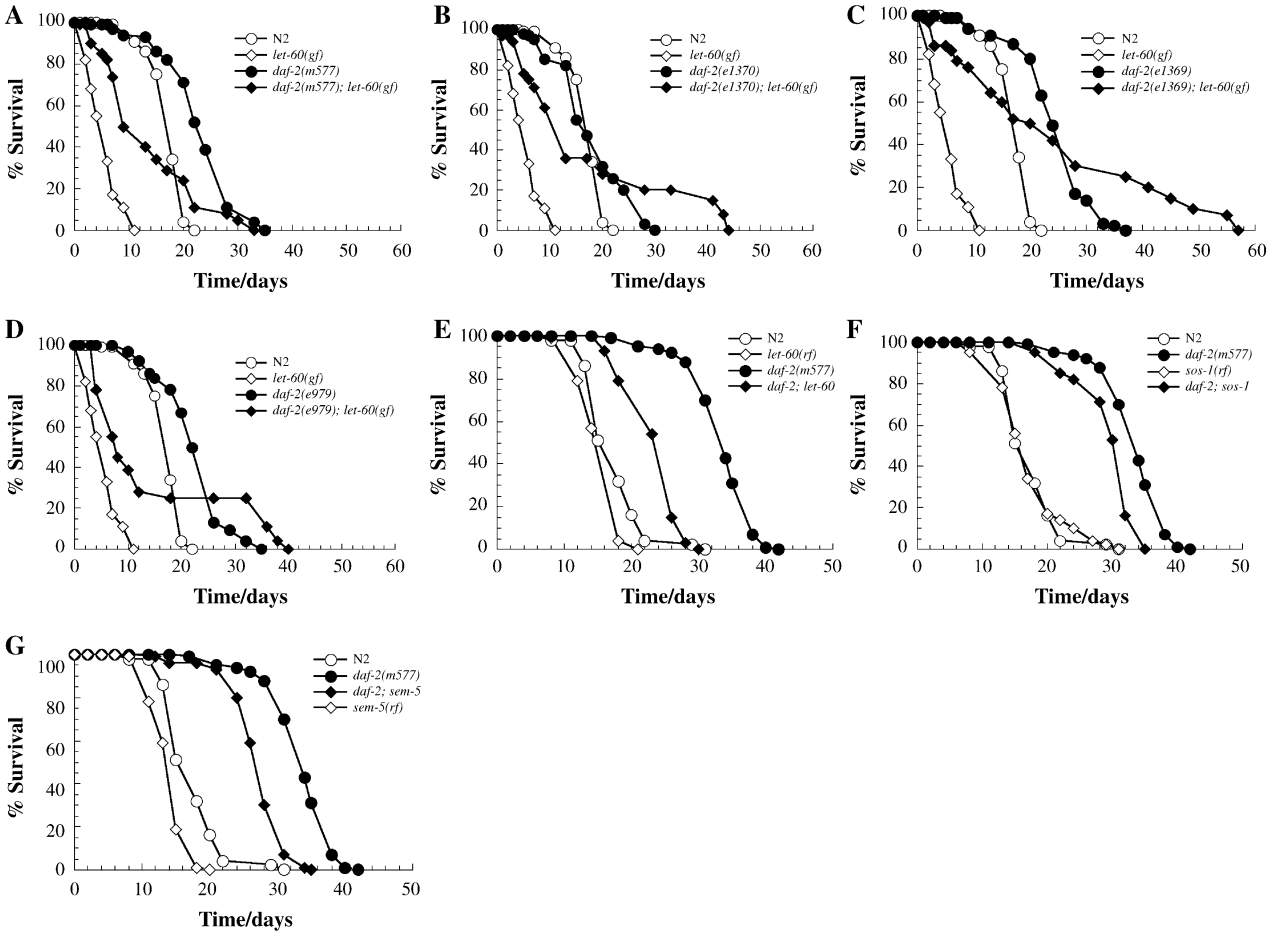

Fig. 3 Effects of mutations in the Ras pathway on adult survival in *daf-2(rf)* and *daf-2(+)* strains (22.5 °C). The zero time point represents the L4 larval stage.

pumps min^{-1} in wild-type L1s, $n = 5$ in each case). Potentially, this failure to feed causes L1 arrest.

daf-2(rf) enhances *let-60(rf)* L1 lethality, but does not suppress *let-60(gf)* excretory duct cell duplication

daf-2(rf) in Ras pathway mutants results in an increased level of L1 arrest (Table 4). But is this due to an increase in the *daf-2* L1 arrest trait or in the *let-60* L1 arrest? We find that it is the latter. *let-60(0)* is lethal due to failure of excretory duct cell fate specification – hence the fluid-filled morphology. All *daf-2; sem-5(n1779)* and *daf-2; let-60(n2021)* arrested animals were fluid filled. This implies that IIS influences Ras pathway function during the specification of the excretory duct cell fate.

To test this further, we investigated whether mutation of *daf-2* suppresses the effects of *let-60(gf)* on excretory duct cell development. Around half of *let-60(gf)* animals show duplication of the duct cell nucleus (Yochem *et al*., 1997), and we observed this too. However, mutation of *daf-2* (four alleles, as above) did not affect this duplication (data not shown). By contrast, a previous study did show that reduced insulin/IGF-1 signaling can suppress a mutant trait generated by *let-60(gf)*: the multivulva phenotype is suppressed by *daf-2(e1370)* (Battu *et al*., 2003).

Discussion

Classical genetic studies imply that signal bifurcation might occur downstream of the DAF-2 receptor. Mammalian receptor investigations suggest that this might involve the Ras pathway. In this study, we explored two possibilities: first, that the LET-60 Ras pathway plays a role in DAF-2 signaling; and second that differences in LET-60 Ras and PI 3-kinase signaling contribute to the difference between class 1 and class 2 *daf-2* alleles. Overall, our findings provide clear support for the first idea, and some evidence for the second.

Evidence that Ras signalling acts downstream of DAF-2

Several findings are consistent with action of LET-60 Ras downstream of DAF-2: (1) *let-60(gf)* weakly suppresses constitutive dauer formation (Daf-c) in *daf-2* mutants; (2) *let-60(gf)* suppresses the embryonic and early larval arrest trait of *daf-2(e979)*; (3) *sem-5(rf)* enhances *daf-2* Daf-c; and (4) lowered Ras pathway

Table 4 Effects of *let-60* Ras, *sos-1* and *sem-5* mutations on aging

Genotype	Trial	Mean lifespan	Proportion of wild type mean lifespan	Maximum lifespan	*N**	*P*1†	*P*2‡
+	–	17.6	1.0	22	47 (34)	–	–
let-60(n1046gf)	–	5.5	0.31	11	70 (6)	< 0.0001	–
daf-2(m577)	–	23.3	1.32	35	48 (32)	< 0.0001	–
daf-2(e1370)	–	18.4	1.05	30	38 (41)	0.20	–
daf-2(e1369)	–	24.6	1.40	37	58 (22)	< 0.0001	–
daf-2(e979)	–	22.8	1.30	35	47 (18)	< 0.0001	–
daf-2(m577); let-60(gf)	–	13.8	0.78	33	61 (22)	0.061	< 0.0001
daf-2(e1370); let-60(gf)	–	18.0	1.02	44	39 (32)	0.066	0.13
daf-2(e1369); let-60(gf)	–	24.5	1.39	57	48 (32)	0.090	0.69
daf-2(e979); let-60(gf)	–	15.1	0.86	40	38 (8)	0.026	0.062
+ (FUdR)	(1)	17.4	1.0	31	85 (3)	–	–
	(2)	15.7	1.0	28	95 (0)	–	–
let-60(n2021)	(1)	16.5	0.95	21	91 (9)	0.055	–
	(2)	14.1	0.90	21	73 (3)	0.009	–
sos-1(n1613)	(1)	17.5	1.01	31	99 (1)	0.91	–
	(2)	17.0	1.08	31	96 (3)	0.13	–
sem-5(n1779)	(1)	14.3	0.82	20	87(10)	< 0.0001	–
	(2)	15.1	0.96	21	87 (16)	0.60	–
daf-2(m577)	(1)	33.8	1.94	42	92 (8)	< 0.0001	–
	(2)	32.2	2.05	41	67 (8)	< 0.0001	–
daf-2(m577); let-60(rf)	(1)	23.8	1.37	30	72 (0)	< 0.0001	< 0.0001
	(2)	27.4	1.75	35	70 (0)	< 0.0001	< 0.0001
daf-2(m577); sos-1(rf)	(1)	29.8	1.71	35	93 (5)	< 0.0001	< 0.0001
	(2)	29.6	1.89	37	96 (6)	< 0.0001	< 0.0001
daf-2(m577); sem-5(rf)	(1)	27.4	1.57	35	68 (7)	< 0.0001	< 0.0001
	(2)	25.7	1.64	34	59 (16)	< 0.0001	< 0.0001

Lifespan measured at 22.5 °C.
*Deaths scored (number of censored values).
†*P*1 = probability of being identical to wild-type lifespan (log rank test).
‡*P*2 = probability of being identical to *daf-2* lifespan.

signaling enhances the *daf-2* Eat trait, while increased Ras pathway signaling weakly suppresses it.

Given that *daf-2* signaling acts via the DAF-16 FOXO transcription factor, these effects of Ras signaling might be exerted via DAF-16. *daf-2*(+) inhibits DAF-16 activity, triggering its phosphorylation and cytoplasmic retention (Henderson & Johnson, 2001; Lee *et al*., 2001; Lin *et al*., 2001). We looked for effects of *let-60(gf)* and *let-60(rf)* on DAF-16 cellular localization in *daf-2*(+) and *daf-2(m577)* backgrounds, but saw none (data not shown). Thus, if LET-60/Ras has any influence on DAF-16 function, it is not exerted via major changes in distribution of DAF-16. Consistent with this, in mammals Ras-RalGEF-Ral-induced phosphorylation of FOXO4 influences transcription without affecting nuclear-cytoplasmic distribution (De Ruiter *et al*., 2001).

Can *daf-2* allele classes be explained by signal bifurcation?

Our initial working hypothesis was that class 1 and class 2 *daf-2* mutants are characterized by greater loss of PI 3-kinase and Ras signaling, respectively. We tested this by examination of several *daf-2* mutant traits: Daf-c, which is seen in all *daf-2* alleles, and several traits which only occur in class 2 alleles: embryonic and early larval arrest, reduced feeding rate (Eat) and late progeny production.

Daf-c

The weak reduction-of-function allele *daf-18(e1375)* suppresses the Daf-c phenotype of class 1 *daf-2* mutants but not class 2 mutants. In this, class 1 mutants resemble severe *age-1* mutants, which are defective in PI 3-kinase signaling alone. This is consistent with a greater reduction in PIP3 synthesis in class 1 mutants. However, both class 1 and class 2 *daf-2* mutants were fully suppressed by *daf-18(0)*. We therefore postulate that alternative pathway modulation of *daf-2* mutant phenotypes is only detectable against a background of reduced PIP3 levels.

Embryonic and early larval arrest

This class 2-specific trait is seen at a low level in several *daf-2* alleles (e.g. *e1370*), and at a high level in *e979* (Gems *et al*., 1998). *e979* early arrest was largely suppressed by *let-60(gf)*, but unaffected by *daf-18(e1375)*. This suggests that reduced LET-60 Ras signaling contributes to this trait. However, the failure of *daf-18(rf)* to suppress in this instance must also reflect the severity of the reduction of PI 3-kinase activity, because *daf-18(0)* fully suppresses *daf-2(e979)*.

Eat and late progeny production

Class 2 mutants show a temperature-sensitive feeding defect (Eat) (Gems *et al*., 1998). We found that this is partially suppressed not only by *let-60(gf)* and *sos-1(gf)*, but also by *daf-18(rf)*. Late progeny production (also class 2 specific) was also suppressed by *daf-18(rf)*. Thus, these two traits do not appear to be attributable to selective loss of PI 3-kinase or LET-60 Ras signaling.

Previously, *daf-2* allele class differences were described in terms of a formal model in which a bifunctional *daf-2* gene contains elements *daf-2A* and *daf-2B*. According to this scheme, class 1 alleles are *daf-2A*(–)*daf-2B*(+) while class 2 alleles are *daf-2A*(–)*daf-2B*(–) (Gems *et al*., 1998). In terms of this scheme, the results of this study suggest a possible correspondence between *daf-2A* and PI 3-kinase signaling. The suppression of the embryonic and early larval arrest traits of *daf-2(e979)* by *let-60(gf)* but not *daf-18(e1375)* suggest a possible correspondence between *daf-2B* and LET-60 Ras signaling. However, other findings presented here fail to support such a correspondence. It seems likely that the properties of *daf-2* may be fully explained only by signal transduction via other insulin-associated signaling moieties, such as IST-1 (IRS) (Wolkow *et al*., 2002), SGK-1 (Hertweck *et al*., 2004), LET-363/CeTOR and DAF-15/raptor (Jia *et al*., 2004), candidate Shc proteins (Luzi *et al*., 2000), or *C. elegans* orthologs of Ral-GEF and Ral (Wolthuis & Bos, 1999).

Ras signaling contributes to longevity assurance

Our results show that Ras signaling can promote longevity. *let-60(gf)* enhances the *daf-2* longevity (Age) phenotype, while reduction of Ras signaling partially suppresses it (Fig. 3, Table 4). As in dauer formation, Ras signaling does not influence longevity where DAF-2 activity is high and DAF-16 low, but it can where DAF-2 activity is low and DAF-16 high. However, while LET-60/Ras potentiates DAF-2 signaling in the control of dauer formation, early larval development and feeding, it acts antagonistically to it in longevity assurance.

The effects of Ras signaling on aging in *C. elegans* were unexpected. In the budding yeast *Saccharomyces cerevisiae*, many studies have shown that Ras activity shortens lifespan, whether replicative or chronological (Longo, 2004). Moreover, in both yeast and mammalian cells, Ras stimulates production of mitochondrial oxidants, which in human cells promote cellular senescence (Lee *et al*., 1999; Hlavata *et al*., 2003). Possibly, in *C. elegans*, Ras signalling reduces longevity where IIS is high, but extends it where IIS is low.

In conclusion, our results demonstrate that Ras pathway signaling influences a number of traits regulated by IIS, including embryogenesis, dauer larva formation and aging. There is also a reciprocal effect of IIS on some Ras pathway-regulated traits: excretion/viability in L1s (this study) and vulval development (Battu *et al*., 2003). We postulate that DAF-2 receptor signaling via SEM-5/Grb-2 to LET-60/Ras contributes to IIS-regulated phenotypes. Moreover, differences in relative levels of disruption of LET-60 Ras and PI 3-kinase might contribute to *daf-2* allele class differences.

Experimental procedures

Growth and culture conditions

All *C. elegans* strains were cultured on nematode growth medium (NGM) plates, on a lawn of *E. coli* strain OP50 (Sulston & Hodgkin, 1988). The following strains were employed: LG II: GR1032 *age-1(mg44)/mnC1 [dpy-10(e128) unc-52(e444)]*; LG III: DR1563 *daf-2(e1370)*; DR1567 *daf-2(m577)*; DR1573 *daf-2(e1369)*; DR1942 *daf-2(e979)*; LG IV: CB1375 *daf-18(e1375)*; NS3227 *daf-18(nr2037)*; MT4866 *let-60(n2021)*; MT2124 *let-60(n1046gf)*; LG V: MT3719 *sos-1(n1613)*; HP17 *sos-1 (pd10gf);* GR1310 *akt-1(mg144gf)*; LG X: GR1318 *pdk-1 (mg142gf)*; MT4185 *sem-5(n1779)*. The N2 used was the CGC male stock; there is variation among N2 lines, and this line best approximates wild type (Gems & Riddle, 2000).

Construction of multiple mutant strains

daf-2(ts); daf-18 strains were constructed as follows: *daf-18(e1375)* males were crossed with *daf-2* hermaphrodites at 15 °C, and F1 progeny selfed at 25 °C (22.5 °C for *e979*). Homozygous *daf-2* segregants were picked as constitutive dauer larvae. Suppression by *daf-18* was not expected, because *daf-18* mutations are maternally rescued (Gil *et al*., 1999). However, a small proportion of dauers maintained at 25 °C (22.5 °C for *e979*) resumed development after several days, and some of the resulting adults exhibited the exploding vulva characteristic of *daf-18* homozygotes. After strain characterization, the presence of *daf-18(e1375)* in the *daf-2(e979); daf-18(e1375)* strain was reconfirmed by checking for the occurrence of the *daf-18* exploding vulva trait. The presence of the *m577* and *e1369* alleles in the double mutants was also confirmed by crossing with wild type (N2), selfing the F1 at 25 °C, and checking for the presence of constitutive dauer larvae among the F2. In the case of *e1369*, these dauers were allowed to recover and self at 15 °C; some dauer larvae were observed, consistent with the allele being *e1369* [most *daf-2*(*ts*) alleles do not form dauer larvae at this temperature].

daf-2; akt-1(mg144) and *daf-2; pdk-1(mg142)* strains were constructed as follows: *akt-1* or *pdk-1* males were mated with *daf-2* hermaphrodites at 15 °C, and F1 heterozygotes selfed at 25 °C (22.5 °C for *e979*). Homozygous *daf-2* segregants were picked as constitutive dauer larvae, and potential *akt-1* or *pdk-1* double mutants identified as dauers that recovered after several days. After propagation, each putative double mutant was selfed again at 25 °C (22.5 °C for *e979*), and in each case, all dauer larvae eventually recovered. Given that the single *daf-2* mutant dauer larvae do not recover under these conditions, it was inferred that (a) the recovering dauer larvae were homozygous for *akt-1* or *pdk-1*, and (b) that neither suppressor mutation causes significant dominant suppression of *daf-2*. After strain characterization, the presence of *e979* in the

daf-2(e979); pdk-1(gf) was confirmed by back-crossing to wild type and resegregation of the ts embryonic lethal/L1 arrest trait.

Construction of all double mutants containing *daf-2* and mutations in the Ras pathway began with the same steps. *daf-2* males were crossed with Ras pathway mutant hermaphrodites, then F1 hermaphrodites were selfed at 25 °C, and F2 dauer larvae picked and allowed to recover. In the case of *daf-2; let-60(n1046gf)*, recovering dauers exhibiting the multivulva (Muv) trait were picked and selfed, and checked for the presence of a high proportion of Muv progeny to ensure that they were homozygous (because *n1046* is semi-dominant). In the case of *daf-2; let-60(n2021rf)*, F2 dauers were allowed to recover and self at 15 °C, and double mutants lines identified as vulvaless (Vul) animals, a proportion of whose progeny died as L1s with a rod-like appearance. In the case of *daf-2; sem-5(n1779)*, recovering dauers were selfed at 22.5 °C and checked for production of more dauers than the *daf-2* single mutant (which forms few dauers at this temperature). The presence of the *n1779* mutation was then confirmed by PCR amplification of the region of the gene containing the lesion, and by sequencing. In the case of *daf-2; sos-1(n1613)*, recovering dauers were selfed at 15 °C, allowed to lay some progeny and then transferred to 25 °C. Double mutant progeny produced all arrested L1s at the higher temperature. In the case of double mutants where the Daf-c phenotype was masked by the L1 Let phenotype, the presence of the *daf-2* mutation was reconfirmed by crossing with wild type and checking the L2 for segregation of dauer larvae at 25 °C.

daf-2; sos-1(pd10gf) strains were constructed as follows. *daf-2* males were crossed with *sos-1(pd10gf) unc-46(e177)* hermaphrodites and non-Unc cross progeny placed at 25 °C. Dauers were picked and allowed to recover at 15 °C. From these, *unc-46* hermaphrodites were selected and mated with *sos-1(pd10gf)* males. Again, non-Unc cross progeny were placed at 25 °C and dauers selected. Upon recovery at 15 °C, non-Uncs were cloned and a line of non-segregating Uncs was picked. *sos-1*(*pd10gf*) was isolated in an independent study as a suppressor of *sem-5(n1619)* lethality (A. Wooller and N.A.H., in preparation).

The strain *age-1(mg44)/mnC1 [dpy-10(e128) unc-52(e444)] II; let-60(gf) IV* was constructed as follows. *age-1/mnC1* males were crossed with *let-60(gf)* hermaphrodites, and then F1 males and hermaphrodites mated. Strongly Muv non-Dpy non-Unc F2 hermaphrodites were then placed individually on plates and allowed to self, and plates segregating Dpy Uncs (i.e. where the F2 hermaphrodite was heterozygous for *mnC1*) were retained. From these plates, a number of hermaphrodites were tested to see whether they were homozygous for *mg44*, by selfing at 25 °C and looking for segregation of 100% dauers (*mg44* homozygotes would not be expected to form dauer larvae in the F3 due to maternal rescue).

Dauer formation and early larval arrest assays

Test strains were raised at 15 °C, and then approximately half a dozen gravid hermaphrodites were transferred to a fresh plate at the test temperature, and allowed to lay eggs for 3–5 h, after which they were removed. Each plate was examined daily to follow development to a terminal phenotype. Any adults or L4 larvae were counted and removed. The numbers of dauer larvae and other arrested progeny were scored approximately 72 h after the midpoint of egg-laying at 25 °C, 80 h at 22.5 °C and 96 h at 21 °C. In some cases dauer recovery was also scored at a later time point (see data tables). Samples compromised by fungal or bacterial contaminants were excluded.

Pharyngeal pumping and late progeny assays

Animals were raised at 20 °C, and shifted to 25 °C as L4s (day 0). Pumping was scored in the presence of food for 15 s for each animal. Typically, 25–30 animals were assayed per genotype per day, using a stereomicroscope. Typically, a small number of animals were not pumping in each assay. This proportion generally increased as the experiment progressed. The pumping rate of the remaining animals also fell as the experiment progressed. As the pumping rates of populations did not fit a normal distribution, statistical analysis was performed using a two-tailed Mann–Whitney test.

To measure late progeny production, animals raised at 20 °C were picked as L4s and placed at 25 °C and moved to fresh plates every day. After 9 days of adulthood animals were placed on individual plates and maintained at 25 °C. Late progeny were defined as those appearing after day 9 of adulthood. N2 hermaphrodites ceased laying eggs between days 4 and 5.

Lifespan measurements

These were conducted as previously described (Gems *et al*., 1998). *let-60(rf)* results in a partially penetrant Vul phenotype, which results in death from internal hatching of larvae. To prevent this, animals were maintained on 5-fluorodeoxyuridine (FUdR), which inhibits DNA replication (Gandhi *et al*., 1980). Survival analyses were performed using the Kaplan Meier method on censored data, and the significance of differences between survival curves calculated using the log rank test. The statistical software used was JMP v.5.1 (SAS Institute Inc., Cary, NC, USA).

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